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UPDATE ON MICROBIOLOGY RESEARCH FOR THE WASTE ISOLATION PILOT PLANT

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UPDATE ON MICROBIOLOGY RESEARCH

FOR THE WASTE ISOLATION PILOT PLANT

This report provides a summary of research that has been conducted to inform the position on microbial effects on the Waste Isolation Pilot Plant. Briefly, it reiterates statements made in previous reports that suggest that the effects that rely on microbial activity (e.g., biodegradation of waste components and subsequent gas generation) are not likely to be as great as predicted in performance assessments. This is due to the unique microbial ecology at the WIPP and the energetic constraints of survival at high salt. For processes that do not necessarily rely on microbial activity (e.g., bioassociation), effects are still being quantified but can be highly variable.

1.0 CHARACTERIZATION OF MICROORGANISMS RELEVANT TO THE WIPP

1.1 Microbial ecology

Two distinct microbial populations exist within any repository environment: indigenous organisms and introduced organisms. In surficial repositories, such as landfills, these populations may initially be very similar. However, a deep subsurface environment supports a unique microbial ecology selected by conditions specific to that site. In a deep geological repository (DGR), each population may have a selective advantage within its own space, as a result of adaptation to the unique geochemical constraints on activity within that given space (for example, halophiles will thrive at high salt concentrations, and desiccation or radiation-resistant organisms may survive within waste drums).

In DGR settings, it is assumed that the two populations can co-exist after drum breach and infiltration by native fluid, and after the establishment of an environment controlled by native and engineered influences. However, in the case of a salt-based repository, this will put the introduced population at a great disadvantage, since few of these organisms will be adapted to the high salt concentrations of infiltrating brine. Nevertheless, the introduced population may have the potential to influence repository performance prior to inundation by degrading waste components within drums and after inundation in ways that do not require their activity. In contrast, although the indigenous population has the advantage of survival at high ionic strengths, their activity under the expected anoxic repository conditions may be limited.

1.1A Introduced population

1.1.A.1. Waste organisms

Samples from two WIPP-bound waste drums were processed for DNA-based identification of microorganisms and also for limited culture-based identification (EDF-10716, 2014; Swanson et al., 2015). Only one drum yielded positive results. For this drum, the culture-independent (DNA sequence-based analysis) and culture-dependent results were very similar. All results depict a population with limited diversity in comparison to an environmental sample, such as soil. In the metagenome, 70.4% of the sequence reads were bacterial, 29.4 % eukaryotic, and less than 1%

were viral, archaeal, or unclassified (Figure 1). Of the bacterial reads, the majority (91%) were from the phylum *Actinobacteria*; while, the eukaryotic reads were predominantly (99%) fungal *Ascomycota*.

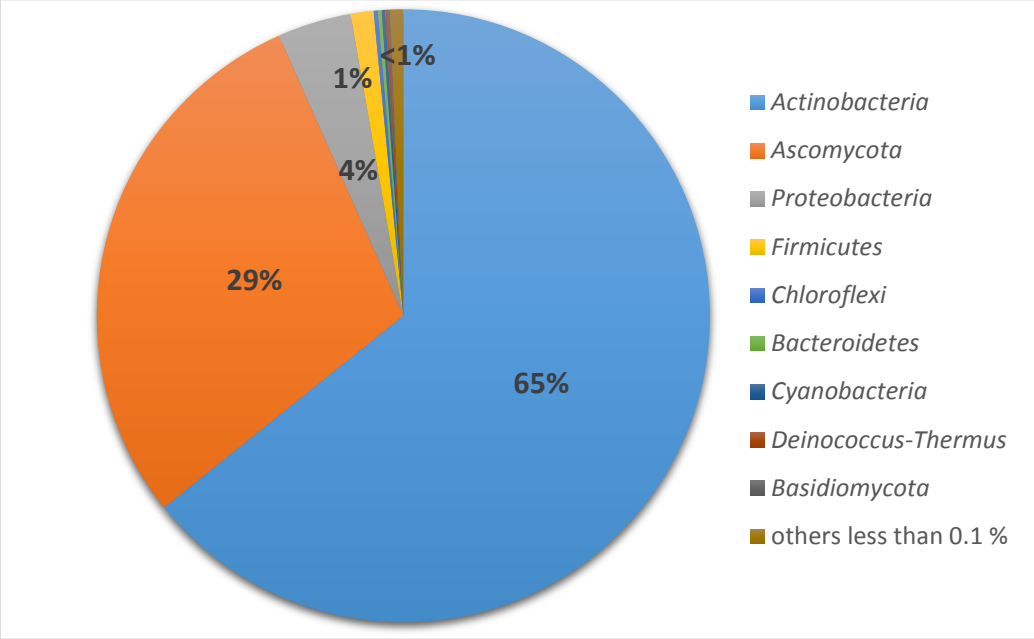


Figure 1. Phylogenetic breakdown of DNA sequence reads in the waste drum metagenome. Note that majority belong to the phyla *Actinobacteria* and *Ascomycota*, bacteria and fungi, respectively.

The organisms isolated from one drum include two members of the *Actinobacteria* (*Arthrobacter* sp., *Brachybacterium* sp.) and three spore-formers (all *Bacillus* spp.). That spore-forming organisms should be isolated but not detected by DNA-based analyses is not unusual and depends on the ability to lyse spores during the extraction process.

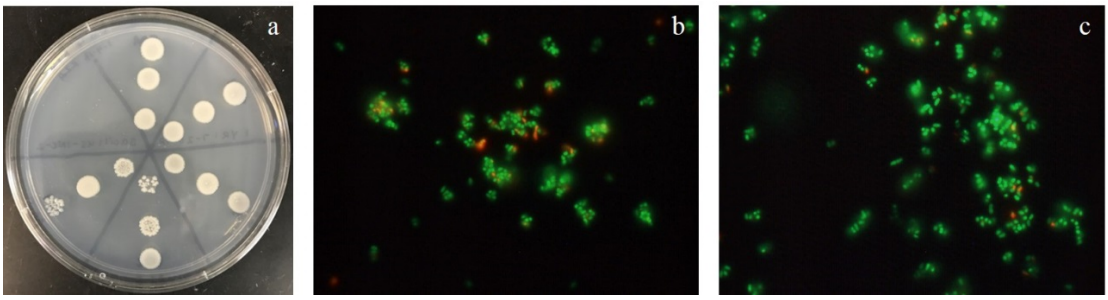
All isolates have been tested for their ability to grow over a range of NaCl concentrations and at best can be classified as halotolerant—i.e. salt is not required for growth but can be tolerated at higher concentrations (Table 1).

Table 1. NaCl optima and tolerance ranges for bacterial isolates from WIPP-bound waste (in Difco Marine Broth 2216 and R2B, see Appendix 2).

Isolate* / M NaCl	*Arthrobacter* sp.	*Brachybacterium* sp.	*Bacillus* sp.2	*Bacillus* sp.3	*Bacillus* sp.4
Growth Range	0-1.62	0-2.04	0-1.62	0-1.62	0-1.19
Growth Optimum	0.76	0.76	0.33	0.33	0.76

*Isolate identifications are based on 16S ribosomal gene-encoding sequence.

Members of the *Actinobacteria* are often (poly)extremophilic or resistant to multiple environmental stressors, and bacilli can resist extreme stress when in spore form. Thus, survival and recoverability of these organisms after some period of time in brine is possible, even if activity is not. For example, spores of *Bacillus* sp. 2 were able to vegetate after a year in 5M NaCl, and *Arthrobacter* sp. viability was verified after 2 months suspension in GWB and ERDA-6 (Figures 2a, 2b, 2c). This suggests that the role of certain waste organisms as biocolloid vectors cannot be ruled out; however, their actual numbers within the waste itself are unknown.



Figures 2a-2c. Growth/survival of waste organisms after exposure to high ionic strength solutions; a) *Bacillus* spore recoverability after 1 year suspension in 1M-5M NaCl; *Arthrobacter* survival at 8 weeks in b) GWB and c) ERDA-6 (green indicates live cells; red cells are dead).

The presence of *Ascomycota* in the waste is also not surprising. Filamentous Fungi are often desiccation and radiation resistant. Many *Ascomycetes* are able to degrade cellulose; thus, it is possible that these organisms have utilized waste components since packaging and are only limited by oxygen, available moisture, and residual levels of radioactivity. However, there are currently no means to measure these effects on any given waste drum.

1.1.A.2. Other introduced organisms

A second subset of introduced organisms comprises those that have come into the WIPP on personnel, equipment, or air-intake shafts. As with the waste organisms, these are unlikely to be extremely halophilic, but some may be desiccation resistant or halotolerant. Attempts to cultivate extreme halophiles from salt mine air (including the WIPP) have been unsuccessful (Norton et al., 1993; Vreeland et al., 1998). This appears to still be the case at the WIPP (Figure 3).

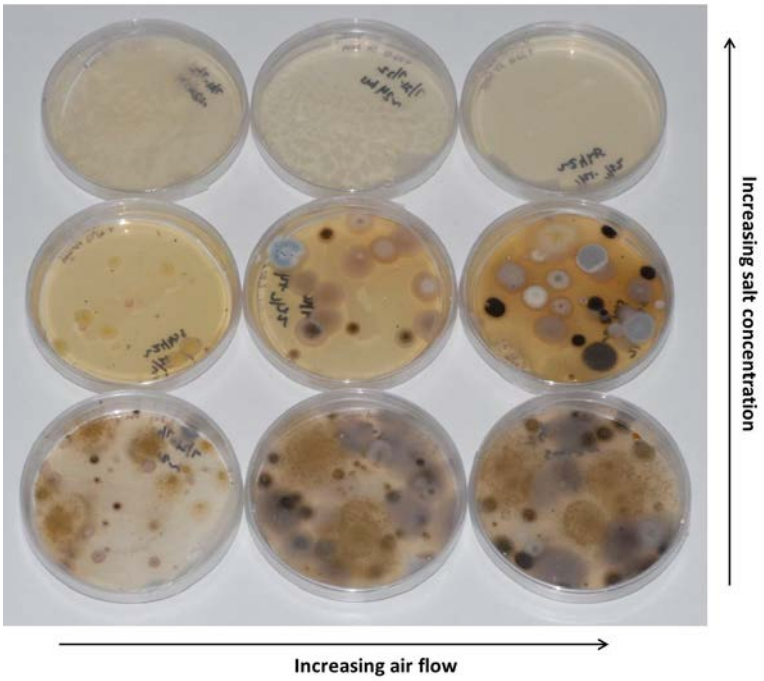


Figure 3. Microbial growth on agar plates exposed to air at the WIPP (SDI area). Note: no growth on plates with the highest salt concentration. Most growth is fungal. Top row, 3.42 M NaCl; middle row, ~1.71 M NaCl; bottom row, ~0.34 M NaCl.

1.1.B. Indigenous population

Approximately 30% of all known haloarchaeal genera have been detected in subterranean halite. The reason for the lower diversity of these organisms in this setting, as compared to surficial hypersaline environments, may be due to the scarcity of carbon and energy sources in the subsurface and the lack of fluid movement to carry such nutrients to microorganisms (Swanson et al., 2016). *Halobacterium* spp. (especially *noricense*) are the most commonly detected and isolated from subterranean halites, and it is hypothesized that this organism is especially adept at long-term survival in salt (Gramain et al., 2011). For this reason, the WIPP strain of *Halobacterium* is used as a representative organism for WIPP studies. Two other haloarchaeal isolates (designated PB-1a and Trans-1b, but as yet unidentified) with slightly different characteristics are also routinely used (Table 2). Microbial characterization work on WIPP halite has been discussed previously (Swanson et al., 2013).

Table 2. Range of growth medium conditions for archaeal halite isolates (see Appendix 2). Optima in parentheses; "sat" = saturated.

Parameter	*Hbt. noricense*	PB-1a	Trans-1b
[NaCl], M	1.71-sat (3.42)	2.14-sat (4.28)	1.71-sat (2.99)
[MgCl2], M	0-1 (0.15-0.2)	0-0.75 (0.15-0.2)	0-1 (0.2)

1.2. Microbial growth and/or survival in WIPP brines

The ability of microorganisms to transform waste depends on their activity; thus, gas generation results only from actively growing cells. As stated earlier, within-drum waste degradation and gas generation has not been systematically investigated. Once the repository becomes infiltrated with brine, only the most halophilic of organisms will grow (Figure 4). Of six halite isolates tested (3 archaea, 3 bacteria), all archaea and the most halophilic bacterium (*Chromohalobacter*) grew in GWB and ERDA-6 amended with carbon sources (acetate, citrate, pyruvate) and nutrients (yeast extract, casamino acids) under aerobic conditions. None of the isolates is capable of anaerobic growth.

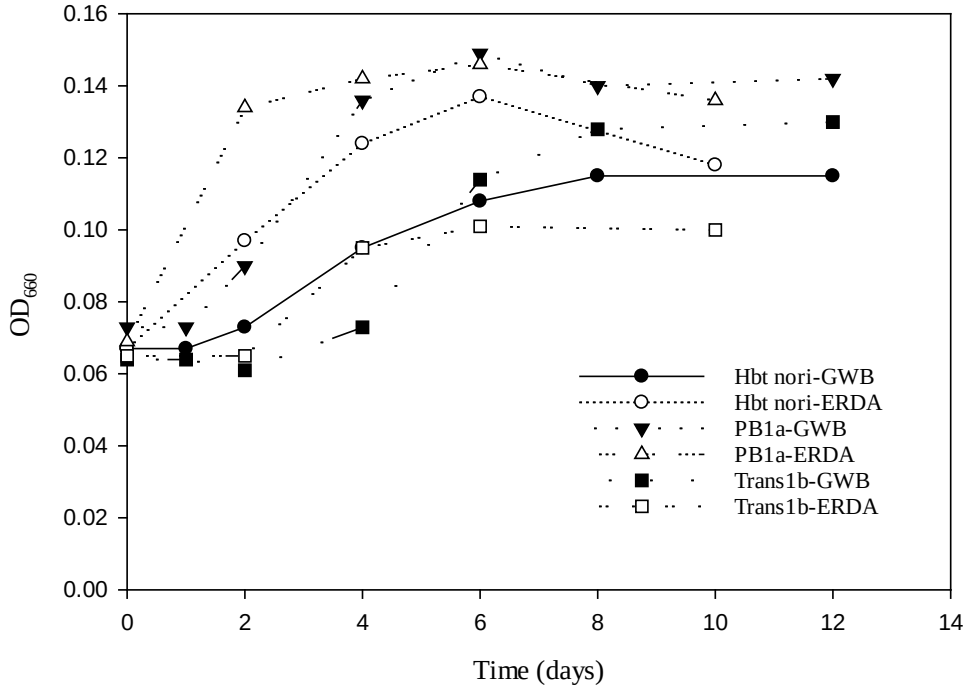


Figure 4. Growth of three archaeal halite isolates in amended WIPP brines, as measured by optical density. Note that under ideal growth conditions, OD readings can approach 0.600.

Because other bacterial isolates were unable to grow in WIPP brines, their ability to survive and be recovered from brine was tested. It appears that the more halotolerant the isolate, the longer it can remain suspended in brine and still recover when transferred to its ideal medium (this does not hold true for *Bacillus* spp. that have already sporulated). The least halotolerant could only be recovered after 1 week of exposure, while the most halophilic has remained viable for over 9 months. The bacteria tested thus far fall into three phyla: *Proteobacteria* (*Chromohalobacter* sp.), *Actinobacteria* (*Nesterenkonia* sp.; *Brachybacterium* sp. and *Enteractinococcus* sp. from Gorleben halite), and *Firmicutes* (*Salinicoccus* sp., *Thalassobacillus* sp.). The latter two phyla

contain many organisms that exhibit (poly)extremophily or tolerance (e.g., to temperature, pH, desiccation, radiation, salt).

1.2.A. Possible microbial effects and the requirement for cell activity versus viability

In many cases, microorganisms must be actively growing in order to have an effect on repository performance. However, this is not universally true and depends on what mode of influence the organism is exerting. In the case of biosorption, both live and dead biomass can adsorb radionuclides; while, microbial activity is necessary for gas generation (Table 3).

Table 3. Reliance of microbial processes on live or dead biomass.

MICROBIAL PROCESS	LIVE AND ACTIVE	LIVE AND INACTIVE	DEAD
Transformation of waste carbon	X		
Gas generation from transformation of waste carbon	X		
Generation of complexing ligands	X		
Degradation of complexing ligands	X		
Alteration of redox conditions	X	?*	
Alteration of pH	X	?*	
Direct redox reactions	X		
Indirect redox reactions	X	X	X
Biosorption	X	X	X
Biomineralization	X	X	X
Actinide Internalization	X	?	

*transient, if at all

2.0 MICROBIAL GAS GENERATION

The gases predicted to be generated from the microbial consumption of waste constituents can include CO_2, N_2, H_2, CH_4, and H_2S. Microbial gas generation is expected to be less than that generated by corrosion of metal canisters and comparable to, or less than, that from radiolysis. However, it is still a large uncertainty in the WIPP PA. The chief reason for this is the preponderance of negative results, i.e. little to no gas was generated in most studies because organisms did not grow under the tested relevant repository conditions. This scenario can be

explained by looking at the microbial ecology of subterranean halite and the bioenergetics of this population (Swanson et al., 2016).

2.1. History of previous work

A summary of past gas generation studies is shown in APPENDIX 1. Key findings from past work include:

- The conspicuous absence of significant gas generation
- Pu inhibitory effect on rates of gas generation
- First demonstration of nitrate-reducing, extreme halophiles in G-seep brine (Francis and Gillow, 1993)
- Positive correlation between gas generation and presence of nutrients (excess nitrate; Francis and Gillow, 1993, 1997, 2006)
- Demonstration of sulfidogenesis, regardless of cause (Francis and Gillow, 1993)

2.2. Negative findings in current work

Little to no progress has been made in the area of microbial gas generation under WIPP-relevant conditions. This is because no anaerobic organisms have been cultivated from WIPP halite to date. Indeed, no strictly anaerobic halophiles have been cultivated from any other subterranean halite. Although anaerobic respiration under hypersaline conditions does occur, this mode of activity has only been shown in sediments of surficial brine lakes or seas (e.g., Dead Sea, Great Salt Lake) or solar salterns, or using isolates therefrom. The closest evidence for anaerobic respiration in subterranean halite is from Francis and Gillow, who found that organisms in G-seep brine were capable of nitrate reduction (Francis and Gillow, 1993).

Nevertheless, the Francis and Gillow results provide the best insight into some realistic conditions: 1) negligible gas generation under humid, anaerobic, uninoculated, unamended incubations ("uninoculated" still contain organisms from G-seep, but none from surficial environments; 2) incubations are both inoculum and nutrient-limited but especially nutrient-limited, as evidenced by the higher rates of gas generation in inoculated, amended, and excess nutrient incubations; 3) fermentation was presumed to be the primary mode of metabolism observed; 4) "biphasic" gas production would be typical of a closed system, where nutrient depletion and build-up of toxic metabolites limit activity.

Within the past few years, several incubations of both WIPP and Gorleben halites were set up under what should have been optimum growth conditions. These incubations were prepared under transitional (sealed under normal atmosphere), nitrate-reducing, iron-reducing (WIPP halite only), and sulfate-reducing conditions, and at both low and high salt concentrations. Gorleben halite containing natural hydrocarbons was also incubated without additional carbon sources. None of these incubations resulted in any growth, as determined by microscopy. Evaluation during the first month suggested that fungal spores were present in some incubations, but these quickly died off.

2.3. CRA implementation

The lack of positive results is not surprising, in light of what is known about the organisms that live at high salt concentrations (Oren, 2011; Swanson et al., 2016). At the time that the WIPP PA was established, much was unknown and therefore assumptions were made based on the known behavior of microorganisms in other settings, i.e. findings at lower ionic strength and projections for other repositories (Table 4). From the CCA through CRA-2004, the assumption was that after the repository becomes anoxic, gas generation from the breakdown of cellulose was predicted to proceed sequentially from denitrification to sulfate reduction and finally methanogenesis, based on the energy yields of each degradation reaction. Methanogenesis was removed for CRA-2009, because sufficient sulfate is present in the repository as anhydrite. Table 4 provides a different view of these assumptions, in light of halophile microbiology, and also raises issues to be addressed.

Table 4. Summary of PA Assumptions Regarding Microbial Activity and Identified Knowledge Gaps (Especially Gas Generation)

ASSUMPTION	REALITY/CURRENT KNOWLEDGE	INFORMATION LACKING
Anaerobic respiration will occur	• Most extreme halophiles (Archaea) are aerobic heterotrophs (Oren, 2011) • Halophilic, anaerobic Bacteria and newly discovered anaerobic haloarchaea exist in surficial hypersaline environments (Oren, 2011; Sorokin et al., 2011, 2015, 2017), but have not been detected in subterranean halites to date (Swanson et al., 2016); all documented anaerobic halophiles are derived from sediments of brine lakes, solar salterns	• Are sulfate-reducers or other sulfidogens present in the WIPP near-field? • Are elemental sulfur, methylsulfides, or methylamines present in the near-field?
Sequential use of terminal electron acceptors (TEAs): oxygen, nitrate, (iron), sulfate, carbon dioxide	• Some few haloarchaea (genus *Haloferax* and *Haloarcula*) will respire nitrate or ferment arginine (genus *Halobacterium*)(Oren, 2011) • Some can reduce dimethylsulfoxide (DMSO) or trimethylamine oxide (TMAO; Oren, 2011) • Anaerobic, halophilic bacteria can reduce nitrate and sulfate at high salt concentrations (Oren, 2011) • Halophilic methanogens cannot reduce carbon dioxide at salt concentrations higher than 120 g/L (2M); if methanogens exist, they will use methylated amines or sulfides, not CO_2 (Oren, 2011) and will not need to compete with SRB • Newly discovered clade of anaerobic haloarchaea can utilize H_2 + TMAO to generate methane; isolated from brine lake sediment (Sorokin et al., 2017) • Most likely modes of metabolism will be fermentation, rather than anaerobic respiration • This assumption may hold true within waste drums, prior to brine inundation, provided all other conditions for growth are met	• Are sulfate reducers present in the near-field? • Are other sulfidogens present? • Are methylated amines or sulfides or elemental sulfur present in the near-field? • Are other TEAs present and usable by halophiles, e.g. uranium, pertechnetate, selenite? • Is PA concerned with gas generation within drums?
Microorganisms will degrade cellulose	• Degradation of laboratory cellulosics (measured as gas generation relative to control and generation of by-products) was observed in Francis and Gillow studies; incubations contained sediment in the inoculum (Gillow and Francis, 2006)	• What is the extent of cellulose hydrolysis that has already occurred within a waste drum?

	• *Halosimplex* sp., *Halorhabdus* sp., and *Halomicrobium* sp. have been enriched from sediment/brine slurries under aerobic conditions using amorphous and microcrystalline cellulose; rates of degradation varied with degree of crystallinity; cell attachment to fibers was prerequisite for activity of hydrolytic enzymes (Sorokin et al., 2015) • Many haloarchaea produce cellulases that can degrade oligosaccharides (e.g. cellobiose) but not insoluble cellulose (Sorokin et al., 2015, 2017) • Microorganisms known to be capable of initial cellulose hydrolysis were present in a sampled waste drum; isolates from the drum were unable to grow at high salt concentrations (Swanson et al., 2015) • 24% of 5,123 bacterial genomes surveyed show capability of degrading cellulose; 56% are opportunists, degrading oligosaccharides and cellobiose; 20% have no capability reflected in genome; suggests cellulose degradation is not a universal capability (Berlemont and Martiny, 2013) • Survey of 20 haloarchaeal genomes shows broad distribution of genes encoding cellulases and related glucanases, but only 2 genera have functional enzymes (Sorokin et al., 2015) • This assumption may hold true within waste drums, prior to brine inundation, provided all other conditions for growth are met	• Will this have generated smaller, more usable substrates (cellulose by-products, CBPs)? • Will cellulase-producing haloarchaea ever come into contact with waste cellulosics during the oxic repository phase?

3.0 ACTINIDE TOXICITY

With respect to actinides, toxic effects can be either chemically or radiologically induced. Chemical toxicity is presumably similar to heavy metal toxicity and, in general, is a function of the free ion concentration in solution. The metal can bind with essential biomolecules, such as proteins and nucleic acids, and alter their structure and/or inhibit their activity. Many factors can affect metal/actinide toxicity—such as speciation, oxidation state, isotope, concentration, matrix, ligand presence, and organism type (Banaszak et al., 1998; Banaszak et al., 1999, Reed et al., 1999; Ruggiero et al., 2005).

Microorganisms combat metal toxicity by using active efflux systems and/or sequestration strategies (sequestration broadly includes complexation, mineralization, precipitation, reduction). Active efflux is the most common mechanism of resistance, but often organisms use a combination of strategies.

In contrast, radiological toxicity of actinides is caused by the generation of oxidizing free radicals (e.g. $OH^{\cdot}$, $HO_2^{\cdot}$, and oxychloride radicals) by ionizing radiation (alpha, beta, or gamma) that results in DNA lesions. Radiation resistance is conferred by nucleic acid repair mechanisms and internal Mn/Fe ratios, among many other characteristics and strategies.

Few studies have been conducted on actinide toxicity, as compared to heavy metal toxicity, and most of those have focused on uranium. Due to the logistical limitations of working with actinides, the parameters most often measured in these studies have been survival and growth.

Preliminary toxicity studies have been undertaken for the WIPP in order to gain a better understanding of microbial activity and/or survival in the presence of actinides. Lethality, or even slight toxicity, could affect those processes listed in Table 3 that require cell activity, such as gas generation. To begin, a series of growth assays was conducted with *Halobacterium* sp. (*noricense*) in the presence of various concentrations of 237-Np(V) and 242-Pu(V/VI). Additional studies were conducted on uranium exposure to a waste drum isolate, *Arthrobacter* sp.

The optimal growth medium for each organism was used in these studies (see Appendix 2), in order to minimize the stress of changing growth conditions and to isolate the stress of actinide exposure. For this reason, assays were not performed under WIPP conditions (i.e., experiments were aerobic and at circumneutral pH). Additionally, these experiments did not use the more active isotopes, such that observed toxicity was most likely chemical rather than radiological. Thus, the results for these experiments (presented below) are valid under these specific conditions. Under strict WIPP conditions, the bioavailability of highly complexed actinide species and the activity of the microorganisms are not as well established; therefore, toxicity may differ.

3.1. Neptunium and plutonium

Because the ideal growth medium used for these studies contained some undefined components (i.e., yeast extract, casamino acids) and possible complexants (e.g., phosphate), spectra of the

spiked medium were checked over time for medium effects on actinide oxidation state or speciation.

3.1.A Neptunium

^{237}Neptunium, as a $Np(V)O_2^+$ complex in 0.01M HCl, was spiked directly into *Halobacterium* growth medium (see Appendix 2; pH 6.97) to achieve a range of concentrations (1.1E-6M to 9.3E-6M). Parallel growth controls with spiked acid were also run to test for pH effects. Samples were incubated at 37°C with constant shaking; growth was measured as the change in optical density over time.

Although Np was added as an aquo species, spectra showed that it rapidly formed a complex in the growth medium and remained stable as Np(V). Under these conditions, neptunium(V) inhibited the overall extent growth of *Halobacterium* sp. by approximately 40% and the initial rate of growth by 47-63% at all concentrations tested (Figure 5). However, due to the narrow range tested, no clear dose-response relationship was evident.

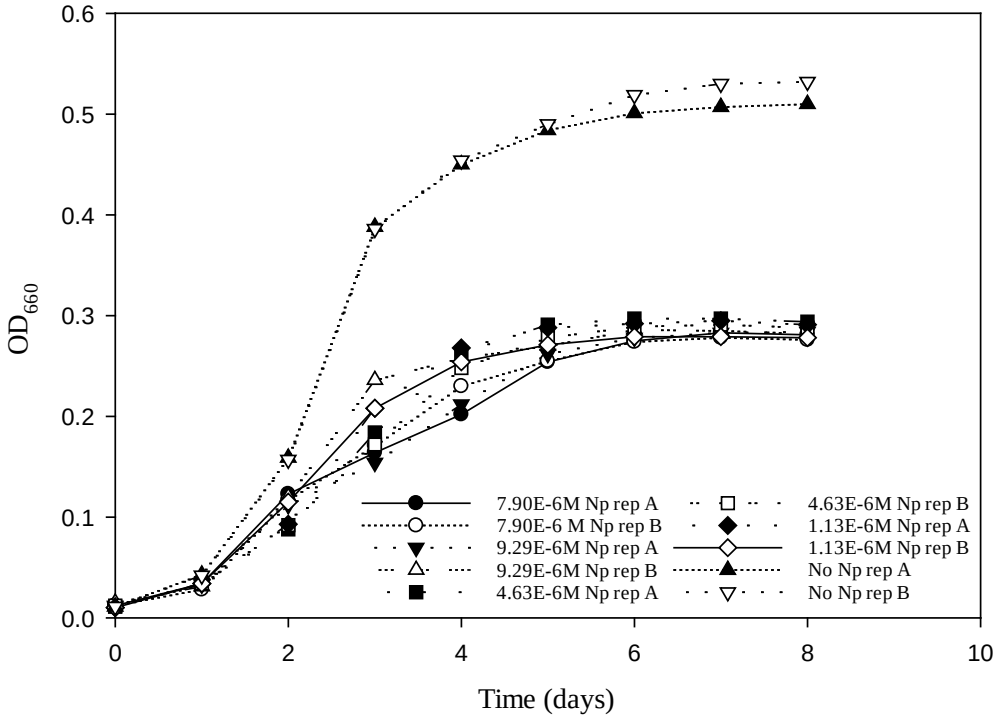


Figure 5. Inhibition of *Halobacterium* growth by neptunium. Concentrations given are those measured by ICP-MS.

There are very few data regarding actinide toxicity to halophilic organisms, so study comparisons are difficult. Francis et al. (1998) found little to no effect on the growth rate of a mixed culture of halophiles exposed to a target concentration of 5×10^{-4} M Np-EDTA under

nitrate-reducing conditions (I ≈ 7 M; [NaCl] = 1.71 M; [Mg] = 1.44 M; pH 6.3). In contrast, the growth rate of a halophilic bacterium exposed to the same Np-EDTA concentration was reduced by ~34% (measured concentration 2.65E-4M; Papenguth, 1996), and the overall extent of growth was reduced by ~80% (I and [NaCl] = 3.1 M; pH 6.3). These data suggest that the archaea were less susceptible than the bacteria in Francis' experiments. In the current study, *Halobacterium*'s growth rate was reduced by ~60% at a much lower concentration, and the overall extent of growth by almost as much. This may be explained by the absence of a less bioavailable EDTA complex in these systems, unlike the Francis studies.

3.1.B. Plutonium

^{242}Plutonium, as $Pu(VI)O_2^{2+}$ in 0.1M perchlorate, was added as a 1:10 dilution to *Halobacterium* growth medium. Serial dilutions were then made from this Pu-spiked solution. A perchlorate control was also run, and the pH of the negative controls measured 6.6. The plutonium in the growth medium was not stable. Spectra taken over time showed a mixture of Pu(VI) colloids (easily filterable), complexed Pu(V) species in solution, and some Pu(IV) that precipitated, which lowered the overall concentration over time.

Under these relatively unstable conditions, plutonium inhibited the growth of *Halobacterium*, but in this case there was a loose dose-response relationship, and growth was significantly inhibited at the ~10^{-4} M target concentration (Figure 6).

Francis et al. (1998) measured the growth rate of halophiles exposed to ^{239}Pu(V)-EDTA (same conditions as Np) and also observed dose-dependent inhibition, but at higher concentrations (1E-5M target; 8E-6M measured, Papenguth, 1996). Although those studies used a higher activity isotope, its complexation with EDTA may have mitigated some of the toxic effect.

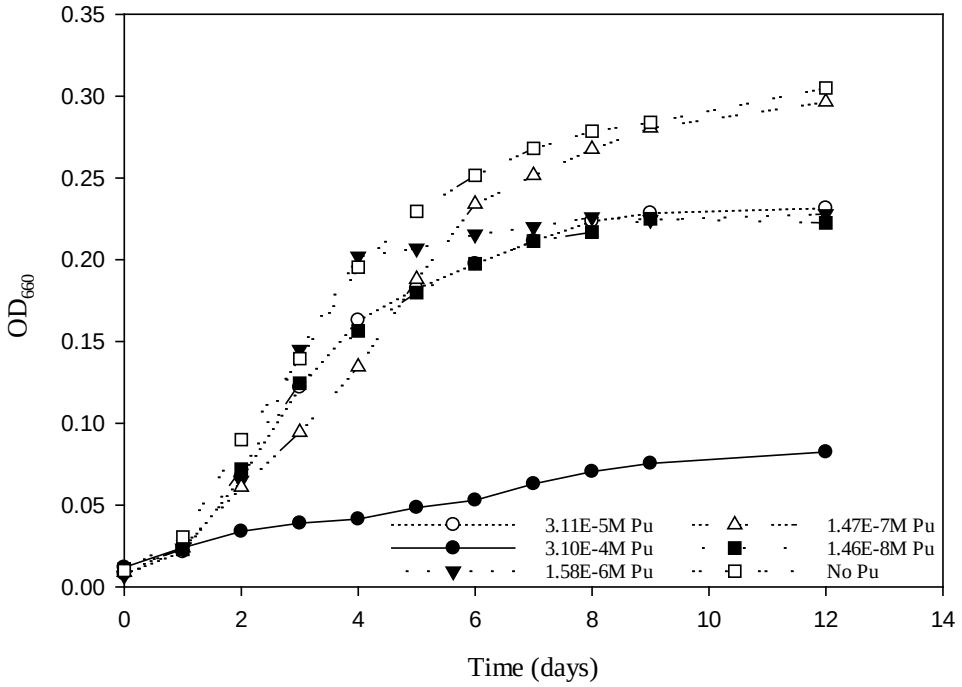


Figure 6. Inhibition of *Halobacterium* growth by plutonium.

3.2. Uranium

Uranium assays were also undertaken to establish procedures for separate molecular investigations. The growth of *Arthrobacter* sp. (see section 1.1.A.1) was tested in the presence of various concentrations of uranium-citrate spiked into its optimal growth medium (see Appendix 2; pH ~7).

This *Arthrobacter* strain can utilize citrate as a sole carbon source, and this resulted in a slight enhancement of the initial growth rate at lower U-citrate concentrations. Growth was inhibited at concentrations of U-citrate greater than 0.5 mM (Figure 7); this was confirmed with citrate only growth controls. However, measurements of optical density were equivocal at the highest concentrations, given that the U-citrate was not soluble.

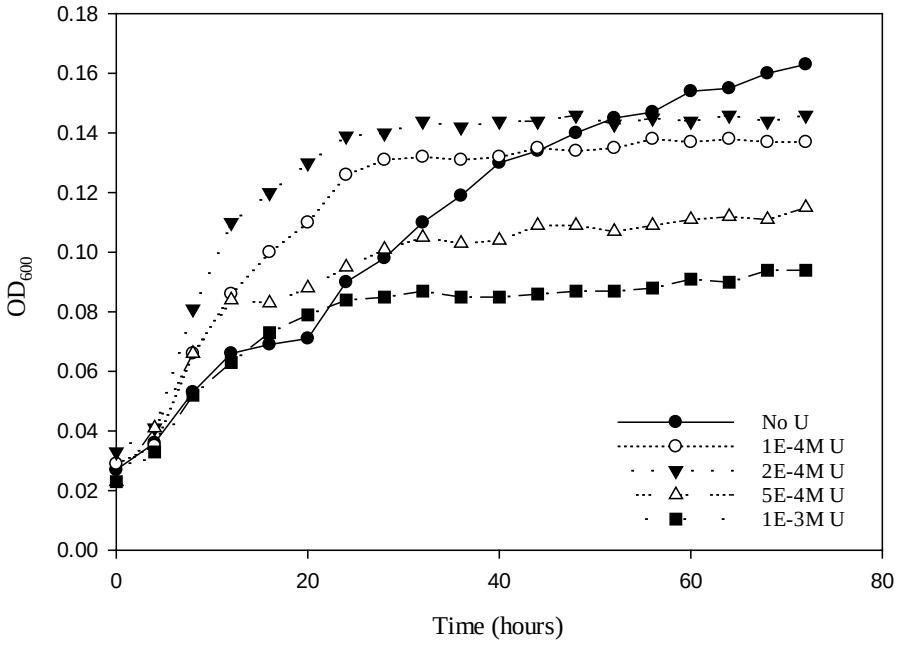


Figure 7. *Arthrobacter* sp. growth in the presence of uranium-citrate; "No U" also has no citrate.

The isolation of *Arthrobacter* spp. from radionuclide contaminated sites is not unusual and its tolerance to uranium has been shown in previous studies (Fredrickson et al., 2004; Cherkouk, 2006). Waste organisms may potentially act as transport vectors, if they are capable of associating with radionuclides in the waste. *Arthrobacter* will be tested further for this capability, especially since it was found to survive for a significant period in WIPP brines (Figure 2b-c).

4.0 BIOASSOCIATION

The term "bioassociation" can be used to describe all types of microbial-metal/actinide associations, including surface sorption, biomineralization, or internal uptake. Bioassociation is influenced by many factors, such as actinide speciation, pH, cell surface composition, and the presence of ligands. The majority of published biosorption experiments have been conducted at lower pH and lower ionic strength (e.g., Moll et al., 2006; Lujaniene et al., 2017). While neither of these conditions describes the WIPP, many experiments have been carried out under non-WIPP conditions to better control and understand the system. These studies are gradually being extended to more WIPP-relevant conditions (e.g., pC_{H^+}-specific brines). This section highlights studies conducted on organisms from each WIPP "compartment": near-field waste drums, near-field salt, and far-field groundwaters.

4.1 Near-field organisms

The near field organisms that have been investigated for their sorption capacity include indigenous halophiles (*Halobacterium noricense, Chromohalobacter* sp.) and an introduced waste organism (*Bacillus* sp., putatively *megaterium*).

4.1.A. Waste organisms

Microorganisms present in WIPP waste are the only organisms initially in contact with radionuclides. Because biosorption does not require microbial viability, even dead biomass can influence radionuclide mobility. Actual biomass concentrations in WIPP waste are unknown but are expected to be low because of the lack of moisture and potentially toxic levels of radioactivity. In the case of a drum breach and brine inundation, there is also no guarantee that cells of non-halophiles will remain intact. However, in the case of spores that can survive high ionic strength, studies on vegetative cells are insufficient for application to performance assessments.

A *Bacillus* sp. was isolated from WIPP-bound waste and tested for its capacity to adsorb neodymium in 0.14 M and 2M NaCl, while in spore form. Neodymium was initially associated with the spores but progressively disassociated over the period of a week (Figure 8). This suggests that adsorption onto spores found in waste will not greatly influence the transport of radionuclides out of drums once they are breached, but further investigations are necessary, as one hypothesis for this phenomenon is the release of a complexing compound (dipicolinate; DPA) that may have resulted in Nd solubilization. This remains to be tested with this organism, but a previous study found that Np could be mobilized by DPA from *Bacillus subtilis* spores (Gorman-Lewis et al., 2013).

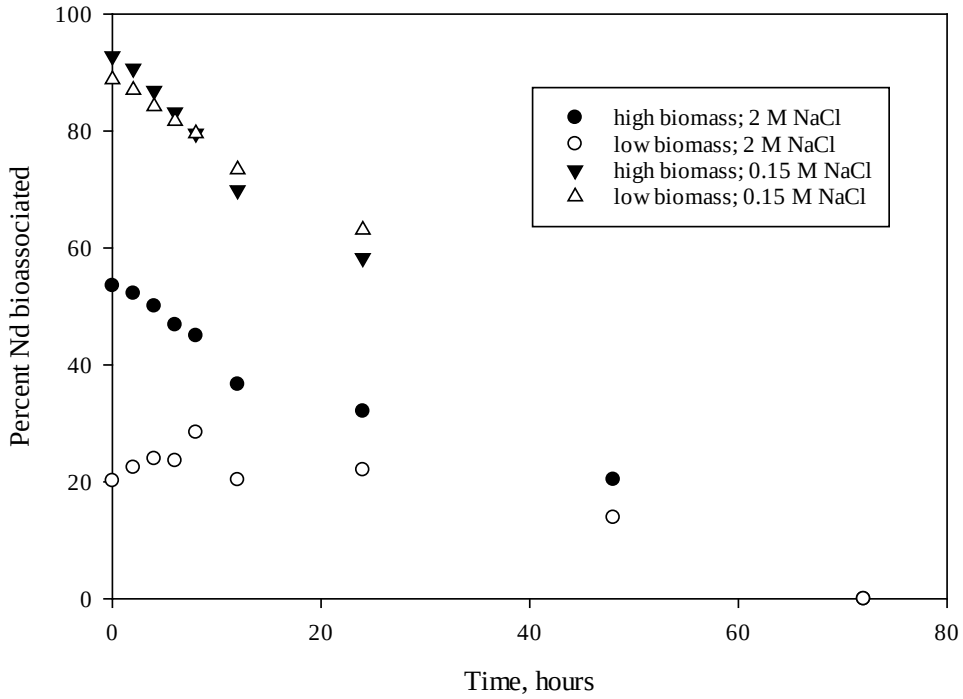


Figure 8. Association of neodymium with spores of a waste drum bacillus. High biomass ≈ 10^7 spores/ml; low biomass ≈ 10^6 spores/ml.

4.1.B. Indigenous halophiles

Previous work with WIPP strain *Halobacterium* sp. showed approximately 40% sorption of added Nd over a pC_{H+} range of 6.5-9.4 in WIPP-specific brine and less sorption (~15%) in the presence of EDTA (Reed et al., 2013). Thorium sorption was also relatively low between pC_{H+} 8-9 (<20%); at higher pH, precipitation dominated thorium loss from solution. This work also showed a distinct decrease in the sorption capacity of *Halobacterium* sp. as ionic strength, specifically magnesium content, increased (pC_{H+} range 6.5-9.3).

Additional experiments have recently been conducted on the WIPP *Halobacterium* sp., although not at WIPP-specific pH (Showalter et al., 2016; Bader et al., 2017). Bader et al showed a pH-dependent, biomass concentration-dependent, and time-dependent association of uranium with cells (~80% association over the period of one week) in 3.4 M NaCl at pH 4 and 6. The association was with carboxyl and phosphoryl groups at the cells' surfaces, as shown by SEM-EDX and ATR-FTIR analyses and was found to be reversible in the presence of citrate. Francis et al. (2004) found surface U-phosphate complexes on the extreme halophile, *Halobacterium salinarum* (pH 5; 4.3 M NaCl). Kinnebrew (as cited in Kenward et al., 2013) found U-carbonate complexes with *Haloferax sulfurifontis* that led to the precipitation of dolomite under certain conditions (1M NaCl, pH 7.2).

In contrast, Showalter et al (2016) found that sulfhydryl groups were responsible for the binding of Cd to the WIPP *Halobacterium* sp. in simplified brine between pH 5-7.5. In this case, the limited number of sites at the cell's surface was found to constrain the binding of Cd, and there was no pH dependence within the tested range. Sulfhydryl groups are limited to certain protein moieties that might be present in the glycoprotein S-layer possessed by most haloarchaea (Sara and Sleytr, 2000). This layer can be shed depending upon experimental conditions. Bader et al found no involvement of sulfhydryl groups in their study.

In addition to the haloarchaeon, a near-field bacterial species was also tested. *Brachybacterium* sp. (*faecium*) was isolated from halite retrieved at the Gorleben site and was tested for its ability to adsorb uranium in simplified brine (Bader et al, 2018). Like *Hbt. noricense*, carboxyl groups were found to be involved in this association, but unlike the haloarchaeon, phosphoryl interactions were not detected in this case. It was hypothesized that the functional groups belonged to sugar moieties on the bacterial surfaces, but amide (protein) involvement was also observed. This organism cannot grow in WIPP brines but is capable of survival for up to 10-13 weeks.

4.2. Far-field

Chromohalobacter sp. has been used frequently as a test organism for the WIPP case (Ams et al., 2013). Because it has been isolated from halite as well as surrounding groundwaters, it can be used as a representative bacterium for both near and far-field experiments. Previous work has shown time-dependent, biomass-dependent, ionic strength-dependent, and pC_{H+}-dependent association of Nd and Np with this organism, as well as a decrease in Nd association in the presence of EDTA. Thorium association was also found to be pC_{H+}-dependent and decreased in the presence of EDTA. Of several bacteria isolated from WIPP by LANL, *Chromohalobacter* is the only bacterium that can grow in WIPP brines.

Chromohalobacter was recently tested in a ternary system containing dolomite and neodymium to investigate competition between organisms and mineral matrices present in the far-field (Zengotita et al, 2017). These experiments were performed in 2.57 M NaCl, 3 mM $NaHCO_3$, at pH 8.3. Flow-through mini-column experiments showed that in the absence of *Chromohalobacter*, neodymium remains attached to the dolomite matrix. When the organisms are present, neodymium can move through the column, albeit at a low recovery rate. However, if neodymium has already sorbed onto the dolomite, the introduction of *Chromohalobacter* did not lead to its removal (Figure 9).

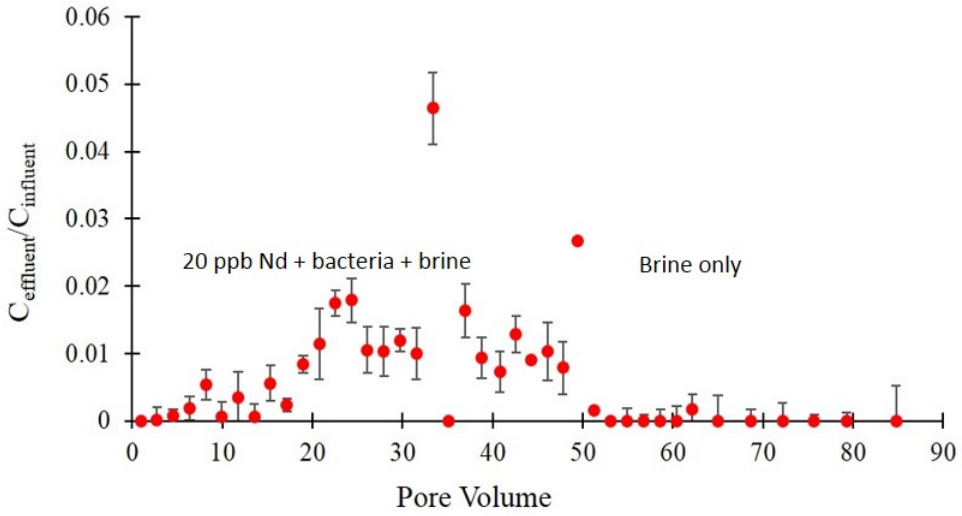


Figure 9. Neodymium recovery after filtration of samples. Second injection occurs at approximately 49 pore volumes.

Bioassociation research under more WIPP-relevant conditions is ongoing and will be the subject of a future report.

5.0 CONCLUSIONS

Based on current and past WIPP-relevant microbiology investigations, we conclude that the influence of microorganisms at the WIPP will differ from other deep geological repositories (Swanson et al., 2016). This is mainly due to the constraints of high ionic strength, high chaotropicity, low water activity, and anoxia on WIPP-relevant microbial populations. While the indigenous extreme halophiles thus far isolated from WIPP halite may survive at high salt concentrations, none can grow anaerobically. As a result, their ability to transform waste may be limited to early oxic periods of the repository. Organisms isolated from WIPP waste cannot grow at high salt concentrations and, therefore, are also unlikely to play a role in waste transformation after drum breach and brine inundation. Nevertheless, both halophilic and non-halophilic organisms may be influential via processes that do not require cell activity.

In contrast to salt-based repository settings, other test DGR sites have measured significant microbial gas generation (CH_4, CO_2) from cellulose degradation (Olkiluoto granite, Finland; Small et al., 2017) and microbially-enhanced actinide reduction and dissolution (Mont Terri clay, Switzerland, Moll et al., 2017; Äspö Hard Rock Laboratory, Sweden, Moll et al., 2006) all under anaerobic conditions. Similarly significant activity has yet to be shown in the extreme halophiles indigenous to the WIPP near-field, suggesting that the negative results obtained by the majority of investigators should be considered meaningful. Nevertheless, efforts are still ongoing to

characterize as many WIPP-relevant samples as possible; to determine the metabolic capabilities of the detected organisms; and to examine their potential for waste transformation, gas generation, and interaction with actinides, in order to support the current PA implementation.

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APPENDIX 1. Summary of past gas generation studies

Reference: Institution	Conditions	Inoculum	Outcome: Observations and Questions
▪ 1978-1980: Barnhart et al. LASL	▪ Simulated organic waste (CPR), sawdust, asphalt ▪ Aerobic vs anaerobic ▪ Humid vs inundated in brine, nutrients ▪ 25 and 70C	▪ Bacilli isolated from simulated waste ▪ Soil (source unknown, possible waste burial site)	▪ CO_2 generated, no methane ▪ Positive correlation with temperature for all anaerobic incubations and most aerobic; is this a result of decreased CO_2 solubility at increased temp? Composting is usually between 55-60C ▪ Rates in sterile controls often > rates in test samples ▪ Greatest rate of gas gen is in 70°C anaerobic, abiotic control ▪ No effect of brine ▪ No check on presence/absence or viability of microbes
▪ 1979: Molecke et al. LASL, UNM, SNL	▪ Simulated organic waste (CPR), sawdust, asphalt ▪ Aerobic, anaerobic ▪ Humid, inundated, Brine B (ERDA-like), nutrients ▪ 25, 40, 50, 60, 70C	▪ See above	▪ CO_2 generated, no methane ▪ Temperature effect, function of microbes or CO_2 solubility? Temperatures of 40, 50, and 60 could suggest composting? ▪ Anaerobic rates > aerobic ▪ Cellulose accounts for all gas generated, not PR ▪ No effect of brine ▪ No check on presence/absence or viability of microbes
▪ 1988: Caldwell et al. UNM, LANL, SNL	▪ See above ▪ Pu added	▪ Soil from TA-54 burial site?	▪ CO_2 only gas detected ▪ Significant inhibitory effect of Pu on CO_2 generation (rate decrease of 70%), presumably decrease in microbial respiration ▪ Both aerobic and anaerobic significant (per authors)
▪ 1990: Brush et al. SNL, Stanford, ANL ▪ 1990: Brush, Grbic-Galic et al. SNL, Stanford	▪ Aerobic, anaerobic (NR, SR, methanogen, fermenter enrichments) ▪ Brine A (high Mg, GWB-like brine)	▪ Sewage sludge, laboratory dust ▪ Halophile co-culture from "WIPP site and vicinity"; uncharacterized mix of organisms from surficial and subterranean environments	▪ First mention of water budget and water activity, micro-niches of microbial activity ▪ No growth of non-halophiles in brine (mentions possible survival, but no evidence is provided; spore recovery?) ▪ Halophile growth in complex medium + glucose with nitrate, sulfate or carbon dioxide as electron acceptors; no data provided on TEA concentrations during course of incubations ▪ CO_2 or (CO_2 + N_2) when glucose is substrate; no gas when cellulose is substrate (G-seep inoculum? Per Brush 1991) ▪ When cellulose was substrate, no gas was produced or the amount produced was indistinguishable from negative control
▪ 1993: Francis and Gillow BNL, SNL ▪ 1997: Francis and Gillow BNL, SNL	▪ Laboratory cellulosics (kimwipes, filter paper, paper towels) ▪ Aerobic, anaerobic	▪ Sediment and brine from local lake, muck pile salt from underground (short-term)	▪ First mention of aerobic, nitrate-reducing and anaerobic bacteria from WIPP underground and surficial environment: "R Vreeland…to be published"; actual publication does not mention anaerobes. This

	▪ Humid, inundated ▪ +/- bentonite ▪ +/- nitrate	▪ Sediment and brine, G-seep brine (long-term) ▪ "uninoculated" controls still contain organisms present in G-seep***	assumption is carried through all subsequent F&G reports and other following papers (Strietelmeier, Brush) ▪ States cellulose degraders isolated by Vreeland; this is equivocal, see below ▪ measured denitrifying capability of lake sediment organisms and brine seep organisms*** ▪ Glucose metabolized aerobically, not anaerobically, in screening tests ▪ Significant CO_2 after 83 days in transitional and anaerobic; in 147 days in anaerobic/excess nitrate ▪ Sulfide production observed after 147 days in two incubations (one transitional, one anaerobic) ▪ First mention of haloarchaeal denitrifier isolate from sediment slurry, but organism not identified or used in future experiments?
▪ 1997: Francis and Gillow BNL, SNL	▪ See above	▪ See above	▪ Gas production rates biphasic: initial rapid (to 600 days) followed by slow ▪ Quantity of gas produced dependent upon presence of nutrients and appropriate inoculum: nutrients/nitrate + mixed inoculum → more gas produced ▪ Evidence for cellulose degradation in metabolites produced; metabolites could support SRB at high salt (lactate, propionate) ▪ Evidence for fermentation (hydrogen production) ▪ "Gas production in anaerobic, uninoculated, unamended samples was indistinguishable from background"; realistic scenario*** ▪ Negligible gas production in humid, anaerobic, uninoculated, amended samples; realistic scenario***
▪ 1998: Vreeland et al. West Chester University	▪ Solka-floc, filter paper ▪ aerobic	▪ Culture enriched from G-seep brine on CG medium (contains solka-floc, citrate, and glucose as possible C-sources	▪ Results of 2-hour cellulose fiber attachment study are equivocal (true attachment versus impingement); 2-year results are qualitative; no organisms shown in images ▪ Cannot verify use of solka-floc as sole carbon source and cannot attribute organic acid production to solka-floc degradation, when both glucose and citrate were also present
▪ 1999: Leonard et al. LANL ▪ 2001: Villarreal et al. LANL	▪ Heterogeneous and homogeneous wastes in containers with brine	▪ Halophile co-culture, designated BAB: muck pile salt solution (30%), hypersaline lake brine and sediment slurry	▪ Tracked cells with different staining techniques and microscopy. Initially tracked subcultures, then examined 66 raw samples: small numbers of viable cells in 3 organic waste containers (out of 54 total containers or 33 sludge containers?)15 x 55-gal drums + 39 x 1L containers

	▪ Subcultures into NR and fermenter enriching media ▪ Variables included Pu (and U, Np, Am, Th?), Brine A versus Castile, Envirostone, melamine, nitrate, cement	(20%), G-seep brine (50%)	▪ Microbial growth tracking stopped after 2 years of no growth ▪ Microbial gas (N_2O) generation was not measured on actual test containers but in separate incubations; experimental design is unclear ▪ Reduction of nitrate noted; reason unknown (microbial, radiolytic, or chemical) but mostly attributed to radiolysis by authors ▪ Gas generated attributed to radiolysis (H_2, CO_2, N_2O)
▪ 2006: Francis and Gillow, BNL, SNL	▪ See above	▪ See above	▪ >10.8 years of incubation ▪ Similar results as 1997: positive correlation between gas gen and presence of nutrients, nitrate ▪ Anaerobic > aerobic ▪ Fermentation by-products detected*** ▪ Methane detected at 7 years ▪ Cell counts at one time-point, t = 6 years: highest cell numbers ~10^8/ml (DAPI stains live and dead), archaea and bacteria; difficult to extrapolate meaningful information based on one time point. Number is greater than snapshot counts of various briny matrices, but sediment counts not performed ▪ DNA analysis at one time point, t = 9.4 years; difficult to extrapolate meaning from results, possible denitrifying archaea present

APPENDIX 2. Growth media

Generic halophile broth (see Table 2 and for toxicity tests)

Component	g/L
NaCl	200 (varies with organism tested)
Yeast extract	2.5
Hy-Case (casamino acids)	2.5
Soluble starch	0.2
$MgCl_2 \cdot 6H_2O$	20
KCl	2.0
$CaCl_2 \cdot 2H_2O$	0.2
Sodium pyruvate	0.11
Trizma base	0.24
ATCC Trace minerals*	1 ml

*American Type Culture Collection

Modified R2B (for toxicity testing of *Arthrobacter* sp. and other growth studies, Table 1)

Component	g/L
NaCl	8.5 (or variable)
Yeast extract	0.5
Hy-Case (casamino acids)	0.5
Proteose Peptone #3	0.5
Dextrose	0.5
Soluble starch	0.5
Sodium pyruvate	0.3
K_2HPO_4	0.3
$MgSO_4.7H_2O$	0.05