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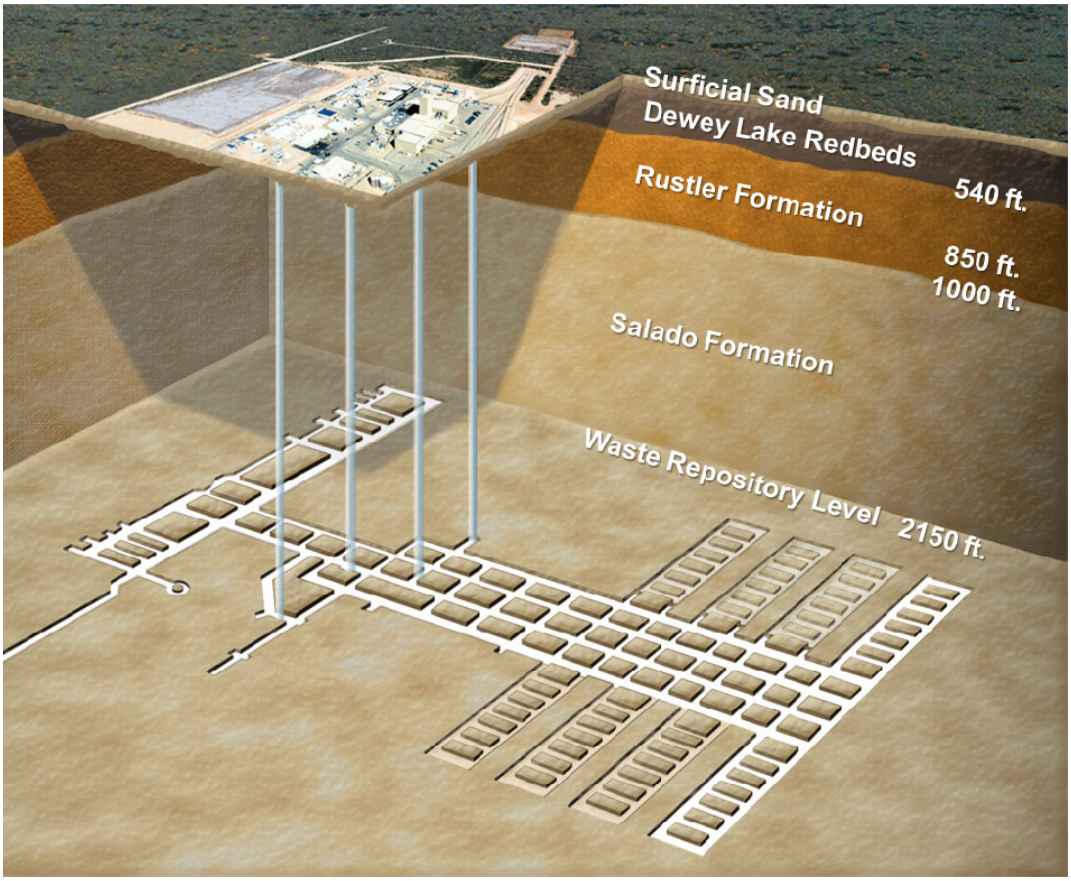

WIPP Facility and Stratigraphic Sequence

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Microbial Characterization of Halite and Groundwater Samples from the WIPP

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EXECUTIVE SUMMARY

This report summarizes the progress made in characterizing the microbial community structures within the WIPP repository, and in surrounding groundwaters. Through cultivation and DNA-based identification, the potential activity of these organisms is being inferred, thus leading to a better understanding of their impact on WIPP performance.

WIPP halite constitutes the near-field microbial environment. Microbial activity in this setting is predicted by WIPP performance assessment (PA) to proceed from aerobic respiration, through nitrate reduction, to focus on sulfate reduction. The role of methanogenesis in the WIPP remains unclear, due to both energetic constraints imposed by a high-salt environment and substrate selectivity, so this role is no longer considered in PA.

Members of the three biological domains — *Bacteria*, *Archaea*, and *Eukarya* (in this case, *Fungi*) — have been found associated with WIPP halite. Thus far, their activity has been limited to aerobic respiration.

Archaea identified in WIPP halite thus far fall exclusively within the family *Halobacteriaceae*. These include *Halobacterium noricense*, a *Halorubrum*-like species, and a *Halopenitus*-like species. Bacterial signatures associated with WIPP halite include members of the phylum *Proteobacteria — Halomonas*, *Pelomonas*, *Limnobacter*, and *Chromohalobacter* — but only the latter has been isolated. Also detected and cultivated were *Salinicoccus* and *Nesterenkonia* spp. Three fungal species were isolated from halite (*Cladosporium*, *Phoma*, and *Engyodontium*). Although these were most likely introduced into the WIPP as contaminants from above-ground, their survival and potential role in the WIPP (e.g., cellulose degradation) is under investigation.

WIPP groundwaters comprise the far-field microbial environment. *Bacteria* cultivated and identified from the overlying Culebra and nearby borehole groundwater are capable of aerobic respiration, denitrification, fermentation, metal reduction, and sulfate reduction, and are distributed across many different phyla. Their structural and metabolic diversity is dependent upon the ionic strength of the sampled groundwater, with a decrease in both at higher strength. Two of the *Bacteria* found in groundwater were also found in WIPP halite (*Chromohalobacter* sp. and *Virgibacillus* sp.). *Archaea* identified in groundwater include *Halococcus saccharolyticus*, *Haloferax* sp., and *Natrinema* sp.

The differences in the microbial communities detected thus far in halite and groundwater suggest that there will be significant differences in the associated metabolic potential of the near- and far-field environments. Whereas the near-field is dominated by *Archaea* with more limited metabolic capabilities, the far-field is dominated by *Bacteria* with extremely broad capabilities.

The authors wish to thank Russ Patterson and Dan Ferguson (DOE-CBFO) for their support, and Washington TRU Solutions for access to sampling. All research was performed by the Los Alamos National Laboratory Actinide Chemistry & Repository Science team at the Carlsbad Environmental and Monitoring Research Center, managed by New Mexico State University (NMSU) and located in Carlsbad, NM. Funding for this research is through the Waste Isolation Pilot Plant project, managed by the Department of Energy—Carlsbad Field Office.

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ACRONYMS AND ABBREVIATIONS

ACRONYM	ABBREVIATION
16S rRNA	16S subunit of ribosomal RNA
Aer	aerobic
ARDRA	amplified ribosomal DNA restriction analysis
ASTP	Actinide Source Term Program
BLAST	Basic Local Alignment Search Tool
CFB	*Cytophaga-Flavobacteria-Bacteroidetes*
CFU	colony-forming unit
CLR	clear halite
CLY	clayey/argillaceous halite
CLR-100	clear halite incubation under low-salt conditions
CLR-175	clear halite incubation under high-salt conditions
CLY-100	clayey halite incubation under low-salt conditions
CLY-175	clayey halite incubation under high-salt conditions
CMC	carboxymethylcellulose
CRA	Compliance Recertification Act
CPR	cellulose, plastic, rubber
Dir	direct (i.e., DNA extracted directly from sample without prior cultivation)
DGGE	denaturing gradient gel electrophoresis
DOE-CBFO	Department of Energy — Carlsbad Field Office
EDTA	ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
ERDA-6	Energy Research and Development Administration Well 6
GC	gas chromatography
GWB	Generic Weep Brine
HPW	high purity water
IC	ion chromatography
ICP-MS	inductively-coupled plasma-mass spectrometry
IR	iron-reducing (incubation to enrich iron-reducing organisms)
Meth	methanogenic (incubation to enrich methanogenic organisms)
MOPS	3-(N-morpholino)propanesulfonic acid (buffer)
NaCl	sodium chloride
NR	nitrate-reducing (incubation to enrich nitrate-reducing organisms)
OTU	operational taxonomic unit
PA	performance assessment
PCR	polymerase chain reaction
RT	room temperature
SEM-EDS	scanning electron microscope-energy dispersive x-ray spectroscopy
SNL	Sandia National Laboratories
SR	sulfate-reducing (incubation to enrich sulfate-reducing organisms)
SRB	sulfate-reducing Bacteria
TEA	terminal electron acceptor
TEM	transmission electron microscope
TOC	total organic carbon
T or Tr	transitional
TRU	transuranic
WIPP	Waste Isolation Pilot Plant
XANES	X-ray absorption near-edge structure

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1.0 INTRODUCTION

1.1 The Waste Isolation Pilot Plant

The Waste Isolation Pilot Plant (WIPP) is located in the northern portion of the Delaware Basin, in southeastern New Mexico. It was certified by the Environmental Protection Agency (EPA) in 1998 as a terminal repository for defense-related transuranic (TRU) nuclear waste, and is currently operated by the Department of Energy's Carlsbad Field Office (DOE/CBFO). According to federal regulations (CRA, 2009a and b), the WIPP must be recertified by the EPA every five years.

This report provides the status of ongoing developmental research in the Los Alamos National Laboratory — Carlsbad Operations' Actinide Chemistry and Repository Science Program to describe the microbial ecology in the WIPP repository and surrounding groundwaters. The goal of this work is to obtain a broader surveillance of the microbial communities within the WIPP environment under different growth conditions in order to better predict their metabolic capability. Predicting this capability will 1) narrow and further define the scope of possible microbial interactions with waste components; and 2) help support the conservatism in the current assumptions about microbial effects in WIPP performance assessment.

In this report, we provide a summary of past work and specifically address the characterization of organisms detected in WIPP halite, in groundwater from the overlying Culebra aquifer, and in borehole groundwater/brine seep proximal to a salt mine tailings pile. The first part of this report will address halite, and the second will focus on groundwater. Methods that are used in characterizing both sample types will be presented in detail in the first part of this report and will be referred to, with any modifications, in the second part.

2.0 BACKGROUND

2.1 The WIPP Conceptual Model for Microbiology

The WIPP mandate is to limit the release of radionuclides from the repository during its regulatory 10,000-year lifetime. Microorganisms can affect repository performance by affecting actinide oxidation state and solubility (McCabe, 1990; Pedersen, 2005; Wang and Francis, 2005). There are many processes through which this can happen, including: 1) the oxidation of waste organics, resulting in CO_2 production and leading to actinide-carbonate complexation; 2) the production of CO_2 or low-molecular weight organic acids, leading to a reduction in pH; 3) the creation of a reduced environment by oxygen consumption; 4) direct actinide reduction; 5) indirect actinide reduction (e.g., via microbially-generated Fe(II) or sulfide, or fermentation); 6) degradation of organic ligands; 7) generation of organic ligands; and 8) surface or internal uptake of actinides. In the WIPP, abiotic processes will generally be more significant (i.e., gas production and the creation of a reduced environment by canister corrosion), but the WIPP conceptual model for microbiology considers production of CO_2 and biosorption to be significant microbial contributions.

The WIPP conceptual model for microbiology is necessarily conservative in its assumptions because of the associated uncertainty. Thus, probability vectors are assigned as follows: significant microbial activity will occur in which all cellulosics, plastics, and rubber (CPR) will be degraded (P = 0.25), and significant microbial activity will occur in which only cellulosics will be degraded (P = 0.75; CRA, 2009a). This microbial activity is assumed to completely oxidize the organics to carbon dioxide, leading to the potential for enhanced actinide solubility, either as a carbonate complex or because of reduced pH. Magnesium oxide is added to the WIPP to sequester the excess CO2 and help buffer the pH to ~9.

The model further assumes that the gas produced will come from denitrification (4%) and sulfate reduction (96%). Methanogenesis is not considered to contribute to biodegradation and gas production.

Biocolloid formation from the sorption of actinides onto microbial surfaces is predicted to be significant. Finally, microbial activity is expected to contribute somewhat to the maintenance of reduced conditions in the WIPP.

2.2 Past WIPP Microbial Characterization Studies

A significant amount of research was performed during the past three decades as part of the WIPP Actinide Source Term Program (ASTP), managed by Sandia National Laboratories (SNL), to support the certification of the WIPP. The primary goals of this early research were to1) predict gas generation from the degradation of organic waste components; 2) identify radiation-resistant organisms in the waste; 3) identify organisms in the environment surrounding the waste; 4) examine the toxicity of actinides to these microorganisms; and 5) evaluate the potential for organisms to degrade waste organics.

Very early work on WIPP-related microbiology was conducted with microorganisms found at a TRU waste burial site in Los Alamos, NM. Although some experiments were carried out using brine, these experiments utilized soil microorganisms from this site as their inocula (Barnhart et al., 1978, 1979a, 1979b, 1979c). Attempts to cultivate organisms from WIPP halite were unsuccessful in those experiments. Later gas generation studies utilized organisms representative of a laboratory environment (e.g., lab dust) but also organisms cultivated from "WIPP environs." These environs included brine and sediments from area salt lakes and G-seep brine from the WIPP (Francis and Gillow, 1993; Leonard et al., 1999). One isolate was identified as belonging to the genus *Halomonas*, and was used later in a series of studies on uranium reduction under denitrifying conditions (Francis et al., 2000). Two other organisms were identified by DNA sequence analysis as a *Clostridium* species and *Halobacter* (now *Halorhabdus*) *utahensis*. The intent of these early studies was not to characterize microorganisms, and because a mixture of surface and subsurface organisms was used as an inoculum, it is not possible to determine the actual source of the organisms that were identified in those incubations.

Only one study was conducted that directly addressed microbial characterization of the WIPP (Vreeland et al., 1998). This study found that microbial distribution within WIPP halite is heterogeneous and sparse, with numbers ranging from zero to ~10^4 colony-forming units (CFU) per gram. This finding is in agreement with those from halite samples from other salt mines worldwide, where up to a kilogram of salt might be required before growth of organisms could be observed (Norton et al., 1993; Radax et al., 2001). Brine seeps tend to have more organisms and, in the WIPP, were found to contain between 10^2 and 10^4 CFU/ml (Vreeland et al., 1998; Francis and Gillow, 1993).

Over two hundred isolates (not necessarily different species) were cultivated from WIPP halite, seeps, and surrounding brine lakes (Vreeland et al., 1998). However, the characterization of these isolates was limited to phenotypic similarity with other known strains of haloarchaea, and DNA sequence-based identification was not performed. Two WIPP isolates have been characterized and published in the literature—a novel genus, *Halosimplex carlsbadense*, and a *Virgibacillus* species derived from a fluid inclusion (Vreeland et al., 2000, 2002).

2.3 Metabolic Diversity of Halophilic Microorganisms

For the purposes of this report, a halophilic microorganism will be defined as one that requires salt (NaCl) for growth and can grow in up to saturated sodium chloride. In contrast, a halotolerant microorganism is one that does not require salt for growth but can survive (and even thrive) in concentrations as high as 2.5 M or greater. Halophiles can be found in the three domains of life—*Bacteria*, *Archaea*, and *Eukarya*. However, this does not imply that the metabolic potential of these organisms is very diverse. Capability is severely limited by the energetic cost of maintaining osmotic balance with the external environment. Halophilic organisms do this by one of two strategies: 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 ("salting out"). The former strategy is limited to the haloarchaea and a few anaerobic bacteria, and is less expensive energetically, while the latter strategy is found in most

halotolerant and halophilic bacteria and all eukaryotes and is energetically costly, especially if these solutes must be synthesized *de novo*.

Because of this cost, metabolic processes are limited to the following at salt concentrations greater than 2.5 M NaCl, which is the cut-off for extremely halophilic microorganisms: oxygenic and anoxygenic photosynthesis; aerobic respiration; denitrification; fermentation; manganese, arsenite, and selenate reduction; dissimilatory sulfate reduction with incomplete organic oxidation; methanogenesis from methylated amines; acetogenesis; and chemolithotrophic oxidation of sulfur compounds (Oren, 1999, 2011). All of these processes are either energetically favorable or are performed by organisms that maintain osmotic balance by the less costly "salting in" strategy.

Apart from these thermodynamic constraints, the repertoire of potential microbial metabolic pathways within the WIPP is limited even further by 1) the physical confinement of the repository without input of exogenous electron acceptors (especially oxygen), moisture (i.e., brine), organisms from external sources, or light; 2) high ionic strength; 3) high pH; and 4) nonideal substrates. These factors may restrict or effectively eliminate many capabilities. As examples, photosynthesis is obviously not an option in the subsurface setting, aerobic respiration will not occur throughout repository history, and methanogenesis from $H_2 + CO_2$ or acetate is thermodynamically infeasible at [NaCl] greater than 120 g/L.

2.4 Predicted Community Composition

From a biological standpoint, the assumption is made that the predominant microbial communities will vary in both time and space.

Aerobic respiration will be predominant immediately after repository closure and will remain so until oxygen levels decrease from the corrosion of iron canisters and--less importantly--due to microbial activity. Once oxygen has been depleted, nitrate, organic acids, and sulfate will be present as potential electron acceptors. Organic complexing agents—acetate, oxalate, citrate, and ethylenediaminetetraacetate (EDTA)—will be the predominant low-molecular-weight carbon substrates, but cellulosics will contribute significantly to the carbon inventory. The availability of these substrates will depend upon their dissolved concentrations in brine, which are expected to be below the inventory-predicted levels (PAIR, 2012; Swanson et al., 2013a, 2013b). The expected pH will range between 8.7-9.0, and [Mg] and [Na] can be between 0.02-0.95 M and 2.9-4.3 M, respectively.

Many haloarchaea are capable of denitrification and fermentation, suggesting that these activities may proceed. Sulfate reducers (*Bacteria*) have so far not been found in subsurface halite, although it is probable that they were never sought. Gillow and Francis (2006) noted a sulfide precipitate in their long-term incubations, which they attributed to the presence of sulfate-reducing bacteria (SRB). These incubations were also inoculated with brine lake sediment, which is the most likely source of these organisms. SRBs have been found in other hypersaline environments (i.e., brine lakes, solar salterns; Porter et al., 2007; Sørenson et al., 2009). Their presence in brine seeps is unknown, but even so, they may be introduced with brine inundation from the Castile formation underlying the WIPP horizon. Sulfate is present in Castile brine and is also formed from the dissolution of anhydrite present in the halite interbeds.

The variation of microbial communities in space concerns the near-field versus far-field and reflects the variation in ionic strength in these spaces. Based on the relative concentrations of Na found in these fields, extreme halophiles (i.e., *Archaea*, some bacteria) will dominate within the repository (near-field). The far-field will be dominated by moderately halophilic and halotolerant *Bacteria*. This distinction is important in that waste transformation will probably occur more readily in the presence of *Bacteria* than *Archaea*. Metabolic activities that may occur in the far-field include aerobic respiration, fermentation, denitrification, metal reduction, sulfate reduction, reduction of other oxyanions, chemolithotrophy (oxidation of ammonium, sulfide), methanogenesis, and methanotrophy.

3.0 PART 1–WIPP HALITE

3.1 Overview

To date, two types of halite ("clear" and "argillaceous" or "clayey") have been used as inocula in aerobic enrichment media, and clear halite has been inoculated into anaerobic enrichment media. The results presented in this report are for aerobic cultures only. From these cultures, DNA has been extracted and sequenced, resulting in the identification of archaeal, bacterial, and fungal species. DNA has also been extracted and processed directly from raw halite without enrichment. Microbial isolates have been obtained and identified. Differences are seen between the two halites that have been attributed to the presence of clay, and other differences arise from the variation in salt concentration in the media.

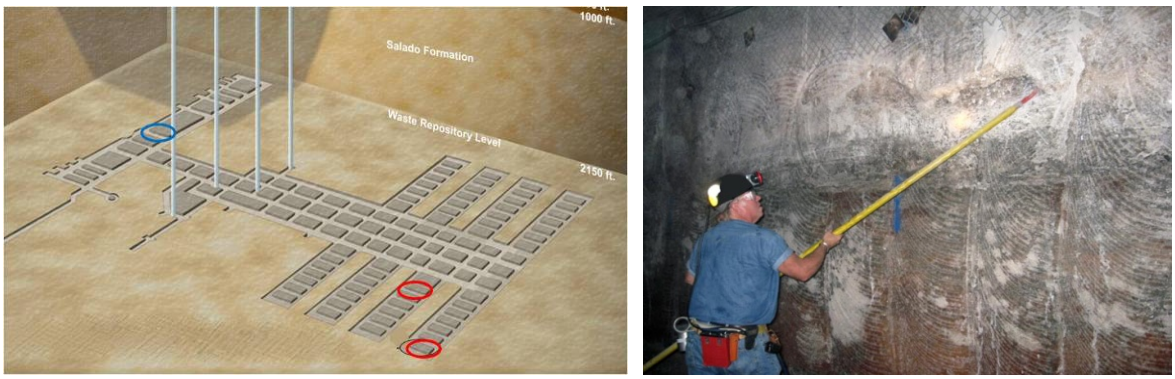
3.2 Objective

Halite samples for earlier studies were obtained from the oldest part of the mine, which has now been sealed (Vreeland et al., 1998; N. Rempe, personal communication; see Figure 1a). Until now, no further characterization has been performed on other parts of the mine. In addition, halophile research has increased dramatically over the past two decades, and new genera and species have been identified. Finally, performance assessments of deep geologic repositories tend to focus on bacteria at low ionic strengths when predicting metabolic capability. Further research and data are needed on halophilic organisms and their more limited lifestyle at high ionic strength.

3.3 Materials and Methods

3.3.1 Halite Sample Collection and Handling

Halite samples were retrieved from three sites along the north wall of Panel 5, Room 7 (see Figures 1a and 1b). A crowbar was used to remove the top layer of halite, which was discarded, and then to pry a layer from underneath. Samples were handled with sterile gloves, placed into sterile bags, and sealed in a dark plastic container to prevent exposure to light. The containers were stored at room temperature (RT; 23 ± 3°C). Halite samples with different physical appearances (e.g. "clear" versus "green/gray") were segregated.



A B

Figure 1: A) Sampling sites for current work are circled in red. The approximate sampling site from the Vreeland study is circled in blue. B) Outer layer of halite being removed to expose underlayer for sampling.

3.3.2 Chemistry

Halite was dissolved (1:10 w/v) in high-purity distilled and deionized water (HPW) and filtered through Millipore MicroCon Y filters (100 kD cut-off). The filtrate was diluted in 2% nitric acid and run on an Agilent 7500ce inductively-coupled plasma-mass spectrometer (ICP-MS) to analyze for metals/cations. Anions were determined by ion chromatography (IC) of the filtrate after dilution in HPW. Analyses were performed on a Dionex DX-500 IC, using a hydroxide gradient and conductivity detection. Total organic

carbon (TOC) analysis was performed on a TekMar Teledyne Carbon Analyzer using 20 ml dissolved samples that had been passed through 0.22 micron nylon membrane filters.

3.3.3 Solid Phase Characterization

Scanning electron microscopy-energy dispersive x-ray spectra (SEM-EDS) of the clay were generated by Peter Cooke of the Core University Research Resources Laboratory at New Mexico State University.

3.3.4 Direct Microscopic Counts

Direct cell counts were made after passing a dissolved halite sample through a black polycarbonate 0.22 µm membrane filter that had been stained using the BacLight Live/Dead viability kit (Invitrogen/Life Technologies, Rockville, MD; Leuko et al., 2004). Microscopic examination of the filter was performed on a Zeiss Axioskop 40 fluorescence microscope fitted with appropriate filters for the stains used.

3.3.5 Cultivation

Because this study was designed to examine what might survive in the repository, regardless of its origins, samples were not sterilized. Approximately 50 gram samples of halite were dissolved in 500 ml of a generic medium for the cultivation of halophilic microorganisms using two different concentrations of NaCl. Medium components were as follows in g/L: NaCl, 100 and 175; yeast extract, 0.5; Hy-Case casamino acids, 0.5; $MgCl_2 \cdot 6H_2O$, 20; KCl, 2; $CaCl_2 \cdot 2H_2O$, 0.2; sodium pyruvate, 0.015; Tris-HCl, pH 8.5, 1.1 ml of 1 M stock; American Type Culture Collection trace elements, 1 ml. The flasks were incubated at RT, in the dark, while stirring. Aliquots were periodically subcultured onto solid media of the same composition as the broth, and onto agar plates with slightly different formulations, in order to enrich for different haloarchaea and halophilic bacteria. Isolated colonies were replated for purity onto the same type of agar that promoted their original growth.

3.3.6 DNA Extraction from Raw Halite

12 and 50-gram samples of halite were dissolved in sterile 15% NaCl and filtered through a 0.22 micron nylon membrane. The filters were then cut with sterile scissors and transferred into bead tubes from MoBio's UltraClean Water DNA Purification Kit (MoBio, Inc.; Carlsbad, CA). Two-gram samples were directly placed into bead tubes from MoBio's UltraClean Soil DNA Purification Kit. Extractions were carried out according to the manufacturer's directions.

3.3.7 DNA Extraction from Broth and Isolate Cultures

One-milliliter aliquots from the broth cultures were centrifuged to concentrate cells, and all but 0.1 ml of the supernatant was decanted. The remaining concentrated suspension was inoculated into bead tubes from MoBio's UltraClean Soil DNA Purification Kit. This same kit was used for extracting DNA from growth of isolates on agar plates by inoculating the bead tube with a loopful of pure culture. Extractions were carried out according to the manufacturer's directions.

3.3.8 Polymerase Chain Reaction (PCR)

A portion of the 16S rRNA-encoding genes, for *Bacteria* and *Archaea*, and the 18S genes, for *Eukarya*, were amplified from the genomic DNA using the primer sets listed (below) in Table 1. A nested reaction was required to obtain a product from some samples. The reaction mix contained 1X AmpliTaq Buffer II (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 250 µM each deoxynucleoside triphosphate, 500 nM each primer, and 2.5 U AmpliTaq *Taq* polymerase (Applied Biosystems; Foster City, CA). Annealing temperatures varied with primer sets (55°C for bacterial; 56°C for archaeal and eukaryotic; 48°C for nested archaeal).

Table 1:
PCR Primers Used for this Research

Primer	Sequence (5'-3')	Group/Target site*	Reference
Bac27f	AGA GTT TGA TCC TGG CTC AG	Bacteria (27-46)	Lane, 1991
Univ1492r	GGT TAC CTT GTT ACG ACTT	Universal (1510-1492)	Lane, 1991
Bac63f	CAG GCC TAA CAC ATG CAA GTC	Bacteria (63-83)	Marchesi et al., 1998
Univ518r	ATT ACC GCG GCT GCT GG	Universal (517-533)	Neefs et al., 1990
Bac907r	CCG TCA ATT CMT TTR AGT TT	Bacteria (926-907)	Lane et al., 1991
Arch21f	TTC CGG TTG ATC CTG CCG GA	Archaea (21-40)	DeLong, 1992
Univ1392r	ACG GGC GGT GTG TRC	Universal (1406-1392)	Lane, 1991
Arch344f	ACG GGG CGC AGC AGG CGC GA	Archaea (344-363)	Raskin et al., 1994
Arch806r	GGA CTA CVS GGG TAT CTA AT	Archaea (825-806)	Takai and Horikoshi, 2000
Euk1Af	CTG GTT GAT CCT GCC AG	Eukarya (4-20)	Sogin & Gunderson, 1987
Euk516r	ACC AGA CTT GCC CTC C	Eukarya (563-548)	Amann et al., 1990

*Bacterial and archaeal target sites refer to *E. coli* numbering; eukaryotic sites use *S. cerevisiae* numbering.

3.3.9 Denaturing Gradient Gel Electrophoresis (DGGE)

When amplifying DNA for DGGE, a GC-rich clamp was attached to the end of the forward primer to prevent complete denaturation of the product (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGGG-3'; Muyzer et al., 1993). Bacterial and archaeal PCR amplicons were resolved on a 6% acrylamide gel with a urea-formamide gradient. The gradient varied depending upon the sample but most often was set at 30-60% or 30-70%. Gels were run at 60°C, 60V for ~16 hours, and then stained for 30 minutes with the nucleic acid stain SYBR Green I.

3.3.10 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Amplicons from archaeal isolates were screened by digestion with *Mbo*I and *Hinf*I (Martinez-Murcia et al., 1995). Each 50 µl reaction contained 5 U of enzyme in the appropriate buffer (New England BioLabs; Ipswich, MA) and was incubated overnight at 37°C. Reactions were stopped by incubating the digest at 96°C and were then resolved on a 3% agarose gel. Isolates were grouped according to pattern, and the purified PCR product of at least one isolate within each group was shipped for sequencing.

3.3.11 Cloning Reaction and Cell Transformation

PCR amplicons were cleaned with Qiagen's PCR Purification Kit (Qiagen; Valencia, CA). Products from the PCR reactions of each raw halite sample were pooled by halite type. Amplicons were ligated into plasmid vector pCR®4-TOPO and transformed into *E. coli* DH5α™-T1^R chemically competent cells using Invitrogen's TOPO TA Cloning® Kit for Sequencing (Invitrogen/Life Technologies; Rockville, MD). Clones were shipped to SeqWright, Inc. (Houston, TX) for sequencing.

3.3.12 DNA Sequence Analysis

All sequences were reviewed and cleaned individually in Sequencher 4.1 (Gene Codes Corporation; Ann Arbor, MI). Clone sequences were screened for vector contamination with VecBase (www.ncbi.nlm.nih.gov) and for chimera formation using the Bellerophon interface in greengenes (greengenes.lbl.gov; Huber et al., 2004). Groupings were made of cloned sequences with 97% similarity. Where appropriate, consensus sequences from the groupings were used in downstream analyses in order to simplify alignment and tree construction. Multiple sequence alignments of consensus sequences, unique singleton clone sequences and isolate sequences were performed with Clustal X, v. 2.0.12

(Higgins and Sharp, 1988; Larkin et al., 2007). A tree was generated using the neighbor-joining algorithm with 1000 bootstrap support. The Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Information database was used to putatively identify organisms based on sequence similarity (Altschul et al., 1997; ncbi.nlm.nih.gov).

3.4 Results

3.4.1 Chemistry of Halite

Figure 2 (below) shows an example of the two types of halite that were investigated: "clear" versus "green/gray."

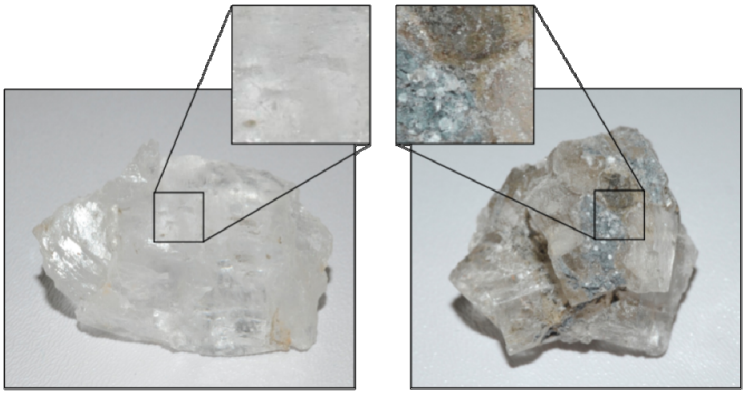


Figure 2: Samples of "clear" and "green/gray" halite.

Results for the significant components of both these sample types are provided in the table below.

Table 2:
Elemental composition of "clear" and "green/gray" halite samples retrieved from WIPP and used for microbial cultivation experiments.

Analyte Measured	Clear Halite (µg/g halite)	Green/gray Halite (µg/g halite)
Cl^-	$5.81 \pm 0.12 \times 10^5$	$4.48 \pm 0.06 \times 10^5$
Br^-	72.4 ± 47	$5.01 \pm 0.33 \times 10^2$
SO_4^{2-}	$4.06 \pm 2.80 \times 10^2$	$4.75 \pm 0.22 \times 10^3$
B (as B_4O_7)	not detected[a]	$5.75 \pm 2.18 \times 10^2$
Na^+	$1.38 \pm 0.01 \times 10^5$	$1.11 \pm 0.08 \times 10^5$
Mg^{2+}	28 ± 35	$8.62 \pm 1.94 \times 10^3$
K^+	$2.02 \pm 0.80 \times 10^2$	$5.34 \pm 1.21 \times 10^3$
TOC (mg/L; at limit of detection)	0.122	---
pH[b]	7.26 ± 0.02	7.17

[a] Due to insufficient sample size, levels of analyte below the detection limit for the given instrumentation, or analyte actually not present in sample.

[b] Operationally defined as the pH measured after 24 hours "dissolution" of a 2:1 (w/v) suspension of crushed halite in deionized water.

A further look at the dark substance contained in the "green/gray" halite by SEM-EDS is shown in Figure 3. This spectrum shows the presence of Si and Al in addition to the other ions detected by ICP-

MS and is consistent with the presence of aluminosilicate clays. This halite is referred to hereafter as "clayey" halite, or CLY.

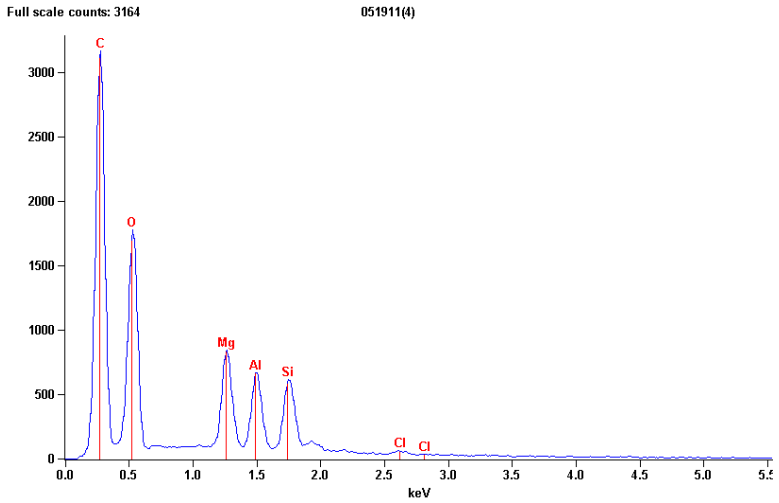


Figure 3: EDS spectrum of green/gray substance in halite showing Al, Si, and O, consistent with the presence of aluminosilicates.

3.4.2 Microbial Characterization Results

3.4.2.1 Summary

A summary of the results for direct microscopy, the cultivation of organisms from halite, the extraction of DNA from halite, and the amplification of extracted DNA by PCR is provided in the table below.

Table 3:
Summary of Microscopy, Cultivation and Molecular Results from Halite Processing

Parameter	Clear (CLR)	Clayey (CLY)
Cells/150 g (by microscopy)	0	0
Growth in medium with 100 g NaCl (+ 50 g halite as inoculum)	+	+
Growth in medium with 175 g NaCl (+ 50 g halite as inoculum)	+	−/+ (positive growth in 2nd attempt)
DNA amplified from raw halite (direct = DIR; 2-50 g clear, 50-150 g clayey)	+ bacterial (nested) –archaeal –eukaryotic	+ bacterial (nested) – archaeal –eukaryotic
DNA amplified from medium with 100 g NaCl	+ bacterial – archaeal –eukaryotic	+bacterial +archaeal ±eukaryotic*
DNA amplified from medium with 175 g NaCl	+ bacterial (nested) + archaeal –eukaryotic	– bacterial –archaeal +eukaryotic
Isolates—100 g NaCl	2 bacterial 0 archaeal 1 fungal	1 bacterial 1 archaeal 2 fungal
Isolates—175 g NaCl	0 bacterial 2 archaeal 0 fungal	0 bacterial 0 archaeal 1 fungal

An average of 8 weeks was required for growth in aerobic cultures, regardless of the concentration or type of salt used. In two cases (CLY-175 bacterial and archaeal), DNA was amplifiable, but the quality was either too poor for downstream analysis, or the amplicon sequences matched to haloarchaeal viruses.

In the clayey halite-high salt incubations, no growth was observed. A second incubation, in a medium containing five times the nutrient content of the original, yielded growth from the clayey halite.

The closest matches to the DNA sequences retrieved through cloning and from isolates are given in Table A-1, in the Appendix section of this report.

3.4.2.2 Archaeal Isolates

Archaea were isolated from both low- and high-salt media (CLR-175 and CLY-100). Since cultivated *Archaea* could not be grouped morphologically due to their identical appearance on plated media and their pleomorphism when viewed microscopically, isolates were screened by ARDRA (see Figure 4). All but one isolate yielded the same pattern, and matched most closely (99% similarity in DNA sequence) to *Halobacterium noricense*. One isolate from CLR-175 matched with low similarity (93%) to a *Halorubrum*-like species. There is currently no cultivated relative listed in the ribosomal RNA databases.

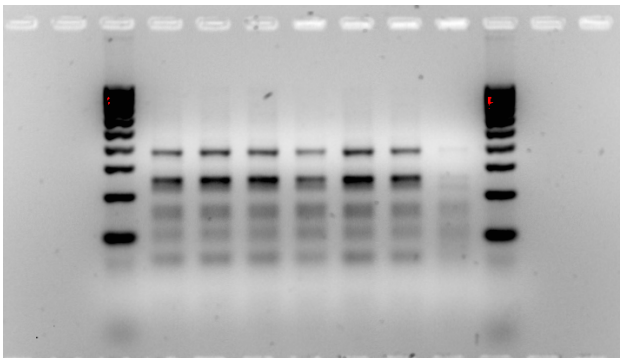


Figure 4: Example of a restriction digest analysis of amplified DNA from *Archaea* isolated from clear halite; similar patterns reflect similar DNA sequence. Reference "ladder" in end lanes denotes size of DNA fragments.

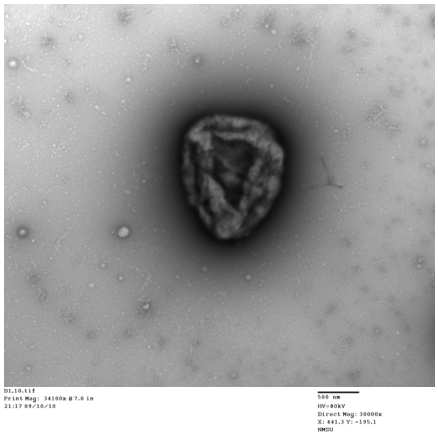


Figure 5: Unique *Halorubrum*-like archaeon isolated from clear WIPP halite.

3.4.2.3 Bacterial Isolates

Bacteria have thus far only been isolated from the incubations of halite at lower salt concentrations. One appeared in both clear and clayey halite incubations (*Nesterenkonia* sp.); one was unique to the clear halite (*Salinicoccus* sp.); and one was unique to the clayey halite (*Chromohalobacter* sp.).

3.4.2.4 Fungal Isolates

Filamentous organisms also appeared in the low-salt incubations and in the nutrient-rich (5X)-high-salt incubation. These were identified by DNA sequence analysis as members of the *Fungi*. One was common to both CLR-100 and CLY-100 (*Cladosporium* sp.); one other was found in CLY-100 (*Engyodontium* sp.); and one was isolated from the second attempt of clayey halite at high salt (CLY-175-2; *Phoma* sp.).

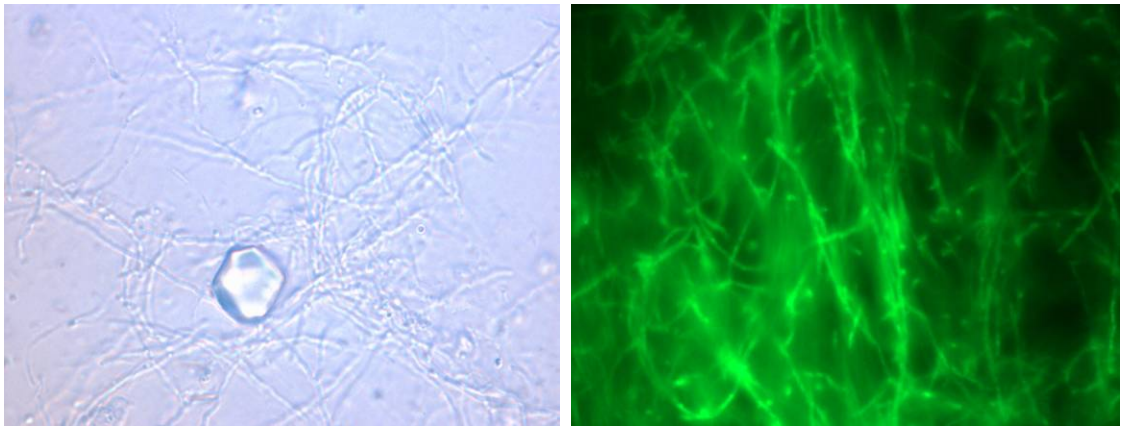


Figure 6: Filamentous organisms in both low- and high-salt incubations; bright field image (left), organisms stained with BacLight Live/Dead stain (right); green = alive.

3.4.2.5 Phylogenetic Distribution of Clones

Clone libraries were constructed from the amplified DNA extracted directly from raw halite (CLR-DIR and CLY-DIR), from both clear halite cultures (CLR-100 and CLR-175), and from CLY-100. Figures 7a and 7b show the makeup of each library constructed for *Bacteria* (a) and *Archaea* (b).

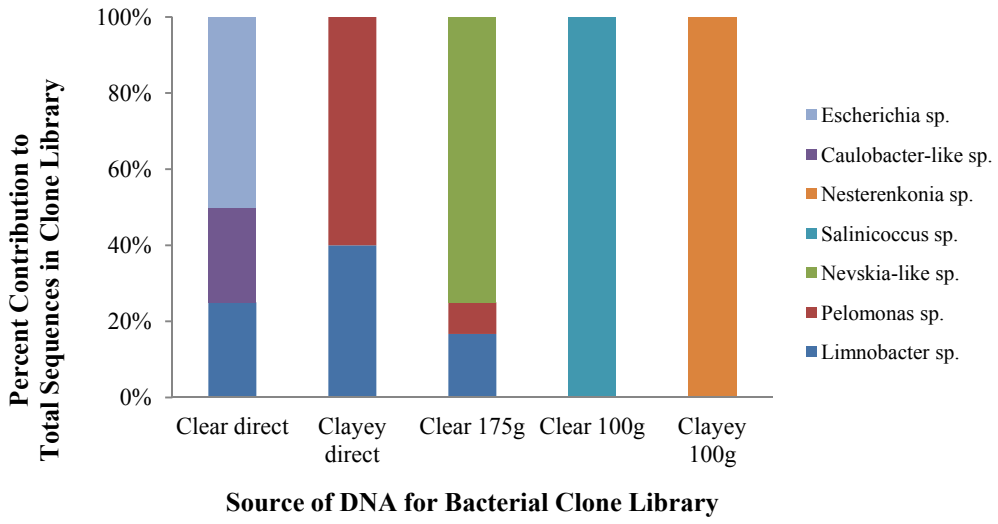


Figure 7a: Bacterial clone libraries from direct DNA extracts of both halites and growth-positive incubations.

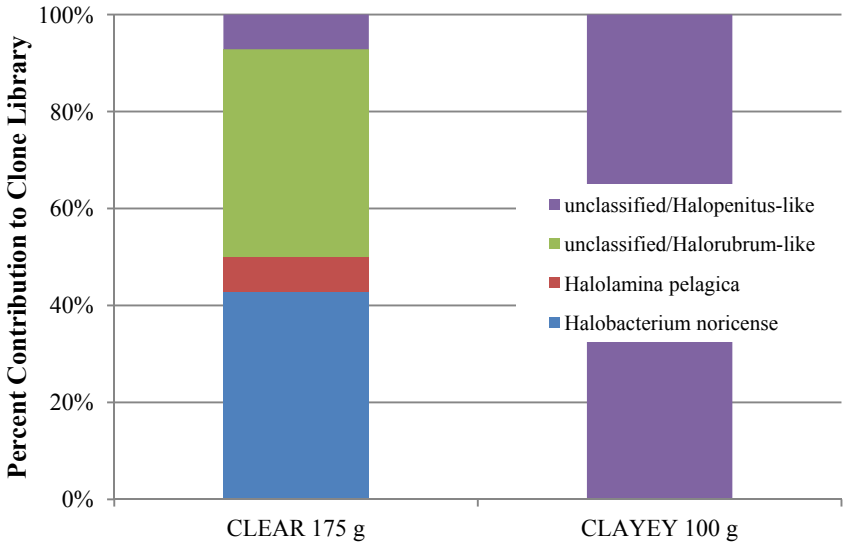


Figure 7b: Archaeal clone libraries from enrichment cultures of clear halite with 175g NaCl and clayey halite with 100g NaCl. Note lack of diversity in clayey halite incubation.

A total of seven bacterial phylotypes were detected between the two halites (4 in clear; 3 in clayey). The raw clayey halite library was distributed evenly between *Limnobacter* and *Pelomonas* spp. *Limnobacter* was also present in the clear halite raw (25%) and high-salt libraries (17% *Limnobacter* and 8% *Pelomonas*). *Caulobacter* and *Escherichia* sequences also appeared in the clear raw library. *Nevskia* sp. signatures appeared in the clear halite-high salt incubation, making up 75% of this library. One hundred percent of the clear halite-low salt library was *Salinicoccus* sp. The clayey halite-low salt incubation comprised only *Nesterenkonia* sp. sequences.

Four archaeal phylotypes were found in the clear halite, high-salt library—*Halorubrum*-like (43%), *Halolamina pelagica* (7%), *Halopenitus*-like (7%), and *Halobacterium* spp. (43%). Only one phylotype was found in the clayey halite incubation; this belonged to the *Halopenitus*-like organism also seen in the clear halite library.

A phylogenetic tree (Figure 8) was constructed from the DNA sequences retrieved from the isolates and libraries for both clear and clayey halites across all three domains (*Bacteria*, *Archaea*, and *Eukarya*). This tree shows the relatedness of the sequences to each other and other reference sequences in rRNA databases.

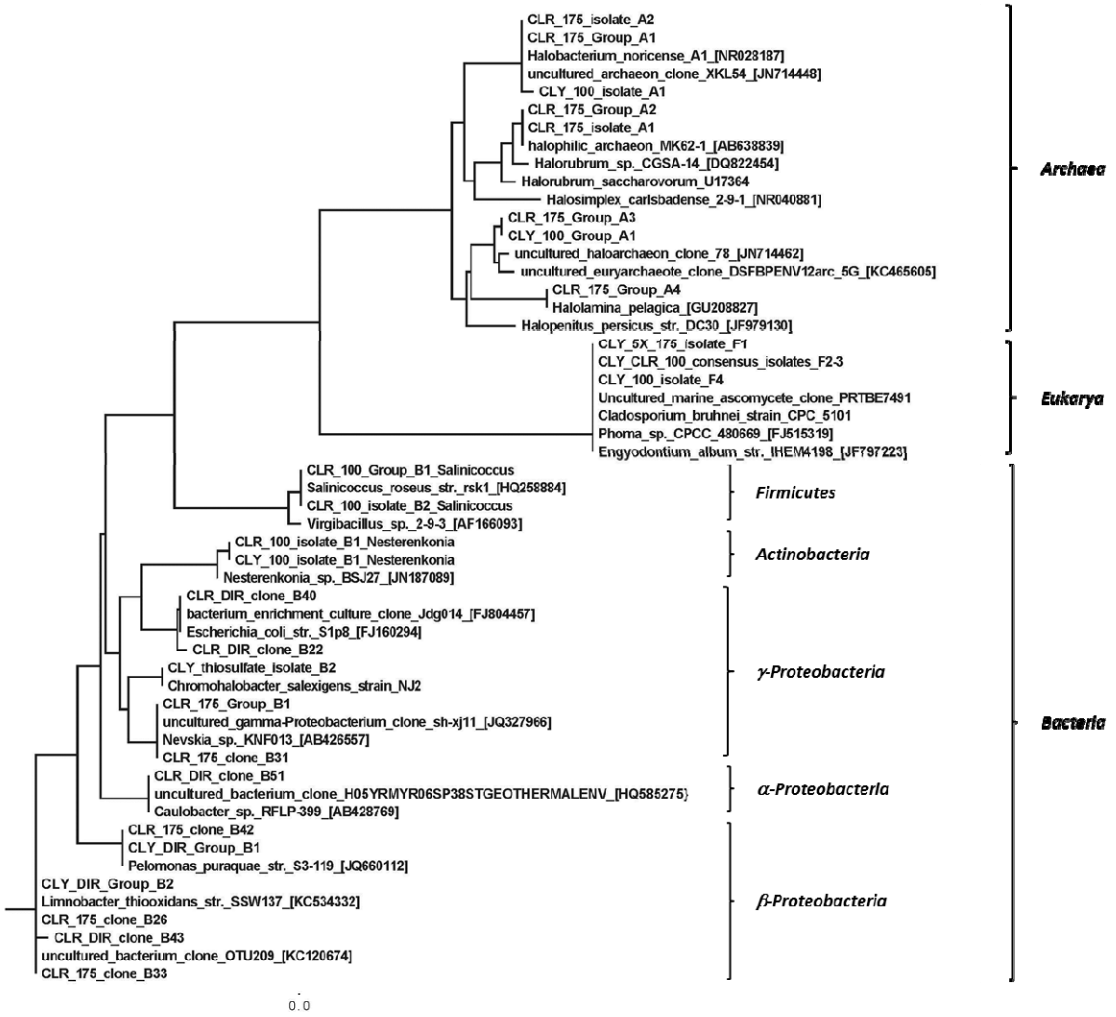


Figure 8: Unrooted phylogenetic tree constructed from clone library and isolate sequences of small subunit ribosomal RNA gene sequences amplified from clear (CLR) and clayey (CLY) halite. DIR indicates raw halite; 100 or 175 indicate grams of NaCl in enrichment culture. Scale bar represents one nucleotide substitution per 10 base positions.

3.5 Discussion

3.5.1 Success of Growth and DNA Amplification

The clear halite yielded positive cultures in both low and high salt media, while the clayey required a second attempt before growth could be obtained in high salt media. This may be due to the additional nutrients in the medium that offset those that may have sorbed onto the clay. Or, more likely, it could be that the original halite inoculum contained no cultivable cells, suggesting an even greater heterogeneity of cell distribution in the clayey samples than in the clear. The length of time required for growth in low-salt versus high-salt and clear versus clayey cultures did not appear to be significantly different.

DNA was more amplifiable from the clear halite than the clayey (i.e. a larger mass of clayey halite was need to obtain amplifiable DNA than clear; 50 g versus 2 g). While this could be due to the inhibitory effects of the clay constituents on molecular techniques, it could also be due to overall lower cell numbers in the clayey halite (possibly due to fewer fluid inclusions or lack of interstitial brines). The effects of clay impurities on DNA extraction and amplification were tested by seeding samples of each halite type with live cells, performing an extraction, and amplifying the DNA. DNA could not be extracted or amplified from seeded clayey halite unless excess EDTA was added during the extraction process.

3.5.2 Community Structural Differences between Clear and Clayey Halite

As shown in Figures 7a and b and in Table A-1 (see the Appendix Tables), there were differences in the community structure between the two halites. These differences are highlighted in Figure 9 (below). Bacterial phylotypes (from both isolates and clones) were shared between the halites, with *Limnobacter*, *Pelomonas*, and *Nesterenkonia* found in both, *Nevskia* and *Salinicoccus* in the clear, and *Chromohalobacter* in the clayey. *Archaea* found in the clayey halite were a subset of those found in the clear, and *Fungi* found in the clear halite were a subset of those found in the clayey.

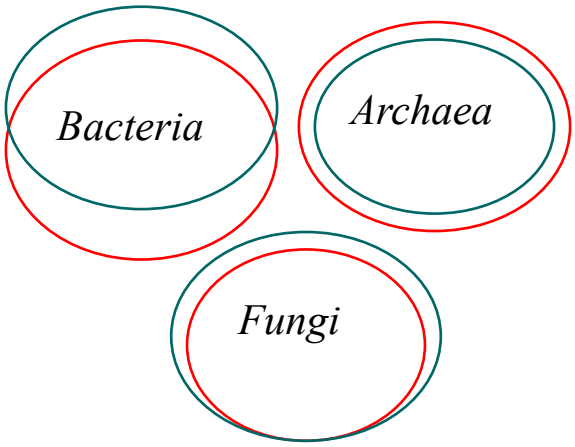


Figure 9: Diagram showing structural relationship between communities found in clear (red) and clayey (green) halites.

The isolation of *Nesterenkonia*, *Halobacterium*, and *Cladosporium*-like organisms from both halites suggests that nothing in the clay is inhibitory to their growth and/or survival. The same is probably true for the *Halorubrum*-like and *Halopenitus*-like organisms, although neither was obtained in pure culture from the clayey halite. The presence of clay above 2% (w/v) was found to inhibit the growth of the *Salinicoccus* isolate, which may explain its absence from the clayey culture. The magnesium-only toxicity screen inhibited *Salinicoccus* but not *Nesterenkonia*, suggesting that Mg is one inhibitor found in clay.

3.5.3 Community Structural Differences between Low- and High-Salt Incubations

Nested PCR was required to detect *Bacteria* in both the clear- and clayey-high salt cultures. This suggests either low numbers of cultivated *Bacteria* or the preservation of their DNA sequences in high salt. *Bacteria* were only isolated from the low-salt incubations. This is not surprising given that *Archaea* tend to dominate communities at high ionic strength. Although both the *Nesterenkonia* sp. and *Chromohalobacter* sp. can survive in high-salt media, it is possible that they were outcompeted by the *Archaea* in the mixed cultures. *Archaea* were neither detected by PCR nor cultured from the clear halite-low salt incubations. One species was present in the clayey halite-low salt library, and a different species was isolated, but the clayey-high salt yielded neither.

3.5.4 Potential Metabolic Capability of Halite Community and Relevance to WIPP

3.5.4.1 Archaea

In contrast to the *Bacteria* and *Fungi*, *Archaea* may be key players at the ionic strengths expected in the repository. They thrive in aerobic Generic Weep Brine (GWB) and Energy Research and Development Administration Well 6 (ERDA-6)-based brines and are capable of degrading three of four organic complexing agents found in the WIPP (Swanson et al., 2013). Many haloarchaea are also able to reduce nitrate. Their ability to thrive and degrade organic complexing agents under more reduced conditions is uncertain. Finally, the reported longevity of these organisms in brine or preserved in halite fluid inclusions (McGenity et al., 2000) suggests that they will be present and viable, under the right

conditions, during the repository's entire lifetime, thereby increasing the opportunity for favorable interaction with the waste.

One of the dominant community members is *Halobacterium noricense*. This organism was originally isolated from Permo-Triassic rock salt obtained from an Austrian mine (Gruber et al., 2004), and it has also been found in halites of various ages from Brazil, Poland, Turkey, and the United Kingdom (McGenity et al., 2000; Vreeland et al., 2007; Weidler, 2007, unpublished submission to GenBank; Ozcan et al., 2009, unpublished submission to GenBank; Park et al., 2009). This suggests that data obtained from this research are relevant to other salt repositories, such as Asse and Gorleben in Germany.

It is also noteworthy that the one archaeal isolate (*Halorubrum*-like) retrieved from the clayey-100g culture was very closely related to those found in the clear-175g halite enrichment culture. This organism also dominated community composition in enrichments in GWB and ERDA-6 containing organic complexing agents, suggesting that it will play a key role in their degradation under aerobic conditions.

3.5.4.2 Bacteria

Many of the identified *Bacteria* fall within the *Proteobacteria*, a phylum consisting of many opportunistic organisms with broad substrate capability. In an aerobic environment, these organisms could play a significant role in waste degradation and, in the case of *Limnobacter,* possible sulfur cycling. However, none of the bacteria thus far identified represent obligate anaerobes. *Escherichia* and *Pelomonas* spp. can be considered facultative and have been shown to reduce nitrate anaerobically, while some *Chromohalobacter* can reduce nitrate aerobically.

The appearance of *Nevskia*-like species in the clear-175g culture is unusual. These organisms have been previously associated with chlorinated waste streams and drinking water distribution systems. It is possible that they arose as contaminants from the water system; however, they have not appeared in any other cultures to date, and an analysis of the water supply after autoclaving yielded no bacterial PCR product. They are obligate aerobes and not known to be halophilic.

Limnobacter, *Pelomonas*, and *Chromohalobacter* have been isolated previously from marine environments, but only *Chromohalobacter* is capable of survival at high salt concentrations. The former two were most likely associated with the halite rock surfaces, and their DNA may have been preserved in the high-salt media, even if the organisms did not grow. It is possible that they may play a role in the initial aerobic, humid environment, where they can colonize surfaces without being suspended in brine.

Of the *Proteobacteria*, *Chromohalobacter* stands the best chance for survival in aerobic WIPP brines. The *Chromohalobacter* isolate retrieved from the clayey halite exhibited growth over a range of NaCl from 0 to 15% (w/v), although some species have a wider range (Arahal et al., 2001). Most are also able to utilize citrate (Sànchez-Porro et al., 2007).

Nesterenkonia spp. fall within the *Actinobacteria*, of which a few members are halophilic, many degrade recalicitrant hydrocarbons, and still others produce antimicrobial agents. Most *Nesterenkonia* spp. are haloalkaliphilic or –tolerant; many can utilize cellulose and acetate; but citrate is only weakly degraded (Collins et al., 2002; Li et al., 2004, 2005). To date, all known species are obligately aerobic. This isolate is also alkaliphilic and is currently maintained on media at pH 9.5 and 25% (w/v) NaCl, although at neutral pH it prefers 5% NaCl. Its ability to grow in WIPP brines is under investigation.

Salinicoccus spp. are members of the phylum *Firmicutes* and have been isolated previously from salted foods, solar salterns, and a salt mine in China (Ventosa et al., 1990; França et al., 2006; Chen et al., submission to GenBank, 2006). The isolate obtained from the clear salt exhibited growth between 0-17.5% (w/v) NaCl with an optimum between 7.5-15%. The most closely matched species to this isolate, *S. roseus*, is able to reduce nitrate under anaerobic conditions but not as a respiratory function. It also utilizes citrate as a sole carbon and energy source.

3.5.4.3 Fungi

All the isolated *Fungi* belong to the *Ascomycota*, which is an order known to contain other extremophilic members, although only *Cladosporium* has been mentioned in the halophile literature. Halophilic fungi are a relatively recent discovery (Gunde-Cimerman et al., 2000), so there is little known about their functional role in environmental high ionic strength matrices. All known species have thus far been isolated from solar salterns and brine lakes, but none from halite. Whether these organisms were introduced from the surface or are actually indigenous to the repository is under investigation, although from the perspective of WIPP performance it is irrelevant, as long as they survive in brine. If they do, their significance in the WIPP may be three-fold. First, like *Actinomycetes*, *Fungi* also produce antimicrobial agents, which may inhibit the growth of some organisms. This has not been observed on plated media thus far. Second, some *Fungi* have been shown to produce low-molecular-weight complexing agents to sequester trace elements in soils; whether or not this will be the case in brine is unknown. Third, *Ascomycota* degrade dead plant matter. Both the *Cladosporium* and *Phoma* isolates are able to grow on solid media with carboxymethylcellulose (CMC) as the sole carbon source, and growth on a minimal nutrient medium is enhanced in the presence of Kimwipes (see Figure 10). However, these organisms have been disappearing from anaerobic incubations, suggesting they are obligately aerobic, so their survival in brine may be limited to early repository history.

All species grow across a range of [NaCl] from 0 to 20%. The *Cladosporium* sp. exhibits a distinct optimum between 5-10%. Growth was also observed on solid media up to pH 10.

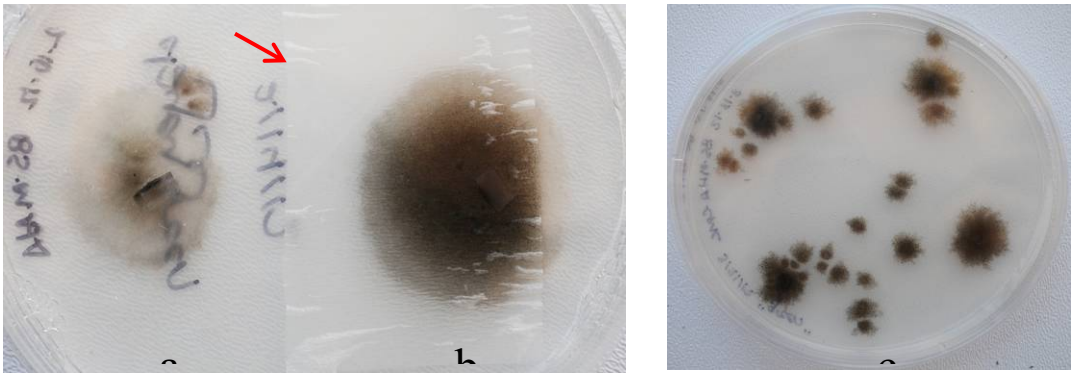


Figure 10: ***Cladosporium*** **sp. growing on minimal nutrient agar with 15% NaCl (a and b) and CMC agar with 9% NaCl (c). Red arrow denotes edge of Kimwipe placed on agar surface. Note increased colony size when on Kimwipe (b) versus no Kimwipe (a).**

It is important to reiterate that the halite samples described in this report were not sterilized. Mine workers, mining equipment, and air-intake shafts can potentially introduce halotolerant organisms from the surface environment, and these organisms will most likely be found on halite surfaces. Colonization of exogenous organisms introduced by air into salt mines has not been shown, and in fact, no organisms could be cultivated from the WIPP air-intake shaft that could survive in high ionic strength media (Vreeland et al., 1998; McGenity et al., 2000). It is not known whether an attempt was made to cultivate organisms on low-salt media that may have supported the growth of *Bacteria*. For the WIPP, the origin of these organisms is irrelevant; what matters is their survival in brine.

In general, harsh environments tend toward a less diverse microbial community structure, and WIPP halite is no exception. Once the repository is closed, the community will likely become even less diverse, with a probable loss of *Fungi* and most *Bacteria*. Whether or not the *Archaea* remain active, especially under anaerobic conditions, is under investigation, but *Archaea* will certainly remain present.

4.0 PART 2–WIPP GROUNDWATERS

4.1 Overview

To date, four groundwaters from the WIPP environs have been sampled (WQSP-1, WQSP-3, H4b-R, PZ-13; see Figure 11).

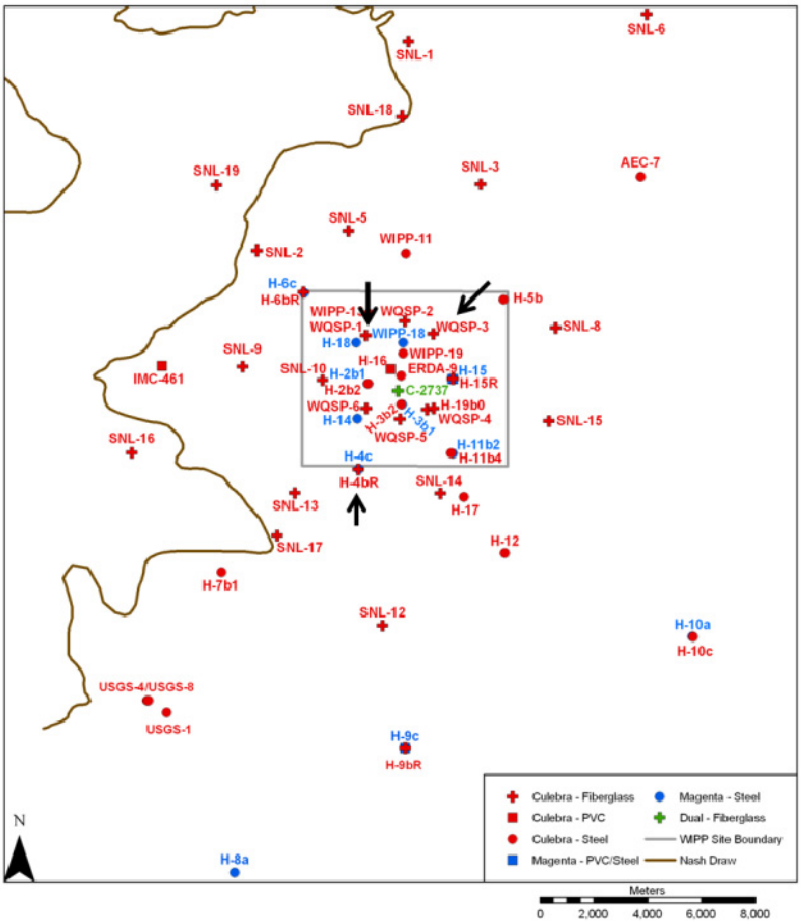


Figure 11: Map of WIPP monitoring wells. The arrows denote wells sampled; the rectangle outlines the WIPP site (CRA, 2009b).

Nucleic acids were directly extracted from these samples, and chemical analyses were also performed. Aerobic and anaerobic incubations were set up. Aerobic and anaerobic moderately halophilic cultures were enriched from WQSP-1 that comprise fermenters, metal reducers, and sulfate reducers. PZ-13 incubations yielded extremely halophilic cultures with metal- and sulfate-reducing capabilities. WQSP-3 incubations resulted in moderately to extremely halophilic aerobes, nitrate reducers, metal reducers, sulfate reducers, and fermenters. Results for H4b-R were ambiguous due to the inability to control for outside contamination and were, therefore, discarded.

4.2 Objective

The goal of this work is to sufficiently characterize the microbial populations within groundwaters surrounding WIPP under different growth conditions, such that valid measurements, determinations, and informed predictions of the impact of these organisms on actinide behavior in the environment surrounding the repository can be made.

The Culebra is considered to be the most likely pathway for actinide migration from the repository, in the low-probability event of a breach into the WIPP horizon (WIPP ASER 2010). Should this occur, indigenous microorganisms may be exposed, and how they interact with the waste (specifically, the organic and actinide components) may affect actinide mobility in the far-field.

Two of the processes through which Culebra microorganisms might influence actinide mobility are oxidation/reduction reactions and actinide uptake (either internalization or surface sorption). Direct reduction of metals by microorganisms occurs in many aqueous environments, such as wastewaters, porewaters, or groundwaters, and indirect reduction of metals may occur in those same environments as a result of the consumption of oxygen through microbial metabolism or fermentation. In general, actinide reduction yields a less soluble form, which after precipitation is effectively immobilized. Metal reduction in hypersaline environments with ionic strengths higher than those in marine settings (i.e. 0.7 M) has been documented for manganese but rarely for iron, due to its low solubility in these matrices and because the higher concentrations of both manganese and sulfate make these preferred electron acceptors (Emmerich et al., 2012).

A second process through which Culebra microorganisms might affect actinide mobility is through uptake. This is viewed in WIPP PA as detrimental to performance, in that it assumes that all microorganisms are planktonic and mobile. Our hypothesis is that 1) these organisms, like most, will remain in nutrient-rich "hot spots" by means of biofilm formation, and that migration occurs far less frequently than assumed; 2) that actual biomass concentrations will be less than those predicted by PA; and 3) that the surface of biomass available for sorption will be far less than that of its pathway.

4.3 Materials and Methods (see also Part I)

4.3.1 Groundwater Sampling and Handling

WQSP-1 and WQSP-3 samples for DNA analysis, chemical analyses, and aerobic culture work were collected in 1 L sterile polypropylene bottles and placed on ice until returning to the laboratory. Samples for anaerobic culture work were obtained after enveloping the source tap in a portable plastic glove bag and filling the bag with nitrogen. Approximately 100 ml of water was collected in 125 ml serum bottles to which small volumes of anaerobic, concentrated stocks of nutrients had been added (see Table 4, below). Bottles were sealed prior to removal from the glove bag. PZ-13 was sampled using an unsterile bailer that had been briefly rinsed with sterile deionized water prior to dropping it into the borehole and then transferred into sterile polypropylene bottles. Upon returning to the lab, one bottle was immediately placed into the anaerobic, nitrogen-filled glove box.

4.3.2 Chemical Analyses

Replicate 0.5 mL aliquots of groundwater were passed through Millipore MicroCon Y filters (100 kD cut-off) prior to chemical analysis. Anion analysis by IC was carried out (as described in Part 1), within 8 hours of sampling. Metal/cation analysis and TOC analysis were performed as described in Part 1. pH was measured with reference to a standard hydrogen electrode.

4.3.3 Solid Phase Characterization

X-ray absorption near-edge structure (XANES) spectroscopy was performed on selected incubation precipitates by Dan Olive at the Illinois Institute of Technology.

4.3.4 Media Preparation

Media were prepared for the following cultivation conditions for the groundwater from WQSP-1 and WQSP-3: aerobic (Aer), transitional (Tr-sealed under aerobic conditions and allowed to proceed through aerobic and anaerobic stages, such that resulting community is more a product of underlying water chemistry), nitrate-reducing (NR), iron-reducing (IR), sulfate-reducing (SR), and methanogenic (M). Fermenting organisms may be enriched from any of the incubations below, except methanogenic.

Because of the limited supply of PZ-13 water and the need at that time to investigate metal reduction, incubations were limited to aerobic, iron-reducing, and sulfate-reducing.

Table 4:
Outline of Media used for Enriching Microorganisms from Groundwater

Medium Component	Aer[a]	Tr[b]	NR[c]	IR[d]	SR[e]	Meth-1[f] (WQSP-1)	Meth-2[f] (WQSP-3)	Meth-3[f] (WQSP-3)
Carbon sources	Acetate, pyruvate, lactate	Acetate, pyruvate, lactate	Acetate, pyruvate, lactate	Acetate, pyruvate, lactate	Acetate, pyruvate, lactate	Formate, methanol	Formate, QSP-1etate	Trimethyla mine oxide
Nutrients and supplements	NH_4Cl, KH_2PO_4 , yeast extract, casamino acids	NH_4Cl, KH_2PO_4 yeast extract, casamino acids, trace elements, vitamins	NH_4Cl, KH_2PO_4, yeast extract, casamino acids, trace elements, vitamins	NH_4Cl, KH_2PO_4, yeast extract, casamino acids, trace elements, vitamins	NH_4Cl, KH_2PO_4, yeast extract, casamino acids, trace elements, vitamins	NH_4Cl, KH_2PO_4, yeast extract, casamino acids, trace elements, vitamins	NH_4Cl, KH_2PO_4, yeast extract, casamino acids, trace elements, vitamins	NH_4Cl, KH_2PO_4, yeast extract, casamino acids, trace elements, vitamins
Terminal electron acceptor	None provided (O_2)	None provided (initially O_2)	KNO_3	Fe(III)-citrate	Na_2SO_4	None provided (CO_2 in headspace)	None provided (CO_2 in headspace)	None provided (CO_2 in headspace)
Buffer	MOPS	MOPS	MOPS	MOPS	MOPS	MOPS	MOPS	MOPS
Headspace	Aerobic	Aerobic, sealed	N_2:CO_2	N_2:CO_2	H_2:CO_2	H_2:CO_2	H_2:CO_2	H_2:CO_2
Reductant	None	None	None	None	Cysteine	Cysteine-sulfide	Titanium citrate	Cysteine-sulfide

[a] Aer = aerobic.

[b] Tr = transitional (sealed aerobically and allowed to proceed to anaerobiasis.

[c] NR = nitrate-reducing.

[d] IR = iron-reducing.

[e] SR = sulfate reducing.

[f] Meth1-3 methanogenic (using different substrate types).

Low-nutrient agar media were prepared using ~500 mL of the groundwater samples as the diluent and adding R2A solids (a low-nutrient medium commonly used for the cultivation of organisms from groundwaters). Broth media contained approximately 3 mM of each organic substrate and ~ 10 mM of each TEA. Methanogenic media contained 5 mM of their respective substrates. MOPS [3-(N-morpholino)propanesulfonic acid] was added to buffer media between 6.8-7.2. Resazurin was added as an indicator of oxygen presence.

4.3.5 Microbial Cultivation

One mL aliquots were removed from the aerobic sample bottles for direct microscopic counts, as described in Part 1. Additional 1 mL aliquots were plated onto R2A agar made with both the groundwater and deionized water. These were allowed to incubate at room temperature in the dark and were checked periodically for growth. Anaerobic serum bottles were returned to the lab, and their headspaces were replaced with the appropriate gas mixture. These were also allowed to incubate in the dark at room temperature and were checked periodically for growth.

4.3.6 DNA-Based Analyses

DNA was extracted directly from the water samples within eight hours of their collection. Cells were concentrated onto 0.22 micron filters, which were then aseptically cut and transferred into sample tubes

for extraction using the MoBio UltraClean Water DNA Purification Kit per the manufacturer's directions. At different time points during the incubation period, aliquots were removed from all incubations for microscopy and, if positive, for DNA extraction. Cells were concentrated by centrifugation, and the pellet was resuspended and transferred into sample tubes for extraction using the MoBio UltraClean Soil DNA Purification Kit. PCR, DGGE, cloning and transformation, and DNA sequencing were carried out as described in Part 1.

4.3.7 Isolate Screening

Numerous isolates were purified and identified by PCR amplification of extracted DNA, followed by sequencing.

4.4 Results

4.4.1 Chemistry

Results of ICP-MS, IC, and TOC analysis on the three groundwaters are provided in Table 5, below.

Table 5:
Groundwater Chemistry

Significant Analyte/ Parameter	WQSP-1		WQSP-3		PZ-13	
	µg/ml	M	µg/ml	M	µg/ml	M
Na^+	7.96×10^3	0.35	8.01×10^4	3.48	8.42×10^4	3.66
Ca^{2+}	1.95×10^3	0.05	1.82×10^3	0.04	2.54×10^3	0.06
Mg^{2+}	1.21×10^3	0.05	2.64×10^3	0.11	1.51×10^3	0.06
K^+	553	0.01	1.74×10^3	0.04	503	0.01
Cl^-	35470	1.00	119360	3.37	555	0.02
SO_4^{2-}	4464	0.05	7047	0.07	1.55×10^5	4.37
pH	7.14	N/A[a]	7.07	N/A	2.54×10^3	0.03
TOC	0.386[b]	N/A	0.162[b]	N/A	nd[c]	nd[c]

[a] N/A = Not applicable.

[b] At limit of detection.

[c] nd = Not done.

4.4.2 Direct Counts

Direct microscopic counts of the raw groundwaters were as follows:

WQSP-1: $(2.37 \pm 0.96) \times 10^5$ cells/ml

WQSP-3: $(8.63 \pm 5.44) \times 10^5$ cells/ml

PZ-13: not determined

4.4.3 Microbial Growth in Incubations

***WQSP-1*:** Growth (as evidenced by turbidity) was observed in aerobic flasks and transitional bottles within 48 hours and in iron-reducing incubations by day 4. Growth was observed in nitrate-reducing incubations at week 4. By week 4, a sulfide precipitate was observed in both the transitional and iron-reducing incubations, with more noted in the IR bottles (see Figure 12). After approximately two years, sulfide precipitation was noted in the sulfate-reducing incubations, along with low numbers of cells.

Methanogenic incubations also showed some cell growth at this time, so samples were withdrawn for DNA analysis, PCR of the *mcrA* gene was performed, and results were negative. Further analysis has not been conducted.

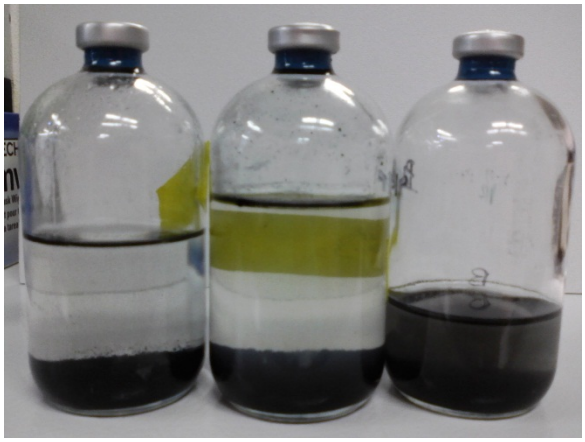


Figure 12: Examples of sulfide precipitation in iron-reducing incubations from (left to right) WQSP-1, WQSP-3, PZ-13.

WQSP-3: Growth (as evidenced by turbidity) was observed in aerobic flasks within 1 week. Transitional and iron-reducing incubations were growing by 8 weeks, as evidenced by direct microscopic observation; although significantly more cells were observed in the IR incubations. A metal sulfide precipitate was also noted in these incubations (Figure 12). NR incubations grew more slowly but had achieved significant numbers by 15 weeks. The cells in these incubations had a different morphology from all other anaerobic incubations. Few to no cells were visualized in the SR and methanogenic incubations until approximately 28 months of incubation, at which time cells were observed in the SR incubations.

PZ-13: Again, because the volume of groundwater obtainable from this well was extremely small, a limited number of incubations were set up (aerobic, iron-reducing, and sulfate-reducing). Growth occurred in the aerobic incubations within 48 hours. Turbidity was also noted in the IR incubations after approximately two weeks. A sulfide precipitate was noted in one of the iron-reducing incubations after approximately one year (Figure 12). No growth was observed in the sulfate-reducing incubations.

4.4.4 Plated Cultures and Isolates

Results for isolate identification by DNA sequence analysis are listed in Tables A-2–A-4, in the Appendix Tables section of this report.

4.4.4.1 WQSP-1

Bacteria

Six isolates were obtained from transitional (2) and iron-reducing (4) incubations. The 16S rRNA-encoding gene sequences of five isolates were able to be grouped together at 97% similarity (i.e. same operational taxonomic unit, OTU, designation). These matched most closely (99% similarity) to *Halanaerobium acetethylicum*. The sixth isolate (from the IR incubations) matched (97% similarity) *Clostridium sediminis*. *Marispirillum* sp. was isolated from the IR incubations grown microaerophilically and maintained aerobically. A *Bacillus* sp. was isolated from aerobic incubations.

4.4.4.2 WQSP-3

Bacteria

Two colony morphologies were cultivated from the aerobic flasks. Three isolated colonies with the same morphology yielded 99% 16S ribosomal RNA-encoding gene sequence similarity to *Chromohalobacter salexigens*. Cultures of the second colonial morphology are still being purified.

4.4.4.3 PZ-13

Bacteria

Plated cultures from the aerobic incubation yielded a *Chromohalobacter* sp. (see Figure 13a). It was also identified in halite enrichment cultures with 100 g NaCl. The range of growth for this organism was found to be between 5-25% NaCl, with an optimum of ~15%. It does not grow in the absence of salt. (This is in contrast to the *Chromohalobacter* sp. isolated from halite.)

A *Virgibacillus* sp. (see Figure 13c) was also isolated from the IR incubation after subculturing under microaerophilic conditions. It was likely in spore form while conditions were anaerobic but germinated upon subculturing. This organism has a range for growth between 2.5-17.5% NaCl with an optimum at 7.5% and does not grow in the absence of salt. A *Virgibacillus* sp. was isolated previously from a fluid inclusion in WIPP halite (Vreeland et al., 2002).

Archaea

Three archaeal isolates were obtained from PZ-13. *Halococcus saccharolyticus* (see Figure 13b) was isolated from the aerobic culture, and *Haloferax* sp. and *Natrinema* sp. were isolated aerobically from the IR culture.

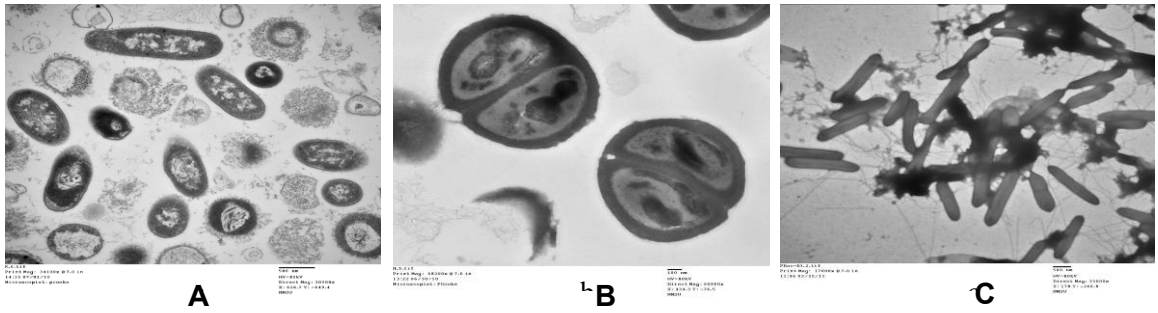


Figure 13: Transmission electron microscope (TEM) thin section of *Chromohalobacter* sp. (A); TEM thin section of *Halococcus saccharolyticus* (B), and TEM negative stain of *Virgibacillus* sp. (C). Scale bars in (A) and (C) denote 500 nm; bar in (B) denotes 100 nm.

4.4.5 DGGE

Resolution of amplified bacterial DNA from the WQSP-1 transitional and iron-reducing incubations is shown in Figure 14 (below).

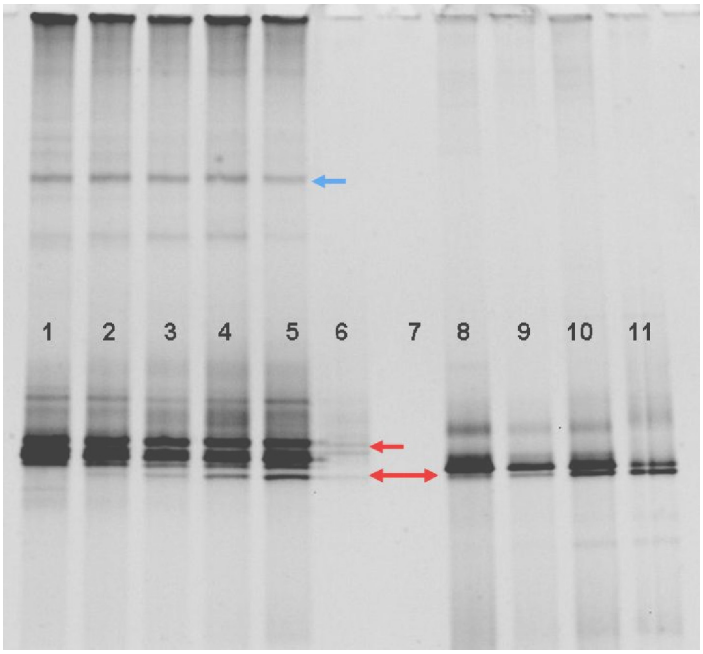


Figure 14: Denaturing gradient gel resolving amplified 16S rRNA-encoding bacterial DNA from WQSP-1 direct sample, transitional incubations, and iron-reducing incubations. Lanes 1-5, transitional incubations over time; lane 6, direct extraction without incubation; lanes 7-11, iron-reducing incubations over time. Note shared bands (red) and unique bands (blue); the double-arrowed, red band increases in intensity over time.

4.4.6 DNA Sequence Analysis of Clone Libraries

Clone libraries were constructed from the amplified bacterial 16S rRNA-encoding genes from incubations and from the DNA extracted directly from the raw groundwaters without any incubation. Tables A2-A4 (see Appendix) show the closest database matches to the retrieved sequence for each groundwater. If the closest match is an uncultured or unidentified organism, the table also lists the closest named relative.

The distribution of clone sequences across microbial phylotypes for the groundwater incubations is shown in Figure 15. Clone libraries were only constructed from PZ-13 IR incubations. A phylogenetic tree showing the relatedness of these sequences to one another and reference organisms is shown in Figure 16.

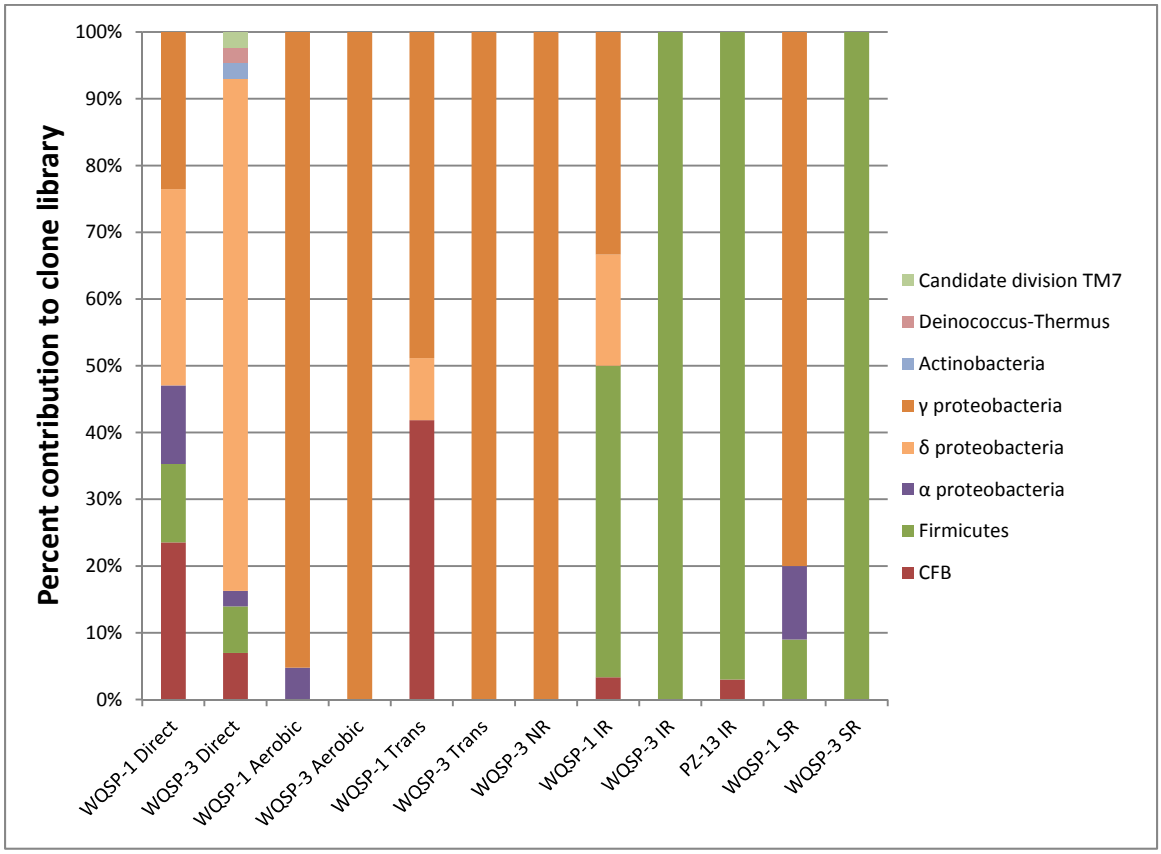


Figure 15: Phylogenetic distribution of clone sequences retrieved from DNA directly extracted from raw groundwater and from aerobic and anaerobic incubations.

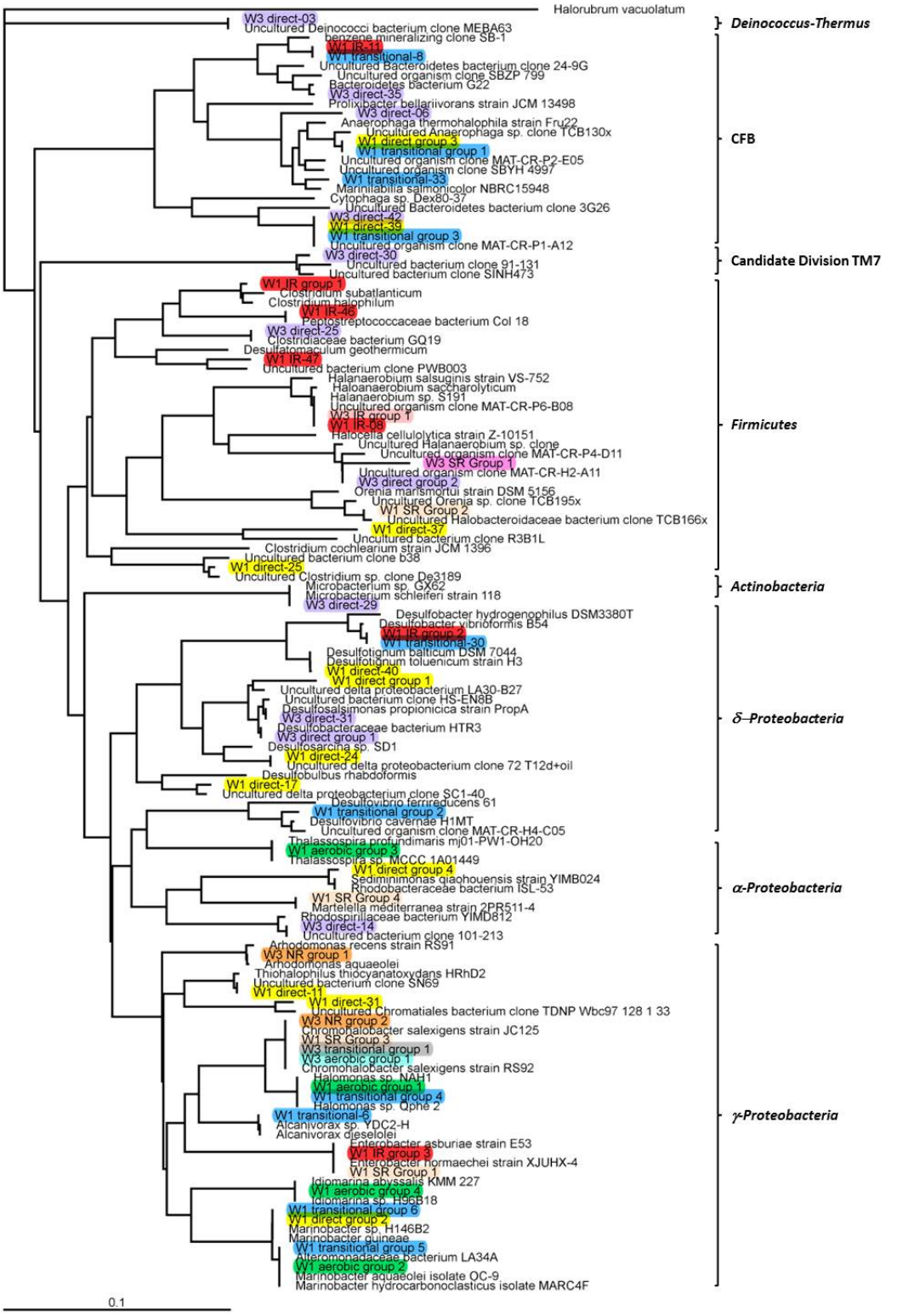


Figure 16: **Phylogenetic tree constructed from WQSP-1 and WQSP-3 16S rRNA encoding sequences retrieved from all samples (raw groundwater = direct (Dir); Aer, IR, and transitional (T))). The tree is rooted to *Halorubrum vacuolatum* as an outgroup. The scale bar indicates one nucleotide substitution per 10 bases.**

4.4.6.1 WQSP-1

Twelve OTU's were detected in DNA extracted directly from WQSP-1. These were distributed evenly among members of the *Proteobacteria*, *Firmicutes*, and CFB, but none of the detected organisms was cultivated in the incubations. Aerobic incubations were limited to the α- and γ-*Proteobacteria* (*Thalassospira*, *Halomonas*, *Marinobacter*). Transitional incubations were a mix of possible facultative γ-*Proteobacteria* and CFB (*Halomonas* sp.; *Anaerophaga* and *Cytophaga* spp.) and anaerobic δ-*Proteobacteria* (*Desulfovibrio* sp.). *Firmicutes* (Clostridia) and other δ-*Proteobacteria* (*Desulfobacter* sp.) appeared in the iron-reducing incubations, along with facultative *Enterobacter* spp. Sulfate-reducing incubations did not appear to select for sulfate-reducers, but instead resulted in a selection for α- and γ-*Proteobacteria* (*Enterobacter* and *Chromohalobacter*, and *Martelella*) and *Halanaerobiaceae* (*Orenia*).

4.4.6.2 WQSP-3

Eleven OTU's were detected in DNA extracted directly from WQSP-3. In contrast to the more even distribution of phyla in WQSP-1, the majority of sequences (77%) in the WQSP-3 direct library belonged to one genus, *Desulfosalsimonas* (originally *Desulfosalina*), which is a newly described member of δ-*Proteobacteria* and a sulfate-reducing bacterium (Kjeldsen et al., 2010). *Firmicutes* (*Halanaerobium*) and CFB (*Cytophaga*-like and *Bacteroidetes*), combined, composed half of the remainder of the WQSP-3 direct library. *Desulfosalsimonas* disappeared during incubation of this groundwater; while, *Halanaerobium* came to dominate the IR incubations. Neither *Chromohalobacter* nor *Arhodomonas* spp. was detected in the raw groundwater; whereas, the aerobic, transitional, and NR incubations were dominated by these organisms. SR incubations yielded only *Halanaerobium* sequences.

4.4.6.3 PZ-13

Clone libraries were constructed from the amplified bacterial and archaeal 16S rRNA-encoding genes in the early IR incubations, but this was prior to the appearance of the sulfide precipitate. 100% of archaeal clones were identified as *Haloarcula* sp. Bacterial clones were divided between two species: *Virgibacillus* (97%), and an uncultured *Bacteroidetes* (3%). When plated, the culture yielded *Pontibacillus* (91%) and *Virgibacillus* (9%).

4.5 Discussion

4.5.1 Chemistry

Results for WQSP-1 and WQSP-3 water chemistry are consistent with those measured by others (WIPP ASER 2010) and reflect a marine origin (WQSP-1) and excess halite (WQSP-3) (Domski et al., 2008). The origins of PZ-13 water are believed to be a mixture of seepage from an above-ground, but capped, mine tailings salt pile and actual groundwater flow through the Santa Rosa and Dewey Lake contact (WIPP ASER 2010). The groundwaters vary significantly in constituent concentrations (especially sodium, potassium, chloride and sulfate) and ionic strength, and these differences are reflected in the resulting community structures.

4.5.2 Microbial Community Structure

In general, diversity decreases with increased environmental constraints. In the case of all groundwaters, an increase in ionic strength would stress the bacterial community, resulting in lower diversity. This is reflected in the lower diversity found in PZ-13 and WQSP-3 relative to WQSP-1.

In the case of anaerobic groundwaters, an increased oxygen concentration could be considered a stressor. Thus, the increase in number of WQSP-1 phylotypes (two in the aerobic incubations to four in the iron-reducing ones) may reflect the stress of aerobiasis (see Figure 15).

The lower diversity in the WQSP incubations, as compared to their direct extracts, is likely due to cultivation bias.

4.5.2.1 WQSP-1

The proportion and diversity of sequences attributed to *γ-Proteobacteria* and CFB (*Cytophaga-Flavobacteria-Bacteroidetes*) decrease as the incubations become more reduced. This would account for a loss of strict aerobes and the selection for strict anaerobes (e.g., *Bacteroides*). The proportion of *δ-Proteobacteria*, of which sulfate-reducing bacteria are members, is higher in the IR incubations than in the transitional, which is likely due to the period of initial aerobiasis in the latter incubations. None of the sequences found in the direct extract were cultivated in the incubations. Both the proportion and diversity of *Firmicutes* sequences were high in the IR incubations, and all belonged to obligate anaerobes. Aerobic incubations were limited to the *γ-Proteobacteria*.

4.5.2.2 WQSP-3

Microbial community structure in the WQSP-3 incubations was far less diverse than that of the raw groundwater. This loss of diversity and community unevenness can be attributed to several different factors—competition, due to differential growth rates or metabolic capabilities, and substrate and/or nutrient limitation — which can act as stressors to the indigenous organisms.

The dominance of *Chromohalobacter* and *Arhodomonas* spp. in aerobic, transitional, and NR incubations suggests that these organisms acted as opportunists and were capable of utilizing the provided substrates. Additionally, the relatively rapid growth rate of *Chromohalobacter*, compared to other halophilic bacteria, would have allowed it near exclusive access to substrates and nutrients, perhaps to the point of depletion. This might explain why no DNA sequences attributable to anaerobes could be found in the transitional library.

The obligately anaerobic population consists of possible metal reducers (*Clostridiaceae*), sulfate reducers (*Desulfosalsimonas* spp.), and fermenters (*Halanaerobium* spp., *Clostridiaceae*). The faster growth in the iron incubations than in other anaerobic incubations suggests that iron may be a limiting nutrient in this groundwater.

4.5.2.3 PZ-13

Because fewer clone libraries were constructed from this sample, it is difficult to compare changes in diversity with incubation condition.

4.5.3 Potential Metabolic Capability of Groundwater Communities and Relevance to WIPP

WQSP-1 and WQSP-3 groundwaters are representative of water moving down gradient through the WIPP site. Characterization of the Culebra groundwaters provides insight into the baseline, or "before," population. In the unlikely event that radionuclides should reach the Culebra, the indigenous organisms identified in this research should be well equipped to directly or indirectly reduce them, thereby decreasing their migration potential.

As expected, the bacterial communities present in WIPP groundwaters reflect the chemistry of their source and the conditions of incubation. At an approximate total ionic strength of 1.5 M and [Na] of 0.35 M, WQSP-1 will support halotolerant and moderately halophilic microorganisms; while, WQSP-3 (3.5 M Na) and PZ-13 (3.7 M Na) will support extremely halophilic microorganisms.

Based on the distribution of phylotypes in the raw WQSP groundwaters (see Figure 13), one would predict a range of metabolic processes, including aerobic and anaerobic respiration and fermentation.

4.5.3.1 WQSP-1

Transitional incubations allow the microbial community to proceed through aerobic respiration, followed by respiration dependent upon the presence of terminal electron acceptors other than oxygen. Because no

nitrate was detectable in this groundwater, denitrification processes were by-passed. Iron and manganese were present in micromolar concentrations in raw groundwater samples, which may have been sufficient to maintain a metal-reducing community and low levels of metal reduction. Also detected in the groundwater were arsenic and selenium and--although this type of respiration was not tested for--it is possible that arsenate and selenate reducers are present as well.

Sulfate is the only natural TEA present in WQSP-1 at sufficient concentrations. *δ-Proteobacteria* were enriched in the SR incubation and may have increased in proportion had the samples been obtained later during the incubation period.

Sulfate-reducing bacteria can also be effective metal reducers. XANES analysis was performed on the sulfide precipitate from the IR incubation and showed a mixture of Fe(II) and (III), suggesting that iron was reduced by both metal and sulfate reducers and, indirectly, by microbially-generated sulfide.

Another potential means of metal and sulfate reduction is by fermentation. Fermentation likely occurred in all anaerobic incubations, although this was not monitored. Lactate is a common substrate for fermenters, and the appearance of a large proportion of *Firmicutes* (e.g., *Clostridium* spp.) supports this supposition. Sequences from an extreme halophile and known fermenter, *Halanaerobium* sp., were retrieved from the IR incubations. This process would lead to lower redox potentials and may indirectly reduce either metal or sulfate.

4.5.3.2 WQSP-3

Again, transitional incubations should allow the community to proceed naturally based on the medium provided and the natural TEAs present (i.e., sulfate). However, in WQSP-3, the transitional incubations did not proceed toward sulfate reduction but were axenic, comprising only *Chromohalobacter salexigens*, a *γ-Proteobacterium*. It is possible that the thriving *Chromohalobacter* may have depleted the nutrient supply such that other organisms could not survive. Alternately, the DNA used to generate this clone library may have been taken too soon in the incubation period, before the onset of true anaerobiasis. The presence of *Chromohalobacter* may be significant early on if an intrusion into the Culebra occurs, when waters may still be oxic or only slightly reduced. *Chromohalobacter salexigens* is capable of anaerobic nitrate reduction and also utilizes acetate and citrate (Arahal et al., 2001).

In contrast to WQSP-1, denitrification was stimulated in WQSP-3 with the addition of nitrate, suggesting that the organisms responsible are capable of other forms of respiration, but that denitrification allowed them to outcompete other organisms. These incubations were monophyletic, comprising only *γ-Proteobacteria*. *Arhodomonas* sp. made up 94% of this library with *Chromohalobacter* sp. composing the remainder. The appearance of nitrate-reducers is of significance, given the expected inventory of nitrate in the WIPP. The detected *Arhodomonas* sp. would be capable of oxidizing acetate at the expense of nitrate but would not utilize citrate (Saralov et al., 2012).

Iron and manganese were also detected in micromolar concentrations in raw WQSP-3 groundwater. However, metal reduction in these IR incubations was not likely due to the presence of direct metal reducers. This incubation comprised only *Halanaerobium* sp., an extremely halophilic and obligately anaerobic fermenter. The lowering of the redox potential within the incubation would have resulted in indirect iron reduction. Sequences belonging to other possible metal reducers were detected in the raw WQSP-3 DNA (e.g. *Clostridium* spp., *Bacteroidetes*), but these organisms were not enriched in the medium. This may be due to culture bias or the ability of *Halanaerobium* to outcompete these other organisms, especially at high ionic strengths. *Halanaerobium* spp. are one of the very few bacteria able to import Na^+, K^+ and/or Cl^- to maintain osmotic balance with their external environment, unlike other bacteria that would use the more energetically costly strategy of intracellular organic osmolyte accumulation (Oren, 2006). *Halanaerobium saccharolyticum* ferments sugars, suggesting it would not affect organic complexing agents but could contribute to cellulose utilization after initial cellulose breakdown has occurred.

The high concentration of sulfate in the raw groundwater (~73 mM) should have supported the growth of sulfate-reducing bacteria. That they are present is evidenced by the fact that the largest portion of the direct clone library was attributed to *Desulfosalsimonas* spp. These organisms would have been capable of utilizing lactate or yeast extract and could have oxidized acetate, since H_2 was in the incubation headspace (Kjeldsen et al., 2010). It is possible that ionic strength was an issue. While *Desulfosalsimonas* spp. can grow in a range of [NaCl], their optima tend to be between 1-2 M (Kjeldsen et al., 2010; Sorokin et al., 2012). However, these organisms dominated the direct clone library, suggesting that culture bias most likely prevented their growth.

4.5.3.3 PZ-13

Unlike WQSP waters, PZ-13 is a shallow, subsurface well for use as a piezometer. It is not associated with the Culebra and, as such, there is no foreseen interaction with the repository. However, the possibility that some of these organisms may have originated from the mine tailings pile, the presence of *Chromohalobacter* and *Virgibacillus* species that were also found in halite, and the overall halophilicity of the organisms found in this water make it relevant for continued study.

4.5.2 Comparison of WIPP Groundwaters at Different Ionic Strengths

For the most part, WQSP-1 yielded more bacterial diversity than WQSP-3. This is consistent with previous findings of lower bacterial diversity as salinity increases. Aerobic cultures of WQSP-1 contained four genera versus only one in WQSP-3. *Archaea* have not yet been analyzed in these samples, but it is probable that the diversity comparison will be the reverse, as haloarchaea will outcompete bacteria at high ionic strength.

Transitional incubations of WQSP-1 yielded 9 operational taxonomic units (OTUs); while WQSP-3 transitional incubations contained only one. This is partially due to the higher ionic strength in WQSP-3 but also to the opportunism practiced by *Chromohalobacter* such that these incubations may not have reached anaerobiasis. That WQSP-1 was approaching anaerobiasis is evidenced by the appearance of δ-Proteobacterial sulfate reducers.

Iron-reducing cultures from WQSP-1 were also more diverse than those from WQSP-3 (6 OTUs versus 1). One hundred percent of the clones from WQSP-3 IR matched to *Halanaerobium saccharolyticum*; whereas *Clostridia*, *Desulfobulbus*, and *Enterobacter* spp. dominated the WQSP-1 IR library. Again, this is likely due to ionic strength differences.

In contrast to all other incubations, no organisms were enriched in nitrate-reducing incubations of WQSP-1; while, *Arhodomonas* dominated the WQSP-3 NR culture. Neither groundwater has detectable levels of nitrate, so it is interesting that only the higher ionic strength groundwater should yield nitrate-reducers.

The only OTU shared between WQSP-1 and WQSP-3 incubations was *Halanaerobium*, while an unidentified member of the CFB phylum was shared in the direct libraries and made up 25% of the WQSP-1 transitional library. *Halanaerobium* made up only a small percentage (3%) of the WQSP-1 IR library, suggesting that it was outcompeted by other organisms. At the higher ionic strength, this organism would outcompete those found in WQSP-1.

Because fewer clone libraries were constructed for PZ-13, it is difficult to compare the diversity in this sample to the other two groundwaters. That being said, all three groundwaters yielded growth of a *Chromohalobacter* sp. No haloarchaea were isolated from incubations of the WQSP groundwaters, suggesting that conditions of the PZ-13 water were more conducive to their survival (e.g., salinity in WQSP-1 is likely too low for haloarchaeal survival; WQSP groundwaters are more reduced and may be inconducive to obligately aerobic haloarchaeal growth).

5.0 Part 3–CONCLUSIONS

There is much in the literature regarding the effects of microbial processes in the low-ionic strength environments generally associated with subsurface nuclear waste disposal sites and, to a lesser extent, the effects on deep geologic repositories. Unfortunately, there are relatively few publications addressing these issues for subsurface, salt-based repositories (Francis et al., 1997, 1998; Gillow et al., 2000; Gillow and Francis, 2006; Swanson et al., 2013a). Because the organisms found in high ionic strength systems are so different from those retrieved from low ionic strength repository sites, it is difficult to extrapolate their behavior, and new data are required.

Extreme halophiles, such as those that will dominate the microbial community in the WIPP near-field, have a metabolic repertoire limited by the thermodynamic constraints of surviving in high salt. Additionally, few haloarchaea are capable of anaerobic metabolism. Finally, apart from acetate and citrate, the carbon sources available to these organisms (i.e., those typically present in WIPP waste) are not their preferred substrates.

It is predicted that microbial activity in the near-field will proceed from aerobic respiration through nitrate reduction to focus on sulfate reduction and fermentation during the repository's 10,000-year lifespan. In the far-field, metabolically more diverse *Bacteria* may engage in all forms of respiration, depending upon ionic strength and nutrient availability. The role of methanogenesis is still uncertain as insufficient evidence for this process exists for the WIPP environs.

None of the *Archaea* thus far found within the groundwater were found in halite. This may be due to a difference in sodium concentration, or this may simply reflect undersampling, or this may be due to actual differences in surficial and subsurface haloarchaeal communities.

Two of the *Bacteria* found in halite were also found in groundwater. A *Virgibacillus* species isolated from PZ-13 was previously isolated from WIPP halite (Vreeland et al., 2002), although not in this study. A *Chromohalobacter* sp. was found in both clayey halite and PZ-13 groundwater. *Chromohalobacter* is a borderline extreme halophile, while *Virgibacillus* forms spores in high salt concentrations. This preliminary finding underscores the potentially significant differences between the microbial communities in the near and far fields.

Not only do significant differences in microbial populations exist between near and far-fields, but differences also exist between groundwaters. Again, these are due to the influence of ionic strength on metabolic capability.

5.1 Ongoing and Future Work

Archaeal DNA signatures have been detected in both WQSP-1 and 3 but are not yet identified. Associations may exist between bacterial and archaeal communities, and the possible growth of haloarchaea under reduced conditions is still under investigation. Additional studies are focusing on the interactions of these indigenous organisms with actinides (e.g. biologically mediated reduction, biosorption, toxicity) and waste organics (e.g. cellulose degradation).

6.0 REFERENCES

Altschul SF, Madden TL Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. *Gapped BLAST and PSI-BLAST: A new generation of protein database search programs*. Nucleic Acids Research 25: 3389-3402.

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. 1990. *Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations*. Applied and Environmental Microbiology 56: 1919-1925.

Arahal DR, Garcia MT, Vargas C, Cànovas D, Nieto JJ, Ventosa A. 2001. *Chromohalobacter salexigens* sp. nov., a moderately halophilic species that includes *Halomonas elongata* DSM 3043 and ATCC 33174. International Journal of Systematic and Evolutionary Microbiology 51: 1457-1462.

Barnhart BJ, Campbell EW, Hardin JM, Martinez E, Caldwell DE, Hallett R. October-December 15, 1978. *Potential microbial impact on transuranic wastes under conditions expected in the Waste Isolation Pilot Plant (WIPP)*. LA-7788-PR Progress Report. Los Alamos, NM: Los Alamos Scientific Laboratory.

Barnhart BJ, Campbell EW, Hardin JM, Martinez E, Caldwell DE, Hallett R. December 15, 1978-March 15, 1979 (1979a). *Potential microbial impact on transuranic wastes under conditions expected in the Waste Isolation Pilot Plant (WIPP)*. LA-7839-PR Progress Report. Los Alamos, NM: Los Alamos Scientific Laboratory.

Barnhart BJ, Campbell EW, Martinez E, Caldwell DE, Hallett R. March 15-June 15, 1979 (1979b). *Potential microbial impact on transuranic wastes under conditions expected in the Waste Isolation Pilot Plant (WIPP)*. LA-7918-PR Progress Report. Los Alamos, NM: Los Alamos Scientific Laboratory.

Barnhart BJ, Campbell EW, Hardin JM, Martinez E, Caldwell DE, Hallett R. October 1, 1978-September 30, 1979 (1979c). *Potential microbial impact on transuranic wastes under conditions expected in the Waste Isolation Pilot Plant (WIPP)*. LA-8297-PR Progress Report. Los Alamos, NM: Los Alamos Scientific Laboratory.

Chen YG, Li HM, Li QY, Chen W, Cui XL, Yang YL, Peng Q, Wen ML, Xu LH, Deng L, Wang ZG, Liu JH, Ren Z, Xiao W. 2006. *Phylogenetic diversity of culturable bacteria in the ancient salt deposits of the Yipinglang salt mine, P.R. China*. Unpublished submission to GenBank; accession number EF177668.

Collins MD, Lawson PA, Labrenz M, Tindall BJ, Weiss N, Hirsch P. 2002. *Nesterenkonia lacusekhoensis sp. nov., isolated from hypersaline Ekho Lake, East Antarctica, and emended description of the genus Nesterenkonia*. International Journal of Systematic and Evolutionary Microbiology 52: 1145-1150.

Compliance Recertification Application (CRA) for the Waste Isolation Pilot Plant. 2009a. Title 40 CFR Part 191; Subparts B and C; Appendix SOTERM-2009, Actinide Chemistry Source Term. United States Department of Energy — Carlsbad Field Office, Waste Isolation Pilot Plant; Carlsbad, NM.

Compliance Recertification Application (CRA) for the Waste Isolation Pilot Plant. 2009b. Title 40 CFR Part 191; Subparts B and C; Appendix HYDRO-2009, Hydrological Investigations. United States Department of Energy — Carlsbad Field Office, Waste Isolation Pilot Plant; Carlsbad, NM.

DeLong EF. 1992. *Archaea in coastal marine environments*. Proceedings of the National Academy of Science, USA 89: 5685-5689.

Domski PS, Beauheim RL. 2008. *Evaluation of Culebra brine compositions*. AP-125 Analysis Report. Sandia National Laboratories; Carlsbad, NM.

Francis AJ, Gillow JB. 1993. *Effects of microbial processes on gas generation under expected Waste Isolation Pilot Plant Repository Conditions*. Report SAND93-7036. Sandia National Laboratories; Albuquerque, NM.

Francis AJ, Gillow JB, Giles MR. 1997. *Microbial gas generation under expected Waste Isolation Pilot Plant repository conditions*. SAND96-2582 Report. Sandia National Laboratories; Albuquerque, NM.

Francis AJ, Dodge CJ, Gillow JB, Papenguth HW. 2000. *Biotransformation of uranium compounds in high ionic strength brine by a halophilic bacterium under denitrifying conditions*. Environmental Science & Technology 34: 2311-2317.

Gillow JB, Dunn M, Francis AJ, Lucero DA, Papenguth HW. 2000. *The potential of subterranean microbes in facilitating actinide migration at the Grimsel test site and Waste Isolation Pilot Plant*. Radiochimica Acta 88: 769-774.

Gillow JB, Francis AJ. 2006. *Microbial gas generation under expected Waste Isolation Pilot Plant Repository Conditions: Final Report*. Report BNL-96148-2011-IR. Sandia National Laboratories; Albuquerque, NM.

Gruber C, Legat A, Pfaffenhuemer M, Radax C, Weidler G, Busse H-J, Stan-Lotter H. 2004. *Halobacterium norcense sp. nov., an archaeal isolate from a bore core of an alpine Permian salt deposit, classification of Halobacterium sp. NRC-1 as a strain of H. salinarum and emended description of H. salinarum*. Extremophiles 8: 431-439.

Gunde-Cimerman N, Zalar P, de Hoog S, Plemenitas A. 2000. *Hypersaline waters in salterns—natural ecological niches for halophilic black yeasts*. FEMS Microbiology Ecology 32: 235-240.

Higgins DG, Sharp PM. 1988. *CLUSTAL: a package for performing multiple sequence alignment on a microcomputer*. Gene 73: 237-244.

Huber T, Faulkner G, Hugenholtz, P. 2004. *Bellerophon: A program to detect chimeric sequences in multiple sequence alignments*. Bioinformatics 20: 2317-2319.

Kjeldsen K.U., Jakobsen T.F., Glastrup J., Ingvorsen K. 2010. *Desulfosalsimonas propionicica, gen. nov., sp. nov., a halophilic, sulfate-reducing member of the family Desulfobacteraceae isolated from a salt-lake sediment*. International Journal of Systematic and Evolutionary Microbiology 60: 1060-1065.

Lane DJ. 1991. *16S/23S rRNA sequencing*. In: Stackebrandt E, Goodfellow M, editors. Nucleic Acid Techniques in Bacterial Systematics. New York: John Wiley & Sons, Inc. p. 133.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. *Clustal W and clustal X version 2.0*. Bioinformatics 23: 2947-2948.

Leonard PA, Strietelmeier BA, Pansoy-Hjelvik ME, Villarreal R. 1999. *Microbial characterization for the Source-Term Test Program (STTP) at Los Alamos*. Proceedings from the Waste Management Conference; Tucson, AZ. CONF-990201.

Leuko S, Legat A, Fendrihan S, Stan-Lotter H. 2004. *Evaluation of the LIVE/DEAD BacLight kit for detection of extremophilic Archaea and visualization of microorganisms in environmental hypersaline samples*. Applied and Environmental Microbiology 70: 6884-6886.

Li W-J, Chen H-H, Zhang Y-Q, Schumann P, Stackebrandt E, Xu L-H, Jiang C-L. 2004. *Nesterenkonia halotolerans sp. nov. and Nesterenkonia xinjiangensis sp. nov., actinobacteria from saline soils in the west of China*. International Journal of Systematic and Evolutionary Microbiology 54: 837-841.

Li W-J, Chen H-H, Kim C-J, Zhang Y-Q, Park D-J, Li J-C, Xu L-H, Jiang C-L. 2005. *Nesterenkonia sandarakina sp. nov. and Nesterenkonia lutea sp. nov., novel actinobacteria, and emended description of the genus Nesterenkonia*. International Journal of Systematic and Evolutionary Microbiology 55: 463-466.

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG. 1998. *Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S RNA*. Applied and Environmental Microbiology 64: 795-799.

Martínez-Murcia AJ, Acinas SG, Rodriguez-Valera R. 1995. *Evaluation of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hypersaline environments*. FEMS Microbiology Ecology 17: 247-256.

McCabe A. 1990. *The potential significance of microbial activity in radioactive waste disposal*. Experientia 46: 779-787.

McGenity TJ, Gemmell RT, Grant WD, Stan-Lotter H. 2000. *Origins of halophilic microorganisms in ancient salt deposits*. Environmental Microbiology 2: 243-250.

Martinez-Murcia AJ, Acinas SG, Rodriguez-Valera F. 1995. *Evaluation of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hypersaline environments*. FEMS Microbiology Ecology 17: 247-255.

Muyzer G, de Waal EC, Uitterlinden AG. 1993. *Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S RNA*. Applied and Environmental Microbiology 59: 695-700.

Neefs J-M, van de Peer Y, Hendriks L, de Wachter R. 1990. *Compilation of small ribosomal subunit RNA sequences*. Nucleic Acids Research 18S: 2237-2317.

Norton CF, McGenity TJ, Grant WD. 1993. *Archaeal halophiles (halobacteria) from two British salt mines*. Journal of General Microbiology 139: 1077-1081.

Oren A. 1999. *Bioenergetic aspects of halophilism*. Microbiology and Molecular Biology Reviews 63: 334-348.

Oren A. 2006. *The Order Haloanaerobiales*. In The Prokaryotes vol. 4, 3rd ed. Dworkin M., Falkow S., Rosenberg E., Schleifer K.-H., Stackebrandt E (eds). Springer; New York.

Oren A. 2011. *Thermodynamic limits to microbial life at high salt concentrations*. Environmental Microbiology 13: 1908-1923.

Ozcan B, Caliskan M, Cokmus C, Yildiz E, Gulyanar S. 2009. *Identification and characterization of halophilic archaea isolates from different parts of Turkey, Cankaya Salt Mine; Halobacterium sp*. CH11 16S ribosomal RNA gene, partial sequence. Unpublished submission to GenBank; accession number FJ686125.

Park JS, Vreeland RH, Cho BC, Lowenstein TK, Timofeeff MN, Rosenzweig WD. 2009. *Haloarchaeal diversity in 23, 121 and 419 MYA salts*. Geobiology 7: 515-523.

Pedersen K. 2005. *Microorganisms and their influence on radionuclide migration in igneous rock environments*. Journal of Nuclear and Radiochemical Science 6: 11-15.Porter D, Roychoudhury AN, Cowan D. 2007. *Dissimilatory sulfate reduction in hypersaline coastal pans: activity across a salinity gradient*. Geochimica et Cosmochimica Acta 71: 5102-5116.

Radax C, Gruber C, Stan-Lotter H. 2001. *Novel haloarchaeal 16S rRNA gene sequences from Alpine Permo-Triassic rock salt*. Extremophiles 5: 221-228.

Raskin L, Stromley JM, Rittman BE, Stahl DA. 1994. *Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens*. Applied and Environmental Microbiology 60: 1232-1240.

Rempe, Norbert. Principal Engineer/Geologist (Ret.), Waste Isolation Pilot Plant.

Saralov A.I., Kuznetsov B.B., Reutskikh E.M., Baslerov R.V., Panteleeva A.N., Suzina N.E. 2012. *Arhodomonas recens sp. nov., a Halophilic Alkane-Utilizing Hydrogen-Oxidizing Bacterium from the Brines of Flotation Enrichment of Potassium Minerals*. Microbiology 81: 630-637.

Sogin ML, Gunderson JH. 1987. *Structural diversity of eukaryotic small sunbunit ribosomal RNAs: evolutionary implications*. Annals of the New York Academy of Sciences 503: 125-139.

Sorokin D.Y., Zacharova E.E., Pimenov N.V., Tourova T.P., Panteleeva A.N., Muyzer G. 2012. *Sulfidogenesis in hypersaline chloride-sulfate lakes of Kulunda Steppe (Altai, Russia)*. FEMS Microbiology Ecology 79: 445-453.

Swanson JS, Norden DM, Khaing H, Reed DT. 2013a. *Biodegradation of organic complexing agents by halophilic microorganisms in brines*. Geomicrobiology Journal 30: 189-198.

Swanson JS, Simmons KA. 2013b. *Update on Microbial Characterization of WIPP Groundwaters*. Report LCO-ACP-20. LA-UR-13-20623. Los Alamos National Laboratory; Carlsbad, NM.

Swanson J.S., Simmons K.A., Norden D.M., Ams D.A., Reed D.T. 2012. *Status Report on the Microbial Characterization of Halite and Groundwater Samples from the WIPP*. LCO-ACP-12, LA-UR-12-22824. Los Alamos National Laboratory; Carlsbad, NM.

Takai K, Horikoshi K. 2000. *Rapid detection and quantification of members of the Archaeal community by quantitative PCR using fluorogenic probes*. Applied and Environmental Microbiology 66: 5066-5072.

Ventosa A, Marquez MC, Ruiz-Berraquero F, Kocur M. 1990. *Salinicoccus roseus gen. nov., sp. nov., a New Moderately Halophilic Gram-Positive Coccus*. Systematic and Applied Microbiology 13: 29-33.

Vreeland RH, Piselli Jr A F, McDonnough S, Meyers SS. 1998. *Distribution and diversity of halophilic bacteria in a subsurface salt formation*. Extremophiles 2: 321-331.

Vreeland RH, Rosenzweig WD, Powers DW. 2000. *Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal*. Letters to Nature 407: 897-900.

Vreeland RH, Straight S, Krammes J, Dougherty K, Rosenzweig WD, Kamekura M. 2002. *Halosimplex carlsbadense gen. nov., sp. nov., a unique halophilic archaeon with three 16S rRNA genes, that grows only in defined medium with glycerol and acetate or pyruvate*. Extremophiles 6: 445-452.

Vreeland RH, Jones J, Monson A, Rosenzweig WD, Lowenstein TK, Timofeeff M, Satterfield C, Cho BC, Park JS, Wallace A, Grant WD. 2007. *Isolation of live Cretaceous (121-112 million years old) halophilic Archaea from primary salt crystals*. Geomicrobiology Journal 24: 275-282.

Wang Y, Francis AJ. 2005. *Evaluation of microbial activity for long-term performance assessments of deep geologic nuclear waste repositories*. Journal of Nuclear and Radiochemical Science 6: 43-50.

Waste Isolation Pilot Plant Annual Site Environmental Report (WIPP ASER) for 2010. United States Department of Energy, DOE/WIPP-11-2225.

Weidler, GW. 2007. *Diversity of halophilic archaeal isolates from rock salt, Austria; Halobacterium sp. BIHGY150/14 partial 16S rRNA gene*. Unpublished submission to GenBank; accession number AM902591.

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APPENDIX TABLES

Table A-1: Closest Matches to Sequences Retrieved from Clear and Clayey Halite

Table A-2: Closest Matches to DNA Sequences Retrieved from WQSP-1 Groundwater (direct extract, incubations, and isolates)

Table A-3: Closest Matches to DNA Sequences Retrieved from WQSP-3 Groundwater (direct extract, incubations, and isolates)

Table A-4: Closest Matches to DNA Sequences Retrieved from PZ-13 Groundwater Incubations (aerobic and microaerophilic isolates and clones)

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Table A-1
Closest Matches to Sequences Retrieved from Clear and Clayey Halite

Clone/Group Designation	Closest BLAST Match/Closest Cultured Relative	Sequence Similarity	Source of Closest BLAST Match
Clear Halite/Direct Extract			
Clone B22	bacterium enrichment culture clone Jdg014	97%	sulfate-reducing enrichment from mine soil
	Escherichia coli str. GCA705	97%	air-borne bacteria
Clone B40	uncultured bacterium clone T2C24	99%	fecal sample
	Escherichia coli str. S1p8	99%	estuarine water sample
Clone B43	uncultured *β-Proteobacterium* clone PRTBB8731	99%	ocean water at 6000 m depth; Puerto Rico Trench
	Limnobacter thioxidans strain CS-K2	99%	sediment, freshwater lake, Germany
Clone B51	uncultured bacterium clone H05YRMYR06SP-38STGEOTHERMALENV	99%	Yellowstone thermal spring origin water
	Caulobacter leidyia str. MQS18	99%	soil
Clear Halite/175 g NaCl Enrichment			
Clone B26	uncultured organism clone ELU0041- T385-S-NIPCRAMgANb_000251	92%	human ileum
	Limnobacter thiooxidans strain SSW137	92%	mountain water, Azores
Clone B31	uncultured γ-Proteobacterium clone sh-xj11	97%	salt water lake, China
	Nevskia sp. KNF013	97%	freshwater lake Kasumigaura, Japan
Clone B33	*Limnobacter thiooxidans* strain SSW137	92%	mountain water, Azores
Clone B42	*Pelomonas puraquae* str. S3-119	99%	plant endophyte
Group B1	uncultured γ-Proteobacterium clone sh-xj11	99%	salt water lake, China
	Nevskia sp. KNF013	99%	Cu-Cr-As treated Douglas fir pole
Group A1	uncultured archaeon XKL54	99%	source not given
	Halobacterium noricense str. H4	99%	salt pan salt water, India
Group A2	haloarchaeon MK62-1	99%	source not given
	Halorubrum sp. CGSA-14	93%	Lake Ejinor, Inner Mongolia, China
Group A3	uncultured haloarchaeal clone 78	99%	salt water lake, China
	Halopenitus persicus str. DC30	93%	salt lake, Iran
Group A4	*Halolamina pelagica*	99%	solar saltern, China
Isolate A1	haloarchaeon MK62-1	99%	Source not given
	Halorubrum sp. CGSA-14	93%	Lake Ejinor, Inner Mongolia, China

Table A-1 (continued)

Clone/Group Designation	Closest BLAST Match/Closest Cultured Relative	Sequence Similarity	Source of Closest BLAST Match
Isolate A2	uncultured haloarchaeon clone XKL54	100%	salt lake, China
	Halobacterium noricense strain H4_DGR	100%	salt pans, India
Clear Halite/100 g NaCl Enrichment			
Group B1	*Salinicoccus roseus* str. MS21	99%	marine snow, 20 m, Adriatic Sea
Isolate B1	uncultured *Nesterenkonia* sp. clone T4317	96	raw cow milk
	Nesterenkonia sp. BSJ27	95%	jeotgal (Korean fermented seafood)
Isolate B2	*Salinicoccus roseus* str. MS21	98%	marine snow, Northern Adriatic Sea
Isolate F2	uncultured marine Ascomycete clone PRTBE749	99%	ocean water at 6000 m depth; Puerto Rico Trench
	Cladosporium bruhnei str. CPC 5101	99%	Cu-Cr-As treated Douglas fir pole
Clayey Halite/Direct Extract			
Group B1	bacterium β-Proteobacterium clone T13M-B1	99%	low-temp hydrothermal oxides, SW Indian Ridge
	Pelomonas aquatica str. R4-315	99%	plant endophyte
Group B2	uncultured bacterium clone OTU209	99%	seawater
	Limnobacter sp. 2A6	99%	Baltic sea water
Clayey Halite/100 g NaCl Enrichment			
Isolate B1	uncultured bacterium clone nbw828g11c1	97%	human skin microbiome
	Nesterenkonia sp. BSJ27	95%	jeotgal, Korean fermented seafood
Group A1	uncultured euryarchaeote clone DSFBPENV-12arc_5G	99%	brine pool water, Salton Sea Geothermal System
	Halopenitus persicus str. DC30	94%	salt lake, Iran
Isolate A1	*Halobacterium* sp. 2-24-7	99%	halite core, Sergipe Basin, Brazil
Isolate F3	uncultured marine *Ascomycete* clone PRTBE7491	99%	ocean water at 6000 m depth; Puerto Rico Trench
	Cladosporium bruhnei str. CPC 5101	99%	Cu-Cr-As treated Douglas fir pole
Isolate F4	*Engyodontium album* str. IHEM4198	100%	source not given
Clayey Halite/5X organics Enrichment			
Isolate B1	uncultured bacterium clone nbw828g11c1	97%	human skin microbiome
	Nesterenkonia alba str. CAAS 252	95%	cotton pulp mill treatment system
Isolate F1	*Phoma* sp. str. CPCC 408669	100%	anti-tumor fungus, source not given

Table A-2:
Closest Matches to DNA Sequences Retrieved
from WQSP-1 Groundwater (direct extract, incubations, and isolates)

Clone/Group Designation	Closest Cultured and Named Relative	Sequence Similarity	Source of Closest BLAST Match
Direct Extract (Dir; i.e. from raw groundwater, no incubation)			
Clone Dir-11	uncultured bacterium clone SN69	98%	pristine soil, Jidong oil field, China
	Thiohalophilus thiocyanatoxydans str. HRhD 2	98%	hypersaline habitat, Russia
Clone Dir-17	uncultured δ-Proteobacterium clone SC1-40	97%	South Atlantic Bight permeable shelf sediment
	Desulfobulbus rhabdoformis str. Mic5C02	91%	water-oil separation system, Norway
Clone Dir-24	uncultured δ-Proteobacterium clone 72 T12d+oil/	99%	oil-contaminated coastal sediment, France
	Desulfosarcina sp. SD1	94%	mangrove sediment
Clone Dir-25	uncultured bacterium clone b38	97%	Cr-contaminated tannery sludge, China
	Clostridium sp. str. P6	93%	brewery wastewater sludge, China
Clone Dir-31	uncultured *Chromatiales* clone TDNP_Wbc97_128_1_33	97%	wetland water, Tabla de Daimiel National Park, Spain
	No cultured relative		
Clone Dir-37	uncultured bacterium clone R3B1L	87%*	low-temperature, anaerobic sewage bioreactor
	Desulfovibrionales bacterium Spi55	83%*	lack rust exposed to hot ridge crustal fluids, Juan de Fuca Ridge, US
Clone Dir-39	uncultured organism clone MAT-CR-P1-A12	99%	hypersaline microbial mat, Candeleria Lagoon, Puerto Rico
	Cytophaga sp. Dex80-37	89%	East Pacific Rise
Clone Dir-40	bacterium enrichment culture clone Tol_45	99%	hydrocarbon-contaminated estuarine sediment, Wadi Gaza, Palestine
	Desulfotignum toluenicum str. H3	98%	toluene-degrading SRB from soil reservoir model column
Group Dir-1	uncultured δ-Proteobacterium clone LA30-B27	98%	lake water, Hawaii
	Desulfosalina sp. HTR2	98%	lake water, Hawaii

Table A-2 (continued)

Clone/Group Designation	Closest Cultured and Named Relative	Sequence Similarity	Source of Closest BLAST Match
Group Dir-2	*Marinobacter guineae* str. LMG 24047T	99%	Antarctica
Group Dir-3	*Anaerophaga* sp. TC371	99%	production water, high-temperature oil field, North Sea, Norway
Group Dir-4	*Sediminimonas qiaohouensis* str. YIM B024	99%	salt mine, Yunnan, China
Aerobic (Aer)			
Group Aer-1	*Halomonas* sp. Qphe 2	99%	petroleum-contaminated soil
Group Aer-2	*Alteromonadaceae* bacterium LA34A	99%	environmental sample, Hawaiian archipelago
Group Aer-3	*Thalassospira* sp. MCCC 1A01449	99%	marine
Group Aer-4	*Idiomarina* sp. FG-5	99%	microalgal culture
Isolate Aer-L1	*Bacillus* sp. J28	96%	black sand
	Bacillus aquimaris str. S6-14	96%	marine sediment, Palk Bay, India
Transitional (Tr)			
Clone Tr-6	*Alcanivorax* sp. YDC2-H	99%	deep-sea hydrothermal vent sediment, Indian Ocean
Clone Tr-8	benzene mineralizing consortium clone SB-1	97%	sulfate-reducing consortium
	Bacteroidetes bacterium M-phe-1	97%	phenanthrene-degrading consortium under methanogenic conditions
Clone Tr-30	uncultured bacterium clone Fe_B_121	96%	methane seep sediment, Eel River Basin, CA
	Desulfobacter vibrioformis str. B54	96%	water-oil separation system
Clone Tr-33	uncultured organism clone MAT-CR-P2-E05	97%	hypersaline microbial mat, Candeleria Lagoon, Puerto Rico
	Marinilabilia salmonicolor str. NBRC15948	96%	isolate type strain
Group Tr-1	*Anaerophaga* sp. TC371	99%	production water, high-temperature oil field, North Sea, Norway
Group Tr-2	*Desulfovibrio cavernae* str. H1MT	96%	deep subsurface brine, Germany
Group Tr-3	uncultured organism clone MAT-CR-P1-A12	99%	hypersaline microbial mat, Candeleria Lagoon, Puerto Rico
	Cytophaga sp. Dex80-37	88%	East Pacific Rise
Group Tr-4	*Halomonas* sp.Qphe 2	99%	petroleum-contaminated soil

Table A-2 (continued)

Clone/Group Designation	Closest Cultured and Named Relative	Sequence Similarity	Source of Closest BLAST Match
Group Tr-5	*Alteromonadaceae* bacterium LA34A	98%	environmental sample, Hawaiian archipelago
Group Tr-6	uncultured bacterium clone BR148	98%	Zhongyuan oil field, China
	Marinobacter guineae str. LMG 24047T	98%	environmental sample, Antarctica
Isolate B1	*Halanaerobium acetethylicum* str. EIGI	99%	DSM culture collection (3532)
Isolate B2	*Halanaerobium acetethylicum* str. EIGI	99%	DSM culture collection (3532)
Iron-reducing (IR)			
Clone IR-8	uncultured organism clone MAT-CR-P6-B08	98%	hypersaline microbial mat, Candeleria Lagoon, Puerto Rico
	Halanaerobium saccharolyticum	98%	type strain isolate
Clone IR-11	benzene mineralizing consortium clone SB-1	97%	sulfate-reducing consortium
	Bacteroidetes bacterium M-phe-1	97%	phenanthrene-degrading consortium under methanogenic conditions
Clone IR-46	*Peptostreptococcaceae* bacterium Col 18	99%	corrosive biofilm, North Sea Harbor, France
Clone IR-47	uncultured bacterium clone PWB00	94%	nitrate-treated production water, oil field, Denmark
	Desulfotomaculum geothermicum	90%	isolate
Group IR-1	*Clostridium* sp. DY192	96%	deep sea sediment, Indian Ocean
Group IR-2	uncultured bacterium clone Fe_B_121	96%	methane seep sediment, Eel River Basin, CA
	Desulfobacter vibrioformis str. B54	96%	water-oil separation system
Group IR-3	uncultured bacterium clone 16slpl112-1e08.p1k	99%	human intestinal pouch
	Enterobacter sp. GW31-6	99%	nvironmental sample, Antarctica
Isolate IR-1	*Marispirillum indicum* str. B1-42	99%	crude oil degrading culture from deep sea water, India
Isolate B3	*Halanaerobium acetethylicum* str. EIGI	99%	DSM culture collection (3532)
Isolate B4	*Halanaerobium acetethylicum* str. EIGI	99%	DSM culture collection (3532)
Isolate B5	*Halanaerobium acetethylicum* str. EIGI	99%	DSM culture collection (3532)
Isolate B6	*Clostridium sediminis*	97%	deep sea sediment, Indian Ocean

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Table A-3:
Closest Matches to DNA Sequences Retrieved
from WQSP-3 Groundwater (direct extract, incubations, and isolates)

Clone/Group Designation	Closest BLAST Match/ Closest Cultured and Named Relative	Accession Number	Sequence Similarity	Source of Closest BLAST Match
Direct				
Group DIR-B1	Uncultured bacterium clone HS-EN8B	FJ536432	97%	Solar saltern lake sediment, Mediterranean Sea
	Desulfosalina sp. HTR2	GQ922848	96%	Soda lake sediment, Altai, Kulunda Steppe, Russia
Group DIR-B2	Uncultured organism clone MAT-CR-P4-D11	EU246154	99%	Hypersaline microbial mat, Cabo Rojo, Puerto Rico
	Halanaerobium hydrogeniformans	CP002304	92%	Fresh water sediment
Clone DIR-B3	Uncultured microorganism clone Group14_b	JN387338	98%	Sulfur spring source sediment, Zodletone Spring, Oklahoma
	Truepera radiovictrix DSM 17093	CP002049	91%	Hot spring runoff, Island of Sco Miguel, Azores, Portugal
Clone DIR-B6	*Anaerophaga* sp. TC371	DQ647061	95%	Produced water, North Sea oil field
Clone DIR-B14	Uncultured α-Proteobacterium clone Y135	EU328078	99%	Moderate saline soil contaminated with crude oil
	Rhodospirillaceae bacterium strain YIM D812	NR_044596	98%	Type strain isolate
Clone DIR-B25	*Clostridiaceae* bacterium GQ6	JQ421331	99%	Yuncheng Salt Lake, China
Clone DIR-B29	*Microbacterium* sp. MOLA 56	AM990831	99%	Sea Water, North Western Mediterranean Sea, France
Clone DIR-B30	Uncultured bacterium clone 91-131	EF157158	97%	Heavy oil seeps, Rancho La Brea tar pits
	Candidate division TM7 clone GL2-37	DQ847438	96%	human skin
Clone DIR-B31	*Desulfosalina* sp. HTR2	GQ922848	99%	Soda lake sediment, Altai, Kulunda Steppe, Russia
Clone DIR-B35	*Bacteroidetes* bacterium G22	JQ683777	98%	Sediment sample

Table A-3 (continued)

Clone/Group Designation	Closest BLAST Match/ Closest Cultured and Named Relative	Accession Number	Sequence Similarity	Source of Closest BLAST Match
Clone DIR-B42	Uncultured organism clone MAT-CR-P1-A12	EU245984	100%	Hypersaline microbial mat, Cabo Rojo, Puerto Rico
	Prolixibacter bellariivorans strain JCM 13498	AB541983	89%	Type strain isolate
Aerobic				
Group AER-B1	*Chromohalobacter salexigens* strain JC125	HE662816	99%	Soil, India
Isolate B1	*Chromohalobacter salexigens* strain L0B	GU397381	99%	hypersaline lake, Iran
Transitional (aerobic → anaerobic)				
Group TR-B1	*Chromohalobacter salexigens* strain JC125	HE662816	99%	Soil, India
Nitrate-reducing (NR)				
Group NR-B1	*Arhodomonas recens* strain RS91	HQ833040	99%	Salt brines of potassium mineral refinement
Group NR-B2	*Chromohalobacter salexigens* strain JC125	HE662816	99%	Soil, India
Iron-reducing (IR)				
Group IR-B1	Uncultured organism clone MAT-CR-P6-B08	EU246275	99%	Hypersaline microbial mat, Cabo Rojo, Puerto Rico
	Halanaerobium saccharolyticum subsp. *saccharolyticum* strain DSM 6643	NR_026257	99%	Type strain isolate
Isolate B1	*Halanaerobium* sp. S191	FJ858788	99%	Northeast Pacific ocean sediment, China

Table A-4:
Closest Matches to DNA Sequences Retrieved from PZ-13
Groundwater Incubations (aerobic and microaerophilic isolates and clones)

Clone/Group Designation	Closest BLAST Match/ Closest Cultured and Named Relative	Sequence Similarity	Source of Closest BLAST Match
Aerobic			
Isolate B1	*Chromohalobacter salexigens* str. SM	100%	salted meat
Isolate A1	*Halococcus saccharolyticus*	98%	isolate, source unknown
Iron-reducing (IR) grown under microaerophilic conditions			
Group IR-B1	*Pontibacillus* sp. R147	99%	solar saltern lake sediment, Mediterranean Sea island
Group IR-B2	*Virgibacillus* sp. BH187	99%	China, no further information provided
Clone IR-B45	uncultured bacterium clone DGS2-40	96%	soil sample above oil and gas field, China
	Bacteroidetes bacterium MO54	94%	soil, Great Salt Plains, Oklahoma
Group IR-A1	*Haloferax* sp. Bej51	99%	salt lake, Algeria
Group IR-A2	*Haloarcula amylolytica*	99%	type strain isolate
Isolate B2	*Virgibacillus* sp. BH187	99%	China, no further information provided
Isolate A2	uncultured *Natrinema* sp. clone VKMM-8	94%	solar saltern, India
	Natrinema versiforme XF10	94%	Aibi salt lake, China

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