

ACRSP Cover Sheet

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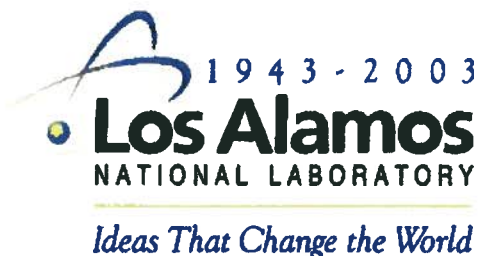
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January 18, 2012
EES-12-CO:11-280

Russ Patterson
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Department of Energy
Carlsbad Field Office
P.O. Box 3090
Carlsbad, New Mexico 88221

Dear Russ,

Enclosed, please find a completed draft of the microbiology update report entitled "Status Report on the Microbial Characterization of Halite and Groundwater Samples from the WIPP". This fulfills our ABC deliverable to provide this report to you by 1/20/12. Our intention is to publish this report as an internal Los Alamos LA-series report. Please provide technical comments to Julie Swanson, who is the lead author of the report. Dan Ferguson is also copied on this since some of the results reported pertain to groundwater samples obtained through his program and we are very grateful for this help.

Please call me (234-5559) if you have any other questions or need additional input.

Sincerely,

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2012

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LCO-ACP-12

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**STATUS REPORT ON THE
MICROBIAL CHARACTERIZATION
OF HALITE AND GROUNDWATER
SAMPLES FROM THE WIPP**

**STATUS REPORT ON THE
MICROBIAL CHARACTERIZATION OF
HALITE AND GROUNDWATER
SAMPLES FROM THE WIPP**

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EXECUTIVE SUMMARY

This report summarizes the progress made in the ongoing task of 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.

Members of the three biological domains—*Bacteria*, *Archaea*, and *Eukarya* (in this case, *Fungi*)—have been identified that are associated with WIPP halite. Thus far, their activity has been limited to aerobic respiration and denitrification; anaerobic incubations are underway.

WIPP halite constitutes the near-field microbial environment. We expect that microbial activity in this setting will proceed from aerobic respiration, through nitrate reduction to focus on sulfate reduction. This is also the current WIPP performance assessment (PA) position. Sulfate reduction can occur at extremely high ionic strengths, and sulfate is available in the WIPP as a brine component and in the anhydrite interbeds. The role of methanogenesis in the WIPP remains unclear, due to both energetic constraints imposed by a high-salt environment and substrate selectivity, and it is no longer considered in PA.

Archaea identified in WIPP halite thus far fall exclusively within the family *Halobacteriaceae*. These include *Halobacterium noricense*, cultivated from both low- and high-salt media, and a *Halorubrum*-like species. The former has also been detected in other salt mines worldwide; the latter likely constitutes a new species. Little is known of its function, but it was prevalent in experiments investigating the biodegradation of organic complexing agents in WIPP brines. Bacterial signatures associated with WIPP halite include members of the phylum *Proteobacteria*—*Halomonas*, *Pelomonas*, *Limnobacter*, and *Chromohalobacter*—but only the latter has been isolated. *Fungi* were also enriched from halite. 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 a nearby borehole groundwater are capable of aerobic respiration, fermentation, metal reduction, and sulfate reduction and are distributed across many different phyla. 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.

Because the majority of the repository's lifetime will be anoxic, ongoing and future work focuses on the presence and role of anaerobic organisms in WIPP. Further tasks on biosorption, cellulose degradation, and bioreduction are being performed using organisms obtained from this characterization work.

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INTRODUCTION

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 May 1998 as a terminal repository for transuranic (TRU) waste and is currently operated by the Department of Energy, Carlsbad Field Office (DOE-CBFO). According to federal regulations (40CFR191/194; USEPA 1998), 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 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 narrow and further define the scope of possible microbial interactions with waste components and 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 a borehole groundwater/brine seep proximal to a salt mine tailings pile. The first part of this report will address halite, and the second focuses on groundwater. Methods that are used in characterizing both sample types will be presented in detail in the first part of this report and referred to with any modifications for the second part.

BACKGROUND

The WIPP Conceptual Model for Microbiology

The WIPP mandate is to limit the release of radionuclides from the repository during its 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 CO₂ production and leading to actinide-carbonate complexation, 2) the production of CO₂ 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; 6) degradation of organic ligands; 7) generation of organic ligands; and 8) biosorption 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 production of CO₂ and biosorption are considered by the model to be significant microbial contributions.

The WIPP conceptual model for microbial activity 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 cellulose, plastics, and rubber (CPR) are degraded ($P = 0.25$); and significant microbial activity will occur in which only cellulose will be degraded ($P = 0.75$; SOTERM, 2009). 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 CO_2 and control its fugacity and to buffer the pH to ~ 9 to minimize actinide-carbonate complexation.

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.

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 to predict gas generation from the degradation of organic waste components, to identify radiation-resistant organisms in the waste, to identify organisms in the environment surrounding the waste, to examine the toxicity of actinides to these microorganisms, and to evaluate the potential for organisms to degrade waste organics.

Very early work on WIPP-related microbiology was conducted with microorganisms found at a TRU burial site in Los Alamos, NM. Although some experiments were carried out using brine, they utilized soil microorganisms from this site as their inocula (Barnhart et al., 1978, 1979). 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 isolated or 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 $\sim 10^4$ 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, 1994).

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. Only two WIPP isolates have been characterized and published in the literature—a novel genus, *Halosimplex carlsbadense* and a *Virgibacillus* species isolated from a fluid inclusion (Vreeland et al., 2000, 2002).

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^+ , 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 some 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, 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 and 2011). All of these processes are either energetically favorable or are performed by organisms who 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) the high ionic strength; 3) the 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.

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 EDTA—will be the predominant low-molecular-weight carbon substrates, but cellulose will contribute significantly to the carbon inventory. The availability of these substrates will depend upon their dissolved concentrations in brine, which are mostly below the inventory-predicted levels (Swanson et al., 2012, in press). 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.

Extreme haloarchaea, similar to those found in WIPP halite, are capable of denitrification and fermentation, suggesting that these activities are likely to proceed. Sulfate reducers (Bacteria) have so far not been found in subsurface halite, although it is probable that they were never sought. Francis et al. (2005) noted a sulfide precipitate in their long-term incubations which they attributed to the presence of SRB; however, these incubations were also inoculated with brine lake sediment, the most likely source of these organisms. SRB have been found in other hypersaline environments (i.e. brine lakes, solar salterns; Porter et al., 2007; Sørensen et al., 2009). Their presence in brine seeps is unknown, but even so, they may be introduced with brine inundation from the Castile. Sulfate is present in the 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 intermediate-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 anaerobic Bacteria) will dominate within the repository; while, both halophilic Archaea and Bacteria will be present in the intermediate-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.

PART 1. WIPP HALITE

Overview

To date, two types of halite (clear and “green/gray”) 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 the raw halite without enrichment. Microbial isolates have been obtained and identified, and some are being used in further studies on metal/actinide sorption and in biodegradation studies. Differences are seen between the two halites that may be due to the presence of a clayey substance in the green/gray halite, but most differences arise from the variation in salt concentration in the media. Anaerobic enrichments are being monitored, but no biological results are yet available.

Objective

Halite samples for earlier study were obtained from the oldest part of the mine that 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. Additionally, 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. This perspective needs to shift to a focus on the more limited metabolic lifestyle exhibited by organisms at high ionic strengths.

Materials and Methods

Halite Sample Collection and Handling. Halite samples were retrieved from three sites along the north wall of panel 5, Room 7 (see Figures 1 a and b). A crowbar was used to remove the top layer of halite which was discarded, 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; $23 \pm 3^\circ\text{C}$). Halite samples with different physical appearances (“clear” versus “green/gray”) were segregated.

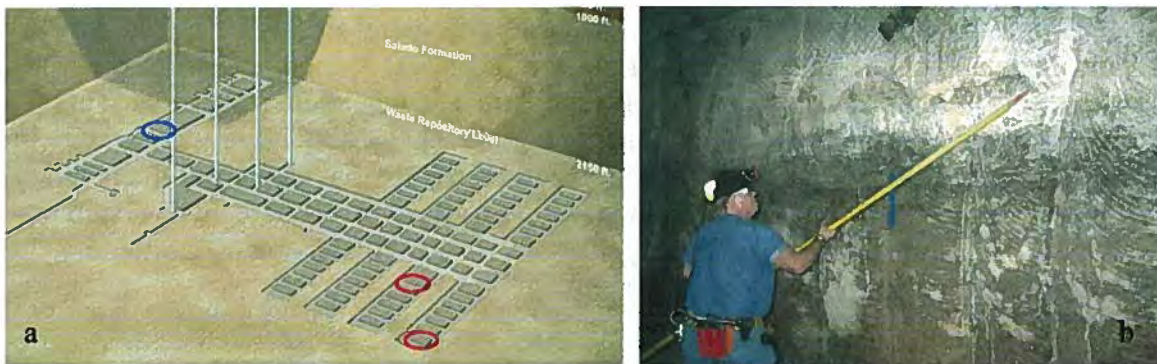


Figure 1. a) sampling sites for current work circled in red. Approximate sampling site from Vreeland study circled in blue. b) outer layer of halite being removed to expose underlayer for sampling.

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 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.

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.

Cultivation. Because this study was designed to see 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. Aerobic medium components were as follows in g/L: NaCl, 100 and 175; yeast extract, 0.5; Hy-Case casamino acids, 0.5; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 20; KCl, 2; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.2; sodium pyruvate, 0.015; Tris-HCl, pH 8.5, 1.1 ml of 1 M stock; ATCC 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 restreaked for purity onto the same type of agar that promoted their original growth.

DNA Extraction from Raw Halite. Twelve and fifty-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.

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.

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 MgCl₂, 250 μM each dNTP, 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).

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., 1985
Arch21f	TTC CGG TTG ATC CTG CCG GA	Archaea (21-40)	DeLong, 1992
Univ1392r	ACG GGC GGT GTG TRC	Universal (1406-1392)	Olsen et al., 1986
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.

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, then stained for 30 minutes with the nucleic acid stain SYBR Green I.

Amplified Ribosomal DNA Restriction Analysis (ARDRA). Amplicons from archaeal isolates were screened by digestion with *Mbo*I and *Hin*fI (Martinez-Murcia et al., 1995). Each 50 μl reaction contained 5 U of enzyme in appropriate buffer (New England BioLabs; Ipswich, MA) and 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.

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* DH5 α [™]-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.

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 Bellerophon (comp-bio.anu.edu.au/bellerophon/bellerophon.pl; 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, as opposed to each individual sequence. 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, and edited in TreeView 1.6.6 (taxonomy.zoology.gla.ac.uk/rod/treeview). The 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).

Results

Chemistry of Halite

The figure below shows an example of the two types of halite that are being investigated: "clear" versus "green/gray".

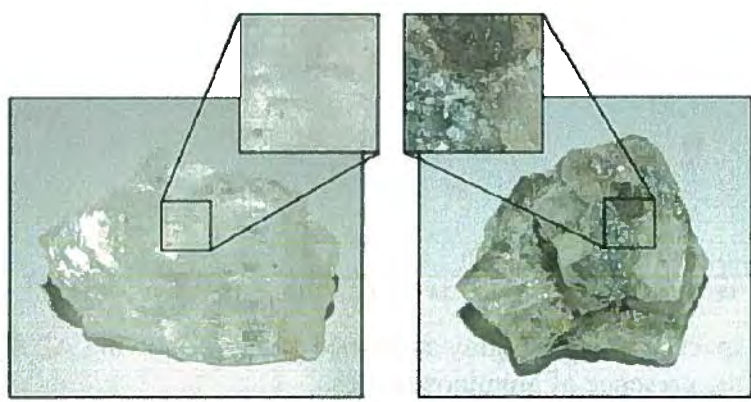


Figure 2. Samples of "clear" and "green/gray" halite.

Results for the significant components of both these sample types are given 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	Green/gray halite
------------------	--------------	-------------------

	µg/g halite	µg/g halite
Cl ⁻	5.81 x 10 ⁵	4.48 x 10 ⁵
Br ⁻	72.4	500
SO ₄ ²⁻	406	4.75 x 10 ³
B (as B ₄ O ₇)	---	575
Na ⁺	1.38 x 10 ⁵	1.11 x 10 ⁵
Mg ²⁺	27	8.62 x 10 ³
K ⁺	202	5.34 x 10 ³
TOC (mg/L; at limit of detection)	0.122	---
pH*	7.26	7.17

--- indicates not detected in the given sample (due to insufficient sample size, levels of analyte below the detection limit for the given instrumentation, or analyte actually not present in sample). *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. As a result, this halite is referred to hereafter as “clayey” halite.

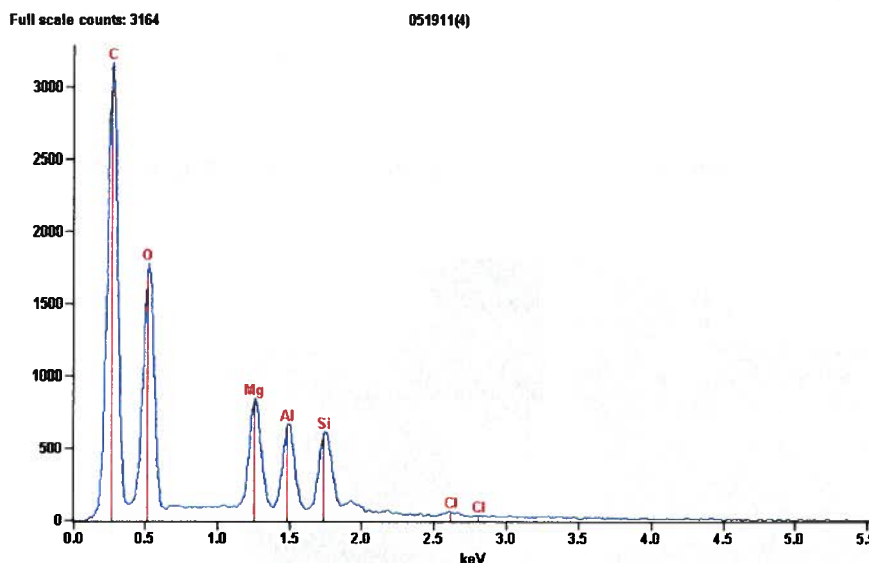


Figure 3. EDS spectrum of green/gray substance in halite showing Al, Si, and O, consistent with the presence of aluminosilicates.

Microbial Characterization Results

Summary

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

Table 3. Summary of cultivation and molecular results from halite processing

DNA extracted from	Clear	Clayey
Raw halite sample (direct) (2-50g clear halite; 50-150g clayey halite)	+ bacterial (nested)* - archaeal (nested) - eukaryotic	+ bacterial (nested) ± archaeal (nested)** - eukaryotic
Halophile Medium with 100g NaCl (+ 50g halite as inoculum)	+ growth (8 weeks) + bacteria + archaeal - eukaryotic	+ growth (8-10 weeks) + bacterial** + archaeal + eukaryotic**
Halophile Medium with 175g NaCl (+ 50g halite as inoculum)	+ growth (6-8 weeks) + bacterial (nested) + archaeal - eukaryotic	- growth (1 st attempt) + growth, (2 nd attempt, 5X substrates added; 8 weeks) + bacterial (5X) - archaeal (5X) - eukaryotic (5X)

*resulting DNA sequences too short for meaningful phylogenetic assignment

**not suitable for downstream analysis (i.e. cloning and sequencing); most likely PCR is inhibited by high metal concentrations, currently attempting to optimized procedure to account for this.

Approximately 8 weeks was required to see growth in aerobic cultures, regardless of the concentration or type of salt used. A nested PCR protocol was required to amplify both bacterial and archaeal DNA from raw crystals of both halite types, and no eukaryotic DNA signatures were found. In low salt medium (100g NaCl + 50g halite inoculum \approx 2.6 M NaCl), bacterial and archaeal DNA was amplifiable from both salt types; while, eukaryotic DNA was amplified from the clayey halite alone. In high salt medium (175g NaCl + 50g halite inoculum \approx 3.9 M NaCl) with clear halite as the inoculum, archaeal DNA amplification did not require a nested protocol; while, bacterial did. In the clayey halite incubations, no growth was observed. A second culture, whose medium contained five times the nutrient content of the original, yielded growth from the clayey halite. Both bacterial and archaeal DNA was amplifiable from this incubation.

The closest matches to the DNA sequences retrieved through cloning and from isolates are given in Table A1 (see Appendix).

Archaeal Isolates

Archaea were isolated from both low and high-salt media. Since the cultivated Archaea cannot be grouped morphologically due to their identical appearance on plated media and their pleomorphism when viewed microscopically, isolates must be screened by ARDRA. Almost all of those screened thus far from both clear and clayey halite have yielded the same pattern, and representatives were submitted for sequencing (see Figure 4).

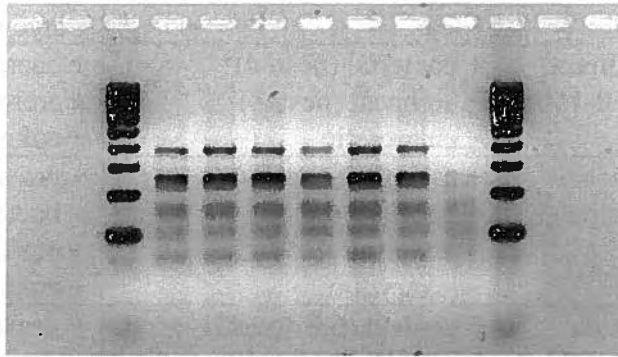


Figure 4. Example of a restriction digest analysis of amplified DNA from Archaea isolated from clear halite; similar patterns reflect similar DNA sequence. Reference “ladder” in end lanes denotes size of DNA fragments.

The closest matches to the DNA sequences retrieved through cloning and from isolates are given in Table A1 (see Appendix). In summary, most match most closely (99% similarity) to *Halobacterium noricense*, although one isolate has matched with low similarity (93%) to a *Halorubrum*-like species, suggesting it is a novel genus. There is currently no cultivated relative listed in the ribosomal RNA databases.

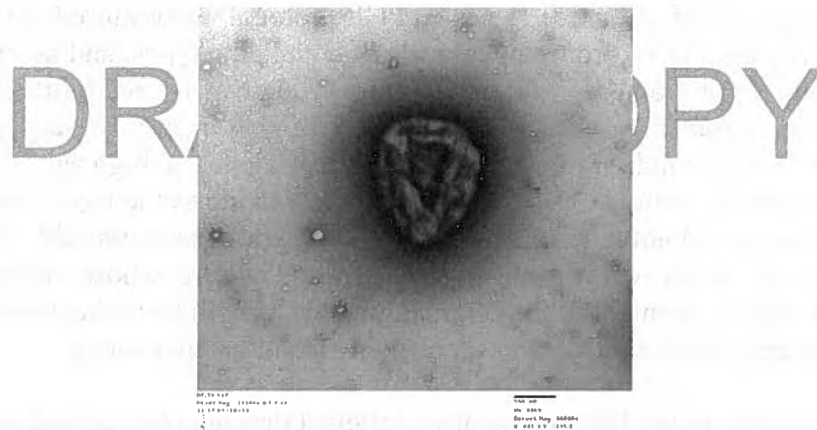


Figure 5. Unique haloarchaeon isolated from WIPP halite.

Bacterial Isolates

Bacteria have thus far only been isolated from the incubations of halite at lower salt concentrations, and three have been sequenced to date. One appeared in both clear and clayey halite incubations (*Nesterenkonia* sp.); one was unique to the clear halite (not yet identified, Gram-positive coccus), and one was unique to the clayey (*Chromohalobacter* sp.). The results for DNA sequencing are provided in Table A1.

Fungal Isolates

Filamentous organisms also appeared in the low-salt incubations and in the nutrient-rich (5X)-high salt incubation. These were identified putatively by DNA sequence analysis as members of the Fungi, but further characterization has not yet been performed. One was

common to both clear and clayey halite low-salt cultures (*Cladosporium* sp.); one other was found in the clayey halite low salt culture (*Tritirichium* sp./ *Engyodontium* sp.); and one was isolated from the clayey halite high salt/5X nutrient culture (*Coniothyrium fuckelii*).

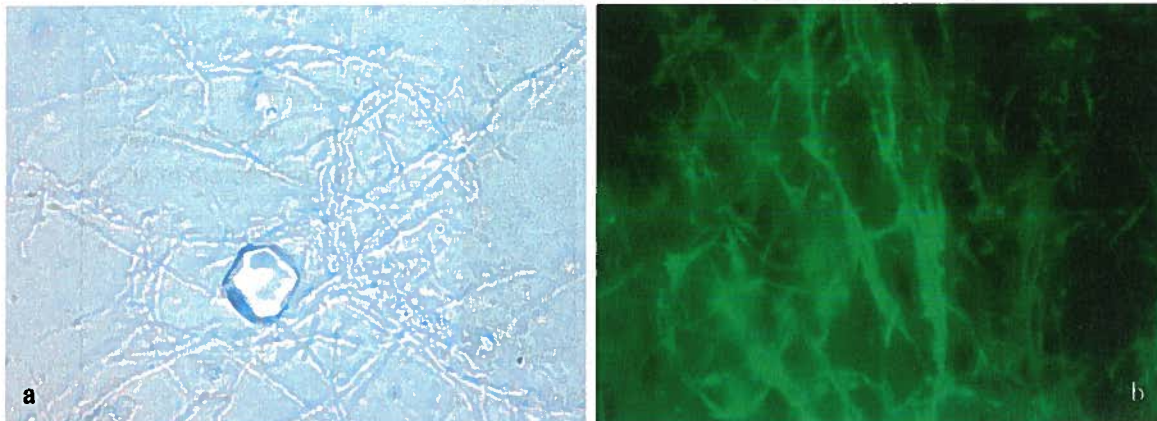


Figure 6. Filamentous organisms in both low- and high-salt incubations; bright field image (left), organisms stained with BacLight Live/Dead stain (right), green = alive.

Phylogenetic Distribution of Clones

Clone libraries were constructed from the amplified DNA extracted directly from raw halite (clear and clayey direct) and from the clear halite culture in 175g NaCl (clear 175g). Libraries for the clear halite in 100g NaCl and clayey-175g-5X are in progress. Figures 7a and 7b show the makeup of each library constructed for Bacteria (a) and Archaea (b).

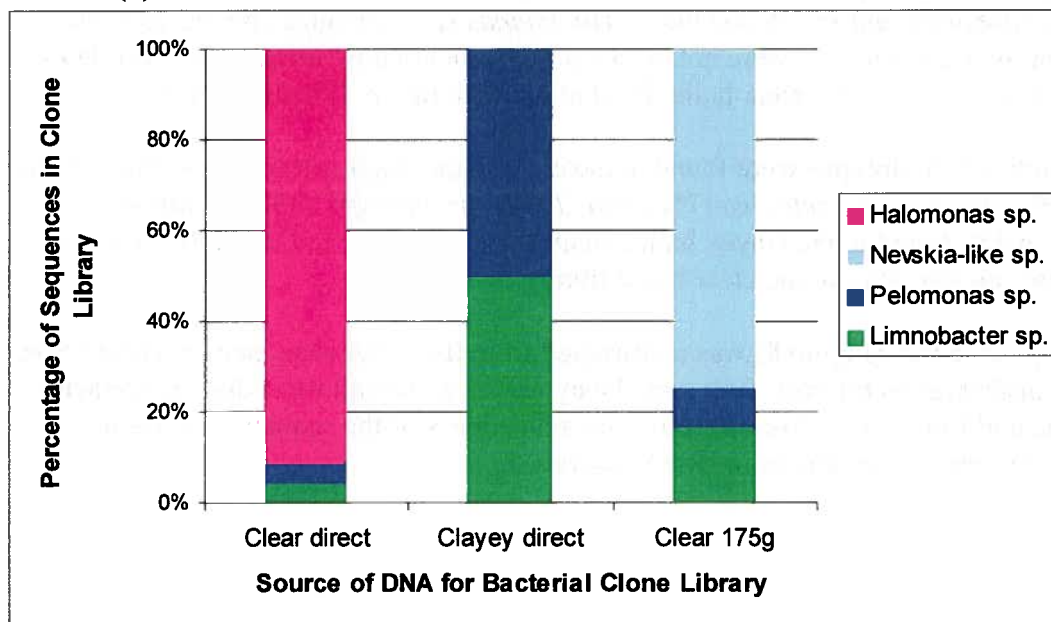


Figure 7a. Bacterial clone libraries from direct DNA extracts of both halites and the clone library from the clear halite-175g NaCl enrichment culture. Note loss of *Halomonas* species in high-salt incubations of clear halite and absence of same in clayey halite.

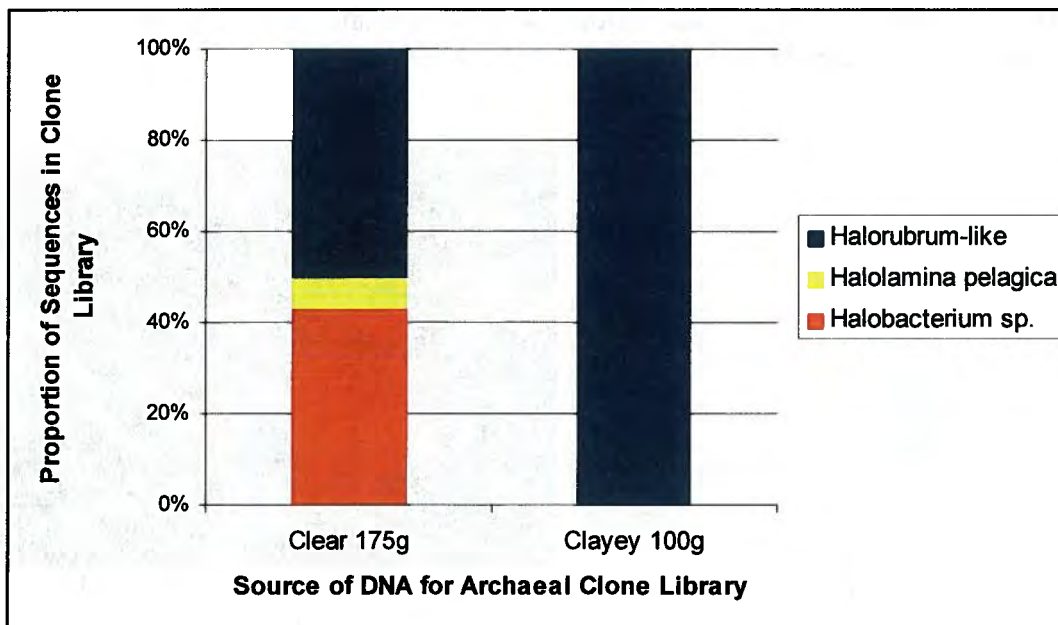


Figure 7b. Archaeal clone libraries from enrichment cultures of clear halite with 175g NaCl and clayey halite with 100g NaCl. Note lack of diversity in clayey halite incubation, possibly due to low salt concentration.

A total of 4 bacterial phylotypes were detected between the two halites. The clayey halite library was distributed evenly between *Limnobacter* and *Pelomonas* spp. These two phylotypes were also present in the clear halite raw (4% each) and high salt libraries (17% *Limnobacter* and 8% *Pelomonas*). *Halomonas* sp. signatures comprised 92% of the raw, clear halite library but were not detected in the clear halite incubation. *Nevskia* sp. signatures appeared in the clear halite incubation, making up 75% of this library.

Three archaeal phylotypes were found in the clear halite, high-salt library—*Halorubrum*-like (50%), *Halolamina pelagica* (7%), and *Halobacterium* spp. (43%). Only one phylotype was found in the clayey halite incubation; this belonged to the *Halorubrum*-like organism also seen in the clear halite library.

A phylogenetic tree (Figure 8) was constructed from the DNA sequences retrieved from 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.

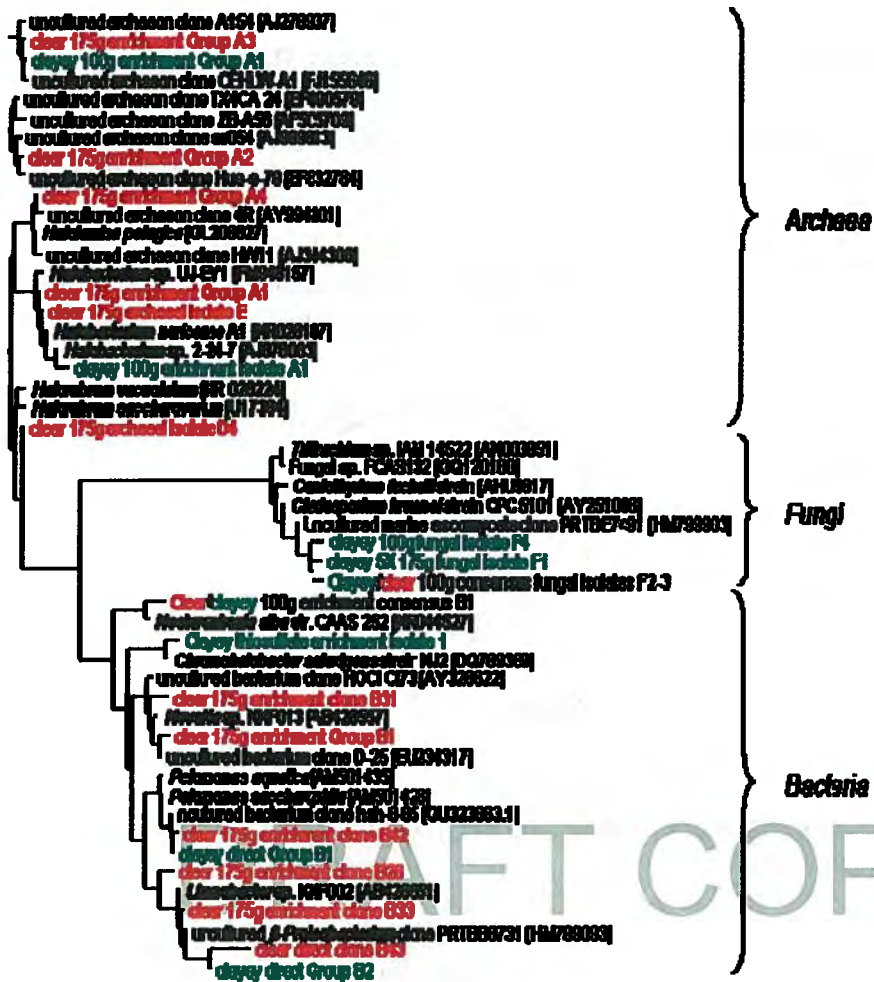


Figure 8. Unrooted phylogenetic tree constructed from clone libraries and isolate sequences of small subunit ribosomal RNA gene sequences amplified from clear (red) and clayey (blue) halite enrichment cultures in medium containing 100 g and 175 g NaCl. Scale bar represents one nucleotide substitution per ten base positions.

Discussion

Success of Growth and DNA Amplification

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. Community structural differences were more likely a result of different salt concentrations rather than halite types, although the potential inhibitory effects of the clayey substance cannot be ruled out. For example, a larger amount of dissolved halite was required in order to obtain amplifiable DNA from the raw, clayey halite than from the clear. While this could easily be due to the inhibitory effects of the clayey substance on PCR, it could also be due to lower cell numbers. Additionally, growth from the clayey halite was only successful on the second attempt. This may be due to the additional nutrients in the medium that offset those that may have sorbed onto the clay. Or, 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.

Community Structural Differences between Clear and Clayey Halite

As shown in Figures 7a and b and in Table A1, there were differences in community structure between the two halites, and 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 an unidentified Gram-positive coccus 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.

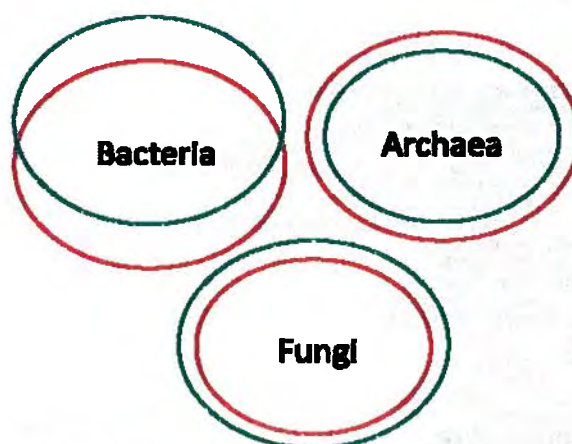


Figure 9. Diagram showing structural relationship between communities found in clear (red) and clayey (green) halites.

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 true for the *Halorubrum*-like organisms, although they were not obtained in pure culture from the clayey halite. Because little is yet known about the Grampink-pigmented isolate from the clear halite, it is not possible to infer anything about its absence from the clayey.

Community Structural Differences between Low- and High-Salt Incubations

Because there are no clone library data yet for bacteria in the low-salt incubations, it is not possible to compare this domain. However, nested PCR was required to detect bacteria in both the clear-175g culture and the 5X organics-175g-clayey culture, suggesting low numbers of organisms in both. Still, bacteria were isolated only from the low-salt incubations. This is not surprising given that Archaea tend to dominate communities at high ionic strength. However, since both the *Nesterenkonia* sp. and *Chromohalobacter* sp. isolates were shown to survive in high-salt media (20-25%) as pure cultures; it is possible that they were outcompeted by the Archaea in the mixed cultures.

Potential Metabolic Capability of Halite Community and Relevance to WIPP

Bacteria

With the exception of *Nesterenkonia*, all of the Bacteria thus far identified 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*, in sulfur cycling. However, none of these bacteria thus far identified represent obligate anaerobes. *Halomonas* sp., *Pelomonas* sp., and some *Chromohalobacter* sp. can be considered facultative, and have been shown to reduce nitrate.

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*, *Halomonas*, and *Chromohalobacter* have been isolated previously from marine environments, but only the latter two, especially *Chromohalobacter* are 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.

Thus, *Chromohalobacter* stands the best chance for survival in 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. It is very likely that isolate WIPP 1A retrieved by Francis et al., (1998), that was previously identified as a *Halomonas* sp. actually belongs to the *Chromohalobacter*. These genera are very similar and had not been separated taxonomically at the time of the original report.

Nesterenkonia sp. fall within the *Actinomycetes*, of which a few members are halophilic. *Actinomycetes* have been shown to degrade recalcitrant hydrocarbons in soil, and many *Nesterenkonia* can utilize cellulose, so this organism may be tested for this ability. They are also common producers of antimicrobial agents, and this may explain the lack of other bacterial isolates, although no antimicrobial effect has been seen on plated media with the Archaea. This organism is also alkaliphilic and is currently maintained on media at pH 9.5 and 25% (w/v) NaCl. The ability of this organism to survive under anaerobic conditions is not well documented in the literature and is under investigation.

Fungi

All the isolated Fungi belong to the *Ascomycota*, an order known to contain other extremophilic members, although only *Cladosporium* has been mentioned in the literature. Halophilic fungi have only recently been discovered (Gunde-Cimmerman et al., 2000), so there is little known about their function 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. Secondly, 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. Thirdly, *Ascomycota* degrade dead plant matter, so their ability to utilize cellulose may be investigated. However, these organisms have been disappearing from the anaerobic incubations, suggesting they are obligately aerobic, so their survival in brine may be a moot point.

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 GWB and ERDA-6-based brines and are capable of degrading three of four organic complexing agents found in the WIPP (Swanson et al., 2012 in press). Many haloarchaea are also able to reduce nitrate. Their ability to thrive and degrade organic complexing agents under more reduced conditions is now being investigated. 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 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 is relevant to other salt repositories, such as Asse and Gorleben in Germany.

It is also noteworthy that the one archaeal sequence (*Halorubrum*-like) retrieved from the clayey-100g culture was very closely related to those found in the clear-175g halite enrichment culture. It 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. This organism seems able to tolerate a broad range of growth conditions in liquid culture (e.g. low vs. high salt, low vs. high Mg).

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 they will certainly remain present.

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PART 2: WIPP GROUNDWATERS

Overview

To date, four groundwaters from the WIPP environs have been sampled (WQSP-1, WQSP-3, H4b-R, PZ-13; see Figure 10).

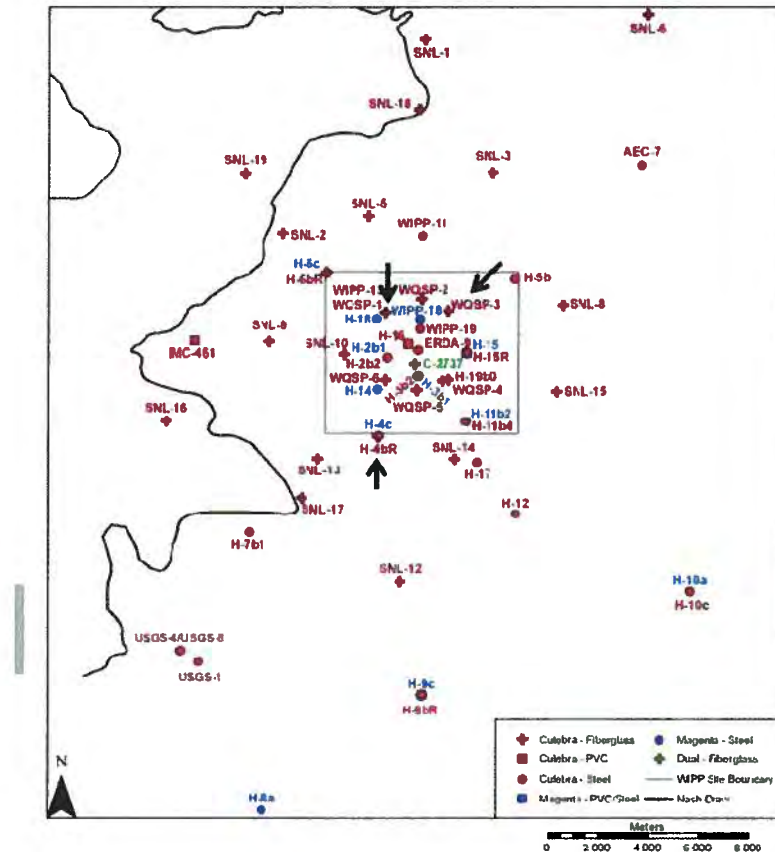


Figure 10. Map of WIPP monitoring wells. Arrows denote wells sampled; rectangle outlines WIPP site (HYDRO-2009).

Nucleic acids were directly extracted from these samples, and chemical analyses were also performed. Aerobic and anaerobic incubations were set up. A complete set of results is available for WQSP-1 and a partial set for PZ-13. 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; one is being investigated for metal reduction and another for sulfate reduction. WQSP-3 incubations are still in progress. Results for H4b-R were ambiguous due to the inability to control for outside contamination and are no longer under consideration.

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 (DOE/WIPP ASER 2010). Should this happen, 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 biosorption. 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. In general, actinide reduction yields a less soluble form which, after precipitation, is effectively immobilized. Metal reduction at ionic strengths higher than those in marine settings (i.e. 0.7 M) has been documented for manganese but not for iron, likely due to its low solubility in these matrices and because the higher concentrations of both manganese and sulfate make these preferred electron acceptors. One of the goals of this work is to obtain metal-reducing microorganisms that prefer higher ionic strengths for growth. These organisms will then be used in further studies on actinide reduction that can simulate conditions in the Culebra under a release scenario.

A second process through which Culebra microorganisms might affect actinide mobility is through biosorption. 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 and 2) that the surface of biomass available for sorption will be far less than that of its pathway. There is little work on biosorption processes at high ionic strengths, and so one goal of this work is to determine the extent of sorption onto organisms obtained from WIPP groundwaters at higher ionic strengths.

Materials and Methods (see also Part I)

Groundwater Sampling and Handling. WQSP-1 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 removing 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.

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 was performed as described in Part 1. pH was measured with reference to a standard hydrogen electrode.

Media Preparation. Media were prepared for the following cultivation conditions for the groundwater from WQSP-1: aerobic, transitional (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, iron-reducing, sulfate-reducing, and methanogenic. Fermenting organisms may be enriched from any of the anaerobic 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	Aerobic (Aer)	Transitional (Tr)	Nitrate-reducing (NR)	Iron-reducing (IR)	Sulfate-reducing (SR)	Methanogenic (M)
Carbon sources	Acetate, pyruvate, lactate	Acetate, pyruvate, lactate	Acetate, pyruvate, lactate	Acetate, pyruvate, lactate	Acetate, pyruvate, lactate	Formate, methanol
Nutrients and supplements	NH ₄ Cl, KH ₂ PO ₄ , yeast extract, casamino acids	NH ₄ Cl, KH ₂ PO ₄ , yeast extract, casamino acids, trace elements, vitamins	NH ₄ Cl, KH ₂ PO ₄ , yeast extract, casamino acids, trace elements, vitamins	NH ₄ Cl, KH ₂ PO ₄ , yeast extract, casamino acids, trace elements, vitamins	NH ₄ Cl, KH ₂ PO ₄ , yeast extract, casamino acids, trace elements, vitamins	NH ₄ Cl, KH ₂ PO 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 ₂ SO ₄	None provided (CO ₂ in headspace)
Buffer	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
Reductant	None	None	None	None	Cysteine	Cysteine-sulfide

A low-nutrient agar medium was prepared using ~500 mL of the groundwater sample 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. Iron(III)-citrate, nitrate, and sulfate were added as concentrated stocks to separate bottles to achieve a final concentration of 10 mM each. Transitional media received no exogenous electron acceptor. Methanogenic media contained 5 mM sodium formate and 5 mM methanol. Vitamins and trace elements were added to anaerobic media; MOPS was added to buffer media between 6.8-7.2. Resazurin was added as an indicator of oxygen presence.

Microbial Cultivation. One mL aliquots were removed from the aerobic sample bottle 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.

DNA-based Analyses. DNA was extracted directly from the water sample within 8 hours of its collection. Cells were concentrated onto a 0.22 micron filter, which was 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 DNA extraction. Cells were concentrated by centrifugation, and the pellet was resuspended in 100 µL of 0.85% (w/v) saline, 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.

Isolate Screening. Numerous isolates were purified (especially from aerobic incubations) and identified by PCR amplification of extracted DNA followed by sequencing. Others of interest were further characterized for biochemical capabilities using the API 20NE biochemical strip (biomérieux; Durham, NC). Others were studied for optimum NaCl concentrations and pH for growth.

Results

WQSP-1

Chemistry

Results of ICP-MS, IC, and TOC analysis on WQSP-1 are given in the table below.

Table 5. WQSP-1 Chemistry

Significant Analyte	µg/ml	M
Na ⁺	7.96 x 10 ³	0.35
Ca ²⁺	1.95 x 10 ³	0.05
Mg ²⁺	1.21 x 10 ³	0.05
K ⁺	553	0.01
Cl ⁻	35470	1.00
SO ₄ ²⁻	4464	0.05
pH	7.14	N/A
TOC	0.386*	N/A

*at limit of detection

Direct Counts

Direct microscopic counts of the raw WQSP-1 groundwater were $(2.37 \pm 0.96) \times 10^5$ cells/ml.

Microbial Growth in Incubations

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. Sulfate-reducing incubations were eventually discarded as no growth occurred. 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 11). No turbidity was noted in methanogenic incubations, other than a chemical precipitate. These incubations were allowed to proceed for a year, at which time a small amount of methane was detected by GC, but not quantified, in each replicate incubation. Samples were withdrawn for DNA analysis.

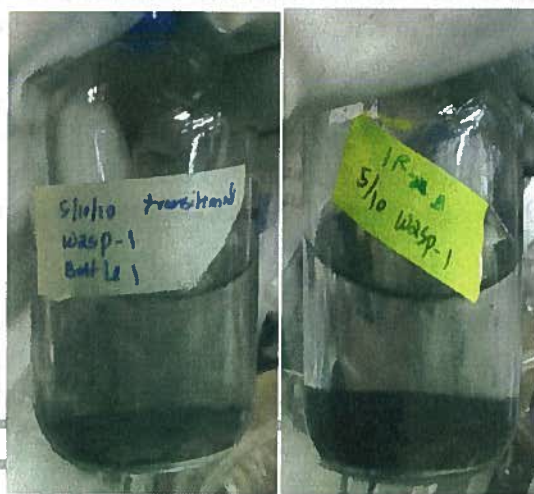


Figure 11. Sulfide precipitation resulting in transitional and iron-reducing incubations, suggesting microbial community ultimately driven toward sulfate reduction.

Plated Cultures and Isolates

Many aerobic and and microaerophilic isolates were obtained from the incubations. These are currently being screened.

DGGE

Resolution of amplified bacterial DNA from the Trans and IR incubations is shown in Figure 12 below.

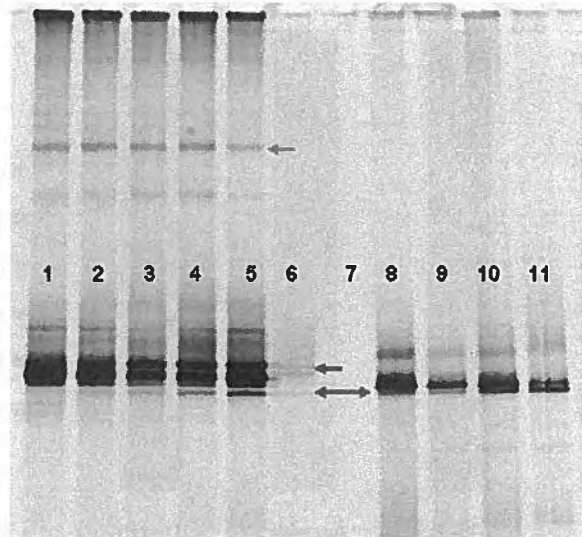


Figure 12. Denaturing gradient gel resolving amplified 16S rRNA-encoding bacterial DNA from WQSP-1 direct sample, transitional incubations, and iron-reducing incubations. Lanes 1-5, transitional incubations over time; lane 6, direct extraction without incubation; lanes 7-11, iron-reducing incubations over time. Note shared bands (red) and unique bands (blue); double-arranged red band increases in intensity over time.

DNA Sequence Analysis of Clone Libraries

Clone libraries were successfully constructed from the amplified bacterial 16S rRNA-encoding genes in aerobic, transitional, and iron-reducing incubations and from the DNA extracted directly from the raw groundwater without any incubation. Archaeal DNA was amplified from the methanogenic incubations, and a clone library has been constructed; sequencing is in progress. Table A2 shows the closest database matches to the retrieved sequence.

The distribution of clone sequences across microbial phylotypes is shown in Figure 13. A phylogenetic tree showing the relatedness of these sequences to one another and reference organisms is shown in Figure 14.

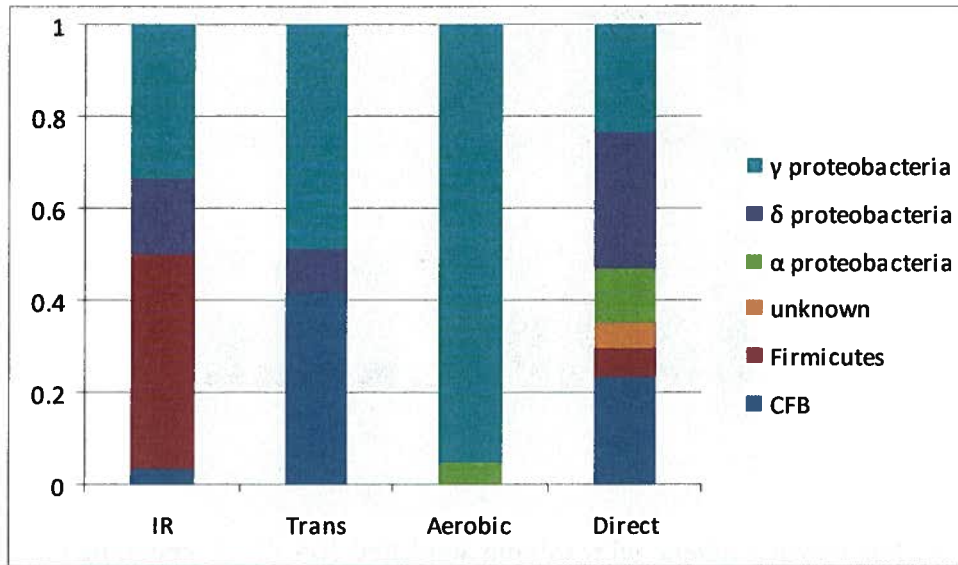


Figure 13. Phylogenetic distribution of clone sequences retrieved from DNA directly extracted from raw groundwater and from aerobic, transitional, and iron-reducing incubations. Each incubation condition contains a subset of sequences detected from the direct extract. Note differences in distribution between conditions (e.g. loss of γ -*Proteobacteria* as conditions become more reduced).

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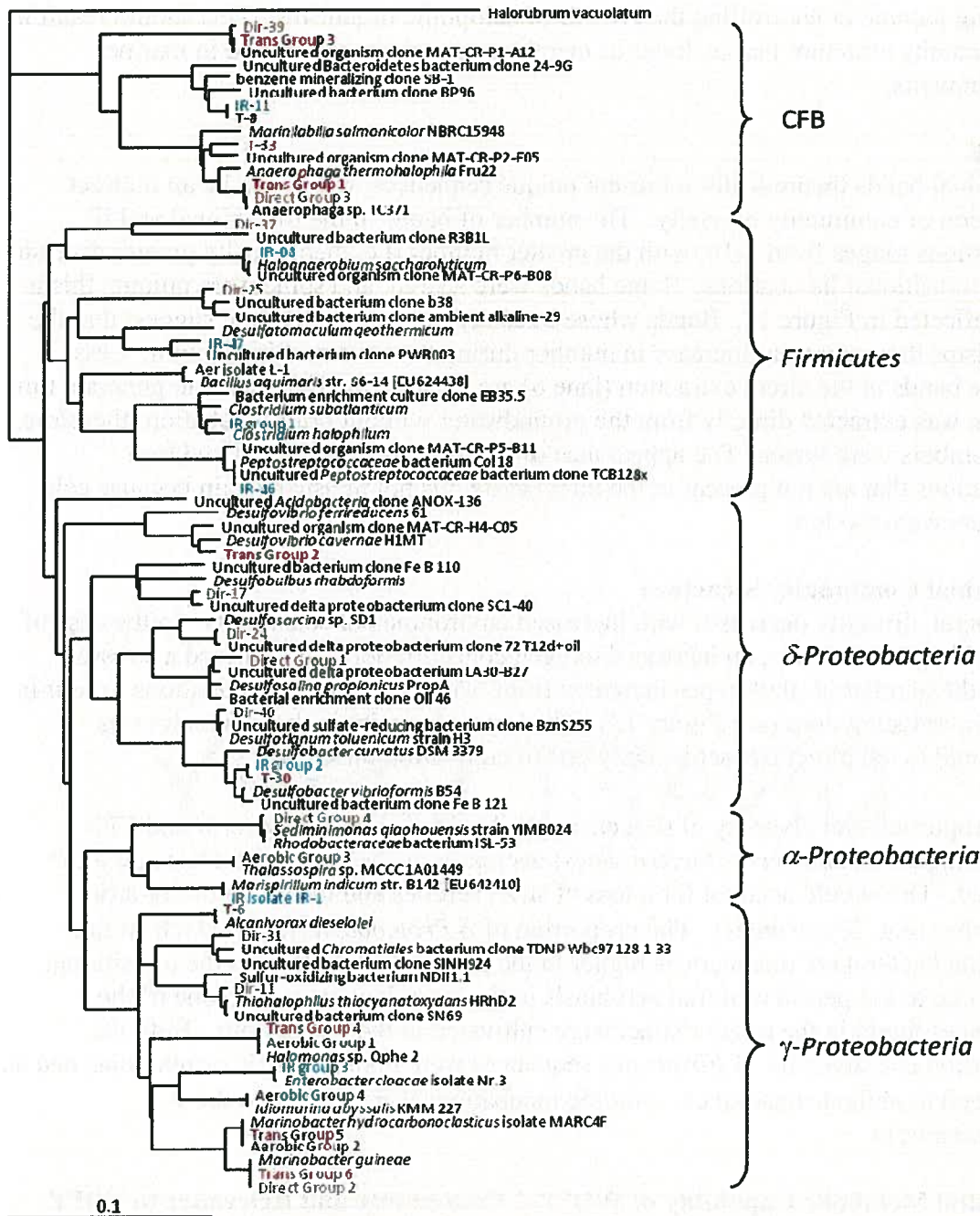


Figure 14. Phylogenetic tree constructed from 16S rRNA encoding sequences retrieved from all samples (raw groundwater = direct (Dir); aerobic (Aer), iron-reducing (IR), and transitional (T)). Tree is rooted to *Halorubrum vacuolatum* as an outgroup. Scale bar indicates one nucleotide substitution per ten bases.

Discussion

Chemistry

Results for water chemistry are consistent with those measured by others (WIPP ASER 2010) and reflect a marine origin (Domski et al., 2008). Sodium concentrations are the

defining parameter controlling the growth of halophilic organisms. This should result in a community structure that includes as members organisms also found in marine environments.

DGGE

Individual bands theoretically represent unique sequences, which can be an indirect reflection of community diversity. The number of bands in the transitional and IR incubations ranges from 5-10, with the greater number (i.e. theoretically greater diversity) in the transitional incubations. Some bands were shared, and some were unique; this is also reflected in Figure 14. Bands whose intensity increases over time suggest that the organisms they represent increase in number during the course of incubation. Less intense bands in the direct extraction (lane 6) are because the DNA used to generate this sample was extracted directly from the groundwater without prior incubation; therefore, cell numbers were lower. The appearance of bands in the transitional and iron incubations that are not present in the direct extract is not unusual, again because cell numbers were too low.

Microbial Community Structure

In general, diversity decreases with increased environmental constraints. In the case of anaerobic groundwaters, an increased oxygen concentration is considered a stressor. Thus, the number of phylotypes increases from two in the aerobic incubations to four in the iron-reducing ones (see Figure 13). The lower diversity in the incubations as compared to the direct extract is likely due to cultivation bias.

The proportion and diversity of sequences attributed to γ -*Proteobacteria* and CFB (*Cytophaga-Flavobacteria-Bacteroidetes*) decreases 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, likely due to the period of initial aerobiosis 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*.

Potential Metabolic Capability of WQSP-1 Community and Relevance to WIPP

As would be expected, the bacterial community present in WQSP-1 is a reflection of the groundwater's chemistry and the conditions of incubation. At an approximate total ionic strength of 1.5 M and [Na] of 0.35 M, this type of groundwater will support halotolerant and moderately halophilic microorganisms.

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

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. This is also reflected in the low concentration of DNA extracted from the NR samples with respect to other incubations. Iron and manganese were detected in raw groundwater samples, and this explains the appearance of a metal-reducing community. Also detected in the groundwater were arsenic and selenium and, although this type of respiration was not tested for, it is likely that arsenate and selenate reducers would be found as well.

The high concentrations of sulfate in the raw groundwater (46 mM) are enough to drive the incubations toward a sulfate-reducing community and maintain that community as the predominant one under anaerobic conditions. Unfortunately, sulfate was also added to incubations meant to enrich SRB, resulting in levels apparently inhibitory to growth.

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). It is likely that the iron was reduced by both metal and sulfate reducers and indirectly by microbially-generated sulfide.

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.

WQSP-1 groundwater is representative of water moving downgradient through the WIPP site. Characterization of this groundwater 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.

Results and Discussion

PZ-13

Chemistry

Results of ICP-MS, IC, and pH analysis on PZ-13 are given in the table below.

Table 6. Chemistry of filtered PZ-13 groundwater.

Significant Analyte	$\mu\text{g/ml}$	M
Na^+	8.42×10^4	3.66
Ca^{2+}	2.54×10^3	0.06
Mg^{2+}	1.51×10^3	0.06
K^+	503	0.01
Si	555	0.02
Cl^-	1.55×10^5	4.37

SO ₄ ²⁻	2.54 x 10 ³	0.03
pH	6.23	N/A

The origins of this 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). This groundwater is much higher in ionic strength than WQSP-1.

Microbial Growth in Incubations

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 15). No growth was observed in the sulfate-reducing incubations.



Figure 15. Sulfide precipitate in PZ-13 iron-reducing incubation.

Plated Cultures and Isolates

Aerobic and microaerophilic isolates have been cultivated from the PZ-13 incubations. These are listed in Table A3.

Bacterial Isolates

Plated cultures from the aerobic incubation yielded a *Chromohalobacter* sp. (see Figure 16a). 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 16c) 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).

Archaeal Isolates

Three archaeal isolates were obtained from PZ-13. *Halococcus saccharolyticus* (see Figure 16b) was isolated from the aerobic culture, and *Haloferax* sp. and *Natrinema* sp. were isolated aerobically from the IR culture.



Figure 16. TEM section of *Chromohalobacter* sp. (a), TEM 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.

DNA Sequence Analysis of Clone Libraries

A clone library was successfully 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. One hundred percent 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%). A library for the aerobic incubation is in progress.

Potential Metabolic Capability of PZ-13 Community and Relevance to WIPP

As with WQSP-1, the microbial community in this groundwater should be a reflection of the water chemistry and the incubation conditions. The sodium concentration alone should have selected for borderline extreme to extremely halophilic organisms. In addition, the level of sulfate (~25 mM) would have been conducive to sulfate reduction by these halophilic organisms. Results from the clone library analysis of the IR incubation with the sulfide precipitate should provide more information.

Some of the organisms cultivated from this groundwater have been used in further studies on metal reduction and biosorption. Reduction of metals other than manganese has not been previously observed at high ionic strengths; however, early results suggest that PZ-13 organisms may be capable of reducing iron. *Chromohalobacter* isolated from this groundwater has been utilized in biosorption studies (Ams et al., 2011, submitted). Both these areas of research should support WIPP performance.

Unlike WQSP-1, 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.



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Part III.

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 and Gillow, 1997, 2005; Francis et al., 1998; Gillow et al., 2000; Swanson et al., 2012, in press). 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 halophile behavior from theirs.

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 our expectation that microbial activity in the near-field will proceed from aerobic respiration through nitrate reduction to focus on sulfate reduction. Sulfate reduction can occur at extremely high ionic strengths, and there will be available sulfate from brine and anhydrite dissolution to serve as a terminal electron acceptor. The role of methanogenesis remains a focus of ongoing and future investigation.

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 may simply reflect undersampling or may be due to actual differences in surficial and subsurface halophilic 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.

Ongoing and Future Work

Because the majority of the WIPP repository's lifetime will be anoxic, it is imperative to define the possible role of anaerobic organisms from WIPP halite. These incubations are currently underway. Many of the organisms identified in this work are being used in further tasks. The current organism inventory is being screened for cellulose degraders, and enrichment cultures for such organisms are being set up. Many iron/metal reducing organisms have been identified and will be used in studies on actinide reduction. Finally, select organisms identified in this work are being used to determine a biocolloid enhancement factor based on their potential surface sorption of actinides.

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.
- Ams DA, Swanson JS, Szymanowski J, Fein J, Richmann MK, Reed DT. 2011. The effect of high ionic strength on neptunium (V) adsorption to a halophilic bacterium. Accepted for publication in *Geochimica et Cosmochimica Acta*.
- 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. 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. 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. 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.
- Compliance Recertification Application (CRA) for the Waste Isolation Pilot Plant. 2009. 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. 2009. 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 Laboratory; 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 Laboratory; Albuquerque, NM.
- Francis AJ, Gillow JB, Giles MR. 1997. Microbial gas generation under expected Waste Isolation Pilot Plant repository conditions. SAND96-2582 Report. Albuquerque, NM: Sandia National Laboratories.
- Francis AJ, Gillow JB, Dodge CJ, Dunn M, Mantione K, Strietelmeier BA, Pansoy-Hjelvik ME, Papenguth HW. 1998. Role of microbes as biocolloids in the transport of actinides from a deep underground radioactive waste repository. Report SAND98-0370J. Sandia National Laboratory; 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.
- 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.
- 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.
- 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.
- 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. 2011. Thermodynamic limits to microbial life at high salt concentrations. *Environmental Microbiology* 13: 1908-1923.
- 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.
- Schubert BA, Lowenstein TK, Timofeeff MN, Parker MA. 2010. Halophilic *Archaea* cultured from ancient halite, Death Valley, California. *Environmental Microbiology* 12: 440-454.
- Snider AC. 2003. Verification of the definition of Generic Weep Brine and the development of a recipe for this brine. Report ERMS 527505. Albuquerque, NM: Sandia National Laboratories.
- Sørensen K, Reháková K, Zapomělová E, Oren A. 2009. Distribution of benthic phototrophs, sulfate reducers, and methanogens in two adjacent salt ponds in Eilat, Israel. *Aquatic Microbial Ecology* 56: 275-284.
- Stan-Lotter H, McGenity TJ, Legat A, Denner EBM, Glaser K, Stetter KO, Wanner G. 1999. Very similar strains of *Halococcus salifodinae* are found in geographically separated Permo-Triassic salt deposits. *Microbiology* 145: 3565-3574.
- Swanson JS, Norden DM, Khaing H, Reed DT. 2012. Biodegradation of organic complexing agents by halophilic microorganisms in brines. Accepted for publication in *Geomicrobiology Journal*.
- 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.
- 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 for 2010. United States Department of Energy DOE/WIPP-11-2225.

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APPENDIX-Table A1.
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 B9*	uncultured bacterium clone 1177-790-14/ no cultured relative	100%	deep marine sediment, Nankai Trough, Japan
Clone B10*	uncultured bacterium clone LARIS 1-01A01/ no cultured relative	87%	sediment, Isis Mud Volcano, Eastern Mediterranean
Clone B11*	uncultured bacterium clone NNP.20/ <i>Limnobacter</i> sp. F3	95% 100%	river sediment mangrove sediment
Clone B34*	uncultured bacterium clone CJRC134/ <i>Bacillus silvestris</i> [AJ550464]	100% 96%	FBR community treating nitrate and uranium contaminated groundwater soybean rhizosphere
Clone B43	uncultured β - <i>Proteobacterium</i> clone PRTBB8731/ <i>Limnobacter thiooxidans</i> strain CS-K2	99% 99%	ocean water at 6000 m depth; Puerto Rico Trench sediment, freshwater lake, Germany
Group B1*	uncultured bacterium clone LARIS 81-01A11/ no cultured relative	100%	sediment, Isis Mud Volcano, Eastern Mediterranean
Clear Halite/175 g NaCl Enrichment			
Clone B26	uncultured bacterium clone 189/ <i>Limnobacter thiooxidans</i> strain TSWCSN35	91% 91%	grass carp symbiont sediment, natural spring, India
Clone B31	uncultured bacterium clone D-25/ <i>Nevskia</i> sp. KNF013	97% 97%	river water, Wang Yang River, China freshwater lake Kasumigaura, Japan

Clone B33	uncultured bacterium clone 189/ <i>Limnobacter thiooxidans</i> strain TSWCSN35	100% 100%	grass carp symbiont sediment, natural spring, India
Clone B42	bacterium MNFS-9/ <i>Pelomonas aquatica</i> strain CCUG 52575T	100% 98%	fruit juice industrial water, Sweden
Group B1	uncultured bacterium clone D-25/ <i>Nevskia</i> sp. KNF013	99% 99%	river water, Wang Yang River, China freshwater lake Kasumigaura, Japan
Group A1	<i>Halobacterium</i> sp. UJ-EY1	99%	salt crust, Arava Desert, Israel
Group A2	haloarchaeon MK62-1/ <i>Halorubrum</i> sp. CGSA-14	99% 93%	source not given Lake Ejinor, Inner Mongolia, China
Group A3	uncultured archaeon A154/ no cultured relative	98%	Alpine Permo-Triassic salt
Group A4	<i>Halolamina pelagica</i> (prev. archaeon TBN49)	99%	solar saltern, China

Clear Halite/100 g NaCl Enrichment

Isolate B1	uncultured bacterium clone nbw828g11c1 <i>Nesterenkonia alba</i> str. CAAS 252	97% 95%	human skin microbiome cotton pulp mill treatment system
Isolate F2	uncultured marine Ascomycete clone PRTBE7491 <i>Cladosporium bruhnei</i> str. CPC 5101	99% 99%	ocean water at 6000 m depth; Puerto Rico Trench Cu-Cr-As treated Douglas fir pole

Clayey Halite/Direct Extract

Group B1	bacterium MNFS-9/ <i>Pelomonas saccharophila</i> ATCC 15946	99% 99%	fruit juice American Type Culture Collection
Group B2	uncultured β - <i>Proteobacterium</i> clone PRTBB8731/ <i>Limnobacter</i> sp. KNF002	99% 99%	ocean water at 6000 m depth; Puerto Rico Trench freshwater lake Kasumigaura, Japan

Clayey Halite/100 g NaCl Enrichment

Group A1	uncultured haloarchaeon clone TX4CA_24/ <i>Halorubrum</i> sp. CGSA-14	94% 93%	alkaline, saline soil of former Lake Texcoco, Mexico Lake Ejinor, Inner Mongolia, China
Isolate F3	uncultured marine Ascomycete clone PRTBE7491 <i>Cladosporium bruhnei</i> str. CPC 5101	99% 99%	ocean water at 6000 m depth; Puerto Rico Trench Cu-Cr-As treated Douglas fir pole
Isolate F4	Fungal species FCAS132	100%	Arabian Sea sediment

Clayey Halite/5X organics Enrichment

Isolate B1	uncultured bacterium clone nbw828g11c1 <i>Nesterenkonia alba</i> str. CAAS 252	97% 95%	human skin microbiome cotton pulp mill treatment system
Isolate F1	<i>Coniothyrium fuckelii</i>	100%	volcanic ash soil

*DNA sequence too short for meaningful phylogenetic assignment; included are best approximations

APPENDIX—Table A2.

Closest Matches to DNA Sequences Retrieved from WQSP-1 Groundwater (direct extract and incubations).

Clone/Group Designation	Closest BLAST Match/ 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/ <i>Thiohalophilus thiocyanatoxydans</i> str. HRhD 2	98% 98%	pristine soil, Jidong oil field, China hypersaline habitat, Russia
Clone Dir-17	uncultured delta proteobacterium clone SC1-40/ <i>Desulfobulbus rhabdoformis</i> str. Mic5C02	97% 91%	South Atlantic Bight permeable shelf sediment water-oil separation system, Norway
Clone Dir-24	uncultured δ -proteobacterium clone 72 T12d+oil/ <i>Desulfosarcina</i> sp. SD1	99% 94%	oil-contaminated coastal sediment, France mangrove sediment
Clone Dir-25	uncultured bacterium clone b38/ <i>Clostridium</i> sp. str. P6	97% 93%	Cr-contaminated tannery sludge, China brewery wastewater sludge, China
Clone Dir-31	uncultured <i>Chromatiales</i> clone TDNP_Wbc97_128_1_33/97% no cultured relative		wetland water, Tabla de Daimiel National Park, Spain
Clone Dir-37	uncultured bacterium clone R3B1L/ <i>Desulfovibrionales</i> bacterium Spi55	87%* 83%*	low-temperature, anaerobic sewage bioreactor black rust exposed to hot ridge crustal fluids, Juan de Fuca Ridge, US
Clone Dir-39	uncultured organism clone MAT-CR-P1-A12/ <i>Cytophaga</i> sp. Dex80-37	99% 89%	hypersaline microbial mat, Candelaria Lagoon, Puerto Rico East Pacific Rise
Clone Dir-40	bacterium enrichment culture clone Tol_45/ <i>Desulfotignum toluenicum</i> str. H3	99% 98%	hydrocarbon-contaminated estuarine sediment, Wadi Gaza, Palestine toluene-degrading SRB from soil reservoir model column
Group Dir-1	uncultured delta proteobacterium clone LA30-B27/ <i>Desulfosalina</i> sp. HTR2	98% 96%	lake water, Hawaii hypersaline soda lake sediment, Kulunda Steppe, Russia
Group Dir-2	<i>Marinobacter guineae</i> str. LMG 24047T	99%	Antarctica

Group Dir-3	<i>Anaerophaga</i> sp. TC371	99%	production water, high-temperature oil field, North Sea, Norway
Group Dir-4	<i>Sediminimonas qiaohouensis</i> str. YIM B024	99%	salt mine, Yunnan, China
Aerobic (Aer)			
Group Aer-1	<i>Halomonas</i> sp. Qphe 2	99%	petroleum-contaminated soil
Group Aer-2	<i>Alteromonadaceae</i> bacterium LA34A	99%	environmental sample, Hawaiian archipelago
Group Aer-3	<i>Thalassospira</i> sp. MCCC 1A01449	99%	marine
Group Aer-4	<i>Idiomarina</i> sp. FG-5	99%	microalgal culture
Isolate Aer-L1	<i>Bacillus</i> sp. J28	96%	black sand
	<i>Bacillus aquimaris</i> str. S6-14	96%	marine sediment, Palk Bay, India
Transitional (Tr)			
Clone Tr-6	<i>Alcanivorax</i> sp. YDC2-H	99%	deep-sea hydrothermal vent sediment, Indian Ocean
Clone Tr-8	benzene mineralizing consortium clone SB-1/	97%	sulfate-reducing consortium
	<i>Bacteroidetes</i> 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
	<i>Desulfobacter vibrioformis</i> str. B54	96%	water-oil separation system
Clone Tr-33	uncultured organism clone MAT-CR-P2-E05/	97%	hypersaline microbial mat, Candelaria Lagoon, Puerto Rico
	<i>Marinilabilia salmonicolor</i> str. NBRC15948	96%	isolate type strain
Group Tr-1	<i>Anaerophaga</i> sp. TC371	99%	production water, high-temperature oil field, North Sea, Norway
Group Tr-2	<i>Desulfovibrio cavernae</i> str. H1MT	96%	deep subsurface brine, Germany
Group Tr-3	uncultured organism clone MAT-CR-P1-A12/	99%	hypersaline microbial mat, Candelaria Lagoon, Puerto Rico
	<i>Cytophaga</i> sp. Dex80-37	88%	East Pacific Rise

Group Tr-4	<i>Halomonas</i> sp.Qphe 2	99%	petroleum-contaminated soil
Group Tr-5	<i>Alteromonadaceae</i> bacterium LA34A	98%	environmental sample, Hawaiian archipelago
Group Tr-6	uncultured bacterium clone BR148/ <i>Marinobacter guineae</i> str. LMG 24047T	98% 98%	Zhongyuan oil field, China environmental sample, Antarctica
Iron-reducing (IR)			
Clone IR-8	uncultured organism clone MAT-CR-P6-B08/ <i>Halanaerobium saccharolyticum</i>	98% 98%	hypersaline microbial mat, Candelaria Lagoon, Puerto Rico type strain isolate
Clone IR-11	benzene mineralizing consortium clone SB-1/ <i>Bacteroidetes</i> bacterium M-phe-1	97% 97%	sulfate-reducing consortium phenanthrene-degrading consortium under methanogenic conditions
Clone IR-46	<i>Peptostreptococcaceae</i> bacterium Col 18	99%	corrosive biofilm, North Sea Harbor, France
Clone IR-47	uncultured bacterium clone PWB00/ <i>Desulfotomaculum geothermicum</i>	94% 90%	nitrate-treated production water, oil field, Denmark isolate
Group IR-1	<i>Clostridium</i> sp. DY192	96%	deep sea sediment, Indian Ocean
Group IR-2	uncultured bacterium clone Fe_B_121/ <i>Desulfobacter vibrioformis</i> str. B54	96% 96%	methane seep sediment, Eel River Basin, CA water-oil separation system
Group IR-3	uncultured bacterium clone 16slpl112-1e08.p1k/ <i>Enterobacter</i> sp. GW31-6	99% 99%	human intestinal pouch environmental sample, Antarctica
Isolate IR-1	<i>Marispirillum indicum</i> str. B1-42	99%	crude oil degrading culture from deep sea water, India

APPENDIX—Table A3.

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	<i>Chromohalobacter salexigens</i> str. SM5 [HQ641752]	100%	salted meat
Isolate A1	<i>Halococcus saccharolyticus</i>	98%	isolate, source unknown
Iron-reducing (IR) grown under microaerophilic conditions			
Group IR-B1	<i>Pontibacillus</i> sp. R147	99%	solar saltern lake sediment, Mediterranean Sea island
Group IR-B2	<i>Virgibacillus</i> 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
	<i>Bacteroidetes</i> bacterium MO54	94%	soil, Great Salt Plains, Oklahoma
Group IR-A1	<i>Haloferax</i> sp. Bej51	99%	salt lake, Algeria
Group IR-A2	<i>Haloarcula amylytica</i>	99%	type strain isolate
Isolate B2	<i>Virgibacillus</i> sp. BH187	99%	China, no further information provided
Isolate A2	uncultured <i>Natrinema</i> sp. clone VKMM-8	94%	solar saltern, India
	<i>Natrinema versiforme</i> XF10	94%	Aibi salt lake, China