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LCO-ACP-20, Revision 0

Update on Microbial Characterization of WIPP Groundwaters and Halite

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ACRONYMS and ABBREVIATIONS

16S rRNA	16S subunit of ribosomal RNA
Aer	aerobic
CFB	*Cytophaga-Flavobacteria-Bacteroidetes*
CMC	carboxymethylcellulose
CRA	Compliance Recertification Application
Dir	direct (i.e., DNA extracted directly from sample without prior cultivation)
IC	ion chromatography
ICP-MS	inductively-coupled plasma-mass spectrometry
LCO-ACP	Los Alamos National Laboratory—Carlsbad Operations-Actinide Chemistry Program
IR	iron-reducing (incubation to enrich iron-reducing organisms)
M	moles per liter
Meth	methanogenic (incubation to enrich methanogenic organisms)
MOPS	3-(N-morpholino)propanesulfonic acid (buffer)
NaCl	sodium chloride
NR	nitrate-reducing (incubation to enrich nitrate-reducing organisms)
OTU	operational taxonomic unit
SR	sulfate-reducing (incubation to enrich sulfate-reducing organisms)
SRB	sulfate-reducing Bacteria
TEA	terminal electron acceptor
TOC	total organic carbon
Tr	transitional
WIPP	Waste Isolation Pilot Plant
WQSP	WIPP Water Quality Sampling Program

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Executive Summary

This report provides a status update of ongoing developmental research in the Los Alamos National Laboratory — Carlsbad Operations' Actinide Chemistry and Repository Science Program to describe the microbial ecology within the WIPP repository and in surrounding groundwaters. Through cultivation and DNA-based identification, the potential activity of these organisms is being inferred, thus leading to a better understanding of their impact on WIPP performance.

WIPP groundwaters comprise the far-field microbial environment. *Bacteria* cultivated and identified from the overlying Culebra groundwater are capable of aerobic respiration, denitrification, fermentation, metal reduction, and sulfate reduction and are distributed across many different phyla. Their structural and metabolic diversity is dependent upon the ionic strength of the sampled groundwater, with a decrease in both at higher strength.

These developments do not have a direct impact on WIPP performance assessment, but they contribute to the overall understanding of the microbial ecology in WIPP that will be reflected in supporting documentation for the Compliance Recertification Application (CRA) in 2014.

Introduction

Overview

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. A detailed description of the methods and initial results of this research is given in LCO-ACP-12 (Swanson et al., 2012). In this report, we provide an update on the characterization of organisms from samples of Culebra groundwater and the enrichment of anaerobic organisms from halite.

Groundwaters

To date, four groundwaters from the WIPP environs have been sampled (WQSP-1, WQSP-3, H4b-R, PZ-13; see Figure 1). H4b was discarded due to the inability to control for outside contamination. Results for WQSP-1 and PZ-13 were presented in LCO-ACP-12. Further results for WQSP-1 and data for WQSP-3 will be presented here.

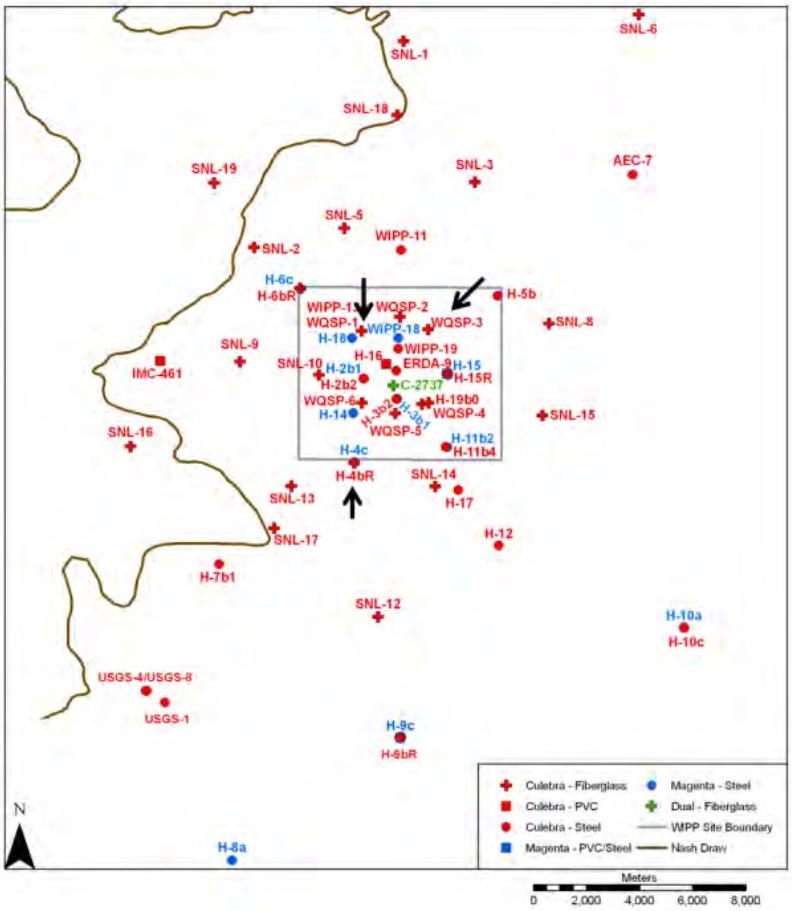

Figure 1. Map of WIPP monitoring wells. Arrows denote wells sampled; rectangle outlines WIPP site (CRA, 2009).

Materials and Methods

Sample collection and handling was as described in LCO-ACP-12. Chemical analyses were also as described in LCO-ACP-12, except that ion chromatography analyses were performed on a Dionex ICS-3000. Media were prepared for the same conditions as listed in LCO-ACP-12 with changes to methanogenic media composition (see Table 1). Microscopy, cultivation, extraction and processing of nucleic acids were all performed as outlined in LCO-ACP-12. Samples were analyzed for bacteria only.

Table 1. Outline of media used for enriching microorganisms from groundwater.

Medium Component	Aer	Tr	NR	IR	SR	Meth1	Meth2
Carbon sources	Acetate, pyruvate, lactate	Acetate, pyruvate, lactate	Acetate, pyruvate, lactate	Acetate, pyruvate, lactate	Acetate, pyruvate, lactate	Formate, acetate	Trimethylamine oxide
Nutrients and supplements	NH_4Cl, KH_2PO_4 , yeast extract, casamino acids	NH_4Cl, KH_2PO_4 , yeast extract, casamino acids, trace elements, vitamins	NH_4Cl, KH_2PO_4 , yeast extract, casamino acids, trace elements, vitamins	NH_4Cl, KH_2PO_4 , yeast extract, casamino acids, trace elements, vitamins	NH_4Cl, KH_2PO_4 , yeast extract, casamino acids, trace elements, vitamins	NH_4Cl, KH_2PO_4 , yeast extract, casamino acids, trace elements, vitamins	NH_4Cl, KH_2PO_4 , yeast extract, casamino acids, trace elements, vitamins
Terminal electron acceptor	None provided (O_2)	None provided (initially O_2)	KNO_3	Fe(III)-citrate	Na_2SO_4	None provided (CO_2 in headspace)	None provided (CO_2 in headspace)
Buffer	MOPS	MOPS	MOPS	MOPS	MOPS	MOPS	MOPS
Headspace	Aerobic	Aerobic, sealed	N_2:CO_2	N_2:CO_2	H_2:CO_2	H_2:CO_2	H_2:CO_2
Reductant	None	None	None	None	Cysteine	Titanium citrate	Cysteine-sulfide

Aer = aerobic; Tr = transitional (sealed aerobically and allowed to proceed to anaerobiasis; NR = nitrate-reducing; IR = iron-reducing; SR = sulfate reducing; Meth1 and Meth2 = methanogenic (using different substrate types).

Results

Chemistry

Results of ICP-MS, IC, and TOC analysis on WQSP-3 are given in the table below.

Table 2. WQSP-3 Chemistry.

Significant Analyte	µg/ml	M
Na^+	8.01×10^4	3.48
Ca^{2+}	1.82×10^3	0.04
Mg^{2+}	2.64×10^3	0.11
K^+	1.74×10^3	0.04
Cl^-	119360	3.37
SO_4^{2-}	7047	0.07
pH	7.07	N/A
TOC	0.162*	N/A

*at limit of detection

Direct Counts—WQSP-3

Direct microscopic counts of the raw WQSP-3 groundwater were $(8.63 \pm 5.44) \times 10^5$ cells/ml.

Microbial Growth in Incubations—WQSP-3

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

Plated Cultures and Isolates—WQSP-3 and WQSP-1

WQSP-3

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

WQSP-1

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

DNA Sequence Analysis of Clone Libraries

Clone libraries were 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. Table A1 (see Appendix) shows the closest database matches to the retrieved sequence. If the closest match is an uncultured or unidentified organism, the table also lists the closest named relative.

The distribution of clone sequences across microbial phylotypes for both WQSP-1 and WQSP-3 is shown in Figure 2. Results for WQSP-1 alone were provided in LCO-ACP-12. A phylogenetic tree showing the relatedness of these sequences to one another and reference organisms is shown in Figure 3.

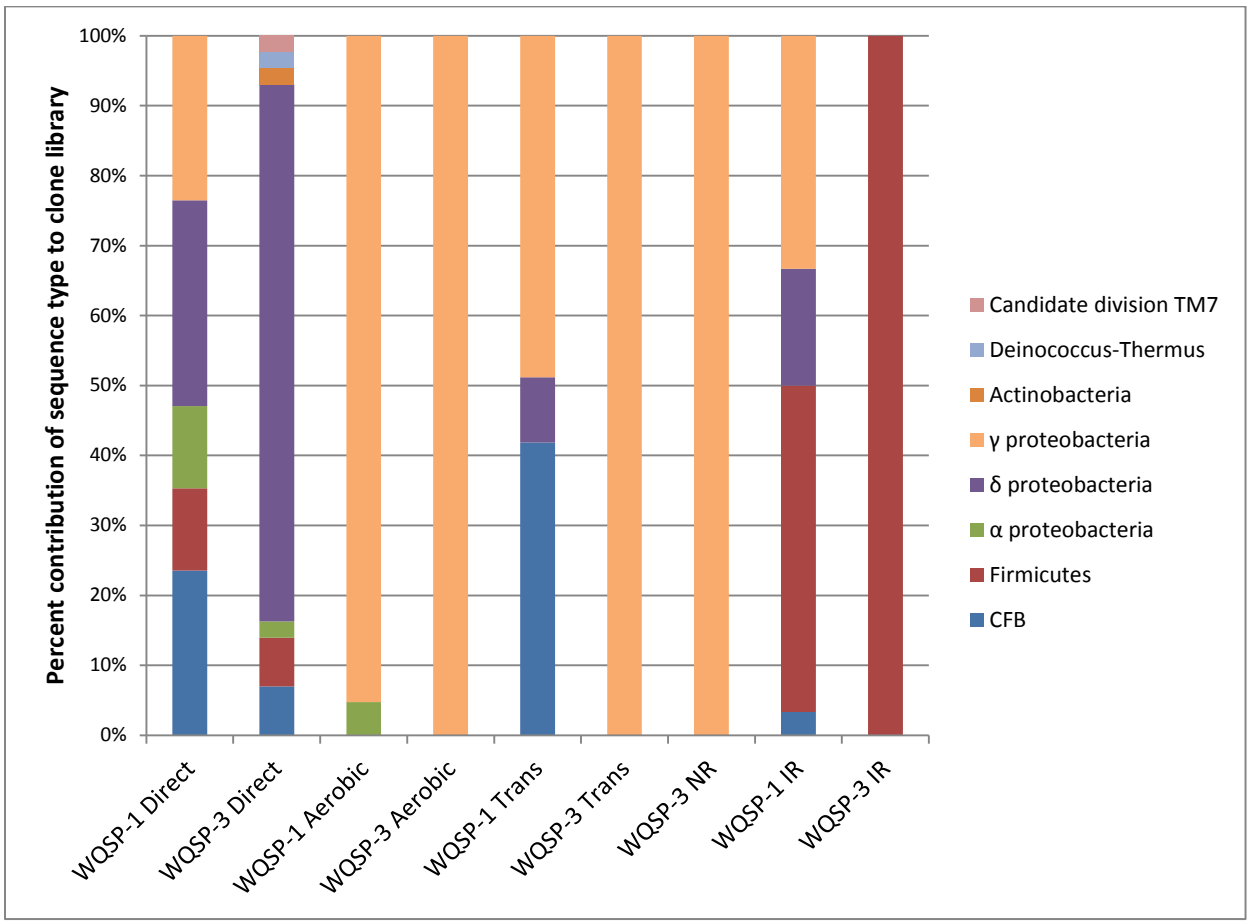

Figure 2. Phylogenetic distribution of clone sequences retrieved from DNA directly extracted from raw groundwater and from aerobic, transitional, and iron-reducing incubations of WQSP-1 and WQSP-3. CFB = *Cytophaga-Flavobacteria-Bacteroidetes*; direct = raw groundwater. "Candidate" indicates taxonomic unit for which no isolates have yet been obtained; all data are from DNA sequences.

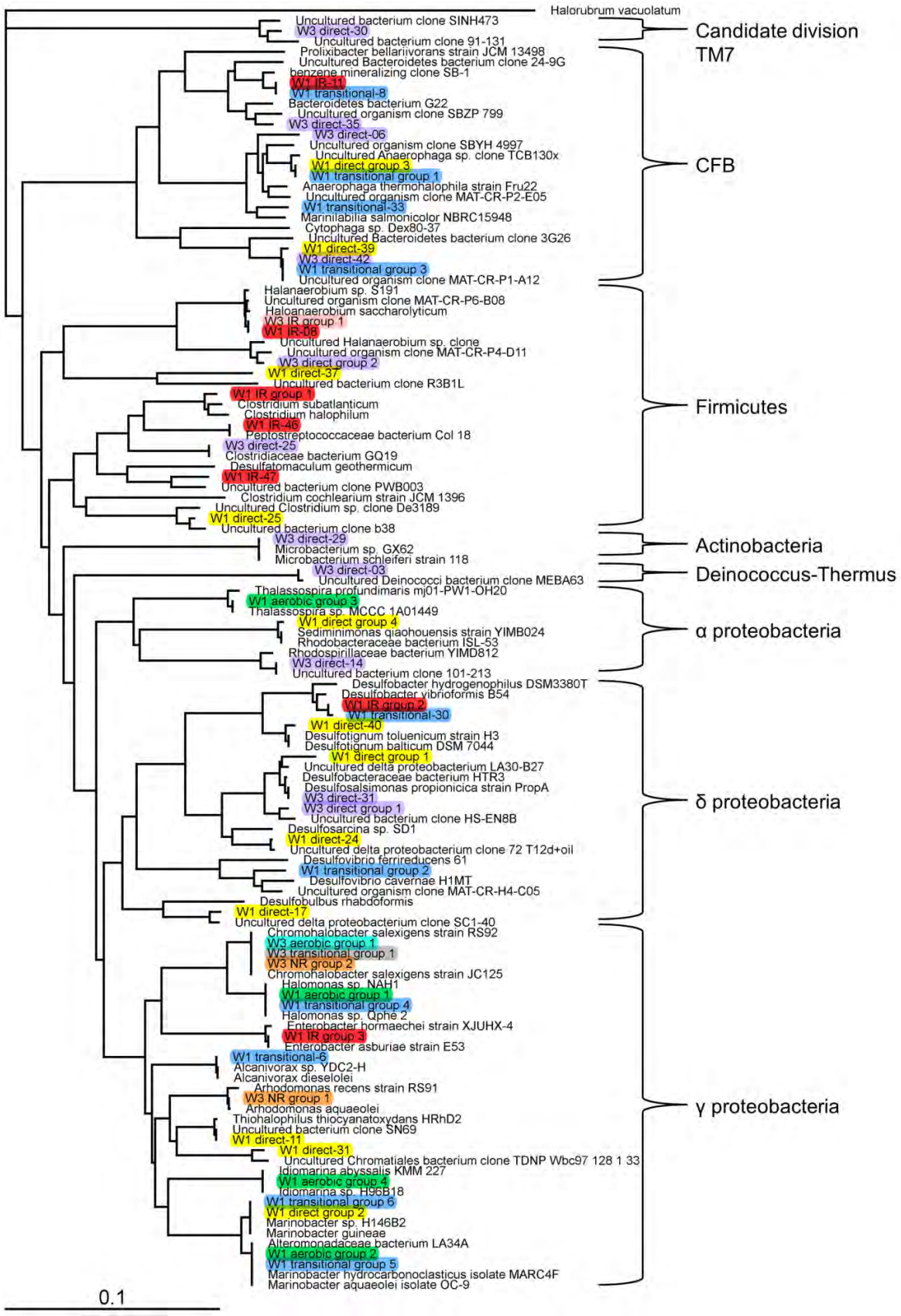
Halorubrum vacuolatum
Uncultured bacterium clone SINH473
W3 direct-30
Uncultured bacterium clone 91-131
Prolixibacter bellariivorans strain JCM 13498
Uncultured Bacteroidetes bacterium clone 24-9G
benzene mineralizing clone SB-1
W1 IR-31
W1 transitional-8
Bacteroidetes bacterium G22
Uncultured organism clone SBZP 799
W3 direct-35
W3 direct-06
Uncultured organism clone SBYH 4997
Uncultured Anaerophaga sp. clone TCB130x
W1 direct group 3
W1 transitional group 1
Anaerophaga thermohalophila strain Fru22
Uncultured organism clone MAT-CR-P2-E05
W1 transitional-33
Marinilabilia salmonicolor NBRC15948
Cytophaga sp. Dex80-37
Uncultured Bacteroidetes bacterium clone 3G26
W1 direct-39
W3 direct-42
W1 transitional group 3
Uncultured organism clone MAT-CR-P1-A12
Halanaerobium sp. S191
Uncultured organism clone MAT-CR-P6-B08
Haloanaerobium saccharolyticum
W3 IR group 1
W1 IR-08
Uncultured Halanaerobium sp. clone
Uncultured organism clone MAT-CR-P4-D11
W3 direct group 2
W1 direct-37
Uncultured bacterium clone R3B1L
W1 IR group 1
Clostridium subatlanticum
Clostridium halophilum
W1 IR-46
Peptostreptococcaceae bacterium Col 18
W3 direct-25
Clostridiaceae bacterium GQ19
Desulfatomaculum geothermicum
W1 IR-47
Uncultured bacterium clone PWB003
Clostridium cochlearium strain JCM 1396
Uncultured Clostridium sp. clone De3189
W1 direct-25
Uncultured bacterium clone b38
W3 direct-29
Microbacterium sp. GX62
Microbacterium schleiferi strain 118
W3 direct-03
Uncultured Deinococci bacterium clone MEBA63
Thalassospira profundimaris mj01-PW1-OH20
W1 aerobic group 3
Thalassospira sp. MCCC 1A01449
W1 direct group 4
Sediminimonas qiaohouensis strain YIMB024
Rhodobacteraceae bacterium ISL-53
Rhodospirillaceae bacterium YIMD812
W3 direct-14
Uncultured bacterium clone 101-213
Desulfobacter hydrogenophilus DSM3380T
Desulfobacter vibrioformis B54
W1 IR group 2
W1 transitional-30
W1 direct-40
Desulfotignum toluenicum strain H3
Desulfotignum balticum DSM 7044
W1 direct group 1
Uncultured delta proteobacterium LA30-B27
Desulfobacteraceae bacterium HTR3
Desulfosalsimonas propionicica strain PropA
W3 direct-31
W3 direct group 1
Uncultured bacterium clone HS-EN8B
Desulfosarcina sp. SD1
W1 direct-24
Uncultured delta proteobacterium clone 72 T12d+oil
Desulfovibrio ferrireducens 61
W1 transitional group 2
Desulfovibrio cavernae H1MT
Uncultured organism clone MAT-CR-H4-C05
Desulfobulbus rhabdoformis
W1 direct-17
Uncultured delta proteobacterium clone SC1-40
Chromohalobacter salexigens strain RS92
W3 aerobic group 1
W3 transitional group 1
W3 NR group 2
Chromohalobacter salexigens strain JC125
Halomonas sp. NAH1
W1 aerobic group 1
W1 transitional group 4
Halomonas sp. Qphe 2
Enterobacter hormaechei strain XJUHX-4
W1 IR group 3
Enterobacter asburiae strain E53
W1 transitional-6
Alcanivorax sp. YDC2-H
Alcanivorax dieselolei
Arhodomonas recens strain RS91
W3 NR group 1
Arhodomonas aquaeolei
Thiohalophilus thiocyanatoxydans HRhD2
Uncultured bacterium clone SN69
W1 direct-11
W1 direct-31
Uncultured Chromatiales bacterium clone TDNP Wbc97 128 1 33
Idiomarina abyssalis KMM 227
W1 aerobic group 4
Idiomarina sp. H96B18
W1 transitional group 6
W1 direct group 2
Marinobacter sp. H146B2
Marinobacter guineae
Alteromonadaceae bacterium LA34A
W1 aerobic group 2
W1 transitional group 5
Marinobacter hydrocarbonoclasticus isolate MARC4F
Marinobacter aquaeolei isolate OC-9
0.1
Candidate division TM7
CFB
Firmicutes
Actinobacteria
Deinococcus-Thermus
α proteobacteria
δ proteobacteria
γ proteobacteria

Figure 3. Phylogenetic tree constructed from 16S rRNA encoding sequences retrieved from all samples. W1 = WQSP-1; W3 = WQSP-3. Tree is rooted to archaeon *Halorubrum vacuolatum* as the outgroup. Scale bar indicates one nucleotide substitution per 10 bases.

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

Discussion

Chemistry

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

Microbial Community Structure of WQSP-3

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

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

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

Potential Metabolic Capability of WQSP-3 Community and Relevance to WIPP

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

As would be expected, the bacterial community present in WQSP-3 is a reflection of the groundwater's chemistry and the conditions of incubation. At a sodium concentration of 3.5 M, this type of groundwater will support extremely halophilic microorganisms.

Based on the distribution of phylotypes in the raw groundwater (see Figure 3), one would predict a range of metabolic processes, including aerobic and anaerobic respiration and fermentation.

Transitional incubations should allow the microbial community to proceed through aerobic respiration, followed by respiration dependent upon the presence of terminal electron acceptors (TEAs) other than oxygen. Sulfate is the only natural TEA present in the WQSP-3 groundwater at sufficient concentrations, but the transitional incubations did not proceed toward sulfate reduction. Instead, they were axenic, comprising only *Chromohalobacter salexigens*, a *γ-Proteobacterium*. As mentioned earlier, it is possible that the thriving *Chromohalobacter* may have depleted the nutrient supply such that other organisms could not survive. Alternately, the DNA used to generate this clone library may have been taken too soon in the incubation period, before the onset of true anaerobiasis. The presence of *Chromohalobacter* may be significant early on if an intrusion into the Culebra occurs, when waters may still be oxic or only slightly reduced. *Chromohalobacter salexigens* is capable of anaerobic nitrate reduction and also utilizes acetate and citrate (Arahal et al., 2001).

Although nitrate was not detected in this groundwater, incubations containing nitrate as the TEA yielded growth. These incubations were monophyletic, comprising only *γ-Proteobacteria*. *Arhodomonas* sp. made up 94% of this library with *Chromohalobacter* sp. composing the remainder. The appearance of nitrate-reducers is of significance, given the expected inventory of nitrate in the WIPP. The detected *Arhodomonas* sp. would be capable of oxidizing acetate at the expense of nitrate but would not utilize citrate (Saralov et al., 2012; Swanson et al., 2013).

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

The high concentration of sulfate in the raw groundwater (~73 mM) should have supported the growth of sulfate-reducing bacteria. That they are present is evidenced by the fact that the largest portion of the direct clone library was attributed to *Desulfosalsimonas* spp. These organisms would have been capable of utilizing lactate or yeast extract and could have oxidized acetate since H_2 was in the incubation headspace (Kjeldsen et al., 2010). It is possible that ionic

strength was an issue. While *Desulfosalsimonas* spp. can grow in a range of [NaCl], their optima tend to be between 1-2 M (Kjeldsen et al., 2010; Sorokin et al., 2012). However, these organisms dominated the direct clone library, suggesting that culture bias most likely prevented their growth.

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.

Comparison of WIPP Groundwaters at Different Ionic Strengths

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

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

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

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

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

Miscellaneous Updates

Anaerobic Incubations of Halite

To date, the incubations of WIPP halite under anaerobic conditions (transitional, nitrate-reducing, iron-reducing, and sulfate-reducing) have yielded no growth. This is most likely due to the inability of anaerobic bacteria to cope with the high concentration of NaCl used in these media but it could also be due to inoculation with "sterile" halite samples. Anaerobic incubations at a lower salt concentration may be attempted.

Conclusions

The previous status report on WIPP microbiology stated that significant differences will exist in microbial populations between the near and far-fields. This will mostly be due to the different strategies for survival at high ionic strength used by microorganisms and the influence of ionic strength on microbial metabolic processes. This current report supports those claims by noting the inability to obtain anaerobically growing haloarchaea and by highlighting differences in bacterial diversity between groundwaters of varying ionic strength, especially under anaerobic conditions.

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

Ongoing and Future Work

Both WQSP-1 and WQSP-3 will be screened for *Archaea*. Associations may exist between bacterial and archaeal communities, and the possible survival of haloarchaea under anaerobic conditions should continue to be investigated. Because the majority of the WIPP repository's lifetime will be anoxic, it is imperative to continue to look for anaerobic organisms within the WIPP near-field (i.e. halite). Additional investigations are focusing on actinide biosorption and bioreduction by isolates obtained during this project. Finally, anaerobic isolates are being screened for the ability to degrade cellulosics.

Acknowledgments

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Appendix A1.
Closest Matches to DNA Sequences Retrieved from WQSP-3 Groundwater (direct extract, incubations, and isolates).

Clone/Group Designation	Closest BLAST Match/ Closest Cultured and Named Relative	Accession Number	Sequence Similarity	Source of Closest BLAST Match
Direct				
Group DIR-B1	Uncultured bacterium clone HS-EN8B	FJ536432	97%	Solar saltern lake sediment, Mediterranean Sea
	Desulfosalina sp. HTR2	GQ922848	96%	Soda lake sediment, Altai, Kulunda Steppe, Russia
Group DIR-B2	Uncultured organism clone MAT-CR-P4-D11	EU246154	99%	Hypersaline microbial mat, Cabo Rojo, Puerto Rico
	Halanaerobium hydrogeniformans	CP002304	92%	Fresh water sediment
Clone DIR-B3	Uncultured microorganism clone Group14_b	JN387338	98%	Sulfur spring source sediment, Zodletone Spring, Oklahoma
	Truepera radiovictrix DSM 17093	CP002049	91%	Hot spring runoff, Island of Sco Miguel, Azores, Portugal
Clone DIR-B6	*Anaerophaga* sp. TC371	DQ647061	95%	Produced water, North Sea oil field
Clone DIR-B14	Uncultured alpha proteobacterium clone Y135	EU328078	99%	Moderate saline soil contaminated with crude oil
	Rhodospirillaceae bacterium strain YIM D812	NR_044596	98%	Type strain isolate
Clone DIR-B25	*Clostridiaceae* bacterium GQ6	JQ421331	99%	Yuncheng Salt Lake, China
Clone DIR-B29	*Microbacterium* sp. MOLA 56	AM990831	99%	Sea Water, North Western Mediterranean Sea, France
Clone DIR-B30	Uncultured bacterium clone 91-131	EF157158	97%	Heavy oil seeps, Rancho La Brea tar pits
	Candidate division TM7 clone GL2-37	DQ847438	96%	human skin
Clone DIR-B31	*Desulfosalina* sp. HTR2	GQ922848	99%	Soda lake sediment, Altai, Kulunda Steppe, Russia
Clone DIR-B35	*Bacteroidetes* bacterium G22	JQ683777	98%	Sediment sample
Clone DIR-B42	Uncultured organism clone MAT-CR-P1-A12	EU245984	100%	Hypersaline microbial mat, Cabo Rojo, Puerto Rico
	Prolixibacter bellariivorans strain JCM 13498	AB541983	89%	Type strain isolate
Aerobic				
Group AER-B1	*Chromohalobacter salexigens* strain JC125	HE662816	99%	Soil, India
Isolate B1	*Chromohalobacter salexigens* strain L0B	GU397381	99%	hypersaline lake, Iran

Transitional (aerobic → anaerobic)				
Group TR-B1	*Chromohalobacter salexigens* strain JC125	HE662816	99%	Soil, India
Nitrate-reducing (NR)				
Group NR-B1	*Arhodomonas recens* strain RS91	HQ833040	99%	Salt brines of potassium mineral refinement
Group NR-B2	*Chromohalobacter salexigens* strain JC125	HE662816	99%	Soil, India
Iron-reducing (IR)				
Group IR-B1	Uncultured organism clone MAT-CR-P6-B08	EU246275	99%	Hypersaline microbial mat, Cabo Rojo, Puerto Rico
	Halanaerobium saccharolyticum subsp. *saccharolyticum* strain DSM 6643	NR_026257	99%	Type strain isolate

APPENDIX—Table A2.
Closest Matches to DNA Sequences Retrieved from WQSP-1 Groundwater Anaerobic Isolates.

Clone/Group Designation	Closest BLAST Match/ Closest Cultured and Named Relative	Accession Number	Sequence Similarity	Source of Closest BLAST Match
Transitional				
Isolate B1	*Halanaerobium acetethylicum* strain EIGI	NR036958	99%	DSM culture collection (3532)
Isolate B2	"			
Iron-reducing				
Isolate B3	*Halanaerobium acetethylicum* strain EIGI	NR036958	99%	DSM culture collection (3532)
Isolate B4	"			
Isolate B5	"			
Isolate B6	*Clostridium sediminis*	HQ696463	97%	deep sea sediment, Indian Ocean