LOS ALAMOS NATIONAL LABORATORY
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Biodegradation of Organic Complexing Agents by WIPP-Indigenous Halophilic Microorganisms in Brines

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0	2-14-13	All	Original release.

ACRONYMS and ABBREVIATIONS

DBR	direct brine release
EDTA	ethylenediamine tetraacetic acid
ERDA-6	Energy Research and Development Administration Well 6
GWB	Generic Weep Brine
IC	ion chromatography
NaCl	sodium chloride
NaOH	sodium hydroxide
NR	nitrate-reducing (incubation to enrich nitrate-reducing organisms)
OCA	organic complexing agent
PA	performance assessment
pC_{H+}	-log of the concentration of the hydrogen ion
QL-1	quality level 1 (quality assurance designation)
WIPP	Waste Isolation Pilot Plant
WQSP	WIPP Groundwater Quality Sampling Program

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EXECUTIVE SUMMARY

The biodegradation of four WIPP-relevant organic complexing agents (OCAs) under aerobic conditions by WIPP-indigenous microorganisms was investigated. Acetate was readily degraded by both a mixed culture of haloarchaea and an axenic bacterial culture. Citrate was degraded by the haloarchaea, and oxalate was degraded by an axenic haloarchaeal culture. Further investigations into citrate degradation under anaerobic (nitrate-reducing) conditions using haloarchaea and halophilic bacteria were negative.

The solubility of complexing agents in WIPP brines was also investigated, as it significantly affected bioavailability and, hence, biodegradation. Preliminary results suggest that all complexing agents, with the possible exception of oxalate, will be soluble at the concentrations predicted by inventory (Van Soest et al., 2012). However, the presence of oxalate in the system affects the dissolved concentrations of acetate and citrate, when all agents are present together. Brine composition and pC_{H+} appear to affect the solubility of all agents except acetate, with decreasing oxalate and EDTA, and increasing citrate concentrations measured at higher pC_{H+}.

Although acetate, citrate, and oxalate should be degradable by WIPP-indigenous microorganisms, this process will be dependent upon which organisms are present and viable. This will vary in time, especially after the disappearance of oxygen, and between near- and far-field environments, where populations will differ significantly. Near-field haloarchaea will degrade acetate and citrate aerobically. Far-field organisms should be able to degrade acetate and citrate under various anaerobic conditions.

The observed solubility of organic complexants in brine provides useful input to plan WIPP-relevant experiments and point to an upper concentration to guide WIPP performance assessment (PA) discussions. Although degradation was observed, these results are not sufficient to change the PA assumption that OCAs are not degraded.

BACKGROUND

Organic complexing agents—including low molecular weight organic acids, such as acetate, oxalate, and citrate, and higher molecular weight anthropogenic chelators, such as ethylenediamine tetraacetic acid (EDTA) and nitrolotriacetic acid (NTA)—were used extensively in chemical separations of radionuclides during past nuclear weapons processing and for decontaminating nuclear facilities. As a result, organic complexing agents (OCAs) often exist as co-contaminants in waste already emplaced in and destined for nuclear repositories.

Effects of biodegradation. Biodegradation of OCAs should be considered when looking at repository performance, as these agents affect actinide solubility and potential mobility. Many microorganisms are capable of degrading these compounds, with or without associated actinides or heavy metals (Francis, 1998; Nörtemann, 1999; Egli, 2001; Lloyd and McCaskie, 2002; Pedersen, 2002; Keith-Roach, 2008; Francis et al., 2007). When associated with metals, degradation can be dependent upon the stability and degree of complexation, both of which affect the free, and therefore bioavailable, organic in solution (Huang et al., 1998; Witschel et al., 1999; Satroutdinov et al., 2000).

OCA biodegradation can lead to seemingly conflicting results within a repository environment. Degradation reduces the concentration of available complexant, thereby reducing the concentration of actinide in solution. Conversely, carbon dioxide (CO_2), generated from the complete mineralization of OCAs, can itself act as a complexant. It also reduces the pH of the surrounding medium, which may convert some actinides to a more soluble oxidation state. Finally, the degradation of some OCAs, especially EDTA, leads to intermediate compounds that also possess complexing ability. To counteract the potential effects of CO_2, magnesium oxide (MgO) is added to the repository. This narrows down the consequences of OCA degradation to two opposing phenomena: 1) a decrease in actinide solubility due to loss of complexants and 2) the generation of complexing metabolites or by-products from the parent OCA.

OCA inventory. The Waste Isolation Pilot Plant (WIPP) is concerned with the presence of four OCAs: acetate, oxalate, citrate, and EDTA. Current inventory amounts are given in Table 1 along with calculated millimolar concentrations of each OCA should a direct brine release (DBR) occur (Clayton, 2008; Van Soest et al., 2012). These values are subject to change with updated inventory records and minimum brine volume calculations.

Table 1. Projected inventory of organic acids and anions in WIPP along with calculated concentrations based on the minimum brine volume DBR (i.e. maximum OCA concentrations).

Organic Complexing Agent	Mass (kg)	Concentration (mM, based on 17,400 m^3 DBR volume)
Acetate	9.96×10^3	9.70
Acetic acid	1.41×10^4	9.88
Citrate	2.55×10^3	0.78
Citric acid	5.23×10^3	1.10
EDTA	3.76×10^2	0.06
Oxalate	6.5×10^2	0.42
Oxalic acid	1.78×10^4	7.75

Importance of OCA solubility. It is well understood that in order for microorganisms to degrade a compound, the compound must be in solution. Thus, solubility and dissolved concentrations affect bioavailability and rates and extent of degradation. Brine is a poor solvent, so the estimated concentration of OCAs in the brine release may be optimistic in some cases.

Objective and scope. The objective of this work was to determine whether WIPP-indigenous microorganisms were capable of degrading the four OCAs under either aerobic or anaerobic conditions. The scope of anaerobic investigations was narrowed to include only citrate under denitrifying conditions. This reduction in scope was deemed more realistic, given the thermodynamic constraints on most other metabolic processes in the near-field. Solubility investigations were also performed in order to estimate substrate availability.

PART 1: SOLUBILITY

Methods. WIPP-relevant brines were prepared over a range of pC_{H+} with formulas derived from model predictions and empirical measurements of each brine constituent (Brush et al., 2011; Lucchini et al., 2013; see Appendix1b). All brines were prepared in a nitrogen-filled glove box in order to minimize carbonate. Concentrated NaOH was used to adjust the pH of each solution. Brines were filtered to remove precipitates and dispensed into sterile 60 ml bottles. Individual OCAs were added as solids to each bottle in order to achieve over-saturation (~10-20% above the predicted OCA dissolved concentration, determined previously; data not shown). Aliquots were removed for analysis of inorganic and organic anions by ion chromatography. Samples were filtered and diluted in high-purity water prior to analysis. The experimental matrix is shown in Appendix 1a.

Results and Discussion. Results of IC analysis of the brines immediately after and one month after the addition of acetate, citrate, and oxalate are shown in Figures 1a-1c; EDTA analysis at the initial timepoint is given in Figure 1d.

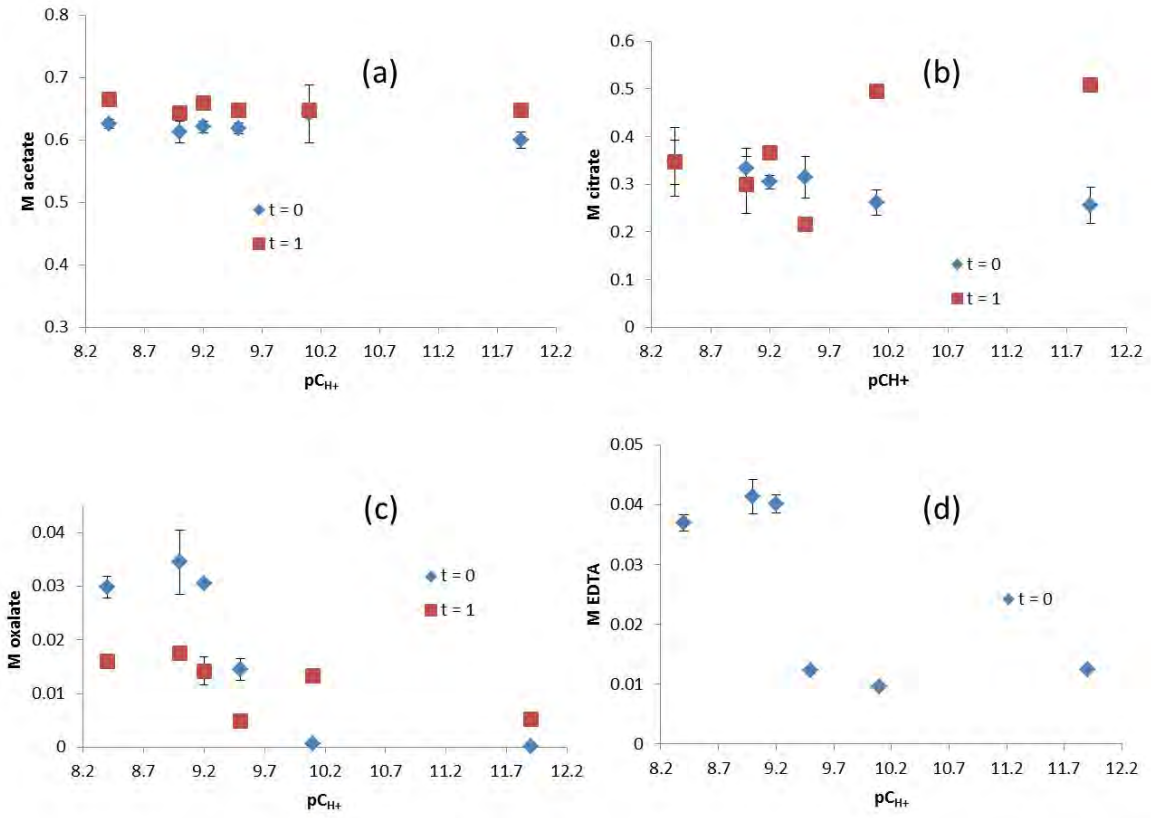

Figure 1. Dissolved concentrations of organic complexing agents in brines as a function of pC_{H+}.

Acetate is the most soluble of the four OCAs and has maintained a relatively constant concentration across all brines, suggesting that its solubility is not affected by brine composition and pC_{H+}. Oxalate concentrations decrease with increasing pC_{H+}, although this observation is not as pronounced at the later time point. A decrease in oxalate solubility with time is consistent with previous findings and is thought to be due to slow kinetics of precipitation (Swanson et al., 2013). Magnesium concentrations also decrease with increasing pC_{H+} and may be a reason for lower oxalate concentrations at higher pC_{H+} (Lucchini et al., 2013). Citrate concentrations showed a decrease with increasing pC_{H+} at the initial time point but an increase with increasing pC_{H+} at the second sampling. This may be due to initial precipitation as a magnesium complex followed by subsequent resolubilization as both calcium and sodium concentrations increase. EDTA concentrations also show a decrease with increasing pC_{H+}.

Individually, all OCAs with the possible exception of oxalate should be soluble, and therefore bioavailable, at the expected WIPP pC_{H+} (9.5) and at the inventory-predicted concentrations (see Table 2). This is in contrast to the case in which all OCAs are present. Previous observations in that case showed that only citrate and EDTA were soluble at their predicted inventory concentrations (see Appendix 2; Swanson et al., 2013). Also, slow precipitation of oxalate from solution affected the dissolution and bioavailability of acetate and citrate.

Should the DBR volume increase, this could result in all OCAs being soluble but their concentrations being lowered by dilution. In this case, solubility is no longer a limiting factor to degradation.

Table 2. Comparison of inventory-based concentrations of OCAs (from Table 1) to measured concentrations of each OCA in brine at WIPP-specific pC_{H+} of 9.5.

Organic Complexing Agent	Concentration (mM, based on 17,400 m^3 DBR volume)	Measured Dissolved Concentration of Individual Anion at pC_{H+} 9.5 (mM)
Acetate	9.70	648 ± 5.00
Acetic acid	9.88	---
Citrate	0.78	216 ± 6.00
Citric acid	1.10	---
EDTA	0.06	12.2 ± 0.60
Oxalate	0.42	4.80 ± 0.70
Oxalic acid	7.75	---

PART 2: AEROBIC DEGRADATION OF ORGANIC COMPLEXING AGENTS BY WIPP-INDIGENOUS HALOPHILIC MICROORGANISMS

Data and conclusions drawn from experiments on the aerobic degradation of OCAs by WIPP halophiles are provided in Appendix 5. In summary, a mixed culture of haloarchaea enriched from WIPP halite utilized both acetate and citrate under aerobic conditions. A haloarchaeon (*Halosimplex carlsbadense*), previously isolated from WIPP halite, was able to utilize oxalate in GWB-based incubations. A halophilic bacterium (*Halomonas halodenitrificans*), similar to one previously isolated from WIPP environs, degraded acetate and citrate in diluted brine incubations. EDTA was not degraded by any of the test organisms.

PART 3: DEGRADATION OF CITRATE BY WIPP-RELEVANT MICROORGANISMS UNDER NITRATE-REDUCING CONDITIONS

The WIPP performance model predicts that once oxygen is consumed, microbial activity will proceed to denitrification as the next respiratory process (CRA SOTERM 2009). Nitrates are present in the WIPP as inorganic co-contaminants (Van Soest et al., 2012). Citrate was tested given that it is, next to EDTA, the strongest complexant of the four agents. EDTA was not tested due to the low probability of degradation by the test organisms. Both halophilic bacteria and haloarchaea are capable of citrate utilization and nitrate reduction; therefore, both were tested.

Part 3a: Haloarchaea enriched from WIPP halite

Methods. WIPP brines (95% formulation) were amended with citrate and nitrate to serve as substrate/electron donor and terminal electron acceptor, respectively. Nutrient supplements were also added in the form of ammonium chloride, potassium phosphate, vitamins, and trace minerals

(see Appendix 3). Media were inoculated with a mixed culture of haloarchaea enriched under aerobic conditions from WIPP halite.

Incubations were sampled periodically for analysis of citrate, nitrate, and nitrite by ion chromatography and for cell numbers by direct microscopy.

***Results and Discussion*.** Several weeks were required for levels of citrate and nitrate to reach steady state. However, no utilization of citrate and no growth of cells were observed during or after this time. The organisms remain viable, and tests for the co-oxidation of citrate with acetate may be conducted.

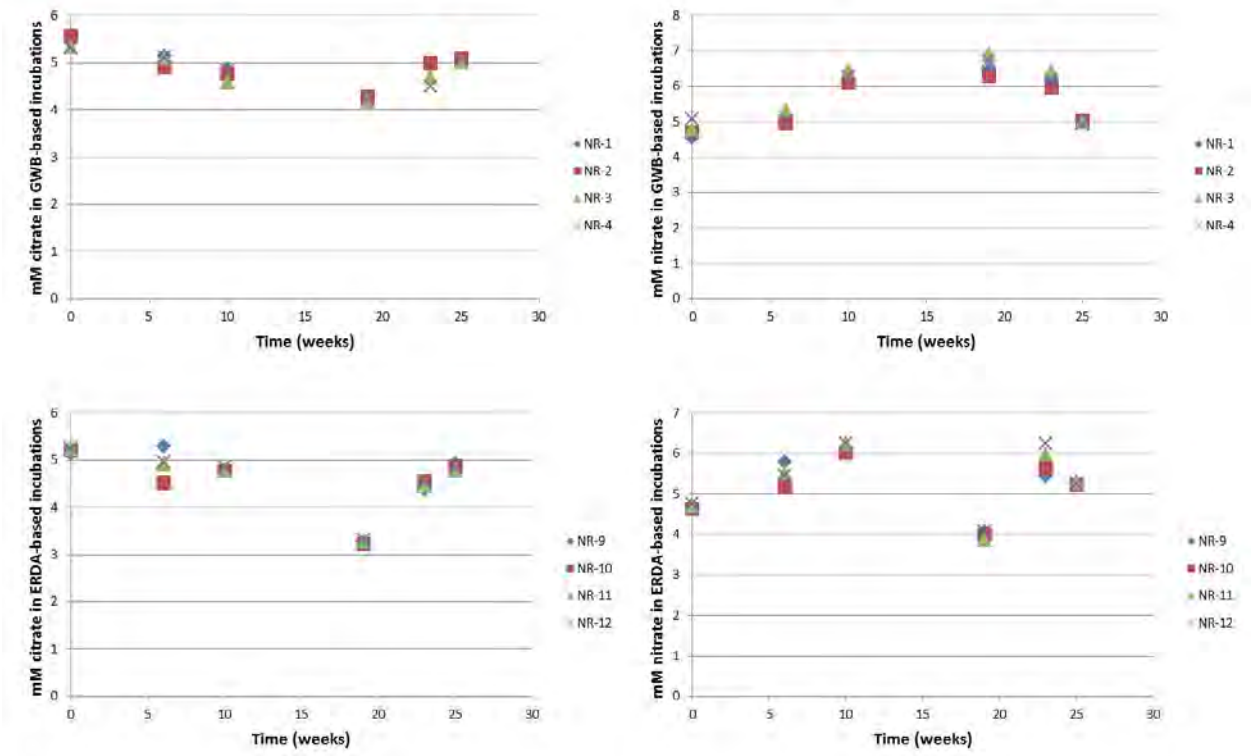

Figure 2. Citrate and nitrate levels over time in brine-based incubations of haloarchaea. NR-1 and NR-2 are replicate biotic incubations in GWB-based medium; NR-3 and NR-4 are replicate abiotic incubations in GWB-based medium. NR-9 and NR-10 are replicate biotic incubations in ERDA-based medium; NR-11 and NR-12 are replicate abiotic incubations in ERDA-based medium.

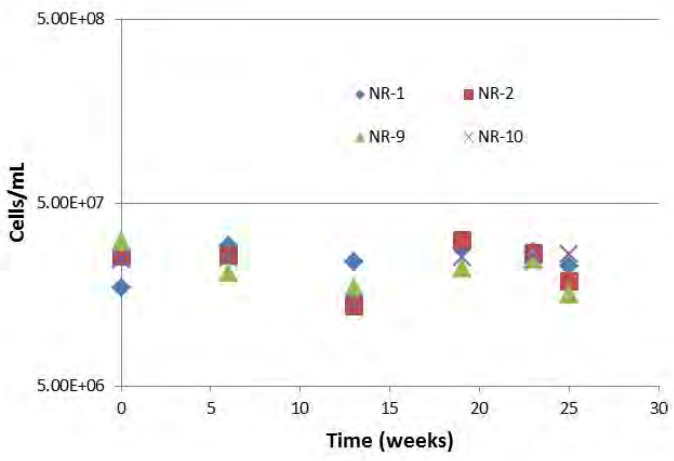

Figure 3. Cell numbers of above incubations over time. NR-1 and NR-2 are GWB-based incubations; NR-9 and NR-10 are ERDA-based incubations. No cells were visible in abiotic incubation. (Note: non-QL-1 data)

Because cell numbers were not increasing and phosphate could not be measured in solution, it was thought that the organisms may not have sufficient nutrients. Additional ammonium chloride and sodium phosphate were added at 19 weeks. This had no effect.

This same culture had previously degraded citrate aerobically in a minimal brine-based medium (Swanson et al., 2013). Because of their inability to do so under nitrate-reducing conditions, it is likely that the archaea in this culture are obligate aerobes. Many haloarchaea are capable of nitrate reduction; thus, the results of these experiments do not preclude the possibility that other haloarchaea indigenous to the WIPP will possess this capability.

Part 3b: Halophilic bacteria enriched from WIPP groundwater

Methods. WIPP brines were diluted to 75% of the 95% formulation. Citrate, nitrate, and nutrient amendments were added (see Appendix 4). Media were inoculated with a presumed mixed culture of bacteria enriched under denitrifying conditions from a high-ionic strength groundwater at the WIPP site (monitoring well WQSP-3). At the time these experiments were begun, this culture had not been characterized. Incubations were sampled periodically for analysis of citrate, nitrate, and nitrite by ion chromatography and for cell numbers by direct microscopy.

Results and Discussion. Cell numbers increased over half an order of magnitude in the ERDA-6-based incubations over a period of six weeks but then decreased again. No definitive difference was measured between biotic and abiotic citrate values during that time. Nitrate concentrations in biotic ERDA-based incubations were consistently lower than abiotic nitrate concentrations after zero time. The production of nitrite in ERDA-based biotic incubations cannot be explained by citrate oxidation.

Recently, the culture used in these experiments was found to possess 99% similarity in its small subunit ribosomal DNA sequence to *Arhodomonas recens*. This genus comprises halophilic bacteria, most commonly associated with petroleum-contaminated saline environments.

Unfortunately, it was discovered that this organism does not utilize citrate, even under aerobic conditions (Saralov et al., 2012). Acetate has been added to these incubations to test for citrate cooxidation.

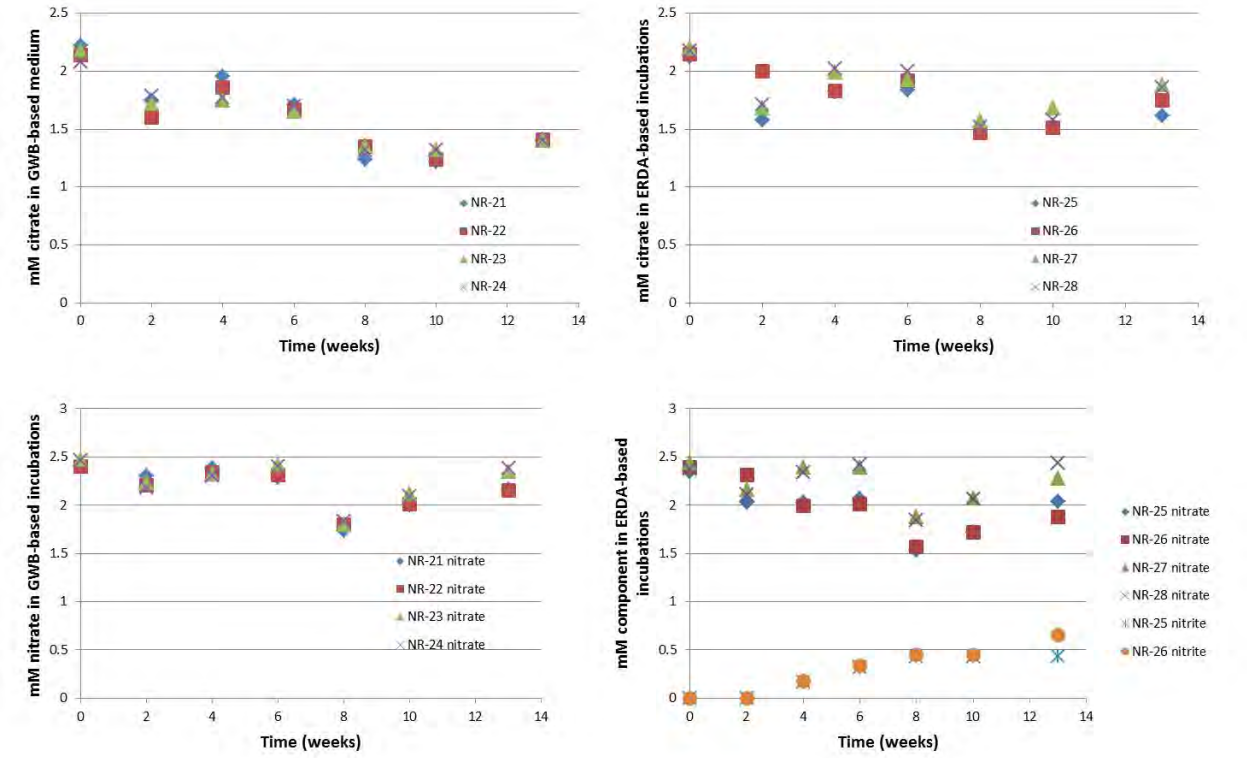

Figure 4. Citrate, nitrate, and nitrite levels over time in brine-based incubations of WIPP groundwater organisms. NR-21 and NR-22 are replicate biotic incubations in GWB-based medium; NR-23 and NR-24 are replicate abiotic incubations in GWB-based medium. NR-25 and NR-26 are replicate biotic incubations in ERDA-based medium; NR-27 and NR-28 are replicate abiotic incubations in ERDA-based medium. Nitrite levels are graphed as cumulative nitrite over time.

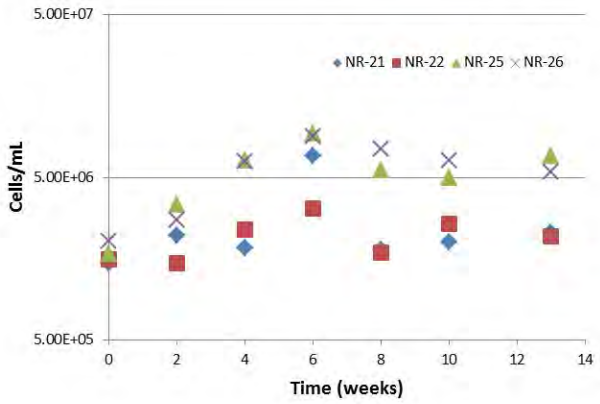

Figure 5. Cell numbers of above incubations over time. NR-21 and NR-22 are GWB-based incubations; NR-25 and NR-26 are ERDA-based incubations. No cells were visible in abiotic incubations. (Note: non-QL-1 data)

PART 4. CONCLUSIONS

At the expected WIPP pC_{H+} of 9.5, citrate and EDTA will be soluble at the levels estimated by inventory, thus making them bioavailable. Oxalate will be at its solubility limit. Dissolved acetate concentrations varied most when present individually and in combination with other OCAs, but acetate is likely to be soluble at the inventory-predicted concentration.

During the initial period of aerobiasis, indigenous microorganisms (i.e. haloarchaea) should be able to degrade acetate, citrate, and oxalate in the following order, based on solubility/bioavailability, degradability, and substrate preference: acetate >> citrate >>> oxalate. EDTA degradation by haloarchaea has not been shown; therefore, complexing metabolites are unlikely to accumulate. Aerobic citrate metabolism is also unlikely to generate complexants.

As conditions proceed toward anaerobiasis, haloarchaea are less likely to predominate. Few halophilic bacteria will survive in the near-field, as the ionic strength within the immediate repository will be too high, but those that will, will most likely be fermenters. In the intermediate- and far-fields, halophilic bacteria should be able to degrade acetate and citrate under a variety of conditions (e.g. acetate oxidation coupled to reduction of nitrate, oxyanions, metals, or sulfate; citrate fermentation; citrate oxidation coupled to nitrate reduction). It is unlikely that EDTA will be degraded until the extreme far-field, where less halophilic organisms possessing greater metabolic capabilities are present. Citrate fermentation may result in the formation of complexing metabolites (e.g. lactate), but these will likely undergo additional fermentation or oxidation.

For the WIPP case, the low inventory (in EDTA), low solubility (oxalate), and potential biodegradability (acetate and citrate) of OCAs combined with the presence of magnesium oxide should mitigate any detrimental effects caused by the presence of organic complexing agents in the waste.

REFERENCES

Brush L.H., Domski P.S., Xiong Y.-L. 2011. Predictions of the Compositions of Standard WIPP Brines as a Function of pC_{H+} for Laboratory Studies of the Speciation and Solubilities of Actinides. Sandia National Laboratories; Carlsbad, NM.

Clayton D.J. 2008. Update to the calculation of the minimum brine volume for a direct brine release. Memorandum to L.H. Brush L. ERMSS 548522. Sandia National Laboratories; Carlsbad, NM.

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

Egli T. 2001. Biodegradation of metal-complexing aminopolycarboxylic acids. Journal of Bioscience and Bioengineering 92: 89-97.

Francis A.J. 1998. Biotransformation of uranium and other actinides in radioactive wastes. Journal of Alloys and Compounds 271-273: 78-84.

Francis A.J., Dodge C.J., Ohnuki T. 2007. Microbial transformations of plutonium. Journal of Nuclear and Radiochemical Sciences 8: 121-126.

Huang F.Y.C., Brady P., Lindgren E.R., Guerra P. 1998. Biodegradation of uranium-citrate complexes: implications for extraction of uranium from soils. Environmental Science & Technology 32: 379-382.

Keith-Roach M.J. 2008. The speciation, stability, solubility and biodegradation of organic co-contaminant radionuclide complexes: a review. Science of the Total Environment 396: 1-11.

Lloyd J.R., Macaskie L.E. 2002. Biochemical basis of microbe-radionuclide interactions. In: Keith-Roach M.J., Livens F.R., editors. Interactions of microorganisms with radionuclides. London: Elsevier-Science Ltd. pp. 313-342.

Lucchini J.F., Borkowski M., Khaing H., Richmann M.K., Reed D.T. 2013. WIPP Actinide-Relevant Brine Chemistry. LCO-ACP-15. Los Alamos National Laboratory; Carlsbad, NM.

Nörtemann B. 1999. Biodegradation of EDTA. Applied Microbiology and Biotechnology 51: 751-759.

Pedersen K. 2002. Microbial processes in the disposal of high-level radioactive waste 500 m underground in Fennoscandian shield rocks. In: Keith-Roach M.J., Livens F.R., editors. Interactions of microorganisms with radionuclides. London: Elsevier-Science Ltd. pp. 279-311.

Saralov A.I., Kuznetsov B.B., Reutskikh E.M., Baslerov R.V., Panteleeva A.N., Suzina N.E. 2012. Arhodomonas recens sp. nov., a Halophilic Alkane-Utilizing Hydrogen-Oxidizing Bacterium from the Brines of Flotation enrichment of Potassium Minerals. Microbiology 81: 582-588.

Satroutdinov A.D., Dedyukhina E.G., Chistyakova T.I., Witschel M., Minkevich I.G., Eroshin V.K., Egli T. 2000. Degradation of metal-EDTA complexes by resting cells of the bacterial strain DSM9103. Environmental Science and Technology 34: 1715-1720.

Swanson J.S., Norden D.M., Khaing H., Reed D.T. 2013. Degradation of Organic Complexing Agents by Halophilic Microorganisms in Brines. Geomicrobiology Journal 30: 189-198.

Van Soest, G.D, McInroy, B. 2012. Performance Assessment Inventory Report-2012. LA-UR-12-26643. Los Alamos National Laboratory; Carlsbad, NM.

Witschel M., Egli T., Zehnder A., Spycher M. 1999. Transport of EDTA into cells of the EDTA-degrading bacterial strain DSM9103. Microbiology 145: 973-983.

APPENDIX 1a. EXPERIMENTAL MATRIX FOR SOLUBILITY STUDY

ORGANIC LIGAND	BRINE					
	pC_{H+} 8.4		pC_{H+} 9.0		pC_{H+} 9.2	
Acetate	8.4-A-ace	8.4-B-ace	9.0-A-ace	9.0-B-ace	9.2-A-ace	9.2-B-ace
oxalate	8.4-A-ox	8.4-B-ox	9.0-A-ox	9.0-B-ox	9.2-A-ox	9.2-B-ox
citrate	8.4-A-cit	8.4-B-cit	9.0-A-cit	9.0-B-cit	9.2-A-cit	9.2-B-cit
EDTA	8.4-A-EDTA	8.4-B-EDTA	9.0-A-EDTA	9.0-B-EDTA	9.2-A-EDTA	9.2-B-EDTA
All	8.4-A-All	8.4-B-All	9.0-A-All	9.0-B-All	9.2-A-All	9.2-B-All
ORGANIC LIGAND	BRINE					
	pC_{H+} 9.5		pC_{H+} 10.2		pC_{H+} 11.9	
acetate	9.5-A-ace	9.5-B-ace	10.2-A-ace	10.2-B-ace	11.9-A-ace	11.9-B-ace
oxalate	9.5-A-ox	9.5-B-ox	10.2-A-ox	10.2-B-ox	11.9A-ox	11.9-B-ox
citrate	9.5-A-cit	9.5-B-cit	10.2-A-cit	10.2-B-cit	11.9-A-cit	11.9-B-cit
EDTA	9.5-A-EDTA	9.5-B-EDTA	10.2-A-EDTA	10.2-B-EDTA	11.9-A-EDTA	11.9-B-EDTA
All	9.5-A-All	9.5-B-All	10.2-A-All	10.2-B-All	11.9-A-All	11.9-B-All

APPENDIX 1b. BRINE FORMULATIONS AT VARIOUS pC_{H+}

Brine / Component	pC_{H+} = 8.4 (measured pH = 7.13)		pC_{H+} = 9.0 (measured pH = 7.77)		pC_{H+} = 9.2 (measured pH = 7.13)		pC_{H+} = 9.5 (measured pH = 7.98)		pC_{H+} = 10.2 (measured pH = 9.18)		pC_{H+} = 11.9 (measured pH = 11.0)	
	g/L	M	g/L	M	g/L	M	g/L	M	g/L	M	g/L	M
$Na_2B_4O_7 \cdot 10H_2O$	14.08	0.0369	14.08	0.0369	8.659	0.0227	2.477	0.0065	1.086	0.0028	1.086	0.0028
$MgCl_2 \cdot 6H_2O$	193.3	0.9509	193.3	0.9509	184.4	0.9069	95.98	0.4721	13.06	0.0643	13.06	0.0643
Na_2SO_4	23.60	0.1661	23.60	0.1661	23.87	0.1680	22.99	0.1618	22.96	0.1616	22.96	0.1616
LiCl	0.168	0.0040	0.168	0.0040	0.082	0.0019	0.134	0.0032	0.141	0.0033	0.141	0.0033
NaBr	2.566	0.0249	2.566	0.0249	2.426	0.0236	2.323	0.2226	2.317	0.0225	2.317	0.0225
KCl	32.57	0.4368	32.57	0.4368	32.47	0.4356	32.67	0.4382	32.31	0.4334	32.31	0.4334
$CaCl_2 \cdot 2H_2O$	1.807	0.0092	1.807	0.0092	2.431	0.0123	2.458	0.0125	2.454	0.0125	2.454	0.0125
NaCl	167.9	2.8723	167.9	2.8723	172.1	2.9440	208.6	3.5688	253.0	4.3284	253.0	4.3284

APPENDIX 2. OCA DEGRADATION PAPER, Table 2. Steady-state concentrations of organic complexing agents in abiotic controls (mean of triplicate samples ± standard deviation). Originally added concentrations are given at the head of each column.

Brine	Oxalate (30 mM)	Acetate (27 mM)	Citrate (3.7 mM)	EDTA (0.12 mM)
1X GWB	7.85 ± 0.34	9.53 ± 0.63	3.65 ± 0.29	0.13 ± 0.01
0.75X GWB	6.02 ± 1.24	10.84 ± 1.53	3.10 ± 0.46	0.10 ± 0.02
0.5X GWB	4.67 ± 0.32	12.14 ± 0.25	3.47 ± 0.47	0.11 ± 0.01
0.25X GWB	4.35 ± 0.51	11.89 ± 0.49	4.19 ± 0.38	0.13 ± 0.01
1X ERDA-6	6.71 ± 1.64	9.99 ± 0.96	2.89 ± 0.33	0.12 ± 0.01
0.75X ERDA-6	11.76 ± 1.00	11.36 ± 0.39	3.35 ± 0.46	0.13 ± 0.01
0.5X ERDA-6	17.80 ± 1.34	13.05 ± 1.32	3.85 ± 0.44	0.13 ± 0.01
0.25X ERDA-6	23.49± 1.61	12.26 ± 1.11	4.60 ± 0.29	0.11 ± 0.02

APPENDIX 3. EXPERIMENTAL SET-UP FOR HALOARCHAEA/CITRATE DEGRADATION

Components were added as solids to pre-prepared 95% brines according to the table below:

Component \ Brine	GWB g/250 mL	ERDA g/250 mL
Trisodium citrate dehydrate	0.3679	0.3677
Ammonium chloride	0.0409	0.0402
Potassium dihydrogen phosphate	0.1020	0.1034
Potassium nitrate	0.1263	0.1253

pH was adjusted to 8.01 for ERDA and 7.04 for GWB. Media were allowed to equilibrate for 5 days prior to sparging with nitrogen. Media were then brought into the nitrogen-filled glove box, passed through a 0.22 µm nylon filter, and allowed to equilibrate for another month prior to inoculation.

Media were inoculated with a mixed haloarchaeal culture originally enriched from WIPP halite (Swanson et al., 2013) that had been maintained in three different media: a generic halophile medium with yeast extract and casamino acids as the predominant substrates, the same generic medium with citrate, and brine-based media containing only acetate and citrate.

IC analyses were performed on a Dionex ICS-3000.

APPENDIX 4. EXPERIMENTAL SET-UP FOR GROUNDWATER CULTURE CITRATE DEGRADATION

Components were added as 100X stocks to 0.75X dilutions of 95% brines with HPW. Nitrogen and phosphorus containing nutrients were prepared as one stock solution.

Component	mass/10 mL
trisodium citrate	0.5763 g
KNO_3	0.1856 g
NH_4Cl	0.0824 g
KH_2PO_4	0.2608 g

Other components:
Trace minerals solution and vitamin solution from ATCC.

pH check: GWB-based medium, 7.436; ERDA-based medium, 8.051; no adjustments made. Media sparged with N_2, sealed, autoclaved, and brought into glove box. Vitamins added.

Media were inoculated with a presumed mixed culture enriched under nitrate-reducing conditions from groundwater at the WIPP site (monitoring well WQSP-3).

Analyses were performed on a Dionex ICS-3000 ion chromatograph.

APPENDIX 5

Manuscript entitled:

Degradation of Organic Complexing Agents by Halophilic Microorganisms in Brines

DEGRADATION OF ORGANIC COMPLEXING AGENTS BY HALOPHILIC MICROORGANISMS IN BRINES

Running title: Degradation of complexants by halophiles in brines

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ABSTRACT

The potential use of geologic salt beds as terminal repositories for nuclear waste has necessitated research on the interaction of the waste with indigenous microbiota. Microorganisms may affect actinide solubility by degrading organic complexing agents present in the waste. A halophilic bacterium and *Archaea* indigenous to a salt formation in New Mexico were examined for their ability to degrade acetate, oxalate, citrate, and ethylenediamine tetraacetate under aerobic conditions in low and high-magnesium brines. All complexing agents, except EDTA, were utilized, suggesting that microorganisms indigenous to such repositories can potentially play a beneficial role in mitigating actinide mobility.

INTRODUCTION

Organic complexing agents—including low molecular weight organic acids, such as acetate, oxalate, and citrate, and higher molecular weight anthropogenic chelators, such as ethylenediamine tetraacetic acid (EDTA) and nitrilotriacetic acid (NTA)—were used extensively in chemical separations of radionuclides during past nuclear weapons processing and for decontaminating nuclear facilities. As a result, organic complexing agents (OCAs) often exist as co-contaminants in soils and groundwaters at many US Department of Energy sites across the nation (Riley and Zachara, 1992) and are also present in waste already emplaced in and destined for nuclear repositories worldwide.

The biodegradation of certain complexing agents has been reviewed for a variety of microorganisms and metals in order to estimate the potential impact on actinide and toxic metal mobility (Francis, 1998; Banaszak and others, 1999; Nortemann, 1999; Egli, 2001; Lloyd and McCaskie, 2002; Pedersen, 2002; Keith-Roach, 2008; Francis and others, 2007; Reed and others, 2010). Degradation can be dependent upon the stability and degree of complexation, both of which affect the free, and therefore bioavailable, organic in solution (Huang and others, 1998; Witschel and others, 1999; Satroutdinov and others, 2000).

Much research has focused on the degradation of aminopolycarboxylic acid chelates, e.g. EDTA and NTA, and citrate, rather than acetate or oxalate, due to their widespread use and higher complexing power (Means and others 1980; Nörtemann, 1992; Bolton and others, 1996; Banaszak and others, 1998; Thomas and others, 1998; Francis and others,

2007). Oxalate degradation by gut bacteria is widely studied by those in the medical field; while, in the environment, it is produced by many soil fungi and degraded therein by those same fungi and other soil bacteria *(*Espejo and Agosin, 1991; Morris and Allen, 1994; Dutton and Evans, 1996). Acetate degradation is not often a focus of study, because it is a common substrate for most microorganisms, and it forms a weak complex with actinides.

Past studies on OCA-actinide-microbial interactions utilized isolates representative of populations found in soils and groundwaters surrounding various existing waste storage sites or sites under consideration for repositories. Since the majority of geologic nuclear waste repositories under investigation are associated with low ionic strength groundwaters (e.g. granitic rock, clay, or tuff formations), the focus of previous research on OCAs has been in low ionic strength matrices. Research in high ionic strength matrices is important to the currently operating transuranic waste repository in bedded salt (the Waste Isolation Pilot Plant-WIPP/USA), the nuclear waste storage facility and a proposed high level waste repository in an underground salt dome (Asse, Gorleben/FDR), and also to discussions of expanded nuclear salt repository options (Fuenkajorn and Wetchasat, 2001).

Early microbial studies on the WIPP repository evaluated the degradation potential of two organisms (isolated from various laboratory-used papers, rubber, and plastics meant to simulate waste inocula) on europium-citrate and –EDTA complexes (Barnhart and others, 1978a, 1978b, 1979a, 1979b). Slow degradation occurred in some incubations but

not in those using brine as the basal medium. This was not surprising, given that none of the isolates was known to be either halotolerant or halophilic. The further intent of these researchers was to look at the degradation potential exhibited by halophilic organisms found in the WIPP; however, their attempts to isolate such organisms were unsuccessful. Later studies did focus on WIPP-relevant halophiles, but OCA degradation by these organisms was not addressed.

To our knowledge, there are no data regarding the impact of OCA degradation by halophilic microorganisms, as this topic is unusual for both the field of repository microbiology (usually concerned with lower ionic strengths) and the field of extremophiles (which is concerned with neither actinides nor their complexants). This study was conducted to examine the potential biodegradation of acetate, oxalate, citrate, and EDTA under aerobic conditions by halophilic microorganisms in order to address the early period in repository history where these conditions may be present, and to define the scope for further investigations centered on anaerobic degradation.

MATERIALS AND METHODS

Organisms

Three inocula were used for this study. *Halomonas halodenitrificans* was obtained from the American Type Culture Collection (ATCC #13511). *Halomonas* species have been isolated from WIPP halite previously (Francis and others, 1997) and, in prior work, were found to make up 96% of a clone library of bacterial 16S rRNA gene sequences obtained from raw halite. This organism was used to represent moderately halophilic *Bacteria*.

Halosimplex (Hsx.) carlsbadense was also isolated from WIPP halite (Vreeland and others, 2002) and was used in this study to represent extremely halophilic *Archaea*. In addition to these isolates, a mixed culture of *Archaea* enriched aerobically from WIPP halite was used as the third inoculum.

Media

The Generic Weep Brine (GWB) simulates interstitial brine and halite inclusion fluid from the Salado Formation. Brine from the Energy Research and Development Administration Well 6 (ERDA-6) represents brine from the Castile Formation, the geologic layer underlying the Salado Formation (Snider, 2003). The elemental composition of each brine is given in Table 1. The GWB basal medium was composed of the following in g/L: $MgCl_2 \cdot 6H_2O$, 193.4; NaCl, 167.8; Na_2SO_4, 23.61; NaBr, 2.565; $Na_2B_4O_7 \cdot 10H_2O$, 14.03; KCl, 32.57; LiCl, 0.174; $CaCl_2 \cdot 2H_2O$, 1.896; NH_4Cl, 0.300; KH_2PO_4, 0.400. The ERDA-6-based medium contained the following in g/L: NaCl, 248.6; Na_2SO_4, 22.52; $Na_2B_4O_7 \cdot 10\ H_2O$, 5.700; NaBr, 1.074; KCl, 6.869; $MgCl_2 \cdot 6H_2O$, 3.667; $CaCl_2 \cdot 2H_2O$, 1.672; NH_4Cl, 0.300; KH_2PO_4, 0.400. The brines were passed through 0.22 µm nylon filters, stored in the dark and allowed to equilibrate for 10 days.

Four organic complexing agents were added to each full-strength (1X) brine at the following concentrations: 38 mM acetate, 5.7 mM citrate, 0.15 mM EDTA, and 45 mM oxalate. These levels approximate those predicted in the low-probability event of a drilling intrusion from the surface into the Castile reservoir, which would inundate the repository horizon and fully dissolve each OCA (Brush and others, 2009). Separate

aliquots of the 1X brines were mixed with distilled, deionized water to obtain 0.75X, 0.5X, and 0.25X dilutions. Amendments from sterile stocks were added to the dilution series to obtain the same concentrations of organics and nutrients as in the 1X media. The measured pH of each medium was adjusted to 7.00 for GWB and 8.00 for ERDA-6 versus a standard hydrogen electrode. This corresponds to a pC_{H+} of 8.23 for GWB and 8.94 for ERDA-6 (Lucchini and others, 2007; Borkowski and others, 2009). Media were again filtered to remove precipitates prior to dispensing 20 mL into sterile 50 mL polypropylene tubes and were allowed to equilibrate for another 5 days prior to inoculation.

Incubation and monitoring

H. halodenitrificans was grown overnight at 37°C prior to inoculation. *Hsx. carlsbadense* had been growing for 10 days at 37°C, and the halite enrichment culture had been growing for 8 weeks at 28°C. The *Halosimplex* and the enrichment culture were centrifuged and washed in either 1X GWB or ERDA-6, depending upon the intended medium. These cultures were inoculated only into 1X brines. *Halomonas* were washed in 6% NaCl prior to inoculation into a dilution series of 1X, 0.75X, 0.5X and 0.25X of each brine. Both the *Halomonas* and *Halosimplex* samples and corresponding abiotic tubes were incubated aerobically at 37°C in the dark and shaken at 175 rpm. The halite enrichment culture and its corresponding abiotic tubes were incubated aerobically in the dark between 26-28°C and also shaken.

Organism growth was monitored by plate counts, for *H. halodenitrificans* and *Hsx. carlsbadense*, and by microscopic direct counts for *Hsx. carlsbadense* and the enrichment culture. Plate counts for *Halomonas* were made on nutrient agar with 6% (w/v) NaCl and for *Halosimplex* on pyruvate-glycerol agar (Vreeland and others, 2002). Direct cell counts were made on a Zeiss Axioskop 40 epifluorescence microscope after incubation with the Live/Dead®BacLight™ viability stain (Leuko and others, 2004). Periodic spot checks were made on abiotic controls by direct microscopy to verify the absence of organisms.

DNA-based analyses

DNA from the enrichment culture incubations was extracted at the end of the incubation period using the Ultra Clean Soil DNA Purification kit (MoBio Laboratories, Carlsbad, CA) per the manufacturer's directions. An aliquot from each incubation was centrifuged and washed with 20% NaCl, prior to extraction of DNA. Purified DNA was pooled from triplicate samples to create the template for polymerase chain reactions (PCR). PCR was performed using primers 21f (5'-TTCCGGTTGATCCTGCCGGA-3'; DeLong, 1992) and 1392r (5'-ACGGGCGGTGTGTRC-3'; Lane, 1991). Each 50 µl reaction contained 1X PCR buffer (10 mM Tris-HCl, 50 mK KCl, pH 8.3), 1.5 mM $MgCl_2$, 250 µM each dNTP, 500 nM each primer, and 2.5 U *Taq* polymerase (Applied Biosystems; Foster City, CA). A three-minute denaturation at 94°C was followed by 25 cycles at 94°C, 56°C, and 72°C, with a final extension step of 10 minutes at 72°C. PCR amplicons were cleaned using Qiagen's PCR Purification Kit (Qiagen; Valencia, CA), inserted into the pCR®4-TOPO® vector using the TOPO TA Cloning® Kit for Sequencing (Invitrogen/Life

Technologies; Rockville, MD), following the manufacturer's directions, and transformed into *E. coli* DH5α^{TM}-T1^{R} chemically competent cells. Clones were shipped to SeqWright, Inc. (Houston, TX) for sequencing. Sequences were screened for chimeras using Bellerophon (comp-bio.anu.edu.au/bellerophon/bellerophon.pl; Huber and others, 2004), and grouped at 97% similarity using Sequencher 4.9 (Gene Codes Corporation; Ann Arbor, MI). Consensus sequences for each group were aligned in Clustal X v. 2.0.12 (Higgins and Sharp, 1988; Larkin and others, 2007), and a phylogenetic tree was constructed using the Neighbor-Joining method with 1000 bootstrap support. Trees were edited in TreeView version 1.6.6 (taxonomoy.zoology.gla.ac.uk/rod/treeview). The BLAST program of the National Center for Biotechnology Information database was used to putatively identify organisms (Altschul and others, 1997; ncbi.hlm.nih.gov). Sequences were deposited in GenBank with accession numbers JN088839 through JN088932.

Chemical analyses

At each sampling point, an aliquot was removed, passed through a 100 kD filter, and diluted 1000-fold in high-purity water (HPW). This dilution factor was necessary due to the potentially deleterious effects of the high ionic strength samples on the instrumentation, and also due to the masking of acetate by the large chloride peak. Analyses were performed on a Dionex DX-500 ion chromatograph. Acetate, oxalate, and citrate were separated by gradient elution through a hydroxide-selective anion exchange column (AS11) and detected by suppressed conductivity (Blau and others, 1998). Samples for EDTA analysis were separated on an anion exchange column (AS7) with an

isocratic nitric acid mobile phase. A post-column conversion to a Fe(III) complex was then measured by UV detection at 330 nm (Kemmei and others, 2006).

RESULTS AND DISCUSSION

Dissolved concentrations of organic complexing agents in study brines

Biodegradability of a compound is dependent upon its bioavailability, and compounds not in solution are generally assumed to be unavailable. This phenomenon was readily apparent in this study and, therefore, a discussion on the solubilization of organic complexing agents in brines is warranted in order to explain the results.

In this study, solution behavior varied with OCA, brine concentration, and brine composition (see Table 2). ERDA-6 appeared to possess a higher solvating capacity than GWB, consistent with its lower ionic strength (4.97 M versus 6.84 M). With the exception of EDTA which was below its solubility limit at the added concentration, the measurements of OCAs were much lower than the levels predicted in the inundation scenario and, in 1X brines, were only a fraction of that originally added.

The most notable finding was the effect of oxalate on citrate and acetate concentrations. This was clearly demonstrated in the long term abiotic equilibration of GWB brine with all four OCAs (see Figure 1). During the first 100 days of equilibration, oxalate continuously precipitated, and citrate and acetate concentrations were very low (0.04 mM and <1 mM, respectively). When the concentration of oxalate approached its steady state (between 7-10 mM), increased solubilization of both citrate and acetate was noted.

Levels of acetate reached 9.53 ± 0.63 mM at steady state; while, citrate resolubilization proceeded more slowly and reached 3.65 ± 0.29 mM after 250 days equilibration.

Oxalate behavior also differed between the two studied brines. Steady state concentrations were relatively stable as a function of GWB dilution but increased significantly with ERDA-6 dilution. This trend can be explained by the difference in magnesium concentrations between GWB (0.95 M) and ERDA-6 (18 mM). In GWB, dissolved oxalate concentrations at t = 0, the initial rate of oxalate precipitation, and the time needed to reach steady state were all dependent upon magnesium concentration. That is, high levels of magnesium led to a rapid formation of metastable Mg-oxalate complexes which then precipitated more slowly from solution than those in diluted GWB. This is in contrast to ERDA-6, where steady oxalate precipitation was only observed in the 1X tubes but was not noted in the dilution series. This suggests that at lower concentrations of magnesium (i.e. ERDA-6 dilutions), dissolved oxalate is dependent upon the overall solvating capacity of the brine rather than on the magnesium itself.

Slow precipitation or coprecipitation has been observed with metals in brine systems and reflects the inherent complexity of brines and the length of time required to establish true solubility-controlling phases (Lucchini and others, 2007; Borkowski and others, 2009). It is likely that this is also true for the organics in this system, especially if their dissolved concentration is linked to Mg complexation. Precipitation in these experiments became a confounding factor in determining whether loss of the organic was actually due to

degradation, and in many cases, citrate and acetate utilization could not be confirmed until oxalate levels had decreased sufficiently. In these cases, degradation was measured as the difference between resolubilized OCA in biotic and abiotic incubations; that is, the steady state abiotic concentration versus final biotic measurements.

OCA degradation by WIPP-relevant isolates

Halomonas halodenitrificans

H. halodenitrificans was viable, but not thriving, for approximately two weeks in 1X GWB. In the diluted brines, biodegradation was readily apparent for acetate. Concentrations in the biotic and abiotic 0.5X brine incubations were significantly different after two weeks ($p < 0.02$; see Figure 2a); while, it only took one week in the 0.25X dilution to observe those differences ($p < 0.05$).

In both 0.5X and 0.25X GWB, citrate degradation was also observed. The precipitation of oxalate from solution to near steady state levels corresponded to an increase in citrate and acetate but only in abiotic incubations. This increase was not observed in the biotic incubations, suggesting that citrate and acetate were both degraded fully. Oxalate and EDTA were not utilized by *H. halodenitrificans* in these incubations.

H. halodenitrificans lost viability after 18 days in 1X ERDA-6 brine. As with the GWB incubations, acetate was degraded in the diluted incubations, with significant differences noted between biotic and abiotic incubations at 0.75X after 4 weeks that corresponded to cell growth ($p < 0.05$; see Figure 2b), and 0.5X dilutions by one week. In both 0.75X

and 0.5X incubations, citrate was also degraded but not until acetate had disappeared. Oxalate and EDTA were not degraded in these incubations.

Halomonas halodenitrificans is a moderately halophilic bacterium that exhibits optimum growth at sodium concentrations between 0.9-1.5 M NaCl but can survive in up to 3.4 M concentrations (Arahal and Ventosa, 2006). It is known to utilize both acetate and citrate in generic growth media, and this study confirms this is also the case in more stringent brine-based media. The lack of citrate utilization in 0.75X GWB was likely due to the fact that it was no longer viable by the time citrate appeared in solution.

Short-term *Halomonas* survival in 1X GWB was not surprising given the high ionic strength and high Mg content of this brine. Although *Halomonas* did survive longer in ERDA-6, its limited term survival in this case was probably due to the high NaCl levels, regardless of the lower concentration of magnesium. Halophilic bacteria maintain osmotic balance with their external environment by accumulating low-molecular-weight organic solutes intracellularly (Oren, 2006). Given that no such compatible solutes were supplied in the medium, *Halomonas* would have had to produce its own. This strategy may not have been effective enough at such high ionic strengths and, in any case, would have been energetically unfavorable.

Halosimplex carlsbadense

Hsx. carlsbadense is an extremely halophilic archaeon. Optimum growth occurs at 4.3 M NaCl with a range of 3.4-5.1 M. This organism has been shown to degrade acetate but

only in the presence of a second substrate, glycerol (Vreeland and others, 2002). Growth of *Halosimplex carlsbadense* in GWB would be especially significant, given the high levels of magnesium. Optimum growth for this organism occurs between 1-2 mM Mg, but no upper limit has been defined (Vreeland and others, 2002).

Hsx. carlsbadense did not appear to utilize acetate or EDTA in either the GWB or ERDA-6 brine-based media. There was a significant difference between the levels of oxalate in biotic versus abiotic incubations in GWB one week that corresponded to cell growth ($p < 0.02$; see Figure 3), and cell numbers decreased as this differential decreased. In order to confirm oxalate utilization as opposed to a microbially-enhanced precipitation, *Halosimplex* from each incubation and from the ATCC stock culture were plated onto solid media based on each brine and containing oxalate as the sole carbon source. Growth was positive for all cultures on both brine media. Colonies from the plates were confirmed to be *Hsx. carlsbadense* by DNA sequence analysis.

Although utilization of citrate in brine was not observed in these incubations, cultures and ATCC stock were also plated onto solid media containing citrate only. Again, growth was noted, and colony identification was confirmed.

OCA degradation by WIPP halite enrichment culture

The mixed archaeal culture enriched from WIPP halite thrived in both the GWB and ERDA-6 brine-based media. Citrate levels steadily decreased to zero in biotic ERDA-6 incubations at four times the overall rate of the abiotic incubations. After citrate

disappeared, acetate utilization began and continued until it was no longer measurable (day 79). At this point, cell numbers began to level off.

Although initially the differences were small, acetate levels in biotic GWB incubations were lower than those in the abiotic incubations and eventually disappeared completely, corresponding to a rise in cell numbers. Oxalate did not appear to be utilized, but as with the *Halomonas* incubations, its precipitation from solution corresponded to an increase in both soluble citrate and acetate in the abiotic incubations. Citrate levels rose slightly in the biotic incubations but then disappeared as it was likely utilized upon resolubilization. This accounts for the second, but smaller, increase in cell numbers around day 130 (see Figure 4).

Citrate utilization was confirmed by growth of subcultures on solid media with citrate as the sole carbon source. Oxalate and EDTA were not degraded by the mixed culture in either brine. However, oxalate utilization was demonstrated by growth of subcultures on solid media containing oxalate alone.

Results of enrichment culture community analysis

The archaeal communities that resulted at the end of incubation in the brine media were different from both the parent community and from each other. A brief description of the closest BLAST matches to consensus DNA sequences of groupings with ≤3% sequence divergence is given in Table 3. The makeup of the clone libraries for the parent and each

outcome culture is given in Figure 5, and the phylogenetic tree containing all groups is provided in Figure 6.

Ninety-one percent of the ERDA-6 outcome library clustered with a *Halorubrum (Hrr.)*-like species (DV427) and 8.6% with unidentified *Natronomonas*-like sequences (2-24-8). This is a decrease in diversity from the original parent culture with a total loss of *Halobacterium* (*Hbt.*) sequences and a significant increase in *Halorubrum*-like sequences (43% in parent culture). The GWB outcome library was slightly more diverse, containing *Hrr*-like sequences similar to ERDA-6 (53%) and *Hbt.* (29%) sequences. Additionally, 18% of the library was made up of clones that were 97% similar to *Halolamina pelagica*, an archaeon isolated from solar salterns in China but show little similarity to any known haloarchaea (Cui and others, 2011). This was an increase from 7% of the parent culture clone library.

The *Halobacterium* sequences in GWB showed a 99% match to both *Halobacterium noricense*, an isolate cultivated from a deep-drilled core of Permian halite in Austria (Gruber and others, 2004) and isolate 2-24-7 from Cretaceous halite in Brazil (Vreeland and others, 2007). The proportion of *Halobacterium* sequences in GWB decreased by 7% from the parent culture. This might be attributed to the levels of magnesium in GWB, although *Halobacterium noricense* survives in up to 1 M Mg (Gruber and others, 2004). The reason for the total loss of *Hbt.* sequences in the ERDA-6 could be due to pH (ERDA-6, pH 8.0). Although *Hbt. salinarum* is tolerant up to pH 8.0, *Hbt. noricense* tolerates a narrower range of 5.2-7.0 (Gruber and others, 2004). *Halobacterium* spp. are

not known for their use of complex substrates and generally require amino acids for growth (Oren, 2006). It is possible that, although these organisms survived in GWB, they did not thrive due to a lack of appropriate substrate or were outcompeted by other groups.

The *Halorubrum*-like sequences in both the outcome cultures most closely match those of DV427, although the sequence similarity ranges from 94 to 98%. DV427 is an isolate cultivated from 22,000 year old fluid inclusions in Death Valley halite (Schubert and others, 2010). The authors grew DV427 on medium containing 4.3 M NaCl and 8 mM Mg, with glycerol and pyruvate as carbon sources, and a pH between 7.3-7.5. ERDA-6 contains a similar makeup (4.25 M NaCl and 17 mM Mg), although the carbon substrates were different, and the pH is adjusted to 8.0. However, these sequences also made up 53% of the GWB library (2.9 M NaCl and 0.9 M Mg; pH 7.0), suggesting that the sodium, magnesium, and pH did not select for this group. The increase in these sequences may have been due to enrichment on the substrates provided.

Natronomonas-like sequences were not detectable in either the parent culture or in the GWB outcome community. Group 1 was most closely related to isolate 2-24-8 cultivated from fluid inclusions in 121 million year old halite from Brazil (Vreeland and others, 2007). Because little is known about these organisms and because they are seemingly distinct from other *Natronomonas* spp., it is difficult to hypothesize what led to their disappearance in GWB. It is possible that the high Mg of GWB inhibits their growth. *Nmn. pharaonis* has a Mg optimum below 0.01 M (Oren, 2006). Or they, like other

Natronomonas spp. and isolate 2-24-8, prefer more alkaline media (Vreeland and others, 2007).

The *Halolamina pelagica* sequences increased in the GWB outcome as compared to the parent culture, but they did not appear in the ERDA-6 outcome library. pH does not easily explain the loss of this organism from ERDA-6, as it exhibits growth over a broad range (5.5-9.5; Cui and others, 2011). *Halolamina pelagica* can utilize acetate but not citrate, so it is possible that it was able to outcompete the *Halorubrum*-like organisms for the available acetate in GWB.

The fact that many of the sequences found match organisms that have been isolated, or similar sequences that have been retrieved, from other halites worldwide shows promise for other salt repositories. For example, the *Halobacterium* sequences obtained from the GWB incubations are 99% similar to isolates and sequences from halite formations in Austria, Brazil, Poland, Turkey, and the United Kingdom (McGenity and others, 2000; Gruber and others, 2004; Vreeland and others, 2007; Weidler, 2007, unpublished submission to GenBank; Ozcan and others, 2009, unpublished submission to GenBank; Park and others, 2009). This type of similarity in organisms and sequences from locations both geographically distant and temporally disparate has been shown previously for strains of *Halococcus salifodinae* (Stan-Lotter and others, 1999) and for *Halobacterium* spp. (Vreeland and others, 2007). Therefore, there is reason to believe that the organisms enriched from WIPP halite in these incubations may also be present in

the halites of other salt-based repositories and may play a role in the degradation of organic complexing agents in those as well.

SUMMARY

Three of the four organic complexing agents tested in this study were degraded aerobically by microorganisms indigenous to the WIPP. Acetate was readily degraded by *Halomonas halodenitrificans* in diluted GWB and ERDA-6 brines and was also utilized by the enrichment culture in 1X ERDA-6 brine. Oxalate was degraded by *Halosimplex carlsbadense*. Citrate utilization by *Halomonas halodenitrificans* and the mixed culture was evident after oxalate levels decreased, and *Hsx. carlsbadense* was able to grow on plates containing citrate, even though it was not utilized in the brine media. EDTA, the strongest complexant, was not degraded by any of the organisms used in this study.

Citrate is the next strongest complexant of those investigated in this study, but its degradation by WIPP-relevant organisms should mitigate the effects of actinide solubilization. Acetate forms relatively weak complexes with actinides and will not likely outcompete hydrolysis and carbonate complexation at the expected repository pH, even if it is not degraded.

OCA degradation was influenced significantly by the solubilization of the complexant which, in turn, was dependent upon the relative brine strengths and the presence of magnesium. The slow precipitation of oxalate from solution influenced both citrate and acetate dissolved concentrations, and as a result their bioavailability.

This study did not address the degradation of OCAs complexed to actinides. Rates of biodegradation may be diminished by complexation with actinides, due to the presumed inability of the complex to be transported across the cell membrane (Banaszak and others, 1999; Satroutdinov and others, 2000; Zhang and others, 2007). Additionally, the type of ligand, bi- versus tri-, may affect rates of degradation, with tridentate complexes being less degradable (Francis and others, 1992).

In summary, whether OCA loss is due to biodegradation or precipitation, the concentrations that will be established in the WIPP when aerobic processes dominate will be much lower than those predicted by inventory. This has the potential effect of decreasing the concentration of dissolved actinides in brine, thereby decreasing any potential release from WIPP. Future studies will focus on anaerobic conditions.

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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.

Arahal DR, Ventosa A. 2006. The Family *Halomonadaceae*. In: Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E, Dworkin M, editors. The Prokaryotes. New York: Springer. pp 811-834.

Banaszak JE, Rittman BE, Reed DT. 1999. Subsurface interactions of actinide species and microorganisms: implications for the bioremediation of actinide-organic mixtures. Journal of Radioanalytical and Nuclear Chemistry 241: 385-435.

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.

Blau N, Matasovic A, Lukasiewicz-Wedlechowicz A, Heizmann CW, Leumann E. 1998. Simultaneous determination of oxalate, glycolate, citrate, and sulfate from dried urine filter paper spots in a pediatric population. Clinical Chemistry 44: 1554-1556.

Bolton Jr H, Girvin DC, Plymale AE, Harvey SD, Workman DJ. 1996. Degradation of metal-nitrilotriacetate complexes by *Chelatobacter heintzii*. Environmental Science & Technology 30: 931-938.

Borkowski M, Lucchini J-F, Richmann MK, Reed DT. Actinide (III) solubility in WIPP brine: data summary and recommendations. 2009. LA-14360 Report. Carlsbad, NM: Los Alamos National Laboratory.

Brush LH, Xiong Y, Long J. Results of the calculations for actinide solubilities for the WIPP CRA-2009 PABC. 2009. ERMS552201 Report. Albuquerque, NM: Sandia National Laboratories.

Cui H-L, Gao X, Yan X, Xu X-W. 2011. *Halolamina pelagica* gen. nov., sp. nov., a novel member of the family *Halobacteriaceae*. International Journal of Systematic and Evolutionary Microbiology 61: 1617-1621.

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

Dutton MV, Evans CS. 1996. Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Canadian Journal of Microbiology 42: 881-895.

Egli T. 2001. Biodegradation of metal-complexing aminopolycarboxylic acids. Journal of Bioscience and Bioengineering 92: 89-97.

Espejo E, Agosin E. 1991. Production and degradation of oxalic acid by brown-rot fungi. Applied and Environmental Microbiology 57: 1980-1986.

Francis AJ, Dodge CJ, Gillow JB. 1992. Biodegradation of metal citrate complexes and implications for toxic-metal mobility. Nature 356: 140-142.

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. 1998. Biotransformation of uranium and other actinides in radioactive wastes. Journal of Alloys and Compounds 271-273: 78-84.

Francis AJ, Dodge CJ, Ohnuki T. 2007. Microbial transformations of plutonium. Journal of Nuclear and Radiochemical Sciences 8: 121-126.

Fuenkajorn K, Wetchasat K. 2001. Rock salt formations as potential nuclear waste repository. 6th Mining, Metallurgical, and Petroleum Engineering Conference, Bangkok, Thailand.

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 descripton of *H. salinarum*. Extremophiles 8: 431-439.

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

Huang FYC, Brady P, Lindgren ER, Guerra P. 1998. Biodegradation of uranium-citrate complexes: implications for extraction of uranium from soils. Environmental Science & Technology 32: 379-382.

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

Keith-Roach M. 2008. The speciation, stability, solubility and biodegradation of organic co-contaminant radionuclide complexes: a review. Science of the Total Environment 396: 1-11.

Kemmei T, Kodama S, Yamamoto A, Inoue Y, Hayakawa K. 2007. Determination of low-level ethylenediaminetetraacetic acid in water samples by ion chromatography with ultraviolet detection. Chromatographia 65: 229-232.

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.

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

Lloyd JR, Macaskie LE. 2002. Biochemical basis of microbe-radionuclide interactions. In: Keith-Roach MJ, Livens FR, editors. Interactions of Microorganisms with Radionuclides. London: Elsevier Science Ltd. pp. 313-342.

Lucchini J-F, Borkowski M, Richmann MK, Ballard S, Reed DT. 2007. Solubility of Nd^{3+} and $UO2^{2+}$ in WIPP brine as oxidation-state invariant analogs for plutonium. Journal of Alloys and Compounds 444-445: 506-511.

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

Means JL, Kucak T, Crerar DA. 1980. Relative degradation rates of NTA, EDTA, and DTPA and environmental implications. Environmental Pollution Series B—Chemical and Physical 1: 45-60.

Morris SJ, Allen MF. 1994. Oxalate-metabolizing microorganisms in sagebrush steppe soil. Biology and Fertility of Soils 18: 255-259.

Nörtemann B. 1992. Total degradation of EDTA by mixed cultures and a bacterial isolate. Applied and Environmental Microbiology 58: 671-676.

Nörtemann B. 1999. Biodegradation of EDTA. Applied Microbiology and Biotechnology 51: 751-759.

Oren A. 2006. Life at high salt concentrations. In: Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E, Dworkin M, editors. The Prokaryotes. New York: Springer. pp. 263-282.

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. 2002. Microbial processes in the disposal of high level radioactive waste 500m underground in Fennoscandian shield rocks. In: Keith-Roach MJ, Livens, FR, editors. Interactions of Microorganisms with Radionuclides. London: Elsevier Science Ltd. pp. 279-311.

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

Riley RG, Zachara JM. 1992. Chemical contaminants on DOE lands and selection of contaminant mixtures for subsurface science research. DOE/ER-0547T. Office of Energy Research, US Department of Energy, Washington, DC.

Satroutdinov AD, Dedyukhina EG, Chistyakova TI, Witschel M, Minkevich IG, Eroshin VK, Egli T. 2000. Degradation of metal-EDTA complexes by resting cells of the bacterial strain DSM 9103. Environmental Science & Technology 34: 1715-1720.

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.

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.

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.

Witschel M, Egli T, Zehnder A, Spycher M. 1999. Transport of EDTA into cells of the EDTA-degrading bacterial strain DSM9103. Microbiology 145: 973-983.

Zhang H, Herman JP, Bolton Jr H, Zhang Z, Clark S, Xun L. 2007. Evidence that bacterial ABC-type transporter imports free EDTA for metabolism. Applied and Environmental Microbiology.

Table 1. Brine Composition (1X brine = 95% saturation)

Species/Element	M in GWB	M in ERDA-6
Mg^{2+}	0.953	0.018
Na^{+}	3.311	4.618
K^{+}	0.437	0.092
Ca^{2+}	0.013	0.011
Li^{+}	0.004	--
Cl^{-}	5.250	4.412
SO_4^{2-}	0.166	0.159
$B_4O_7^{2-}$	0.038	0.015
Br^{-}	0.025	0.011
Total Ionic Strength	6.839 M	4.965 M

Table 2. Steady-state concentrations of organic complexing agents in abiotic controls (mean of triplicate samples ± standard deviation). Originally added concentrations are given at the head of each column.

Brine	Oxalate (45 mM)	Acetate (38 mM)	Citrate (5.7 mM)	EDTA (0.15 mM)
1X GWB	7.85 ± 0.34	9.53 ± 0.63	3.65 ± 0.29	0.13 ± 0.01
0.75X GWB	6.02 ± 1.24	10.84 ± 1.53	3.10 ± 0.46	0.10 ± 0.02
0.5X GWB	4.67 ± 0.32	12.14 ± 0.25	3.47 ± 0.47	0.11 ± 0.01
0.25X GWB	4.35 ± 0.51	11.89 ± 0.49	4.19 ± 0.38	0.13 ± 0.01
1X ERDA-6	6.71 ± 1.64	9.99 ± 0.96	2.89 ± 0.33	0.12 ± 0.01
0.75X ERDA-6	11.76 ± 1.00	11.36 ± 0.39	3.35 ± 0.46	0.13 ± 0.01
0.5X ERDA-6	17.80 ± 1.34	13.05 ± 1.32	3.85 ± 0.44	0.13 ± 0.01
0.25X ERDA-6	23.49± 1.61	12.26 ± 1.11	4.60 ± 0.29	0.11 ± 0.02

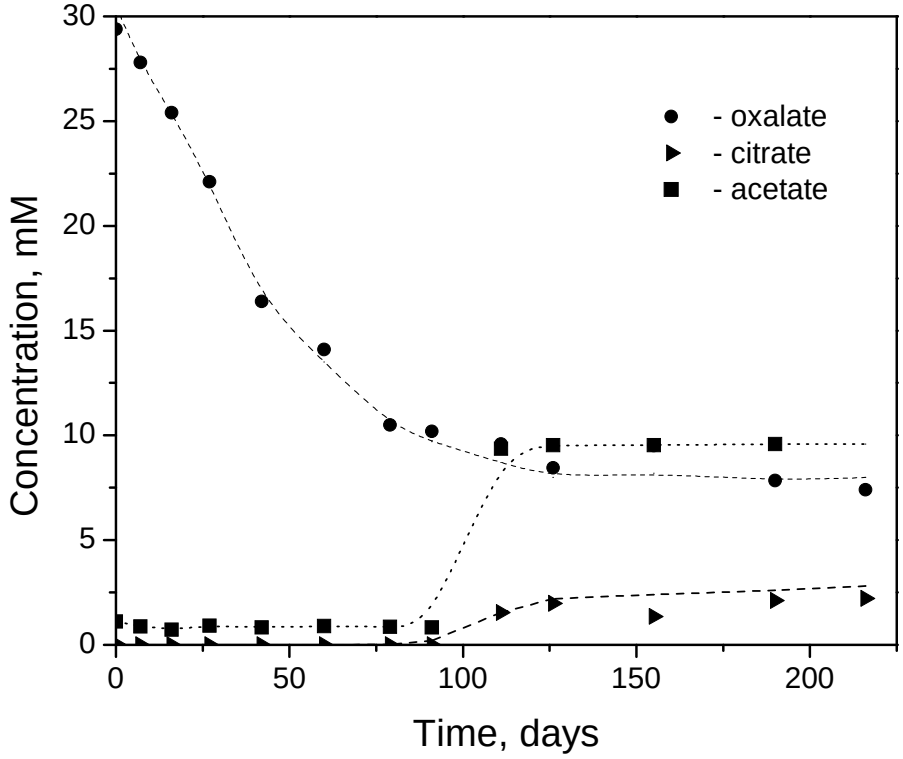

Figure 1. Citrate, oxalate, and acetate in 1X abiotic GWB over time. Plotted are the average of triplicate incubations and corresponding trendlines.

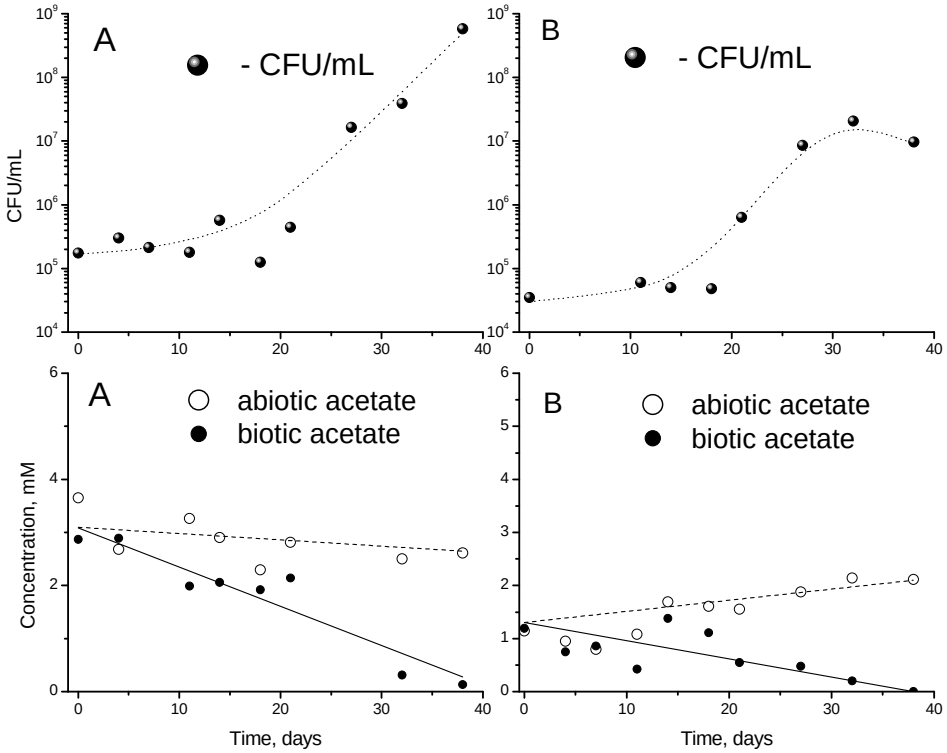

Figure 2. Acetate degradation and corresponding cell growth in *Halomonas halodenitrificans* cultures in 0.75X ERDA-6 (A) and 0.5X GWB (B). Plotted are the average of triplicate incubations and corresponding trendlines.

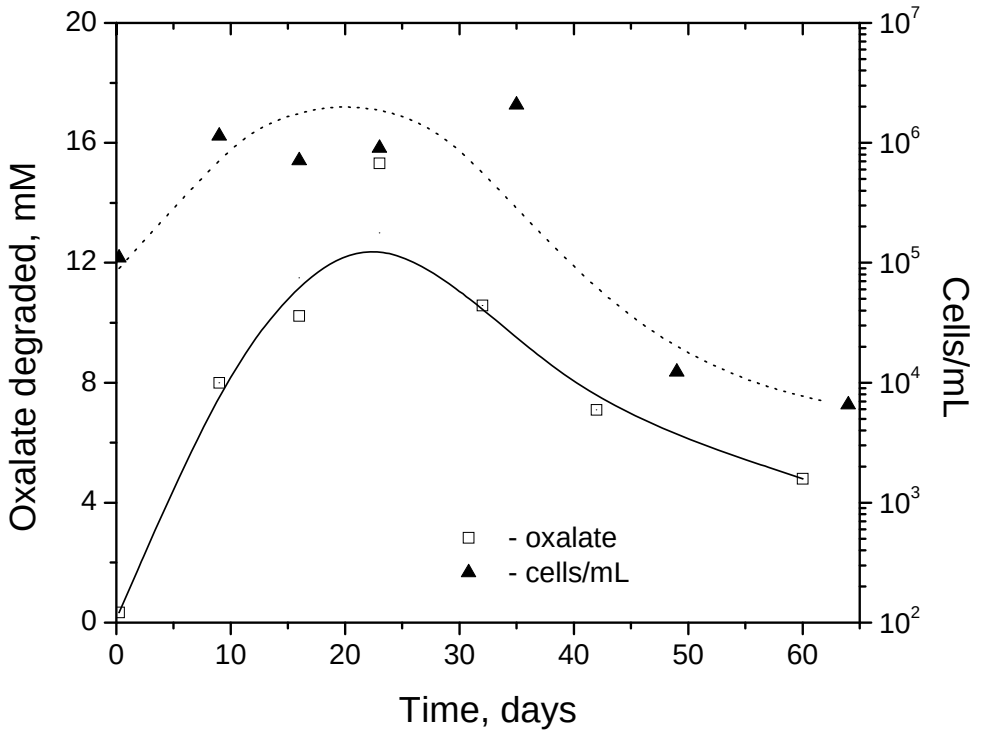

Figure 3. Correlation between cell numbers of *Halosimplex carlsbadense* and degraded oxalate. Plotted are the average of triplicate incubations and corresponding trendlines.

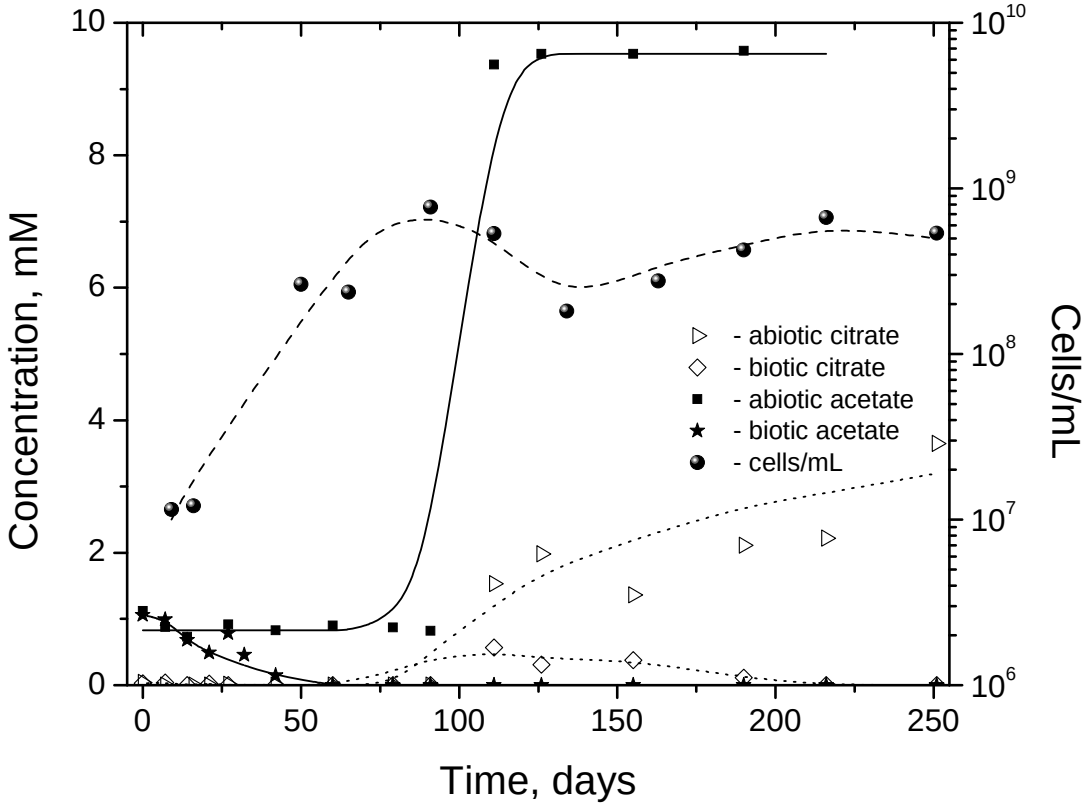

Figure 4. Citrate and acetate utilization in GWB by halite enrichment culture. Plotted are the averages of triplicate incubations and corresponding trendlines.

Figure 5. Comparison of clone library compositions for the parent inoculum and GWB and ERDA outcome cultures.

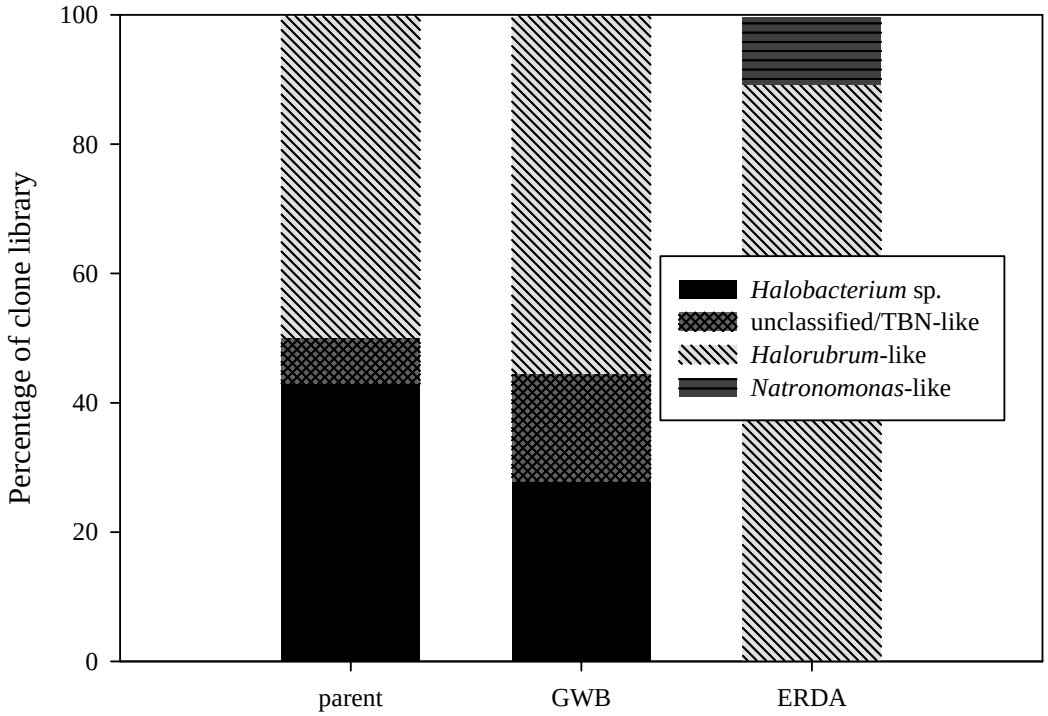

Figure 6. Phylogenetic tree constructed from consensus groups and clone sequences for parent culture, GWB outcome culture, and ERDA-6 outcome culture. Tree is rooted to *Methanospirillum hungatei*. Bootstrap values are provided on select branches relevant to study sequences.

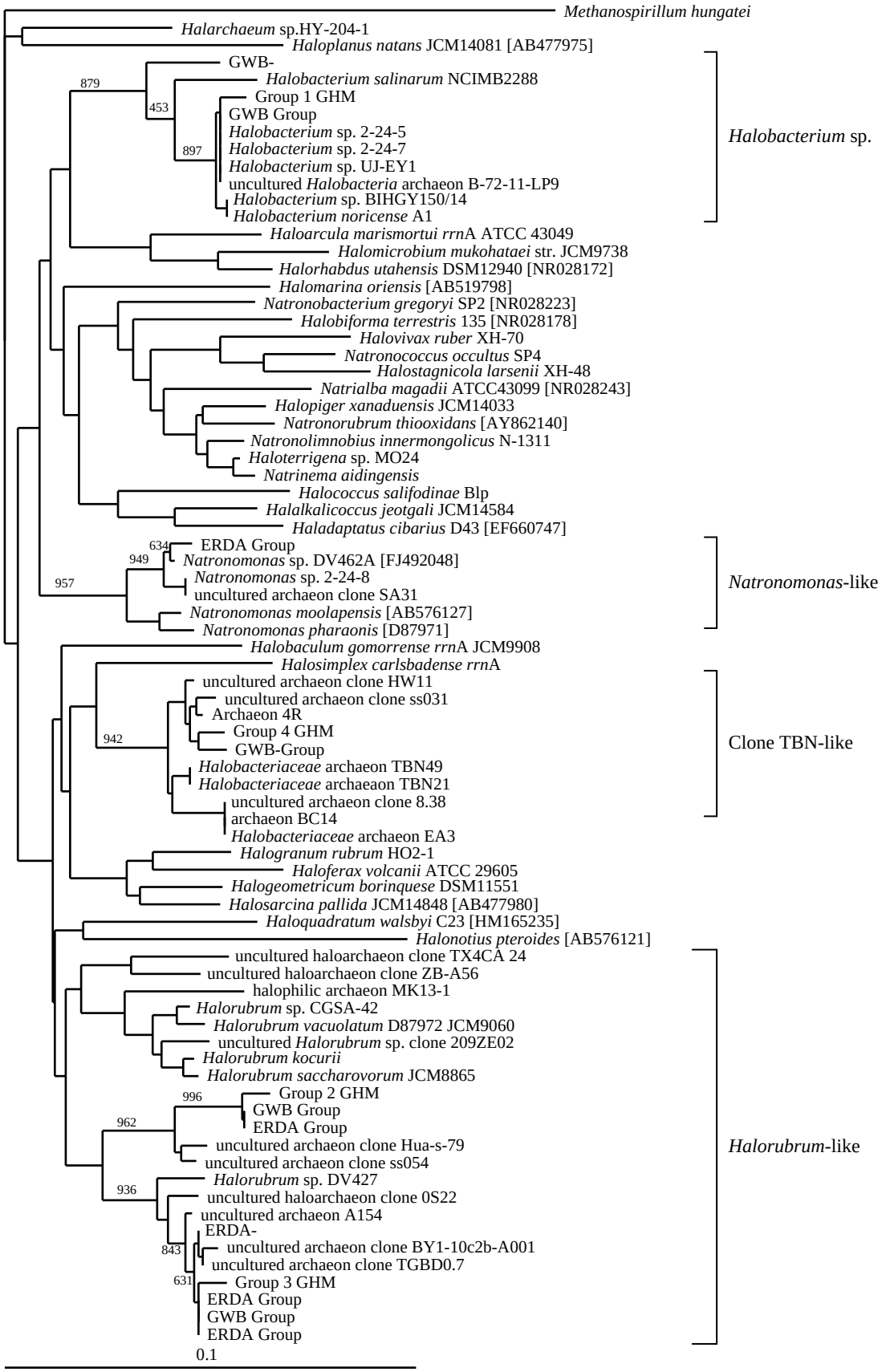
Methanospirillum hungatei
Halarchaeum sp.HY-204-1
Haloplanus natans JCM14081 [AB477975]
GWB-
Halobacterium salinarum NCIMB2288
Group 1 GHM
GWB Group
Halobacterium sp. 2-24-5
Halobacterium sp. 2-24-7
Halobacterium sp. UJ-EY1
uncultured *Halobacteria* archaeon B-72-11-LP9
Halobacterium sp. BIHGY150/14
Halobacterium noricense A1
879
453
897
*Haloarcula marismortui rrn*A ATCC 43049
Halomicrobium mukohataei str. JCM9738
Halorhabdus utahensis DSM12940 [NR028172]
Halomarina oriensis [AB519798]
Natronobacterium gregoryi SP2 [NR028223]
Halobiforma terrestris 135 [NR028178]
Halovivax ruber XH-70
Natronococcus occultus SP4
Halostagnicola larsenii XH-48
Natrialba magadii ATCC43099 [NR028243]
Halopiger xanaduensis JCM14033
Natronorubrum thiooxidans [AY862140]
Natronolimnobius innermongolicus N-1311
Haloterrigena sp. MO24
Natrinema aidingensis
Halococcus salifodinae Blp
Halalkalicoccus jeotgali JCM14584
Haladaptatus cibarius D43 [EF660747]
Halobacterium sp.
ERDA Group
Natronomonas sp. DV462A [FJ492048]
Natronomonas sp. 2-24-8
uncultured archaeon clone SA31
Natronomonas moolapensis [AB576127]
Natronomonas pharaonis [D87971]
634
949
957
Natronomonas-like
*Halobaculum gomorrense rrn*A JCM9908
*Halosimplex carlsbadense rrn*A
uncultured archaeon clone HW11
uncultured archaeon clone ss031
Archaeon 4R
Group 4 GHM
GWB-Group
Halobacteriaceae archaeon TBN49
Halobacteriaceae archaeaon TBN21
uncultured archaeon clone 8.38
archaeon BC14
Halobacteriaceae archaeon EA3
942
Clone TBN-like
Halogranum rubrum HO2-1
Haloferax volcanii ATCC 29605
Halogeometricum borinquese DSM11551
Halosarcina pallida JCM14848 [AB477980]
Haloquadratum walsbyi C23 [HM165235]
Halonotius pteroides [AB576121]
uncultured haloarchaeon clone TX4CA 24
uncultured haloarchaeon clone ZB-A56
halophilic archaeon MK13-1
Halorubrum sp. CGSA-42
Halorubrum vacuolatum D87972 JCM9060
uncultured *Halorubrum* sp. clone 209ZE02
Halorubrum kocurii
Halorubrum saccharovorum JCM8865
Group 2 GHM
GWB Group
ERDA Group
uncultured archaeon clone Hua-s-79
uncultured archaeon clone ss054
Halorubrum sp. DV427
uncultured haloarchaeon clone 0S22
uncultured archaeon A154
ERDA-
uncultured archaeon clone BY1-10c2b-A001
uncultured archaeon clone TGBD0.7
Group 3 GHM
ERDA Group
GWB Group
ERDA Group
996
962
936
843
631
Halorubrum-like
0.1

Table 3. Phylogenetic affiliation of clone groupings generated from each brine outcome community and from parent inoculum culture. Groups are based on clones with ≤ 3% sequence divergence.

Clone/Group Designation	Closest BLAST Match	Sequence Similarity	Source of Closest BLAST Match
GWB Group 1	*Halorubrum* sp. DV427 [FJ492047]	97%	Death Valley salt core (22KYr)[a]
GWB Group 2	*Halobacterium* sp. 2-24-7 [AJ878083]	99%	Sergipe Basin salt core, Brazil (121MYr)[b]
GWB Group 3	DV427	94%	
GWB Group 4	*Halobacteriaceae* archaeon TBN49 [GU208827]	97%	marine solar saltern, Eastern China[c]
Clone GWB-A44	TBN49	96%	
ERDA Group 1	*Natronomonas* sp. 2-24-8 [AJ878077]	98%	Sergipe Basin salt core, Brazil
ERDA Group 2	DV427	98%	
ERDA Group 3	DV427	97%	
ERDA Group 4	DV427	94%	
Clone ERDA-A33	DV427	94%	
GHM Group 1	*Halobacterium* sp. UJ-EY1 [FM946157]	99%	Arava Desert salt crust, Israel[d]
GHM Group 2	uncultured archaeon clone Hua-s-79 [EF632784]	98%	saline wetland sediment, Salar de Huasco, Chile[e]
GHM Group 3	uncultured archaeon A154 [AJ278937]	98%	Alpine Permo-Triassic rock salt, Austria[f]
GHM Group 4	TBN49	99%	

[a]Schubert and others, 2010
[b]Vreeland and others, 2007
[c]Cui, C.L. submitted to GenBank 2010
[d]Buchvarova and others, submitted to GenBank 2008
[e]Dorador and others, submitted to GenBank 2007
[f]Radax and others, 2001