INVESTIGATION INTO THE POST-EXCAVATION SOURCES OF METHANE FROM INL TRU WASTE DRUMS ARP-60106 and 60109

Final Report: 8/24/15

Julie Swanson, Donald Reed, Michael Richmann, and Danielle Cleveland

Actinide Chemistry and Repository Science Program

Repository Science and Operations

Los Alamos National Laboratory

Prepared for:

Thomas Clements

Transuranic Waste Projects

CH2M-WG Idaho, LLC

LA-UR 15-26657

Intentionally Blank

EXECUTIVE SUMMARY

Detailed microbial and qualitative radiolytic assessments were performed on INL TRU samples from drums ARP-60106 and ARP-60109 to determine the cause of methane gas production observed in these two WIPP-bound TRU waste drums. Currently, these drums exhibit methane gas generation rates that prevent their shipment to the WIPP site. Since other such drums may be found, it was important to evaluate the mechanism of gas generation with the idea of developing a treatment strategy to mitigate or eliminate methane production.

In all, six samples of approximately 10 grams each were removed by INL staff from each of the two drums. These were taken with relatively few precautions to minimize microbial contamination, and drums had been splayed in air twice to inhibit what was thought to be anaerobic microbial gas generation prior to this sampling. The samples obtained were shipped to the Los Alamos labs located at the Carlsbad Environmental Monitoring and Research Center in Carlsbad NM and affiliated with the WIPP project. It was verified by LANL, prior to our subsequent analyses performed, that significant methane generation continued to occur under both oxic and anoxic conditions, and that rates of methane generation were not dependent on levels of oxygen.

The first mechanism evaluated was the possibility that the gas generated was due to methanogenic microorganisms. DNA was extracted from ARP 60106, and reactions were performed to amplify target genes that are used as signatures for Archaea, in general, and methanogens, specifically. No DNA signatures for methanogens were detected, and general archaeal signatures were extremely low. These results suggest that there were no methanogens present at the time of sampling. However, these results did not preclude past presence or activity of methanogens, especially since the drums had been buried in soils likely to contain such organisms. DNA could not be extracted from ARP 60109.

Additional analyses were conducted on ARP 60106 to determine what organisms were present. DNA analyses showed a low-diversity, monophyletic community composed of *Actinobacteria*. Culture-based analyses yielded two members of this phylum, in addition to three *Bacillus* isolates. The organisms detected and/or isolated in this drum have also been found in other radionuclide-contaminated environments. None of these organisms is capable of methanogenesis.

A second mechanism, evaluated qualitatively, was the possibility that the source of methane production was the radiolytic decomposition of the organic content in the waste drums. This had been initially discounted based on back-of-the-hand calculations, but there were no good quantitative data on the moles of methane production as a function of the mass of waste. Experiments were performed to evaluate this mole-to-mass ratio, and steps were taken to distinguish chemical and radiolytic sources from microbial ones. Results from these tests showed that radiolysis is also not the immediate source of high methane in the two TRU waste samples. The methane production observed does not correlate with measured alpha activity (high activity samples have lower methane production). The calculated G values are too high for radiolysis to be the source of methane.

The overall conclusions are that microbial and radiolytic pathways cannot account for the levels of methane production observed in samples ARP 60106 and ARP 60109. Therefore, it is hypothesized that the observed methane is a result of slow outgassing from the waste matrix. The original methane source may have been either biogenic or radiolytic. Fluctuations in the measured methane levels could be due to changes in headspace volume or to newly exposed waste surface area after repackaging. Although this interpretation explains much of our data, it remains speculative and requires more definitive corroboration.

TABLE OF CONTENTS

Intentionally Blank

INVESTIGATION INTO THE POST-EXCAVATION SOURCES OF METHANE FROM INL TRU WASTE DRUMS ARP-60106 and ARP-60109

Samples from the Idaho National Laboratory (INL) transuranic (TRU) waste drums, ARP-60106 and ARP-60109, were analyzed to establish the presence or absence of methane-generating microorganisms. The high methane generation measured in these drums disqualified them from shipment to the Waste Isolation Pilot Plant (WIPP) for their permanent geologic disposal. Additional gas generation studies were also performed to evaluate the possibility that the source of methane gas was radiolytic in origin.

In this report we provide, along with some background on the history of the drums and the methane generation observed, a summary of the results of the microbial and radiolytic assessments performed. The observations and conclusions, based on these results, are provided as input that can be used to determine the final disposition of TRU drums where high methane gas generation is present.

1.0 INTRODUCTION AND BACKGROUND

Waste Isolation Pilot Plant (WIPP) Transuranic Repository

The WIPP is located in the northern portion of the Delaware Basin in southeastern New Mexico east of Carlsbad and continues to be the only repository in the US for the permanent disposal of TRU waste. It was first certified as a TRU waste repository by the Environmental Protection Agency (EPA) in May 1998 and is currently operated by the Department of Energy, Carlsbad Field Office (DOE CBFO). The regulatory guidelines for the WIPP are given in 40CFR191/194 [U.S. EPA 1998]. Although the WIPP is currently awaiting resolution of a number of operational issues that have surfaced in the past year, there has been much success in TRU waste disposal with over 11,000 shipments received, > 380,000 loaded-drum equivalent containers disposed, and >86,000 m^3 of TRU waste emplaced. Six of the originally designed nine panels are full.

TRU waste coming to the WIPP must go through a rigorous certification process and meet the shipment criteria established in the WIPP Waste Acceptance Criteria [WIPP-WAC] and the CCP Transuranic Waste Certification Plan [CCP-PO-002]. A critical acceptance criterion for shipment of TRU waste to the WIPP is that a prior-to-shipment headspace gas analysis of the drum shows methane concentrations below 1250 ppm. This is a flammability criterion to assure that there are no potential problems during shipment. The motivation for the experimental work described in this report is that a small number of drums recovered from the ARP by INL had higher than acceptable methane generation that persisted even after re-packaging. The goal of this work was to evaluate potential contributors to the observed gas generation so that a path forward for current and future, if any, TRU containers could be properly configured to meet the WIPP-WAC requirements for shipment to the WIPP repository.

Historical Perspective: Drums ARP 60106 and ARP 60109

TRU waste drums ARP 60106 and ARP 60109 are high methane generating drums that do not pass the WIPP-WAC criteria. More detailed accounts of the origin of these drums and the headspace methane issue are provided in the Acceptable Knowledge (AK) report for this waste stream [CCP-AK-INL-001, rev. 12] and in the Engineering Design File for this project [EDF-10716, Rev 0].

Briefly, the methane-generating TRU waste was excavated from ARP-IV (Pit 5) and ARP-VI (Pit 4-West) retrieval areas at the INL site (see Figures 3A and 3C in the CCP-AK-INL-001). Most of this waste originated from the Rocky Flats Plant (RFP), but some is also from INL and a few small generator sites. These were shipped to INL for disposal. Pit 4 was open to receive waste from January 1963 through September 1967. The ARP-VI retrieval area contains waste shipments from RFP buried from March 1964 to March 1965 and from INL generators buried from August 1964 to September 1965. Pit 5 was open to receive waste from June 1963 to December 1966. The ARP-IV retrieval area contains waste shipments from RFP buried from May 1964 to December 1966 and from INL generators buried from May 1964 to December 1966. These containers remained buried in the underground for almost 50 years prior to their recovery and repacking in 2012.

The contents of the two drums that were sampled and analyzed had undergone repackaging a number of times due to the presence of high methane. This included splaying and allowing to sit in open-air trays for several days in an attempt to reduce the methane concentrations to levels acceptable for shipping. Still, these drums did not pass the criterion for methane concentration.

The estimated radionuclide and waste content of the test drums are given in Table 1-1. The predominant nuclides in these two containers were Am-241 and Pu-239. These wasteforms are organic-rich (>40% by mass) residues that have the appearance of fine, unreacted concrete.

A history of headspace gas sampling is given in Table 1-2. The predominant gas component is methane with lesser, but significant, amounts of hydrogen, carbon dioxide (not shown), and light hydrocarbons (not shown).

Table 1-1. Activity Content of TRU Waste Drums ARP-60106 and ARP-60109

Isotope	*Average Ci/g	#ARP-60106 mCi	#ARP-60109 mCi
Am-241	7.84E-6	55.3 ± 26.3	72.9 ± 33.6
Np-237	8.88E-11	ND	ND
Pu-239	4.53E-6	7.64 ± 4.85	16.1 ± 9.18
Pu-238	3.67E-8	0.337	0.709
Pu-240	1.90E-7	1.75	3.67
Pu-241	1.82E-6	ND	ND
Pu-242	3.69E-11	1.25E-4	2.64E-4
U-238	1.27E-9	ND	ND
U-234	7.61E-10	ND	ND
U-235	2.22E-11	ND	ND
Cs-137	2.00E-12	ND	ND
Sr-90	2.99E-12	ND	ND
Container Properties			
Container Weight (Kg)	NA	132.33	122.33
Density (g/cm^3)	NA	0.746	0. 817
TRU content (nCi/g)	12,600	491	763

NA – Not applicable
ND – not detected
* - averaged values based on the IWTS database

- Content based on NDA analysis (NDA-03-21-14_0018.spc for ARP-60106; NDA-03-21-14_0011.spc for ARP-60106) – Pu-238, Pu-240 and Pu-242 are estimated based on Pu-239 ratios

Table 1-2. INL Head-space Gas Analysis Data for TRU Waste Drums

Container	FGA BDR or machine	Sample Date	Hydrogen ppmv (5% limit)	Methane ppmv (1250 limit)
ARP60808 (03/20/14) (ARP60106) (organic) (ARP70245)	FIO-unit5	7/3/2012	201.70	36,881.91
	FIO-unit5	7/11/2012		116,702.94
	FIO-unit5	7/12/2012		106,484.65
	FIO-unit9	7/12/2012		115,583.78
	FIO/Dragger	7/19/2012		140,693.91
	IN12FG9016	7/31/2012	321.07	169,789.35
	IRC LAB	7/31/2012		120,000.00
	IN13FG10032	5/9/2013		15,911.72
	IN13FG6030	10/8/2013		19,002.75
	Unit-5	3/18/2014	528.51	6,029.70
	Unit-5	5/20/2014	218.21	9,756.22
ARP60829 (03/20/14) (ARP60109) (organic) (ARP70230)	IN12FG9016	7/31/2012	4,027.53	137,315.97
	IN13FG10032	5/9/2013		20,892.65
	IN13FG6030	10/8/2013		24,551.18
	Unit-5	3/18/2014		2,602.76
	Unit-5	5/20/2014	945.37	21,893.30
ARP60110 (08/09/14) (organic) (ARP70232)	IN12FG5083	8/15/2012	182.28	15,241.20
	IN13FG10032	5/9/2013		3,946.22
	IN13FG6030	10/8/2013		4,896.52
	Unit-5	3/18/2014		2,857.06
	Unit-5	5/20/2014	104.62	6,269.99
ARP60112 (08/09/14) (organic) (ARP70246)	IN12FG5083	8/15/2012	80.83	30,763.48
	IN13FG10032	5/9/2013		6,571.50
	IN13FG6030	10/8/2013		8,639.20
	Unit-5	3/18/2014		2,606.04
	Unit-5	5/20/2014		8,876.57

2.0 DRUM SAMPLING AND SAMPLE SHIPMENT, HANDLING, AND ANALYSIS

2.1 TRU Waste Drum Sampling at INL

The TRU waste drums ARP-60106 and ARP-60109 were sampled on 3/20/14 to obtain six ~ 10 g samples in 50 mL sterile, polypropylene tubes for shipment to the Carlsbad Environmental Monitoring and Research Facility (CEMRC) for microbial analysis. Pictures detailing this overall process are shown in Figure 2-1. Specific notes on the sampling process are given in this section.

Drum to Tray Sampling (3/20/14)

Prior to sampling, both drums were intact and sitting in the open bay area. These were sampled sequentially (first ARP60106, then after sampling and re-packaging complete ARP60-109). The lids on these drums are partially crushed to loosen them and then removed. This leaves a partly distorted drum with an intact, plastic bag within. The plastic liner was partially shaken out, then slit open on its side and dumped into the tray for transport. This emptied the bag through the side with little spatial resolution. The tray was then lifted and inserted into the glovebox to be sampled.

Microbial Samples from Drum ARP 60106 (re-packaged to drum ARP 60808 after sampling):

The waste contents appeared relatively homogeneous with a light gray color and "damp" sand feel. A few small "clumps" were noted at depth (approximately 12" deep in the tray). Sampling was done in the following way with a new, sterile scoopula for each set of two:

Bottles 1 and 2: ~ 10 g from top 2" of the waste – this was all very homogeneous and samples are equivalent

Bottles 3 and 4: scraped off the top ~ 6" and then sampled – these are equivalent samples. Some 1-2" "clumps" were noted.

Bottles 5 and 6: scraped off all but the bottom 2", took equivalent samples from this level

In the above, the sample is inverted relative to the container, so bottles1 and 2 correspond to the bottom of the waste drum, and bottles 5 and 6 correspond to the top.

Overall, the waste was homogeneous and unavoidably mixed (top to bottom in the sampling process). Since this was at least the second sampling of each container, this type of mixing had already been done before. For this reason, the results presented in this report cannot rely too heavily on the zonation for interpretation. The waste was not powdery dry (few visible airborne particulates) and seemed to be slightly wetted which caused the slight clumping noted. The dumping process may have broken some of the clumps, so the amount of "clumping" noted may not be representative of the original form of the waste.

Microbial Samples from Drum ARP 60109 (re-packaged to drum ARP 60829 after sampling):

The appearance of the waste contents in drum ARP 60109 was also very homogeneous but slightly lighter in color (khaki-colored) than the prior (gray-colored) waste. Samples were taken according to the following:

Bottles 1 and 2:	equivalent samples from the top 2" of the waste.
Bottles 3 and 4:	Bottle 3: finer material after scraping off ~ 6" of the waste
	Bottle 4: Handpicked small clumps from this zone (1/2 to 1")
Bottles 5 and 6:	Bottle 5: finer material after scraping down to ~ 2"
	Bottle 6: Handpicked small clumps from this zone (1/2 to 1")

Final Packaging:

In each case, after all six samples were taken, they were wiped down to remove residual surface contamination, placed into individual bags and then combined into sets of six (all from one drum) into a single zip-lok bag. The two zip-lok bags were combined into a secondary for shipment. In all cases, weights were estimated by volume and not explicitly measured. These were stored at room temperature while sealed and then shipped to the CEMRC facility the next week.

Initial Sampling/Opening of Waste Drum

Dumping of waste into tray

Glovebox view, showing final repackaging station

Glovebox with TRU waste tray in place

Appearance of TRU waste

Sampling of TRU waste

Figure 2-1. Photographs showing the TRU waste sampling process

2.2 Handling, Storage and Sampling of TRU Waste Samples at CEMRC

The 12 samples (six from each drum) were received at the CEMRC facility on 3/27/14. They were opened and surveyed immediately after placement in a once-through air glovebox. Some residual activity was noted on the outside of the sample tubes and wiped down to < 20 dpm alpha. When not in process, the samples were stored at room temperature in the air glovebox antechamber to prevent exposure to light.

The sixth sample from each drum was separated on 3/28/14 and transferred to an anoxic (nitrogen-filled) glovebox (typically < 0.1 ppm O_2). Once in this box, they were opened for ~ 20 seconds to make the tube headspace suboxic. These two sample tubes were resealed and placed in a sealed 500 cc polycarbonate container and set aside in the back of the glovebox. This was done to slowly re-establish anoxic conditions in the sample tubes to stimulate possible anaerobic microbial growth and check for methane generation.

The production of methane and CO_2 was established for the two anoxic samples and selected oxic ("as-received") samples. This was done using a Bruker model 430 GC equipped with an FID detector and a CO_2-methane reaction cell using high-purity nitrogen as the carrier gas. In all cases, the gas was sampled using a gas-tight Hamilton syringe, and 2 cc were injected at room pressure into the GC. Conversion to ppmV was done by comparison to NIST-traceable gas samples that were analyzed in the same way.

The continued generation of methane was confirmed for both oxic and anoxic samples. In the case of the anoxic samples, where the residual methane was removed from the sample tube, the production of methane was shown to initially be linear with time. The data for the anoxic tubes are shown in Figure 2-2 and given in Tables 2-1 and 2-2. The volume occupied by the TRU sample was approximately 15% of the total vessel volume. This is a much larger "headspace" volume relative to the waste and cannot be directly compared to the results obtained in the drum headspace analysis. The actual ppm that would have been observed under the same conditions of the WIPP headspace analysis would have been significantly higher since the headspace-to-waste volume ratio is much lower. It is also important to note that the overall amount of methane generated was about 5 times higher for drum ARP 60106 relative to ARP 60109. The fact that similar and relatively high methane gas generation rates were observed

under both anoxic and oxic conditions provides initial evidence that methanogenesis was probably not the source of methane, as the presence of oxygen should inhibit this process

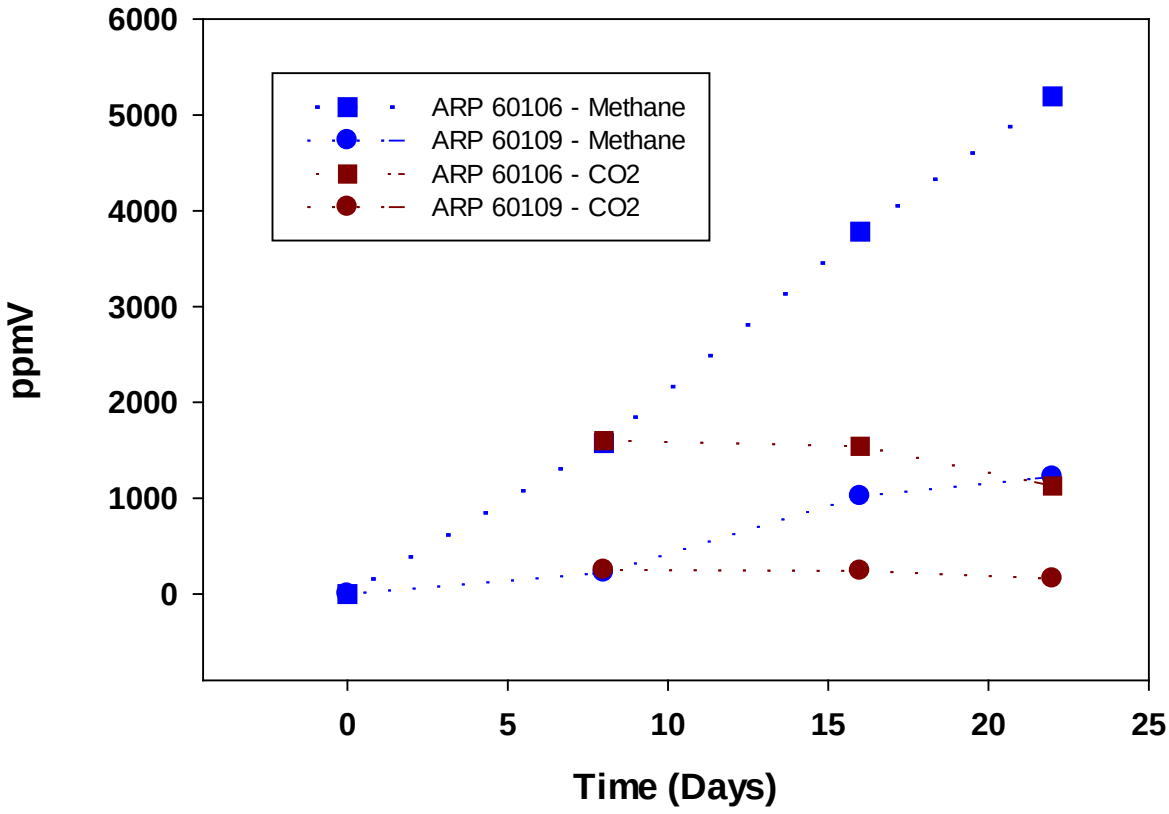

Figure 2-2 Methane and CO_2 gas production for the anoxic sample #6 from both drums ARP 60106 and ARP 60109 as a function of time.

Table 2-1. Gas Sampling from Sample 6 from Drum ARP 60106 (all are in ppmV and ~ ± 15%)

Gas	GC RT (minutes)	[1]Initial Sampling		[2]Post DNA Extraction Analysis		
		5/27/14 60 days	5/28/14 61 days	1st (6/6/14) T = 8 days	2nd (6/14/14) T = 16 days	3rd (6/20/14) T = 22 days
Methane	0.7	5551	5188	1575	3783	5194
CO_2	1.4	3874	3042	1600	1542	1128
Acetylene	2.36	25	19	5.9	14	17
Ethane	3.2	57	47	7.4	28	18
Propylene	15.3	18	16	4.3	0	6.9
Propane	17.6	14	12	3.0	0	4.6

(1) this was lightly degassed on 3/28/14 and left in Nitrogen GB, Sampled on 5/27. A sampling on 5/28 was also taken to confirm earlier results – but should show decrease due to sample removal in the sampling process.
(2) second DNA extraction was done on 5/29/14—sample opened to glovebox atmosphere

Table 2-2. Gas Sampling from Sample 6 from Drum ARP 60109 (all are in ppmV and ~ ± 15%)

Gas	GC RT (minutes)	[1]Initial Sampling		[2]Post DNA Extraction Analysis		
		5/27/14 60 days	5/28/14 61 days	1st (6/6/14) T = 8 days	2nd (6/14/14) T = 16 days	3rd (6/20/14) T = 22 days
Methane	0.7	464	360	220	1019	1223
CO_2	1.4	193	184	150	241	159
Acetylene	2.36	1.2	1.1	0.5	2.2	2.5
Ethane	3.2	4.1	1.5	0.8	2.9	3.4
Propylene	15.3	1.1	0.8	0.0	0.4	1.3
Propane	17.6	0.5	0.3	0.0	0.0	0.7

(1) this was lightly degassed on 3/28/14 and left in Nitrogen GB, Sampled on 5/27. A sampling on 5/28 was also taken to confirm earlier results – but should show decrease due to sample removal in the sampling process.
(2) second DNA extraction was done on 5/29/14—sample opened to glove box atmosphere

3.0 MICROBIAL CHARACTERIZATION AND ANALYSIS

The TRU waste in tubes 1-5 from each drum were sampled directly for DNA analysis. An overview of the process is shown in Figure 3-1. This initial sampling has been the focus of the DNA analysis that was performed.

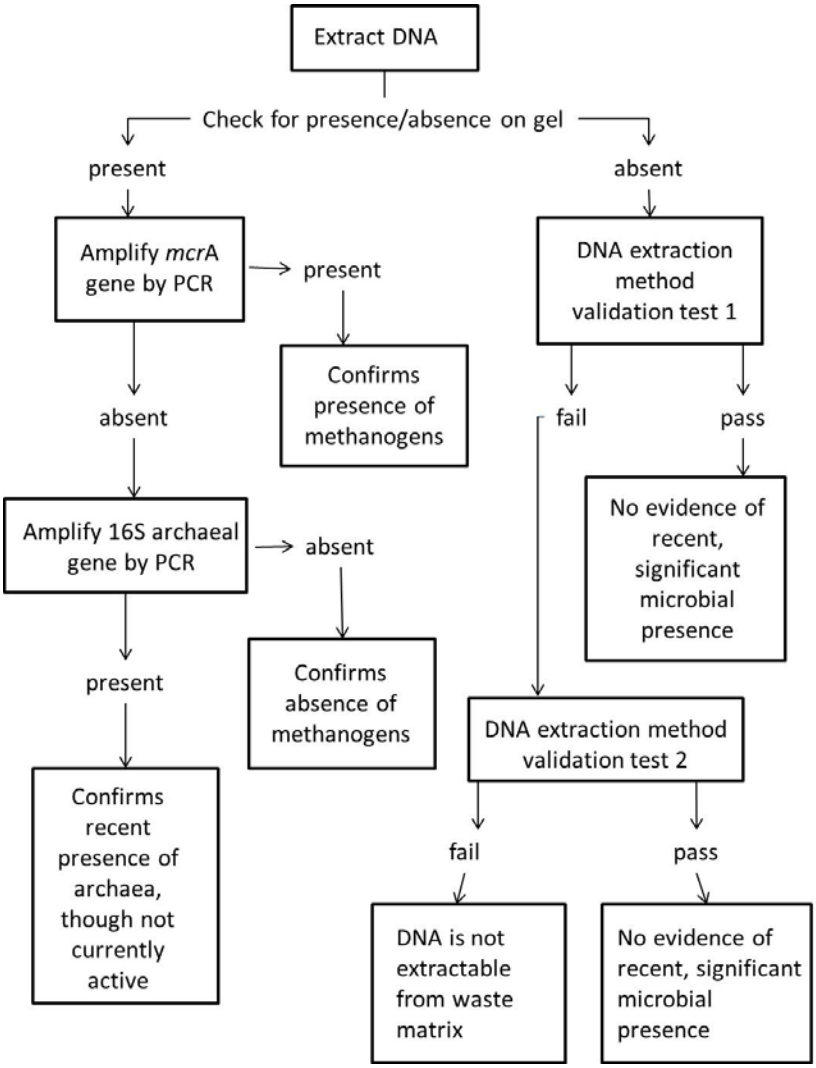

Figure 3-1. Planned Flow Chart for Microbial Analyses of INL Waste Drums.

Once it was observed that methane generation was occurring in the two anoxic samples (Sample tubes 6 from both waste drums), further DNA extractions were performed on these samples.

3.1 DNA Extraction of As-Received Aerobic Samples

DNA was extracted from ~0.25-0.35 g of waste using the PowerSoil DNA Purification Kit from MoBio Laboratories, Inc. Duplicate extractions were performed from each of 5 tubes from each drum. The replicate extractions were amended with additional EDTA (100 mM), but otherwise the manufacturer's directions were followed. This addition was to account for co-extracted metals and radionuclides, which may have adverse effects on downstream molecular techniques, and the metal content of the wastes was unknown. Final extracts were resolved on an agarose gel. No DNA was detectable in the extracts from Drum ARP60109.

DNA was observed in the samples from Drum ARP60106 and these results are shown in Figure 3-2. Some shearing was noted, suggesting DNA degradation, but whether this was due to the extraction process or waste matrix cannot be determined. The extent of shearing does not appear significant enough to preclude downstream molecular analyses.

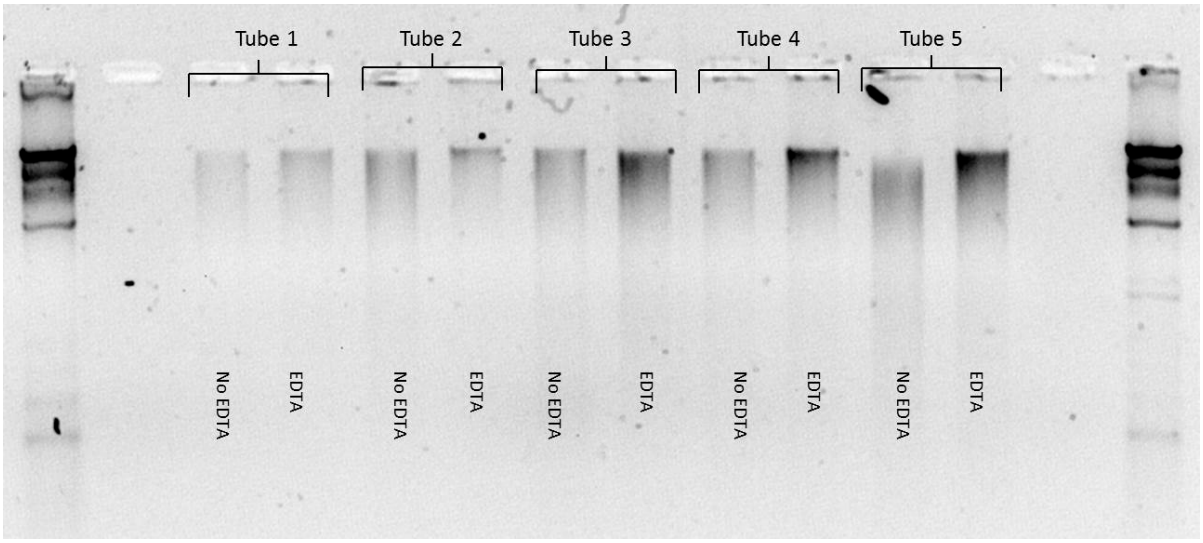

Figure 3-2. Genomic DNA extracted from tubes 1-5 of Drum ARP60106. Outer lanes, λHindIII DNA digest (size marker). The image shows an apparent increase in DNA concentration in the higher-numbered tubes and in tubes where EDTA was used during the extraction process.

Although the extracts with EDTA yield bands that are denser than those without, it is difficult to say whether the addition of EDTA enhanced DNA recovery, since the extracts were not quantified. Also, the quantity of DNA extracted appears to increase with tube number (i.e. with decreasing depth), as evidenced by increased density moving from left to right on the gel. Again, since the DNA was not quantified, this is a qualitative assessment only. Because of the difficulty in controlling for drum zones during the splaying process, it is not possible to say whether the increase in DNA extracted with decreasing depth is at all valid.

PCR Amplification of Methyl Coenzyme Reductase Encoding Gene

Methyl-coenzyme M reductase is an enzyme present in all methanogens and catalyzes the final step in methanogenesis. Amplification of the gene encoding a subunit of this enzyme (*mcr*A) was carried out using the polymerase chain reaction (PCR) on Drum ARP60106 extracts. Results were negative, suggesting that methanogens were not active. This does not rule out their presence at some point in the past.

PCR Amplification of 16S Ribosomal RNA-Encoding Gene of Archaea

Since it was possible that methanogens were present and active at one point during the waste lifetime but were not currently active, a second round of PCR was used to detect the presence of the domain *Archaea*, of which methanogens are members. Results for this test were also negative. Thus, it is unlikely that methanogens were present in the waste in the recent past.

This concludes processing of Drum ARP60106 for methanogens. There is no evidence of recent or current methanogen presence or activity in this drum. This does not preclude past presence and/or activity, but it is highly unlikely that the methane generation currently being observed is due to microbial methanogenesis.

3.2 Method Validation of DNA Extraction for Samples from Drums ARP60106 and ARP 60109

Method Validation Test 1: Spiked DNA (also performed on Drum ARP60106)

In order to validate the DNA extraction method for use on the waste samples, test extractions were performed. Aliquots of waste (~0.2-0.35 g) were spiked with known

concentrations (10, 100, and 500-1000 ng) of purified methanogen DNA from a reputable culture collection (*Methanococcus maripaludis*, American Type Culture Collection BAA-1333D-5). EDTA was also added to the lysis solution for these extractions. All samples were taken from tube 5 of each respective drum. DNA was extracted using the same DNA purification kit listed previously and according to the manufacturer's directions, but with the above modifications (addition of EDTA; spiked DNA). Samples were run on an agarose gel (results shown in Figure 3-3).

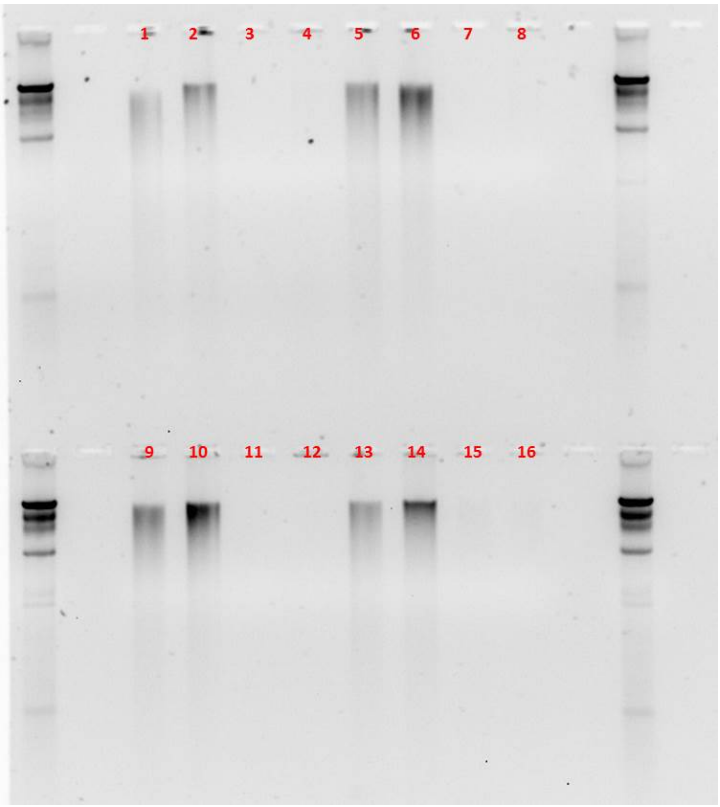

Figure 3-3. DNA extracts from method validation test 1. Outer lanes are λHindIII DNA digest (size ladder). These data confirm that the method used for DNA extraction is valid for ARP 60106 but are inconclusive for ARP 60109.

DNA was only visible in Drum ARP60106 extracts, both spiked (lanes 5, 6, 9, 10, 13, 14) and unspiked (1, 2). Unspiked Drum ARP60109 extracts contain no DNA (lanes 3, 4). Drum ARP60109 extracts with 10 and 100 ng spiked DNA show no visible bands (lanes 7, 8, 11, 12). Drum ARP60109 extracts with the maximum DNA spike yielded very faint bands (lanes 15, 16; see figure 3-4). The fact that spiked DNA was barely detectable in Drum ARP60109 could

suggest any of the following: insufficient DNA spike, DNA degradation, sorption onto the sample matrix, or the inhibition of reagents by the matrix. Because this test was inconclusive, the second method validation test was performed.

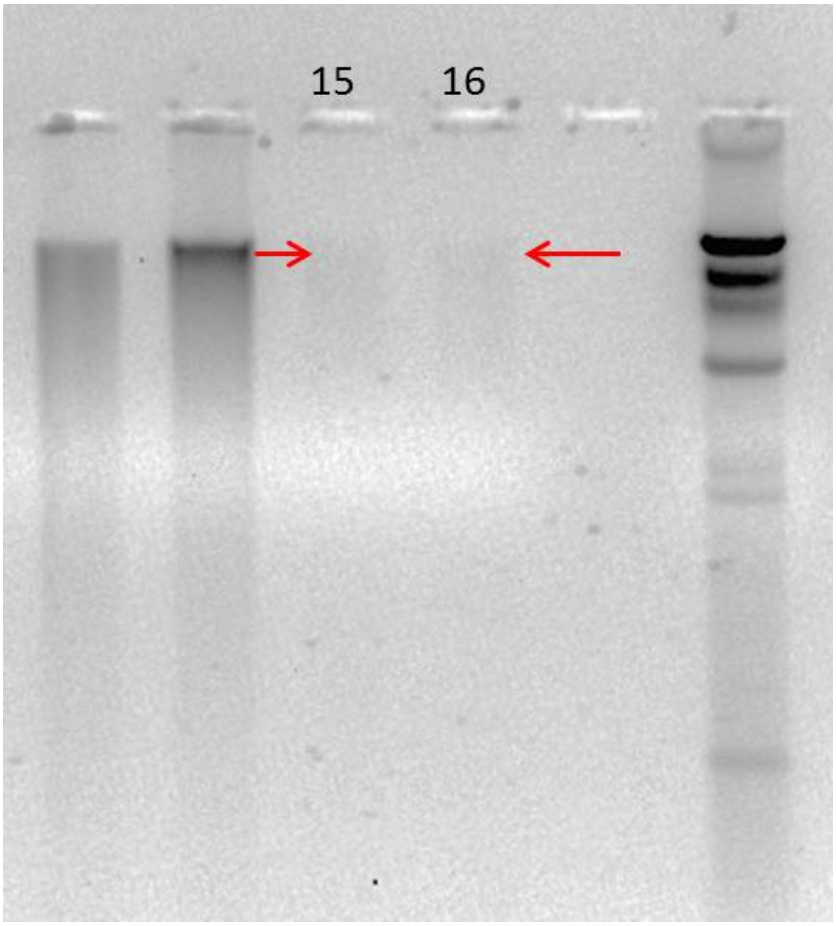

Figure 3-4. Close-up of lanes containing samples 15 and 16 showing faint DNA (red arrows).

Method Validation Test 2: Seeded Whole Cells

Representative waste samples from Drum ARP60109 (~0.25-0.35 g) were seeded with a saturated inoculum of cells from an environmental bacillus isolate (ID based on DNA sequence: *Bacillus firmus*). This organism forms spores which are more easily protected from the waste matrix but are difficult to lyse during DNA extraction procedures. The addition of lysozyme

enhances spore lysis. DNA was extracted per manufacturers' directions with the above modifications. Extracts were resolved on an agarose gel and the results are show in Figure 3-5.

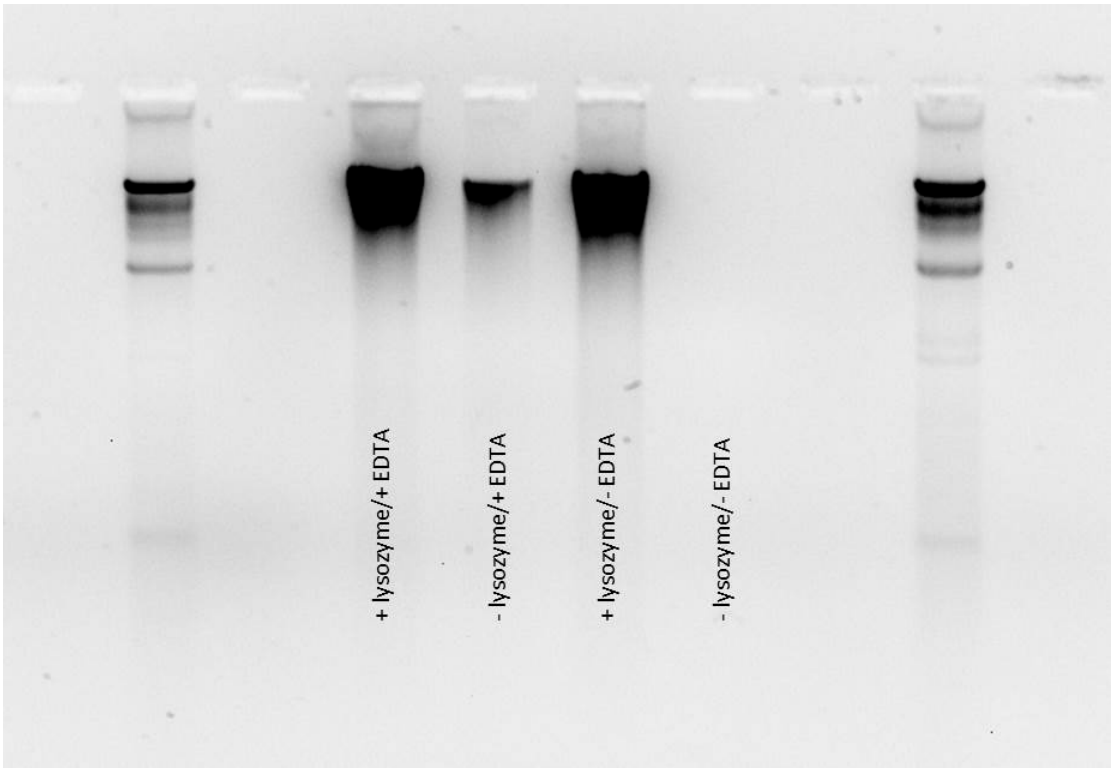

Figure 3-5. Agarose gel showing DNA extracted from seeded Drum ARP60109 samples. These results confirm the DNA extraction method is valid for this drum.

DNA was extractable from all samples except that which was not spiked with either EDTA or lysozyme. This test shows that DNA is extractable from the Drum ARP60109 waste matrix and supports the previous results that showed no detectable DNA in this drum.

This concludes processing of Drum ARP60109 for methanogens. There is no evidence of recent or current microbial presence in this drum.

3.3 Analysis of Anoxic Samples: Addendum to processing for methanogens

Upon receipt of the samples from INL, one tube from each drum (designated tube 6) was moved into the nitrogen-filled glove box. These tubes had not been used for DNA extraction previously. These were monitored for methane generation after receiving notice from INL that methane was still being detected in the drums. A significant amount of methane, with some CO_2 and lesser amounts of longer-chain alkanes were noted when the headspace gas in these tubes was analyzed (see Figure 2-2). In light of this, samples were withdrawn from these tubes for another DNA extraction (see Figure 3-6) and subsequent re-analysis for methanogens (following same flow chart outline). Neither EDTA nor lysozyme was used. As before, DNA was extracted from Drum ARP60106 but not from Drum ARP60109.

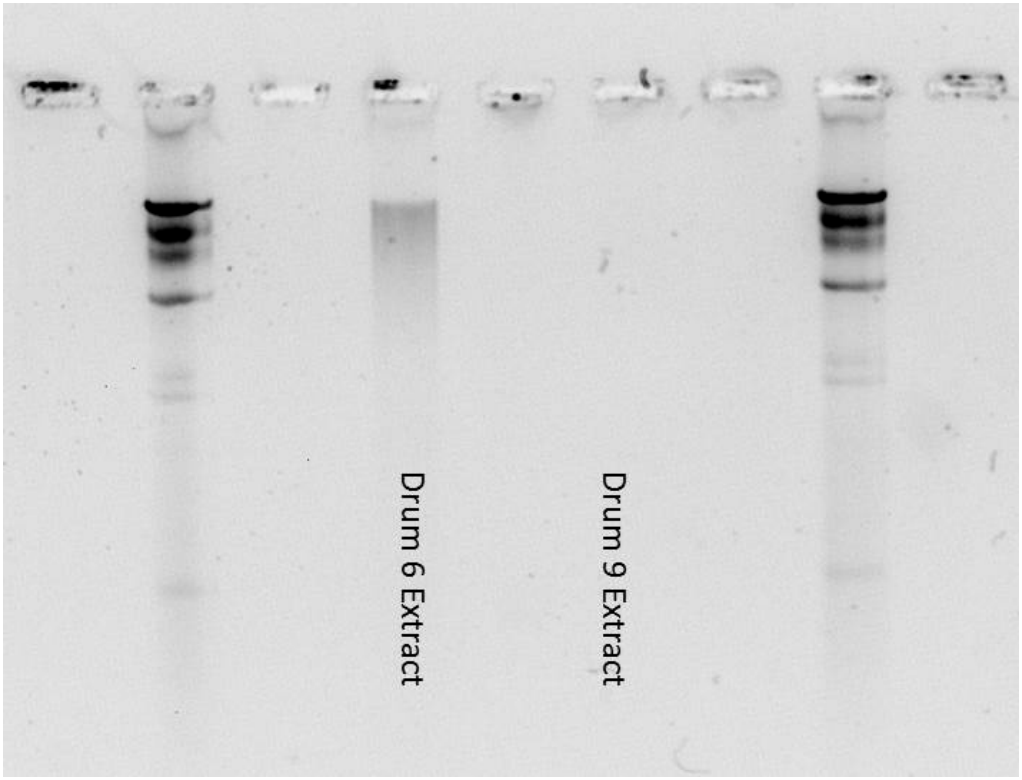

Figure 3-6. DNA extracts from Drum ARP60106, tube 6, and Drum ARP60109, tube 6, stored under nitrogen. These results continue to confirm that there is no DNA in Drum ARP60109, even after continued methane generation was noted.

Archaeal and *mcr*A PCR were performed on the new extracts from Drum ARP60106 and Drum ARP60109 (see Figure 3-7), and results were still negative. This confirms previous results that no active biomethanogenesis is occurring.

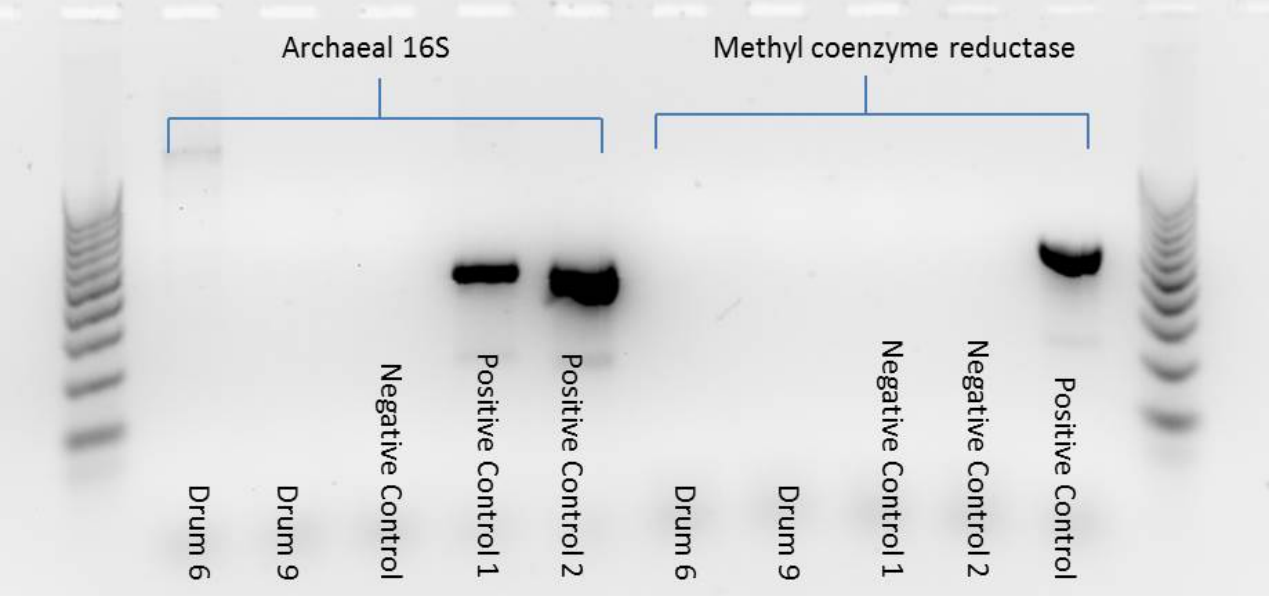

Figure 3-7. PCR amplification of archaeal 16S rRNA gene and mcrA gene. Two Archaea were used as controls for PCR: one is not a methanogen. These results show that there are no detectable Archaea in the samples and no genetic signature specific for methanogens.

As a final attempt to look for methanogens in these samples, a nested PCR was performed on the tube 6 extracts. This process amplifies a large portion of a gene and then uses this product as the template for another round of amplification of a portion of the same gene contained within the larger portion (i.e., "nested"). Nested PCR has been shown to be successful on samples with extremely low cell numbers.

Results for the nested PCR of archaeal 16S RNA gene were only faintly positive after significant image adjustment (see Figure 3-8). This suggests that archaea were present in this sample at extremely low numbers. It is not known whether these were methanogenic archaea.

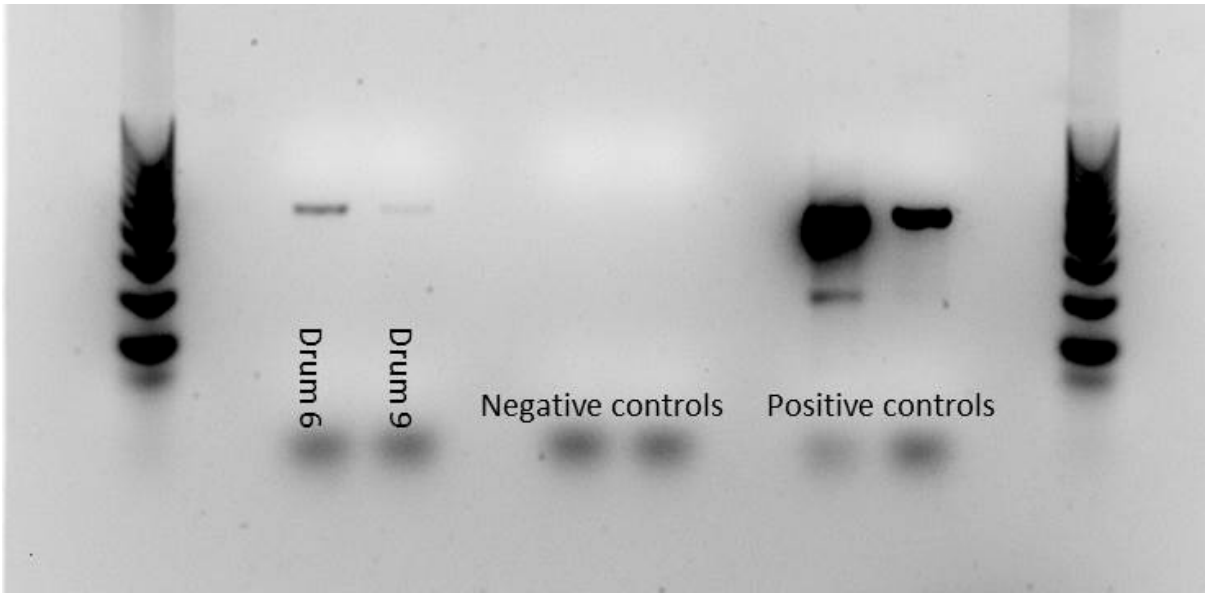

Figure 3-8. Results for nested PCR reaction of archaeal 16S RNA gene. Note blurring of size ladders and positive controls is due to need for maximum adjustment of image to distinguish product in drums ARP60106 and ARP60109.

4.0 GAS GENERATION STUDIES AND QUALITATIVE ASSESSMENT OF RADIOLYSIS AS A POTENTIAL MECHANISM FOR METHANE PRODUCTION

In recognition of the lack of evidence for a microbial source for the observed methane production, the possibility that radiolysis may be the methane-generating mechanism was evaluated in two ways. First the initial rate data (see Figure 2-1) was evaluated from the point of view of mole production of methane to estimate radiolytic yields and see if these are reasonable. Second, sample ARP-60106 sample 6 was split into four samples that were treated in different ways to evaluate initial gas generation. These results, although somewhat qualitative, were used to assess the possibility of a radiolytic mechanism and are summarized in this section.

4.1 Analysis of Radiolytic Content of Samples ARP 60106 and 60109

The main source of radiolysis is the actinide content, primarily Pu-239 and Am-241 (See Table 2-1) which are given in terms of initial inventory and what was determined based on NDA analysis. To more directly measure the alpha activity of the samples we received, a known quantity of samples from ARP 60106 and 60109 were dissolved in acid and counted using liquid scintillation counting (Beckman-Coulter LS6500 multipurpose scintillation counter) to estimate the alpha emitting content.

The dissolution of pre-weighed samples was not straightforward. The following were done: 1) taking to dryness in 8 M nitric acid (twice), 2) taking to dryness in concentrated HBr (once), 3) re-dissolving in 8 M nitric acid and stirring for ~ 2 weeks. Even after this, there was still some undissolved residue. Some of this residue was recovered and tested for alpha activity and found to have essentially no alpha emitters (no actinides). The final dissolution was done quantitatively into 3 mL of 8 M nitric acid and this was sampled (25 µL) quantitatively for LSC counting. The samples, masses dissolved, and counting results are all summarized in Table 4-1. These numbers are significantly higher than the NDA-predicted activity loading but agree reasonably well (within 50%) with the source term content data.

Table 4-1. Summary of Counting Data for Samples ARP 60106 and 60109				
Sample	**Amount Dissolved (g)**	**[1]LSC results (d/m)**	**[2]Estimated Source Concentration (d/m per gram of TRU Waste)**	**[3]Dose to Sample (MeV/Day per g of TRU Waste)**
ARP 60106 #4	0.0881	235.5 / 239.6	326356	2.59E+09
ARP 60106 #6	0.0581	216.1 / 224.3	305517	2.42E+09
ARP 60109 #4	0.0641	732.6 / 730.9	995551	7.89E+09
ARP 60109 #6	0.0629	465.7 / 488.2	664972	5.27E+09
1 - 25µL LSC sample, uncertainty ± 5%, first number is unfiltered, second is 100 kD (20 nm) filtered 2 – Based on filtered data (no suspended solids to dilute result) 3 – Assumption of 5.5 MeV/alpha particle event was made				

4.2 Estimated Radiolytic Yields from the Initial Whole-Sample Gas Generation Rates

The initial methane gas generation data from the whole sample was shown in Figure 2-1, and the data were summarized in Table 2-1. These correspond to the whole ~ 10 g sample as received from INL after the gas phase had been equilibrated with the nitrogen glovebox atmosphere. In this case, the residual methane generated since sample preparation at INL and shipment had been removed. These were done in the original 50 cc centrifuge tubes that were fitted with a modified lid to allow for sampling and were not rigorously leak tight. It was noted previously that the highest gas generation rates were noted for ARP 60106, which is counterintuitive from a radiolytic point of view, since this has a significantly lower activity (~ 47% for sample #6 that was used in the gas generation studies). This is the first qualitative observation that argues against this being a primarily radiolytic pathway.

If one assumes that radiolysis is the source of the methane produced, the estimated moles of methane “produced” in these samples can be used to determine G-values. Whether or not these values are reasonable reflects the likelihood that the observed gas generation is radiolytic.

These calculations are summarized in Table 4-2. In both cases the G-values estimated (1048 and 113 molec/100 eV) are well above the G(methane) < 1 molec/100 eV.

Table 4-2 Estimated G(values) for methane generation on the assumption that this is a radiolytic process

Sample	[1]Estimated Dose to Sample (MeV)	[2]Moles of Methane Observed	[3]Estimated G(value) for Methane Production (molec/100 eV)
ARP 60106 #6	5.32 E+11	9.26 E-06	1048
ARP 60109 #6	1.16 E+12	2.18 E-06	113

1 – dose/day from Table 4-1 x 10 g (approximate weight of sample) x 22 days
2 – moles based on final ppmV measurements at 22 days and an estimated free volume of 48 cc
3 – G-value is the molecules of methane / # of 100 eV

4.3 Observations from the ARP-60106 Sample Split

A final attempt to directly measure radiolytic yields more quantitatively was performed. Sample ARP 60106 #6 (highest methane generating sample) was split into four ~ 1-2 gram samples to measure methane gas production. The matrix for these experiments is given in Table 4-3. One sample (ARP6 6-1) was simply transferred to new 50 cc centrifuge tubes, a second sample (ARP6 6-2) was pumped down in the glovebox antechamber for ~ 2 hours and held at < 10 mtorr vacuum to promote outgassing, a third sample (ARP6 6-3) was mixed with paraformaldehyde as a biological fixative, and the fourth sample (ARP6 6-4) was mixed with dry BES (an inhibitor of microbial methanogenesis). The idea was to effectively eliminate microbial contributions and measure only radiolytic gas production.

The results of experiment ARP6 6-3, where paraformaldehyde was added, were anomalous and showed ~ 10X the amount of methane in an initial pulse. The methane gas analysis for experiments ARP6 6-1, 6-2 and 6-4 are shown in Figure 4-1. These show initial rapid methane gas production followed by a slow decrease with time. These data do not achieve the goal of measuring radiolytic gas production, since this was masked by the relatively high initial release of gas. The long term decrease in the methane concentration is most likely due to

sampling (5 cc out of 48 is removed at each sampling) and a slow leak into the glovebox. These data seem to be consistent with a slow desorption process that is not greatly affected even when a vacuum is pulled for a short time. Further tests will be done to confirm and/or establish a sorption process, but these data also support the conclusions in section 4.2 that radiolysis is not likely to be the immediate source of methane outgassing/production in these samples.

Table 4-3. Matrix: Follow up GC Studies with ARP Waste Samples

Drum/Sample	Experiment designation	Amount*	Description	Comments
ARP-60106 (6)	ARP6 - R	Remainder	What is left after start of experiments	Refreshed gas, so ~ zero start – 16:40 on 7/2/14
	ARP6 6-1	1.4 g	As is – transferred to new container	Start time: 16:40 on 7/2/14
	ARP6 6-2	1.8 g	Pumped down and held at vacuum for 75 minutes in GB2 antechamber (<0.8 relative units) – opened to GB atm, then resealed	Start time: 16:45 on 7/2/14
	ARP6 6-3	2.2 g	Mixed with fixative (wet) – 0.5 mL of paraformaldehyde (40%, EMS Lot 081006)	Start time: 16:45 on 7/2/14
	ARP6 6-4	1.4 g	Mixed with inhibitor (dry) – 0.59 g of BES (2-bromoethansulfonic acid – Na salt ACROS Lot A0248707)	Start time: 16:45 on 7/2/14
ARP-60106 (4)	ARP6 4 -1	All ~ 10 g	Transferred to GB2 and made anoxic	Made “suboxic” at 18:00 on 7/2/14
ARP-60109 (4)	APR9 4-1	All ~ 10 g	Transferred to GB2 and made anoxic	Made “suboxic” at 18:00 on 7/2/14

*For ARP6/9 sample 4 – did not weigh. For ARP6 6-1 to 6-4, this was done by difference in GB2

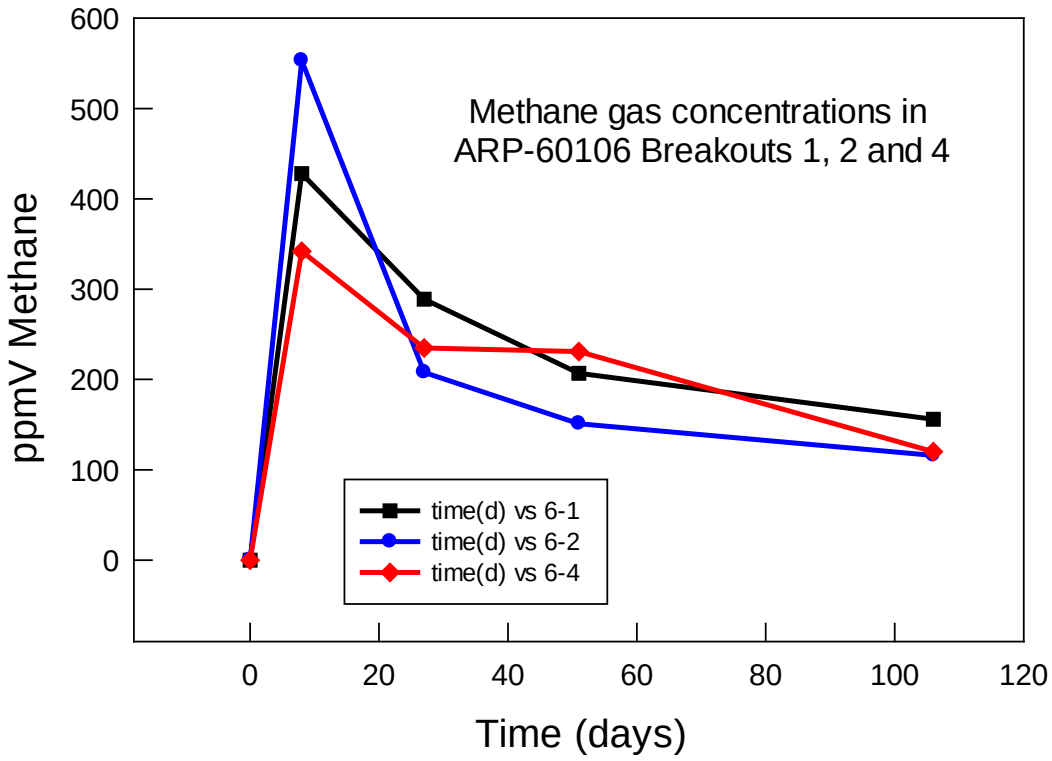

Figure 4-1. Methane gas concentration as a function of time for samples ARP6 6-1, 6-2 and 6-4. Although a rapid outgassing (re-equilibration) is noted, there is no long-term buildup of methane gas (decreases noted are mostly due to methane removal during sampling).

Although methane gas was observed to be near linear for a few days after samples were mixed, this did not persist over the long term and appeared to peak. Along with the yield data and lack of correlation with sample activity, this is inconsistent with a predominantly radiolytic pathway, as this would have shown a steady rate of gas generation. This is further confirmed by the very low methan concentrations noted after rigorous pump-down, which presumably removed the sorbed methane but did not change the radiolytic pathway.

5.0 SUMMARY OF CONCLUSIONS

Overall, we conclude that microbial and radiolytic pathways cannot account for the methane levels and production that are observed in samples ARP 60106 and ARP 60109.

The results for the microbial analyses are shown in Figure 5-1. This conclusion is based on the absence of microorganisms that directly account for methane gas generation in ARP60106 and the overall absence of a microbial signature in ARP60109. These results do not discount that the origin of methane could be biogenic in nature from some time in the past, but methanogens are currently not present in the TRU waste and cannot account for what is seen in the headspace analysis and in our laboratory studies.

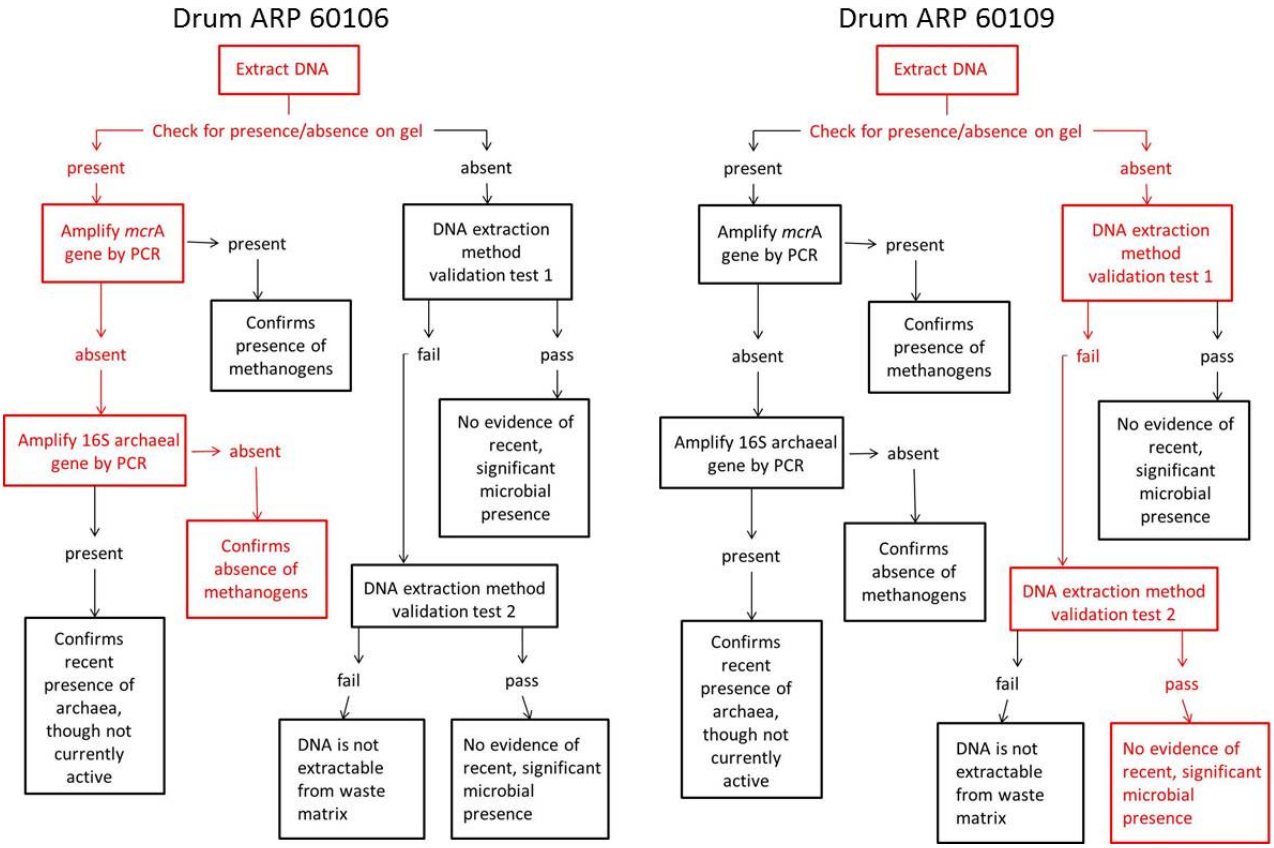

Figure 5-1. Summary of DNA analysis experiments. Red highlighted areas delineate paths taken for each drum.

The radiolytic pathways are also discounted as the immediate source of high methane in these two TRU waste samples. The methane "production" observed does not correlate with measured alpha activity (high activity samples have the lower methane production). The G values calculated for radiolysis are unreasonably high, supporting the assertion that radiolysis is not the immediate source of methane, although over time in the subsurface this could be a source that was somehow trapped in the TRU waste.

The best, albeit speculative, interpretation of our data is that there is a slow outgassing step that is occurring: regardless of the source of methane, it has been trapped in the waste and is released when new surface area is exposed. The best illustration of this is shown in Figure 4-1. This interpretation is, again, quite speculative and needs more definitive corroboration.

Intentionally Blank

Appendix

Supplemental Information: Continued work on Drum ARP60106

PCR Amplification of 16S Ribosomal RNA-Encoding Gene of Bacteria

Because archaeal DNA was not detected but DNA was present, a second round of PCR was used to amplify the bacterial 16S gene. Results were positive.

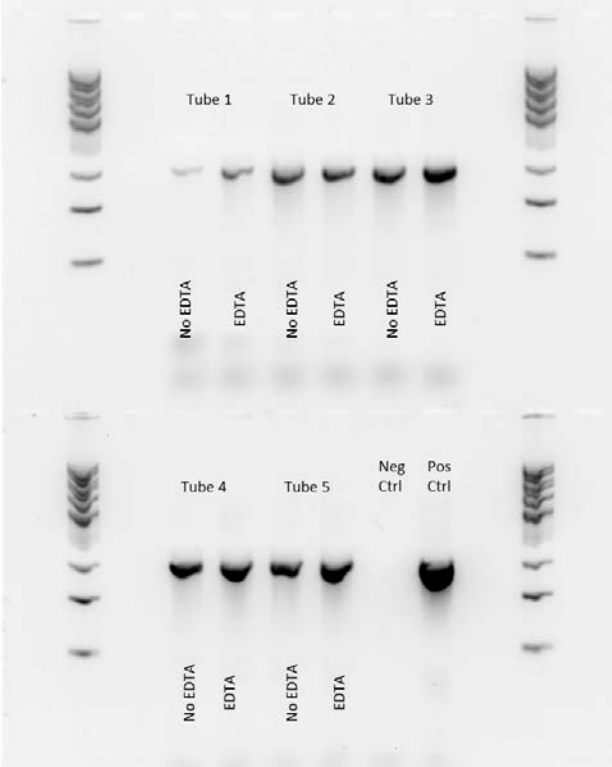

Figure A-1. Results for the PCR amplification of a target segment within the bacterial 16S rRNA-encoding gene.

Two clone libraries were constructed from purified bacterial PCR products (+ EDTA and – EDTA). Each library was a collection of the 16S target genes present within the sample. This permitted screening for unique DNA sequences each putatively identifying a different organism. Samples were sent for DNA sequencing to SeqWright, Inc. of Houston, TX.

Sequencing results show both libraries to be monophyletic, i.e. composed of only one phylum—*Actinobacteria*. Lower diversity is often associated with environmental stressors (in

this case, probably radioactivity and lack of moisture). Still, the presence of only one phylum is rare.

Actinobacteria are ubiquitous organisms, found in environments ranging from soil to human skin. One genus, *Rubrobacter*, has been described as gamma radiation-resistant, but no mechanism was identified in that study (Ferreira et al., 1999). Resistance may be due to the presence of carotenoid pigments that help to scavenge reactive oxygen species and/or to the high G+C content of their DNA. Factors that confer resistance to desiccation and increased salt content also appear to confer resistance to radiation, in some organisms. It is possible that this is also the case with the *Actinobacteria* found in this sample. *Actinobacteria* have been detected in biofilms exposed to radiation fallout from Chernobyl (Ragon et al., 2011) and were the dominant organisms found in soils contaminated with radioactive waste (Fredrickson et al., 2004).

Phylogenetic distribution results are shown in Figures A-2 and A-3 for both individual libraries (with and without EDTA) and pooled.

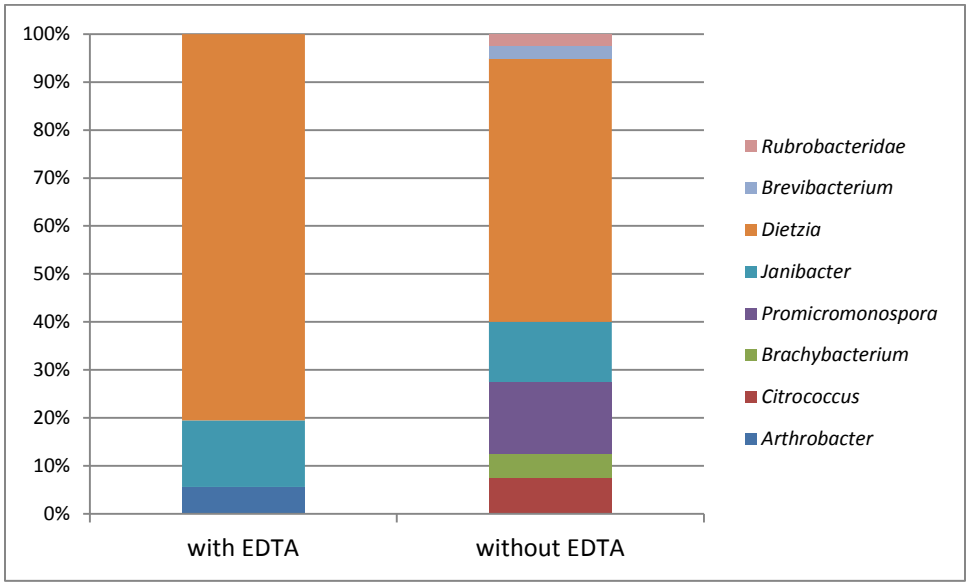

Figure A-2. Phylogenetic distribution of 16S sequences from each clone library.

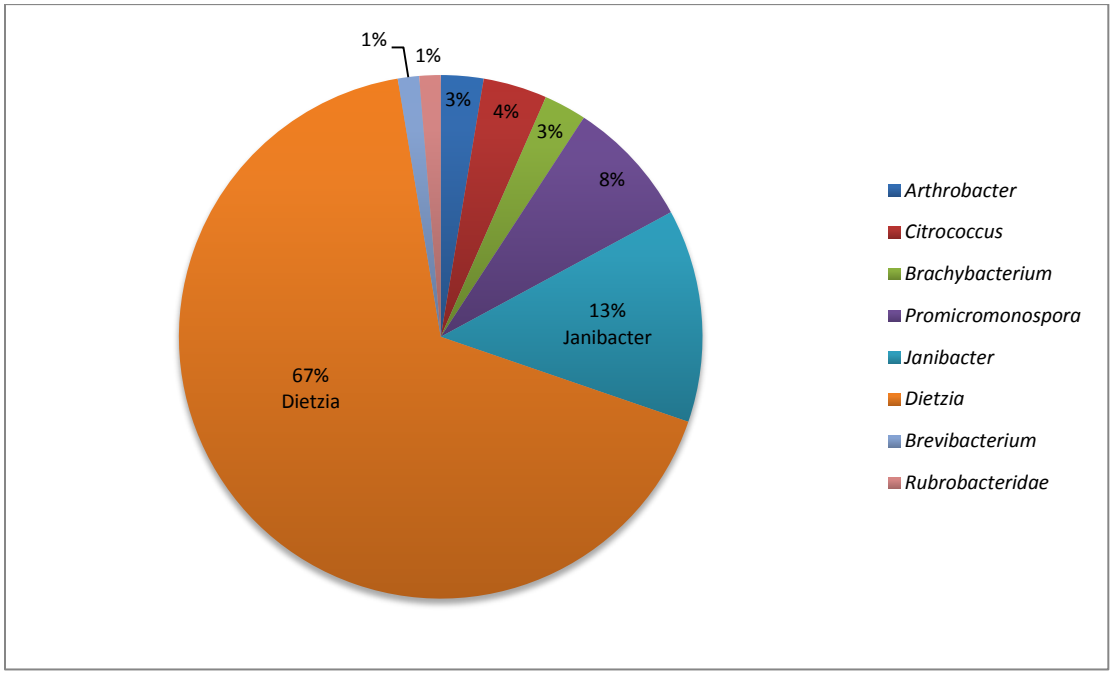

Figure A-3. Distribution of sequences in pooled libraries.

The sequence diversity in the library that did not use EDTA during the extraction protocol was greater than when EDTA was used (7 versus 3 genera). When the libraries were pooled, two-thirds of the DNA sequences were from a single genus, *Dietzia*, with *Janibacter* and *Promicromonospora* spp. comprising smaller percentages (13% and 8%, respectively). The remainder of the pool was composed of low-frequency sequences from *Citrococcus*, *Brachybacterium*, *Arthrobacter*, *Brevibacterium*, and *Rubrobacteridae* spp.

A phylogenetic tree showing the relatedness of these bacteria to one another is shown in Figure A-4. Because of the lack of phyletic diversity, it is difficult to get a perspective on their relation to other bacteria from other phyla.

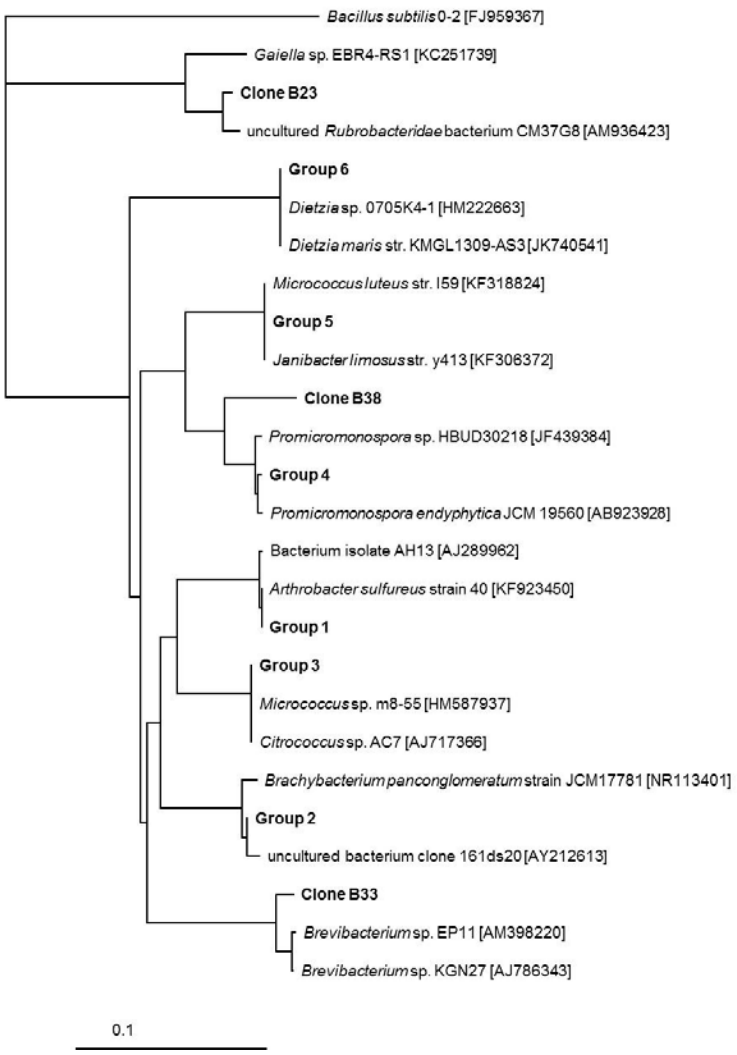

Figure A-4. Phylogenetic tree showing relatedness of DNA sequences to each other. Sequences from this study are in bold; all others are close matches to serve as references. Tree is rooted to *Bacillus subtilis* as an outgroup. Scale bar represents 1 nucleotide substitution per 10 bases. Numbers in brackets are GenBank database accession numbers.

Cultivation work on Drum ARP60106:

Given the unexpected amount of DNA obtained from Drum 6, further work was conducted to see if any organisms could be cultivated from the waste. Only aerobic conditions were used, since anaerobic enrichments require amending the waste chemically. Approximately ~0.5 g of waste were suspended in ~2 mL of sterile normal saline (0.85% w/v NaCl), vortexed,

and plated onto the following agars: R2A (regular strength), 0.5X R2A, marine agar, R2A + 10% NaCl, carboxymethycellulose agar + 10% NaCl, and generic halophile agar (20% NaCl).

R2A is a common medium used for the routine cultivation of environmental organisms. The NaCl-rich agars were used to see if any organisms from the waste might be halotolerant, as they are destined for the WIPP. The CMC agar also selects for cellulase-producing organisms (i.e., those that may contribute to cellulose degradation, also of interest to the WIPP).

All plates were incubated at ambient temperature in the air-filled glove box. Growth occurred on all R2A and CMC plates within 48-72 hours, after which fungal growth made colony purification difficult. Subsequent serial transfers have been made from each plate to purify colonies and to reduce any radioactivity that may be associated with the cells. Five distinct colony morphologies have been isolated. All are Gram-positive rods of varying sizes (see Figure A-5).

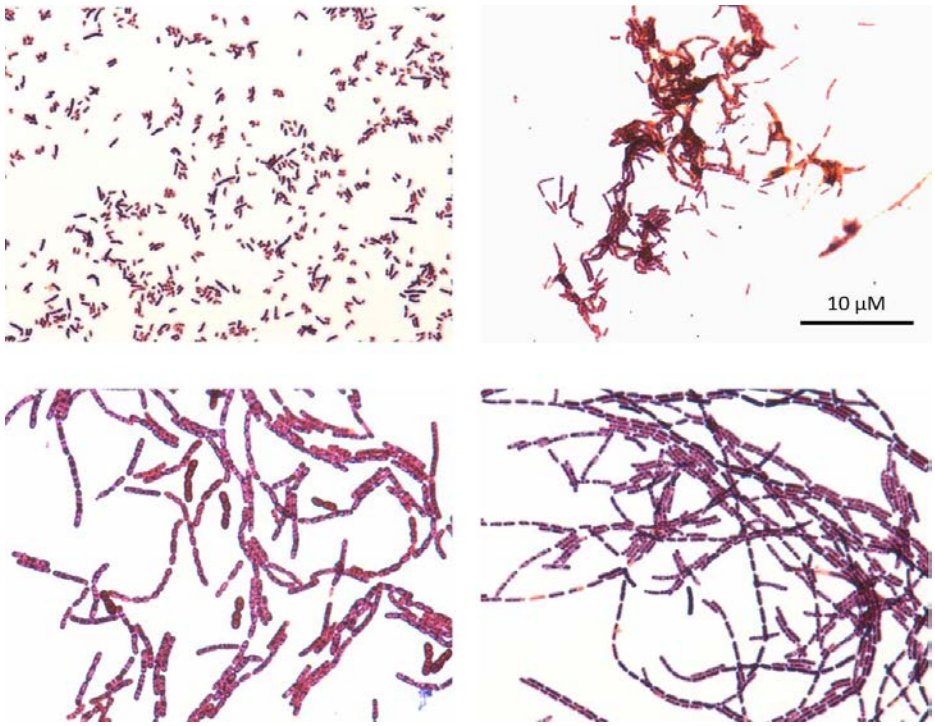

Figure A5. Gram-stained images of four bacterial isolates from Drum ARP60106 under aerobic conditions.

DNA was extracted and the 16S gene was amplified from all the isolates and shipped for sequencing. Sequencing revealed isolate 1 to be of the genus *Arthrobacter* (similar to Group 1 clones; see Figure A-6). These are ubiquitous soil organisms with diverse degradative capabilities and resistance to various stressors, such as desiccation. These organisms were also dominant in radionuclide-contaminated soils at Hanford (Fredrickson et al., 2004). The closest relative to this isolate (based on DNA sequence) is capable of degrading organic solvents, which may explain its presence in the waste. Isolate 5 is of the genus *Brachybacterium* (also a member of the *Actinobacteria*), similar to Group 2 clones. Its closest relative was isolated from chromium-contaminated sludge.

Isolates 2-4 are of the genus *Bacillus*. This genus was not detected in the clone libraries. These organisms form spores which can be difficult to lyse during DNA extraction procedures and may be the reason why they were not detected in the libraries. It is very common to have differences between cultivation-dependent and independent analyses; hence the reason for pursuing both.

Bacillus spp. were prevalent in cultures of soil samples affected by the Chernobyl incident and exhibited increased UV and radiation resistance as compared to those from a non-affected area (Zavilgelsky et al., 1998).

The presence of *Bacillus* spp. in Drum ARP60106 introduces a second phylum (*Firmicutes*) into the sample set. A second phylogenetic tree was generated using all the clone and isolate sequences (see Figure A-6).

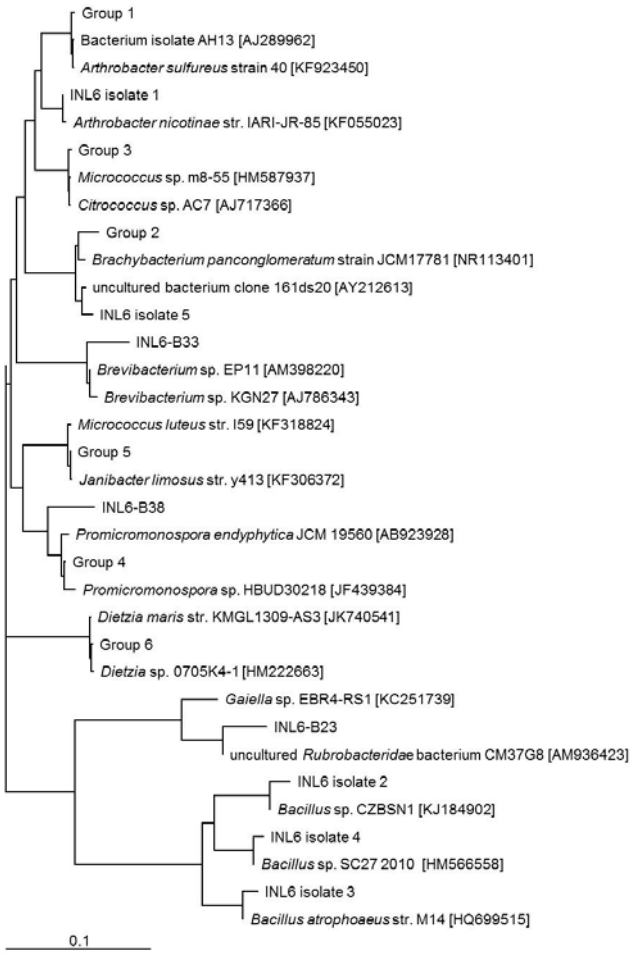

Figure A-6. Phylogenetic tree showing relatedness of DNA sequences (clones and isolates) to each other. Sequences from this study are in bold; all others are close matches to serve as references. Tree is not rooted. Scale bar represents 1 nucleotide substitution per 10 bases. Numbers in brackets are GenBank database accession numbers.

References

Fredrickson JK, Zachara JM, Balkwill DL, Kennedy D, Li S-MW, Kostandarithes HM, Daly MJ, Romine MF, Brockman FJ. 2004. Geomicrobiology of High-Level Nuclear Waste-Contaminated Vadose Sediments at the Hanford Site, Washington State. Applied and Environmental Microbiology 70: 4230-4241.

Zavilgelsky GB, Abilev SK, Sukhodolets VV, Ahmad SI. 1998. Isolation and Analysis of UV and Radio-Resistant Bacteria from Chernobyl. Journal of Photochemistry and Photobiology B: Biology 43: 152-157.