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Report, General

INFLUENCE OF MICROBIAL DEGRADATION ON GAS PRESSURE EVOLUTION AND TERMINAL GAS COMPOSITION IN BATCH ANAEROBIC CONTAINERS OF SURROGATE LOW-TO-INTERMEDIATE LEVEL RADIOACTIVE WASTE

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Report, General

Influence of Microbial Degradation on Gas Pressure Evolution and Terminal Gas Composition in Batch Anaerobic Containers of Surrogate Low-to-Intermediate Level Radioactive Waste

Research and Development

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APPENDICES

1. INTRODUCTION

This work is the final report for work performed under the Atomic Energy of Canada Limited (AECL) and the Canadian Nuclear Safety Commission's (CNSC) Memorandum of Understanding (AECL Ref #008794-0081 Revision 1, CNSC #87055-15-0148). The information within this report is also available as a draft manuscript (153-121241-021-000, CW-121241-CONF-023, Revision D2).

Many countries plan to confine mixed low and intermediate-level radioactive waste in terrestrial subsurface environments to protect the public and the environment via the long-term containment and isolation in deep geologic repositories (DGRs) (Aikas and Anttila, 2008; Brewitz et al., 2008; Delay et al., 2008; Gartner et al., 2008; Olsson et al., 2008; Powers and Holt, 2008; Woller, 2008). The biogeochemical activity of microorganisms within the subsurface is expected to have an impact on the design and long-term safety of a DGR; therefore, predictions based on knowledge of the microbial ecology have formed part of the safety assessments for commissioning a DGR (Arter et al., 1991; Humphreys et al., 1997; Avis et al., 2014).

An important biogeochemical cycle within a DGR is the *in situ* anaerobic biodegradation of the organic components of the waste. These reactions are expected to produce gases such as hydrogen, carbon dioxide, methane and organic acids -- these reactions are listed in Table 1-1. Corrosion of the carbon steel containers that hold the waste will generate additional hydrogen gas and the aqueous solubility of carbon dioxide (Duan and Mao, 2006) will result in formation of carbonic acid. This acid production is in addition to the organic acids formed by fermentation (Table 1-1, reaction 2). Because the carbonate minerals present in the limestone of some DGR host formations and in the cement in shaft seals are prone to acid dissolution, they are inherently sensitive to the partial pressure of carbon dioxide and other acids. The host geological formation and the shafts sealing materials, therefore, represent the ultimate barrier to mitigate aqueous and gaseous radionuclide migration (Toulhoat, 2002). It is through the formation of acids like these that the microbial communities within a DGR may mediate mineral dissolution (Moyce et al., 2014). However, in DGR safety assessments, methane is assumed to be the dominant gas formed (Avis et al., 2014; Small et al., 2008). If methane were the dominant gas, the acidity of any carbon dioxide produced would be reduced by the activity of hydrogenotrophic methanogens (Table 1-1, reaction 3), and any associated mineral dissolution of the host rock and shaft seal would also be reduced.

Table 1-1
Reaction involved in the biodegradation of cellulose and resulted gas formation.

Cellulose → soluble carbohydrate (CH_2O)	Hydrolysis of solid cellulose	reaction 1
CH_2O → organic acids + CO_2 + H_2	Hydrolysis of carbohydrate and fermentation	reaction 2
4 H_2 + CO_2 → CH_4 + 2 H_2O	Hydrogenotrophic methanogenesis	reaction 3
CH_3COOH → CH_4 + CO_2	Aceticlastic methanogenesis	reaction 4
4 H_2 + 2 CO_2 → CH_3COOH + 2 H_2O	Acetogenesis	reaction 5

To test the assumption that methane will be the terminal gas produced, we explored the dynamics of gas and microbial evolution within surrogate waste under different conditions (either with or without amendments and at different initial pH values). Amendments included nutrients and microbes, or nutrients and enzymes. The starting pH was either slightly acidic (in a range optimal for cellulase activity) or at neutral pH (in a range optimal for methanogenesis). The evolution of gas pressure was continuously monitored and the headspace gas was opportunistically monitored. The source of gas evolution was only from surrogate organic waste without corrosion as a source of hydrogen. We measured the microbiology directly at the end of the gas monitoring period by testing for cellulase activity and by targeting specific gene markers for phylogeny and the functions of interest (cellulose hydrolysis, methanogenesis and acetogenesis, and other functional markers related to microbial ecology).

2. MATERIALS AND METHODS

2.1 Experimental Approach

To test whether methane is likely to be the terminal gas produced, we used a surrogate cellulosic waste material that was kept within closed glass containers for approximately 5 and 6.5 years, respectively. Initial starting conditions were either un-amended or amended to accelerate the evolution of gases. The amendments consisted of compost aids or added cellulase enzymes from a fungal source. The surrogate wastes were emplaced within sealed glass containers that were instrumented for pressure and temperature monitoring. Headspace volumes were sampled periodically for gas composition analysis. The containers were located on the laboratory bench under ambient room temperature and light conditions. The pressure measurements were corrected for temperature. After this monitoring period, the glass containers were opened and the waste was tested for the microbial biomass associated with any gas pressure build-up and head space gas compositions.

2.2 Sample Preparation and Monitoring

Mops are used for routine maintenance of facilities located within radiological zones areas. Because of the work performed within these zones, used mops would be deemed either low or intermediate-level waste. Mops are also as source of cellulosic material and have a high water holding capacity. For each test container, approximately 100 g of mop material was used in testing. The water holding capacities of the mop samples were determined by weight. The samples were then wetted with sterile buffer (pH 7 or pH 5) or with sterile water weight to achieve at least 60% of the moisture holding capacity. The wetted samples were then inserted into separate sterile 1 L glass bottles. Before sealing the bottles, some of the samples were amended to test the effects of additives on the rate of gas generation. These were commercial compost additives, Ringer All Purpose Compost Plus Compost Maker and Jobes Organics Compost Starter, mixed in proportion to the manufacturer instructions, and 1 unit of purified cellulase enzyme from *Trichoderma viride* (lyophilized powder, 0.3-1.0 unit/mg solid, Sigma) at a starting pH of 7. For those samples with a set starting pH, the pH was established using either phosphate buffer (pH 7) or citrate buffer (pH 5). Details of the starting conditions for each sample are listed in Table 2-1.

The containers were sealed using media bottle lids that had been modified to allow temperature and pressure gauges to be situated inside of the bottle. These penetrations were sealed with stainless steel tubing. The temperature and pressure inside the containers were monitored continuously using thermocouples and differential pressure cells (Cole Parmer, 14.7 to 15 psig). These penetrations also provide the means for periodic sampling of the container headspace for gases. Data from the thermocouples and pressure cells were logged automatically using a Keithley data logger. To prevent over pressurizing the containers as the mop heads degrade, each container was fitted with a 68.95 kPa (10 psi) pressure relief valve. The experimental set up is shown in Figure A-1.

Table 2-1 lists the sample names, their starting conditions and the duration between headspace gas sampling. Three of the samples, called "Un-amended", "Compost Accelerator" and "Compost Maker" were sampled for headspace gas once, after 464 days, and then removed from continuous monitoring to start the monitoring of the other three samples. These containers were stored sealed until the headspace gas from the next three samples was analyzed for the final time, after 1965 days, at which time all six samples were processed for gene abundances by digital drop PCR (ddPCR).

Table 2-1
Sample names, starting condition and the elapsed time in days when the headspace gases were sampled.

Sample Name	Starting Condition	Headspace Sampling: elapsed time in days
Un-amended	No additive	464
Neutral, pH7	Starting pH: neutral*	150, 730 and 1965
Acidic, pH5	Starting pH: acidic**	150, 730 and 1965
Cellulase, pH7	Starting pH: neutral* Cellulase from *Trichoderma viride*	150, 730 and 1965
Compost Accelerator	Commercial blend: nutrients and enzymes	464
Compost Maker	Commercial blend: nutrients and microorganisms	464

* phosphate buffer, ** citrate buffer

2.3 Headspace Gas Analysis

Headspace gas analysis was performed using Agilent 6890N gas chromatograph (GC) by connecting the pressurized sample containers (vessels) to a vacuum line, and then evacuating the line to a pressure <1 torr absolute, as measured by an MKS Baratron Capacitance Manometer. The vacuum pump was then isolated, and a sample of the headspace gas within the container was bled into the vacuum system by a low flow metering valve to a pressure of roughly 760 torr. The sample inlet was then isolated and time was given for the pressure to stabilize in the vacuum system. This process was repeated 3 times on each sample to ensure sample consistency. A 100 µL sample of the analyte gas was then directed to the GC using a 6-port, 2-position sampling valve. The GC was configured with an Agilent HP-PLOT Molsieve 15 m column for separation of permanent gases (Ar, O_2, N_2, H_2, CH_4), and an Agilent GASPRO 30 m column for separation of corrosive gases (CO_2, H_2S); a valve was installed to allow for column selection midway through the GC run. The analysis was performed independently using both He and Ar carrier gases for each sample to ensure detection of hydrogen gas. Detection of the gases was performed using a Thermal Conductivity Detector (TCD) and a reference gas matching the carrier (both He and Ar, respectively). Calibration of the GC was performed using certified standard mixes of the analyte gases using the same sample introduction scheme at

sample pressures ranging from 10 to 760 torr. Examples of the analysis output are provided in Figure A-2.

2.4 Sample Preparation for ddPCR

Following the final headspace gas sampling, the sample bottles were opened, and two approximately 1 cm pieces of material were cut out and processed for DNA extraction using the protocol from the PowerSoil® DNA Isolation kit (MoBio, 12888). DNA extracts were also obtained from liquid leachate collected from the samples. The extracted DNA was quantified by fluorescence using the intercalating dye, PicoGreen, from the Quant-iT PicoGreen dsDNA assay kit (Life Sciences, P7589). The detected concentration ranged from 0.08 to 0.39 ng/µL, with two samples that failed to quantify (Table A-1). These extracts were stored at -20°C until they were analyzed by ddPCR.

2.5 Primers

To gauge the cellulolytic, fermentative, sulfate-reducing, acetogenic and methanogenic microbes, a literature research was performed to find primers that targeted the genes of interest. The cellulase enzymes are involved in the early steps of cellulose degradation by catalysing the hydrolysis of glycosidic bonds. There are several different types of cellulases based on the reaction to be catalyzed. The glycoside hydrolase family of cellulose enzymes consist of 131 protein families based on their sequence and structure information (Henrissat and Bairoch, 1996). To evaluate the potential for cellulose degradation, the set of primers used spanned genes for the exo- and endo-glucosidases that are found within bacteria and fungi (Table A-1). The primers also covered four of the families within the glycoside hydrolase enzyme class (family 4, 5, 6 within bacteria (Canizares et al., 2010), and family 61 within fungi (Busk and Lange, 2013)) (Table A-1). The primers, mlas and mcrA, are directed to the alpha-subunit of the methyl co-enzyme M reductase gene (*mcr*A) and detect methanogenic Archaea (Steinberg and Regan, 2008). This enzyme catalyzes the last step of methanogenesis, is conserved among all methanogens (Steinberg and Regan, 2008) and is absent in non-methanogens except for the anaerobic methane oxidizing Archaea (Hallam et al., 2003). We also tested the primers dsr1-F and dsr-r (LeLoup et al., 2007) that are directed to the dissimilatory bisulfite reductase gene (*dsr*AB) as a functional marker to evaluate the sulfate reducing community (Baker et al., 2003). The dissimilar sulfate reduction trait is patchily distributed in the Tree of Life, with five bacterial and two Archaeal phyla containing recognized members of this guild. Members within this guild also represent a fermentative trait. Primers for acetyl co-enzyme synthase (Aydin et al., 2015) and acetyl co-enzyme synthetase (Aydin, 2015) were applied to gauge for genes involved in acetate formation from hydrogen and carbon dioxide, and for genes involved in acetate consumption to form methane. Quantification of the three Kingdoms were determined using primer pairs for the 16S rRNA gene for Archaea (Baker et al., 2003), glutamine synthetase for bacteria (Hurt et al., 2001) and the 18s rRNA gene for fungi (Zhu et al., 2005).

Details of the primer sequences, targets, expected amplicon size and thermal cycler conditions are provided in Table A-2. The goal was to gauge the proportions of cellulolytic, fermentative and methanogenic biomasses. Primers were ordered from Integrated DNA Technologies (IDT). Upon receipt, the primers were solubilized to a final concentration of 100 µM in sterile water and stored at -20°C. Multiple working stocks were diluted to 10 µM and stored at -20°C. All reagents were made up and stored in autoclaved DNase, RNase, Pyrogens, DNA and PCR inhibitor and Endotoxin free tubes. For all reactions, Sarstedt Biosphere DNA, RNase and Pyrogen free filter tips were used.

2.6 ddPCR

All ddPCR reactions were set up inside of a laminar flow hood that had been sterilized with 70% ethanol and ultraviolet light for 3 min prior to entering the hood. Individual reactions for ddPCR contained a final primer concentration of 150 nM with 2x QX200 ddPCR EvaGreen Supermix (Bio-Rad #186-4033) following manufacturer's instructions. Two microlitres of DNA were loaded into a total reaction volume of 21 µL. All ddPCR runs included a no template negative control and a positive control. Table A-2 provides a description of the positive controls. Twenty microlitres of the ddPCR reaction were transferred to a DG8 Cartridge (Bio-Rad #186-4008) with 70 µL of Droplet Generation Oil for EvaGreen (Bio-Rad #186-4006) covered with a DG8 Gasket (Bio-Rad #186-3009) and converted to droplets with the QX200 Droplet Generator (Bio-Rad #186-4002). Droplets were then transferred to a 96-well plate (Eppendorf #0030128.575) and heat sealed at 180°C for 6.5 seconds with Pierceable Foil Heat Seal (Bio-Rad #1814040) using the Bio-Rad PX1 PCR Plate Sealer (#181-4000). The samples were then cycled in a Bio-Rad C1000 Touch Thermal Cycler (#185-1196) using a 2-step or a 3-step cycling program (see Table A-2). The cycled plate was then transferred and read on the QX200 reader (Bio-Rad #186-4003), and data were analyzed with the QuantaSoft Software (Bio-Rad #186-4011).

2.6.1 Optimization of ddPCR

To determine the optimal concentration of DNA for ddPCR, a dilution series was constructed for two of the samples (one sample that displayed high expression of the target and one that displayed low expression of the target from the PCR, see Table A-3).

To determine the optimal annealing temperature (T_A) for all primers, a temperature gradient ddPCR was performed. *Glomus irregularis* gDNA at 0.1 ng/µL was used across a range of 52°C to 62°C in a 3-step cycle.

2.6.2 Analysis of ddPCR Results

For the ddPCR, the gene abundance (concentration) was reported as copy number per µL. The positive droplets and the total accepted droplets were also reported. To fulfill Poisson's distribution rules, samples must have more than 13,000 droplets to be analyzed. If the samples did not reach this threshold, individual samples were removed. The threshold to determine

quantification of target was set manually using samples with a well-discriminated fluorescence difference between positive and negative droplets.

2.7 Cellulase Activity Assay

Cellulase activity within the leachate was determined after opening the sample bottles. The mop materials were removed from the bottles and the liquid was extracted from the top, middle and bottom portions of each sample by inserting each portion into a sterile 30 mL syringe and squeezing liquid into sterile plastic tubes. The leachates were stored at -20°C until analyzed for cellulase activity. We used the fluorometric-based Cellulase Activity Assay Kit (Abcam, ab189817) that detects the release of a fluorescent compound, resorufin, from a cellulase substrate, resorufin-β-D-cellobioside (Coleman et al., 2007). Detection of resorufin was by excitation at 530 nm and emission at 595 nm using a Varioskan Flash Spectral Scanning Multimode plate reader (Thermo Fisher). The detected cellulase activity was compared to the cellulase activity from *Trichoderma ressei* of 6.7×10^{-4} µmole/mL/min (Coleman et al., 2007) as a reference. Samples having fluorescence values that exceeded the range of the standard curve were diluted and re-analyzed.

3. RESULTS

3.1 Evolution of Gas Pressures and Gas Compositions

Changes in gas pressure were continuously monitored. The results of the gas pressure evolution for the 1965-day study (Figure 3-1) shows the evolution of gas pressure for the starting conditions of neutral pH, slightly acidic pH and by the additional of cellulases (Table 2-1). The results of the gas pressure evolutions for the 464-day study (Figure 3-1) show that pressure development differed depending on the starting condition: un-amended; amended with nutrients and enzymes; or amended with nutrients and microorganisms (Table 2-1). The headspace gas compositions for the test containers in the 1965-day study were determined at three time points (after 150, 730 and 1965 days of continuous monitoring); the results for these analyses are also shown in Figure 3-1. The headspace gas compositions for the test containers in the 464-day study were determined at one time point (after 464 days of continuous monitoring); the results for this analysis is also shown in Figure 3-1. The development of gas pressure inside each of the test containers varied in terms of the timing of gas pressure onset, the rate of gas pressure onset and the rate of gas pressure change over the monitoring period. The test container that displayed the slowest onset of gas pressure (the container with the un-amended waste (Figure 3-1)) also had the lowest total pressure at the time that the test was stopped, but was the test container that had the highest proportion of methane gas amongst all the samples.

The test containers from the 1965-day study displayed very different patterns of gas pressure evolution (Figure 3-1). The test containers for the acidic starting condition and the added cellulase as the starting condition displayed an initially rapid rate of gas pressure rise followed by a rapid leveling off within the first week of starting the gas pressure monitoring, the duration of which lasted about 50 days for the test container with the acidic waste and for over 300 days for the test container with added cellulose. The gas pressure within these containers continued to evolve as either a continuous decline (acidic waste) or a second rapid rise to 185 kPa followed by a period of variable pressure changes before displaying a continuous decline (added cellulase). The test container with waste exposed to neutral conditions at the start of gas pressure monitoring displayed a gas pressure evolution that first declined then rose, following a rate of change that slowly leveled off over approximately 1500 days and remained unchanging for the remainder of the monitoring period. The headspace gas compositions determined for these containers was mainly hydrogen, carbon dioxide and nitrogen gas (Figure 3-1). The carbon dioxide content of the headspace ranged from 14% to 69%, the nitrogen content ranged from 30% to 79%, hydrogen ranged from 0.1 to 11% and argon was measured at up 1.8%. Although the headspace gas analysis included methane, hydrogen sulfide and oxygen, these gases were not detected. The relative composition of the major gases in dry air are also shown (Figure 3-1). The decline of gas pressure detected within the test containers for the acidic and added cellulase starting conditions, and the variation in carbon dioxide and hydrogen without the formation of methane in these containers, suggest that acetogenic metabolism was established. Acetogenesis, like methanogenesis, consumes

carbon dioxide and hydrogen, but forms acetate rather than methane (Ragsdale and Pierce, 2008).

Addition of the compost additives, Compost Maker (with microorganisms within its composition) and Compost Accelerator (with enzymes within its composition), increased the rate of gas pressure onset (Figure 3-1). Compared to the un-amended waste sample, the waste amended with the additive that included enzymes within its composition (Compost Accelerator, Figure 3-1) displayed the quickest onset of gas pressure and the fastest rate of gas pressure rise; these periods were preceded by an initial decline in the headspace gas pressure. By about day 50, the rate of gas pressure rise started to slow and had reached a constant level by about day 250 onward to the end of the test period at 464 days. The waste amended with the additive that included microorganisms within its composition (Compost Maker, Figure 3-1) displayed an initial decline in headspace gas pressure, a slower onset of gas pressure rise and a slower initial rate of gas pressure rise compared to the Compost Accelerator. By the end of the test period, the rate of gas pressure rise was slowing but had not yet leveled off. The rate of change of pressure within the test container for the un-amended sample also displayed an initial decline in gas pressure followed by a slight increase starting at about day 80; the gas pressure rate increased again starting at about day 130 then leveled off for the next approximately 120 days before displaying another period of increasing pressure (Figure 3-1). This variation (sequential low rate of pressure rise then a faster rate of pressure rise) was also seen in the large-scale gas generation experiment (GGE), performed at Olkiluoto, Finland (Small, 2008). In this experiment, methane was the main gas formed, making up 98% or higher of the partial pressure within the test vessel (Small, 2008).

The headspace gases in the 464-day study were tested for nitrogen, oxygen, carbon dioxide, hydrogen, methane and argon. The gas present in the highest proportion within all three of the containers was nitrogen, making up 62% to 67% of the gas partial pressures (Figure 3-1). The next highest headspace gas was carbon dioxide, making up 25% to 35%. The headspace gases, argon, hydrogen and methane, made up the remainder of the gas composition, at 1% to 1.9%, up to 0.24% and at 0.6% to 12%, respectively (Figure 3-1). The lack of hydrogen detected within the test containers coupled with the formation of methane suggest that hydrogenotrophic methanogenesis had established within these containers, a reaction that has a low hydrogen threshold and would thus maintain a lower hydrogen gas partial pressure than the threshold for acetogens (Cord-Ruwisch et al., 1988).

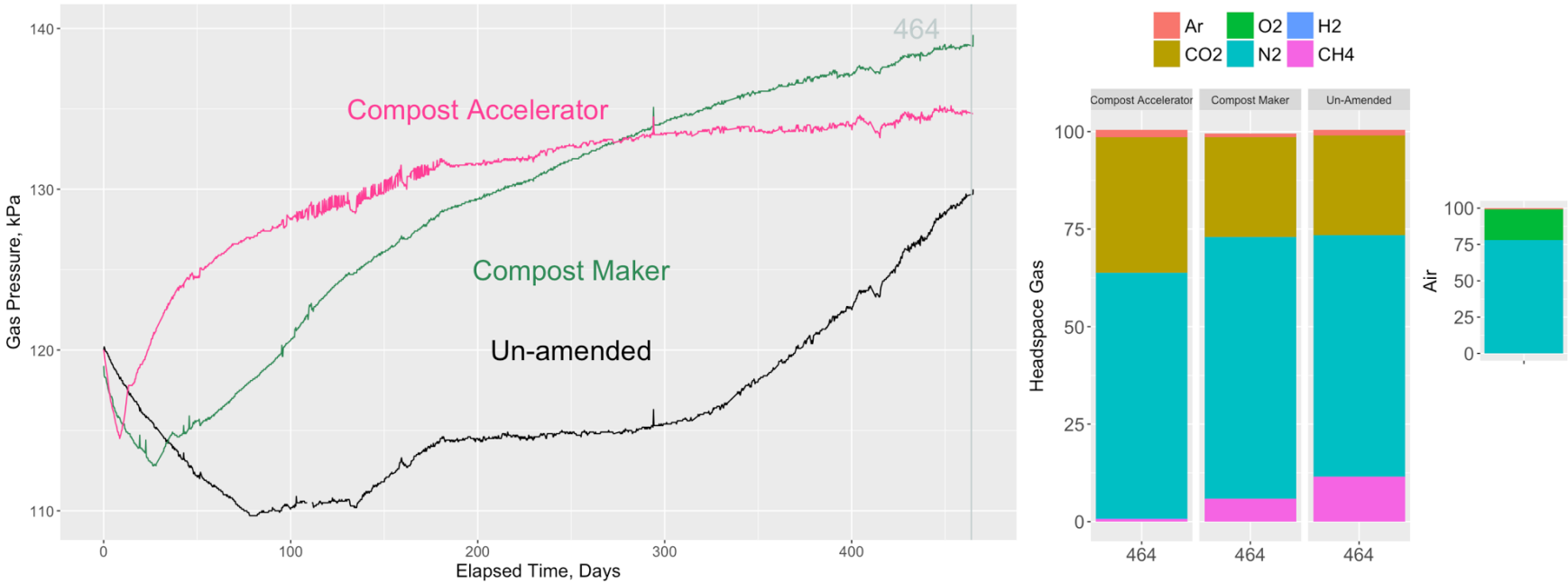

Figure 3-1 Evolution of gas pressure and headspace gas compositions for surrogate waste. Numbers in grey correspond to the vertical grey lines marking when the headspace gases were sampled for analysis at 150, 464, 730 and 1965 days elapsed time. Also shown are the composition of the major gases in the headspaces and in dry air: nitrogen, oxygen and argon and the minor gases, carbon dioxide, hydrogen and methane.

3.2 Glycoside Hydrolase Activity in Surrogate Waste Leachate

The complete biodegradation of solid wastes involves multiple enzymatic activities. Degradation of cellulose-based wastes involves several carbohydrate active enzymes, such as the glycoside hydrolases (Berlemont and Martiny, 2016), that comprise several families (Henrissat and Bairoch, 1996). Within a microbial community, most of the members are secondary fermenters that carry only the genes for degradation of the more easily hydrolyzed carbohydrates (Berlemont and Martiny, 2013). These include reactions catalyzed by the glycoside hydrolase families 1 and 3 (Davies and Henrissat, 1995) that degrade the carbohydrate β-D-cellobiose. Only a small fraction of the community members would be primary fermenters that carry the genes for both cellulose and β-D-cellobiose degradation (Berlemont and Martiny, 2013). Cellulase activity extracted from the surrogate wastes was determined by the fluorescently labelled disaccharide substrate, resorufin-β-D-cellobiose (Coleman et al., 2007). Upon hydrolysis of this compound, the fluorescence of the liberated resorufin is proportional to the concentration of glucose molecules that are also released. The results of this assay are shown in Table 3-1 as the cellulase activity (µmole/mL/min) and as cellulase activity relative to the maximum enzyme activity determined for a characterized cellulase from *T. reesei* of 6.7×10^{-4} µmole/mL/min (Coleman et al., 2007). The extracted activity leached from the surrogate waste was highest in the sample with the added cellulase (Table 3-1), from which was measured an activity three times higher than the maximum activity determined for *T. reesei*. Dilution of this leachate by 20-fold relieved some inhibition of the cellulase activity that, after dilution, displayed six to seven times higher activity than the maximum activity for *T. reesei*. The enzyme activity was still four times greater than that from *T. reesei* even after dilution by 100-fold (Table 3-1). The sample with the neutral starting condition and the sample amended with the Compost Maker (that included enzymes within its composition (Table 2-1)) each displayed between 3 and 29% of the maximum cellulase activity of *T. reesei*. The two test containers, whose rates of gas pressure changes had leveled off (the sample with an acidic starting condition and the sample with the added Compost Accelerator (Figure 3-1)), each had no measured cellulase activity (Table 3-1). The gas pressure changes within the test container holding the neutral sample had also leveled off (Figure 3-1), but this sample displayed measureable cellulase activity (Table 3-1).

Table 3-1
Cellulase activity based on the glycoside hydrolase family 1 and 3 activities detected in the sample leachates. Cellulase activity is based on the hydrolysis of resorufin-β-D-Cellobiose (Coleman et al., 2007).

Sample	Portion of the Sampled Extracted	Emission 595 nm	Concentration of Resorufin	Cellulase Enzyme Activity	Fraction of Enzyme Activity*
		RLU	nM	µmole/mL/min	%
Cellulase	Top	1492.8	1288.4	2.15×10^{-3}	319
Cellulase, 20x dilution	Top	297.0	2454.6	4.09×10^{-3}	608
	Middle	384.1	3220.1	5.37×10^{-3}	797
	Bottom	339.4	2827.5	4.71×10^{-3}	700
Cellulase, 100x dilution	Top	58.6	1800.9	3.00×10^{-3}	446
	Middle	60.4	1878.3	3.13×10^{-3}	465
	Bottom	59.2	1823.9	3.04×10^{-3}	452
Compost Accelerator	Top	20.7	Not detected	--	--
	Middle	20.7	Not detected	--	--
	Bottom	20.5	Not detected	--	--
Compost Maker	Top	35.0	15.2	2.54×10^{-6}	0.4
	Middle	50.4	28.8	4.79×10^{-5}	7.1
	Bottom	39.1	14.0	2.34×10^{-5}	3.5
Acidic Un-amended, pH5	Top	21.4	Not detected	--	--
	Middle	21.7	Not detected	--	--
	Bottom	21.7	Not detected	--	--
Neutral Un-amended, pH7	Top	60.9	33.1	5.52×10^{-5}	8
	Middle	105.4	77.1	1.29×10^{-4}	19
	Bottom	152.0	118.1	1.97×10^{-4}	29
Un-amended	Top	25.0	1.7	2.87×10^{-6}	0.4
	Middle	24.6	1.4	2.28×10^{-6}	0.3
	Bottom	23.8	0.7	1.10×10^{-6}	0.2

* Values for the fraction of activity are relative to the maximum rate (Vmax) for cellulase activity from T. ressei of 6.7×10^{-4} µmole/mL/min (Coleman et al., 2007).

3.3 Quantification of Marker Genes

Anaerobic microbial communities that utilize cellulose as the primary carbon source include sulfate-reducing, methanogenic and, possibly, acetogenic microbes. These communities are challenging to characterize due to the phylogenetic and functional diversity required to mineralize solid wastes. The fungal and bacterial compositions comprise the primary and secondary fermenters that produce the carbon dioxide, hydrogen and volatile organic acids from the solid waste, and the bacterial and archaeal compositions utilize the resulting fermentation products to produce hydrogen sulfide (in the case of sulfate reducers), methane (in the case of methanogens) and acetic acid (in the case of acetogens). To quantify the phylogenetic diversity present within each of the test containers, primer pairs directed to bacteria (glutamine synthetase, glnA (one gene copy)), archaea (the Archaeal 16S rRNA gene, A16s) and fungi (the Eukaryal 18S rRNA gene, 18s) were employed. To quantify any functional genes involved in anaerobic biodegradation, primer pairs directed to genes associated with sulfate reduction (the dissimilatory sulfite reductase gene, *dsr*AB, primer pair dsr1), methane formation (the methyl co-enzyme M gene, *mcr*A; acetyl co-enzyme synthetase, *acas*), acetogenesis (acs) and cellulose degradation (a suite of degenerate primer pairs directed to genes representing the bacterial and fungal glycoside hydrolases (endo- and exo- glucanases and β-glucosidases) were used. The details of each of these primers are provided in Table A-2.

Phylogenetic and functional diversity was determined for both the solid surrogate waste, as small pieces taken from the top of each of the samples within the test containers, and for the leachate, taken from whole waste separated into the top, middle and bottom sections in the orientation of the material in the test containers. The results for diversity of bacteria, Archaea and fungi, are shown in Figure 3-2 and the diversity associated functional genes are shown Figure 3-3.

The Archaea were numerically dominant on the solid material across the samples except the sample amended with added cellulase (A16s, Figure 3-2). The Archaea associated with methanogenesis, by the *mcr*A gene, were a minor component of all the solid samples (*mcr*A, Figure 3-3). The gene copies representing the fungal diversity, the 18s gene (Figure 3-2), were higher in number or similar in number to the gene copies representing the bacterial diversity, the glnA gene (Figure 3-2). Along with a more abundant fungal community on the solid waste relative to the bacteria, only the fungal gene for cellulose degradation was detected in the samples. These were represented by the primer pairs gh61.2/gh61.3 and gh61.5/gh61.4 (Figure 3-3) and are from the glycoside hydrolase family 61. The number of copies of these genes were approximately equal across each of the samples. Genes for the bacterial cellulolytic glycoside hydrolases were not detected, but the copies of the functional gene associated with sulfate reduction made up a large proportion of the bacterial abundance. The dsr1 gene copies were higher in the samples (un-amended, un-amended (neutral) and un-amended (acidic)) than in the amended samples (Figure 3-3). The microbiology of the solid waste suggests that the biodegradation is mediated by fungal celluloytic cellulases. The bacterial cellulases, such as the

cellulases from glycoside hydrolase families 1 and 3, would contribute to fermentation reactions that result in the hydrogen and carbon dioxide gas production.

Bacteria or fungi were numerically dominant in the leachate across the samples and the one that dominated depended upon initial conditions: bacteria were dominant in leachate from treated waste (Compost Maker, Compost Accelerator) and in the container with an acidic starting condition (Figure 3-2, blue); fungi were dominant in leachate from un-amended, and for the neutral and cellulase treated starting condition (Figure 3-2, green). The genes for fungal cellulases were detected within the leachate, but the dsrAB gene was most prevalent as were the genes for acetyl co-enzyme synthetase (acas, Figure 3-2, pink). The microbiology of the leachate suggests that the waste biodegradation is aided by the fermentation by sulfate reducing prokaryotes and by the glycoside hydrolase families 1 and 3 (Table 3-1).

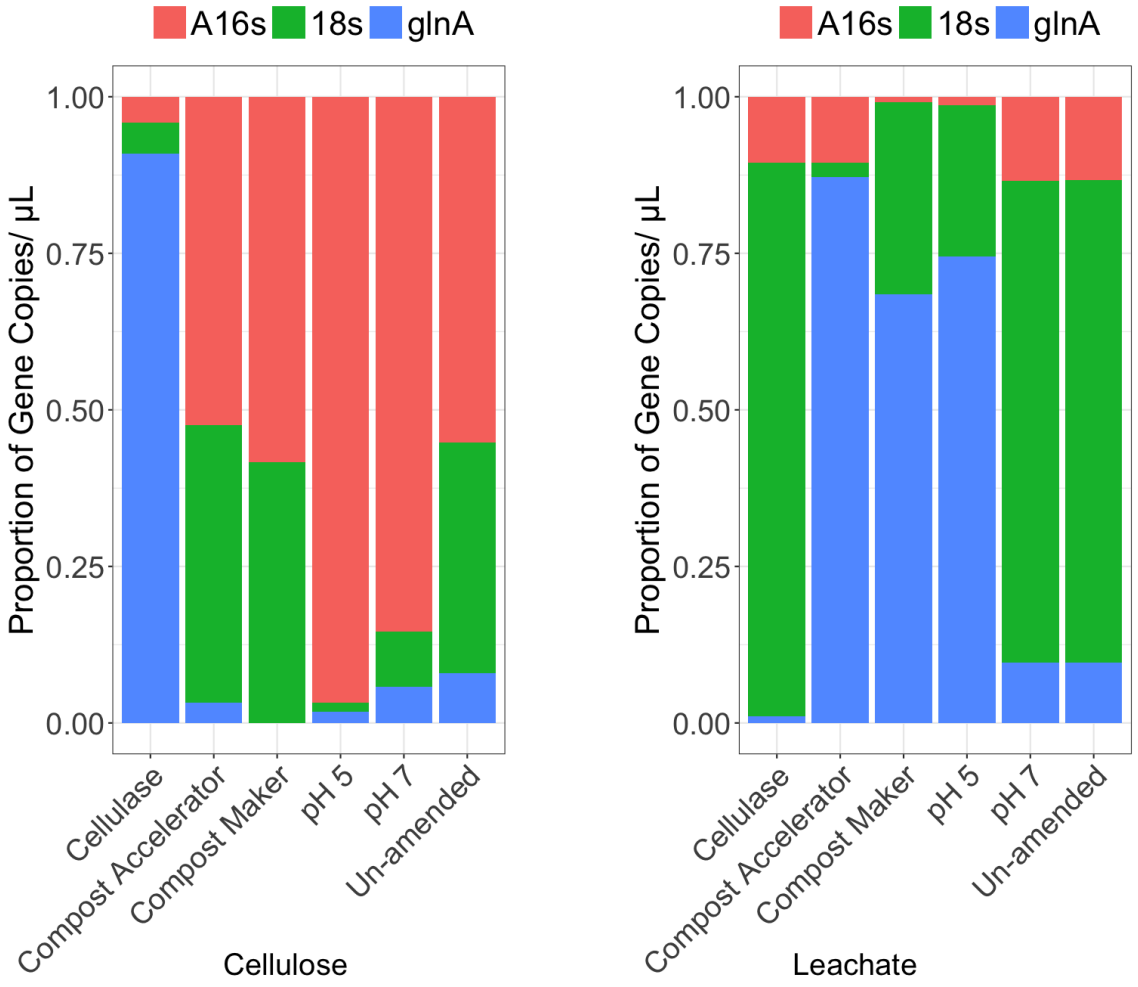

Figure 3-2 Community differences for cellulose and leachate by differential relative abundances of gene copies detected within the surrogate waste for: Archaeal rRNA (A16S), fungal rRNA (18s), bacterial glutamine synthetase (glnA) genes.

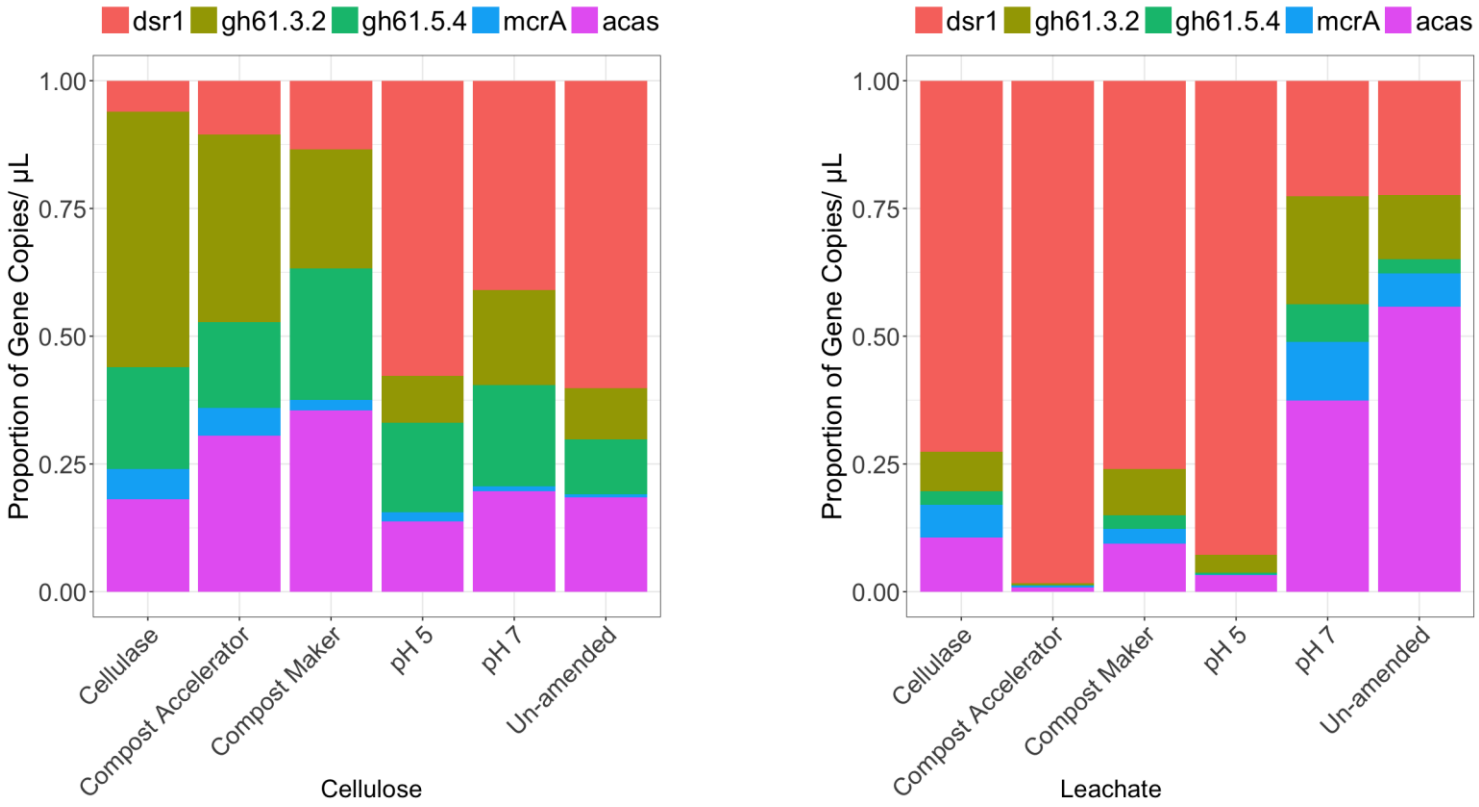

Figure 3-3 Community differences for cellulose and leachate by differential relative abundances of gene copies detected within the surrogate waste for: dissimilatory sulfate reductase (dsr1), glycoside hydrolase family 61 (gh61.3.2, gh61.5.4), methyl co-enzyme M (mcrA) and acyl co-enzyme A synthase (acas) genes.

Included in the analysis but not detected: Ubiquitin carrier protein, RNA polymerase Beta, cellulase genes except for the glycoside hydrolase family 61 (gh61.3.2 and gh61.5.4).

4. DISCUSSION

In this study, we tested whether methane becomes the exclusive terminal gas product of cellulose biodegradation by evaluating gas pressure onset, gas pressure evolution and headspace gas compositions from within sealed test containers housing surrogate waste. We also tested the microbiology of the waste indirectly by measuring cellulase activity and directly by quantifying specific marker gene targets.

The complex dynamics occurring within a DGR means the cavern may be subject to periods of water infiltration (that promotes microbial gas generation and acid formation), and periods of water efflux by the resulting gas pressure build-up. This build-up, within a repository, is predicted to delay the re-saturation of the cavity chambers (Shaw, 2013). The formation of gases in a DGR is desirable because it would allow for a longer decay time of the radioactive inventory and so would also delay the possible aqueous transport of soluble radionuclides through the host rock and shaft seals. The formation of methane as the dominant terminal gas is also desirable as it would reduce mineral dissolution of the host rock and shaft seal and better impede migration of gaseous and aqueous radionuclides.

Methane formation by the activity of hydrogenotrophic methanogens by reaction 3, Table 1-1 has a threshold for hydrogen of ~0.01% (Cord-Ruwisch, 1988). The hydrogen produced within a DGR that goes above this partial volume would support methane formation by this pathway. A second possible reaction pathway for methane formation is by the activity of aceticlastic methanogens by reaction 4, Table 1-1 (formed from the acetate produced by either fermentation of the waste or from the acetate produced by hydrogen consuming acetogens). This pathway produces carbon dioxide, and thus contributes to the potential impact of carbon dioxide transport and carbonic acid formation. Even so, this reaction can limit the effects of lower pH in a DGR associated with acetate production and in doing so, support pH sensitive reactions like hydrogenotrophic methanogenesis (Batstone et al., 2002).

Ultimately, the rates of biodegradation and resulting gas build-up will depend upon the composition and abundances of the endogenous bacteria and Archaea within a DGR cavity and on the bacteria (Archaea and fungi that enter the cavern with the waste itself).

4.1 Microbiology of the Surrogate Wastes

Within natural and engineered anaerobic environments, co-existing microbial processes involve cellulose degradation, fermentation, sulfate reduction, methanogenesis and acetogenesis (Pereyra et al., 2010; Lever, 2012). The breakdown of complex carbohydrate structures like cellulose release short carbohydrate compounds to the environment. This is achieved by the activity of cellulolytic glycoside hydrolase enzymes (Henrissat and Bairoch, 1996). In our tests, among the primer set designed to encompass bacterial and fungal celluloytic glycoside hydrolase marker genes (Table A-2), the only cellulolytic gene associated with the solid waste was from the fungal glycoside hydrolase family 61 (now called Auxiliary Activity Family 9). Copies of these genes were associated with both the solid cellulose (Figure 3-3, cellulose,

gh61.3.2 and gh61.4.5) and the leachate (Figure 3-3, leachate, gh61.3.2 and gh61.4.5). These genes were present within all the containers. Their relative abundances were higher within the amended solid samples (cellulase from *Trichoderma viride*, the commercial blends of microbes and enzymes (compost accelerator), and of microbes and nutrients (compost maker)). The proportion of glycoside hydrolase family 61 marker genes (Figure 3-3, gh61.3.2 and gh61.4.5) were also more abundant than the marker gene for fungi (Figure 3-2, 18s). These cellulases, therefore, may be a mixture of cellulases from the additive compositions and cellulases that were endogenous to the surrogate waste itself.

The next most abundant genes were those genes from the acetyl co-enzyme synthetase, for aceticlastic methanogens and from the dsrAB gene for sulfate reducing bacteria and Archaea (Figure 3-3, acas and dsr1). The containers with these additives differ in microbial composition (Figure 3-2, A16s, 18s and glnA). The amendment without added microbes (the cellulase treated sample) had fewer Archaea (Figure 3-2, A16s, red) and fungi (Figure 3-2, 18s, green), and had more bacteria (Figure 3-2, glnA, blue). Amendments with compost accelerator and compost maker were most abundant in Archaea (red) and fungi (green) with relatively few bacteria (blue).

The community compositions within the leachate were different from the community compositions from the solid waste (Figure 3-2 and Figure 3-3). Leachate from cellulase amended waste was dominated by fungi (Figure 3-2, 18s, green) rather than bacteria. Leachate from the compost accelerator and compost maker amendments were higher in bacteria (Figure 3-2, glnA, blue) rather than the corresponding proportions of Archaea and fungi on the solid wastes. All leachates had relatively fewer Archaea.

Separately, we also detected glycoside hydrolase activity, from families 1 and 3, at various levels in the leachate (Table 3-1). Activity of these enzymes are responsible for the further breakdown of the small carbohydrate compounds (Batstone et al., 2002) released from the waste. The enzyme activity across the samples ranged from undetected to levels over four times the maximum velocity of a known fungal enzyme (Coleman et al. 2007). The waste amended with added cellulase had the highest glycoside hydrolase activity (Table 3-1); inhibition of activity was relieved by dilution. The amendment of microbes and enzymes (compost accelerator, Table 3-1) and the un-amended waste with a starting pH of 5 had no detectable glycoside hydrolase activity. The containers between these two extremes displayed glycoside hydrolase activities from <1 to 29% of the maximum rate determined for a characterized enzyme (Table 3-1)

Overall, the marker gene for sulfate reduction, the dsrAB gene (Figure 3-3, dsr1), made up a large proportion of the un-amended waste, in the leachate from the amended waste and the un-amended waste that had an acidic starting condition. The phyla represented by this trait also ferment carbon and may account for some of the gas production (Figure 3-1). The Archaeal 16S rRNA gene (Figure 3-2, A16s) was present in all samples and was the main phylogenetic marker gene detected, but the marker gene for methanogens, *mcr*A (Figure 3-3, *mcr*A) were the lowest within all the containers. The methane that was detected (within only

two of the containers) suggests methane could be the dominant gas, on average, as is assumed, but its production may actually only occur in patches across a DGR.

4.2 Gas Pressure and Headspace Gases

In our tests, surrogate cellulosic waste was subjected to various treatments establishing initial conditions for added microbes, enzymes and initial pH. The resulting gas evolution was monitored throughout. In one set of tests, lasting 464 days, there was evidence of methane formation at 0.6 to 11.5 % of the gas composition (Figure 3-1); the other main headspace gases were nitrogen and carbon dioxide. Hydrogen gas was a minor component of this gas composition, making up to 0.2% of the gas volume. This was above the level of the measured threshold for methanogenesis (Cord-Ruwisch et al., 1988). In a second set of tests lasting 1965 days, methane was a minor component of the gas composition, making up less than 0.01% of the headspace volume with hydrogen making up 0.1 to 10.7% of the gas volume (Figure 3-1). The other main headspace gases were nitrogen and carbon dioxide.

In a repository, corrosion of carbon steel containers would also be a significant source of hydrogen gas partial pressure. Since glass containers fitted with stainless steel components were used in this study, corrosion would not have been a significant source of hydrogen in these tests. The source of the hydrogen within the test containers would have been from fermentation of the soluble carbohydrates formed by the hydrolysis of the cellulosic surrogate waste material. The thermodynamics of the syntrophic relationship between the hydrogen-producing reactions and hydrogen-consuming reactions means interspecies hydrogen transfer occurs only within a narrow range of hydrogen concentrations (Batstone et al., 2002; Cord-Ruwisch et al., 1988). Hydrogen accumulation in the headspace in one set of test containers and not another set of test containers suggests that the rate of hydrolysis of the waste determined the rate of subsequent fermentation reactions and the rate of gas pressure rise (Noike et al., 1985), including the accumulation of hydrogen gas.

Hydrogen consumption also crosses phylogenetic classes. Competition for hydrogen gas in anaerobic environments by sulfate-reducing, methanogenic and acetogenic microbes means that the members of the proximal hydrogen consuming group that has a higher affinity for hydrogen will predominate over the members of the proximal group that has a lower hydrogen affinities (Cord-Ruwisch et al., 1988). It is possible to have spatially distinct hydrogen-producing reactions and hydrogen-consuming groups within a repository. The measured hydrogen threshold for acetogenesis, 0.09%, is higher than that for hydrogenotrophic methanogenesis, 0.01% (Cord-Ruwisch et al., 1988). The higher affinity for hydrogen by methanogens means that active methanogenesis can limit hydrogen gas accumulation in the headspace, and thus keep conditions unfavorable for acetogenic hydrogen consumption and slow the rate of gas pressure rise. The thermodynamics of hydrogen gas on these reactions also means that the hydrogen and carbon dioxide consuming pathway of acetogenesis, the Wood-Ljundahl pathway (Ragsdale and Pierce, 2008; Lever, 2012), can operate in a forward direction at high hydrogen concentration and in reverse at low hydrogen concentration (Hoehler et al., 1999). At elevated

hydrogen concentrations, acetogenesis from hydrogen and carbon dioxide could become thermodynamically possible (Hoehler et al., 1999). We posit that within the 464-day study, syntrophic hydrogen producing fermentation reactions supplied the hydrogen and the carbon dioxide to hydrogenotrophic methanogens, both groups being present within the test container. These groups were either endogenous to the waste itself (un-amended) or were supported by the addition of nutrients and enzymes present within the Compost Maker formulation (Figure 3-1). The slower rate of gas pressure rise and the higher proportion of headspace gas methane in the un-amended sample suggest that the products of the rate of gas pressure rise from the hydrolysis of crystalline cellulose can limit the later onset of methane formation.

4.3 Effect of Additives on Gas Pressure

The rate determining step in the biodegradation of solid cellulose is its hydrolysis (Noike et al., 1985). The enzymes present in the formulation of the Compost Accelerator (microbes and enzymes) stimulated a faster rate of gas pressure rise than the rate that was seen for the un-amended test-container (Figure 3-1) or for the waste amended with Compost Maker (microbes and nutrients). Waste amended with enzymes (Compost Accelerator, Figure 3-1) also displayed a slower onset of methane formation compared to the un-amended (un-amended, Figure 3-1) and amended with nutrients (Compost Maker, Figure 3-1).

The rate of gas formation is not associated with methane production. Within the 1965-day study, the addition of cellulase enzymes, rather than Compost Accelerator, produced a rapid increase in gas pressure rise that levelled off before a second phase of rapid increase in gas pressure rise (Figure 3-1). An acidic starting condition, that was amenable to optimal cellulase activity, also produced a rapid gas pressure increase (Figure 3-1). Neither starting condition resulted in methane formation (Figure 3-1), but both starting conditions eventually displayed a gas pressure deceleration indicating that the gases that were produced had begun to be consumed. None of the 1965-day test containers displayed methane formation as a terminal gas (Figure 3-1) and none of these containers had detectable hydrogen sulfide (Figure 3-1). However, the presence of hydrogen within the headspace of each of the test containers at day-150 and day-730 but not at day-1965 (Figure 3-1) could suggest that hydrogen was produced in sufficient quantity to favor lower affinity hydrogen consuming pathways, like the acetogenic pathway, and that other higher affinity hydrogen consuming pathways, like those of sulfate reduction and hydrogenotrophic methanogenosis, were not active enough to maintain a low hydrogen partial pressure at these time points. For these tests, we posit that the rate of gas formation, initiated by either the added cellulase or by a pH permissive for cellulase endogenous to the surrogate waste, produced sufficient hydrogen gas to promote acetogenic activity. The neutral test container within the 1965-day test also produced hydrogen but similarly failed to display methanogenesis. This further suggests that stochasticity with the microbiology of the waste has an influence on the gases produced.

4.4 Acetogenic Activity and Safety Assessment of DGRs

The suggestion that other hydrogen consuming activities can be prevalent over methanogenesis is surprising. Although methanogenesis can and does occur, methane formation is not the only process to consider. Like methanogenic Archaea, acetogenic Archaea and bacteria can consume carbon dioxide and hydrogen, in this case to form acetate (Schuchmann and Müller, 2014; Ragsdale and Pierce, 2008; Lever, 2012). Acetogens, however, are often thought to be outcompeted by microorganisms performing energetically more favorable metabolic pathways (Lever, 2012). When modelling the microbial ecology of a DGR (Avis et al., 2014), a redox tower of free energy change is used to model sequential processes; the model assumes that the most energetically favorable metabolic processes in the sequence will occur first, followed by the next most favorable process in the sequence. In this concept of metabolism, oxygen is consumed first followed by oxides of nitrogen, metals, sulfate and eventually carbon fixation by methanogens. In organic carbon limited oligotrophic environments, however, the net consumption of electron acceptors like nitrate and sulfate may not occur (D'Hondt et al., 2009). Furthermore, while sulfate reducers and methanogens gain more energy than acetogens from shared energy substrates, acetogens co-occur and engage in organic carbon cycling in the terrestrial subsurface (Pedersen et al., 2008; Kotelnikova and Pedersen, 1997; Griebler and Lueders, 2008), a process that is thought to be possible by a metabolic strategy of pooling the free energy gain through substrate flexibility and simplified maintenance costs (Lever, 2012). The presence of acetogens in significant quantities in a DGR would imply production of acidity, and the potential for dissolution of limestone or cement in shaft seals that represent the barrier delaying migration of soluble and gaseous radionuclides.

5. CONCLUSIONS

Long-term pressure monitoring of sealed containers and periodic analysis of the headspace gas composition showed that methane may not be the terminal gas, as is assumed in models for DGR gas production. The assumption of methane being the terminal dominant gas is linked to a reduced acidity within a DGR cavern.

Predicting gas generation and production of acidity within a DGR is complicated by the biodegradation having multiple steps and multiple constraints. The thermodynamic influence of hydrogen gas on both the methanogenic and acetogenic hydrogen consumption may provide improved prediction of the gases generated under certain conditions and on the expected formation of carbonic acid, which can then be used to improve the shaft seal designs.

Corrosion is an important gas generating process in any DGR hosting low-to-intermediate level waste because of the large number of steel containers. Given that corrosion produces hydrogen gas and the important role of hydrogen on gas consumption processes in anaerobic environments, analysis of the microbiology of actual low-level waste housed in steel containers of various ages and degrees of corrosion degradation would help gauge the long-term impact of all sources of hydrogen within a DGR.

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Appendix A

SUPPLEMENTARY INFORMATION

Table A-1
Quantification of DNA from replicate 1 cm piece of mop. The quantification was performed in duplicate for each piece.

ID	Sample Name	Replicate 1		Replicate 2	
		ng/µL DNA			
A	**Un-ammended, pH7**	0.39	0.39	0.27	0.26
B	**Un-ammended, pH5**	Below detection		Below detection	
C	**Cellulase, pH7**	0.06	0.05	0.12	0.13
D	**Un-ammended**	0.27	0.25	0.68	0.63
E	**Compost Accelerator**	Below detection		Below detection	
F	**Compost Maker**	0.08	0.08	0.11	0.10

Table A-2
Details of positive control, annealing temperature for ddPCR and the dilution of the DNA. For details on the specific primers and their targets, please see Table 2-1.

TARGET	PRIMER SET	POSITIVE CONTROL	T_A (^oC)	2-step or 3-step	DNA DILUTION
Bacteria	glnA F/R	glnA plasmid dil#5 or 6	59	2	1:10
Archaea	A16s 340/806	A16s plasmid dil#4	58	2	1:3
Fungi	18s euk345F/euk 499R	Arbuscular mycorrhizal Glomus irregularis, 0.1ng/µl or 0.01ng/µl	59	2	1:10
Methanogens	Mlas/mcrA	ME plasmid dil#6	60	2	1:3
Sulfate Reducers	dsr1-F-RT/dsr-R-RT	SRB plasmid dil#6	60	2	1:3
Acetyl co-A synthestase	acas For/acas Rev	Heliobacterium gDNA 22ng/µl	53	3	
Cellulase	gh61.3/61.2	none	54	3	1:3
	gh61.5/61.4	none	54	3	1:3
	bglu	none	54	3	1:3

Table A-3
Samples and the dilution series used to determine the optimal conditions for ddPCR. For details on the specific primers and their targets, please see Table 2-1

PRIMER SET	SAMPLES (high, low)	DILUTION SERIES
glnA	F and A	1:25, 1:100
A16s 340/806	A and F	1:5, 1:10
Mlas/mcrA	F and A	1:5, 1:10
dsr1-F-RT/dsr-R-RT	D and B	1:5, 1:10

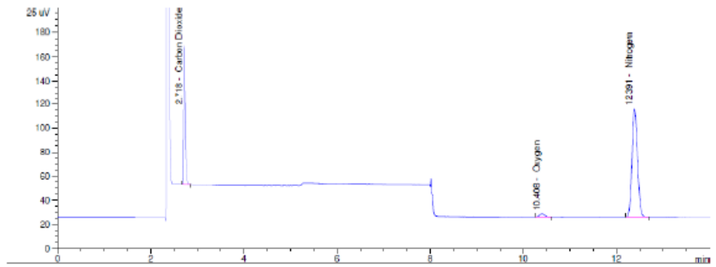
Figure A-1 An example (left image) of the sample container layout and data logger that was used for continuous collection of the internal temperature and pressure within each bottle, and a view of three of the samples before they were sealed.

Bottle "A" – Mop, Phosphate Buffer (pH 7), Air			
Analyte	**Peak Area (uV·min)**	**Partial Pressure (torr)**	**Mol. %**
Carbon Dioxide	262.201	179	25.3 %
Hydrogen	144.338	5.55	0.78 %
Oxygen / Argon	19.631	12.9	1.82 %
Nitrogen	738.74	510	72.1 %
Methane	< 2.520	< 0.868	< 0.12 %
Hydrogen Sulfide	< 3.477	< 0.672	< 0.01%

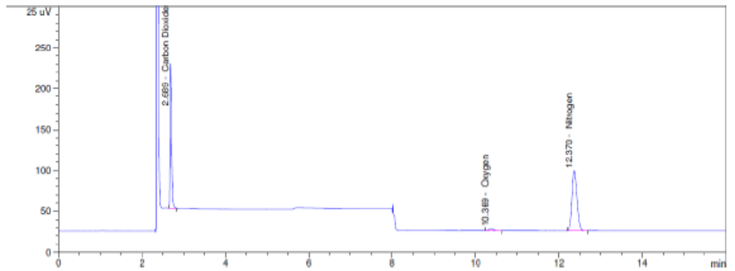
Bottle "B" – Mop, Phosphate/Citrate Buffer (pH 5), Air			
Analyte	**Peak Area (uV·min)**	**Partial Pressure (torr)**	**Mol. %**
Carbon Dioxide	436.678	300	41.2 %
Hydrogen	87.666	3.33	0.46 %
Oxygen / Argon	14.570	9.52	1.31 %
Nitrogen	602.394	415	57.02 %
Methane	< 2.520	< 0.868	< 0.12 %
Hydrogen Sulfide	< 3.477	< 0.672	< 0.01%

Bottle "C" – Mop, Phosphate Buffer (pH 7), Cellulase, Air			
Analyte	**Peak Area (uV·min)**	**Partial Pressure (torr)**	**Mol. %**
Carbon Dioxide	849.651	587	68.6 %
Hydrogen	30.395	1.10	0.13 %
Oxygen / Argon	13.111	8.55	0.99 %
Nitrogen	376.063	259	30.3 %
Methane	< 2.520	< 0.868	< 0.12 %
Hydrogen Sulfide	< 3.477	< 0.672	< 0.01%

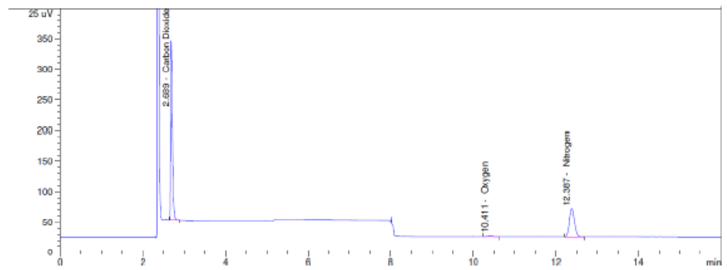
Helium Carrier Chromatogram:



Helium Carrier Chromatogram:



Helium Carrier Chromatogram:



Argon Carrier Chromatogram:

Argon Carrier Chromatogram:

Argon Carrier Chromatogram:

Figure A-2 Gas chromatography headspace gas results at day 1965 for un-amended samples, pH 7 (left) and pH 5 (middle), and for the sample amended with cellulase enzyme from Trichoderma viride (right).

A.1 Effects of Corrosion and Acidity in a DGR

Microbial communities may mediate mineral dissolution (Moyce et al., 2014). Carbonate minerals in limestone are prone to acid dissolution and are inherently sensitive to the partial pressure of CO_2, as well as total pressure. A general rule of thumb is that, as CO_2 partial pressure and total pressure increase, so does carbonate mineral solubility (Langmuir, 1997). This means that microbial production of CO_2 and pressure build-up within a DGR may promote carbonate mineral dissolution in the host rock.

As indicated in our experiment, additional sources of acid from microbial processes include organic acids such as acetic acid from fermentation of cellulosic material. Formation of acetic acid will be confined initially to the inside of waste containers.

Another impact of gas pressure build-up induced by microbial communities is the creation of new rock fractures in a DGR cavity (Harrison et al., 2011; Wragg et al., 2012). Such newly formed rock fractures could create preferential transport pathways for gases when the DGR has not resaturated and for soluble species when a DGR has resaturated. However, it is very unlikely that newly created rock fractures would extensively develop over several meters of rock above the isolated low and intermediate level waste.

On the other hand, generation of acidity from CO_2 and acetic acid production could affect the integrity of shaft seal material if they are made of cement. Perfettini et al. (1991) and Fomina et al. (2007) demonstrated that microorganisms can produce acids and degrade cement. The mineralogy of cements is complex, but portlandite ($Ca(OH)_2$) is one of the main crystalline phases. The reactivity of portlandite towards CO_2, which is generated from the degradation of cellulosic material, is well documented. It can be entirely recrystallized into calcite in a short period of time following the general reaction (Beruto and Botter, 2000):

$$Ca(OH)_2 + CO_2 \rightarrow CaCO_3 + H_2O \quad (1)$$

As noted by Beruto and Botter (2000), the progress of portlandite carbonation by CO_2 is of concern with respect to the deterioration of cements, even under conditions of low relative humidity (i.e., 70%). Furthermore, the release of water from the carbonation reaction can lead to condensation with relative humidity rising to the dewpoint (Regnault et al., 2009).

In fact, studies of limestone dissolution at total pressures up to 10 MPa with pCO_2 values close to the total pressure show that mineral dissolution results in the formation of non-uniform highly conductive channels within the rock (Luquot and Gouze, 2009). This is of particular concern with respect to the interface between concrete shaft seals and host rock where focused mineral dissolution processes may substantially increase porosity and permeability. In view of these considerations, microbial production of CO_2 presents an element of risk for concrete degradation.

A.1.1 Uncertainties: dilute groundwater vs brines

Oren (2011) discusses that life at high salt concentrations (i.e., low a_w levels) is energetically expensive and problematic for many microbes. At the same time, there is a growing body of evidence demonstrating that diverse populations of highly specialized or specific bacteria not only survive in hyperarid environments within halite crusts, but also manage to grow and divide (Davila et al., 2008; Wierzchos et al., 2012). This means that access to water is a critical and limiting factor, but it is not possible to discount entirely the possibility of some microbial activity at the low water activity level anticipated for any repository, unless the water activity drops below 0.6 (Davila et al., 2008).

A.1.2 Uncertainties: glass containers meaning absence of corrosion

In our experiment, we used glass containers instead of the carbon steel used to store low and intermediate level waste. Microbiologically influenced corrosion is an important gas generation process in any DGR hosting low and intermediate level waste because of the large number of steel containers. Corrosion of steel also produces hydrogen gas that is important in methanogenesis.

The involvement of microorganisms in corrosion processes relates to a number of factors. First, under both aerobic and anaerobic conditions, microbial acid production increases the availability of protons in the form of carbonic acid from CO_2 (eq. 2) and organic acids, such as acetic acid (eq. 3). Second, under aerobic and anaerobic condition using either oxygen (eq. 4) or nitrate (eq. 5), respectively as electron acceptors, microbial oxidation of Fe^{2+} causes subsequent precipitation of hydrous ferric-oxide corrosion products. Third, under anaerobic conditions, microbial oxidation of H_2 coupled to processes such as acetogenesis (eq. 6), with the reduction of SO_4^{2-} produces S^{2-} with concomitant precipitation of FeS corrosion products (eq. 7):

$$2Fe^0 + O_2 + 4H_2CO_3 \rightarrow 2Fe^{2+} + 4HCO_3^- + 2H_2O \quad (2)$$

$$2Fe^0 + O_2 + 4CH_3CO_2H \rightarrow 2Fe^{2+} + 4CH_3CO_2^- + 2H_2O \quad (3)$$

$$4Fe^0 + 3O_2 + 6H_2O \rightarrow 4Fe(OH)_3\ (s) \quad (4)$$

$$10Fe^0 + 6NO_3^- + 6H^+ + 12H_2O \rightarrow 10Fe(OH)_3\ (s) + 3N_2 \quad (5)$$

$$4Fe^0 + 2CO_2 + 8H^+ \rightarrow 4Fe^{2+} \rightarrow + CH_3CO_2H + 2H_2O \quad (6)$$

$$4Fe^0 + SO_4^{2-} + 8H^+ \rightarrow FeS\ (s) + 3Fe^{2+} + 4H_2O \quad (7)$$

Two corrosion scenarios exist initially in a DGR. These are (i) external corrosion on exposed metal surfaces, and (ii) internal corrosion within waste containers. The time course evolution of these two different corrosion regimes will eventually converge, at some point in time after closure, as waste containers ultimately fail and release their contents into the DGR.

Figure A-3 pH of leachate after the containers were opened. Amendments: Compost Maker (microbes and nutrient), Compost Accelerator (microbes and enzymes), Cellulase (enzyme).