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Memo

date: *January 6, 2000*

to: *Y. Wang, Sandia National Laboratories*

from: *A.J. Francis and Jeff Gillow*

subject: *Progress Report: Microbial Gas Generation Program*

The following summarizes work performed during the period February to September, 1999, under SNL Test Plan TP-99-01, Rev. 0 (2/4/99) for the project titled "Re-evaluation of Microbial Gas Generation Under Expected Waste Isolation Pilot Plant Conditions."

OBJECTIVE

I. Re-evaluate existing microbial gas generation data and develop appropriate technical approaches to reducing the conservatism in the current gas generation model.

II. Perform scoping experiments to test the effect of crystallinity on cellulose degradation under hypersaline conditions: a diminishing microbial gas generation rate with time may be related to the degree of crystallinity of cellulose.

III. Re-examine and improve the experiment for cellulose degradation under humid conditions to derive a more realistic rate for humid microbial degradation.

IV. Determine the rate and extent of methanogenesis by halophilic microorganisms and the effect of MgO on methanogenesis.

PROGRESS SUMMARY

I. **Literature Review**

A literature review was performed in order to assess the current state of knowledge of processes and phenomena that may affect the gas generation rates in the long-term gas generation studies at BNL. These include: i) the occurrence of methanogens and sulfate reducers in hypersaline natural environments, ii) the effect of nitrate on gas generation (specifically methanogenesis), and iii) the role of hydrogen, organic acids, and methylated compounds as pre-cursors for methanogenesis. In addition, literature related

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to cellulose characterization techniques was reviewed in order to examine the effect of crystallinity on biodegradation. Pertinent recent references are included in Appendix A of this report.

II. Gas Analysis

Gas production data up to 1228 days incubation for cellulose biodegradation under inundated conditions has been presented in the report titled "Microbial Gas Generation Under Expected Waste Isolation Pilot Plant Repository Conditions" (SAND96-2582). We analyzed the total volume of gas produced (ml g^{-1} cellulose) carbon dioxide (µmoles g^{-1} cellulose) and methane (nmoles g^{-1} cellulose) in inundated samples after 2718 days of incubation (~7.5 years) at 30 ± 2°C. Gas volume was analyzed using a digital pressure gauge, CO_2 by gas chromatography (GC) with thermal conductivity detection and CH_4 by GC-flame ionization detection. Appendix B provides the data. All samples have been corrected for the gas produced in control treatments (without cellulose). Negative values for total gas are due to continual gas loss due to sampling. Methane was analyzed in all samples and the results are summarized in Appendix B. Samples were photographed at 2718 days (Appendix C).

Gas has continued to be produced in some samples over the 1490 days (4 years) since these samples were last analyzed (Appendix B). In anaerobic unamended inoculated samples g^{-1} cellulose was present at 2718 days, 2.45 ml of total gas and 24.0 µmol CO_2 g^{-1} cellulose were detected. This is 0.22 ml and 10.1 µmol more total gas and CO_2, respectively, since the 1228 day analysis (Table 1). Most significant is the carbon dioxid produced in the anaerobic amended inoculated + excess nitrate sample (57 µmoles g^{-1} cellulose produced over 4 years, from 1228 to 2718 days, Table 1). In initially aerobic samples (these samples were sealed with air in the headspace at t=0), carbon dioxide production is most significant in the amended inoculated + excess nitrate sample containing bentonite (79 µmoles g^{-1} cellulose produced over 4 years, Table 2).

Methane was produced in small quantities in most anaerobic samples except those with excess nitrate (Appendix B, Table 3). Nitrous oxide was detected in the headspace of samples containing excess nitrate (data not shown). The lack of methane production in samples that contain nitrogen-compounds is consistent with the recent information in the literature related to the inhibitory effect of nitrate on methanogenic bacterial activity. This effect has been found to be bacterial species-specific and it is unknown at this time whether the inhibitory effect observed in these samples is reversible or irreversible; additional data is required to determine this. Most of the methane detected was in samples that were not amended with any nitrogen-containing compounds at all (NH_4NO_3, KNO_3) specifically the unamended and unamend/inoculated samples. This is an additional line of evidence for the biogenic origin of methane in these samples. Further analyses will include an attempt at subculturing the methanogens in these samples and/or molecular biological detection of methanogens.

Of the intially aerobic samples, only two treatments contained methane (unamended and unamended/inoculated (without bentonite), Appendix B, Table 3). In all of the other initially aerobic treatments, the combination of oxygen, nitrogen-compounds, and other

alternate electron acceptors (Fe^{3+} provided by bentonite) may have an effect on the survival and growth of methanogens.

Although the quantities of methane are small, this information is significant because of the paucity of data in the literature on laboratory observations of methanogenesis under hypersaline conditions (Oren, 1999). **This data may in fact be the first to show methanogenesis under hypersaline (>20% w/v NaCl) conditions in the laboratory.**

III. SNL Technical Information Exchange on Microbial Gas Generation.

On July 28, 1999, a meeting was organized by SNL (Hans Papenguth, Yifeng Wang and Kathy Knowles) to discuss the relevant information gaps with respect to gas generation and performance assessment. The objectives of the meeting were as follows:

1. Provide a forum to update WIPP near-field participants on microbial gas generation and related activities including gas generation research, evolution and status and modeling activities.
2. Improve focus of related research programs.
3. Improve defensibility of related research programs.
4. Promote cross disciplinary approaches.

A major thrust of the meeting was to compile a list of assumptions or approaches that resulted in conservatism and to develop a list of activities to pursue within the existing program to improve defensibility and incorporate more realism. The meeting provided a forum to clearly state where the microbial gas generation experiments and data interpretation have been conservative. These conservatisms, means of resolution, and outcomes that reduce conservatism are summarized as follows:

1. *The "maximum rate" provided to PA for microbial gas generation was conservative given that we don't know if gas generation was due to biodegradation of what may be a relatively minor component of the mixed cellulosics (easily leachable dissolved organic carbon (DOC) or amorphous cellulose). If this is the case, then the "maximum rate" will certainly not be maintained over the period of performance.*

   Resolution:
   a) Develop an understanding of the structural character of the mixed cellulosics, both at time = 0 and 7.5 years, to attribute gas generation to what may possibly be a less important fraction of the cellulose.
   b) In a parallel study, determine the biodegradability of amorphous cellulose and crystalline cellulose by using radiolabeled compounds.
   c) Investigate whether nutrient limitation, and not cellulose crystallinity, was the cause for a lower gas production rate after approximately 500 days incubation.

Conservatism can be reduced if:

i. The result of (a) is that <5% of the mixed cellulosics is composed of amorphous cellulose, and amorphous cellulose is missing from 7.5 year incubations that have shown gas production.
ii. The result of (b) is that amorphous cellulose is degraded faster than crystalline, perhaps more efficiently converted to carbon dioxide.
iii. Nutrient limitation did not cause the lowered gas production rate.

2. *The rate of microbial gas generation under humid conditions is 0 – 0.04 mole C/kg/year. These rates were obtained from studies using a humidity (70%) that is higher than that currently expected in the presence of MgO. In addition, the design of the experiment was not optimum, that is, 1-2 ml of liquid was added to wet the cellulose as a means of introducing inoculum and nutrients to the cellulose.*

Resolution:

a) Assemble an experiment that does not require the presence of standing or adsorbed liquid to introduce inoculum and nutrients. This may be accomplished by adding inoculum and nutrients, air drying the samples, and then incubating in a closed system. MgO can be added to the system, and a radiolabelled substrate, with a removable (via syringe) NaOH trap can be included to trap radioactive carbon dioxide. With this system, much lower water activities can be studied, with a very low detection limit for gas production using liquid scintillation counting.

Conservatism can be reduced if we show a lower rate of gas production or even no gas production under conditions of very low water activity.

3. *Over a 3 year period, methane was never detected in microbial gas generation studies. Recently, we have found evidence of small amounts of methane produced in specific samples. Methane is included in the gas generation model for PA, however, if methanogenesis occurs only very slowly under non-MgO constrained conditions, perhaps it will not occur under more aggressive (less favorable for biological systems due to high pH) MgO constrained conditions. Thus methane production rates in the CCA may be very conservative. In addition, nothing is known about the reaction* $4H_2 + CO_2 \Rightarrow CH_4 + 2H_2O$ *under these conditions. This reaction consumes* CO_2 *but may also provide* H_2O *that might be available for more gas production.*

Resolution:

a) Examine methane production under MgO constrained conditions (hypersaline, pH 9, $[Mg^{2+}]$ = ~2 M (?)). Use enrichment cultures from cellulose samples, cultures from hyperalkaline hypersaline lakes in New Mexico, or pure cultures, with cellulose, acetate, or CO_2 and H_2 as the substrate for methanogenesis.

Conservatism can be reduced if we show that methane is not produced under MgO constrained chemical conditions, or the rate of production is extremely low. Also, if

methane is produced from carbon dioxide and hydrogen, it may be useful to model this reaction to take credit for carbon dioxide removal.

Appendix A

References

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Appendix B

Table 1. Total gas and carbon dioxide gas analysis of the anaerobic inundated cellulose samples

Sample	Total Gas ($ml\ g^{-1}$ cellulose)		Carbon Dioxide ($\mu mol\ g^{-1}$ cellulose)	
	1228 Days	2718 Days	1228 Days	2718 Days
Anaerobic				
Unamended/ Uninoculated	-0.24 ± 0.05	-0.30 ± 0.08	3.13 ± 0.02	8.29 ± 3.77
Unamended/ Inoculated	2.23 ± 12	2.45 ± 0.27	13.9 ± 1.0	24.0 ± 1.7
Amended/ Inoculated	3.78 ± 0.09	4.21 ± 0.04	49.2 ± 0.8	66.9 ± 1.1
Amended/Inoc. + Exc. Nitrate	12.12 ± 0.44	11.03 ± 0.43	194 ± 4	251 ± 5
Anaerobic + Bentonite				
Unamended/ Uninoculated	0.00 ± 0.04	0.26 ± 0.06	4.70 ± 4.90	8.72 ± 0.55
Unamended/ Inoculated	2.39 ± 0.20	2.48 ± 0.31	55.2 ± 1.4	59.0 ± 7.1
Amended/ Inoculated	3.62 ± 0.56	3.72 ± 0.63	99.4 ± 4.4	83.6 ± 8.2
Amended/Inoc. + Exc. Nitrate	14.9 ± 0.6	12.0 ± 0.4	370 ± 14	350 ± 36

Appendix B (continued)

Table 2. Total gas and carbon dioxide gas analysis of the initially aerobic inundated cellulose samples

Sample	Total Gas (ml g^{-1} cellulose)		Carbon Dioxide (µmol g^{-1} cellulose)	
	1228 Days	2718 Days	1228 Days	2718 Days
Initially Aerobic				
Unamended	-0.04 ± 0.08	-0.02 ± 0.00	4.43 ± 0.06	4.61 ± 0.14
Unamended/ Inoculated	0.30 ± 0.07	0.64 ± 0.04	14.4 ± 0.1	16.2 ± 0.1
Amended/ Inoculated	1.42 ± 0.28	1.33 ± 0.56	22.0 ± 2.9	21.9 ± 2.1
Amended/Inoc. + Exc. Nitrate	10.3 ± 1.5	8.42 ± 1.40	186 ± 8	165 ± 44
Initially Aerobic + Bentonite				
Unamended	0.33 ± 0.13	-0.97 ± 0.26	11.0 ± 0.2	11.7 ± 0.8
Unamended/ Inoculated	1.47 ± 0.22	-0.09 ± 0.04	69.6 ± 4.8	73.9 ± 14.7
Amended/ Inoculated	6.09 ± 0.04	4.02	169 ± 11	120 ± 6
Amended/Inoc. + Exc. Nitrate	8.10 ± 0.75	7.76 ± 2.34	154 ± 7	233 ± 5

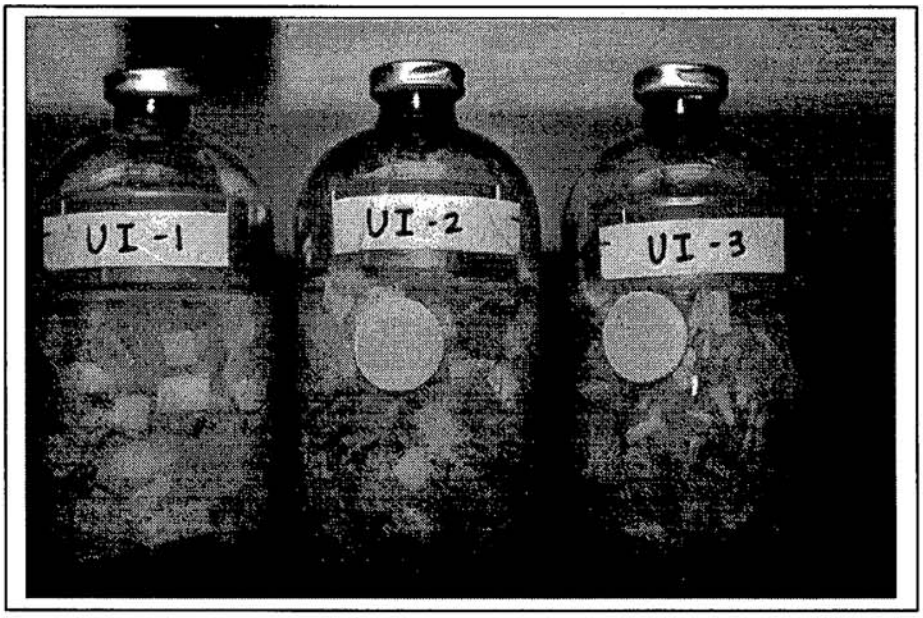
Appendix B (continued)

Table 3. Methane analysis of inundated cellulose samples.

Sample	1228 Days (nmol g^{-1} cellulose)	2718 Days (nmol g^{-1} cellulose)
Anaerobic		
Unamended	nd	3.92 ± 0.27
Unamended/Inoculated	nd	4.03 ± 1.38
Amended/Inoculated	nd	0.85 ± 0.7
Amended/Inoc. + Exc. Nitrate	nd	nd
Anaerobic + Bentonite		
Unamended	nd	3.84 ± 0.40
Unamended/Inoculated	nd	3.52 ± 0.20
Amended/Inoculated	nd	1.12 ± 0.03
Amended/Inoc. + Exc. Nitrate*	nd	nd
Initially Aerobic		
Unamended	Nd	1.25 ± 0.29
Unamended/Inoculated	Nd	1.10 ± 0.13

nd = not detected; methane was not detected in initially aerobic samples with nutrient amendments and excess nitrate nor in initially aerobic samples with bentonite.

Appendix C
Photographs of Samples

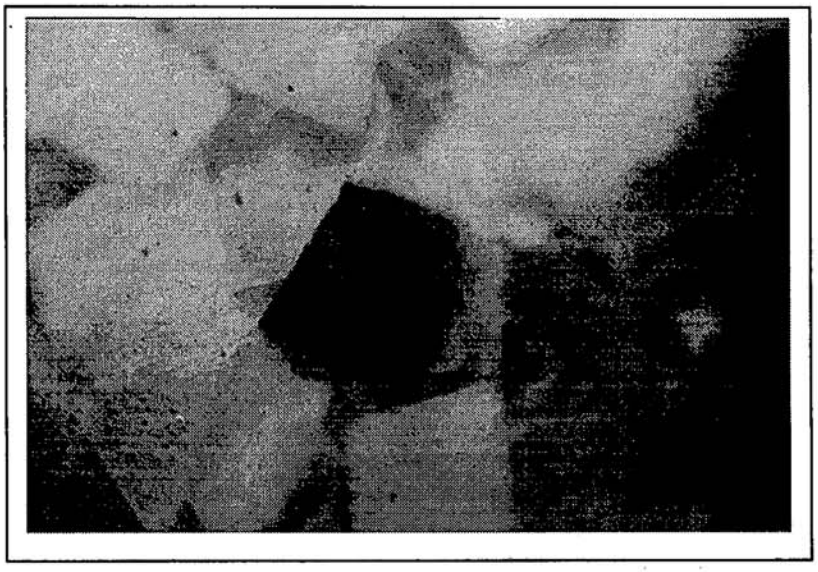


Anaerobic unamended inoculated samples contain paper that remains intact.

Anaerobic amended inoculated samples + excess nitrate contain paper that has been partially turned into pulp.

Appendix C (continued)
Photographs of Samples

Anaerobic amended inoculated sample at 2718 days showing pulping of cellulose due to microbial



Blackening of paper in anaerobic unamended inoculated sample containing bentonite due to reaction of iron with biogenic sulfide.