Effect of Steam Treatment on Microbial Activity at Guadalupe. Final Summary: Pre-steam and Post Steam Microbial Assays

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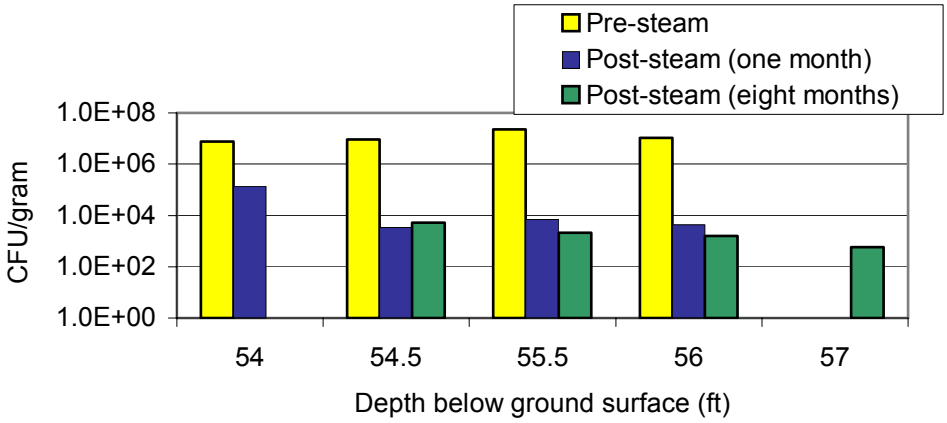
Yarrow Nelson, Ph.D., Principal Investigator
Christopher Kitts*, Ph.D., Co- Principal Investigator
Barbara Orchard, M.S. Graduate Student
Lynne Maloney, M.S. Graduate Student

Department of Civil and Environmental Engineering
California Polytechnic State University
San Luis Obispo, CA 93407

***Department of Biological Sciences**



Executive Summary

The effect of steam-driven hydrocarbon contaminant extraction/treatment on subsurface aerobic and anaerobic microbial communities was investigated at the former Guadalupe Oil Field (GOF) using multiple microbial assays before and after steam treatment. Soil samples were collected and analyzed prior to, one month after, and eight months after a five-month field pilot test of steam injection and extraction. Aerobic soil samples were analyzed by respirometry, plate counts, and direct microscopic counts. Anaerobic microbial activity was examined by monitoring methane generation in anaerobic microcosms with gas chromatography. Terminal restriction fragment (TRF) analysis was used to determine the diversity of the microbial community before and after steam treatment.

The pre-steam assays were run as part of a 2002-2003 Unocal-funded project, and detailed results can be found in our pre-steam report dated December 2003. One-month post-steam assays were run during the 2003-2004 funding cycle and reported December 2004. To check for microbial recovery following steam treatment, the eight-month samples were analyzed during the 2004-2005 academic year. Comprehensive results of all three studies are presented in this report.

Respirometry showed pre-steam CO_2 production varied with depth and ranged from 0.06 to 0.23 uL/g-hr, compared to blank controls with 0.00 uL/g-hr. One month after steam treatment CO_2 production was below the detection limit of the respirometer. Eight months after steam treatment, CO_2 production was detectable, but in this analysis the blank control also exhibited significant CO_2 production calling into question the validity of the measured respiration rate for eight months post-steam. Post-steam plate counts were one to four orders of magnitude lower than the pre-steam samples for both one month and eight months after steam. Direct microscopic counts showed post-steam (one and eight month) cell numbers were higher than the pre-steam counts, but based on plate counts these cells were mostly non-viable. Significant amounts of methane and hydrogen were generated from pre-steam anaerobic microcosms, but post-steam microcosms had no detectable methane, and only trace amounts of hydrogen. Pre-steam TRF analysis showed distinct differences in the microbial communities above and below the smear zone. Post-steam TRF analyses were not possible because insufficient DNA could be extracted from the soil.

Collectively, these results show that the field-scale test of steam extraction at the GOF dramatically reduced or eliminated both aerobic and anaerobic soil microbial activity. It is likely that microbial activity will eventually recover after additional cooling takes place, but the rate of this ultimate recovery is beyond the scope of this project.

Acknowledgements

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Table of Contents

Introduction

Steam-enhanced soil vapor extraction (SESVE) is an experimental method of improving non-aqueous phase liquids (NAPLs) extraction by injection of steam into the subsurface, while vapor and liquid are recovered through extraction wells. SESVE works via two main mechanisms: distillation of subsurface contaminants and the displacement of NAPLs. When applied appropriately, SESVE may significantly reduce the time required for the remediation of sites contaminated with volatile organic compounds (VOCs) and semi-volatile organic compounds (SVOCs) (Bouchard 2003). Even after successful SESVE, a site will probably have residual contamination, especially where SVOCs are the target. In these cases, and when the site closure or cleanup goal has not been met by SESVE alone, natural attenuation is important as a polishing step. However, SESVE may be detrimental to the indigenous microbial communities due to the high temperatures employed. The effect of SESVE on the microbial activity of soil has been studied with bench-top experiments such as those of Huesmann et al. (2002) and Richardson et al. (2002). The number of active microorganisms in soil prior to cooling following bench-top steaming was below the detection limit (Richardson et al. 2002). There have been a few field experiments to determine microbial activity directly following treatment, but in these experiments the subsurface remained at elevated temperatures (Krauter et al. 1996). The soil (still at elevated temperatures) experienced significant decreases in total microbial populations of up to 98%. The microbial community also shifted from gram negative to gram-positive organisms, most likely because some gram-positive cells form spores, which are capable of surviving high temperatures. Two years after steam treatment commenced, the groundwater temperature was still elevated to temperatures ranging from 45-75°C (Krauter et al. 1996). Thus, although microbial populations may rebound from SESVE after the subsurface cools, during this extended cooling period the microbial population and activity are still decimated. Anaerobic microbial activity was not addressed in these studies.

To further test the effect of SESVE on soil microbial communities, aerobic and anaerobic microbial activity were assayed before and after a steam pilot test at the former Guadalupe Oil Field (GOF). This pilot test was conducted to evaluate SESVE for removal of light non-aqueous phase liquid (LNAPL) hydrocarbons. The test lasted five months and employed subsurface temperatures reaching 115°C.

The objectives of this study were to determine to what extent SESVE reduces aerobic and anaerobic microbial activity, and to determine if this microbial activity would rebound as either one month or eight months after treatment. The microbial assays used in this study included:

1. Plate counts to quantify active aerobic populations,
2. Direct microscopic counts,
3. Respirometry to quantify aerobic microbial activity by measuring carbon dioxide production,
4. Methane and hydrogen generation to quantify anaerobic microbial activity,
5. Terminal restriction fragment (TRF) analysis to characterize the diversity of the aerobic and anaerobic microbial populations.

Pre-steam soil samples were collected at the SESVE site before operating the steam pilot test, and post-steam soil samples were collected one and eight months after cessation of steam injection. One month after cessation of steam the temperatures were still greater than 80°C, and after eight months temperatures had decreased to 40°C. Complete aerobic and anaerobic microbial assays were conducted on each soil core as listed above.

The pre-steam microbial assays were part of a research project conducted during the 2002-2003 academic year. Detailed results of these pre-steam assays can be found in our December 2003 report titled "Effect of Steam Treatment on Microbial Activity at Guadalupe. Part 1: Pre-Steam Microbial Assays." Additional information may also be found in the MS thesis of Lynne Maloney (Maloney, 2003). The pre-steam results were also presented in a poster presentation at the Battelle Conference in June 2004 (Maloney et al. 2004).

The post-steam assays conducted one month after cessation of steam injection were part of our 2003-2004 research program, and are reported in our December 2004 final reported titled "Effect of Steam Treatment on Microbial Activity at Guadalupe. Part 2: Post-Steam Microbial Assays," aka "Steam Kills." Finally, the eight-month post-steam assays were done during the 2004-2005 academic year and are reported here along with a comparison to pre-steam assays. Additional details on both post-steam assays may be found in the MS thesis of Barbara Orchard (Orchard, 2005). The current report is a comprehensive coverage of all pre-steam and post-steam assays and results.

Methods

Pre-steam and post-steam (one month) soil samples were gathered from multiple depths at two boring locations (Core Locations 6 and 8) for aerobic assays. An additional boring (Core Location 8) was drilled and sampled under nitrogen to maintain anaerobic conditions for the methanogenic assays and was stored in a nitrogen atmosphere. Each sleeve was six-inches long, two-inches in diameter and was treated as one sample (depth). Since post-steam (one month) soil samples exhibited reduced microbial populations and activity, further studies were necessary to determine the recovery of the microbial community following cooling of the subsurface. Eight months after the pilot test ended, four aerobic sleeves and six anaerobically handled sleeves were gathered from the same location as Core Location 6.

Prior to any microbial assays, soil from each sleeve was homogenized by mixing all of the soil from the sleeve. Soil for TRF analysis and total petroleum hydrocarbon (TPH) analysis was frozen until analysis. Aerobic samples were analyzed using plate counts, respirometry, and direct epifluorescent microscopy. For plate and direct counts, microbes were transferred from the soil particles into solution by combining the soil sample, phosphate buffer solution (pH 7.2, Aldrich), water, and sodium pyrophosphate in a 125-mL Erlenmeyer flask. The flask was covered and mixed using a magnetic stir bar. The TPH concentrations of pre-steam aerobic soil samples were as high as 73,000 mg/kg. Post-steam aerobic cores had decreased TPH levels from 31 to 7100 mg/kg. Pre-steam anaerobic soil sample TPH concentrations ranged from non-detect (ND) to 150,000 mg/kg, while post-steam TPH concentrations ranged from ND to 23,000 mg/kg.

Aerobic Respirometry. CO_2 production rates in the aerobic samples were measured using a Columbus Instruments (Columbus, OH) Micro-Oxymax respirometer with a CO_2/CH_4 detector. Duplicate 50-g soil samples of each depth and a blank containing no sample were tested in the respirometer for 48 hours. An external water bath was used to maintain a constant temperature of 20°C.

Plate Counts. Plate counts were performed using general R2A agar (Becton Dickinson, Sparks, MD). The stock solution was serially diluted with autoclave-sterilized phosphate buffer solution (PBS). Using sterile technique, 100 µl of each dilution was plated in triplicate. Control plates were inoculated with 100 µl of sterilized PBS. The plates were incubated aerobically at 20°C for four days before counting.

Direct Microscopic Counts. Direct microscopic counts of bacteria were performed using a method modified from (Bhupathiraju et al. 1999) to enumerate the total number of cells per gram of soil, using epifluorescent microscopy and 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF), which stains the cell walls of both respiring and non-respiring cells. Stained bacterial cells on five randomly selected fields were counted for one slide for each depth. An Olympus BX50 Microscope, BX-FLA Reflected Light Fluorescence Attachment, and BH2-RFL-T3 Power Supply Unit were used with Omega Optical filter XF115-2.

For the pre-steam samples an attempt was made to enumerate live bacteria using a CTC stain reported by Bhupathiraju et al. (1999). Unfortunately there were too many false positive readings for live bacteria when using this method on soils from Guadalupe. An autoclaved control was run which showed significant fluorescence from particles in the soil. Therefore, only the DTAF method was used on all subsequent samples. The DTAF method provides total cell counts and does not distinguish live cells from dead cells.

Anaerobic Microcosms. Anaerobic soil microcosms were setup in a nitrogen-purged glovebox by placing 40 g of soil into each 150 mL glass serum bottle and sealing with Teflon®-lined septa using a crimper. The anaerobic microcosms were incubated at 20°C until analysis. The headspaces of duplicate microcosm bottles were analyzed using gas chromatography (Inland Empire Analytical, Norco, CA) with a thermal conductivity detector (TCD) for CH_4, O_2, H_2, CO_2, N_2 and N_2O.

TPH Analysis. Zymax Envirotechnology (San Luis Obispo, CA) performed the TPH analysis on the soil samples using EPA Method 3550 extraction, followed by gas chromatography/mass spectrometry (GC/MS). The analytical range was C8 to C40. TPH was quantified against standards prepared from Guadalupe hydrocarbons. Results below the practical quantification limit of 10 mg TPH per kg soil are reported as Non-Detect (ND).

TRF Analysis. Terminal Restriction Fragment (TRF) analysis was performed using the method described by Kitts (2001). TRF analysis is a method based on polymerase chain reaction (PCR). DNA was extracted from the soil and amplified with PCR. PCR and gel electrophoresis were performed on the aerobic soil cores.

Results

Aerobic microbial activity before and after steam treatment is quantified by respirometry, and aerobic microbial populations are quantified by plate counts and direct microscopic counts. Anaerobic microbial activity is quantified by methane and hydrogen production.

Aerobic Respirometry.

Pre-steam average respiration rates ranged from 0.06 to 0.23 μL/g-hr (Tables 1 and 2, and Figure 1A). The average carbon dioxide production rates of all the one-month post-steam samples were zero or below zero (Tables 1 and 2, and Figure 1B). These results show a dramatic decrease in aerobic metabolic activity following steam extraction.

Respiration results for the eight-month post-steam samples showed cumulative CO_2 production of up to 10 μL/g (Figure 1C). However, the blank control run with these samples also exhibited significant CO_2 production (Figure 1C), so the respirometry results for these samples are inconclusive. It is probably not appropriate to simply subtract the CO_2 production of the blank from that of the samples because the high CO_2 production of the blank is an indication of possible respirometer malfunction. Therefore the CO_2 production observed for the eight-month post steam samples cannot necessarily be interpreted as recovery of the microbial community. The respirometry results for the eight-month post steam samples are not included in Table 1.

TABLE 1. Comparison of pre and post-steam respiration rates for Core Location 6. Note: respirometry data for eight months post-steam were not reliable because the blank control exhibited significant CO2 production, and thus respiration results for eight months post-steam are not included in this table.

CO_2 Production Rate (µL/g-hr)				
Depth (ft bgs)	Pre-steam Average	Post-steam (one month)		
		Sample A	Sample B	Average
54	0.07	0.00	*	0.00
54.5	0.08	0.00	0.00	0.00
55.5	0.21	0.00	0.00	0.00
56.5	0.15	0.00	0.00	0.00
57				
Blank	0.005	0.00	---	0.00

TABLE 2. Comparison of pre and post-steam respiration rates for Core Location 8.

	CO_2 Production Rate (µL/g-hr)			
Depth (ft bgs)	Pre-steam Average	Post-steam (one month)		
		Sample A	Sample B	Average
57	0.23	-0.04	-0.03	-0.04
57.5	0.06	-0.04	0.01	-0.02
58.5	0.16			
59	0.20			
61.5		-0.01	-0.02	-0.01
62		-0.02	0.02	0.00
Blank	0.003	-0.01	---	-0.01

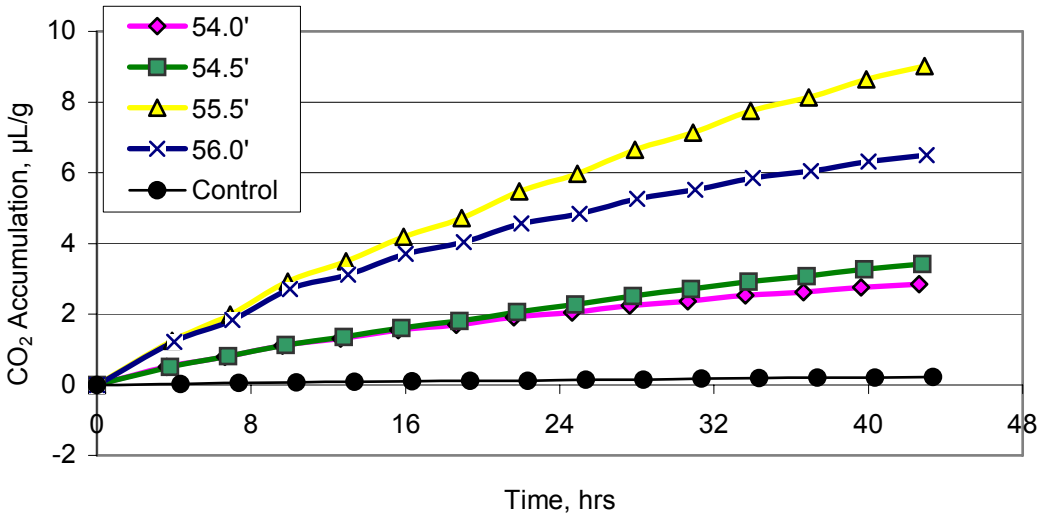


FIGURE 1A. Pre-steam cumulative carbon dioxide production.

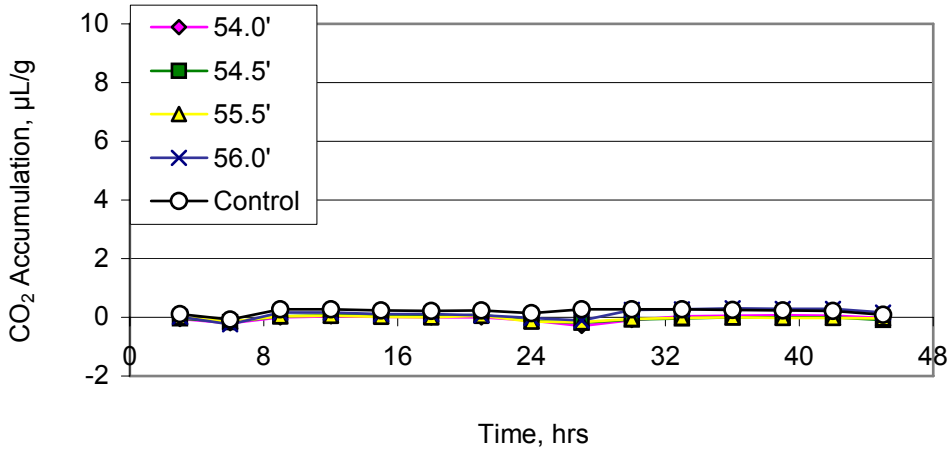


FIGURE 1B. Post-steam (one month) cumulative carbon dioxide production.

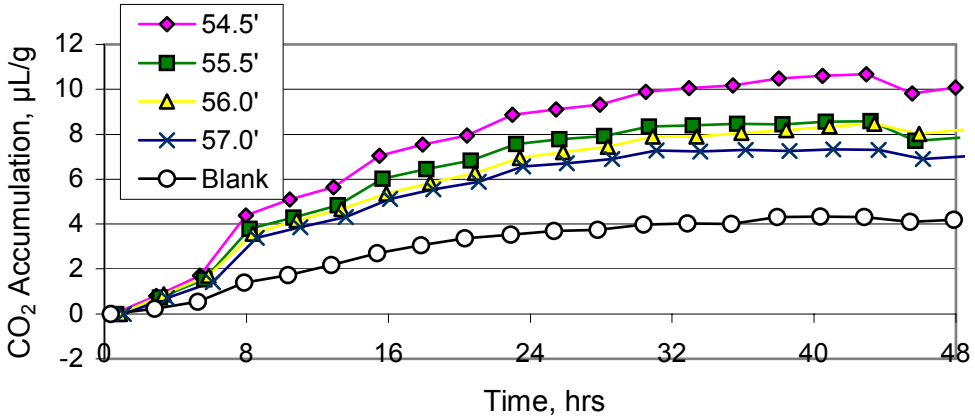


FIGURE 1C. Post-steam (eight months) cumulative carbon dioxide production (note high respiration rate of blank control).

Aerobic Plate Counts.

Pre-steam plate counts varied from 1.6 x 10^6 to 2.2 x 10^7 colony-forming units (CFU) per gram of soil (Table 3). The post-steam (one month) counts ranged from 3.4 x 10^3 to 1.98 x 10^5 CFU/g (Table 3). The post-steam plate counts were lower by one to four orders of magnitude than the pre-steam counts, and the percent reduction ranged from 97.87% to 99.96% (Table 3 and Figure 2). The post-steam plate counts at eight months were even lower, ranging from 5.7 x 10^2 to 5.4 x 10^3 CFU/g (Table 4 and Figure 2). These results show conclusively that SESVE killed the vast majority of subsurface bacteria and that these populations had not recovered 8 months after treatment.

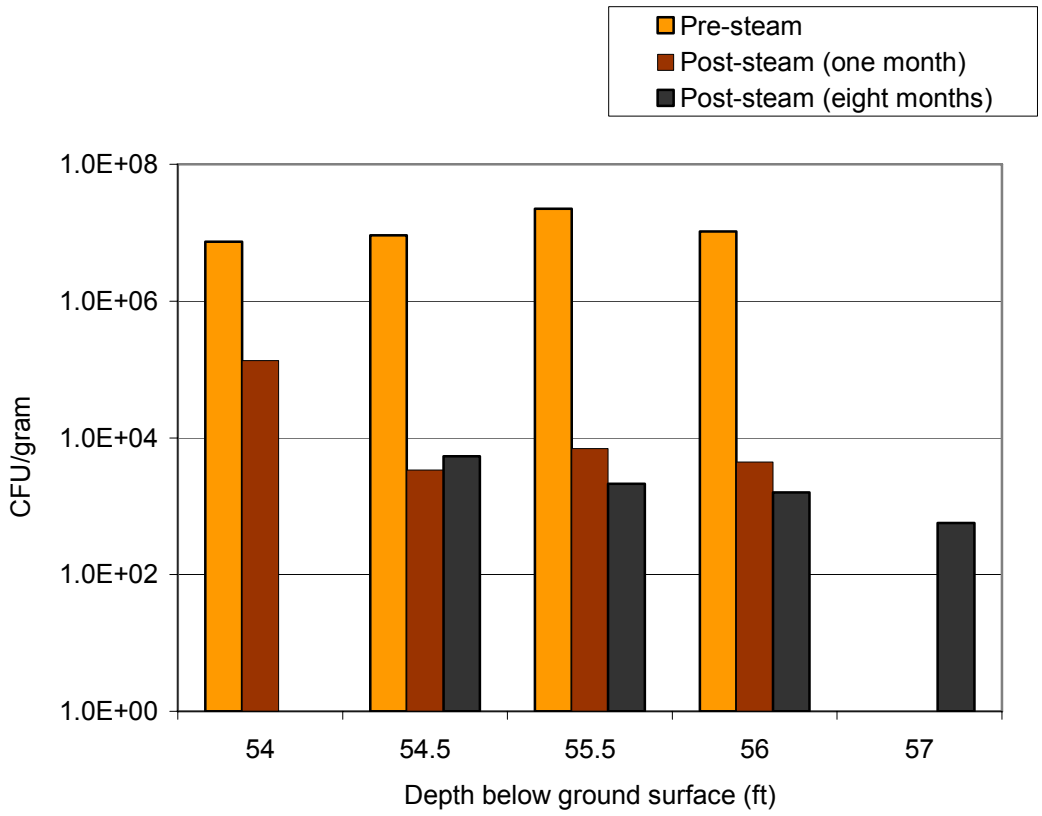
TABLE 3: Comparison of pre-steam and post-steam plate counts (Core Locations 6 and 8)

Core 6	Pre-steam	Post-steam (one month)	% Reduction
Depth	Average CFU/gram		
54	7.40E+06	1.35E+05	98.17
54.5	9.20E+06	3.40E+03	99.96
55.5	2.25E+07	6.95E+03	99.97
56	1.04E+07	4.43E+03	99.96
Core 8	Pre-steam	Post-steam (one month)	% Reduction
Depth	Average CFU/gram		
57	1.60E+06	6.45E+03	99.60
57.5	6.30E+06	1.34E+05	97.87
58.5	4.40E+06		
59	1.05E+07		
61.5		2.77E+04	99.37
62		1.98E+05	98.12

TABLE 4: Comparison of Pre-steam and Post-steam Plate Counts at one-month and eight months for Core Location 6.

Core 6	Average CFU/gram		
Depth (ft bgs)	Pre-steam	Post-steam (one month)	Post-steam (eight months)
54	7.40E+06	1.35E+05	---
54.5	9.20E+06	3.40E+03	5.37E+03
55.5	2.25E+07	6.95E+03	2.13E+03
56	1.04E+07	4.43E+03	1.60E+03
57	---	---	5.67E+02

FIGURE 2. Comparison of plate counts from Core Location 6 before and after steam treatment.



Direct Microscopic Counts.

Pre-steam direct counts varied from 2.8 to 33.6 million cells per gram and increased with increasing depth (Table 5). Post-steam direct counts at one month ranged from 29.5 to 99.4 million cells per gram, and post-steam direct counts at eight months ranged from 10.2 to 74.4 million cells per gram (Table 5). Overall, the post-steam counts were significantly higher than pre-steam counts, but based on the plate counts described above these cells are likely inactive or dead (Figure 3).

TABLE 5: Direct count results for Core Location 6

	Average 10^6cells/gram		
Depth (ft bgs)	Pre-steam	Post-steam (one month)	Post-steam (eight months)
54	7.5	29.5	---
54.5	2.8	38.1	10.2
55.5	12.0	64.2	64.8
56	33.6	61.3	60.8
57	---	---	74.4

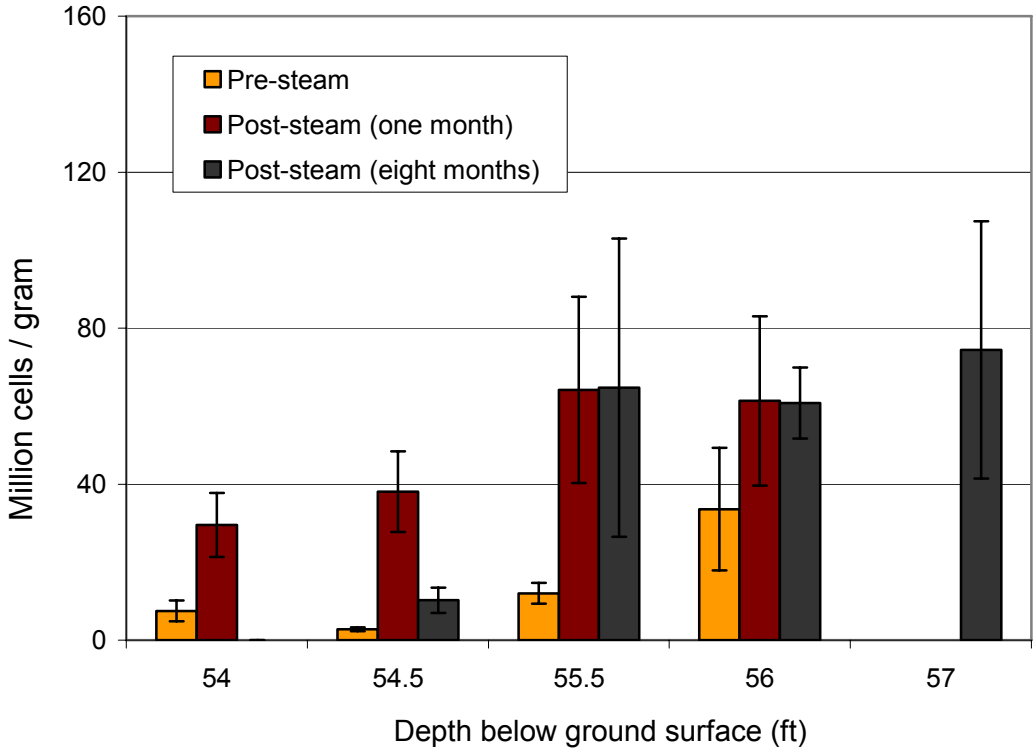


FIGURE 3. Comparison of all Core Location 1 direct count results. Error bars indicate +/- one standard deviation.

Anaerobic Microcosms.

Gas headspaces of pre-steam soil microcosms were incubated up to 135 days, and all of the samples collected below the NAPL smear zone showed significant methane production (up to 2700 ppm) at each sampling date (Table 6). Pre-steam hydrogen levels were as high as 20,000 ppm (Table 6). The headspaces of the one-month and eight-month post-steam microcosms were analyzed after incubating for up to 211 days and 129 days, respectively. None of the post-steam microcosms (one or eight-month) showed detectable methane (Tables 7 and 8). Most of the post-steam microcosms had no detectable hydrogen production (Tables 7 and 8). One post-steam (one month) sample had 124 ppm hydrogen and post-steam hydrogen levels around 2 to 16 ppm were observed for a few samples for soil depths of 54 to 57 feet bgs (Tables 7 and 8). These results indicate that virtually all anaerobic microbial activity was arrested by the steam process.

Some of the post-steam (one month) microcosms were found to have leaked as indicated by oxygen concentrations of over 1%. Samples exhibiting oxygen leakage were excluded from the data analysis and are not included in Table 7. All other samples exhibited oxygen concentrations less than 1 %, which is similar to the background of measurement.

TABLE 6: Pre-steam anaerobic microcosm GC results

Incubation time before analysis	Depth ft bgs	H_2 ppm	CH_4 ppm	CO_2 %
42 days	37.5	7152.4	126	1.153
	38	8.2	109	1.425
	38.5	1612.9	120	0.639
	38.5	23914.7	139	0.464
	39	0.7	148	1.057
	39	1759.3	92	0.840
	39.5	4.5	151	0.955
	40	<0.5	105	1.105
	40	48.3	266	0.912
	40.5	1.8	719	1.050
	41	<0.5	265	0.835
	41.5	17.4	497	1.205
	42	0.6	594	0.909
	42.5	11804.3	576	2.111
109 days	11	5537.6	69	1.301
	31	1	<5	1.377
	34	9288.5	<5	1.649
	34.5	2239.1	<5	1.453
	35	3495.7	<5	1.410
	35.5	94.7	116	0.700
	36	1.1	<5	1.184
	36.5	128.2	<5	2.168
	37	1362.9	121	1.068
	37.5	1988.7	114	1.059
	38	4.5	86	2.064
	38.5	<0.5	123	0.677
	38.5	20299.1	156	0.377
	39	6	593	1.228
	39	0.5	96	1.037
	39.5	<0.5	158	1.249
	40	0.6	167	1.159
	40	5820.7	964	0.911
	40.5	23	1175	1.160
	41	0.5	304	0.998
	41.5	53.2	738	1.043
	42	0.8	155	1.391
	42.5	<0.5	2699	1.976

TABLE 6 (continued): Pre-steam anaerobic microcosm GC results

Incubation time before analysis	Depth	H_2	CH_4	CO_2
	ft bgs	ppm	ppm	%
135 days	11	<0.5	12	1.520
	31	<0.5	<5	1.427
	34	10315.2	10	1.492
	34.5	553.1	<5	1.405
	35	2725.7	<5	1.388
	35.5	13.8	130	0.686
	36	<0.5	<5	1.164
	36.5	26.4	26	1.915
	37	25	115	0.771
	37.5	5124.9	125	1.122
	38	0.8	93	1.951
	38.5	<0.5	131	0.730
	38.5	13790.8	178	0.298
	39	1.2	667	1.283
	39	<0.5	101	1.101
	39.5	<0.5	191	1.235
	40	0.6	175	1.336
	40	4694.0	1369	0.897
	40.5	40.4	1817	1.148
	41	0.6	319	1.174
	41.5	15.4	1561	1.052
	42	0.9	814	1.147
	42.5	<0.5	2568	2.210

TABLE 7: Post-steam (one month) anaerobic microcosm GC results

Incubation time before analysis	Depth	H_2	CH_4	CO_2
	ft bgs	ppm	ppm	%
34 days	51.5	<0.5	<5	0.140
	52.5	<0.5	<5	0.183
	54	122.4	<5	0.269
	54.5	1.8	<5	0.522
	55.5	1.8	<5	0.385
	56	1.7	<5	0.405
	61.5	<0.5	<5	0.121
	62	0.8	<5	1.396
	62.5	<0.5	<5	0.490
	62.5	<0.5	<5	0.535
	63	<0.5	<5	0.093
	63	<0.5	<5	0.176
50 days	51.5	0.7	<5	0.109
	52.5	<0.5	<5	0.188
	53	1.7	<5	0.268
	54.5	3.5	<5	0.503
	54.5	5.7	<5	0.543
	55.5	2.7	<5	0.630
	56	2.5	<5	0.584
	61.5	<0.5	<5	0.156
	61.5	<0.5	<5	0.698
	61.5	<0.5	<5	0.143
	62	<0.5	<5	1.816
	62	<0.5	<5	3.113
	62.5	<0.5	<5	2.413
	62.5	<0.5	<5	0.187
	62.5	<0.5	<5	0.223
	63	<0.5	<5	0.135
	63	<0.5	<5	0.127
90 days	51.5	1.0	<5	0.188
	52.5	<0.5	<5	0.244
	53	2.2	<5	0.394
	54.5	4.8	<5	0.679
	54.5	6.2	<5	0.746
	55.5	3.5	<5	0.779
	56	3.7	<5	0.959
	61.5	<0.5	<5	0.252
	61.5	<0.5	<5	1.009
	61.5	<0.5	<5	0.207
	61.5	<0.5	<5	1.340
	61.5	<0.5	<5	0.224
	62	<0.5	<5	2.491
	62	<0.5	<5	3.437

TABLE 7 (continued): Post-steam (one month) anaerobic microcosm GC results

Incubation time before analysis	Depth	H_2	CH_4	CO_2
90 days	ft bgs	ppm	ppm	%
	62	<0.5	<5	0.349
	62.5	<0.5	<5	2.498
	62.5	<0.5	<5	0.279
	62.5	<0.5	<5	0.318
	62.5	<0.5	<5	0.241
	62.5	<0.5	<5	0.248
	63	<0.5	<5	0.229
	63	<0.5	<5	0.223
	63	<0.5	<5	0.244
	63	<0.5	<5	0.211
	63	12.3	<5	0.642
211 days	51.5	<0.5	<5	0.219
	54.5	6.1	<5	1.273
	54.5	6.9	<5	1.275
	55.5	3.8	<5	0.572
	55.5	3.4	<5	0.936
	56	2.2	<5	3.325
	56	3.6	<5	1.133
	61.5	<0.5	<5	0.495
	61.5	<0.5	<5	0.504
	61.5	<0.5	<5	1.382
	61.5	<0.5	<5	0.409
	61.5	<0.5	<5	1.601
	61.5	<0.5	<5	0.447
	62	<0.5	<5	8.015
	62	<0.5	<5	2.720
	62	<0.5	<5	3.724
	62	<0.5	<5	0.687
	62.5	<0.5	<5	1.218
	62.5	<0.5	<5	4.139
	62.5	<0.5	<5	2.770
	62.5	<0.5	<5	0.516
	62.5	<0.5	<5	0.603
	62.5	<0.5	<5	0.477
	62.5	<0.5	<5	0.528
	63	<0.5	<5	0.270
	63	<0.5	<5	0.360
	63	<0.5	<5	0.348
	63	<0.5	<5	0.330
	63	<0.5	<5	0.355
	63	<0.5	<5	1.100

Table 8: Post-steam (eight months) anaerobic microcosm GC results

Incubation time before analysis	Depth	H_2	CH_4	CO_2
	ft bgs	ppm	ppm	%
30 days	58.5	<0.5	<5	0.525
	58.5	<0.5	<5	1.008
	59	<0.5	<5	0.364
	59	<0.5	<5	0.343
	60	<0.5	<5	0.461
	60	<0.5	<5	0.389
	60.5	<0.5	<5	0.392
	60.5	<0.5	<5	0.313
61 days	54	<0.5	<5	1.017
	54	<0.5	<5	4.158
	57.5	3.9	<5	1.139
	57.5	15.8	<5	0.978
	58.5	<0.5	<5	0.665
	58.5	<0.5	<5	0.694
	59	<0.5	<5	0.394
	59	<0.5	<5	0.446
	60	<0.5	<5	0.485
	60	<0.5	<5	0.499
	60.5	<0.5	<5	0.496
	60.5	<0.5	<5	0.343
92 day s	58.5	<0.5	<5	0.698
	58.5	<0.5	<5	0.792
	59	<0.5	<5	0.411
	59	<0.5	<5	0.443
	60	<0.5	<5	0.512
	60	<0.5	<5	0.504
	60.5	<0.5	<5	0.401
	60.5	<0.5	<5	0.563
129 days	54	<0.5	<5	1.342
	54	1.4	<5	3.083
	57.5	7.7	<5	1.016
	57.5	6.7	<5	0.927
	58.5	<0.5	<5	0.884
	58.5	<0.5	<5	9.604
	59	<0.5	<5	0.528
	59	<0.5	<5	0.560
	60	<0.5	<5	0.615
	60	<0.5	<5	0.681
	60.5	<0.5	<5	0.575
	60.5	<0.5	<5	0.481

Terminal Restriction Fragment (TRF) Analysis Results.

Soil TRFs: TRF analysis of pre-steam soil samples revealed 37 different TRF peaks representing 37 different organism types for samples above the air-oil interface and 54 TRF peaks below the air-oil interface. TRF analysis revealed the presence of three distinct microbial communities at the site. The aerobic and anaerobic non-contaminated zone included *Actinomyces*, *Pseudomonas*, and other microorganisms. The transition zone included *Streptomyces*. Microorganisms including *Mycobacteria* and *Actinobacteria* characterized the zone with the highest TPH concentrations. TRF analysis was attempted on post-steam (one and eight month) soil cores, but these samples did not have enough DNA material for the analysis.

Groundwater TRFs: Sufficient DNA was extracted from pre-steam and post-steam (one-month) groundwater samples to allow for TRF analysis. These TRF analyses showed a shift in the type of dominant gamma proteobacteria compared to pre-steam samples and a complete loss (below detection) of the *Actinobacteria* associated with high TPH in pre-steam analyses (Figure 4).

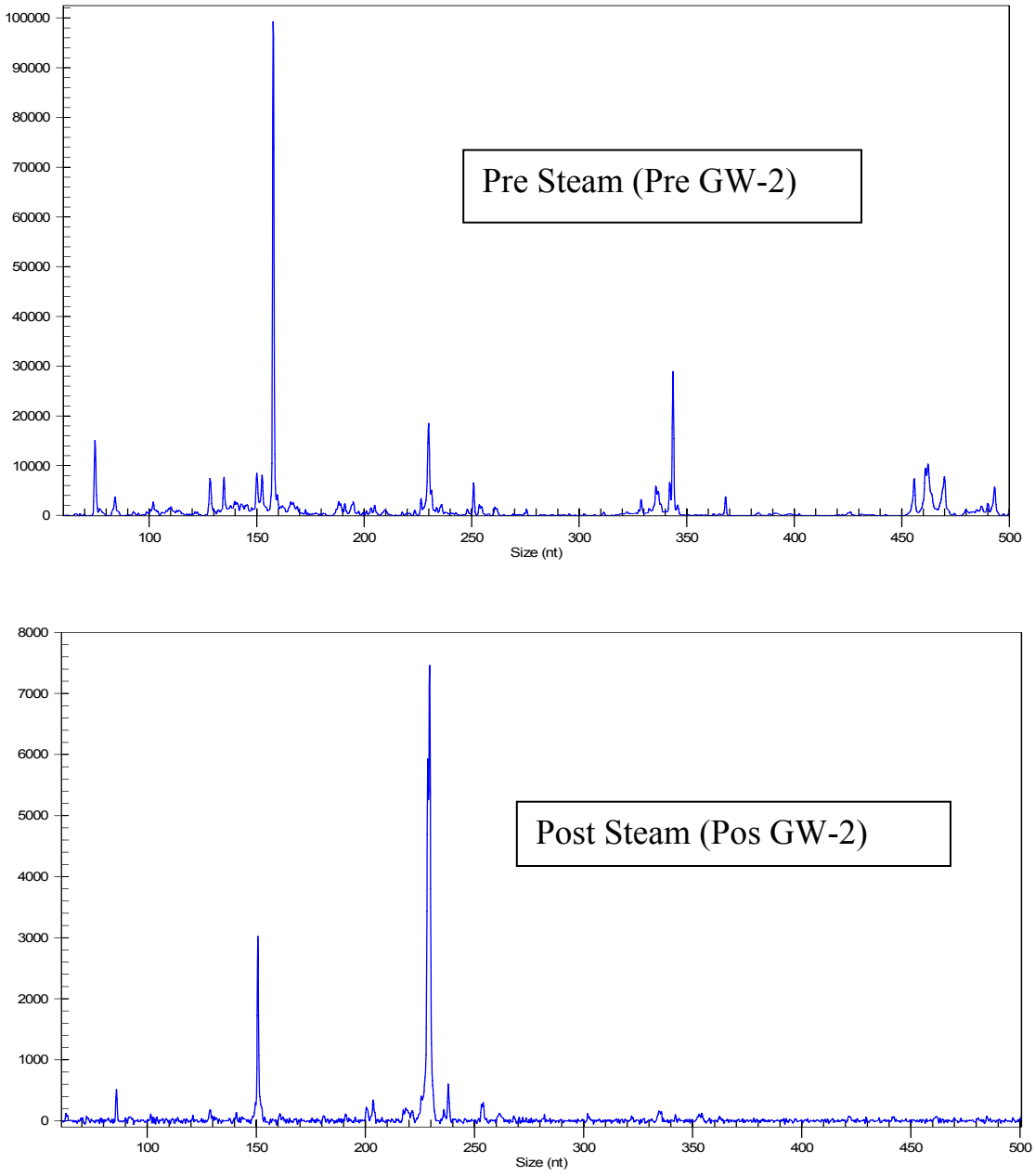


FIGURE 4. Comparison of TRF patterns for pre-steam and post-steam (from Dec 2004 Final Report)groundwater.

Discussion

The pre-steam microbial analyses provided a baseline of the microbial community and population present in the TPH contaminated subsurface. The post-steam analyses show a dramatic decrease in the active microbial population and significant shift in the community species diversity (in groundwater) at one month and eight months after steam treatment. Results from respirometry and plate counts were consistent for the pre-steam one-month post-steam assays, suggesting immediately after steam treatment the aerobic microbial population was drastically reduced and the aerobic microbial activity was minimal. Post-steam plate counts at eight months were of the same order of magnitude as the post-steam counts at one month, indicating little or no recovery of the active microbial population after eight months. The post-steam respirometry at eight months showed some CO_2 production, but the blank control for this run also showed significant CO_2 production, which makes the eight-month post steam respirometry measurements inconclusive.

Post-steam direct microscopic counts (one and eight months) were much higher than the pre-steam direct counts. However, since the plate counts for the post-steam soils were orders of magnitude lower than the pre-steam plate counts, it is highly unlikely that the cells counted microscopically were active. Instead, it is likely that the vast majority of cells counted under the microscope are dead. One possible explanation for the high microscopic cell counts could be that there had been rapid growth of bacteria as the soil temperatures passed through an optimal range, on the way up to the lethal temperatures. This scenario would lead to high cell counts of mostly dead bacteria.

Direct counts are usually expected to be much higher than plate counts because only a fraction of the cells are expected to be culturable on plates (Amann et al. 1995). Further, plate counts enumerate colonies and several cells may be clumped to form a colony, which would also lead to direct counts higher than plate counts. In contrast to these arguments, the pre-steam plate counts were similar to or higher than the pre-steam direct counts (Tables 4 and 5). These results suggest that the cells observed under the microscope were mostly viable and culturable in the case of the pre-steam assays. This provides further assurance that the cells observed microscopically for the post-steam assays were mostly dead rather than being unculturable.

Anaerobic microbial activity was also virtually eliminated by steam treatment. While pre-steam anaerobic microcosms generated methane and hydrogen, no methane and only very minimal amounts of hydrogen were detected in the headspaces of post-steam one-month and eight- month microcosms even after incubating for up to seven months at 20°C.

The very low microbial activity observed following steam treatment could be attributed to the high soil temperatures at the time of soil sampling. After one month, the soil temperatures were still up to 80°C, and after eight months the soil temperatures were still up to 40°C. Similarly, previous studies have shown recovery of the microbial community (with exception of thermo-tolerant species) is limited while the subsurface remains at elevated temperatures (Krauter et al. 1996; Richardson et al. 2002). It is possible, and even likely, that microbial activity would eventually recover after temperatures reach ambient levels, but such a study is currently beyond the scope of this project.

Conclusions

Immediately following steam treatment (one month):

- Aerobic microbial activity was not detectable by respirometry.
- No anaerobic microbial activity was observed in anaerobic microcosms (no methane or hydrogen production)
- Active aerobic microbial populations (plate counts) were reduced by 2-3 orders of magnitude relative to pre-steam plate counts.
- The species diversity of the microbial community in groundwater was significantly reduced below the air/oil interface.

Eight months after steam treatment:

- Aerobic microbial activity was difficult to quantify by respirometry because the blank control exhibited CO_2 production.
- Aerobic microbial populations (plate counts) were still at the same drastically reduced level observed at one month.
- Anaerobic microbial activity is still essentially zero (no methane production and very minimal hydrogen production).
- The composition of the microbial community is unknown because sufficient DNA could not be extracted for TRF analysis.

Possible follow-up studies could include:

- Follow up on steam pilot test site when groundwater temperature cools to 20°C.
- Conduct laboratory microbial assays at both pre and post-steam groundwater temperature, to account for thermophiles, if present.
- Evaluate alternative methods for characterizing the microbial community, which will be effective even for samples with little genetic material.

In conclusion, the overall effect of the steam treatment at the GOF pilot test was to drastically reduce the aerobic and anaerobic microbial activity and active microbe populations for up to 8 months after cessation of steam injection. Further studies would be required to determine how long it would take for the microbial activity to recover after soil temperatures reach ambient temperatures.

References

Amann, R. I., W. Ludwig, et al. 1995. "Phylogenic identification and in situ detection of individual microbial cells without cultivation." *Microbiological Reviews. 59*(1): 143-169.

Bhupathiraju, V. K., M. Hernandez, et al. 1999. "A new direct microscopy based method for evaluating in-situ bioremediation." *Journal of Hazardous Materials.* (B67): 299-312.

Bouchard, D. P., Musterait, Todd M., and Sobieraj, James A. 2003. "A Practical Approach to Steam-Enhanced Dual-Phase Extraction: A Case Study." *Remediation. Summer 2003*: 39-57.

Huesmann, M. H., T. S. Hausmann, et al. 2002. "Evidence of thermophillic biodegradation for PAHS and diesel fuel in soil." Paper 2G-09. In: A. R. Gavaskar and A. S. C. Chen (Eds.), *Proceedings of the Third International Conference on Remediation of Chlorinated and Recalcitrant Compounds*. Battelle Press, OH (CD format).

Kitts, C. L. 2001. "Terminal restriction fragment patterns: A tool for comparing microbial communities and assessing community dynamics." *Current Issues in Intestinal Microbiology. 2*(1): 17-25.

Krauter, P., D. MacQueen, et al. 1996. "Effect of Subsurface Electrical Heating and Steam Injection on the Indigenous Microbial Community." *Proceedings of the International Topical Meeting on Nuclear and Hazardous Waste Management. 2*: 1270-1274.

Maloney, L. C., Y. M. Nelson, et al. 2004. "Characterization of Aerobic and Anaerobic Microbial Activity in Hydrocarbon-Contaminated Soil." Paper 3E-02. In: A. R. Gavaskar and A. S. C. Chen (Eds.), *Proceedings of the Fourth International Conference on Remediation of Chlorinated and Recalcitrant Compound*. Battelle Press, OH (CD Format).

Maloney, Lynne, 2003. Characterization of Aerobic and Anaerobic Microbial Activity in Hydrocarbon-Contaminated Soil, MS Thesis, California Polytechnic State University, San Luis Obispo. Available on-line at http://ceenve.ceng.calpoly.edu/nelson/Theses.htm

Orchard, Barbara, 2005. Microbial Activity of Soil Following Steam-Enhanced Soil Vapor Extraction of Hydrocarbons, MS Thesis, California Polytechnic State University, San Luis Obispo. Available on-line at http://ceenve.ceng.calpoly.edu/nelson/Theses.htm

Richardson, R. E., C. A. James, et al. 2002. "Microbial activity in soils following steam treatment." *Biodegradation. 13*(4): 285-295.