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Microbial Activity of Soil Following Steam Treatment

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ABSTRACT: The effect of steam treatment on subsurface aerobic and anaerobic microbial communities was investigated using multiple microbial assays. Soil samples were gathered 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. Respirometry showed pre-steam CO_2 production was consistent with natural attenuation, post-steam (one month) CO_2 production was below detection, and post-steam (eight months) CO_2 production was about half of pre-steam. Post-steam (one and eight month) plate counts were one to four orders of magnitude lower than the pre-steam samples. 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. Terminal restriction fragment (TRF) analysis was performed to determine the diversity of the microbial community before and after steam treatment. 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.

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 can 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, since 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). Anaerobic microbial activity was not addressed in these studies. 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.

To further test the effect of SESVE on soil microbial communities, aerobic and anaerobic microbial activity were measured before and after a steam pilot test. The pilot test was conducted at the former Guadalupe Oil Field site to remove light, nonaqueous-phase liquid (LNAPL) hydrocarbons and lasted five months with subsurface temperatures reaching 115°C. Post-steam test soil samples were collected one and eight months after cessation of steam injection when the temperatures were greater than 80°C and 40°C, respectively. Soil samples from multiple depths were analyzed to determine the aerobic and anaerobic microbial activity, population, and species diversity. This comparison was used to determine the effect of steam treatment on the microbial activity of soil. This research included several objectives: (1) quantify the aerobic populations using plate counts, and direct microscopic counts; (2) quantify aerobic microbial activity by measuring carbon dioxide production; (3) quantify anaerobic microbial activity by monitoring methane generation in anaerobic microcosms; (4) characterize the diversity of the aerobic and anaerobic microbial populations using terminal restriction fragment (TRF) analysis; (5) compare the pre-steam results to the post-steam results; and (6) evaluate recovery of the microbial community during cooling.

MATERIALS AND METHODS

Pre-steam and post-steam (one month) soil samples were gathered from multiple depths at two boring locations (Core 1 and 2) for aerobic assays. An additional boring (Core 3) 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 1.

Prior to any experiments, 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.

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

Pre-steam and post-steam microbial assays are compared in this section. Additional details of pre-steam experiments can be found in Maloney et al. (2004). Aerobic microbial activity is defined 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 (Table 1, Figure 2A). The average carbon dioxide production rates of all the post-steam (one month) samples are zero (Figure 2B). The cumulative CO_2 production in µL/g for post-steam (eight months) samples showed evidence of aerobic microbial activity (Figure 2C). The average carbon dioxide respiration rates of the post-steam (eight months) samples ranged from 0.06 to 0.12 µL/g-hr. The CO_2 production rates, like the cumulative CO_2 production, show some recovery of aerobic microbial activity.

TABLE 1. Comparison of pre and post-steam respiration rates (Core 2)

Average CO_2 Production Rate (µL/g-hr)		
Depth (ft bgs)	**Pre-steam**	**Post-steam (one month)**
57	0.23	0.00
57.5	0.06	0.00
58.5	0.16	
59	0.20	
61.5		0.00
62		0.00
Control	0.00	0.00

Aerobic Plate Counts. Pre-steam plate counts varied from 1.6×10^6 to 2.2×10^7 colony forming units (CFU) per gram of soil. The post-steam (one month) counts ranged from 3.4×10^3 to 1.98×10^5 CFU/g. 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% (Figure 1). The post-steam plate counts at eight months were even lower, ranging from 5.7×10^2 to 5.4×10^3 CFU/g.

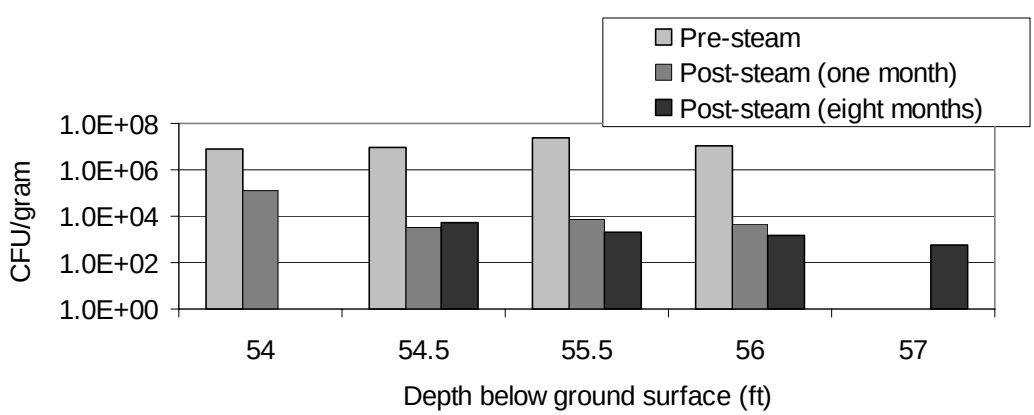

FIGURE 1. Comparison of all Core 1 plate counts.

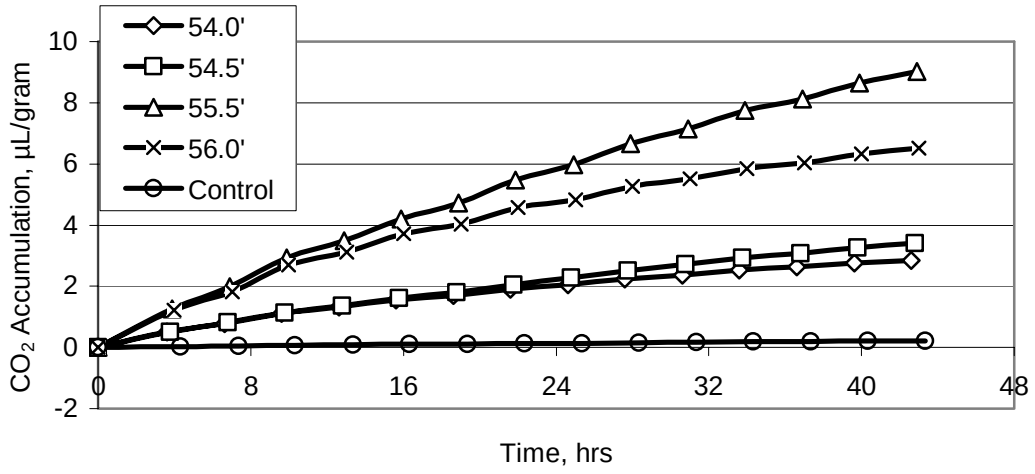

FIGURE 2A. Pre-steam cumulative carbon dioxide production.

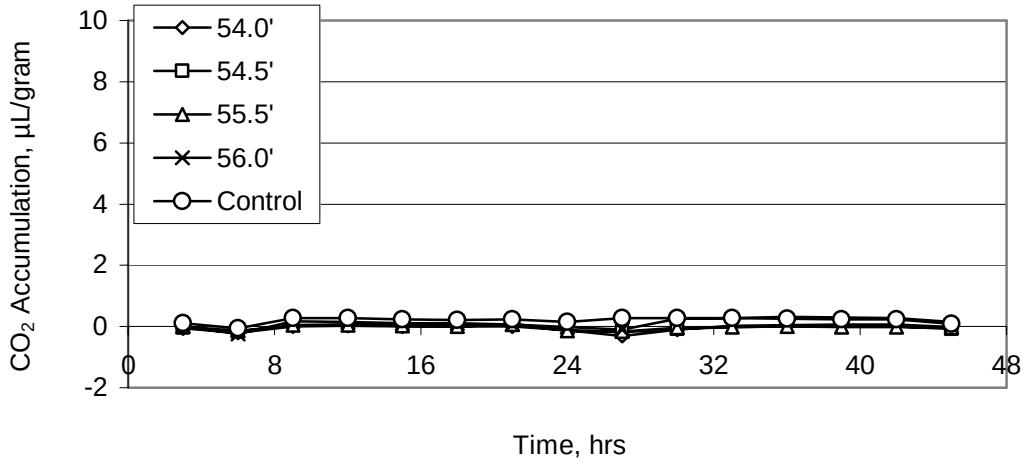

FIGURE 2B. Post-steam (one month) cumulative carbon dioxide production.

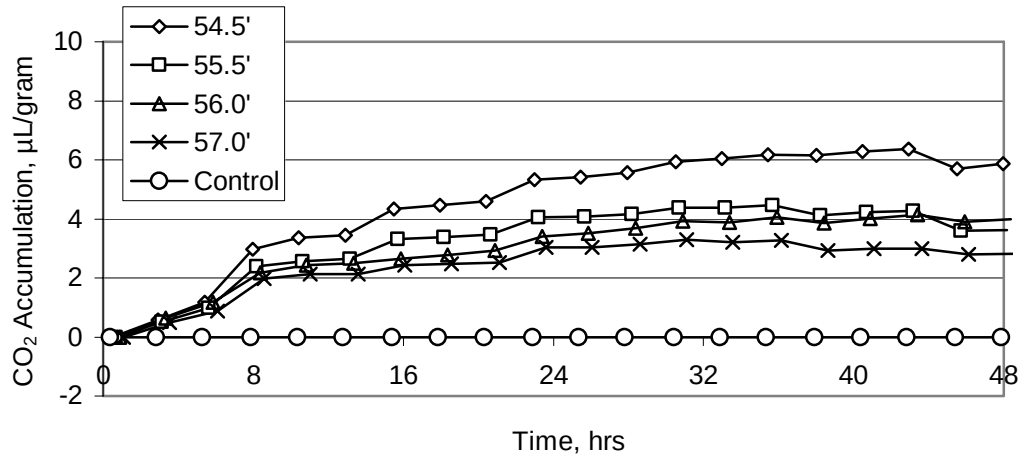

FIGURE 2C. Post-steam (eight months) cumulative carbon dioxide production.

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

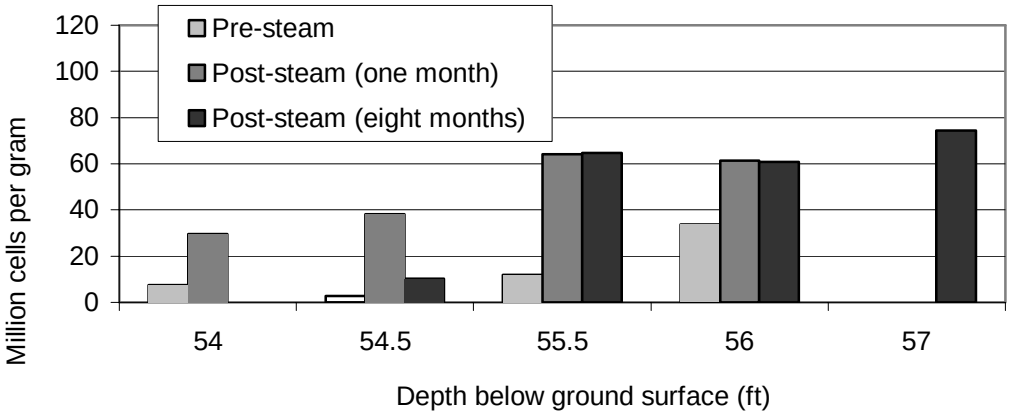

FIGURE 3. Comparison of all Core 1 direct count results.

Anaerobic Microcosms. Pre-steam microcosm samples were analyzed up to 135 days after incubation began and all of the samples below the NAPL smear zone showed significant methane production (up to 2700 ppm) at each of these sampling dates. Pre-steam hydrogen levels were as high as 20,000 ppm. 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. Very low levels of hydrogen (2 to 6 ppm) were detected in several of the microcosms.

Terminal Restriction Fragment (TRF) Analysis. 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. Sufficient DNA was extracted from 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.

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) at one month and eight months after steam treatment. Eight months following steam treatment the temperatures remained above 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).

Anaerobic microbial activity was significantly impacted by steam treatment. While pre-steam anaerobic microcosms generated methane and hydrogen, no methane and only minimal amounts of hydrogen were detected in the headspaces of post-steam one-month and eight-month microcosms even after incubating at 20°C for seven months and four months, respectively. Results from respirometry and plate counts were consistent, suggesting immediately after steam treatment the aerobic microbial population was drastically reduced and the aerobic microbial activity was minimal. While the post-steam (eight months) respirometry suggested some recovery of aerobic microbial activity, post-steam plate counts at eight months were of the same order of magnitude as the post-steam counts at one month. Post-steam (one and eight month) direct microscopic counts were generally the same or higher than the pre-steam counts. However, this does not necessarily mean that the total number of cells increased after steam, since the direct microscopic method counts total cells, dead or alive, these cells may be dead. . Direct counts are expected to be several orders of magnitude higher than are plate counts because only a fraction of the cells are expected to be culturable (Amann et al. 1995). Further, plate counts enumerate colonies and several cells may be clumped to form a colony. The post-steam and post-steam recovery direct counts were greater than the plate counts as expected. However, the pre-steam plate counts were higher than the pre-steam direct counts, possibly indicating error in the pre-steam direct counts or that the number of cells increased during the heating of the soil and then died as temperatures rose above the optimal range.

CONCLUSIONS

Immediately following steam treatment, when compared with pre-steam results:

- Both aerobic and anaerobic microbial activity is essentially zero.
- Active aerobic microbial populations are drastically reduced.
- The species diversity of the microbial community in groundwater was significantly reduced.

Eight months after steam, when compared with pre and one-month post-steam results:

- Aerobic microbial activity rebounded to about half of pre-steam.
- Aerobic microbial populations are still drastically reduced.
- Anaerobic microbial activity is still essentially zero.
- The composition of the microbial community is unknown.

Further studies should include the following:

- Conduct experiments at both pre and post-steam groundwater temperature, to account for thermophiles, if present.
- Follow up on steam pilot test site when groundwater temperature cools to 19°C.
- Evaluate alternative methods for characterizing the microbial community, which will be effective even for samples with little genetic material.

Therefore, the overall effect of the steam treatment is to drastically reduce the activity of aerobic and anaerobic microbes up to 8 months after cessation of steam injection.

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