

Final Report: Biodegradation of Petroleum Hydrocarbons in Salt-Impacted Soil by Native Halophiles or Halotolerants and Strategies for Enhanced Degradation

EPA Grant Number: R827015-01-0

Title: Isolation and Characterization of Aerobic and Anaerobic Hydrocarbon Degrading Bacteria From Oil-Brine Soil

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Project Amount: \$20,000

Research Category: Remediation

Objectives of the Research Project:

There are many exploration and production (E&P) sites in Oklahoma and throughout the world that are contaminated with both oil and salt. This poses a problem for cleaning up these sites using bioremediation technologies since high salinity will not support externally added bacteria. On the other hand, though degradation of petroleum compounds has been extensively studied, information on their degradation in oil-brine soil is scant. Therefore, the main purpose of this project was to demonstrate the potential of indigenous halophilic or halotolerant bacteria to degrade petroleum hydrocarbons in oil-brine soil and to try to optimize conditions for enhanced rates.

Project Summary:

Oil-Brine Soil: This project was initiated with soil samples obtained from the vadose zone at five contaminated E&P sites in Seminole and Stephens counties in Oklahoma. They are labeled Sem-1, Sem-2, Sem-3, Sem-4, and Stephens. Table 1 shows concentrations of benzene, toluene, ethylbenzene, total petroleum hydrocarbon (TPH), and chloride for each oil-brine soil used in this study.

Table 1. Soil Analysis

Soil Analysis*	Method (EPA)	Stephens	Sem-1	Sem-2	Sem-3	Sem-4
Sample depth (ft)		0-1.5	4	0-3	4	4
Benzene (mg/Kg)	8021B	BDL	4.48	3.41	1.72	0.149
Toluene (mg/Kg)	8021B	0.241	9.72	10.4	7.48	0.715
Ethylbenzene (mg/Kg)	8021B	BDL	30.2	7.95	14.1	3.04
TPH (mg/Kg)	8015M	125580	72744	6071	25198	120276
Chloride (mg/Kg)	325.3	1270	42200	14800	3740	220

*Soil analyses were performed by the Beacon Environmental Assistance, Corp., Edmond, OK.

We have used benzene as a model compound to assess the degradation of low molecular weight petroleum compounds in oil-brine soil under both aerobic and anaerobic conditions. Since, Sem-2 soil contained considerably less TPH and also was taken from the top (0 to 3 feet

depth), it was used to study benzene degradation under aerobic conditions. The other four soils were used to evaluate benzene degradation under anaerobic conditions since the samples were taken from greater depths (4 feet, except Stephens sample) and also had high levels of TPH.

Microcosm Preparation: The initial screening study was done using 70-ml capacity serum bottles containing 10 g (wet weight) brine soil and 40-ml mineral salts medium (MSM). Bottles were closed with Teflon-lined septa and aluminum caps. The bottles for the aerobic studies were supplemented with 100 mg of magnesium peroxide (MgO₂) and air in the headspace as the source of oxygen. Anaerobic microcosms were prepared similarly in an anaerobic glove box filled with N₂.

Mineralization of ¹⁴C-Benzene: The degradation potential of bacteria present in the brine soils was assessed by injecting a known amount of ¹⁴C-Benzene in microcosms under both aerobic and anaerobic conditions. A large number of both active and autoclaved control bottles were prepared in glove box using Sem-1, Sem-3, Sem-4, or Stephens. The ability of benzene mineralization under aerobic conditions was evaluated by using the Sem-2 culture instead of soil. All bottles were spiked with 100-μL stock ¹⁴C-benzene (specific activity of ¹⁴C-benzene = 33.2 mCi/mmol). This amounts to 27,500 dpm/ml. Bottles were closed with Teflon-coated septa and aluminum caps and incubated static at 30°C in the dark. At the end of 0, 4, 8, and 12 weeks of incubation, triplicate active and duplicate control bottles were sacrificed by injecting approximately 0.2-ml of 10 N HCl (pH < 2). It is anticipated that under strong acidic conditions, all aqueous ¹⁴CO₂ (¹⁴C-HCO₃⁻) is converted to gaseous ¹⁴CO₂. The bottles were then purged with N₂ (80-100 ml/min) for 40 min and the ¹⁴CO₂ was trapped in 5 glass vials connected in series. Each vial contained 10-ml of Harvey's ¹⁴C-Cocktail and was sealed with a Teflon-coated septum and an aluminum cap. Radioactivity in all 5 traps was measured using a Beckman LS 6000SC liquid scintillation counter (LSC) (Beckman Instruments, Inc., Fullerton, CA). The ability of benzene mineralization under aerobic conditions was evaluated by using the Sem-2 culture instead of soil.

As shown in Table 2, at the end of three months of incubation, about 5 to 10% of the initially added ¹⁴C-benzene was mineralized to ¹⁴CO₂ above the control in microcosms prepared with Sem-1, Sem-3, and Sem-4 under anaerobic conditions. However, no degradation was seen in microcosms prepared with soil from the Stephens County. Although, the removal of benzene under anaerobic conditions seem poor, the rate and extent of degradation could be enhanced under more optimal growth conditions. Results show that a significant amount of benzene was mineralized by the Sem-2 enrichment culture under aerobic conditions.

Table 2. Mineralization of Benzene by Halophiles Under Aerobic & Anaerobic Conditions

Soil Type	Sem-1	Sem-2	Sem-3	Sem-4	Stephens
Incubation	Anaerobic	Aerobic	Anaerobic	Anaerobic	Anaerobic
Time	12 weeks	4 weeks	12 weeks	12 weeks	12 weeks
% ¹⁴C-Benzene converted to ¹⁴CO₂*	6 ± 1	46 ± 14	10 ± 6	4.6 ± 0	< 1%

* ¹⁴C-CO₂ recoveries are percents above the control bottles.

Development of Sem-2 Enrichment: A highly enriched microbial culture was developed from the Sem-2 soil using benzene as the sole carbon source in MSM containing 2.5 M NaCl. After more than 7 months of continuous enrichment process, the Sem-2 consistently degraded added benzene below GC detection level within 2.5 weeks (Figure 1). All optimization studies were conducted using the sem-2 culture as the inoculum.

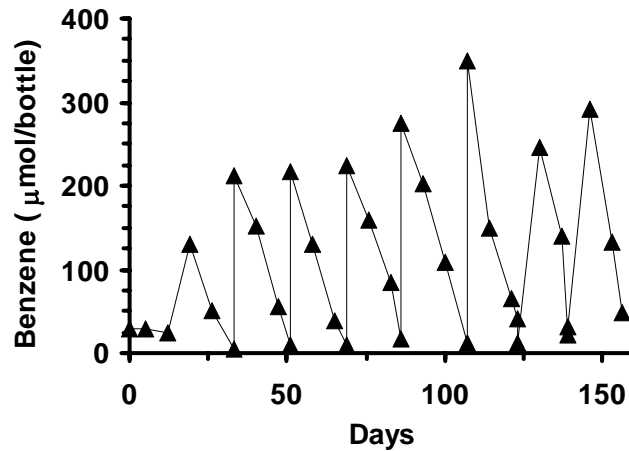


Figure 1 shows the degradation of benzene after repeated spikes by the Sem-2 enrichment culture. Results for only one bottle are shown, duplicate enrichment degraded similarly.

Effect of Osmolytes: Studies were carried out to test if the addition of commercially available osmolytes stimulated benzene degradation. Our data revealed that none of the tested osmolytes showed enhanced benzene degradation. On the contrary, the rates were lower than when no osmolyte was present in the growth medium (Table 3). The exact reason for the lack of stimulation by the tested osmolytes is not known. It is possible that perhaps bacteria were able to synthesize their own osmolytes or the added osmolyte was used a carbon source thus resulting in the inhibition of benzene degradation. Alternatively, the tested osmolytes were not specific to indigenous halophiles or halotolerants.

Table 3. Effect of Osmolytes on Benzene Biodegradation

Osmolyte	Concentration	Percent Degradation	Days
No osmolyte	NA	97.77 ± 0.9	12
Glycine	1M	62.69 ± 0.2	61
Proline	1M	42.30 ± 4.0	61
Betain	1M	67.25 ± 2.0	61
KCl	1M	56.41 ± 2.0	61

Degradation of Alkylated Benzenes: We also evaluated the ability of the Sem-2 culture to degrade other low molecular weight petroleum compounds including toluene, ethylbenzene, and *o*-, *m*-, and *p*- xylene. Triplicate active microcosms were established in 120-ml serum bottles with 45-ml MSM and 5-ml (10%) of the Sem-2 culture. Autoclaved control bottles were prepared similarly. As shown in Figure 2, the culture degraded benzene, toluene, ethylbenzene, or xylenes as the sole carbon source. Among the tested compounds, toluene was degraded fastest. Complete degradation of toluene occurred in < 1 week, while, benzene, ethylbenzene, or xylenes needed roughly 2 to 3 weeks.

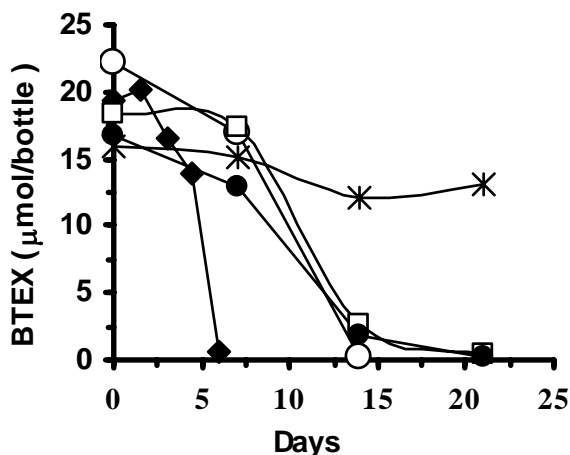


Figure 2. Biodegradation of benzene, (○); toluene, (◆); ethylbenzene, (□); xylenes, (●) in bottles containing 45-ml MSM containing 2.5M NaCl and inoculated with 5-ml of Sem-2 enrichment. The data are averages of triplicate active and duplicate control bottles. Since all controls behaved similarly, only control for xylenes (★) is shown.

Biodegradation of Benzene at Varied Salt Concentrations: Further tests included determining the ability of the Sem-2 culture to degrade benzene in the presence of varied NaCl concentrations ranging from 0 to 4 M. As seen in Figure 3, benzene was degraded in the presence of 0.5, 1.0, 2.0 and 2.5 M NaCl. No degradation occurred in the bottles amended with higher concentrations including 3 and 4 M NaCl. Also, no degradation of benzene occurred in the absence of added salt suggesting that the culture is a true halophile and requires salt for growth.

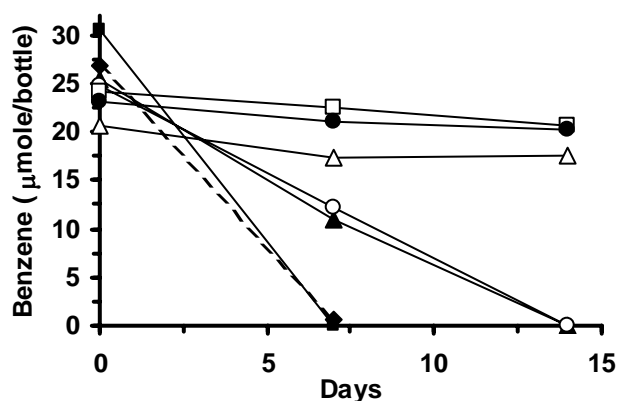


Figure 3. Biodegradation of benzene by the Sem-2 enrichment at different salt concentrations (NaCl). All microcosms were spiked with benzene (22 $\mu\text{mol}/\text{bottle}$) and varied concentrations of NaCl, including 0 M, (Δ); 0.5 M, (\blacklozenge); 1 M, (\blacksquare); 2 M, (\blacktriangle); 2.5 M, (\circ); 3 M (\square); or 4 M (\bullet). The results are means of triplicate active microcosms.

Impact of Salt on Microbial Community Structure: The purpose of this work was to identify the impact of salt on the dominant microbial populations of the Sem-2 enrichment. Bacterial DNA was extracted from the enrichment culture grown with 0 M and 2.5 M NaCl and amplified using a 16S rDNA universal primer and polymerase chain reaction (PCR). The PCR products were then separated using denaturing gradient gel electrophoresis (DGGE). DGGE profiles of the culture showed the presence of four major bands labeled A, B, C, and D when grown in the presence of 2.5 M NaCl and 30 ppm of benzene as the sole carbon source (Figure 4). However, similar bands were missing in the DGGE profile obtained from the culture grown 30 ppm benzene in the absence of added salt. The bands were excised, reamplified and sequenced. The sequences were compared to sequences in the GenBank database. The results show that all the four bands showed > 99% sequence homology with the genus *Marinobacter*. The bands labeled F did not yield enough information for sequence identity. The *Marinobacter* spp have been isolated from geographically different locations including the French Mediterranean coast, at the mouth of a petroleum refinery outlet, from deep-sea sediments in the Western Pacific, and from oil wells off the coasts of Vietnam and California

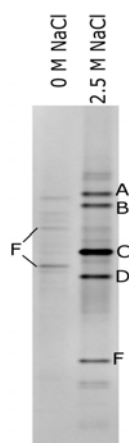


Figure 4. The differing banding patterns between the 0M and 2.5M NaCl samples indicate different microbial communities at the two salt concentrations.

Conclusions:

Our work clearly demonstrated that benzene could be mineralized to CO_2 by aerobic and anaerobic bacteria under hypersaline conditions. Although the extent of benzene degradation under anaerobic conditions is not significant, it could be improved through optimization studies. We have successfully enriched halophilic microbial populations capable of assimilating benzene, toluene, ethylbenzene and xylenes under aerobic conditions. The microbial community structure analysis using DGGE revealed that *Marinobacteria* spp. were the dominant organisms in the

Sem-2 when grown in the presence of 2.5 M salt and these bacteria were not detected (not dominant) in the same culture when grown at 0 M salt. Thus suggesting that perhaps *Marinobacteria* are responsible for the observed benzene degradation under hypersaline condition. These observations provide hope that cost-effective technologies can be designed to treat EP sites that have been contaminated with high concentrations of salt and low molecular weight petroleum compounds.

Publications and Presentations:

1. Carla A. Nicholson and Babu Z. Fathepure (2004) Biodegradation of benzene by halophilic and halotolerant bacteria under aerobic conditions. *Appl. and Environ. Microbiol.* 70: (*In Press*).
2. Caral A. Nicholson and Babu Z. Fathepure (2004) Aerobic biodegradation of benzene and toluene by extremophiles from the Great Salt Plains, Oklahoma. *FEMS. Microbiol. Ecol.* (*Submitted*).
3. Caral Nicholson and Babu Fathepure (2004) Aerobic Biodegradation of Benzene by Halophilic and Halotolerant Bacteria at the Great Salt Plains, OK. American Society of Microbiology 104th General Meeting, New Orleans, LA. May 23-27 (*abstract submitted*)
4. Carla Nicholson and Babu Fathepure (2003). Biodegradation of benzene under aerobic and anaerobic conditions by halophilic bacteria. Poster Q-035 at American Society of Microbiology 103rd General Meeting, Washington, D.C. May 18-22.
5. Babu Fathepure and Carla Nicholson (2003) Aerobic Biodegradation of benzene, toluene, ethylbenzene, and xylenes by halophilic and halotolerant bacteria. Abstract. International Petroleum Environmental Conference. Houston, TX. November 11-14.
6. Babu Fathepure and Carla Nicholson (2002) Biodegradation of benzene in salt-impacted soil by halophilic and halotolerant bacteria. Abstract. International Petroleum Environmental Conference, Albuquerque, NM. October 22-25.

Supplemental Keywords:

bioremediation, halophilic, halotolerant, BTEX, enrichment culture, osmolytes, denaturing gradient gel electrophoresis