A KINETIC STUDY OF AEROBIC PROPANE UPTAKE AND COMETABOLIC DEGRADATION OF CHLOROFORM, *CIS*-DICHLOROETHYLENE AND TRICHLOROETYLENE IN MICROCOSMS WITH GROUNDWATER/AQUIFER SOLIDS

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Abstract. The focus of this study was to compare the behavior of different consortiums of aerobic propane-utilizing microorganisms, with respect to both the lag time for growth after exposure to propane, and their ability to transform three chlorinated aliphatic hydrocarbons (CAHs): chloroform (CF), cis-dichloroethylene (c-DCE) and trichloroethylene (TCE). Thirty-three slurry microcosms, representing seven combinations of aquifer solids and groundwater were constructed for this study. The lag time required for establishing propane-utilizing consortiums ranged between 24 and 29 days in 6 of the 7 combinations. Kinetic tests were performed with respect to propane utilization and CAH transformation. After CAH exposure, the ability of the microorganisms to metabolize propane was significantly reduced. CF and TCE were transformed more slowly than c-DCE, the average values of the initial transformation rates being equal to 0.10 ± 0.04, 0.09 ± 0.05 and 0.98 ± 0.18 μmol/(L h), respectively. CF caused the greatest reduction in propane uptake rates, whereas c-DCE exhibited an apparently reversible negative effect on propane uptake rates. The estimates of the Monod half-saturation constants relative to CF, TCE and c-DCE resulted in the 2–3 μmol/L range, but were characterized by a high degree of uncertainty.

Keywords: bioremediation, chlorinated solvents, cometabolism, microcosms, propane

1. Introduction

Chlorinated solvents, such as trichloroethylene (TCE), *cis*-dichloroethylene (c-DCE) and chloroform (CF) are widely observed as subsurface contaminants. The employment of propane as a cometabolic substrate for the aerobic transformation of chlorinated aliphatic hydrocarbons (CAHs) has been investigated in several studies, with specific reference to trichloroethylene (TCE), chloroform (CF), 1,2-dichloroethane (1,2-DCA), *cis*-dichloroethylene (c-DCE) and 1,1,1-trichloroethane (1,1,1-TCA), both at laboratory and field scale (Chang and Alvarez-Cohen, 1995; Tovanabootr and Semprini, 1998; Kim *et al.*, 1997; Keenan *et al.*, 1994; Wilcox *et al.*, 1995; Wackett *et al.*, 1989). These studies report longer lag

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times for the adaptation of unspecialized biomasses to propane (24–25 days) than to methane (6–10 days) or butane (10–20 days), and show that propane-grown mixed cultures have a higher ability to deplete CAHs such as TCE after propane is consumed, if compared to methane-grown cultures. This behavior might be ascribed to a higher capacity to store energy reserves. Propane presents several advantages over more conventional growth substrates for cometabolic processes such as methane, toluene and phenol: it has a high solubility in water (62.4 mg/L at 1 atm and 25 °C; McKay and Shiu, 1981); it is nontoxic and not regulated (Tovanabootr and Semprini, 1998); biomass yields with propane are higher than with other substrates (0.8–0.9 mg/mg; Chang and Alvarez-Cohen, 1995). The available literature on the use of propane as a carbon source reports that most propane-utilizing bacteria are Gram-positive bacteria such as *Corynebacterium, Nocardia, Mycobacterium* or *Rhodococcus*, and that at least four pathways of terminal or sub-terminal propane oxidation are possible, all of which are mediated by oxygenase enzimes (Perry, 1980; Ashraf *et al.*, 1994).

The purpose of this study was to compare different consortiums of aerobic propane-utilizing microorganisms with regard to their ability to metabolize propane and to degrade TCE, c-DCE and CF. More specifically, the focus was on determining: (1) lag times for growth after exposure to propane; (2) rates of propane utilization; (3) ability to cometabolically transform TCE, c-DCE and CF, and (4) the effect transformation of CAHs had on the microorganisms' ability to metabolize propane. For these tests, thirty-three slurry microcosms were constructed that combined two types of aquifer solids from contaminated sites and four different groundwaters, yielding seven combinations of conditions. These combinations were tested to determine optimum conditions to use in assembly of a large-scale physical aquifer model given limited availability of specific aquifer solids and groundwater. Propane utilization rates and CAH transformation rates were chosen as the most important indicators for successful, stable operation of the large-scale model. Particular attention was given to specific aspects poorly investigated in previous studies, such as the effectiveness of different ways of bioaugmenting aquifer materials with unspecific and propane-grown cultures and the bacterial ability to recover after the exposition to toxic products of CAHs degradation.

During the first phase of the experiment, all of the microcosms were repeatedly exposed to high doses of propane (3% *vol* in headspace); during the second phase, some of the microcosms were exposed to alternated pulses of propane and either TCE, c-DCE or CF, whereas others were fed propane only as control microcosms. The concentrations of propane and CAHs were frequently monitored over a period of 4 months. The results were fit with a kinetic model of aerobic cometabolism in order to estimate transformation rate coefficients.

2. Materials and Methods

2.1. Microcosm preparation

The microcosms were prepared using 155-mL amber serum bottles with Teflon-lined rubber septa. Each microcosm contained 25 g of wet solids, 70 mL of groundwater and 75 mL of headspace air. Bottles, caps, synthetic groundwater and all the tools utilized for constructing the microcosms were autoclaved prior to use. Microcosms were constructed under a sterile laminar-flow hood. Propane (2.25 mL; 94 μmoles), corresponding to a headspace concentration of 3%, was added to each microcosm using a 1-mL gastight syringe (Hamilton Co., Reno, NV).

2.2. Microcosm composition

Aquifer solids were obtained from two different CAH-contaminated sites: McClellan Air Force Base (CA), and the Hanford Department of Energy Site (WA). CF is present at the Hanford site as a transformation product of carbon tetrachloride contamination, and c-DCE and TCE are primary contaminants of the McClellan AFB site. Limited solids were available from McClellan AFB, where a demonstration of cometabolic air sparging with 2% propane was being conducted in an aquifer zone contaminated with TCE, c-DCE and trace amounts of CF. Large volumes of solids were available from the Hanford Site, but their potential for propane-utilization and CAH transformation was untested. At each site, several cores were obtained during the construction of different monitoring wells at different depths. The cores were highly homogeneous. Core samples were mixed to yield a representative sample for each site. Groundwater was available from McClellan Air Force Base, but not from the Hanford Department of Energy Site. Both the McClellan and Hanford solids, as well as the McClellan groundwater, contained only negligible traces of the original CAH contamination.

Table I reports the 7 combinations that were utilized in order to establish the different consortiums of propane-utilizing microorganisms. Three microcosms were set up for each combination, except for combination #3 (9 microcosms). The first combination was prepared by combining McClellan aquifer solids and McClellan groundwater. In order to keep nitrogen from becoming a limiting nutrient, sodium nitrate was added to yield a groundwater nitrate concentration of 50 mg/L. Since Hanford groundwater was not available, in the second combination, Hanford aquifer solids were mixed with filtered (0.1 μm) McClellan groundwater amended with nitrate, to provide appropriate micronutrients, but devoid of McClellan microorganisms. In the third combination, Hanford aquifer solids were mixed with a synthetic groundwater that had the same anion-cation concentration as the McClellan groundwater, and a nitrate concentration of 50 mg/L. The synthetic groundwater was prepared by adding the following salts (mg) to 1 L of deionized water: $CaCl_2$ 52.59, $MgCl_2$ 34.48, $NaHCO_3$ 56.97, $NaNO_3$ 59.00, $MgSO_4$

TABLE I

Combinations of aquifer solids and groundwater tested; lag times and average rates of propane uptake

Microcosm set	Type of aquifer solids	Type of groundwater	Number of microc.	T_{50} (d)		R_m (μmol/L h)	
				Average value	95% Conf. int.	Average value	95% Conf. int.
1	100% McClellan	McClellan	3	24.4	2.2	13.2	5.0
2	100% Hanford	McClellan filtered 0.1 μm	3	54.2	13.7	2.2	3.0
3	100% Hanford	Synthetic	9	24.0	4.5	9.2	0.9
4	95% Hanford, 5% McClellan	McClellan	3	23.9	4.2	8.5	2.1
5	90% Hanford, 10% McClellan	McClellan	3	23.6	2.2	10.7	3.4
6	50% Hanford, 50% McClellan	McClellan	3	25.7	3.8	10.9	2.9
7 [a]	100% Hanford	Synthetic	3	29	4.5	19.7	1.7

[a] With 100-μL inoculum from combination #1. T_{50}, time for consumption of 50% of the mass of propane initially spiked; R_m, average rate of propane uptake during the first pulse.

17.84, KH_2PO_4 3.45, $Fe(NO_3)_3$ 0.43, $Mg(NO_3)_2$ 4.26, $MnSO_4$ 0.06, $Ni(NO_3)_2$ 0.47, $CoCl_2$ 0.004; NaOH 0.001 N was added to raise the pH to 7. In combinations #4, 5 and 6 McClellan groundwater enriched with nitrate was utilized, and Hanford solids were inoculated with increasing amounts of McClellan solids. This was done to enrich the Hanford microbial population with varying amounts of McClellan microorganisms. This choice was based on the assumption that the McClellan solids contained a significantly higher microbial population than the Hanford solids. Finally, the seventh combination was obtained by combining Hanford solids and synthetic groundwater (like in combination #3), and adding a 100-μL inoculum obtained from the microcosms of combination #1, after complete development of a propane-utilizing microbial consortium. This combination evaluated the potential for bioaugmenting Hanford aquifer material with microorganisms from the McClellan site.

2.3. MICROCOSM OPERATION

Microcosms were incubated at 20 °C and placed on a shaker table at 120 rpm. After the initial propane was utilized, four more additions of propane (2.25 mL) were made, in order to obtain the complete development of a propane-utilizing consortium in each microcosm. Before each addition of propane, atmospheric pressure was re-established in the headspace by addition of pure oxygen, in order to maintain aerobic conditions. Subsequently, sets #5 and 6 were used to test for CF transformation, whereas microcosm sets #1, 3 and 7 were used initially for an experiment of c-DCE transformation, and subsequently for an experiment of TCE transformation. In each experiment, the addition of propane (3.5 to 9 μmoles) was followed by one or two additions of CAH (0.1 to 0.8 μmoles), which in turn was followed by a final spike of the same amount of propane initially provided. The simultaneous presence of propane and contaminant was avoided, so that the estimation of the kinetic parameters was not affected by enzyme competition between propane and the CAH. The details of each experiment are provided in Table II. The three solvents were taken from saturated solutions obtained by adding 4 mL of pure CAH in a 125-mL capped serum bottle, which was shaken and allowed to settle for 12 hours before use. Propane and CAH concentrations were measured every 30–60 min during each experiment, corresponding to 8–15 analysis for each degradation curve.

2.4. ANALYSIS AND CHEMICALS

Propane, c-DCE, TCE and CF were measured in the headspace samples from the microcosms. The total mass of propane and CAH in each microcosm was calculated by mass balances, assuming Henry's law equilibrium partitioning between liquid and gas phase. The Henry's Law constants used are those reported by Mackay and Shiu (1981). Propane, c-DCE and TCE were measured with a Hewlett

TABLE II
CAH transformation tests

	CAHs tested		
	CF	c-DCE	TCE
Microcosm sets	5, 6	1, 3, 7	1, 3, 7
Number of microcosms	4	9	8
Sequence of amendments	Propane/CF/ propane	Propane/c-DCE/ c-DCE/propane	Propane/TCE/ propane
Propane added (μmol)	9	3.8 to 4	3.5
CAH added (μmol)	0.71 to 0.80	0.42 to 0.58 (each time)	0.10
Initial CAH concentration in aq. phase (μmol/L)	8.0 to 9.0	4.7 to 5.5	1.5

Packard 6890 GC equipped with a capillary J&W GS-Q column (divinylbenzene homopolymer, 30 m, 0.53 mm), a photo ionization detector (for c-DCE and TCE) and a flame ionization detector (for propane), using helium as a carrier gas (15.1 mL/min). Inlet temperature and pressure were 250 °C and 15 psi respectively; the oven had an initial temperature of 105 °C (2 min), and was raised to 185 °C (1 min) with a 40 °C/min rate; the FID was operated at 250 °C, with a 15 mL/min make-up flow (He), a 165 mL/min air flow and a 35 mL/min H_2 flow. CF was measured using a Hewlett Packard 5890 GC equipped with a capillary J&W GS-Q column (divinylbenzene homopolymer, 30 m, 0.53 mm) and an electron capture detector, using an argon/methane (95/5) mixture as a carrier gas (6.5 mL/min). Inlet temperature and pressure were 250 °C and 60 psi respectively; the oven was operated isothermally at 80 °C, whereas the ECD was operated at 200 °C. For all compounds, a 100-μL headspace sample was taken from the microcosms using a 100-μL gastight syringe (1700 series, Hamilton Co., Reno, NV). All of the methods were calibrated using external standards. Calibrations were made in the following ranges (μmol/L): CF: 0.1–20; c-DCE: 0.07–10; TCE: 0.07–1.7. Detection limits were, respectively, 0.05, 0.01 and 0.008 μmol/L. Propane (>99.9%), TCE (>99%), c-DCE (>99.5%) and CF (>99.9%) were purchased from Aldrich Chemical Co. (Milwaukee, WI).

3. Results and Discussion

3.1. Determination of the lag time to establish propane-utilizing consortiums

The first part of the study focused on the determination of the lag time for the onset of utilization of the propane initially spiked. The lag time for substrate utilization is an important design parameter for *in situ* and *on-site* cometabolic bioremediation treatments, and can be ascribed to either low numbers of specialized microorganisms in the indigenous biomass or to slow growth rates (Tovanabootr and Semprini, 1998). The time required for 50% uptake of the initial amount of propane (T_{50}) was chosen as the best parameter to characterize the behavior of the different propane-utilizing microbial consortiums during the initial phase. In addition, average propane uptake rates obtained during the initial pulse were measured. The results are reported in Table I in terms of average values obtained in each combination and 95% confidence intervals. Microcosm sets #1, 3, 4, 5, 6 and 7 had similar lag times, ranging from 24 and 29 days, and average rates in the 9–20 μmol/(L-h) range; the behavior of the microcosms was relatively uniform within each group. On the contrary, set #2 was characterized by a significantly longer T_{50} (54 $\pm$ 14 days), a lower rate of propane uptake (2.2 $\pm$ 3.0 μmol/(L-h)) and an inhomogeneous behavior of the microcosms. These results indicate that, within the sets of microcosms tested, the time lag for the establishment of a propane-utilizing consortium was weakly affected by the type of solids and groundwater utilized, with the exception of set #2.

3.2. Evaluation of kinetic parameters relative to the uptake of propane and to the transformation of CF, C-DCE and TCE

As an example, the data relative to the experiment of c-DCE transformation in the 3 microcosms corresponding to set #3 are represented in Figure 1. In order to obtain estimates of kinetic parameters, the experimental data relative to the 3 experiments described in §2.3 and Table II were fit to a model of aerobic cometabolism which, in the absence of competition between substrate and contaminant, can be expressed by the following Monod-type equation, valid for both the substrate and the contaminant:

$$\frac{dm}{dt} = -\frac{K_{max} \cdot X \cdot C_L}{K_s + C_L} \cdot V_L \,, \tag{1}$$

where m is the total mass of substrate or contaminant in the microcosm (μmol), C_L is the concentration in aqueous phase (μM), V_L is the volume of the aqueous phase (L), X is the concentration of active biomass (mg/L), K_s is the half-saturation constant (μM) and K_{max} is the maximum specific transformation rate (μmol/mg cell/hr).

TABLE III
Average estimates of initial propane uptake rates (R_i) before and after CAH transformation, with % variation

CAH	Microcosm set	$C_{s,i}$ (μmol/L)	$R_{i,1}$ (before CAH transf.) (μmol/L h)		$R_{i,2}$ (after CAH transf.) (μmol/L h)		Variation of R_i	
			(1)	(2)	(1)	(2)	(1)	(2)
CF	#5	4.5	5.7	5.7	1.3	1.2	–76%	–79%
	#6		5.7		1.0		–82%	
c-DCE	#1	2.1	11.6	9.4	5.9	4.3	–49%	–55%
	#3		10.1		3.6		–65%	
	#7		6.6		3.3		–50%	
TCE	#1	1.8	11.8	11.0	9.2	8.8	–21%	–20%
	#3		13.7		8.9		–47%	
	#7		7.9		8.5		+7%	

$C_{s,i}$, initial propane concentration in the aqueous phase.
(1) Average estimate in the microcosm set; (2) overall average estimate.

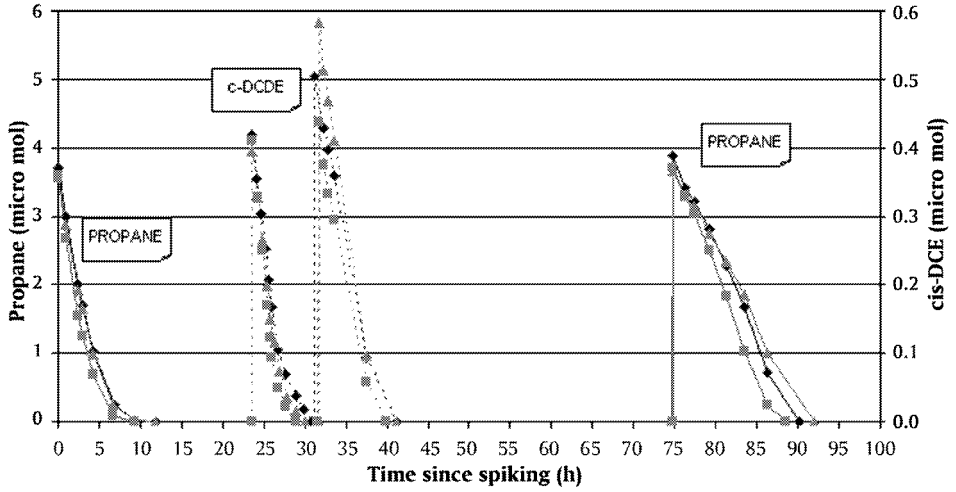

Figure 1. Experiment of c-DCE degradation in the 3 microcosms corresponding to set # 3 (Hanford soil and synthetic groundwater). Propane (continuous line) and c-DCE (broken line) are expressed as total mass in the microcosm (μmol).

Table III reports the average estimates of the initial rates of propane uptake obtained in each experiment, before and after the transformation of the chlorinated solvent of interest. In all cases except one, there was a decrease of the microbial ability to utilize propane; the highest average decrease was associated with the transformation of CF (79%), and corresponded to the most contaminant transformed in a single spike (0.71 to 0.80 μmol), whereas the lowest average decrease was associated with the transformation of TCE (20%), and corresponded to the least contaminant transformed (0.10 μmol). The diminished propane uptake rates after CAH transformation may have been due to toxic effects of the CAH transformation products on the propane-utilizing microorganisms (Chang and Alvarez-Cohen, 1995). In each experiment, two control microcosms were utilized. The first one was subjected to the same sequence of propane additions, without any addition of CAH, in order to check for possible other reasons of decrease in microbial ability to metabolize propane; no decrease in propane uptake rates was observed in these microcosms. The second one was exposed to the CAH without any prior exposition to propane, in order to test for non-cometabolic degradation processes; negative results were obtained in all the three experiments.

After conclusion of the c-DCE transformation test, 50 μmoles of propane were added to microcosm sets #1, 3 and 7 to aid in the recovery of the microbial consortiums. In all the microcosms except one, propane was utilized with average rates comparable to that observed during initial propane uptake (13 to 31 μmol/(L-h)). Only one microcosm, from microcosm set #1, was significantly affected by c-DCE transformation, resulting in a reduced rate of propane uptake of 0.5 μmol/(L-h). Subsequently, 3.5 μmoles of propane were added in the same microcosms (except

the one that had proved most damaged), and the initial rate of propane uptake was again estimated. The average values obtained for each microcosm set proved slightly higher than the ones obtained before the degradation of c-DCE, with an average increase equal to 17%. These results indicate that, in 8 out of the 9 microcosms tested in this experiment, the uptake of one high dose of propane was sufficient to provide full recovery of the propane-utilizing microbial consortiums.

The average estimates of initial transformation rates for c-DCE, TCE and CF are reported in Table IV. The transformation rate for c-DCE was approximately one order of magnitude higher than those for TCE and CF. This can be related neither to the initial solvent concentration in the liquid phase (CF>c-DCE>TCE), nor to significant differences of active biomass for microcosm sets #1, 3 and 7, based on initial propane uptake rates before c-DCE and TCE transformation. Therefore, the differences in the transformation rates of the three chlorinated solvents must be ascribed to differences in the microorganisms' capacity for transforming the different CAHs.

If initial CAH concentrations are high enough to approximate zero-order transformation kinetics (as predicted in Monod-type Equation (1)), half-saturation constants (K_s) can be estimated by comparing the observed rates at high CAH concentrations [pseudo-zero-order transformation: $(C_L/(C_L + K_s) \sim 1]$ to those observed at low CAH concentrations [pseudo-first-order transformation: $(C_L/(C_L + K_s) \sim C_L/K_s]$. In this manner, average estimates of the half-saturation coefficient (K_s) for CF, c-DCE, and TCE were calculated and are reported in Table V. The comparison between the initial CAH concentrations and the estimated K_s values indicates that actual zero-order behavior was not achieved in any of the microcosm sets tested. Initial CAH concentrations for CF and c-DCE represent approximately 2.5 to 3 times the estimated K_s values, indicating that the measured initial rates correspond to about 75% of the maximum specific transformation rate ($X\ K_{max}$). On the contrary, initial TCE concentrations were below the estimated K_s value, which indicates that in no way the measured initial rates represent maximum transformation rates. The K_s values estimated in this manner contain a high degree of uncertainty and represent a first-cut approximation that could be refined with transformation tests over greater CAH concentration ranges. Nonetheless, the estimated K_s values are similar to the ones obtained in previous studies relative to the aerobic degradation of both TCE and c-DCE (Smith *et al.*, 1997). K_s provides a useful indication of the microbial capacity to degrade low concentrations of CAHs.

4. Conclusions

The results confirm that unspecialized biomasses are characterized by long lag times of adaptation to propane (25–30 days, almost independently of the aquifer materials utilized) in comparison with the lag times typically observed for growth on other substrates (such as methane, toluene or phenol), and that propane-grown

TABLE IV
Average estimates of CAH initial transformation rates

CF				c-DCE				TCE			
Microcosm set	$C_{c,i}$ (μmol/L)	Average estimate (μmol/Lh)	95% Confidence interval	Microcosm set	$C_{c,i}$ (μmol/L)	Average estimate (μmol/Lh)	95% Confidence interval	Microcosm set	$C_{c,i}$ (μmol/L)	Average estimate (μmol/Lh)	95% Confidence interval
#5	8.0	0.10	[a]	#1	5.0	0.74	0.11	#1	1.5	0.02	[a]
#6	9.0	0.10	[a]	#3	5.5	1.39	0.25	#3	1.5	0.17	0.02
				#7	4.7	0.81	0.18	#7	1.5	0.07	0.04
Overall average	8.5	0.10	0.04	Overall average	5.1	0.98	0.18	Overall average	1.5	0.09	0.05

[a] Not enough data are available to calculate the 95% confidence interval; $C_{c,i}$ initial aqueous concentration.

TABLE V
Average estimates of Monod half-saturation constants (K_s) for c-DCE, TCE and CF

CF			c-DCE			TCE [c]		
Microcosm set	Average estimate (μmol/L)	95% Confidence interval	Microcosm set	Average estimate (μmol/L)	95% Confidence interval	Microcosm set	Average estimate (μmol/L)	95% Confidence interval
#5	2.3	[a]	#1	1.9	0.7	#1	[b]	[b]
#6	3.7	[a]	#3	1.9	0.2	#3	1.9	[a]
			#7	2.1	0.2	#7	2.3	0.3
Overall average	2.7	1.4	Overall average	2.0	0.2	Overall average	2.1	0.2

[a] Not enough data are available to calculate the 95% confidence interval.
[b] No first-order behavior was observed.
[c] High degree of uncertainty in estimate.

mixed cultures have a potential to effectively transform several chlorinated alkenes (c-DCE and TCE) and alkanes (CF), even in the absence of the growth substrate. After adaptation to propane, no further lag time was required for the onset of CAH transformation. The propane-utilizing cultures exhibited a high capacity to regain the ability to metabolize propane after the damage inflicted by exposure to CAH transformation products.

Little difference in performance was seen over the 7 combinations of aquifer materials tested. The Hanford solids exhibited a high potential for the development of propane-utilizing cultures and for the biodegradation of several CAHs, which was not initially expected on the basis of the low moisture tenor.

The experimental method utilized in this study proved an effective tool for evaluating the CAH transformation potential of aquifer materials and the effectiveness of bioaugmentation techniques. The method was also useful in providing a first-cut approximation of the design parameters required for large-scale physical aquifer models and *in situ* bioremediation treatments of CAH-contaminated sites with propane-utilizing microbes.

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