



## Push–pull tests evaluating in situ aerobic cometabolism of ethylene, propylene, and *cis*-1,2-dichloroethylene

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### Abstract

In situ aerobic cometabolic transformations of ethylene, propylene, and *cis*-1,2-dichloroethylene (c-DCE), by microorganisms stimulated on propane, were examined in groundwater contaminated with c-DCE and trichloroethylene (TCE). In situ measurements were performed by conducting field push–pull tests, which consisted of injecting site groundwater amended with a bromide tracer and combinations of propane, dissolved oxygen (DO), nitrate, ethylene, propylene, c-DCE, and TCE into existing monitoring wells and sampling the same wells over time. Mass balance and transformation rate calculations were performed after adjusting for dilution losses using measured tracer concentrations. Initial rates of propane utilization were very low; rates increased substantially following sequential additions of propane and DO. Evidence that propane and DO additions had stimulated organisms expressing a propane monooxygenase enzyme system and that had the capability to transform chlorinated aliphatic hydrocarbons (CAHs) included: (1) the transformation of injected ethylene and propylene to the cometabolic byproducts ethylene oxide and propylene oxide, (2) the transformation of c-DCE, and (3) the inhibition of these transformations in the presence of coinjecting acetylene, a known monooxygenase mechanism-based inactivator. These results suggest that a series of push–pull tests performed with nontoxic chemical probes can be useful for detecting and monitoring in situ aerobic cometabolism of CAHs.

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## 1. Introduction

Stimulation of the propane monooxygenase enzyme system in laboratory studies has been shown to initiate the aerobic cometabolic transformations of chlorinated aliphatic hydrocarbons (CAHs) including trichloroethylene (TCE) and *cis*-1,2-dichloroethylene (c-DCE) (Vanderberg et al., 1995; Vanderberg and Jerry, 1994; Wackett et al., 1989). Cometabolism of ethylene and propylene to their corresponding epoxides (ethylene oxide and propylene oxide) by the propane monooxygenase enzyme has also been reported in laboratory studies (Hou et al., 1983; Stephen and Dalton, 1987). The ability to cometabolize ethylene and propylene to their corresponding oxides has been observed with CAH-transforming methanotrophic cultures (Hou et al., 1979; van Hylckama Vlieg et al., 1996). The production of CAH epoxides during aerobic cometabolic transformations of CAHs has also been observed in laboratory studies. For example, van Hylckama Vlieg et al. (1996) found that both c-DCE and TCE were transformed to their corresponding epoxides by *M. trichosporium* OB3b expressing soluble methane monooxygenase (sMMO). Woods and Murrell (1989) and de Bont and Peck (1980) reported that most propane-oxidizing microorganisms cannot grow on ethylene or propylene. Moreover, microorganisms that do utilize alkenes (e.g., ethylene and propylene) as the sole carbon and energy source are known to express the enzyme epoxidase, which further metabolizes epoxides produced during growth (Ensign, 1996; Allen and Ensign, 1998). This suggests that ethylene and propylene can be used as chemical probes to detect and quantify aerobic cometabolic activity in CAH contaminated groundwater. If ethylene or propylene is injected into a CAH contaminated aquifer under aerobic conditions and ethylene oxide or propylene oxide production is observed, we can conclude that these compounds have been cometabolized and that the indigenous microbial community may have the metabolic capability to also cometabolize CAHs. Ethylene and propylene are well suited for use in field tests as they are inexpensive, easily detectable at low concentrations, nontoxic, and not normally present in CAH contaminated groundwater at high concentrations.

Acetylene has been known to be an irreversible inactivator of methane monooxygenase (MMO) from *Methylococcus capsulatus* (Bath) (Prior and Dalton, 1985), AMO from *N. europaea* (Keener and Arp, 1993), butane monooxygenase (BMO) from butane-grown *Pseudomonas butanovora*, an environmental isolate, CF8 (Hamamura et al., 1999), and propane monooxygenase (PMO) from propane-grown *Mycobacterium vaccae* JOB5 (Vanderberg and Jerry, 1994). This phenomenon has also been observed in studies with mixed cultures grown on methane and propane (Alvarez-Cohen and McCarty, 1991; Tovanabootr and Semprini, 1998). Radiolabelled [<sup>14</sup>C] acetylene has also been used to bind with polypeptides to differentiate butane monooxygenases of different butane-utilizing microorganisms (Hamamura et al., 1999). They also showed that all three microorganisms oxidized ethylene to ethylene oxide, and that acetylene blocked the transformations. In the push–pull tests reported here, acetylene was used to evaluate the involvement of a monooxygenase enzyme in propane degradation, the transformation of ethylene, and the CAHs of interest.

In a previous study we developed a simple field method, the single-well, push–pull test for quantifying in situ rates of propane and DO utilization during aerobic cometabolism (Kim et al., 2004). In addition we demonstrated that it is possible to monitor aerobic cometabolic transformations of injected ethylene and propylene to their corresponding oxides as a way to detect the presence of microorganisms with the metabolic capability to transform CAHs. However, the previous study was conducted in an aquifer that was not contaminated with CAHs and thus we were unable to confirm the direct relationship between transformations of CAH-

surrogates (e.g., ethylene and propylene) and CAHs transformation by field testing at that site. In the study presented here we performed a series of field tests in an aquifer contaminated with TCE and c-DCE. Through a series of propane and DO injections we were able to stimulate propane and DO utilization, the transformation of injected ethylene and propylene to their corresponding epoxides, and the cometabolic transformation of c-DCE. Because it is well known that a monooxygenase enzyme initiates propane utilization, ethylene and propylene transformation, and CAH transformation under aerobic conditions, we developed protocols for conducting push–pull tests with coinjected acetylene, a known monooxygenase mechanism-based inactivator. By injecting acetylene we were able to inhibit propane utilization, ethylene transformation, and c-DCE transformation and thus confirm that monooxygenase is mainly responsible for observed microbial activity. The study was also conducted in a relatively deep aquifer, at a depth of 30 m, illustrating the ability to conduct controlled push–pull experiments with dissolved gas components at this depth.

## 2. Materials and methods

### 2.1. Site description

Push–pull tests were performed in two monitoring wells (MW2 and MW3) at former McClellan AFB, CA. The aquifer at this site is mainly contaminated with c-DCE (20–40 µg/L) and TCE (200–400 µg/L), and is aerobic (~6.3 mg/L DO). The aquifer consists primarily of alluvial deposits, and is unconfined with a water table depth ranging from 30 to 32 m below ground surface. The two monitoring wells were constructed of 5.1 cm polyvinyl chloride casing with a 2.9 m long screen.

### 2.2. Field tests

Push–pull Transport Tests were conducted in each well. These tests were followed by a biostimulation period consisting of five sequential additions of propane and DO to each well; followed by a series of Activity Tests and Acetylene Blocking Tests (Table 1). Field equipment consisted of compressed or liquefied gases, gas flow meters, two carboys (500 L and 50 L), a collapsible metalized-film gas-sampling bag (Chromatography Research Supplies, Addison, IL), a peristaltic pump to inject the test solution into the well, and a submersible pump to extract groundwater from the same well. Site groundwater was used to prepare three solutions: 1) 500-L with known concentrations of bromide (KBr, Spectrum Chemical Mfg. Corp. Gardena, CA) to serve as a nonreactive tracer, nitrate (NaNO<sub>3</sub>, Mallinckrodt Chemical, Inc. Paris, KY) as a trace nutrient, and DO as an electron acceptor; 2) 50-L with known concentrations of one or more dissolved gases [(propane (99.5%), ethylene (>99.9%), and/or propylene (>99.0%); Airgas inc., Randor, PA) to probe for microbial activity; and 3) 5-L with known concentrations of dissolved acetylene (99.6%, Airgas inc., Randor, PA) in a collapsible metalized-film gas-sampling bag. Specified dissolved gas concentrations in the 500 and 50 L carboys were achieved by controlling the flow rates of each gas to ceramic sparging stones placed in the bottom of the carboys. Gas flow rates were controlled using rotameters fitted to a gas proportioner multitube frame that contained direct reading flow tubes (Cole-Parmer Instrument Co., Vernon Hills, IL). After dissolved gas concentrations had stabilized, the contents of the carboys and metalized bag were combined to obtain the desired solute concentrations using calibrated peristaltic and piston pumps and injected into the well. The composition of the test solution was monitored during

Table 1  
Test solution composition for field push–pull tests using well MW 2

Test type	Injection volume (L)	Propane (mg/L)	Propylene (mg/L)	Ethylene (mg/L)	<sup>1</sup> DO (mg/L)	<sup>2</sup> NO <sub>3</sub> <sup>-</sup> N (mg/L)	Br <sup>-</sup> (mg/L)	<sup>3</sup> c-DCE (μg/L)	<sup>3</sup> TCE (μg/L)
Transport Test	264	2.0 ± 0.1	4.0 ± 0.2	4.1 ± 0.2	22 ± 0.8	NI	34 ± 1.5	3.7 ± 1.0	27 ± 5.1
Biostimulation (5 Additions)	498 ± 15	7.6 ± 3.0	<sup>7</sup> NI	NI	30 ± 3.5	7.7 ± 0.6	108 ± 20	2.5 ± 0.5	28 ± 2.5
<sup>5</sup> 1st Propane Activity Test	238	2.4 ± 0.1	NI	NI	30 ± 0.8	1.9 ± 0.1	40 ± 1.5	4.4 ± 1.1	54 ± 1.1
2nd Propane Activity Test	250	1.3 ± 0.1	NI	NI	16 ± 0.6	4.4 ± 0.2	22 ± 0.1	2.1 ± 0.2	36 ± 3.2
Ethylene Activity Test	255	NI	NI	0.67 ± 0.02	17 ± 0.45	5.8 ± 0.3	68 ± 1.5	1.3 ± 0.01	32 ± 2.0
3rd Propane Activity Test	251	1.6 ± 0.1	NI	NI	18 ± 1.0	6.0 ± 0.2	122 ± 4.3	1.4 ± 0.1	31 ± 2.6
Propylene Activity Test	255	NI	1.6 ± 0.1	NI	16 ± 0.6	4.9 ± 0.1	228 ± 3.5	1.4 ± 0.2	33 ± 2.1
4th Propane Activity Test	317	1.6 ± 0.2	NI	2.0 ± 0.18	35 ± 0.95	3.8 ± 0.1	37 ± 1.4	5.2 ± 0.8	44 ± 4.2
<sup>6</sup> Acetylene Blocking Test	346	1.2 ± 0.9	NI	2.2 ± 0.15	31 ± 2.2	7.3 ± 0.4	77 ± 3.2	4.8 ± 0.3	35 ± 1.0

<sup>1</sup>: Background average DO concentration of 6.3 mg/L. <sup>2</sup>: Background average NO<sub>3</sub><sup>-</sup> (as N) concentration of 1.1 mg-N/L. <sup>3</sup>: Average concentrations of c-DCE and TCE concentrations in the injected test solution (C<sub>0</sub>). <sup>4</sup>: Average values obtained during 1st through 5th Biostimulation tests. <sup>5</sup>: The 1st propane Activity Test was performed just prior to the 2nd Biostimulation test. <sup>6</sup>: Tests were performed in MW3 only. Injected acetylene concentration was ~0.5 mM (10 mg/L). <sup>7</sup>: NI indicates not included.

injection by collecting samples from the well using a submersible pump (GRUNDFOS Pumps Co, Fresno, CA).

Samples of the injected test solution were collected by pumping the groundwater from the wells using a submersible pump placed down-hole in the screened interval of the well. Thus, the actual concentration of injected solutes entering the aquifer was monitored. This down-hole sampling method provided very reproducible concentrations of the dissolved gases in the injected fluid, and permitted the tests to be performed in a deep aquifer.

### 2.3. Transport test

A short-duration Transport Test was conducted in each well to compare the relative mobility of bromide, nitrate, and dissolved propane, oxygen, propylene, and ethylene in the aquifer prior to subsequent tests (Table 1). Two hundred and sixty-four liters of test solution (prepared as described above) were injected at 2 L/min. After a 16 h rest phase with no pumping, the test solution/ground water mixture was extracted from the well at a rate of 2.5 L/min. Samples collected during the extraction phase were analyzed and used to prepare breakthrough curves for each injected solute.

### 2.4. Biostimulation period

During the Biostimulation Period, five sequential additions of propane and DO were performed in each well to stimulate the activity of indigenous propane oxidizing bacteria. Test solutions were prepared and injected as described above and contained known concentrations of

bromide, dissolved propane and oxygen, and nitrate (Table 1). Since commercial grade propane can contain ethylene and propylene, high purity propane (99.5%) was used to insure the stimulation of propane-utilizing microorganisms, and not ethylene-utilizing or propylene-utilizing microorganisms. Periodic sampling of the test solution/groundwater mixture was used to quantify rates of propane and DO utilization.

### 2.5. Activity tests

Following the Biostimulation Period, a series of five Activity Tests were conducted to quantify rates of propane utilization, ethylene and propylene transformation, and c-DCE and TCE transformation (Table 1). Test solutions were prepared and injected as described above. After a 16 h rest phase with no pumping, the test solution/ground water mixture was extracted from the well at a rate of 2.5 L/min. Samples collected during the extraction phase were analyzed and used to prepare breakthrough curves for each injected solute and transformation products detected in situ.

### 2.6. Acetylene blocking test

Acetylene Blocking tests were conducted using the same procedures used in the Activity Tests, except that dissolved acetylene (10 mg/L) was included in the injected test solutions (Table 1). Acetylene Blocking tests were performed last, since acetylene is an irreversible inactivator of monooxygenase enzymes. The test was performed only in monitoring well MW3, since monitoring well MW2 was to be used for other field tests.

### 2.7. Analysis

Test samples were collected in 40-mL VOA vials with a Teflon/neoprene septum and a polypropylene-hole cap (Supelco, Bellefonte, PA). Triplicate samples were obtained periodically during the tests for quality control purposes. For all the compounds of interest standard deviations of triplicate samples were typically less than  $\pm 5\%$ . Samples were not preserved with acid, since the transformation of potential cometabolic by-products, ethylene oxide and propylene oxides, are acid catalyzed. DO concentrations were measured in the field with a Clark (Yellow Springs, Ohio) O<sub>2</sub> electrode mounted in a glass water-jacketed cell (1.8 mL) to maintain a constant temperature. Samples for laboratory analysis were stored at 4 °C and analyzed within one week. Bromide and nitrate concentrations were determined using a Dionex (Sunnyvale, CA) model DX-120 ion chromatograph equipped with an auto-sampler, an electrical conductivity detector and a Dionex AS14 column. The EPA 502.2 purge-and-trap method (Slater and Ho, 1986) was adapted for use in determining the dissolved concentrations of gaseous substrates. Five to eight mL aqueous samples from the VOA vials were introduced into an HP 7695 purge-and-trap system, and the volatile compounds were sorbed onto a tenax/silica gel/charcoal trap (Supelco, Bellefonte, PA). A sample purging time of 15 min was used. Chromatographic separations were achieved with a 30-m megabore GSQ-PLOT column from J and W Scientific (Folsom, CA) installed on a HP5890 series GC connected to a photo ionization detector (PID) followed by a flame ionization detector (FID).

Ethylene oxide and propylene oxide were identified by retention time comparisons with authentic ethylene oxide (>99.5%, Aldrich, Milwaukee, WI) and propylene oxide (>99.5%,

Fluka, Milwaukee, WI) standards. Under the same GC operating conditions as described above, the retention times for ethylene oxide and propylene oxide standards were 14.4 and 21.7 min., respectively. To supplement this identification, authentic standards were assayed with chromatographic separation using a HP624 capillary column under the same GC operating conditions. The retention times for ethylene oxide and propylene oxide were 6.31 and 7.98 min., respectively. To further confirm the identification of test samples, the method of standard addition was used where specific amounts of authentic standards were added to the test samples, and resulting concentration increase measured. Ethylene oxide and propylene oxide were quantified using the FID detector.

The stability of ethylene oxide and propylene oxide during sample storage prior to analysis was evaluated by performing batch laboratory tests. Specific amounts (~1 mg/L) of the oxide of interest were added into 150 mL glass serum bottle containing autoclaved groundwater (140 mL) and 25 mg/L of HgCl<sub>2</sub> and incubated on a rotary shaker at 20 °C. Liquid samples from the bottle were periodically taken and analyzed using the methods described above.

### 3. Data analysis

Mass balance calculations were performed by integrating measured solute concentrations and injection and extraction volumes. For plotting purposes, normalized concentrations,  $C^*$ , were computed using

$$C^* = [(C - C_{BG}) / (C_o - C_{BG})] \quad (1)$$

where  $C$  is a measured concentration in an extraction sample,  $C_o$  is the average injected concentration of the same solute, and  $C_{BG}$  is the background (pre-injection) concentration of the same solute. Overall zero-order reaction rates ( $r$ ) for injected solutes were calculated using the method of Istok et al. (1997):

$$r = \frac{M_{inj} - \{M_{ext} / R_{tracer}\}}{(V_{inj})(t^*)} \quad (2)$$

where  $M_{inj}$  is total mass of solute injected,  $M_{ext}$  is the total mass of solute injected or produced in situ during the test (e.g., ethylene oxide and propylene oxide),  $V_{inj}$  is volume of injected test solution (L),  $R_{tracer}$  is the mass recovery fraction of the conservative tracer (extracted tracer mass divided by injected mass) and  $t^*$  is the mean residence time defined as the elapsed time from the midpoint of the injection phase to the centroid of the conservative tracer breakthrough curve during the extraction phase. Additional details of this calculation are in Istok et al. (1997) and Haggerty et al. (1998).

## 4. Results

### 4.1. Transport tests

Extraction phase breakthrough curves for all injected solutes were similar and essentially all injected solute mass was recovered (Table 2) indicating conservative transport of all injected solutes prior to biostimulation (Fig. 1). These results are important because they mean that measured concentrations of the gaseous substrates and metabolites can be adjusted for dilution using measured bromide concentrations (Haggerty et al., 1998). Slight temporal variations in

Table 2  
Summary of mass balance and rate calculations

Test type	Quantities	Propane		Ethylene		Propylene		Br <sup>-</sup>	
		MW2	MW3	MW2	MW3	MW2	MW3	MW2	MW3
Transport Test	% recovery	104	105	99	99	103	105	99	98
	rate(μmol/L/hr)	≈ 0	≈ 0	≈ 0	≈ 0	≈ 0	≈ 0	–	–
1st Propane Activity Test	% recovery	94	94	–	–	–	–	96	88
	rate (μmol/L/hr)	0.09	≈ 0	–	–	–	–	–	–
2nd Propane Activity Test	% recovery	31	7	–	–	–	–	<sup>2</sup> 107	92
	rate (μmol/L/hr)	1.1	0.8	–	–	–	–	–	–
Ethylene Activity Test	% recovery	–	–	<sup>1</sup> 59 (3.1%)	<sup>1</sup> 75 (3.8%)	–	–	102	90
	rate (μmol/L/hr)	–	–	0.51	0.35	–	–	–	–
3rd Propane Activity Test	% recovery	44	17	–	–	–	–	99	90
	rate (μmol/L/hr)	1.0	1.8	–	–	–	–	–	–
Propylene Activity Test	% recovery	–	–	–	–	<sup>1</sup> 75 (2.3%)	<sup>1</sup> 69 (0.45%)	92	88
	rate (μmol/L/hr)	–	–	–	–	0.34	0.46	–	–
4th Propane Activity Test	% recovery	–	40	–	<sup>1</sup> 60 (5.2%)	–	–	–	107
	rate (μmol/L/hr)	–	0.82	–	1.2	–	–	–	–
Acetylene Blocking Test	% recovery	–	90	–	<sup>1</sup> 86 (0.12%)	–	–	–	107
	rate (μmol/L/hr)	–	≈ 0	–	≈ 0	–	–	–	–

<sup>1</sup>: Numbers in parenthesis indicate percentage of ethylene molar mass transformed to oxides molar mass extracted.

<sup>2</sup>: When bromide recovery is greater than 100%, a value of  $R_{\text{tracer}}$  in Eq. (2) is assumed as 1.00.

the concentration of the injection solution, caused by non-ideal mixing or perturbations in the flow of metering pumps, may have resulted in several normalized concentrations being above 1.0.

#### 4.2. Activity tests

Six Activity tests were performed to confirm the stimulation of indigenous propane-oxidizers through Biostimulation tests and to quantify the rates of propane degradation and CAH-surrogate transformation. The 1st propane Activity Test was performed after the 1st propane and DO additions during the Biostimulation Period, and the 2nd Activity Test was conducted after the 5th propane and DO additions during the Biostimulation Period, followed by an ethylene Activity Test, a 3rd propane Activity Test, a propylene Activity Test, and the 4th propane Activity Test (Table 1).

Propane utilization was not detected during the 1st propane Activity Test as normalized concentrations of injected propane, DO, and bromide were all similar (Fig. 2A). However, substantial propane and DO utilization were observed during the 2nd propane Activity Test (Fig. 2B). The 1st activity test was performed after a single addition of dissolved propane and oxygen. Dilution of the injected solution resulting from groundwater flow likely resulted in residence times that were too short for effective biostimulation to be achieved with a single addition of dissolved propane and oxygen. These results suggest that biostimulation was progressively achieved by successive injections of dissolved propane and oxygen. Similar results were observed

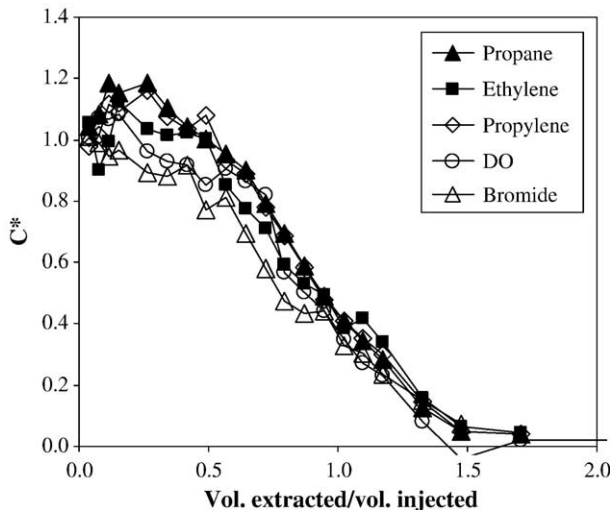


Fig. 1. Pull phase normalized concentrations at MW2 during the Transport Test showing conservative transport of dissolved gases. A DO concentration in a groundwater sample lower than the background DO concentration results in the negative value of  $C^*$  on the abscissa.

in tests at MW3 (data not shown). Estimated zero-order rates of propane utilization were also similar between wells MW2 and MW3 (Table 2).

The results of the ethylene Activity Test indicated that injected ethylene was transformed in situ to ethylene oxide at MW2 (Fig. 3B); a similar amount of ethylene oxide production was also observed at MW3. The ethylene Activity Test was conducted in the absence of added propane. DO utilization during this test was less than observed in the presence of propane (Fig. 2B). In both wells the estimated zero-order rate of ethylene transformation was ~45% of the estimated zero-order rate of propane utilization obtained from the 2nd propane Activity Test at both wells (Table 2). Percentages of the molar mass of ethylene oxide produced to the ethylene molar mass transformed at MW2 were ~3.1% at MW2 and ~3.8% at MW3 (Table 2. Note that percentages of ethylene molar mass transformed to oxides molar mass extracted are presented beside the values for ethylene recovery in the parenthesis). The results indicate that not all the ethylene transformed can be accounted for by ethylene oxide production. One possible explanation for the apparent incomplete mass balance is that a portion of the ethylene oxide produced was further biologically transformed to non-detected products. van Hylckama Vlieg et al. (1996) showed that the epoxide formed during c-DCE transformation was biologically transformed. Another possibility is abiotic processes are removing the epoxide. The abiotic transformation of ethylene oxide in sterilized site ground water samples was observed in a laboratory batch tests (Fig. 4). The rates of ethylene oxide disappearance were slow with an estimated half-life of 18 days. Thus the abiotic transformation of ethylene oxide is not likely occurring in this aquifer at a rate sufficient to effect ethylene oxide concentrations during these relatively short duration tests (~24–30 h). Ethylene oxide has a very high aqueous solubility in water and a very low  $\log_{10}$  octanol water partition ( $K_{ow}$ ) of  $-0.3$  (Laws, 1999), thus sorption would also be minimal.

Propylene was transformed to propylene oxide during the propylene Activity Test (Fig. 5). The computed zero-order rate of propylene transformation at MW2 was about a factor of 1.5 lower than the ethylene transformation rate, while both rates are comparable at MW3 (Table 2). The ratios of mass of propylene oxide detected to propylene transformed were ~2.3% for MW2 and ~0.45% for



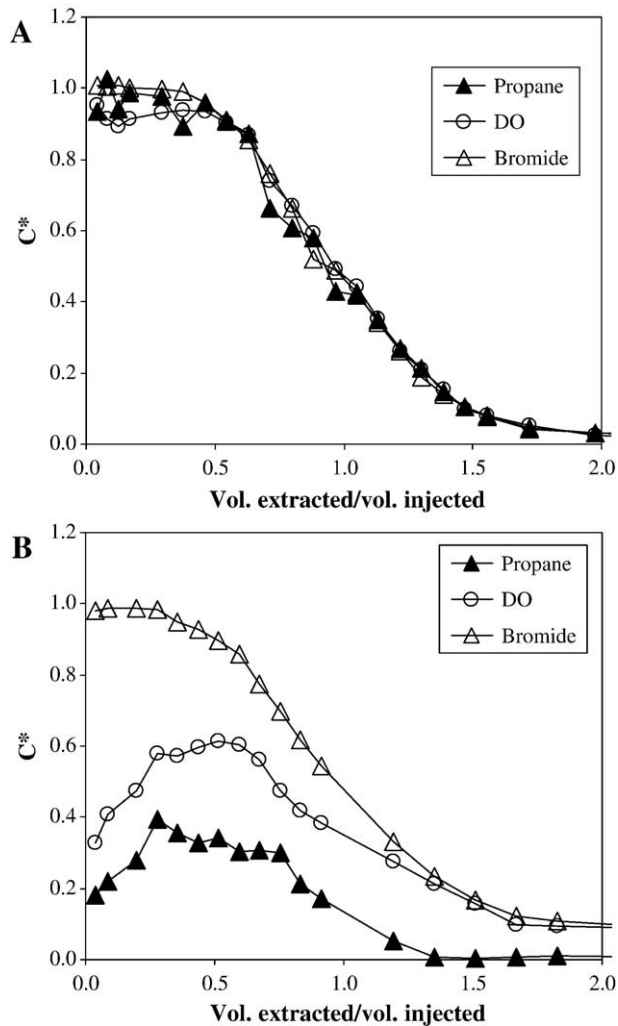


Fig. 2. Extraction phase normalized concentrations at well MW2 during (A) 1st propane Activity Test, and (B) 2nd propane Activity Test.

MW3, which are lower than the 3.1–3.5% observed for ethylene oxide at both wells during the ethylene Activity Test (Table 2). Similar to the ethylene results, the amount of propylene oxide observed was less than can be accounted by the amount of propylene transformed. However, the small concentrations of ethylene and propylene oxides observed in these tests combined with the limited transformation of ethylene and propylene permitted only a qualitative comparison between these substrates. Nevertheless, the results of these tests showed that propane-utilizers stimulated by successive propane additions were able to cometabolize ethylene and propylene.

#### 4.3. Acetylene blocking tests

The 4th propane Activity Test was performed with both propane and ethylene present in the injected groundwater. Simultaneous utilization of propane, ethylene, and DO were observed (Fig.

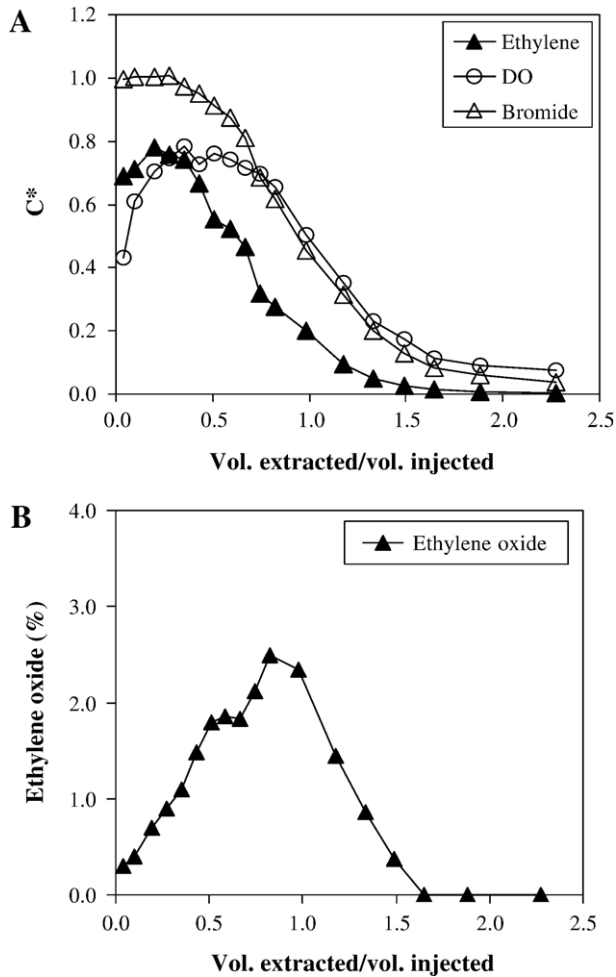


Fig. 3. (A) Normalized concentrations for ethylene, DO, and bromide at well MW2 during the ethylene Activity Test and (B) ethylene oxide concentration in the extracted groundwater as a percentage of average ethylene concentration in injected test solution.

6A), and ethylene oxide was again produced with a ratio of ethylene oxide detected to ethylene injected of  $\sim 5.2\%$  (Table 2). The zero-order rate of ethylene oxidation production was about a factor of three greater than achieved in the earlier test at MW3, while the propane utilization rate was similar to that achieved in the in the 2nd propane Activity Test, and slower than achieved in the 3rd propane Activity Test. These diverse results may be due to several factors such as the effect of substrate concentration on rate, inhibition between the growth substrate (propane) and cometabolic substrate (ethylene), and activity of the stimulated propane-oxidizing microorganisms. Substrate conditions differed among the tests, with only propane present in 2nd and 3rd Propane Activity Tests, while both propane and ethylene were present in the 4th test. Thus, it is difficult to make strong conclusions related to inhibition and the causes of enhancements in rates.

An Acetylene Blocking Test was then performed using the same conditions of the 4th propane Activity Test, but with acetylene added to the injection solution. In the presence of acetylene,

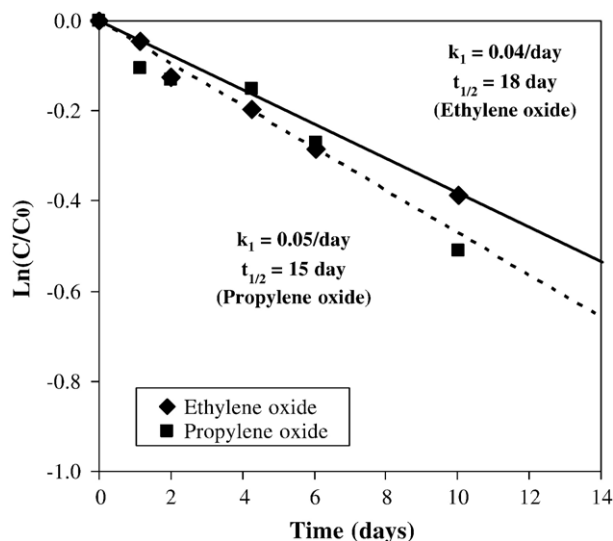


Fig. 4. Abiotic hydrolysis of ethylene oxide and propylene oxide in laboratory incubation at 20 °C.

substrate utilization was highly inhibited (Fig. 7A), and very little ethylene oxide was produced with a ratio ethylene oxide formed to ethylene injected of  $\sim 0.12\%$  that is a factor of 43 less than observed in the absence of acetylene (Table 2). Zero-order rates of propane-utilization and ethylene oxidation production decreased by a factor of 4.7 and 2.4, respectively, in the Acetylene Blocking Test compared to the 4th propane Activity Test (Table 2). These results indicate that the propane monooxygenase enzyme is likely responsible for propane degradation and cometabolism of ethylene.

Concentrations of c-DCE and TCE in the injected and extracted fluids were also measured during the Activity Tests. In Fig. 8, extraction phase breakthrough curves for propane, ethylene, c-DCE, TCE, and bromide are plotted as  $1 - C^*$ , that is,  $1 - [(C - C_{BG}) / (C_o - C_{BG})]$  because, unlike the other substrates, c-DCE and TCE concentrations were lower in the injected test solution than in the background groundwater. For a non-reactive compound, such as bromide, this method of normalization should provide zero values during the early phase of test solution extraction and should increase to unity later in the test as concentrations approach background values. This method of normalization leads to similar responses for non-reactive compounds with high background concentrations compared to the injection concentration. A reactive component that has an injection concentration much greater than background (i.e., propane or ethylene) should have normalized values greater than zero during the early phase of extraction, as concentrations are reduced below the injected concentration, and then increase to one as extraction proceeds. For reactive compounds with high background concentrations (c-DCE or TCE) compared to the injection concentration, negative normalized concentrations could result during the early phase of extraction, with values potentially remaining below unity as extraction proceeds.

During the 4th propane Activity Test, the normalized propane and ethylene concentrations were greater than zero during the early phase of extraction, and increased to one as extraction continued, suggesting significant degradation of propane and ethylene occurred during the rest phase. The normalized c-DCE concentrations were lower than those of bromide, indicating that c-DCE was cometabolically transformed during the test. Normalized concentrations for TCE were

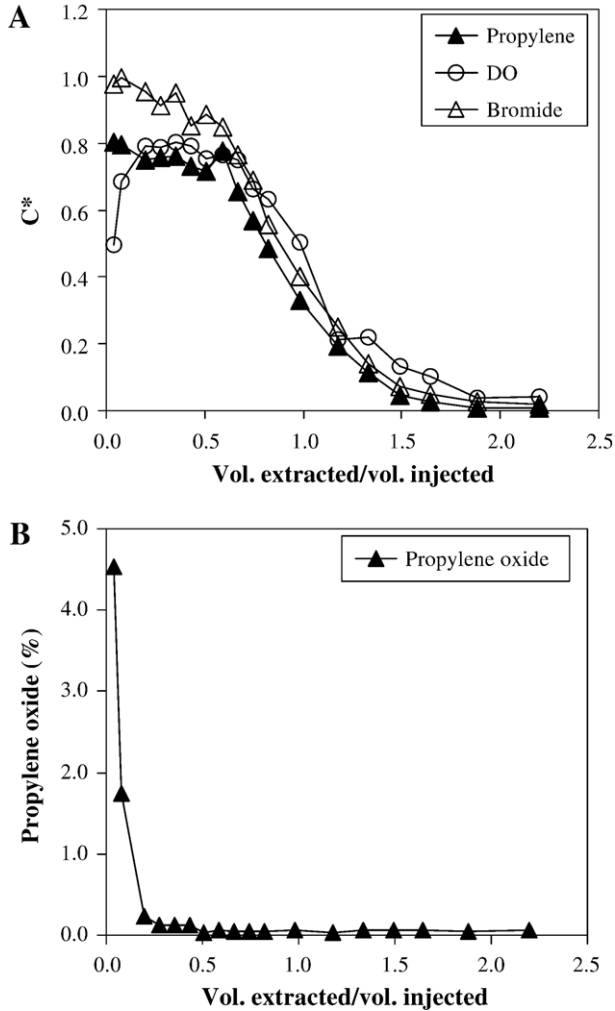


Fig. 5. (A) Normalized concentrations for propylene, DO, and bromide at well MW3 during the propylene Activity Test and (B) propylene oxide concentrations in the extracted groundwater as a percentage of average propylene concentration in injected test solution.

essentially identical to those of bromide suggesting that no detectable TCE transformation occurred (Fig. 8). However, during the Acetylene Blocking Test, normalized concentrations for all solutes showed similar trends as bromide. Towards the end of the test, the *c*-DCE normalized concentrations approached unity indicating *c*-DCE transformation was also inhibited by acetylene.

## 5. Discussion

In situ rates of propane utilization, ethylene, and propylene transformation were quantified using push-pull test methods. Biostimulation was achieved by sequential additions of propane and oxygen dissolved in groundwater. Injected ethylene and propylene were transformed to

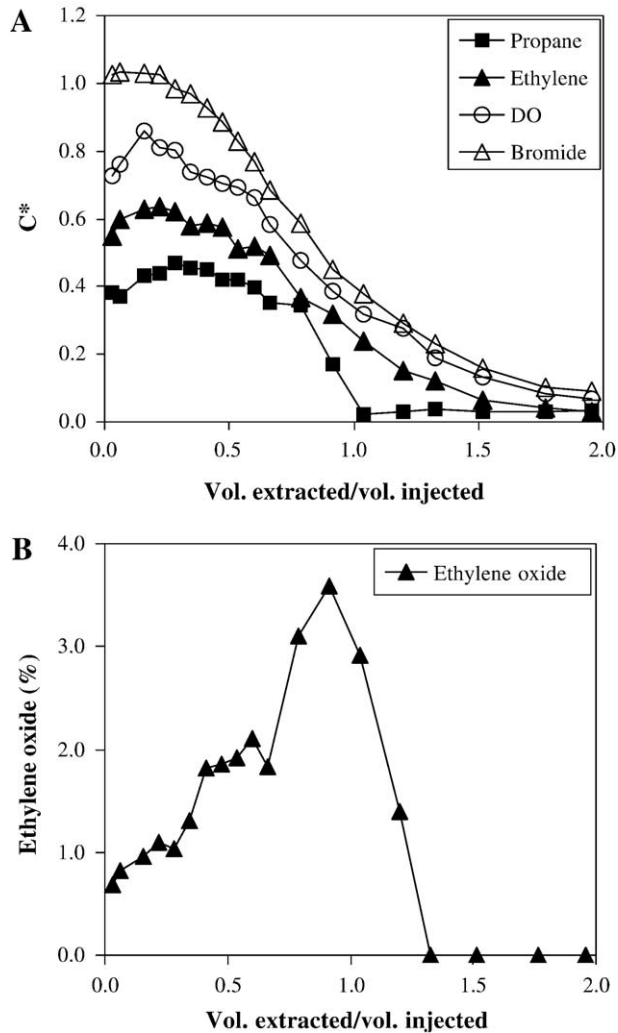


Fig. 6. Extraction phase breakthrough curves at well MW3 during the 4th propane Activity Test (A) injected solutes (B) ethylene oxide concentrations expressed as a percentage of average ethylene concentration in injected test solution.

ethylene oxide and propylene oxide, respectively, which provides direct evidence that these substrates are being cometabolized, and provides indirect evidence that CAHs could be similarly transformed. Acetylene effectively blocked both propane utilization and ethylene transformation further indicating the stimulation of propane monooxygenase activity. Transformations of *c*-DCE was indicated by the normalization plot,  $1 - C^*$ , that is,  $1 - [(C - C_{BG}) / (C_o - C_{BG})]$  (Fig. 8). This method of normalization is useful to qualitatively assess transformation when high background concentrations were present and lower concentrations were present in the injected test solution.

The results are consistent with microcosm laboratory tests using groundwater and soil cores from the former McClellan AFB that showed *c*-DCE was cometabolized more rapidly than TCE (Timmins et al., 2001; Tovanaboot and Semprini, 1998) by propane grown microorganisms. Results of a large scale propane cometabolic sparging demonstration conducted at the same test

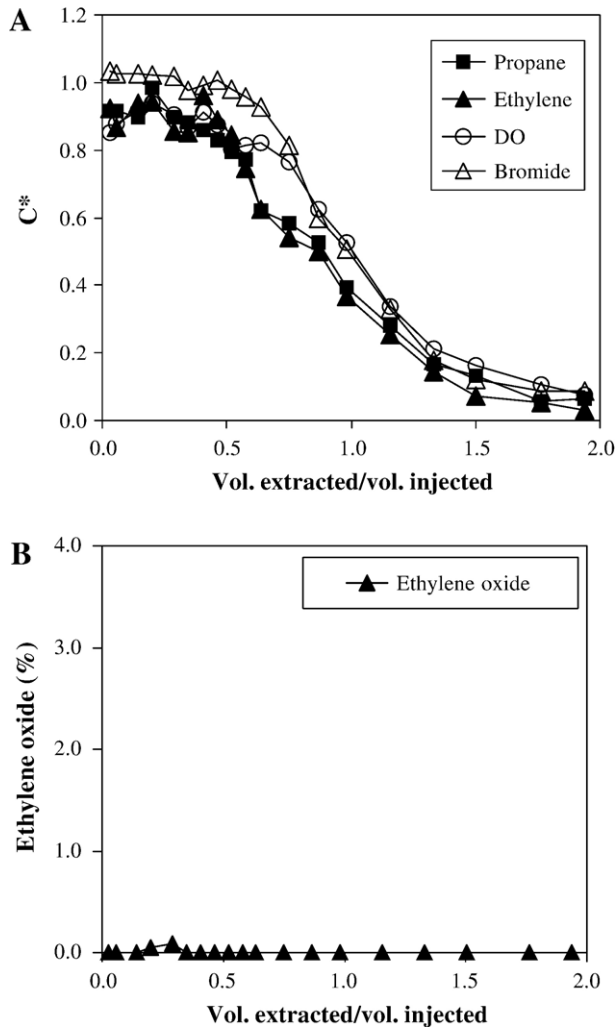


Fig. 7. Extraction phase breakthrough curves from well MW3 during the Acetylene Blocking Test (A) injected solutes (B) ethylene oxide concentrations expressed as a percentage of average ethylene concentration in injected test solution.

site, McClellan AFB (Tovanaboot et al., 2001; Cannon et al., 2005) also showed that c-DCE was transformed at a faster rate than TCE. Thus, our observations are consistent with both microcosm and field tests. If TCE transformation was occurring it was likely at a rate that could not be detected during the time scale of our Activity tests with a reaction period of less than 24-h. The results further illustrate the usefulness of adding the surrogate compounds because they were transformed at a faster rate than TCE. Changes in the ethylene responses in the normalized plots with and without acetylene blocking (Fig. 8) are greater than observed with c-DCE which was present with high background concentrations.

Relationships between growth substrate utilization rates and cometabolic transformation rates are also of interest. Tovanaboot et al. (2000, 2001) reported higher propane utilization rates were associated with higher c-DCE and TCE transformation rates in the cometabolic gas sparging field

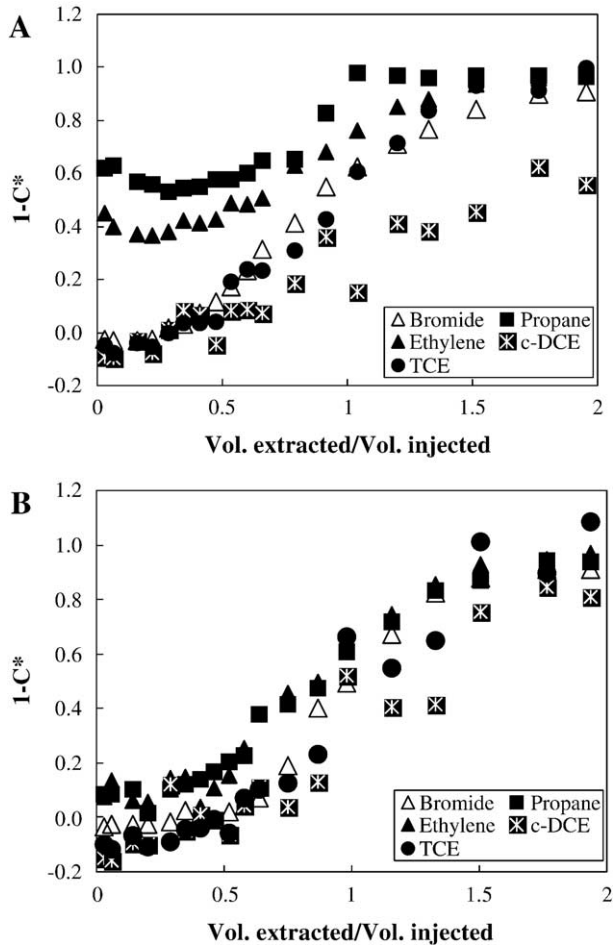


Fig. 8. Extraction phase breakthrough curves during from well MW3 (A) the 4th propane Activity Test, and (B) Acetylene Blocking Test.  $C_0$  values for propane, ethylene, c-DCE, and TCE were  $\sim 1.4$  mg/L, 2.1 mg/L, 5.0  $\mu\text{g/L}$  and 40  $\mu\text{g/L}$ , respectively.  $C_{BG}$  values for bromide, propane and ethylene were zero, while  $C_{BG}$  values for c-DCE and TCE were 15 and 160  $\mu\text{g/L}$ , respectively.

study performed at the same site. In our push–pull Activity tests, we also observed that higher propane utilization rates were associated with higher ethylene and propylene transformation rates. For example, higher propane utilization rates were observed at MW2 during the 2nd propane Activity Test than at MW3. During the ethylene Activity Test that followed, higher ethylene transformation rates were observed at MW2. A similar correlation in rates between tests at MW2 and MW3 was observed during the 3rd propane Activity Test and the propylene Activity Test.

The results reported here extend the work previously presented by Kim et al. (2004). The tests were conducted in a much deeper aquifer that is contaminated with TCE and c-DCE. Although degassing of the dissolved gaseous substrates can occur during the test solution injection into the deep aquifer, methods were developed to overcome this problem. By collecting samples of the injected test solution down-hole at the screen level of the well, the actual concentrations of dissolved gases entering the aquifer could be determined. Very stable

injection concentrations were also obtained using this method. This permitted very controlled push–pull tests to be conducted at depth, even with dissolved gases present in the injected fluid.

The results support the observations of Kim et al. (2004) on the use of ethylene and propylene as surrogate compounds. The current study also permitted a comparison among the two wells, and showed reasonable agreement in measured rates. Acetylene was also demonstrated to be an effective in situ blocking agent of propane utilization and the cometabolic transformations. The tests also demonstrated a potential method for evaluating cometabolism when CAHs are present as background contaminants.

## 6. Conclusion

The Push–Pull tests developed in this study is useful for evaluating the feasibility for in situ CAHs bioremediation through aerobic cometabolism. The method provides direct evidence that propane-utilizers were stimulated and monooxygenase enzymes, were present that are capable of cometabolizing ethylene and propylene as CAH surrogates. Although high background concentrations of c-DCE and TCE were present, the results qualitatively support transformation of c-DCE but minimal transformation of TCE occurred.

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