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# Bacterial community composition determined by culture-independent and -dependent methods during propane-stimulated bioremediation in trichloroethene-contaminated groundwater

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## Summary

An *in situ* co-metabolic air sparging (CAS) study was carried out at McClellan Air Force Base (MAFB), Sacramento, CA, USA, in a trichloroethene- (TCE) and *cis*-dichloroethene (*cis*-DCE)-contaminated aquifer where one test zone received 2% propane in air and the other served as a control and received only air. As part of that study, bacterial population shifts were evaluated by length heterogeneity polymerase chain reaction (LH-PCR). The results showed that an organism(s) that had a fragment size of 385 bp was positively correlated with propane removal rates. The 385 bp fragment consisted of up to 83% of the total fragments in the analysis when propane removal rates peaked. A 16S rRNA clone library made from the bacteria sampled from the propane-sparged groundwater included clones of a TM7 division bacterium that had a 385 bp LH-PCR fragment; no other bacterial species with this fragment size were detected. Both propane removal rates and the 385 bp LH-PCR fragment decreased as nitrate levels in the groundwater decreased. Extinction culturing in natural unamended groundwater medium was used to assess the bacterial diversity of the culturable fraction of microorganisms in both CAS and air-sparged groundwater and to bring novel species into culture for further study. The dominant cultures acquired from the CAS groundwater were from the Herbaspirillum/Oxalobacter clade. The dominant cultures from the air-sparged groundwater were from a novel beta-Proteobacterial clade, which we named after isolate HTCC333.

## Introduction

Trichloroethene (TCE) is a chlorinated aliphatic hydrocarbon (CAH) that has been widely used as a degreasing agent for aircraft engines. This practice led to extensive groundwater contamination at numerous military bases around the country. TCE is listed as a top priority pollutant by the Environmental Protection Agency (Federal Register, 1989). TCE undergoes reductive dehalogenation under anaerobic conditions to form *cis*-dichloroethene (DCE), 1,1-DCE, *trans*-DCE and vinyl chloride (VC), which are also common co-contaminates at these sites (Vogel and McCarty, 1985; Vogel *et al.*, 1987; McCarty, 1997; Lee *et al.*, 1998). Traditionally, CAH-contaminated groundwater has been remediated by: (i) pumping and treating, *ex situ*, via air stripping or chemical means; or (ii) *in situ* air sparging in order to volatilize the CAHs (Norris *et al.*, 1994).

Co-metabolic air sparging (CAS) is an innovative variation of conventional *in situ* air sparging that has the potential to remediate CAH-contaminated groundwater more effectively and less expensively. CAS has the advantage of reducing the off-gassing of CAHs during sparging by enhancing CAH biodegradation (Marley and Bruell, 1995) through the introduction of co-metabolic growth substrates such as methane (Travis and Rosenberg, 1997), propane or butane. Another advantage of CAS is the avoidance of VC production. VC is a potent carcinogen that only accumulates in a reducing environment (Code of Federal Regulations, 1985; Eder, 1991; Bouwer, 1994; McCarty and Semprini, 1994).

Propane was used as a co-metabolic substrate at the CAS demonstration site located at McClellan Air Force Base (MAFB), Sacramento, CA, USA. The demonstration aquifer was contaminated with a mixture of primarily TCE and *cis*-DCE, with levels as high as 2.68 mg l<sup>-1</sup> and 2.27 mg l<sup>-1</sup> respectively. A microcosm study conducted using soil cores taken from the site indicated that propane was an effective co-metabolic substrate for the bioremediation of a complex mixture of CAHs when compared with methane and butane (Tovanabootr and Semprini, 1998). Propane also contributed to better long-term TCE removal activity after the propane substrate was consumed. Details of the co-metabolic sparging tests conducted at

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MAFB are provided by Tovanabootr and colleagues (2000; 2001) and ESTCP (2001). The test zone receiving propane began to show propane utilization *in situ* 4–6 weeks after propane sparging began, and TCE and *cis*-DCE levels began to decrease in proportion to propane usage 6 weeks after sparging began (Tovanabootr *et al.*, 2000).

To detect shifts in the bacterial community composition, we characterized bacterial assemblages using both culture-dependent and -independent methods before and during the CAS demonstration. The groundwater bacterial community was analysed by length heterogeneity polymerase chain reaction (LH-PCR) and by construction of a 16S rRNA gene clone library to detect community shifts that occurred during the CAS demonstration. We were primarily interested in the shifts that were related specifically to the addition of propane to the groundwater and, to this end, the bacterial community shifts resulting solely from the addition of air were used as a control. We investigated the relationships between community shifts and rates of propane utilization, nitrate levels and TCE and *cis*-DCE removal. Extinction culture methods were used in both the CAS and the air-sparged groundwater. The extinction culturing method that we used (Button *et al.*, 1993) is similar to the most probable number (MPN) method refined by Haas (1989), but differs in that the microbial density of the inoculum is first determined by direct microscopic counting, and then a predetermined volume of inoculum is added to each culture tube such that the average number of cells each tube receives is of the order of 1–100. We used a variation of the extinction culturing method, which was designed for high-throughput processing (Connon and Giovannoni, 2002).

## Results

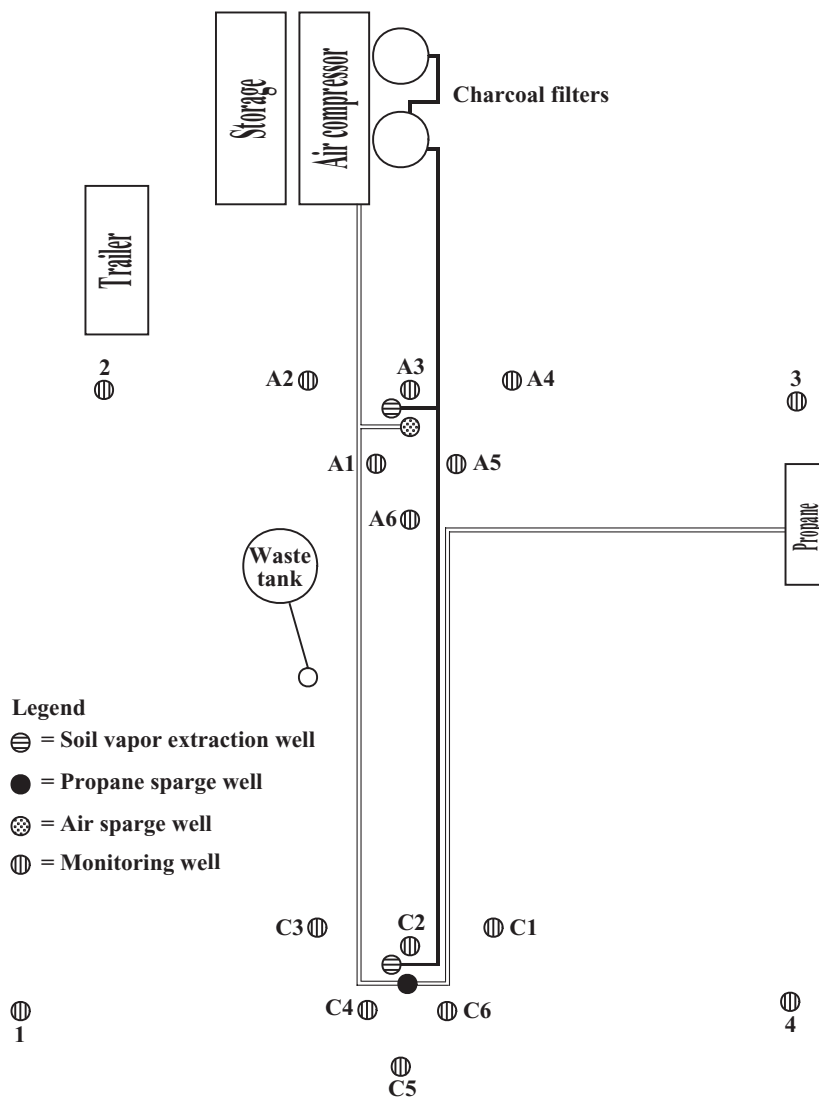
The C-zone received 2% propane in air, and the A-zone received air alone (Fig. 1). The first sparging event occurred on day 36 of the field demonstration. A significant increase was seen in the abundance of microorganisms in the C-zone groundwater after the addition of propane. The average cell count, with a 95% confidence interval for six wells and eight samples collected in the C-zone before propane sparging (day 20), was  $8.9 \times 10^4$  cells ml<sup>-1</sup> ( $\pm 3.0 \times 10^4$ ) and after sparging (day 128) including three wells and four samples was  $1.1 \times 10^6$  cells ml<sup>-1</sup> ( $\pm 0.82 \times 10^6$ ), which is a significant increase in cellular abundance. Groundwater from the individual wells C2-113 and C4-113 showed an increase from  $7.6 \times 10^4$  to  $1.9 \times 10^6$  and from  $4.5 \times 10^4$  to  $8.6 \times 10^5$  cells ml<sup>-1</sup>, respectively, after propane sparging. The addition of propane to the groundwater at MAFB caused a general increase in microbial abundance, whereas adding air alone showed no such increase. The average cell count,

with a 95% confidence interval for five wells and seven samples in the A-zone before air sparging (day 20), was  $1.1 \times 10^5$  cells ml<sup>-1</sup> ( $\pm 3.6 \times 10^4$ ) and after sparging (day 128) including two wells and two samples was  $1.9 \times 10^5$  cells ml<sup>-1</sup> ( $\pm 1.8 \times 10^5$ ).

Direct count data collected from groundwater also showed that large, brightly stained filamentous rods,  $\approx 0.5 \mu\text{m}$  wide and up to  $11 \mu\text{m}$  long, were seen only in the wells that had received propane. An accurate count of these filamentous rods could not be assessed because of severe clumping of these cells. Representatives of typical microbes seen in groundwater collected from C2-113 before propane sparging (day 20) and representatives of the large filamentous rods seen after propane sparging (day 128) are shown (Fig. 2). The small rods and cocci, generally 1–2  $\mu\text{m}$  in length, seen in the presparged C-zone and A-zone groundwater, continued to be observed in the C-zone after propane sparging and are visible as faint cells around the filamentous rods in the image.

LH-PCR of the 16S rRNA gene was used to detect the bacterial composition shifts that occurred in both the C-zone and the A-zone control. Both air and propane sparging induced shifts in the bacterial community of the A-zone and C-zone, respectively, and can be observed in the LH-PCR electropherograms for groundwater from wells C2-113, C4-113 and A1-113 (Fig. 3). One peak with a fragment size of 385 bp is consistently and unambiguously related to the addition of propane. The only other fragment size that correlated with propane sparging was a 366 bp fragment, which was seen in several, but not all, groundwater samples that received propane. This 366 bp fragment is visible in the sample taken from well C4-113 on day 128 of the study (Figs 3 and 5). All other fragments that occurred in the post-propane-sparged C-zone samples occurred at least once in an A-zone and/or a presparged C-zone sample; this includes data from electropherograms not shown in Fig. 3.

The LH-PCR fragment of 385 bp was detected from all wells that were successfully injected with propane including C2-113 (0.78), C2-117 (2.4), C3-113 (0.93), C3-117 (0.52), C4-113 (1.17) and C4-117 (1.09). Parentheses after each well indicate the average of the first propane measurements (mg l<sup>-1</sup>) taken after a propane sparge. Well C5-117 (0.12) received very little propane, and wells C5-113, C1-113, C1-117, C6-113 and C6-117 did not successfully receive propane as a result of aquifer heterogeneities as discussed by Tovanabootr and colleagues (2001). Well C2-117, which received at least a twofold higher propane concentration than any other well, also showed the highest proportion of the 385 bp peak on day 96: 83% of the total LH-PCR fragments. The percentage of the 385 bp fragment was determined by dividing the 385 bp fragment peak area by the total of all peak areas in the sample. Wells C2-113 and C4-113 were chosen at



**Fig. 1.** Layout of study site at McClellan Air Force Base, Sacramento, CA, USA, showing location of sampling wells.

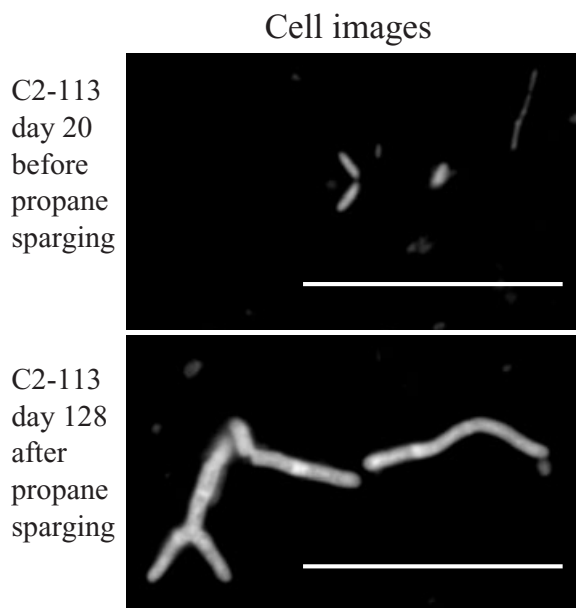
the outset of this project for the most intensive biological sampling while other wells were sampled less frequently.

The proportion of the 385 bp fragment in the LH-PCR products increased as the propane removal rate increased and diminished when propane removal slowed (Fig. 4A). TCE, *cis*-DCE and nitrate levels were all inversely correlated with the increase in the 385 bp LH-PCR fragment and the propane removal rate. TCE, *cis*-DCE and nitrate levels began to drop as the propane removal rate and the percentage of the 385 bp fragment increased (Fig. 4B and C). However, as the nitrate level continued to drop, the proportion of the 385 bp LH-PCR fragment and propane removal rate also decreased, and the TCE concentration then began to increase. Interestingly, although the TCE concentration rebounded as a result of naturally occurring inputs of fresh groundwater, the *cis*-DCE and nitrate levels showed no increase in well C4-113 and very little increase in C2-113 (Fig. 4C).

Dissolved oxygen (DO) levels varied between 5.2 and

6.8 mg l<sup>-1</sup> until day 89, when the DO levels dropped dramatically 9 days into the biweekly sparging events, which started with the second sparge event on day 80. This was an indicator that microbial respiration of oxygen had increased, which occurred as propane removal rates also increased. The DO levels then fluctuated between 1.0 and 8.1 mg l<sup>-1</sup> throughout the rest of the study, but remained at higher levels, overall, in well C4-113 than in C2-113 (Fig. 4C).

A 16S rRNA gene clone library consisting of 91 clones was made from well C4-113 (day 128) and screened for the 385 and 366 bp LH-PCR fragments, as they were the only fragments that could be unambiguously correlated to propane sparging. Three clones had an LH-PCR fragment size of 385 bp, as determined by ABI GeneScan analysis, and had identical *Hae*III and *Mbo*I restriction digest patterns. One clone had a fragment size of 366 bp, as determined by ABI GeneScan analysis. Clones MAFB-C4-28 (385 bp) and MAFB-C4-52 (366 bp) were sequenced, and



**Fig. 2.** Cell images. Scale bar is 10  $\mu\text{m}$ . Groundwater was 10% (v/v) formalin fixed on site and stored at 4°C for up to 1 week before staining with 5  $\mu\text{g ml}^{-1}$  DAPI and filtering down on to 0.2  $\mu\text{m}$  black polycarbonate membranes for counting. Images were taken with a Princeton Instruments MicroMax 1300Y 5MZ cooled interline CCD camera mounted on a Leica DMRB epifluorescence microscope using a 100 $\times$  PL Fluotar 1.3 NA oil objective.

the sequences showed that the actual sequence lengths, between and including the two LH-PCR primers, were 384 bp for MAFB-C4-28 and 365 bp for MAFB-C4-52. Both clones represent TM7 candidate division of bacteria (Rheims *et al.*, 1996; Hugenholtz *et al.*, 1998) and are members of subdivision 3 of the TM7 bacterial division as defined by Hugenholtz and colleagues (2001). MAFB-C4-28 (385 bp) has a sequence similarity of 92.8% to its closest relative in GenBank, clone NoosaAW89 (AF269022), which was acquired from a sewage treatment plant. Clone MAFB-C4-52 (366 bp) is phylogenetically most closely related to clone MAFB-C4-28 (385 bp) with a sequence similarity of 97.1%.

Sixty-eight of the 91 clones in the clone library were represented more than once in the library and were also sequenced and analysed to determine their phylogenetic relationships to known sequences in GenBank using the ARB and PAUP programs (Table 1; trees not shown). These 68 clones included 47 beta-Proteobacteria, 10 alpha-Proteobacteria, four gamma-Proteobacteria, four OP11 and three TM7. Interestingly, several clones are closely related to previously cultured microorganisms that are known to degrade toxic or recalcitrant compounds, which are shown in Table 1.

**Table 1.** Sequence-based projection of LH-PCR fragment length and known carbon degradation abilities for isolates in the same phylogenetic clade as clone.

MAFB-C4 clones <sup>a</sup>	Sequence-based determination of LH-PCR fragment <sup>b</sup>	Phylogenetic clade of clones	Toxic or generally bioresistant compounds degraded by isolates in each phylogenetic clade <sup>c</sup>
		Beta-Proteobacteria	
3, 24, 25, 67, 70, 73, 83, 85, 89	342	Herbaspirillum/Oxalobacter	6-methylnicotinic acid, chlorophenols <sup>d</sup>
1, 30, 37, 55, 57, 64	342	Ramlibacter	–
9, 15, 27, 62, 72	342	PM1 (isolate)	Methyl <i>tert</i> -butyl ether (MTBE) <sup>e</sup>
10, 82, 94	342	HTA10 (clone)	–
38, 60, 92	342	JS666/Polaromonas	<i>cis</i> -Dichloroethene ( <i>cis</i> -DCE) <sup>f</sup>
2, 4, 17, 19, 33, 45, 50, 53, 56, 63, 65, 69, 71, 87, 95	340	MAFB-C4-17 (clone this study)	–
5, 35, 43, 79, 86, 96	340	Ideonella	–
		Alpha-Proteobacteria	
13, 20, 21, 22, 36, 44, 48, 58	329	SM2C02 (clone)	–
14, 84	322	Holospora (endosymbiont)	–
		Gamma-Proteobacteria	
29, 31	342	Acinetobacter	Aliphatic polyesters, chlorinated guaiacols <sup>g</sup>
32, 34	348	Legionella	–
		TM7	
28, 61, 76	384	subdivision 3	–
		OP11	
11, 12, 68, 80	365	subdivision 5	–

**a.** Numbers are clone names, which are otherwise appended by MAFB-C4-. Only clones represented more than once in the library are listed. Library was constructed from water collected from well C4-113 on day 128.

**b.** LH-PCR fragment length as determined by running on ABI equipment differed by up to 1 base pair from actual sequence length.

**c.** Carbon compounds, many known to be toxic or generally bioresistant, which can be utilized or degraded by at least one isolate in the clade.

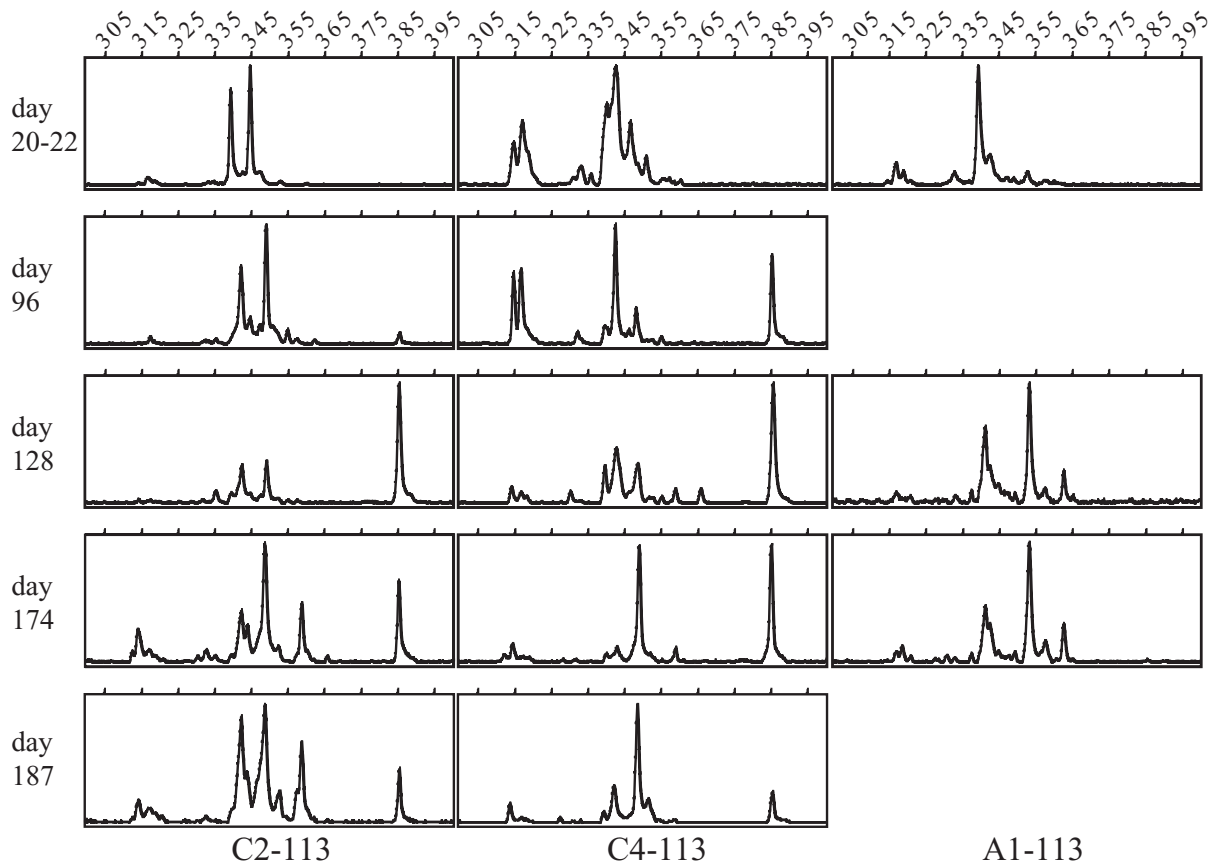
**d.** Tinschert *et al.* (1997); Ueda and Sashida (1998); Mannisto *et al.* (2001).

**e.** Bruns *et al.* (2001).

**f.** Coleman *et al.* (2002).

**g.** Gonzalez *et al.* (1993); Suyama *et al.* (1998).

–, Not determined.



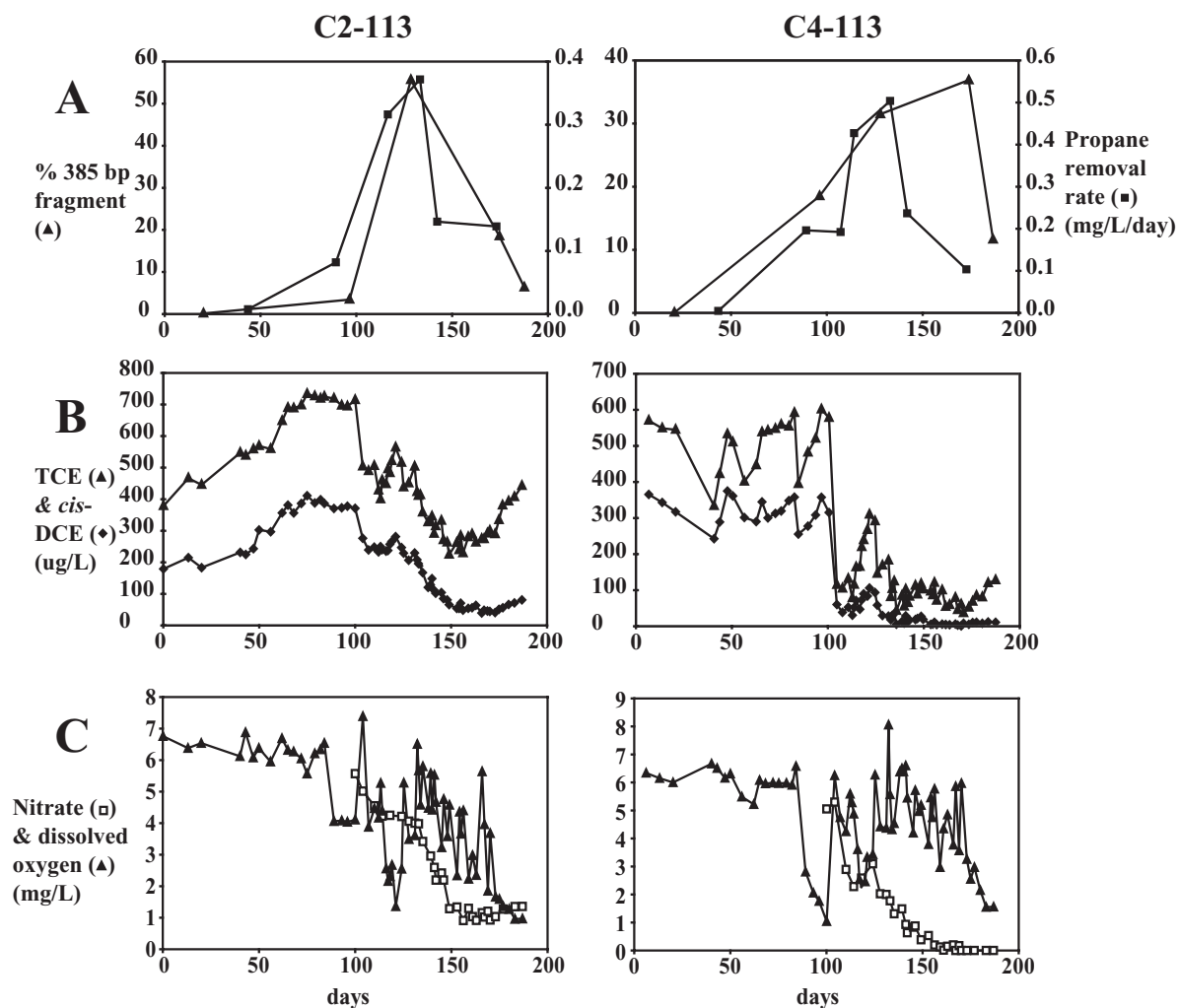
**Fig. 3.** The LH-PCR electropherograms show the amplified fragments represented as peaks. The fragment size is shown on the x-axis. The y-axis is relative peak intensity where the sum of the peak areas for each sample represents the total LH-PCR product. The shift in the bacterial community is shown over time for wells C2-113, C4-113 and A1-113. Propane sparging was initiated in the C-zone on day 36 of this study. Data were not collected for well A1-113 on days 96 and 187.

The number of bases between and including the LH-PCR primers 8F and 338R for each sequenced clone was determined and compared with the LH-PCR fragments from the same sample (Fig. 5). Six prominent fragments in the LH-PCR corresponded to clones with sequence-determined fragment sizes within 1 bp of an LH-PCR fragment. Most of the LH-PCR fragments were 1 bp longer than the most probable corresponding sequences determined from the clone library. The 385 bp LH-PCR fragment from this sample was 31.5% of the total LH-PCR fragments. Other dominant LH-PCR fragments represented in the clone library included 343 bp (23.7%), 349 bp (13.9%) and 340 bp (8.4%), 366 (3.1%) and 330 bp (2.7%). *Herbaspirillum/Oxalobacter*, the most commonly isolated group, and PM1-related isolates are categorized under the 343 bp fragment, which is the second most abundant fragment size in this sample.

Growth was detected in 112 out of 576 extinction culture attempts from A- and C-zone groundwater using high-throughput processing. Culturability of the groundwater microbes using this culturing method ranged from 0.6%

to 5.0% in these zones (Table 2). No significant difference in culturability was detected for the ambient air versus propane growth chamber treatments, with either A- or C-zone groundwater. Bacteria were also isolated from the C-zone using traditional mineral medium agar plates in the presence of 2% propane and two other potential carbon sources, carbon dioxide and unpurified agar. The culturability on agar was 1.5% and 11.1% from groundwater collected from wells C2-113 and C4-113 respectively (Table 2).

Extinction cultures and minimal agar medium isolates were identified by phylogenetic analysis of the 16S rRNA gene and by comparing these sequences with sequences in GenBank to determine the closest matches (Table 3). Novel isolates recovered by extinction culturing were determined to be members of the beta-Proteobacteria and alpha-Proteobacteria. Novel isolates recovered by traditional agar plating were determined to be members of the beta-Proteobacteria and the Flexibacteraceae family. Fifteen extinction cultures from the C-zone and six from the A-zone were determined to be mixed cultures based



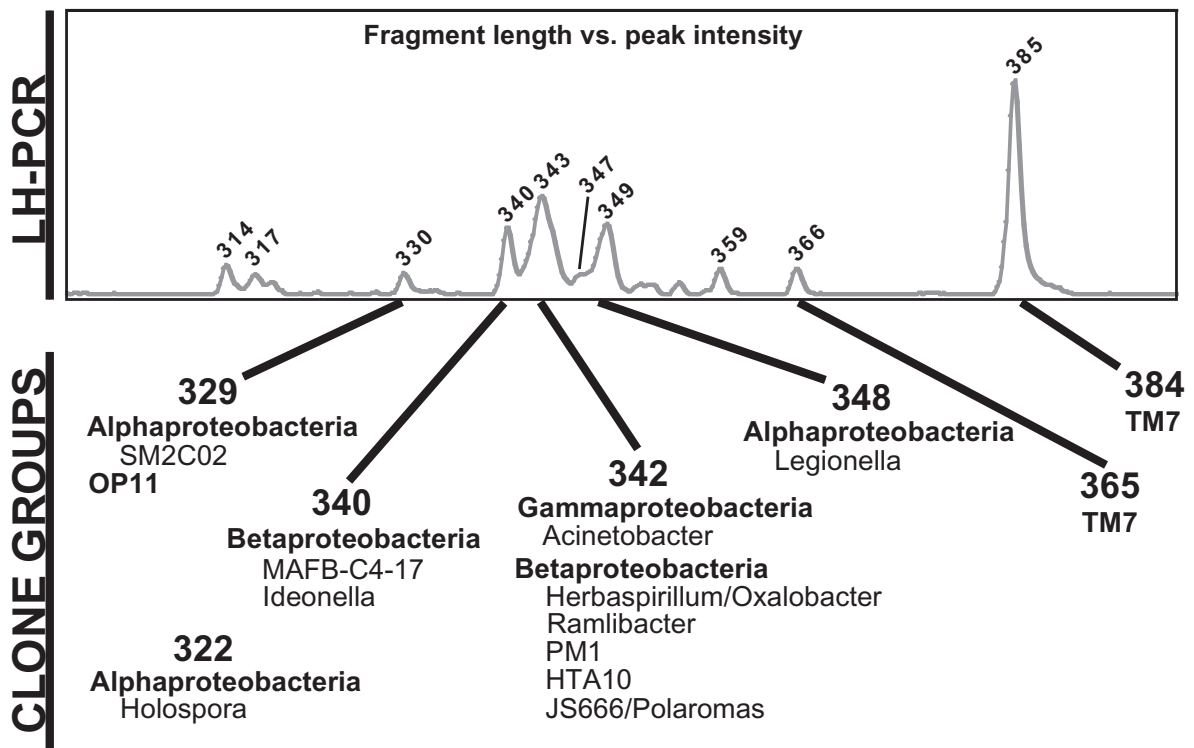
**Fig. 4.** Wells C2-113 and C4-113 over the course of the 187 day CAS demonstration. A. Propane utilization rates and percentage of 385 bp LH-PCR fragment. B. Concentration of TCE and *cis*-DCE. C. Nitrate and dissolved oxygen.

on restriction fragment length polymorphism (RFLP) analysis and were not identified. One extinction culture from the C-zone failed to amplify by PCR, and its identity was not determined.

Although the species cultured by extinction methods from the propane-sparged C-zone and the air-sparged A-zone were clearly different, incubation in a propane or air atmosphere did not have a noticeable effect on the diversity of species cultured. The dominant cultures acquired from the C-zone groundwater, *Herbaspirillum*/*Oxalobacter* clade isolates, and the A-zone groundwater, HTCC333 clade isolates from the beta-Proteobacteria, were isolated at similar frequencies with both propane and ambient air conditions. The rest of the isolates recovered from the A-zone and C-zone were not recovered often enough for it to be clear whether or not they dominated in either the

propane or the ambient air growth conditions. At the end of the 7 weeks of incubation, *Herbaspirillum*/*Oxalobacter* clade isolates grew, on average, to a cell density of  $1.9 \times 10^6 (\pm 5.9 \times 10^5)$  cells  $\text{ml}^{-1}$ , and the HTCC333 clade isolates grew to  $2.0 \times 10^5 (\pm 2.1 \times 10^5)$  cells  $\text{ml}^{-1}$ .

A comparison was made among the extinction cultures, the minimal agar medium isolates and clones that appeared more than once in the environmental 16S rDNA library (Tables 1 and 3). Thirteen major phylogenetic groups were recovered in the 16S rRNA gene clone library made from DNA collected from propane-stimulated groundwater in the C-zone. Microorganisms represented more than once in the clone library were considered to be dominant members of the microbial community. The clones and the extinction cultures were both dominated by representatives of the beta-Proteobacteria. Two of



**Fig. 5.** Comparison of LH-PCR and clone library made from water collected from well C4-113 on day 128. The sequence-determined LH-PCR fragment sizes of the clones are correlated with the most likely LH-PCR fragment peak on the electropherogram. Only clones represented more than once in the library were analysed with the exception of the clone represented by the 366 bp peak, MAFB-C4-52, which was only detected once in the library. The phylogenetic clades to which these clones belong are listed with the corresponding sequence-determined fragment length of the MAFB clones in that group.

these groups, PM1 and Herbaspirillum/Oxalobacter clades, were cultured using extinction culture techniques, whereas a traditional agar medium failed to recover any of the dominant groups that were represented by the clone

library. There was also very little overlap between the cultures acquired on agar medium and those acquired using the extinction culturing technique. Under the conditions used, the extinction culturing technique, which more

**Table 2.** Extinction culturability statistics compared with traditional counts.

Inoculation sample <sup>a</sup>	Av. cells per well	Total wells inoculated	Positive wells <sup>b</sup>	Culture designations	Percentage culturability <sup>c</sup>	Culturability on agar <sup>d</sup> (%)
Propane atmosphere growth chamber						
C2-113	3.0	48	3	HTCC 301–303	2.2 (1.0, 3.4)	1.5
	10	48	11	HTCC 304–314	2.6 (1.8, 3.4)	
C4-113	3.0	48	3	HTCC 315–317	2.2 (1.0, 3.4)	11.1
	10	48	12	HTCC 318–329	2.9 (2.1, 3.7)	
A1-113	10	48	3	HTCC 330–332	0.6 (0.2, 1.0)	–
A2-113	10	48	19	HTCC 333–351	5.0 (3.8, 6.2)	–
Ambient air atmosphere						
C2-113	3.0	48	3	HTCC 352–354	2.2 (1.0, 3.4)	–
	10	48	14	HTCC 355–368	3.4 (2.5, 4.3)	
C4-113	3.0	48	5	HTCC 369–373	3.7 (2.1, 5.3)	–
	10	48	18	HTCC 374–391	4.7 (3.6, 5.8)	
A1-113	10	48	5	HTCC 392–396	1.1 (0.6, 1.6)	–
A2-113	10	48	16	HTCC 397–412	4.1 (3.1, 5.1)	–

**a.** Groundwater samples were collected from the well indicated on day 128 of the co-metabolic sparging demonstration at MAFB, which was 92 days after propane and air sparging began.

**b.** Wells were scored for growth after 7 weeks of incubation at 16°C.

**c.** Exact lower and upper 95% confidence limits are shown in parenthesis and were determined in part with the SAS package 6.12 (SAS Institute) as described by Connon and Giovannoni (2002).

**d.** Inocula were the same as those used for the microtitre plates; – indicates not determined.

**Table 3.** Sequenced isolates and their 16S rRNA gene sequence similarities to nearest cultured neighbour or nearest clone.

Sequenced isolate	A-zone isolates <sup>a</sup>	C-zone isolates <sup>a</sup>	No. of nucleotides <sup>b</sup>	Phylogenetic clade <sup>c</sup>	Closest match in GenBank	% sequence similarity
Beta-Proteobacteria						
HTCC333	21		1439	*HTCC333	Clone HC-38 (AY168745)	98.0
HTCC410	1		1414	*HTCC410	Clone CLi22 (AF529318)	96.5
HTCC303		2	765	*PM1	MAFB-C4-9 (AY435506)	100
HTCC304		1	1335		Isolate PM1 (AF176594)	99.5
HTCC349	1		1392	*OM43	Clone Gitt-KF-149 (AJ532688)	98.6
HTCC379		2	1404	*Boom-7 m-04	Clone HOCiCi25 (AY328574)	99.9
W8		2	867		Clone HOCiCi25 (AY328574)	99.9
HTCC302		35	1424	*Herbaspirillum/Oxalobacter	MAFB-C4-83 (AY435503)	98.9
HTCC315		1	1432		Isolate COL (AF214642)	96.5
HTCC347	4		788	Variovorax	<i>Variovorax</i> sp. HAB-30 (AB051691)	100
HTCC332	2		1436	Ramlibacter	Clone C-CF-23 (AF443568)	98.5
HTCC356		1	667	Acidovorax	<i>Acidovorax</i> sp. smarlab 133815 (AY093698)	100
W4		1	874		<i>Acidovorax</i> sp. smarlab 133815 (AY093698)	99.2
HTCC392	2		792		<i>Acidovorax</i> sp. UFZ-B530 (AF235013)	99.2
HTCC329		2	640	Hydrogenophaga	<i>Hydrogenophaga taeniospiralis</i> (AF078768)	98.9
HTCC319		2	475	Ralstonia	<i>Ralstonia insidiosa</i> (AF488779)	100
HTCC376		1	1407	Limnobacter	<i>Limnobacter thiooxidans</i> (AJ289885)	99.2
Alpha-Proteobacteria						
HTCC309		1	806	*MHP14	Isolate AP-12 (AY145551)	99.6
HTCC396	1		899	*4-Org1-14	Clone 4-Org1-14 (AF143833)	94.3
HTCC353		1	421	Bradyrhizobiaceae	<i>Blastobacter denitrificans</i> (AF338176)	100
HTCC381		2	653		<i>Bradyrhizobium elkanii</i> (U35000)	100
HTCC407	1		900	Afipia/Oligotropha	<i>Afipia massiliensis</i> (AY029562)	100
HTCC354		2	771		<i>Oligotropha carboxidovorans</i> (AB099659)	99.5
W10		1	845		<i>Oligotropha carboxidovorans</i> (AB099659)	99.8
HTCC339	1		421	Methylobacterium	<i>Methylobacterium</i> sp. G296-15 (AF395034)	99.8
HTCC335	1		416	Sphingomonas	Isolate F0917 (AF235994)	99.3
HTCC399	1		896		<i>Sphingomonas</i> sp. ACM-3962 (AF411072)	99.4
Y8		2	926	Xanthobacter	<i>Xanthobacter flavus</i> (X94199)	99.8
W7		1	588	Agrobacterium/Rhizobium	Clone ccspost215 (AY133098)	99.3
W5		1	939	Mesorhizobium	<i>Mesorhizobium loti</i> (U50165)	98.1
Actinobacteria phylum						
HTCC345	1		453	Arthrobacter	<i>Arthrobacter</i> sp. SMCC ZAT262 (AF197055)	99.3
Y1		6	902	Mycobacterium	<i>Mycobacterium senegalense</i> (AF480596) and <i>M. fortuitum</i> (AF480580)	98.0
P5		1	878		<i>Mycobacterium tokaiense</i> (AF480590)	100
P2		2	927	Rhodococcus	<i>Rhodococcus koreensis</i> (AF124342)	99.7
W1		3	926	Nocardioidea	<i>Nocardioidea</i> sp. C157 (AF253509)	97.7
W6		1	878		<i>Nocardioidea</i> sp. V4-BE-17 (AJ244657)	97.7
P3		2	627	*Flexibacteraceae	Clone SM1E06 (AF445684)	96.7

a. Total number of cultures with the same RFLP pattern as the sequenced culture, including sequenced culture, isolated from each test site.

b. Sequenced length; ambiguous bases were removed from the phylogenetic analysis.

c. Isolates from the clades that have a \* preceding the name are less than 97% similar to any described species.

closely mimicked the natural environment by using natural groundwater as a medium, was clearly better at bringing into culture the dominant species as determined by molecular techniques.

## Discussion

All microbial community analyses in this study were performed on the groundwater or aqueous-borne fraction of the microbial community. Microorganisms that adhere tightly to the soil matrix may not be represented. Some sediment was released during pumping of the groundwater, and sparging itself could possibly release attached bacteria, so the attached portion of the microbial community cannot be strictly excluded in these analyses.

An increase in the size and numbers of microorganisms in the propane-sparged groundwater compared with the controls is an indication that this carbon source was being utilized. Biofouling due to nutrient amendments and subsequent increase in microbial biomass is a common problem in the field of groundwater bioremediation (Baveye *et al.*, 1998). While the microbial numbers increased dramatically after the addition of propane, this increase was not enough to interfere with pumping of groundwater for sample collection.

### LH-PCR community analysis before and after treatment

The addition of propane to the groundwater at MAFB caused a strong bacterial community shift in species com-

position to a TM7 division bacterium with an LH-PCR fragment of 385 bp, which was not detectable in the A-zone control that received only air or the presparged C-zone. This strong shift was clearly seen in groundwater from well C2-117, which received at least two times more propane than any other well and showed the highest proportion of the 385 bp peak on day 96, 83% of the total LH-PCR fragments. The only other fragment size that was found exclusively in the propane-sparged site was the 366 bp fragment, which matched the fragment length of TM7 division 16S rRNA genes recovered from the site. The proportion of the 385 bp LH-PCR fragment from groundwater sampled from well C4-113 on day 128 was 31.5% of the total fragments, but only 3.3% of the clones (three out of 91) had this LH-PCR fragment size. PCR bias of the LH-PCR analysis was minimized by restricting the final concentration of the amplified product, as this effectively reduces kinetic bias (Suzuki and Giovannoni, 1996; Suzuki *et al.*, 1998). The clone library was made with the same forward primer, but the reverse primer, 1522R, was used instead of 338R. No TM7 sequences were found in GenBank that included the 1522R primer site, indicating that this primer is not likely to be a good match for the TM7 bacterial division, which could explain its low yield in the clone library.

The TM7 division of bacteria currently has no cultured representatives and was named after clone TM7 found in a peat bog (Rheims *et al.*, 1996). TM7 division bacterial 16S rRNA genes are found in many diverse environments including activated sludge (Bond *et al.*, 1995), arid soils (Dunbar *et al.*, 1999; 2002), Amazonian soils (Borneman and Triplett, 1997) and a subsurface aquifer (Cho and Kim, 2000). Of particular interest is the fact that TM7 clones have been recovered previously from a TCE- and *cis*-DCE-contaminated site (Lowe *et al.*, 2002). Hugenholz and colleagues (2001) presented an in-depth investigation of the TM7 bacterial division and reported fluorescence *in situ* hybridization data indicating that TM7 organisms from activated sludge had various morphologies that included cocci, small rods and filamentous rods.

#### *TM7 correlation with propane removal rates*

The strong correlation of the 385 bp fragment with propane removal rates suggests that a TM7 division bacterium may be using propane or a metabolic byproduct of propane oxidation by another microorganism. The sharp drop in TCE and *cis*-DCE concentrations in response to stimulation of the microbial community by propane sparging also suggests that the microorganism(s) responsible for propane utilization may be co-oxidizing TCE and *cis*-DCE. It is possible that correlations to propane utilization by other microorganisms went undetected because of the nature of the LH-PCR analysis. The primers used for the

LH-PCR do not amplify archaeal 16S rRNA genes, and multiple microorganisms can be represented by one fragment size, potentially obscuring correlation patterns.

#### *Bacteria detected in propane-sparged groundwater by clone library analysis*

Of the 91 clones recovered in the clone library, 68 were represented more than once based on RFLP analysis and were investigated further. The 16S rDNA clones shown in Table 1 are members of phylogenetic clades that include isolates with known physiologies. It is interesting to note the physiologies of cultured species that have close sequence similarities to the 16S rDNA clones from this library. However, the physiologies of the species represented by the clones cannot be known conclusively without a cultured representative.

Three of the 47 beta-Proteobacterial clones were included in the JS666/*Polaromonas* clade and had a sequence-determined LH-PCR fragment size of 342 bp. The sequenced clone, MAFB-C4-60, is 98.2% similar to the 16S rRNA gene from isolate JS666, which is known to use *cis*-DCE as a sole source of carbon and energy (Coleman *et al.*, 2002). After sparging ended, TCE, *cis*-DCE, nitrate and DO levels continued to be measured (Tovanabootr *et al.*, 2001). TCE levels increased to pre-propane-sparged levels, but *cis*-DCE levels continued to remain low. It would be interesting to investigate further whether clone MAFB-C4-60 represents an organism that can use *cis*-DCE aerobically as a carbon and energy source, thus explaining these continued low *cis*-DCE levels in the absence of a co-oxidative substrate.

Nine beta-Proteobacterial clones belonged to the *Herbaspirillum*/*Oxalobacter* clade. *Herbaspirillum* sp. have a respiratory metabolism and fix nitrogen (Elbeltagy *et al.*, 2001; Kirchof *et al.*, 2001). Nitrogen fixation may be important for TCE co-oxidation. TCE removal by a nitrogen-fixing, methane-oxidizing mixed community was found consistently to outperform a non-nitrogen-fixing, methane-oxidizing community that was supplied with nitrogen (Chu and Alvarez-Cohen, 2000).

#### *Potential nitrogen limitation*

The decrease in both the propane removal rates and the proportion of the 385 bp fragment was correlated with a decrease in nitrate levels (Fig. 4C). Nitrogen limitations may have been responsible for this decrease in the rate of propane removal and subsequent decrease in TCE transformation. The groundwater flow at the site continually brought nitrate, TCE and *cis*-DCE, which complicates the analysis of the nitrate, TCE and *cis*-DCE concentration trends. As nitrate concentrations decreased below 2.0 mg l<sup>-1</sup> in groundwater from wells C2-113 on day 146

and C4-113 on day 131, TCE concentrations began to increase. Another consideration is the fact that no additional propane was added from day 160 to 188, which was the end of this phase of the demonstration, although measurable levels of propane were still being detected during this time. However, the increase in TCE was clearly occurring before day 160. It is possible that this increase in TCE was the result of nitrate limitation on the microorganisms responsible for TCE remediation.

Nitrogen limitation in the natural unamended groundwater, which had a starting nitrogen concentration of 5 mg l<sup>-1</sup>, may be the reason why the cultures from the *Herbaspirillum*/*Oxalobacter* clade grew to much higher densities than the HTCC333 clade isolates. Members of the genus *Herbaspirillum* can fix nitrogen. Nitrogen limitation was also a limiting factor in the bioremediation of TCE and *cis*-DCE at the study site where the water was collected for the growth medium from a nearby well. It is therefore interesting that members of the *Herbaspirillum*/*Oxalobacter* clade were frequently cultured from the C-zone groundwater where propane sparging and bioremediation were occurring.

#### *Bacteria detected in the C-zone and A-zone study site by culturing methods*

The extinction culturing approach resulted in isolates from eight previously uncultured or undescribed phylogenetic groups of bacteria, including two dominant groups detected in the environmental clone library, PM1 and *Herbaspirillum*/*Oxalobacter* clades. The PM1 clade was named for the isolate PM1, which is the only previously recovered isolate in this clade. Based on 16S rDNA phylogeny, it is a clade within the Comamonadaceae family of the beta-Proteobacteria. PM1 is able to use methyl *tert*-butyl ether (MTBE) as a sole carbon source (Bruns *et al.*, 2001). A member of the TM7 division of bacteria was clearly a dominant bacterium in the propane-sparged zone based on LH-PCR, yet it was not found among the isolates that we retrieved, suggesting that the chemistry of the medium or culturing conditions could not support the growth of this strain.

Mimicking natural environmental conditions as closely as possible, as in these experiments, has been a successful strategy for culturing new and relevant microbial species. However, variations in culturing conditions, especially those that mimic natural variation in nature, could be pursued in an attempt to bring more species into culture (e.g. additions of nitrogen sources, additions of TCE and *cis*-DCE and variations in the incubation time). Also, as a solid surface could be important to groundwater microorganisms, providing a solid surface for microbial attachment, such as gellan gum, which was used successfully by Janssen and colleagues (2002), or addition

of soil into the bottom of each extinction culture well could be adapted to existing culturing procedures. Interactions with other microorganisms in the environment have also been shown to be an important factor for culturing some species (Kaeberlein *et al.*, 2002). As more than one bacterial cell is put into each well of the microtitre plate, this high-throughput culturing method is well adapted to culturing microorganisms in co-culture.

Culturing microorganisms that are dominant at sites undergoing bioremediation is an important experimental strategy for understanding bioremediation processes. The role of the dominant species detected at the study site during active bioremediation can be more fully understood by investigating their physiology in pure culture. Further study is needed to evaluate the cultures acquired in this study for their ability to co-metabolize TCE and *cis*-DCE and for their nitrogen-fixing capabilities. Also needed is further investigation into the links between nitrogen fixation and enhanced bioremediation at the study site.

## Experimental procedures

### *Study site*

Two identical test zones were located ≈100 feet apart at MAFB (Fig. 1). Monitoring wells were located in both the A-zone and the C-zone at depths of 113 feet below ground surface (bgs) and 117 feet bgs. The water table started at a depth of ≈100 feet bgs. The A-zone was sparged only with air, and the C-zone received 2% propane in air (Tovanabootr *et al.*, 2000; 2001). The CAS demonstration, during which this study was conducted, was started on 12 May, 1999 and ended 15 November, 1999 lasting 187 days. Sparging was initiated on day 36 (17 June, 99) and performed weekly starting with the second sparging event on day 80 until day 120 and twice a week from day 120 to 160. No sparging was performed from day 160 to 187. Each sparge event was conducted over a 5–10 h period.

### *Analytical procedures for the measurement of propane, TCE, cis-DCE, DO and nitrate*

Groundwater samples were tested for the presence of propane, TCE, *cis*-DCE, DO and nitrate concentrations using methods described by Tovanabootr and colleagues (2000; 2001). Collection of nitrate data began on day 100 of this study. Propane removal rates were estimated based on three temporal propane measurements made after sparging. For accuracy, only measured propane levels above 0.01 mg l<sup>-1</sup> were used.

### *Microbial direct counts*

Groundwater samples from the A-zone and C-zone wells were collected for direct counts before propane sparging (day 20) and after sparging began (day 128). Direct cell counts were done by staining with 4',6-diamidino-2-phenylindole (DAPI) as described by Turley (1993), except that 1% form-

aldehyde was used. At least 300 cells were counted per filter, and triplicate filters were counted for each sample.

#### Sample collection for DNA recovery and purification

Total microbial DNA was purified from microbial cells collected from the groundwater samples for subsequent bacterial community analyses. The groundwater was sampled using low flow techniques as described in ASTM (1999) after purging with a minimum of three well volumes. Groundwater samples from the A-zone at 113 feet bgs (A1-113, A2-113 and A3-113) and the C-zone at 113 feet bgs (C2-113, C3-113, C4-113 and C5-113) were collected before propane sparging (day 20) and after the onset of propane uptake (days 96, 128, 174 and 187). Groundwater samples from the A-zone at 117 feet bgs (A1-117, A2-117 and A3-117) and C-zone at 117 feet bgs (C2-117, C3-117, C4-117 and C5-117) were collected before propane sparging (day 21) and after sparging began (day 126, and 187). Samples of groundwater (1 l) from each well were filtered on to 0.2 µm Supor membranes, placed into 10 ml cryovials (Midwest Scientific) with 5 ml of GES (5 M guanidine thiocyanate, 100 mM EDTA, 0.5% sarkosyl) and immediately stored in liquid nitrogen dewars. Samples in cryovials were then thawed and incubated at 37°C for 30 min in a hybridizer HB-1 (Techne) to ensure complete cell lysis before the DNA was extracted. The DNA was purified by a standard phenol chloroform protocol followed by ethanol precipitation (Sambrook *et al.*, 1989).

#### Bacterial community analysis by LH-PCR

LH-PCR was used to measure the change in the bacterial community composition of the groundwater (Suzuki *et al.*, 1998). The primers 8F (5'-(6-FAM)AGAGTTTGATCMTG GCTCAG-3') (Genset) and 338Rpl (5'-GCWGCCWCCCG TAGGWT-3') were used to amplify the 5' end of the 16S rRNA genes from each extracted DNA sample. The LH-PCR mixture contained 0.025 U µl<sup>-1</sup> *Taq* (Promega or MBI Fermentas), 5% acetamide, 1.5 mM Mg<sup>2+</sup>, 200 nM each primer, 220 µM dNTP and 1× PCR buffer (Promega or MBI Fermentas). The amplification conditions were 94°C denaturation for 30 s, 55°C annealing for 1 min and 72°C extension for 1 min, except the final extension, which was run for 20 min. The number of cycles of LH-PCR varied from 16 to 26 in order to restrict the final amplified product concentration to a range of 0.5–1 ng µl<sup>-1</sup>, as a low final concentration of amplified product reduces kinetic PCR bias (Suzuki and Giovannoni, 1996; Suzuki *et al.*, 1998). The LH-PCR samples were run on an ABI 373A or 377 gel-based automated sequencer (Applied Biosystems) in GeneScan mode and analysed using Applied Biosystems GENESCAN analysis software.

#### Clone library construction

A clone library was made from a sample collected on day 128 from monitoring well C4-113 of the propane-sparged test zone and consisted of 91 clones. The primers 8F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1522R (5'-AAGGAG GTGATCCANCCRCA-3') were used to amplify the 16S rRNA gene for cloning. The PCR mixture was identical to that used

for the LH-PCR above. The amplification conditions were 35 cycles of 94°C denaturation for 30 s, 55°C annealing for 1 min and 72°C extension for 2 min. Amplification products were cloned into the pGEM-T-easy vector (Promega) according to the manufacturer's instructions.

#### Phylogenetic analysis reported in tables and text

Sequences were aligned and masked in ARB (Strunk *et al.*, 1996). Phylogenetic analyses were performed using ARB and PAUP\* (Swofford, 2001). Phylogenetic trees were inferred by neighbour joining using the Jukes and Cantor model to estimate evolutionary distances. Bootstrap values were obtained in PAUP\* from a consensus of 1000 neighbour-joining trees. The percentage similarity of sequences was determined using the distance matrix tool in ARB; ambiguous bases were not included.

#### Media preparation

Groundwater was collected for extinction culturing medium on 23 May, 1999 from monitoring well 244, located 400 feet SW of the test site. Both the site and well 244 are within the 500 µg l<sup>-1</sup> TCE contour in operational unit (OU-A). Chlorinated solvents present in the groundwater were removed by sparging with air for several hours. The groundwater was then filtered through a 0.2 µm Supor membrane, autoclaved and sparged with CO<sub>2</sub> and air as described by Connon and Giovannoni (2002). The groundwater medium was not amended with any nutrients. The groundwater contained nitrate at 5 mg l<sup>-1</sup> as an available nitrogen source.

Traditional mineral medium agar plates were used in an attempt to culture propane oxidizers. This medium, described previously by Wiegant and deBont (1980), consisted of 0.5 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>, 23.5 mM NaNO<sub>3</sub> and 757 µM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and was phosphate buffered to a pH of 7.5 with K<sub>2</sub>HPO<sub>4</sub> and NaH<sub>2</sub>PO<sub>4</sub> for a final PO<sub>4</sub> concentration of 631 mM. The following trace elements were added: 22.6 µM FeSO<sub>4</sub>, 1.52 µM MnCl<sub>2</sub>, 0.514 µM ZnSO<sub>4</sub>, 1.0 µM H<sub>3</sub>BO<sub>3</sub>, 0.45 µM Na<sub>2</sub>MoO<sub>4</sub>, 0.1 µM NiCl<sub>2</sub>, 0.1 µM CuCl<sub>2</sub> and 0.1 µM CoCl<sub>2</sub>. Fifteen grams of agar was added per litre of medium to make agar plates.

#### Culture collection using extinction culturing methods

Groundwater was sampled from wells C2-113, C4-113, A1-113 and A2-113 on day 128, 92 days after propane and air sparging began. An extinction culturing approach was used to culture microorganisms from the groundwater as outlined by Connon and Giovannoni (2002). Inoculum water was collected in 1 l polycarbonate bottles that had been washed with 10% HCl followed by a NANOpure (Barnstead) water rinse. They were stored in a cooler with blue ice packs for transport to Corvallis, OR, USA. Direct cell counts of the inocula were performed immediately before diluting into the prepared medium by staining with DAPI as described above. Cells were diluted to either 3 or 10 cells ml<sup>-1</sup>, and 1 ml of the dilution was added to the wells of 48-well plates within 12 h of collection. Two 48-well plates were made with 10 cells per well for each of the A-zone and C-zone wells. Two 48-well

plates were made with three cells per well for each of the C-zone wells. Two control 48-well plates were also made with only medium added. One set of 48-well plates was incubated in an acrylic desiccant cabinet (Nalge Nunc International) that contained 2% propane, and several trays of water were added for humidity. The propane was added through a syringe port. This propane chamber was opened and recharged with propane every week. Propane levels in the chamber never fell below 0.75%, except briefly each week when the propane-air mixture was renewed. Propane was measured by methods outlined by Tovanabootr and Semprini (1998). The other set of 48-well plates was wrapped in Parafilm and incubated in ambient air. All plates were incubated at 16°C in the dark for 7 weeks. Growth in the wells was detected and cultures were identified by methods outlined by Connon and Giovannoni (2002). Briefly, each chamber in the 48-well plate was filtered on to a corresponding 48-spot filter array and DAPI stained. The chambers positive for growth were then detected by microscopy, and the density of the culture was also determined.

#### Culture collection using traditional culture methods

Mineral medium agar plates were also used to culture propane oxidizers from groundwater collected from wells C2-113 and C4-113 on day 128. The same groundwater inocula described above were spread on to the agar plates. The plates were inoculated in duplicate with 100 µl, 10 µl and 1 µl of undiluted groundwater inocula; 1 µl was diluted into 9 µl of sterile groundwater before spreading. Plates were incubated in the propane chamber, and colonies were counted after 7 weeks of growth. Colonies were streaked for isolation at least three times before the colonies on the plate were rinsed with 1 ml of sterile mineral medium broth to collect cells for sequence identification and cryopreservation. Twenty-three colonies were chosen randomly (10 from C2-113 and 13 from C4-113) for subsequent isolation and identification. The names are indicative of the colony colour: a 'W' prefix indicates isolates that formed white or clear colonies, 'P' indicates coral pink colonies and 'Y' indicates yellow colonies.

#### Identification and storage of cultures

A Qiagen DNeasy kit was used to extract the genomic DNA from 200 µl of the cell suspensions collected from the agar plates. A Vivaspin concentrator and cycles of freeze-thawing were used to prepare genomic DNA, as described by Connon and Giovannoni (2002), from cultures acquired by the extinction culturing method. Conditions for PCR were the same as those used for the clone library construction. The PCR product from each isolate was screened with the restriction enzymes *Hae*III and *Mbo*I or *Bsh*1236I (MBI Fermentas), and a representative of each isolate indicated by a unique restriction digestion pattern was sequenced.

Glycerol at a final concentration of 5% was added to 200 µl of cell suspension, and multiple aliquots were frozen to -80°C in 1.5 ml cryovials (Nalge Nunc International) using Mr Frosty (Nalge Nunc International), which controls the rate of freezing to -1°C min<sup>-1</sup>. The cells were then transferred to liquid nitrogen storage dewars.

#### Sequences submitted to DDBJ/EMBL/GenBank

Sequencing was done on an ABI 377 automated sequencer (Applied Biosystems). All sequenced clones have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AY435496 to AY435513. All sequenced isolates have been submitted under accession numbers AY429685-AY429721.

#### Acknowledgements

We wish to acknowledge Adisorn Tovanabootr, affectionately known as Merf, for his invaluable contribution to the collection of these data, while unbeknown to him he suffered from leukaemia, and for his relentless courage during his illness, which claimed him on 17 December, 2000. This work was supported by the Environmental Security Technology Certification Program of the Department of Defense and by McClellan Air Force Base through a contract with Battelle, Columbus, OH, USA. This work was also supported by National Science Foundation grants OIA-9977469 and MCB-9977930 and a grant from the Murdock Charitable Trust. In addition, Tartar Research fellowships and general grants and funds were provided by Oregon State University. We would also like to thank Victor Magar and Andre Leeson who were project managers for the co-metabolic air sparging project.

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