9-1989

Biological Reductive Dechlorination of Tetrachloroethylene and Trichloroethylene to Ethylene under Methanogenic Conditions

David L. Freedman
Clemson University, dfreedm@clemson.edu

James M. Gossett
Cornell University

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1989, p. 2144–2151
0099-2240/89/092144-08$02.00/0

Vol. 55, No. 9

Biological Reductive Dechlorination of Tetrachloroethylene and Trichloroethylene to Ethylene under Methanogenic Conditions

DAVID L. FREEDMAN AND JAMES M. GOSSETT*

School of Civil and Environmental Engineering, Cornell University, Ithaca, New York 14853-3501

Received 13 February 1989/Accepted 8 June 1989

A biological process for remediation of groundwater contaminated with tetrachloroethylene (PCE) and trichloroethylene (TCE) can only be applied if the transformation products are environmentally acceptable. Studies with enrichment cultures of PCE- and TCE-degrading microorganisms provide evidence that, under methanogenic conditions, mixed cultures are able to completely dechlorinate PCE and TCE to ethylene, a product which is environmentally acceptable. Radiotracer studies with [^{14}C]PCE indicated that [^{14}C]ethylene was the terminal product; significant conversion to $^{14}CO_2$ or $^{14}CH_4$ was not observed. The rate-limiting step in the pathway appeared to be conversion of vinyl chloride to ethylene. To sustain reductive dechlorination of PCE and TCE, it was necessary to supply an electron donor; methanol was the most effective, although hydrogen, formate, acetate, and glucose also served. Studies with the inhibitor 2-bromoethanesulfonate suggested that methanogens played a key role in the observed biotransformations of PCE and TCE.

The contamination of groundwater with tetrachloroethylene (PCE) and trichloroethylene (TCE) is widespread. PCE and TCE are among 14 volatile organic compounds regulated under the Safe Drinking Water Act Amendments of 1986. The maximum contaminant level for TCE is currently 5 μg/liter; a standard for PCE is expected by June 1989 (23). Physical and chemical processes (e.g., air stripping and carbon adsorption) are used most frequently for remediation. However, interest has been growing in biological processes because they offer the prospect of converting the contaminants to harmless products, rather than transferring them from one part of the environment to another.

Biotransformation of PCE and TCE under anaerobic conditions has been observed in field studies (22), in continuous-flow fixed-film reactors (6, 7, 25, 26), in soil (18), sediment (2–4, 19–21), and aquifer microcosms (27, 28), and to a limited degree in pure cultures (9–12). General agreement exists that transformation of PCE under anaerobic conditions proceeds by sequential reductive dechlorination to TCE, dichloroethylene (DCE), and vinyl chloride (VC). Each chlorine is replaced by hydrogen. Of the three possible DCE isomers, 1,1-DCE is the least significant intermediate; several studies have reported that *cis*-1,2-DCE predominates over *trans*-1,2-DCE (3, 4, 19–21).

Conversion of PCE and TCE to less chlorinated alkenes is of little or no benefit. *cis*-1,2-DCE, *trans*-1,2-DCE, and VC are also regulated under the 1986 Safe Drinking Water Act Amendments, precisely because they, too, pose a threat to public health. For anaerobic bioremediation to be useful, PCE and TCE must be degraded to nonchlorinated, environmentally acceptable products. Very few studies have demonstrated that this is possible. Vogel and McCarty (25, 26) have reported 24 and 27% conversions of low concentrations of PCE (31 to 60 μg/liter) and VC, respectively, to CO_2.

In this study, we present evidence that anaerobic enrichment cultures which support methanogenesis are capable of completely dechlorinating PCE and TCE to ethylene (ETH), without significant conversion to CO_2. Degradative pathways were inferred from studies with [^{14}C]PCE. We also investigated the potential need of each microbial system for an auxiliary electron donor to sustain reductive dechlorination and the possible link between degradation of the chlorinated compound and methanogenesis.

* Corresponding author.

MATERIALS AND METHODS

Chemicals and radioisotopes. The following compounds were obtained in neat liquid form: PCE (high-pressure liquid chromatography grade, 99.9+%; Aldrich Chemical Co., Inc., Milwaukee, Wis.); TCE (certified American Chemical Society grade; Fisher Scientific Co., Pittsburgh, Pa.); *cis*-1,2-DCE (97%; Aldrich Chemical Co.); *trans*-1,2-DCE (5-g ampoule; Supelco, Inc.); and 1,1-DCE (5-g ampoule; Supelco, Inc.). VC, ETH, and CH_4 were obtained as gases in lecture bottles (99+%; Scott Specialty Gases). PCE and TCE were added to cultures from saturated-water stock solutions, containing approximately 1.2 μmol of PCE and 8.6 μmol of TCE per ml, respectively. 2-Bromoethanesulfonic acid (BES; sodium salt, 98%) was obtained from Aldrich Chemical Co. [^{14}C]PCE (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 150 ml of distilled deionized water and stored in a 160-ml serum bottle, capped with a Teflon-lined rubber septum. The [^{14}C]PCE stock solution was initially 1.1×10^7 dpm/ml (990 nmol of PCE per ml); with extensive use and losses over time, this gradually declined to 7.6×10^6 dpm/ml (680 nmol of PCE per ml). ScintiVerse-E (Fisher Scientific) liquid scintillation cocktail was used.

Cultures and enrichment procedures. All experiments were conducted in 160-ml serum bottles containing 100 ml of liquid. The bottles were sealed with Teflon-lined rubber septa and aluminum crimp caps. Incubation was conducted at 35°C, under quiescent conditions, with the liquid in contact with the septum (to minimize loss of volatiles). Degradations of PCE and TCE were initially achieved in "first-generation" cultures, prepared by anaerobically transferring 100-ml mixed-liquor samples directly from a laboratory reactor to 160-ml serum bottles. The laboratory reactor was a 15-liter, stirred, semicontinuous, anaerobic digester operated at 35°C with a residence time of 20 days. It was initially seeded with digested sludge from the Ithaca wastewater treatment plant and batch fed daily a substrate consisting of (per liter of tap water): Ensure, 31.5 ml; $NaHCO_3$, 3.36 g; $K_2HPO_4 \cdot 3H_2O$, 0.35 g; $MgSO_4 \cdot 7H_2O$, 0.20 g;

TABLE 1. Operation of bottles used for [^{14}C]PCE analysis

Bottle no.	Electron donor	Length of time operated (days)		No. of PCE + [^{14}C]PCE additions	Total PCE added (μmol)	Total [^{14}C]PCE added (10^6 dpm)
		Semicontinuously	Incubation			
VI-M1	Methanol	16	26	6	2.44	3.24
VI-M2	Methanol	26	21	11	4.47	5.83
VI-M3	Methanol	45	20	16	6.26	8.27
VI-M4	Methanol	61	31	22	8.56	11.0
VI-M5	Methanol	80	30	28	10.7	13.5
VI-G1	Glucose	18	7	4	1.63	2.15
VI-G2	Glucose	40	16	9	3.53	4.66
VI-G3	Glucose	58	16	15	5.76	7.41
VI-G4	Glucose	76	29	21	7.88	9.95
VI-G5	Glucose	90	24	24	9.03	11.1

$FeCl_2 \cdot 4H_2O$, 0.010 g; and $CoCl_2 \cdot 6H_2O$, 0.007 g (14). The 15-liter digester was not fed for several days prior to use as an inoculum source; nevertheless, it doubtless contributed unknown quantities of complex substrates to the first-generation cultures.

Typical initial doses were 300 to 450 nmol/100 ml for PCE (0.50 to 0.75 mg/liter) and 550 to 700 nmol/100 ml for TCE (0.72 to 0.92 mg/liter). When the initial dose was degraded, the bottles were repetitively respiked by adding PCE- or TCE-saturated stock solution, after first withdrawing an equivalent volume of the cultures. Samples from several of the first-generation bottles were then used to inoculate the second-generation cultures, which were then used to inoculate the third-generation cultures, and so on, through the development of sixth-generation cultures. A 2 to 10% (vol/vol) inoculum was used each time, the balance consisting of basal medium plus 50 mg of auxiliary substrate (glucose, methanol, acetic acid, or sodium formate) per liter. The basal medium (modified from Zeikus [29]) consisted of (per liter of distilled deionized water): NH_4Cl, 0.20 g; $K_2HPO_4 \cdot 3H_2O$, 0.10 g; KH_2PO_4, 0.055 g; $MgCl_2 \cdot 6H_2O$, 0.20 g; trace metal solution (per liter, 0.1 g of $MnCl_2 \cdot 4H_2O$; 0.17 g of $CoCl_2 \cdot 6H_2O$; 0.10 g of $ZnCl_2$; 0.20 g of $CaCl_2$; 0.019 g of H_3BO_4; 0.05 g of $NiCl_2 \cdot 6H_2O$; and 0.020 g of $Na_2MoO_4 \cdot 2H_2O$, adjusted to pH 7 with NaOH or HCl), 10 ml; resazurin, 0.001 g; $Na_2S \cdot 9H_2O$, 0.50 g; $FeCl_2 \cdot 4H_2O$, 0.10 g; $NaHCO_3$, 5.0 g; and yeast extract, 0.050 g. It was prepared by boiling the first six components, cooling under an N_2 purge, adding the remaining components, switching the purge gas to 30% CO_2–70% N_2, and adjusting the pH to 7 to 7.5. The medium was dispensed by the method of Holdeman et al. (17). Whenever PCE or TCE was degraded, 4.0 ml of well-mixed liquid was removed and replaced with fresh basal medium (containing 50 mg of the auxiliary substrate per liter) plus PCE or TCE stock solution. The interval between each removal and respiking of fresh basal medium plus chlorinated compound was never less than 2 days, making the minimum retention time in the serum bottles 50 days. Operation of the bottles in this semicontinuous reactor mode often exceeded 100 days.

Each set of experiments with inoculated bottles was accompanied by duplicate water controls (100 ml of distilled deionized water plus the chlorinated compounds) and duplicate inoculated bottles which were autoclaved (121°C, 30 min) and cooled before addition of the chlorinated compounds.

Ten sixth-generation bottles were used for the [^{14}C]PCE degradation experiments (Table 1). In five of the bottles, methanol was the auxiliary substrate; the other five used glucose. They were operated semicontinuously, as described above, except that each addition of unlabeled PCE was accompanied by 50 μl of [^{14}C]PCE stock. Samples of the [^{14}C]PCE stock were counted along with each addition. Aliquots (2.0 ml) of the mixed liquid removed were also counted. Within each set of five bottles, the length of total operation time varied (42 to 110 days for the methanol bottles, 25 to 114 days for the glucose bottles). In all of the bottles, semicontinuous operation was followed by an incubation period (7 to 31 days) when PCE and [^{14}C]PCE additions were stopped. At the end of the incubation period, the bottles were sacrificed for complete ^{14}C analysis.

Analysis of volatile organic compounds. Volatile organic compounds (CH_4, ETH, VC, 1,1-DCE, 1,2-DCEs, TCE, and PCE) were routinely determined by gas chromatographic (GC) analysis of a 0.5-ml headspace sample, using a flame ionization detector in conjunction with a stainless-steel column (3.2 mm by 2.44 m) packed with 1% SP-1000 on 60/80 Carbopack-B (Supelco, Inc.), as described previously (15). Detection limits for the chlorinated compounds ranged from 3.60 nmol/100 ml for 1,1-DCE to 14.3 nmol/100 ml for *cis*-1,2-DCE; the limits for ETH and methane were 2.00 and 3.52 nmol/100 ml, respectively. When necessary, separation of *cis*- and *trans*-1,2-DCE was achieved on a stainless-steel column (3.2 mm by 2.44 m) packed with 20% SP-2100 on 80/100 Supelcoport (Supelco, Inc.), operated isothermally at 60°C. More refined separation of CH_4 and ETH was obtained on a stainless-steel column (3.2 mm by 3.2 m) packed with 100/120 Carbosieve S-II (Supelco, Inc.), operated at 150°C for 10 minutes, ramped 10°C/min to 200°C, and held for 10 min.

Identification and confirmation of PCE, TCE, DCEs, and VC was made by GC-mass spectrometry (MS; Finnigan model 3500 GC-MS, coupled with a Teknivent Interactive GC-MS Data System). Identification was subsequently confirmed by injection of authentic material into the same GC system routinely used for headspace monitoring (SP-1000/Carbopack-B column). For ETH (and for low levels of CH_4), the detection limit of the MS system precluded its use. Identification was accomplished by matching the retention times of authentic material with unknown peaks from headspace samples of enrichment cultures, using four GC columns operated under different temperature conditions. At a carrier gas (helium) flow of 30 ml/min, retention times for CH_4 and ETH were 0.58 and 0.85 min on SP-1000/Carbopack-B (60°C isothermal), 0.49 and 1.51 min on Chromosorb 101 (40°C isothermal), 4.03 and 10.8 min on Carbosieve G (60°C for 2 min, ramped to 150°C at 20°C/min, ramped to 200°C at 10°C/min, held for 4.5 min), and 4.42 and 16.9 min

on Carbosieve S-II (150°C for 5 min, ramped to 200°C at 10°C/min, held for 15 min).

GC calibration factors were measured to relate directly the total mass of compound present in a serum bottle to the GC peak area obtained from a 0.5-ml headspace injection. Known masses of PCE, TCE, and DCEs were added to replicate serum bottles containing 100 ml of distilled deionized water, allowed to equilibrate at 35°C, and then analyzed (0.5-ml headspace) by GC (15). Standard additions of CH_4, ETH, and VC were effected from neat gaseous stocks. Coefficients of variation [(standard deviation/mean) × 100] for the calibration factors ranged from 0.58 to 2.9%.

The headspace monitoring method relied on the volatile compounds being freely available, i.e., unsorbed and uncomplexed. This was a reasonable assumption, judging from a comparison of water controls to inoculated bottles. Soon after preparation of a new generation of enrichments, the GC peak areas obtained for PCE and TCE in inoculated bottles were consistently close to those in the water controls.

Analysis of ^{14}C-labeled compounds. Volatile ^{14}C-labeled compounds (PCE, TCE, DCEs, VC, ETH, and CH_4) were analyzed with a GC-combustion technique. A 0.5-ml headspace sample was injected into the GC. All of the GC column effluent was routed (1.59-mm stainless-steel tubing) to a quartz combustion tube (6-mm inside diameter by 10-mm outside diameter by 39-cm length) held at 800°C by means of a tube furnace (Lindberg, Inc.). CuO catalyst pellets occupied approximately 24 cm of the tube, the ends of which were fitted with Thermogreen LB-1 cylindrical thru-hole septa (6 by 9-mm; Supelco, Inc.). Passing through the combustion tube, the well-separated peaks of volatile compounds were oxidized to CO_2 and then conveyed through a porous-glass diffuser into separate glass test tube traps (13 by 100 mm) containing 3.0 ml of 0.5 M NaOH. These traps were manually exchanged as each peak eluted; their contents were subsequently counted in 15 ml of liquid scintillation cocktail.

Two samples were used to determine the distribution of ^{14}C-labeled compounds in the headspace of a bottle. The first was injected onto the SP-1000/Carbopack-B column. CH_4, CO_2, and ETH were trapped together in the 0- to 1.5-min fraction. The well-separated chlorinated compounds were then trapped between the following times (minutes): 2.8 to 5.2 for VC; 5.8 to 6.8 for 1,1-DCE; 6.9 to 8.4 for 1,2-DCEs; 9.9 to 12.0 for TCE; and 14.4 to 18.0 for PCE. The second sample was injected onto the Carbosieve S-II column, which separated CH_4 (trapped between 4.1 and 6.5 min) and CO_2 (trapped between 7.8 and 10.5 min) from ETH (eluted between 16.6 and 20.6 min); volatile chlorinated compounds did not elute from this column. The disintegrations per minute for ETH were obtained by using the total disintegrations in the 0- to 1.5-min fraction off the Carbopack-B column and subtracting the disintegrations per minute for CH_4 and CO_2 from the Carbosieve S-II column (which was the most effective at separating CH_4, CO_2, and ETH, among the four columns examined). The trapping time needed for each compound was determined by injecting nonlabeled samples of authentic material to each column. Instead of trapping the effluent from the combustion tube in NaOH, it was routed to a hot-wire detector (175°C, 240 mA), and beginning and ending times of the resulting CO_2 peaks were noted. In general, the combustion tube added approximately 0.2 min to the retention time of each compound, with no significant impact on peak width.

The disintegrations per minute (dpm) measured for each volatile compound were related to the total in the bottle by $dpm_{total} = 2(S - B)[(V_l/H_c) + V_g]$, where S is disintegrations per minute trapped from a 0.5-ml headspace injection; B is disintegrations per minute for a blank (fractions trapped from a 0.5-ml injection of air across the same time intervals); V_l is volume of liquid (100 ml); V_g is volume of headspace (60 ml); and H_c is "dimensionless" Henry's constant [(gas concentration in moles per cubic meter)/(aqueous concentration in moles per cubic meter)]. Previously reported values for H_c at 35°C were used for PCE, TCE, and DCEs (15). H_c values for VC, ETH, and CH_4 at 35°C were measured by the technique described by Gossett (15). Four serum bottles were prepared with 100 ml of distilled deionized water, and four were prepared with 10 ml. Each compound was added as a gas, necessitating the assumption that equal masses were added to all bottles. The H_c values obtained, with their 95% confidence intervals, were 1.38 ± 0.01 for VC, 9.06 ± 0.45 for ETH, and 33.1 ± 6.9 for CH_4.

The overall efficiency of the GC-combustion system was tested by comparing the cumulative disintegrations per minute measured from an NaOH trap with those obtained by injecting a replicate headspace sample directly into a scintillation vial containing 15 ml of liquid scintillation cocktail. Loss of volatile compounds was minimized by injecting samples through a Teflon-lined rubber septum placed beneath the vial's cap, which had a 3-mm hole drilled in it. The average recovery was 104.0% (range, 96.0 to 114.0%).

After analysis of ^{14}C-labeled volatile compounds was completed, $^{14}CO_2$ and ^{14}C-labeled nonvolatile compounds were measured. A 1.5-ml amount of 5 M NaOH was injected into a serum bottle, which raised the pH above 10.5 and forced virtually all CO_2 into the aqueous phase (14). Separation of $^{14}CO_2$ and ^{14}C-labeled nonvolatile compounds was performed in an apparatus consisting of a 30-ml stripping chamber (glass test tube, 20 by 150 mm, fitted with a two-hole rubber stopper, a porous-glass diffuser, and a septum) connected to a 30-ml gas absorption chamber (glass test tube, 20 by 150 mm, fitted with a two-hole rubber stopper and a porous-glass diffuser) by a piece of tubing partially filled with 0.25 g of Tenax (to trap those ^{14}C-labeled volatile chlorinated compounds having relatively low Henry's constants). A 20-ml aliquot was transferred to the gas stripping chamber, which was then closed. The pH was lowered to 4.4 by injecting 0.4 ml of glacial acetic acid through the septum, and the contents were sparged for 30 min with nitrogen (50 to 60 ml/min). Gaseous effluent from the stripping chamber passed through the Tenax into the gas absorption chamber, which contained 20 ml of 0.5 M NaOH to trap $^{14}CO_2$. The contents of each chamber were then diluted to 25.0 ml, and ^{14}C activity was measured in 2.0-ml aliquots. Confirmation of $^{14}CO_2$ in the absorption chamber was made by adding $Ba(OH)_2$, shaking vigorously, centrifuging (26,600 × g for 10 min), and then counting a sample of the supernatant. The supernatant disintegrations per minute never exceeded 2% of the presumptive $^{14}CO_2$ disintegrations. The disintegrations per minute remaining in the stripping chamber represented what we termed "nonstrippable residue" (NSR). It was further partitioned into soluble and insoluble fractions by centrifugation (26,600 × g for 10 min) and then by counting an aliquot of the supernatant.

^{14}C activity was assayed with a model 9800 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Corrections for counting efficiency were made according to a quench curve (sample H# versus efficiency).

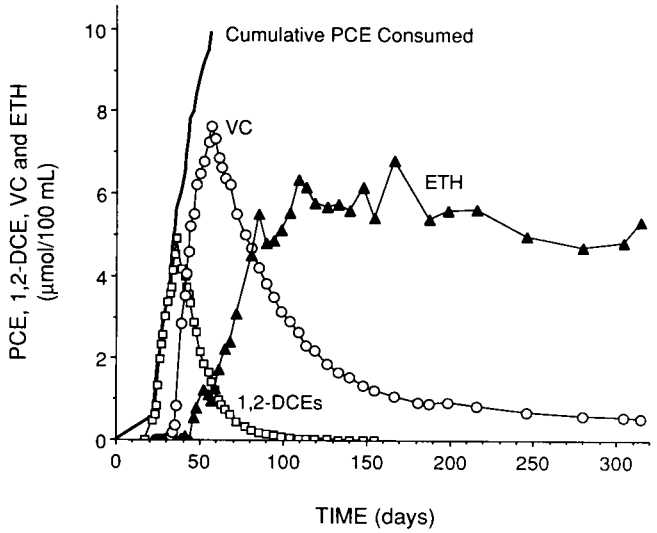

FIG. 1. Reductive dechlorination of PCE in a first-generation culture (bottle I-1), which was obtained directly from a laboratory anaerobic digester.

RESULTS

Biotransformation pathway. Formation of ETH by reductive dechlorination of PCE was initially observed in first-generation bottles I-1 and I-2. In bottle I-1, a total of 9.93 μmol of PCE was degraded (25 repetitive additions) between days 0 and 55, at which time PCE additions were stopped (Fig. 1). VC, which had accumulated to 7.64 μmol, immediately began to decline while ETH rose. Beyond day 85, ETH levels remained more or less the same, while VC continued to decline. This was most likely a consequence of leakage of VC and ETH through the bottle's septum, which had been punctured numerous times. In bottle I-2, 84 PCE additions were degraded. TCE and 1,2-DCEs were the major intermediates formed in the first 31 days. Thereafter, TCE disappeared entirely, 1,2-DCEs crested and then declined, and VC and ETH became the principal products. After 309 days of operation, 36.4 μmol of PCE was consumed; 4.90 μmol of VC and 12.0 μmol of ETH remained. The difference between PCE consumption and VC and ETH production was most likely a consequence of leakage losses. Over the same period, PCE decreased from 618 to 228 nmol in duplicate water controls and from 556 to 207 nmol in duplicate autoclaved-inoculated controls.

Routine GC analysis of headspace samples was performed with the SP-1000/Carbopack-B column, which did not separate *cis*- from *trans*-1,2-DCE very well. Analysis on the 20% SP-2100 column of samples from four different bottles indicated that the predominant isomer was *trans* (100, 100, 89, and 56%). On this basis, 1,2-DCE amounts were subsequently calculated assuming all *trans*. Although this approach underestimated the total amount of 1,2-DCE, it was not a major concern because, in most of the enrichment cultures past the second generation, 1,2-DCE did not accumulate; instead, only VC and ETH accumulated.

[^{14}C]PCE experiments were undertaken to determine whether the ETH formed was a consequence of PCE degradation and whether products not detectable by headspace monitoring (e.g., CO_2) with a flame ionization detector were also being formed. The 10 sixth-generation bottles described in Table 1 were used to assess the fate of [^{14}C]PCE. Routine

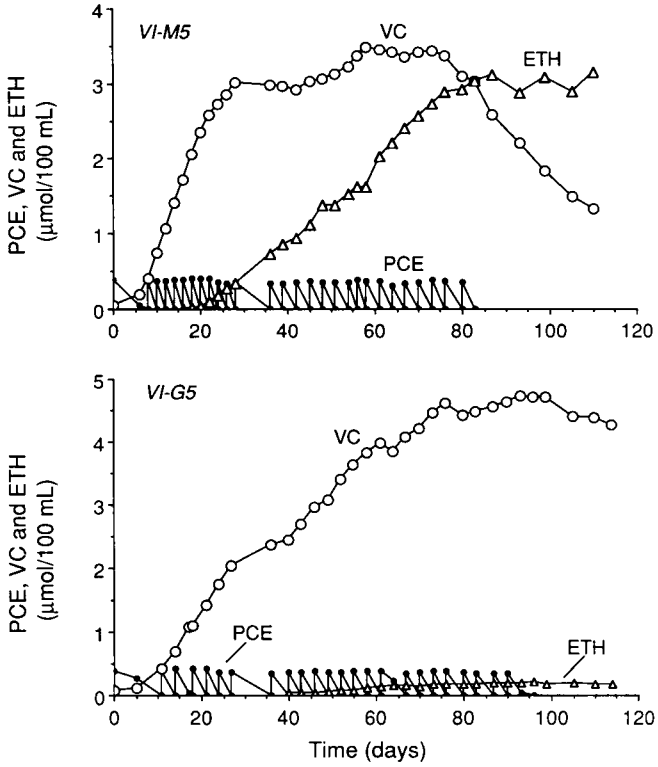

FIG. 2. Reductive dechlorination of PCE in sixth-generation enrichment cultures, using methanol (VI-M5) or glucose (VI-G5) as the electron donor. Each spike of PCE was accompanied by [^{14}C]PCE.

GC headspace monitoring was performed on all of the bottles. Results for bottles VI-M5 and VI-G5 are presented in Fig. 2. A typical pattern emerged: PCE was rapidly converted to VC, with ETH accumulating more slowly; during the incubation period which followed cessation of PCE additions, VC levels declined while ETH rose. Much more ETH was formed in the bottles receiving methanol as an auxiliary carbon source compared with the bottles receiving glucose. The other eight bottles (VI-M1 through -M4 and VI-G1 through -G4) were operated for shorter periods of time. The shorter the period of operation, the lower the conversion of VC to ETH.

Through the combined processes of effluent removal (in semicontinuous operation) and leakage of volatile ^{14}C constituents, the total ^{14}C remaining in a bottle at the time of radiotracer analysis was sometimes considerably less than the cumulative [^{14}C]PCE disintegrations per minute added. Leakage losses (as determined by comparing cumulative stock addition counts with the sums of disintegrations per minute obtained from bottle headspaces, liquid contents, and cumulative effluents) ranged from 12 to 36%, depending on the total length of operation. On the other hand, the GC-combustion method and CO_2 stripping technique gave a very good mass balance for the ^{14}C remaining in the bottles. For the 10 inoculated bottles analyzed, the sum of radioactivity measured (CH_4, PCE, TCE, DCEs, VC, ETH, CO_2, and NSR) ranged from 94.2 to 121.6% (mean, 103.7%; coefficient of variation, 7.3%) of the total radioactivity obtained by directly counting the gaseous and liquid phases of each bottle.

Table 2 presents the distribution of ^{14}C in each of the inoculated bottles at the end of its incubation period. The

TABLE 2. Distribution of ^{14}C in bottles spiked with [^{14}C]PCE[a]

Bottle no.	% of total dpm remaining after semicontinuous operation and incubation							
	ETH	VC	1,1-DCE	1,2-DCEs	TCE	PCE	CO_2	CH_4
VI-M1	24.3	71.9	0.59	1.43	0.23	0.33	0	1.24
VI-M2	34.6	62.5	0.57	0.65	0.19	0.29	0	1.19
VI-M3	44.9	52.8	0.49	0.44	0.08	0.21	0	1.04
VI-M4	60.9	34.0	0.47	0.73	0.27	0.25	2.65	0.73
VI-M5	68.7	26.7	0.53	0.72	0.30	0.42	1.64	0.96
VI-G1	1.89	93.0	0.71	2.32	0.34	0.40	0.39	0.94
VI-G2	8.28	87.6	0.65	1.52	0.26	0.26	0	1.43
VI-G3	8.49	85.5	1.23	2.13	0.68	0.81	0	1.20
VI-G4	11.1	83.4	1.21	1.78	0.50	0.52	0	1.49
VI-G5	7.67	87.9	0.72	1.33	0.19	0.15	0.41	1.64

[a] See Table 1 for a description of how each bottle was operated. Formation of ^{14}C-labeled NSR was negligible in all of the bottles.

data of Table 2 have been corrected for the contaminated condition of the [^{14}C]PCE stock solution. Our analysis indicated that the ^{14}C stock contained an average of only 80.1% [^{14}C]PCE; the balance consisted of approximately 8.8% $^{14}CO_2$ and 11.1% ^{14}C-labeled NSR (all soluble). No further attempt was made to characterize this ^{14}C-labeled NSR contaminant; however, we later observed that the ^{14}C-labeled NSR content of inoculated samples markedly diminished with extended incubation. Though we cannot be absolutely certain, we believe the product was $^{14}CO_2$, since no corresponding increase in $^{14}CH_4$ was observed, and the sum of $^{14}CO_2$ plus ^{14}C-labeled NSR tended to be rather constant. Therefore, we assumed that the sum of $^{14}CO_2$ plus ^{14}C-labeled NSR contributed by the contamination of the stock behaved conservatively. Furthermore, at the total concentration of carbonates (and the neutral pH) in the basal medium, virtually all of the $^{14}CO_2$ (and, of course, the ^{14}C-labeled NSR) was partitioned into the aqueous phase of a serum bottle. Knowledge of stock volumes added to serum bottles and of effluent volumes removed allowed the correction of observed disintegrations per minute for contaminant contributions.

The principal products of [^{14}C]PCE degradation by these anaerobic cultures were [^{14}C]VC and [^{14}C]ETH; neither $^{14}CH_4$ nor $^{14}CO_2$ was noted in any significant quantities (Table 2). Most important, Table 2 indicates that (i) [^{14}C]ETH was produced from [^{14}C]PCE (conversion was highest in those bottles fed methanol as auxiliary substrate) and (ii) the "net" quantity of $^{14}CO_2$ in the inoculated bottles never exceeded 2.7%, regardless of auxiliary substrate or the total time of operation.

The specific activities of PCE, VC, and ETH were essentially the same, providing further evidence that VC and ETH were produced by reductive dechlorination of PCE. On average, the specific activities (± their 95% confidence intervals) were 0.466 ± 0.019, 0.492 ± 0.024, and 0.464 ± 0.034 mCi/mmol for PCE, VC, and ETH, respectively.

Our need to correct radiotracer results to account for the initially contaminated condition of the stock [^{14}C]PCE solution caused some concern, though we had confidence in our correction methods and our accounting procedure. Nevertheless, to reinforce conclusions drawn from these earlier studies, a further experiment was undertaken. A sixth-generation methanol enrichment culture (VI-M6) degraded 2.83 μmol of TCE during 17 days of semicontinuous operation. Most of the TCE was converted to VC, which accumulated to a peak of 2.54 μmol on day 21. No further additions of TCE were made after day 21. Methanol was occasionally added to facilitate conversion of VC to ETH. On day 53, a 5.0-ml headspace sample from bottle VI-G5 (used in previous [^{14}C]PCE studies; Table 1) was injected into VI-M6. This headspace sample was essentially free of $^{14}CO_2$ or ^{14}C-labeled NSR, because it was obtained after adding NaOH to VI-G5. Thus, the headspace injection made to VI-M6 on day 53 constituted a source of [^{14}C]VC and [^{14}C]ETH uncontaminated by $^{14}CO_2$ or ^{14}C-labeled NSR. After further incubation, the bottle was analyzed on day 119 for distribution of ^{14}C species. Although losses were significant (33%), there was substantial conversion of [^{14}C]VC to [^{14}C]ETH, without a corresponding increase in $^{14}CH_4$ or detectable appearance of $^{14}CO_2$ (Table 3).

In sum, results of the [^{14}C]PCE experiments indicate that biological dechlorination terminated primarily at ETH; significant transformation to $^{14}CO_2$ was not observed. The pathway can be summarized as follows:

$$\text{PCE} \xrightarrow{2[H]\ HCl} \text{TCE} \xrightarrow{2[H]\ HCl} \text{1,2-DCEs} \xrightarrow{2[H]\ HCl} \text{VC} \xrightarrow{2[H]\ HCl} \text{ETH}$$

Electron donor requirements. Each step in the pathway described above exhibits a reductive dechlorination. To determine what may serve as the reductant (i.e, electron donor), several fifth-generation PCE enrichment cultures were examined. A typical result for a PCE-methanol enrichment culture is given in Fig. 3. After 17 days, the initial PCE dose was consumed, followed by semicontinuous operation for the next 28 days. This meant that methanol and yeast extract were present along with each new addition of PCE (approximately 300 nmol/100 ml), which was degraded (to VC and smaller amounts of ETH) within 3 days. On day 45, PCE was added without any basal medium and, hence, without any auxiliary carbon source. At the same time,

TABLE 3. Distribution of ^{14}C in bottle VI-M6[a]

Compound	Day 53		Day 119	
	10^3 dpm	% of dpm	10^3 dpm	% of dpm
CH_4	4.92	3.52	4.03	4.28
ETH	22.4	16.0	41.9	44.5
VC	112.5	80.5	48.0	51.0
CO_2	0	0	0	0
NSR	0	0	0.19	0.20

[a] The ^{14}C added to this bottle (on day 53) was from the headspace of bottle VI-G5 after it had been spiked with NaOH, so the sample was free of CO_2 and NSR.

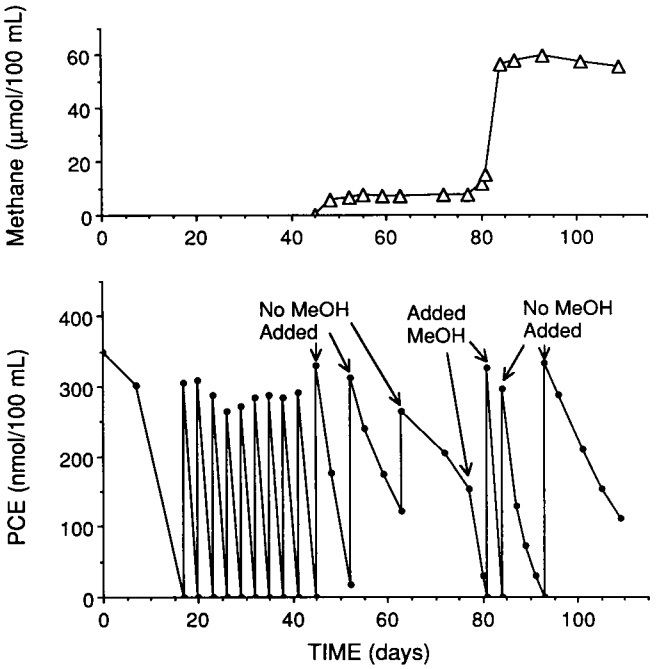

FIG. 3. Effect of MeOH on rate of PCE dechlorination in a fifth-generation enrichment culture. The dechlorination products were VC and smaller amounts of ETH.

cumulative methane output was monitored as a measure of any electron donor still available to the culture. As soon as methane output leveled off, the rate at which PCE degraded slowed considerably. On days 77 and 81, 1 mg of methanol was added to the bottle. This restored the faster rate of PCE degradation, and methane output rose accordingly. Without any further methanol additions, the rate of PCE degradation once again slowed considerably and at the same time methane production virtually stopped.

The process of providing an electron donor and then withholding it, providing it again and withholding it again, etc., was tested with other cultures, using glucose, acetate, or formate as the electron donor, with the same results. It was also examined with TCE-dechlorinating cultures, with TCE spikes as high as 9 μmol/100 ml. As long as the electron donor was provided, TCE conversion to VC and ETH was sustainable.

Hydrogen was also shown to be an effective electron donor for PCE and TCE dechlorination. Two bottles were set up identically (inoculated with a fifth-generation PCE-degrading enrichment culture, using glucose as the auxiliary carbon source). One received 1 ml of H_2 each time it was respiked with PCE; the other received only PCE. Over a 216-day period, the bottle receiving H_2 consumed a total of 13.7 μmol of PCE (with VC the principal product formed; ETH remained below 0.60 μmol). In the bottle which received no H_2, the rate of PCE degradation slowed considerably over time; only 3.48 μmol of PCE was consumed in 198 days (Fig. 4). PCE degradation in the absence of added H_2 was probably supported by the yeast extract initially present in the basal medium. Duplicate bottles behaved similarly.

Effect of BES. The effect of BES on reductive dechlorination of PCE and TCE was examined with fourth-generation enrichments receiving formate, acetic acid, glucose, methanol, or no auxiliary substrate. Duplicate bottles were operated semicontinuously in the usual manner. Once repetitive

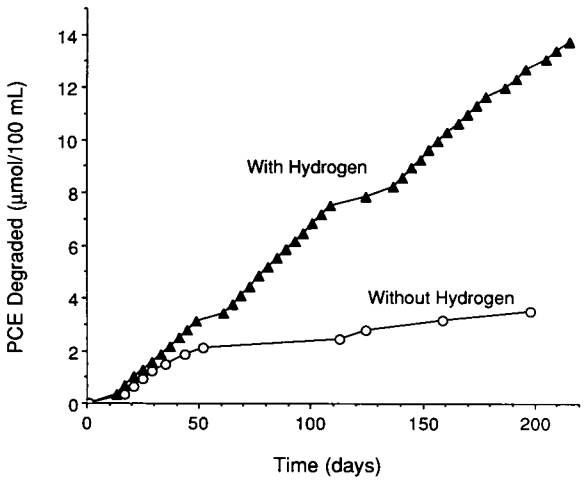

FIG. 4. Effect of hydrogen on rate of PCE dechlorination in sixth-generation enrichment cultures.

degradation was demonstrated, 0.5 mmol of BES was added to one of the bottles in each pair. In all cases, BES immediately stopped TCE degradation and methane production (Fig. 5). Bottles not receiving BES continued to degrade TCE and produce methane.

BES added to PCE-degrading bottles also immediately stopped methane production; however, repeated BES additions were required before PCE degradation ceased (Fig. 6). Although BES did not immediately stop PCE degradation, it did cause 1,2-DCE and TCE to accumulate. In bottles receiving no BES, VC was the principal degradation product.

DISCUSSION

Sequential reductive dechlorination of PCE and TCE to VC under methanogenic conditions has been demonstrated previously (3, 19, 21, 25, 26). We have demonstrated that PCE and TCE can be further dechlorinated to ETH. This

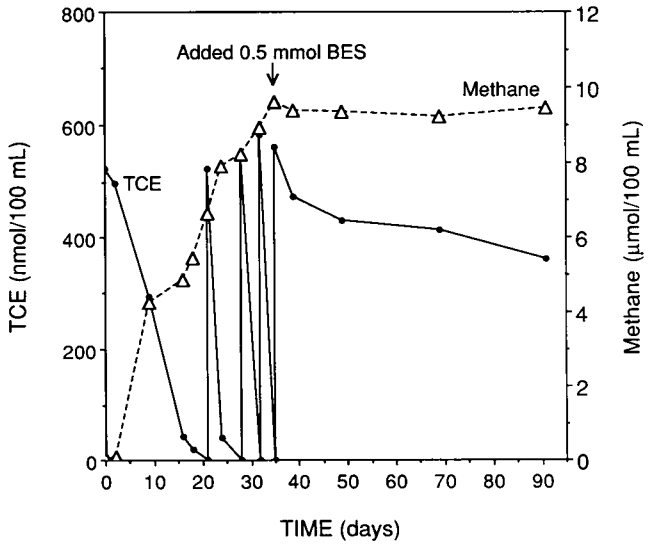

FIG. 5. Effect of BES on reductive dechlorination of TCE in a fourth-generation enrichment culture (electron donor = formate).

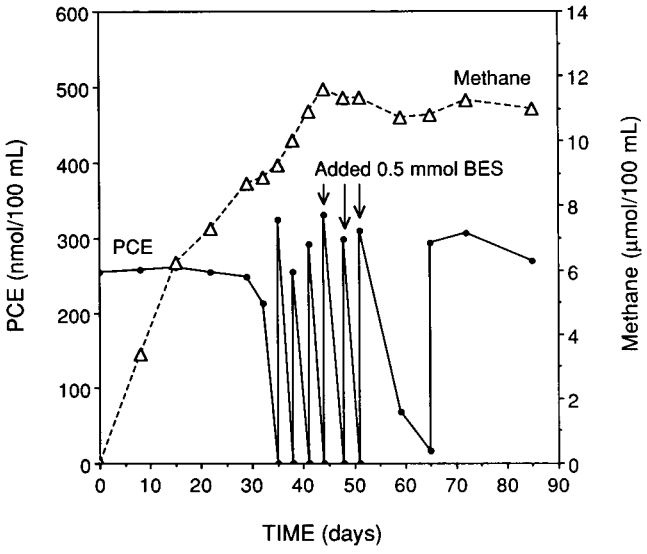

FIG. 6. Effect of BES on reductive dechlorination of PCE in a fourth-generation enrichment culture (electron donor = formate).

finding is significant because, unlike VC, ETH is an environmentally acceptable biotransformation product. ETH is sparingly soluble in water, and it has not been associated with any long-term toxicological problems (1). It is also a commonly occurring plant hormone. Identification of ETH as a product was due mainly to use of a flame ionization detector and repetitive respiking of the cultures. Flame ionization detectors are far more sensitive to nonhalogenated hydrocarbons than electron capture or electrolytic detectors. Although MS is adequately sensitive, distinguishing ETH from N_2 is complicated by their virtually identical mass numbers. Repetitively respiking the cultures with PCE and TCE allowed ETH to accumulate to detectable levels.

Formation of ETH under anaerobic conditions from halogenated alkanes has been observed previously, but not from halogenated alkenes. Pure cultures of *Methanobacterium thermoautotrophicum*, *Methanococcus deltae*, and *Methanococcus thermolithotrophicus* have been used to produce ETH from 1,2-dichloroethane, 1,2-dibromoethane, and bromoethanesulfonate (5, 9). 1,2-Dibromoethane has also been reductively dechlorinated to ETH in mixed-culture soil-water suspensions (8). Further degradation of ETH was not reported, which is in agreement with our finding that PCE and TCE were degraded to ETH, but not to CO_2. In an attempt to determine whether ETH could be degraded by methanogens (in pure and mixed cultures), Schink (24) observed that not only was ETH recalcitrant, but it was a potent selective inhibitor of methanogenesis.

The rate-limiting step in complete reductive dechlorination of PCE and TCE to ETH was conversion of VC to ETH. At 35°C, it took enrichment cultures only 2 to 3 days to continuously convert 0.5 to 1.0 mg of PCE and TCE per liter to VC, which accumulated. ETH was formed when PCE and TCE were repeatedly added, but at a much slower rate. Dechlorination of all of the VC present in a bottle was never achieved, even after additions of PCE or TCE ceased (Fig. 1 and 2). Improvement in the rate and extent of ETH formation, especially at temperatures closer to those of groundwaters, is needed before anaerobic bioremediation can be implemented. Fathepure et al. (12) have pointed out that, in anaerobic systems, the fewer chlorine atoms remaining on an alkene, the more difficult they are to remove. The reverse occurs in aerobic environments; e.g., Fogel et al. (13) were able to observe degradation of TCE, but not PCE, with a mixed culture containing methanotrophs.

Reductive dechlorination was not sustainable unless an electron donor was provided. When PCE or TCE was added to an enrichment culture without auxiliary substrate, the rate of degradation slowed considerably as soon as readily degradable electron donors were exhausted. Fathepure and Boyd (11) demonstrated a similar dependence of the dechlorination of PCE to TCE on the amount of substrate provided to pure cultures of *Methanosarcina* sp. strain DCM. Other studies reporting dechlorination of PCE and TCE under anaerobic conditions also supplied an electron donor (in one form or another). For example, Vogel and McCarty (25, 26) used acetic acid and traces of other organic compounds in their work with continuous-flow, fixed-film reactors; Kleopfer et al. (18) added soybean meal to soil samples; and Parsons et al. (19) added methanol (used to prepare stock solutions of PCE and TCE) to their sediment samples, which undoubtedly also contributed degradable organic matter.

The most effective enrichment cultures were those fed methanol, in terms of both maintaining the rate at which repetitive additions of PCE or TCE were degraded (for periods exceeding 3 months of semicontinuous operation) and the extent to which VC was converted to ETH. Fathepure et al. (11, 12) also achieved better success dechlorinating PCE with pure cultures of methanogens grown on methanol, rather than acetate. Barrio-Lage et al. (4) were unable to improve TCE degradation by adding acetate to sediment microcosms. It is not yet clear why methanol is most effective as a source of reducing equivalents. Metabolism of methanol by methanogens does involve enzymes and pathways not involved in the metabolism of other common methanogenic substrates such as hydrogen, formate, and acetate (30).

A major operational cost of anaerobic bioremediation will be the supply of an electron donor. At this point, the amount required to completely dechlorinate PCE or TCE remains to be defined. During semicontinuous operation of the enrichment cultures, each 79-μg/100 ml (600-nmol/100 ml) addition of TCE was accompanied by 200 μg of auxiliary substrate per 100 ml. In the case of methanol, this provided more than 13 times the minimum reducing equivalents needed to dechlorinate TCE to ETH, yet complete conversion of VC to ETH was never reached. Either higher ratios of electron donor to TCE or PCE must be provided (thereby increasing costs), or a way must be found to channel more of the donor into reductive dechlorination and less into methane production.

All of our reductive dechlorination studies were conducted with mixed cultures, so it was not possible to specify the class of organisms (methanogens, other obligate anaerobes, facultative anaerobes, etc.) responsible for PCE or TCE degradation. Indirect evidence strongly suggests that methanogens played a key role. First, degradation of TCE was completely stopped, as was methane output, when BES (a selective inhibitor of methyl-coenzyme M reductase, the enzyme which catalyzes the final step in methanogenesis [16]) was added to enrichment cultures. A similar effect was observed with PCE, but repeated additions of BES were required, possibly because these cultures were able to degrade BES. Schink (24) has noted that this is a drawback to the use of BES. Second, reductive dechlorination was stimulated by electron donors typically used by methanogens: hydrogen, methanol, formate, and acetate. Fathepure and

Boyd (10) also observed inhibition of PCE dechlorination with BES and have proposed a model of PCE dechlorination based on electrons transferred during methanogenesis. If methanogens are indeed responsible, and ETH is the desired end product of dechlorination, then care must be taken in design of treatment systems to ensure that ETH does not reach high enough levels to inhibit methanogenesis.

Other investigators have demonstrated biotransformation of PCE to TCE with pure cultures of methanogens, including *Methanosarcina mazei*, *Methanobacterium thermoautotrophicum*, and *Methanosarcina* sp. strain DCM (9–12). However, further reductive dechlorination occurred only with a methanogenic consortium, which included an obligate anaerobe capable of dechlorinating 3-chlorobenzoate (12). Thus, mixed cultures may be essential to achieve complete dechlorination of halogenated alkenes under methanogenic conditions. More information is needed on the specific organisms involved, so that environmental conditions, including nutritional requirements, can be optimized.

ACKNOWLEDGMENTS

This research was supported by the U.S. Air Force Engineering and Services Center, Tyndall AFB, Fla., under contract F08635-86-C-0161.

We gratefully acknowledge the assistance of Mike Henley, U.S. Air Force Engineering and Services Center, for providing GC-MS support.

LITERATURE CITED

1. **Autian, J.** 1980. Plastics, p. 531–556. *In* J. Doull, C. D. Klaassen, and M. O. Amdur (ed.), Casarett and Doull's toxicology. Macmillan Publishing Co., Inc., New York.
2. **Barrio-Lage, G., F. Z. Parsons, and R. S. Nassar.** 1987. Kinetics of the depletion of trichloroethene. Environ. Sci. Technol. **21**:366–370.
3. **Barrio-Lage, G., F. Z. Parsons, R. S. Nassar, and P. A. Lorenzo.** 1986. Sequential dehalogenation of chlorinated ethenes. Environ. Sci. Technol. **20**:96–99.
4. **Barrio-Lage, G. A., F. Z. Parsons, R. S. Nassar, and P. A. Lorenzo.** 1987. Biotransformation of trichloroethene in a variety of subsurface materials. Environ. Toxicol. Chem. **6**:571–578.
5. **Belay, N., and L. Daniels.** 1987. Production of ethane, ethylene, and acetylene from halogenated hydrocarbons by methanogenic bacteria. Appl. Environ. Microbiol. **53**:1604–1610.
6. **Bouwer, E. J., and P. L. McCarty.** 1983. Transformations of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions. Appl. Environ. Microbiol. **45**:1286–1294.
7. **Bouwer, E. J., and J. P. Wright.** 1988. Transformations of trace halogenated aliphatics in anoxic biofilm columns. J. Contam. Hydrol. **2**:155–169.
8. **Castro, C. E., and N. O. Belser.** 1968. Biodehalogenation. Reductive dehalogenation of the biocides ethylene dibromide, 1,2-dibromo-3-chloropropane, and 2,3-dibromobutane in soil. Environ. Sci. Technol. **2**:779–783.
9. **Egli, C., R. Scholtz, A. M. Cook, and T. Leisinger.** 1987. Anaerobic dechlorination of tetrachloromethane and 1,2-dichloroethane to degradable products by pure cultures of *Desulfobacterium* sp. and *Methanobacterium* sp. FEMS Microbiol. Lett. **43**:257–261.
10. **Fathepure, B. Z., and S. A. Boyd.** 1988. Reductive dechlorination of perchloroethylene and the role of methanogens. FEMS Microbiol. Lett. **49**:149–156.
11. **Fathepure, B. Z., and S. A. Boyd.** 1988. Dependence of tetrachloroethylene dechlorination on methanogenic substrate consumption by *Methanosarcina* sp. strain DCM. Appl. Environ. Microbiol. **54**:2976–2980.
12. **Fathepure, B. Z., J. P. Nengu, and S. A. Boyd.** 1987. Anaerobic bacteria that dechlorinate perchloroethene. Appl. Environ. Microbiol. **53**:2671–2674.
13. **Fogel, M. M., A. R. Taddeo, and S. Fogel.** 1986. Biodegradation of chlorinated ethenes by a methane-utilizing mixed culture. Appl. Environ. Microbiol. **51**:720–724.
14. **Gossett, J. M.** 1985. Anaerobic degradation of C_1 and C_2 chlorinated hydrocarbons. NTIS no. AD-A165 005/0. Engineering & Services Laboratory, U.S. Air Force Engineering and Services Center: Tyndall AFB, Fla.
15. **Gossett, J. M.** 1987. Measurement of Henry's law constants for C_1 and C_2 chlorinated hydrocarbons. Environ. Sci. Technol. **21**:202–208.
16. **Gunsalus, R. P., J. A. Romesser, and R. S. Wolfe.** 1978. Preparation of coenzyme M analogues and their activity in the methyl coenzyme M reductase system of *Methanobacterium thermoautotrophicum*. Biochemistry **17**:2374–2377.
17. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobic laboratory manual. Virginia Polytechnic Institute and State University, Blacksburg.
18. **Kleopfer, R. D., D. M. Easley, B. B. Haas, T. G. Deihl, D. E. Jackson, and C. J. Wurrey.** 1985. Anaerobic degradation of trichloroethylene in soil. Environ. Sci. Technol. **19**:277–280.
19. **Parsons, F., G. Barrio-Lage, and R. Rice.** 1985. Biotransformation of chlorinated organic solvents in static microcosms. Environ. Toxicol. Chem. **4**:739–742.
20. **Parsons, F., and G. B. Lage.** 1985. Chlorinated organics in simulated groundwater environments. Am. Water Works Assoc. **71**:52–59.
21. **Parsons, F., P. R. Wood, and J. DeMarco.** 1984. Transformations of tetrachloroethene and trichloroethene in microcosms and groundwater. Am. Water Works Assoc. **76**:56–59.
22. **Roberts, P. V., J. Schreiner, and G. D. Hopkins.** 1982. Field study of organic water quality changes during groundwater recharge in the Palo Alto baylands. Water Res. **16**:1025–1035.
23. **Sayre, I. M.** 1988. International standards for drinking water. Am. Water Works Assoc. **80**:53–60.
24. **Schink, B.** 1985. Inhibition of methanogenesis by ethylene and other unsaturated hydrocarbons. FEMS Microbiol. Ecol. **31**: 63–68.
25. **Vogel, T. M., and P. L. McCarty.** 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. **49**:1080–1083.
26. **Vogel, T. M., and P. L. McCarty.** 1987. Abiotic and biotic transformations of 1,1,1-trichloroethane under methanogenic conditions. Environ. Sci. Technol. **21**:1208–1213.
27. **Wilson, B. H., G. B. Smith, and J. F. Rees.** 1986. Biotransformations of selected alkylbenzenes and halogenated aliphatic hydrocarbons in methanogenic aquifer material: a microcosm study. Environ. Sci. Technol. **20**:997–1002.
28. **Wilson, J. T., J. F. McNabb, B. H. Wilson, and M. J. Noonan.** 1983. Biotransformation of selected organic pollutants in ground water. Dev. Ind. Microbiol. **24**:225–233.
29. **Zeikus, J. G.** 1977. The biology of methanogenic bacteria. Bacteriol. Rev. **41**:514–541.
30. **Zeikus, J. G., R. Kerby, and J. A. Krzycki.** 1985. Single carbon chemistry of acetogenic and methanogenic bacteria. Science **227**:1167–1173.