APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1988, p. 1709–1714
0099-2240/88/071709-06$02.00/0

Vol. 54, No. 7

Mineralization of Trichloroethylene by Heterotrophic Enrichment Cultures

C. B. FLIERMANS,[1]* T. J. PHELPS,[2] D. RINGELBERG,[2] A. T. MIKELL,[2] AND D. C. WHITE[2]

Savannah River Laboratory, E. I. du Pont de Nemours & Co., Inc., Aiken, South Carolina 29808,[1] and Institute for Applied Microbiology, University of Tennessee, Knoxville/Oak Ridge National Laboratory, Knoxville, Tennessee 37932[2]

Received 19 January 1988/Accepted 15 April 1988

Microbial consortia capable of aerobically degrading more than 99% of exogenous trichloroethylene (TCE) (50 mg/liter) were collected from TCE-contaminated subsurface sediments and grown in enrichment cultures. TCE at concentrations greater than 300 mg/liter was not degraded, nor was TCE used by the consortia as a sole energy source. Energy sources which permitted growth included tryptone-yeast extract, methanol, methane, and propane. The optimum temperature range for growth and subsequent TCE consumption was 22 to 37°C, and the pH optimum was 7.0 to 8.1. Utilization of TCE occurred only after apparent microbial growth had ceased. The major end products recovered were hydrochloric acid and carbon dioxide. Minor products included dichloroethylene, vinylidine chloride, and, possibly, chloroform.

Large portions of subsurface aquifers in the United States have been contaminated by chlorinated hydrocarbons (8, 19) and groundwaters near several industrial sites contain over 1,000 mg of chlorocarbons, such as tetrachloroethylene and trichloroethylene (TCE), per liter (6). These extremely high levels of contamination pose technologically difficult problems of containment and remediation. Since subsurface aquifers are not in contact with the atmosphere, volatilization or photolysis of these chemicals is unlikely. Chemical degradation is often prohibitively expensive, charcoal adsorption requires further disposal of the contaminant and adsorbent, and air stripping releases the contaminant into the atmosphere, where it may pose alternative environmental and health concerns. Biological treatments which degrade contaminants of the site without generating toxic wastes may be suitable for purifying large volumes of contaminated groundwater.

Soil microbiology studies conducted during the early part of the twentieth century did not detect large microbial populations in subsurface soils (16, 21, 22); this suggested that little microbial life existed in the deep subsurface. Even though evidence of deep subsurface microbial populations and activities had been reported (4, 26), it was generally accepted that the role of microorganisms in subsurface environments was insignificant (1). It has recently been established that aquifers and vadose-zone sediments contain substantial populations of microorganisms which can influence groundwater quality (5, 9, 23, 24).

Degradation of TCE or its degradation products has been reported by many investigators; some of these reports have included mechanisms and kinetic models for TCE degradation (2, 3, 7, 10, 11, 14–18, 20, 25). Wilson and co-workers (25) have established TCE-degrading soil columns amended with methane. Methanotrophs were observed to increase in biomass in the columns (15), and it was hypothesized that methanotrophs were integral to the TCE mineralization process. The hypothesis was based in part on the observation that methanotrophs contain mixed-function oxidases capable of degrading chlorinated ethenes (7).

Others (3, 20) have demonstrated anaerobic metabolism of low concentrations of TCE in digester microcosms. Anaerobic utilization of TCE appears to result in the formation or the accumulation of vinyl chloride (20), which is mutagenic and recalcitrant. Degradation of TCE by an aerobic heterotrophic microorganism has been recently reported (14). Although TCE was converted to carbon dioxide, there was a requirement for an aromatic compound, such as phenol, and only low concentrations of TCE were utilized (less than 1 mg/liter). To date, investigators have used concentrations of TCE approximately 3 orders of magnitude lower than those observed in many contaminated aquifers.

The purpose of this investigation was to examine the activity and community structure of microorganisms in subsurface sediments which had been heavily contaminated with short-chain chlorinated hydrocarbons. Studies included radioisotope activity measurements, microcosm enrichment studies, and isolation enrichments for TCE-degrading microorganisms. This report describes enrichment cultures which are capable of degrading TCE at concentrations greater than 50 mg/liter by using a variety of energy sources.

MATERIALS AND METHODS

Gases, chemicals, and isotopes. Nitrogen and N_2-CO_2 (9:1, vol/vol) were more than 99.9% pure. In the laboratory, all gases were passed through copper-filled Vycor furnaces (Sargent-Welch Scientific Co., Skokie, Ill.) to remove traces of oxygen. All chemicals were of reagent grade and were obtained from Mallinckrodt, Inc. (Paris, Ky.) or Sigma Chemical Co. (St. Louis, Mo.). Glass-distilled solvents and reagents were purchased from J. T. Baker Chemical Co. (Phillipsburg, N.J.). [1,2-^{14}C]TCE (10 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, Mass.).

Laboratory techniques. Descriptions of the field site, sampling methods, and handling of samples have been given elsewhere (T. J. Phelps, D. Ringelberg, D. Hedrick, J. Davis, C. B. Fliermans, and D. C. White, Geomicrobiol. J., in press). Subsurface sediment samples were aseptically collected from depths of 5 to 50 m from an area contaminated with short-chain chlorinated hydrocarbons at the Savannah River Plant near Aiken, S.C. Sediment samples recovered from subsurface formations were placed into sterile Whirl Pak bags (NASCO, Fort Wilkinson, Wis.) and stored under a N_2 atmosphere. Enrichment and incubation mixture experiments were either immediately initiated in the field or

* Corresponding author.

subsequently initiated in the laboratory under both aerobic and anaerobic conditions. Media typically contained 10 mM carbonate, 2 mM phosphate buffer, trace minerals, and vitamins (12) with or without 300 to 500 mg of yeast extract per liter and one or more of the following potential energy sources: 10% methane, 5% propane, 15% hydrogen, TCE (50 mg/liter), acetate (400 to 800 mg/liter), glucose (100 to 300 mg/liter), methanol (300 mg/liter), 10% ethylene, Trypticase (300 to 500 mg/liter; BBL Microbiology Systems, Cockeysville, Md.), or other chlorinated hydrocarbons at concentrations of 50 mg/liter. Electron acceptors included 20 mM nitrate, sulfate, or carbon dioxide or 40 mM oxygen; resazurin served as a redox indicator. Solidifying agents included Noble agar, purified agar, silica gel, and Whatman filter paper. Unless otherwise stated, each transfer moved 0.3 ml of broth into 10 ml of fresh medium.

All manipulations involved syringe techniques for the transfer of solutions or inocula. Enrichments utilized crimp-top tubes or serum vials (Bellco Glass, Inc., Vineland, N.J., or Wheaton Industries, Millville, N.J.) sealed with Teflon-lined septa (Alltech, Deerfield, Ill.). Incubations were conducted at ambient temperatures (23°C) for 3 weeks to 3 months. Unless otherwise stated, all tubes contained 50 to 150 mg of TCE per liter (calculated assuming all of the TCE was in the liquid phase) with a gas to liquid volume ratio of 2.0:1 to 2.5:1.

Analytical procedures. Nonradioactive and radioactive carbon dioxide were examined by the gas chromatography-gas proportional counting technique described by Nelson and Zeikus (13). A Packard 417 gas chromatograph equipped with a thermal conductivity detector was connected to a Packard 894 gas proportional counter (Packard Instruments Co., Inc., Downers Grove, Ill.). The gas chromatograph operated at 85°C; the helium carrier gas flow was 45 ml/min, with a stainless steel column (0.3175 cm by 2 m) packed with Carbosieve (80/100 mesh). Carbon dioxide formation from TCE was confirmed by gas chromatography-gas proportional counting techniques. Duplicate enrichment vials were inoculated with 30 mg of TCE per liter plus 700,000 dpm of [1,2-^{14}C]TCE in 3 ml of medium in a 12-ml vial. Vials were incubated for 4 weeks at 22°C. Loss of total TCE was measured by purge and trap analysis with the electron capture detector. Chloride concentrations were determined with a chloride test kit (LaMotte Chemical Products Co., Chestertown, Md.).

Short-chain chlorinated hydrocarbons were analyzed on a Hewlett-Packard 5890 capillary gas chromatograph with a split-splitless injector, a 50-m methyl silicone-fused silica capillary column, an electron capture detector, and the liquid nitrogen cryogenic cooling option with hydrogen as the carrier gas (Hewlett-Packard, Avondale, Pa.). When direct headspace gas injections were made onto the gas chromatograph, the oven was operated isothermally at 60°C. Autosampling was performed with a Tekmar liquid sampler and liquid sample concentrator equipped with a Tenax-charcoal-silica gel trap (Tekmar, Co., Cincinnati, Ohio). Liquid nitrogen was provided from a 25-liter, high-pressure Dewar flask (Cryofab Inc., Kenilworth, N.J.), and ultra-high-purity N_2 was the purge gas. Purge and trap analyses typically included purging with N_2 at 44 ml/min for more than 3 min, heating the trap to 180°C, and desorbing for 0.9 min. The gas chromatograph oven temperature was increased from approximately −70°C to −30°C at 20°C per min, and then to 10°C at 2°C per min, and finally to 100°C. Data were analyzed with a Hewlett-Packard 3350 series laboratory data system.

TABLE 1. Degradation of TCE by enrichment cultures[a]

Inoculum	Substrate	Enrichment culture[b]	TCE degradation after successive transfer[c]:			
			1	2	3	4
805-180	CH_4	III	99	50	0	0
805-180	CH_4	M1III	41	0	27	54
805-180	CH_4	M1II	96	90	86	50
805-180	Acetate	BIII	32	35	79	21
805-mix[d]	TY	102	99	30	50	99
805-mix	Methanol	I	99	34	43	0
805-mix	CH_4	I	99	70	99	71
805-mix	TY	I	99	65	75	75
805-mix	Propane	III	99	80	99	0
805-mix	Propane-CH_4	II	30	75	40	24
Sewage sludge	Propane	I	38	57	20	74

[a] Experiments were performed as described in Materials and Methods.
[b] Enrichment cultures were incubated at ambient temperature for 4 to 6 weeks prior to analysis.
[c] Percent decrease in TCE compared with control.
[d] Parent of 5TY104.

RESULTS

Previous studies demonstrated that subsurface sediments from M-area at the Savannah River Plant contained zones of intense biological activity and zones saturated with TCE where biological activity was not detected (Phelps et al., in press). Sediments collected 3 to 15 m beneath the TCE-saturated zone exhibited increased activities and biomass compared with those measured in either near-surface sediments or sediments from similar formations in other wells. Sediment microcosms and enrichment experiments were initiated with inocula from the TCE-contaminated but biologically active subsurface sediments. More than 400 enrichments and incubation mixtures were examined for TCE utilization after 1 month of incubation. The ability to degrade TCE was transferable, and after six or more transfers, stable TCE-degrading consortia were obtained which utilized several energy sources for growth.

The data in Table 1 show the percent decrease of TCE in 11 of the enrichment cultures as compared with that observed in control tubes. Enrichments were coded by site, substrate, and number. All enrichments contained 50 mg of TCE per liter; TCE was added at the beginning of each experiment. Methanol, methane, propane, and Trypticase plus yeast extract (TY) served as energy sources. Unexpectedly, TY stimulated the degradation of TCE. Of the 11 enrichments, 10 were obtained from subsurface sediments underlying the TCE-saturated zone of well MSB-805. Four enrichments were derived from water-bearing sands at a depth of 60 m.

It was not uncommon for transferred inocula to consume 98 to 99.5% of the TCE as compared with controls. Although the ability to degrade TCE was transferable, enrichment cultures lacked consistency and reproducibility. Enrichment culture M1III did not consume TCE on transfer two but did consume TCE during transfers three and four. Most enrichment cultures lost TCE-degrading potential upon subsequent transfers. When triplicate transfers were made from positive cultures, one or more were generally successful.

Enrichment culture T/Y102 degraded 99% of the TCE in transfers one and four but degraded only 39 and 50% of the TCE in transfers two and three, respectively. Culture T/Y102 was unique in that the TCE-degrading population was capable of being diluted 100-fold more than were other

TABLE 2. Effect of energy sources on aerobic TCE degradation by an enrichment culture (5TY104)[a]

Electron donor	Degradation of TCE at concn of[b]:	
	50 mg/liter	150 mg/liter
TY	98	37
Glucose	93	18
Acetate	99	24
Methanol	99	0
TCE	0	0
H_2-CO_2	0	0
Propane	0	8
Methane	91	23

[a] Substrate concentrations are as described in Materials and Methods. Experiments were performed in duplicate with a 3-week incubation at 22°C. Controls were inhibited with Formalin at t_0, and the culture was routinely maintained on 0.05% TY.

[b] Average percent decrease in TCE compared with controls.

enrichments. A subsequent dilution of transfer four of T/Y102 readily degraded TCE and was used in subsequent experiments.

Experiments were undertaken to determine the upper concentration limits of TCE degradation with various energy sources and enrichment culture 5TY104. Teflon-sealed tubes containing 10 ml of medium were incubated for 3 weeks at 22°C with 50, 150, or 300 mg of TCE per liter. Controls were inhibited with Formalin at time zero (t_0). The data in Table 2 demonstrate that methane, methanol, acetate, glucose and TY stimulated the degradation of TCE at a concentration of 50 mg/liter. All successful energy sources resulted in the reduction of the TCE concentration by 1 order of magnitude. Enrichments often degraded more than 99.8% of TCE at a concentration of 50 mg/liter. At a TCE concentration of 150 mg/liter, TCE consumption varied from 0 to 50%. Low levels of degradation (less than 20%) occurred at TCE concentrations of 200 mg/liter, while TCE at 300 mg/liter approached the upper limits of tolerance for TCE in these enrichments (data not shown).

The data in Fig. 1 demonstrate the gas-chromatographic quantification and separation of TCE degradation intermediates by the purge and trap system. Water blanks typically contained chloroform, a solvent heavily used in our laboratory, as well as trace quantities of TCE and tetrachloroethylene. Several experimental samples exhibited production of dichloroethylene and trace amounts of vinylidine chloride. Although vinyl chloride was assayed on aerobic samples by gas chromatography and mass spectroscopy, none was detected in the limited analyses.

Thirteen enrichment cultures which degraded more than 90% of TCE at a concentration of 50 mg/liter were examined by purge and trap analyses and gave results similar to those shown in Fig. 1, with the production of 1 to 5 mg of dichloroethylene per liter. On one occasion, chloroform was not detected in the water blank, controls, or the TCE blank but was observed at concentrations of 0.5 to 2.0 mg/liter in eight cultures, indicating that it may have been a product of TCE degradation in some enrichment cultures. Measured chlorinated organic degradation products accounted for less than 10% of the TCE loss.

Time course experiments were performed to determine TCE consumption over time. The data indicated that incubations longer than 2 weeks were required to observe TCE utilization. The results in Fig. 2 show that cultures of 5TY104 did not consume TCE until after day 6, while

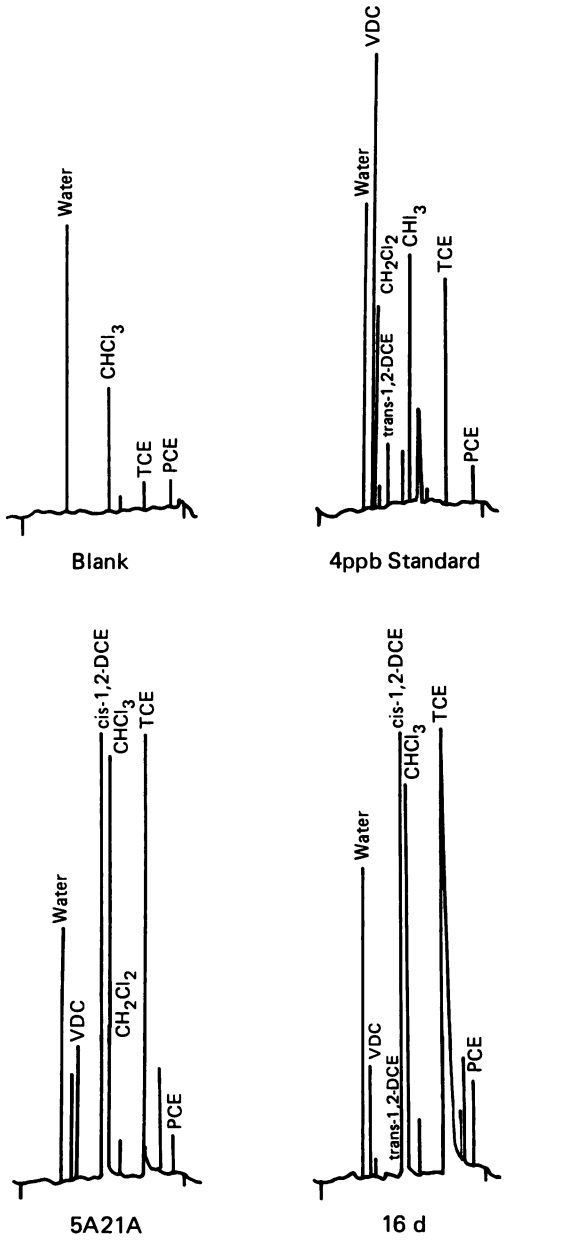

FIG. 1. Chromatograms of chlorinated compounds subjected to purge and trap analyses. Chromatograms of two samples from aerobic enrichment cultures, 5A12A and 16d, showed considerable production of *cis*-dichloroethylene and a small amount of vinylidine chloride.

growth, as indicated by turbidity, was completed by day 3 (data not shown).

Considerable effort was directed towards developing indirect indicators of TCE utilization. Although assays based upon chloride formation were rapid and sensitive, growth did not occur in incubation mixtures free of exogenous chloride. It appeared that production of >50 mg of chloride per liter from TCE was not enough to allow growth in the enrichment cultures, although 10 mg of chloride per liter was sufficient for community growth. These results support the hypothesis that TCE utilization occurred after growth had ceased. To date, no evidence from this work suggests that

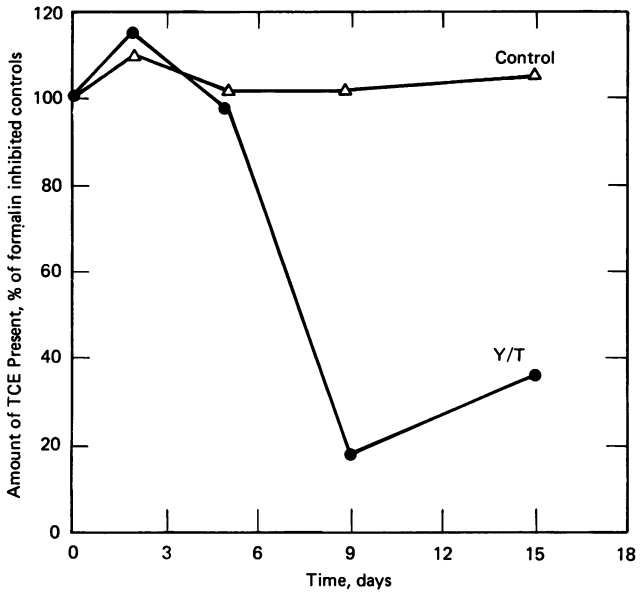

FIG. 2. Utilization of TCE during a time course experiment. Aerobic enrichments of 5TY104 were incubated at 22°C with 50 ppm (50 μg/ml) of TCE and 0.03% TY in the phosphate- and bicarbonate-buffered medium. At each time point, two test microcosms and two Formalin controls were sacrificed.

TCE degradation is related to energy formation or growth in any of the enrichment cultures.

Biological utilization of TCE was dependent on temperature and pH (data not shown). The optimum pH range for TCE utilization by the TY cultures was 7.0 to 8.1. At pH values less than 6.0 or greater than 8.5, loss of TCE was not observed, although some enrichment cultures exhibited growth, as evidenced by turbidity. The optimum temperature appeared to be between 22 and 37°C. At temperatures less than 12°C or greater than 60°C, no significant loss of TCE was observed.

Enrichment cultures 5TY104 and M1III consumed 99% of the TCE and converted 34 and 21%, respectively, of the radiolabeled TCE to carbon dioxide (Fig. 3). The thermal conductivity detector on the gas chromatograph measured the increase in headspace carbon dioxide from vials while radioactive carbon dioxide was quantified by the gas proportional counter. The chromatographic separation and quantification of $^{14}CO_2$ from [1,2-^{14}C]TCE definitively demonstrated that enrichment cultures utilizing a variety of energy sources were capable of mineralizing substantial concentrations of TCE to carbon dioxide. Controls consisted of cultures inhibited with Formalin and indicated no radioactive transformations of TCE to carbon dioxide.

DISCUSSION

Stable, aerobic microbial consortia capable of consuming TCE at a concentration of 100 mg/liter were obtained from subsurface sediment enrichment cultures from a contaminated waste site. The aerobic TCE-degrading enrichment cultures were obtained from sediments below a plume with a TCE concentration of 1,000 mg/liter. Sediments from the zone of highest contamination did not show biological activity, while deeper sediments containing TCE at concentrations of less than 100 mg/kg provided TCE-degrading inocula. Water-bearing sands 20 m below the TCE plume were the source of four successful enrichment cultures. These enrichment cultures were stable and reproducible and used a variety of energy sources for growth but could not use methane as a sole source of carbon and energy and did not have the phospholipid biomarkers typical of methanotrophs (15).

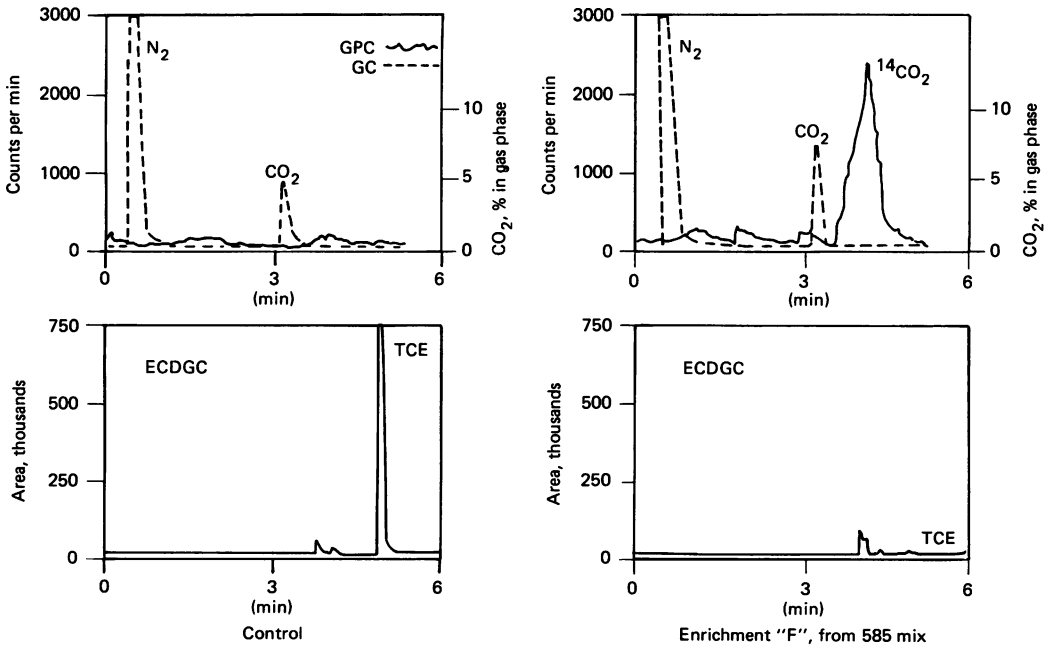

FIG. 3. Aerobic enrichments containing 700,000 dpm of [^{14}C]TCE and 30 ppm (30 μg/ml) of TCE in 3 ml of medium incubated for 1 month in 12-ml serum vials at 20°C. Gaseous CO_2 was measured by gas chromatography (GC) and thermal conductivity detection. Gaseous $^{14}CO_2$ was quantified by gas proportional counting (GPC), and TCE was measured by electron capture detection gas chromatography (ECDGC).

Considerable effort was required to design enrichment conditions which would allow cell biomass to reach 10 to 30 mg/liter without depleting the oxygen from the headspace. It was assumed that Trypticase and yeast extract represented cellular biomass with a molecular weight of 104 g/mol of nitrogen with 17 electron equivalents per mol. Thus, the Trypticase and yeast extract medium could have led to anaerobic conditions had one-half of the carbon been oxidized to carbon dioxide. Gas chromatographic analysis of incubation mixtures (Fig. 2) indicated little increase in the headspace carbon dioxide concentrations. Adsorption coefficient calculations suggested that less than 20% of the energy source was oxidized to carbon dioxide; thus, less than one-third of the oxygen concentration in the headspace was utilized. To deplete the oxygen concentration, the cell biomass would have to exceed 100 mg/liter, and the gaseous carbon dioxide concentration would have to increase from 5 to 15%. Similarly, the energy sources were added at levels which would not deplete the oxygen from the headspace.

In addition to calculating available electron equivalents, monitoring increases in biomass, and measuring carbon dioxide accumulation, we used resazurin as a redox indicator. When resazurin was added to media prior to autoclaving, the media turned pink, but when added at the end of the experiments, the media remained blue. When nutrient levels were increased substantially above those reported in this study, resazurin became colorless, demonstrating that the enrichment cultures were capable of reducing the redox potential of the medium.

Production of carbon dioxide from TCE has been quantitatively demonstrated by radioisotopic studies in which labeled carbon dioxide was chromatographically separated and measured via gas proportional counting without the use of trapping solutions. Our experiences suggested that trapping solutions were not appropriate for volatile organic radioisotopes. Microbial degradation resulted in increased production of chloride and carbon dioxide and minor production of dichloroethylene and, possibly, chloroform. The evidence suggests that TCE degradation is not associated with growth or energy production but probably occurs by cometabolism. Time course experiments indicated that TCE utilization occurred only after measurable growth ceased.

Pure cultures of microorganisms capable of consistently and reproducibly degrading TCE at concentrations of 100 mg/liter have been elusive in this study. Single colonies chosen from our cultures were either sluggish, unpredictable, or mixed cultures. No growth has been associated with TCE oxidation. To date, all evidence suggests that TCE is consumed late in the stationary phase after microbial growth has ceased. It is possible that microbial populations that degrade TCE are slow to grow and only attain adequate biomass long after the community ceases obvious growth.

Degradation of low concentrations of TCE (<1 mg/liter) has been reported by a number of investigators. Wilson and co-workers (15, 25) have established methane-amended soil columns which degrade TCE. Methanotrophic biomass within the column increased (15), and it was hypothesized that methanotrophs were an integral part of the TCE mineralization process. The hypothesis was based in part on the observation that methanotrophs contained mixed-function oxidases capable of degrading chlorinated ethenes (7). Degradation of low concentrations of TCE by heterotrophic microorganisms has been reported recently (13, 14). Although TCE was converted to CO_2, there was a requirement for an aromatic compound, such as phenol. Anaerobic degradation of TCE has been demonstrated in methanogenic microcosms (3, 20). Anaerobic utilization of TCE appears to result in the formation and/or accumulation of vinyl chloride, which is mutagenic and recalcitrant.

We sought to isolate methanotrophic enrichment cultures but were unsuccessful. Trypticase (BBL), yeast extract, or substrates supporting other microbial populations were essential for TCE degradation. Although glucose enrichment cultures grew rapidly, within a couple of transfers TCE-degrading activity was lost. The best enrichment substrates were those which slowly stimulated the bacterial community. Methane stimulation of TCE-degrading consortia provides a role for methanotrophs irrespective of their potential for degrading contaminants. It is likely that methane additions slowly enrich the community, allowing the expression of populations capable of tolerating and detoxifying TCE. When economics, availability, and suitability of energy sources are considered, methane may well be the substrate of choice for in situ biological remediation.

Success has been demonstrated at degrading TCE at concentrations of 150 mg/liter, and the biological tolerance to TCE appears to be approximately 200 to 300 mg/liter in microcosms, cultures, and contaminated environments. When TCE concentrations approached 1,000 mg/liter in water or 300 mg/kg in sediments, biological activity was not detected (Phelps et al., in press). Enrichment cultures inoculated with materials from the toxic zone were not successful. Immediately below the TCE plug, biological activities were orders of magnitude greater than in near-surface soils (Phelps et al., in press). These metabolically active sediments underlying the TCE plug provided TCE-degrading inocula for this study. Enrichment cultures obtained through this work demonstrate degradation and mineralization of TCE at concentrations greater than 50 mg/liter and represent a significant step towards the development of strategies for the biological remediation of environments severely contaminated with chlorinated ethenes.

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