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Mineralization of Trichloroethylene by Heterotrophic Enrichment Cultures

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Abstract.

Microbial consortia capable of aerobically degrading greater than 99% of 50mg/l exogenous trichloroethylene (TCE) have been enriched from TCE contaminated subsurface sediments. Concentrations of TCE greater than 300mg/l were not degraded nor was TCE used as a sole energy source. Successful electron donors for growth included tryptone-yeast extract, methanol, methane or propane. The optimum temperature for growth was 22°-37°C and the pH optimum was 7.0-8.1. 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.

Introduction.

Large portions of subsurface aquifers in the United States have been contaminated by chlorinated hydrocarbons (8, 20), and groundwater near several industrial sites contain over 1000mg/l chlorocarbons (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 is unlikely. Chemical degradation is often prohibitively expensive, since charcoal adsorption requires further disposal of the contaminant and adsorbant, and air stripping releases the contaminant into the atmosphere where it may pose alternative environmental concerns. Biological treatments which degrade contaminants on-site without generating toxic wastes may exhibit potential for rectifying large volumes of groundwater.

Soil microbiology studies conducted during the early part of the twentieth century did not detect large microbial populations in subsurface soils (17, 22, 23) suggesting little microbial life existed in the deep subsurface. Even though evidence of deep subsurface microbial populations and activities had been reported (4, 27), 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 significant populations of microorganisms which can influence groundwater quality (5,9,24,25).

Degradation of TCE or their degradation products has been reported by many investigators under a variety of conditions and have hypothesized mechanisms and kinetic models (2,3,7,10,11,14,15,17-19,21,26). Wilson and co-workers (26) have obtained 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 in the TCE mineralization process. The hypothesis was based in part on the observation that methanotrophs contain mixed-function oxidases which were capable of degrading chlorinated ethenes (7). Although aerobic and anaerobic transformations of TCE have been demonstrated, anaerobic metabolism results in production and accumulation of vinyl chloride, a known mutagen (21).

McCarty et al. (3, 21) have demonstrated anaerobic oxidation of TCE in digester microcosms and partial metabolism of low concentrations of TCE. Anaerobic utilization of TCE appears to result in the formation and accumulation of vinyl chloride (21) which is mutagenic and recalcitrant. Degradation of TCE by an aerobic heterotrophic microorganism was 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 1mg/L). To date investigators have used concentrations of TCE often three orders of magnitude lower than 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 chained 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 greater than 50mg/l concentrations using a variety of electron donors.

MATERIALS AND METHODS

Gases, chemicals and isotopes. Nitrogen and N_2-$C0_2$(90:10%) were greater than 99.9% pure. In the laboratory all gases were passed through copper-filled Vycor furnaces (Sargent-Welch Scientific Co., Skokie, IL) to remove traces of oxygen. All chemicals were of reagent grade and were obtained from Mallinckrodt (Paris, KY) or Sigma Chemical Co. (St. Louis, MO). Resianalyzed glass distilled solvents and reagents were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). [1, 2-^{14}C] Trichloroethylene (10mCi/mmol) was purchased from New England Nuclear Corp. (Boston, MA).

Laboratory techniques. Description of the field site, sampling methods and handling of samples were discussed elsewhere (Phelps, et al., 1987). Subsurface sediment samples were aseptically collected from depths of 5-50m from an area contaminated with short chained chlorinated hydrocarbon at the Savannah River Plant near Aiken, South Carolina. Sediment samples recovered from subsurface formations were placed into sterile Whirl Pak@ bags (NASCO, Ft. Wilkinson, WI) bags and stored under a N_2-gas atmosphere. Enrichment and microcosm experiments were immediately initiated in the field or subsequently in the laboratory under both aerobic and anaerobic conditions. Media typically contained a carbonate or phosphate buffer, trace minerals and vitamins (12), with or without

0.05% yeast extract and one or more potential electron donors including: methane, propane, hydrogen, trichloroethylene, acetate, glucose, methanol, ethylene, trypticase or other chlorinated hydrocarbons. Electron donors were added at concentrations of less than 10mmol/L. Electron acceptors included oxygen, nitrate, sulfate or carbon dioxide. Solidifying agents included Noble agar, purified agar, silica gel or Whatman filter paper.

All manipulations employed syringe techniques for the transfer of solutions or inocula. Enrichments utilized crimp-top tubes or serum vials (Bellco Glass Co., Vineland, NJ or Wheaton Scientific, Millville, NJ) sealed with teflon lined septa (Altech, Deerfield, IL). Incubations were conducted at ambient temperatures (23°C) for three weeks to three months. Unless stated otherwise all tubes contained 50-150mg/L TCE (calculated assuming all in the liquid phase) with a gas to liquid volume ratio of 1.5-2.5:1. All anaerobic studies were performed using strictly anaerobic techniques with a N_2-CO_2 (90:10%) atmosphere. Reductants included 0.05% cysteine-HCL or sodium sulfide with resazurin as the redox indicator.

Analytical Procedures. Carbon dioxide and radioactive carbon dioxide were examined by the gas chromatography-gas proportional counting (GC-GPC) 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. The GC operated at 85°C, helium carrier gas flow of 45ml/min with 1/8in x 6ft stainless steel column packed with carbosieve (80-100 mesh). Chloride concentrations were determined using a Chloride Test Kit (LaMotte Chemical Products Co., Chesterron, MD).

Short chained chlorinated hydrocarbon analyses were performed on a Hewlett-Packard 5890 capillary gas chromatograph with a split-splitless injector, 50m methyl silicone fused silica capillary column, electron capture detector and the liquid nitrogen cryogenic cooling option with hydrogen as the carrier gas. When direct headspace injections were made onto the GC the oven was operated isothermally at 60°C. Autosampling was accomplished by a Tekmar Liquid Sampler and liquid sample concentrator equipped with a tenax/charcoal/silica gel trap. Liquid nitrogen was provided by a 25L high pressure dewar flask (Cryofab Inc., Kenilworth, NJ) and ultra high purity N_2 was the purge gas. Purge and trap analyses typically included N_2 purging at 44ml/min for greater than 3min; heating the trap to 180°C; and desorbing for 0.9min. The GC oven temperature was increased from approximately -70°C to -30°C at 20°C per min then to 10°C at 2°C per min, and then to 100°C. Data were analyzed by a Hewlett Packard 3350 series laboratory data system.

RESULTS

Previous studies demonstrated that subsurface sediments of the M-area at Savannah River Plant contained zones of intense biological activity and zones saturated with TCE where biological activity was not detected (Phelps, et al. 1987). Sediments collected 3 to 15m beneath the TCE saturated zone exhibited increased activities and biomass as compared to near surface sediments or similar formations in other wells. Sediment microcosms and enrichment experiments were initiated using inocula from the TCE contaminated but biologically active subsurface sediments. More than four hundred microcosms and enrichments were examined for TCE utilization after one month of incubation. Degradation of TCE was noted in aerobically and anaerobically incubated enrichments utilizing a variety of electron donors and electron accepters. The ability to degrade TCE was transferable and after six or more transfers stable TCE degrading consortia were obtained which utilized several electron donors for growth.

The data in Table 1 show the percent decrease of TCE as compared to control tubes for eleven enrichment cultures. Enrichments were coded by site, substrate and number. All enrichments contained 50mg/L TCE which was added at the beginning of each experiment. Methanol, methane, propane, and trypticase plus yeast extract were successful electron donors. Unexpectedly trypticase plus yeast extract (T/Y) stimulated the loss of TCE. Ten of the eleven enrichments 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 meters.

It was not uncommon for transfered inocula to consume 98-99.5% of the TCE as compared to controls. Although the ability to degrade TCE was transferable, enrichments lacked consistency and reproducibility. Enrichment M III did not consume TCE on the second transfer but did the third and fourth. Most enrichments lost

TCE degrading potentials upon subsequent transfers. When triplicate transfers were made from positive cultures one or more were generally successful.

Enrichment T/Y 102 degraded 99% of the TCE the first and the fourth transfer but only 39% and 50% in the second and third transfers, respectively. Culture T/Y 102 was unique in that the TCE degrading population was capable of being diluted one hundred fold more than other enrichments. A subsequent dilution of the fourth transfer of T/Y 102, readily degraded TCE and was used in subsequent experiments. Unless otherwise stated, each transfer contained 0.3ml broth into 10ml fresh medium.

Experiments were undertaken to determine the upper concentration limits of TCE degradation using various electron donors. Enrichment T/Y104, was incubated with a variety of electron donors. Teflon sealed tubes containing 10ml medium and 40-60mmol of electron donor per L were incubated three weeks at 22°C with 50, 150 or 300mg/L TCE. Controls were inhibited with formalin at t=0. As shown in Table 2, methane, methanol, acetate, glucose or T/Y were capable of stimulating the degradation of 50mg/L TCE. All successful electron donors reduced the TCE concentration by an order of magnitude. Enrichments were often capable of degrading greater than 99.8% of 50mg/L TCE. At 150mg/L TCE consumption varied from 0.0-50%. Low levels of degradation (less than 20%) occurred at 200mg/L TCE while 300mg/L approached the upper limits of tolerance for TCE in enrichments (data not shown).

The data in Figure 1 demonstrate the gas-chromatographic quantification and separation of TCE degradation intermediates using 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 (PCE). Several experimental samples exhibited production of dichloroethylene (DCE) and trace amounts of vinyldine chloride (VDC). Although vinyl chloride was assayed on aerobic samples by gas chromatography-mass spectroscopy, none was detected in the limited analyses.

Thirteen enrichment cultures which degraded greater than 90% of 50mg/L TCE were examined by purge and trap analyses, and showed similar results as shown in Figure 1, with the production of 1-5mg/l dichloroethylene. On one occasion chloroform was not detected in the water blank, controls or the TCE blank, but was observed at 0.5-2.0mg/L in eight cultures indicating that it may have been a product of TCE degradation in some enrichments. 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 two weeks were required to observe TCE utilization. As shown in Figure 2, cultures of T/Y104 did not consume TCE until after day six, while growth, as indicated by turbidity, was completed by day 3 (data not shown).

A 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 tubes free of exogenous chloride. It appeared that chloride production from TCE was not enough to allow growth even though 10mg/L chloride was sufficient for community growth. These results supported the hypothesis that TCE utilization occurred after growth. To date no evidence from this work suggests that TCE is related to energy formation or growth in any of the enrichments.

Biological utilization of TCE was supported by temperature and pH profiles (data not shown). Optimum pH for TCE utilization by the T/Y culture was 7.0-8.1. At pH values less than 6.0 or greater than 8.5 loss of TCE was not observed although some tubes exhibited growth as evidenced by turbidity. Temperature optimum 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.

Confirmation of carbon dioxide formation from TCE was demonstrated using gas chromatography-gas proportional counting techniques. Duplicate vials of several enrichments were inoculated with 30mg/L TCE plus 700,000dpm of [1-2^{14}C] TCE in three ml of medium in a 12ml vial. Vials were incubated for three weeks at 22°C. Loss of total TCE was measured by purge and trap analysis using the electron capture detector. Enrichment T/Y104 and MIII consumed 99% of the TCE and respectively converted 34% and 21% of the radiolabeled TCE to carbon dioxide (Figure 3). The thermal conductivity detector on the GC measured the increase in headspace carbon dioxide from vials while radioactive carbon dioxide was quantified by the gas proportional counter. These results definitively demonstrated that enrichments utilizing a variety of electron donors 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 100mg/L concentrations of TCE have been obtained from subsurface sediment enrichments from a contaminated waste site. The aerobic TCE degrading enrichments were obtained from sediments below a 1000mg/L TCE plume. Sediments from the zone of highest contamination did not show biological activity, while deeper sediments containing TCE concentrations of less than 100mg/Kg provided successful inocula. Water bearing sands 20m below the TCE plume were the source for four successful enrichments. The enrichments are stable, reproducible, use a variety of electron donors for growth but are not methanotrophic. The evidence suggests that TCE metabolism is not associated with growth or energy production. Time course experiments indicated that TCE utilization occurred only after measurable growth ceased. Production of carbon dioxide from TCE has been quantitatively demonstrated by radioisotopic studies where labeled carbon dioxide was chromatographically separated and measured via gas proportional counting enabling the gas to be assayed for radioactivity without using trapping solutions. Our experiences suggested that trapping solutions were not appropriate for volatile organic radioisotopes. Microbial degradation products included hydrochloric acid, carbon dioxide and minor production of DCE, and likely chloroform.

Pure cultures of microorganisms capable of consistently and reproducibly degrading 100mg/L TCE have been elusive in this study. Single colony picks are 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 had ceased. It is possible that TCE degrading populations are slow to grow and only maintain adequate biomass long after the community ceases obvious growth.

Methanotrophic enrichments were actively pursued in this study but were unsuccessful. Trypticase, yeast extract or substrates supporting other microbial populations were essential for TCE degradation. Although glucose enrichments grew rapidly, within a couple of transfers TCE degrading activity was lost. The best enrichment substrates were those which slowly stimulated the bacterial community. These findings provide a role for methanotrophs irrespective of their contaminant degradative potential. It is likely that methane additions provide a slow enrichment of the community allowing the expression of populations capable of tolerating the detoxifying TCE. When economics, availability and suitability of electron donors are considered, methane may well be the substrate of choice for in situ biological remediation.

Success has been demonstrated at degrading 150mg/L TCE and the biological tolerance to TCE appears to be approximately 200-300mg/L in microcosms, cultures and the contaminted environment.

When TCE concentrations approached 1000mg/L in water or 300mg/kg in sediments,biological activity was not detected (manuscript submitted). Enrichments 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. These underlying sediments provided successful inocula for this study. Enrichments obtained through this work represent a significant step in the development of strategies for the bioremediation of environments severely contaminated with TCE.

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Table 1.

Degradation of trichloroethylene (TCE) by enrichment cultures.

Decrease in TCE (% decrease from controls)

			successive	transfers		
Inoculum	Substrate	I. D.	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*	T/Y	102	99	30	50	99
805-mix	Methanol	I	99	34	43	0
805-mix	CH_4	I	99	70	99	71
805-mix	T/Y	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

Experiments performed as described in Materials and Methods. Enrichments were incubated at ambient temperature 4-6 weeks prior to analysis.

*Parent of 5TY104.

Table 2.

Effect of electron donors on aerobic TCE degradation by an enrichment culture (5TY104)

	Average decrease in TCE (% decrease form controls)	
Electron donor	50 mg/l TCE	150 mg/l TCE
T/Y	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

Substrates were added at 40-60mmloles of available electron equivalents per liter of media. Experiment performed in duplicate. Incubation was 3 weeks at 22°C. Controls were inhibited with formalin at t=0. Culture was routinely maintained on TRY/YE at 0.05% each.

Figure 1.

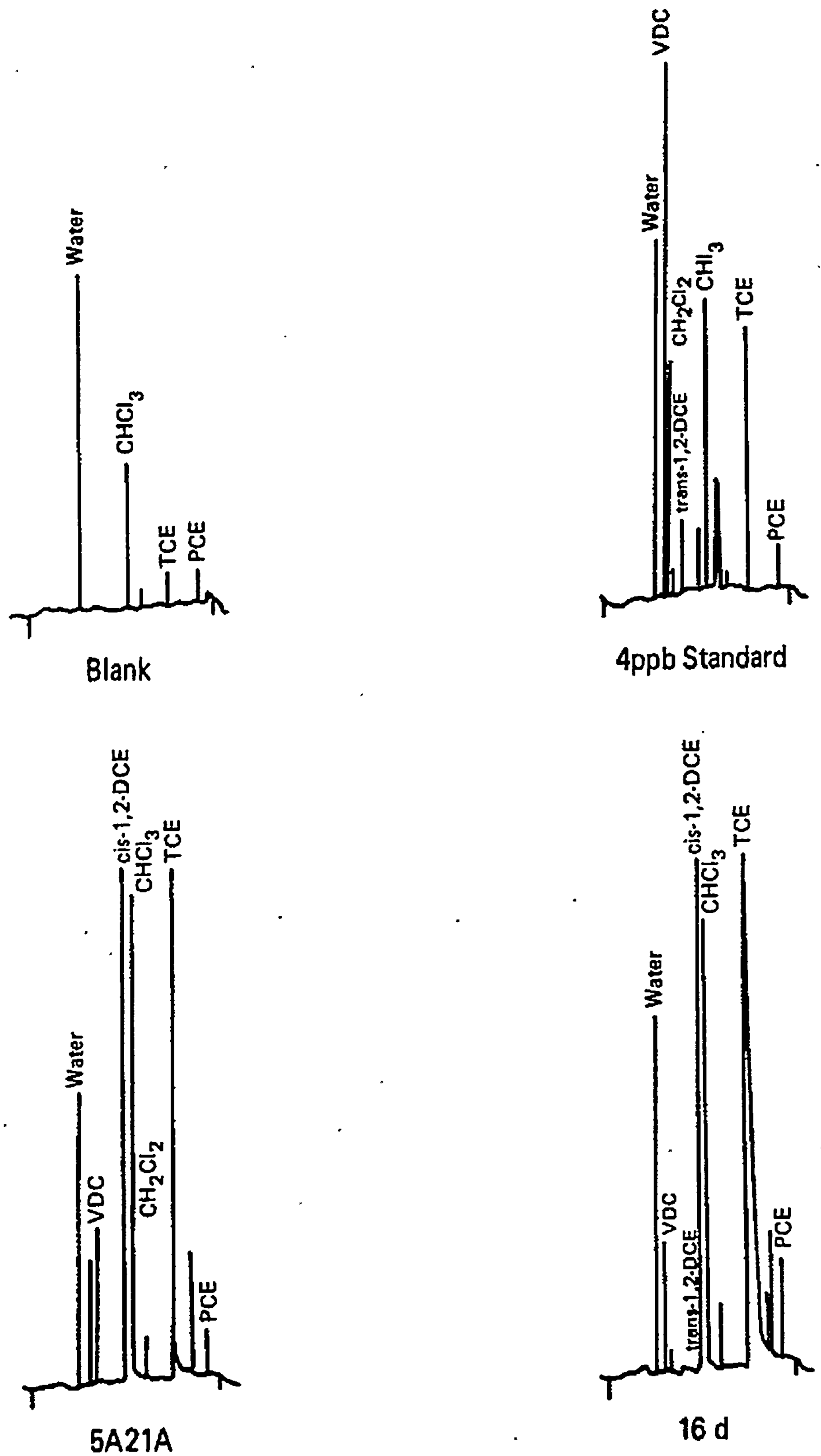
Chromatograms of chloro-compounds analyzed by purge and trap analyses. Chromatograms of two samples from aerobic enrichment cultures. 5A12A and 16d, showed considerable production of cis-DCE and a small amount of VDC.

Figure 2.

Utilization of TCE during a time course experiment. Aerobic enrichments of 5TY10^4 were incubated at 22°C with 50 ppm TCE and 0.03% yeast extract and trypticase in a phosphate and bicarbonate buffered medium. At each time point, two test microcosms and two formalin controls were sacrificed.

Figure 3.

Aerobic enrichments containing 700,000 dpm of ^{14}C-TCE and 30 ppm TCE in 3 milliliters of medium, incubated for one month in 12 milliliter serum vials at 20°C. Gaseous $C0_2$ was measured by gas chromatography and thermal conductivity detection. Gaseous $^{14}C0_2$ was quantitated by a gas proportional counter and TCE was measured by electron capture detection gas chromatography.



Blank

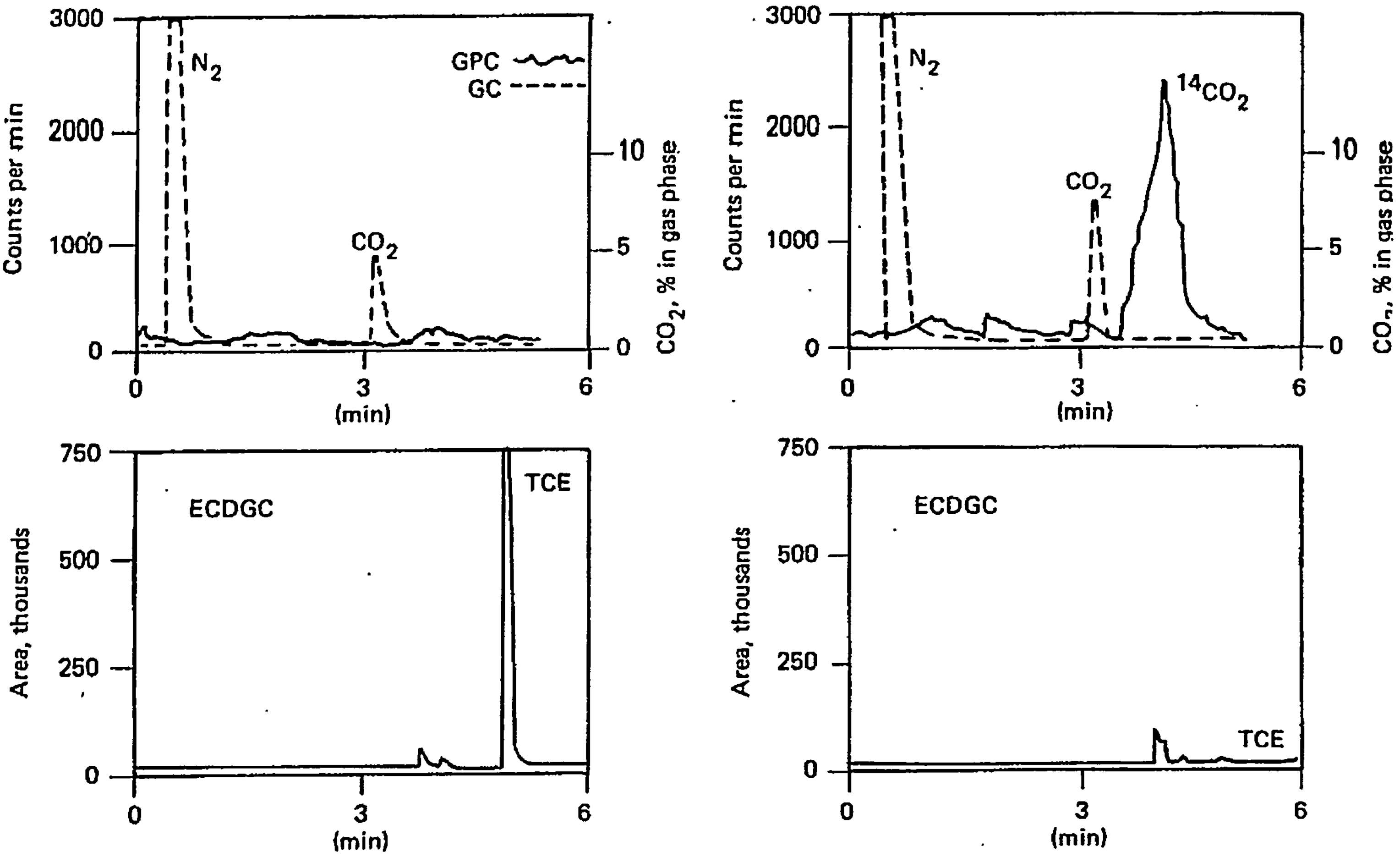
4ppb Standard

5A21A

16 d

Chromatograms of chloro-compounds analyzed by purge and trap analyses. Chromatograms of two samples from aerobic enrichment cultures, 5A12A and, 16d, showed considerable production of cis-DCE and a small amount of VDC.

FIGURE 1. Purge and Trap Gas Chromatography of Trichloroethylene and Associated Chlorinated Hydrocarbons



Control

Enrichment "F", from 585 mix

Aerobic enrichments containing 700,000 dpm of ^{14}C-TCE and 30 ppm TCE in 3 milliliters of medium, incubated for one month in 12 milliliter serum vials at 20° C. Gaseous CO_2 was measured by gas chromatography and thermal conductivity detection. Gaseous $^{14}CO_2$ was quantitated by a gas proportional counter and TCE was measured by electron capture detection gas chromatography.

FIGURE 3. The Degradation of Trichloroethylene to Carbon Dioxide