213

Geomicrobiology Journal, Volume 6, pp. 157–170
Printed in the UK.

0149-0451/88 $3.00 + .00

Microbial Biomass and Activities Associated with Subsurface Environments Contaminated with Chlorinated Hydrocarbons

T. J. PHELPS[1]
D. RINGELBERG[1]
D. HEDRICK[1]
J. DAVIS[1]
C. B. FLIERMANS[2]
D. C. WHITE[1]*

[1]Institute for Applied Microbiology, University of Tennessee/Oak Ridge National Laboratory, Knoxville, TN 37932
[2]Savannah River Laboratory, E. I. duPont de Nemours and Co., Aiken, SC 29808

Abstract *Soil microcosms and enrichment cultures from subsurface sediments and groundwaters contaminated with trichloroethylene (TCE) were examined. Total lipids, [1-^{14}C]acetate incorporation into lipids, and [Me-^{3}H]thymidine incorporation into DNA were determined in these subsurface environments. In heavily TCE-contaminated zones (greater than 500 mg/L) radioisotopes were not incorporated into lipids or DNA. Radioisotope incorporation occurred in sediments both above and below the TCE plume. Phospholipid fatty acids (PLFA) were not detected, i.e., less than 0.5 pmol/L in heavily contaminated groundwater samples. In less contaminated waters, extracted PLFA concentrations were greater than 100 pmol/L and microbial isolates were readily obtained. Degradation of 30–100 mg/L TCE was observed when sediments were amended with a variety of energy sources. Microorganisms in these subsurface sediments have adapted to degrade TCE at concentrations greater than 50 mg/L.*

Groundwater contamination by chlorinated hydrocarbons represents a serious challenge in potable and agricultural waters in the United States (Council on Environmental Quality 1981; Westerick *et al.* 1984). Predominant contaminants include short-chained aliphatic halogenated hydrocarbons, notably trichloroethylene (Schwarzenbach and Giger 1985; USEPA 1982). Trichloroethylene (TCE) has been used for decades as a degreaser in the dry cleaning and metal finishing industries. Initially, accepted treatment technologies included the disposal of the volatile solvent into clay-lined pits for evaporation. However, chlorinated short-chained hydrocarbons migrated through permeable subsurface formations and into groundwater aquifers.

Decontamination of polluted groundwaters is a formidable task. Subsurface contaminants are difficult to quantify. They are unlikely to disappear by volatilization and are

* Corresponding author.
Received January 9, 1988; accepted June 28, 1988

often stable (Bellar and Litchenberg 1974; Corapcioglu and Baehr 1985; Schwarzenbach and Giger 1985; Phelps *et al.* 1988). Biological transformation on site has been considered as a method of remediation (Bouwer and McCarty 1983; Kobayashi and Rittman 1982; Wilson and Wilson 1985).

Biological transformations of short-chained, chlorinated hydrocarbons under a variety of conditions have been reported by several investigators (Barrio-Lage *et al.* 1986; Bouwer and McCarty 1983; Fogel *et al.* 1986; Nelson *et al.* 1986, 1987; Schwarzenbach and Giger 1985). Recent studies suggested that anaerobic degradation produced vinyl chloride, a toxic and recalcitrant mutagen (Vogel and McCarty 1985). However, aerobic mineralization appears to avoid accumulation of vinyl chloride through epoxide formation (Kline *et al.* 1987), and subsequent breaking of the carbon–carbon double bond. To date, cometabolism of TCE at concentrations at or below 1 mg/L (Fogel *et al.* 1986; Nelson *et al.* 1986, 1987; Nichols *et al.* 1987; Vogel and McCarty 1985; Wilson and Wilson 1985) has been examined. A degradative role has been suggested for methanotrophs which possess methane monooxygenase (Colby *et al.* 1977; Fogel *et al.* 1986; Nichols *et al.* 1987; Wilson and Wilson 1985). Although degradation of TCE occurs in methane-amended soil columns containing large populations of methanotrophs (Nichols *et al.* 1987), it is not clear whether the major role of the methane-oxidizing bacteria in this degradation involves stimulation of the microbial community or the direct consumption of TCE. Heterotrophic microorganisms which degrade micromolar concentrations of trichloroethylene have been isolated (Nelson *et al.* 1986, 1987). Populations of heterotrophs and sulfate-reducing microorganisms from groundwater and subsurface sediments have been identified (Dockins *et al.* 1980; Ghiorse and Balkwill 1983; Smith *et al.* 1986b; Wilson *et al.* 1983), suggesting that TCE degrading communities could exist in subsurface environments.

Groundwater may contain TCE concentrations approaching 1000 mg/L, and vadose zone formations may contain more than 500 mg/kg. If bioreclamation were to be successful, microorganisms and technologies must be optimized to facilitate remediation at somewhat higher concentrations than have been previously reported. Early priorities include chemical and biological characterization of contaminated sites and the development of criteria for evaluating their suitability for in situ treatment. This project was undertaken as a collaborative effort to characterize TCE contaminated and control sites at the Savannah River Plant (SRP) Aiken, South Carolina. The characterization included microbiological, chemical, geological, and hydrological studies of a particular site (M-Area) and the adjoining watershed.

Materials and Methods

Description of Experimental Site

The Savannah River Plant (SRP) is a restricted access facility which is 768 km^2 in size and operated until April 1, 1989 by the E. I. duPont de Nemours and Co. for the U.S. Department of Energy. In 1958 an unlined surface retention impoundment was constructed at M-Area for settling metal processing wastes. Waste waters entering the basin also contained substantial quantities of solvents common to metal finishing industries. For over two decades liquids overflowed and leached through the basin, contaminating the local subsurface and groundwater with short-chained chlorinated hydrocarbons. The principal contaminants were tetrachloroethylene and trichloroethylene. These were present at concentrations greater than 1 mg/L in several subsurface formations including

the water-bearing zones. Five core holes were examined in this study in addition to groundwater from several test monitoring wells. Cores were collected from well MSB-805 and groundwater screen zone MSB-3A, which were approximately 10 m from the M-Area basin boundary. The depth to the water table is approximately 37 m. Water was sampled from well MSB-4A which bordered an adjoining side of the basin. Other wells were 1 to 5 km away (Fig. 1).

Sampling Techniques

Professional Service Industries, Inc. (PSI Jackson, SC) provided the drilling services. Wells were cored to a depth of approximately 100 m and screen zones developed in the major drinking water aquifer. Site MSB-805 was cored to a dense clay layer 25 m above the major aquifer. Quik-gel (NL Baroid/NL Industries, Inc., Houston, TX), a sodium bentonite viscosifying drilling fluid, was recirculated through the wells to facilitate drilling. Undisturbed subsurface materials were obtained using Pitcher barrel (Pitcher Drilling Co., Palo Alto, CA), Shelby tubes, or Dennison corer samplers (Acker, Scranton, PA) depending on sediment type. All samples were collected in core liner tubes constructed of either brass or stainless steel which were steam cleaned prior to use (Phelps *et al.* 1989).

Retrieved materials were removed from the sampler and immediately carried into the Mobile Microbiol Ecology Laboratory (MMEL) in order to handle the sediment samples aseptically as previously described (Phelps *et al.* 1989).

Gases, Chemicals, and Isotopes

The purity of nitrogen and N_2–CO_2 (90:10%) gases were greater than 99.9%. 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 reagent grade, supplied by either Mallinckrodt (Paris, KY) or Sigma Chemical Co. (St. Louis, MO). Glass-distilled solvents and reagents were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). [1-^{14}C[Acetate (56 mCi/mmol), [1,2,-^{14}C]trichloroethylene (10 mCi/mmol), and [*Me*-^{3}H]thymidine (79.9 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, MA). [2-^{14}C]Acetate (56 mCi/mmol) was obtained from Amersham Corp. (Arlington Heights, IL).

Laboratory Techniques

Enrichment and microcosm experiments were done in crimp-top tubes or serum vials (Bellco, Vineland, NJ, or Wheaton, Millville, NJ) sealed with Teflon-lined septa (Altech, Deerfield, IL). Incubations were at ambient temperature for three weeks to three months. Unless otherwise stated, all tubes contained 50–150 mg/L TCE (calculated assuming that all of the TCE was in the liquid phase) with a 1.5–2.5:1 gas to liquid volume ratio. Potential energy sources were added at 5–10 mM concentrations and included methane, propane, ethylene, hydrogen, trichloroethylene, methanol, glucose, yeast extract, peptone, acetate, and chlorinated hydrocarbon mixes. Subsamples were incubated aerobically and anaerobically with exogenous electron acceptors including carbon dioxide, nitrate, and sulfate. 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.

Field Studies

Sediment aliquots were inoculated for aerobic and anaerobic microbial activity experiments in the MMEL within 30 min of core extrusion. Time course experiments were performed in duplicate using sterile polypropylene centrifuge tubes or crimp-top tubes (Phelps *et al.* 1988). All incubations were kept at a temperature similar to the in situ temperature of 20–23 °C. Acetate incorporation experiments contained 2 g sediment, 5.0 μCi of [1-^{14}C]acetate, and 1.0 mL sterile distilled water.

Preliminary experiments were performed on four subsurface sediments to determine optimum incubation times. Time course experiments were performed in duplicate and consisted of at least eight time points between 20 min and 30 days. The earliest time point that provided measurable data for three of the four preliminary sediment analyses was 24 h. Consequently, time course experiments ending with 24-h time points were selected for these studies. During the time course experiments, the reactions in duplicate tubes were inhibited with 3.0 mL methanol and the samples were frozen. Thymidine incorporation experiments contained 1.0 g sediment, 30 μCi of [*Me*-^{3}H]thymidine, and 2 nmol of nonradioactive thymidine in 1.0 mL of sterile distilled water. At appropriate time points the reactions were stopped by the addition of 3.0 mL of 80% ethanol and frozen at −20 °C.

Enrichment and microcosm experiments were also initiated in the field under both aerobic and anaerobic conditions. Media typically contained a carbonate or a phosphate buffer (Lynd *et al.* 1982) with or without 0.05% yeast extract and one or more energy sources, including methane, propane, hydrogen, ethylene, acetate, glucose, methanol, trichloroethylene, and other chlorinated hydrocarbons. Energy sources were added at concentrations less than 10 mM. Electron acceptors included oxygen, nitrate, sulfate, or carbon dioxide. All media and microcosm experiments contained trace minerals, including selenium and molybdenum, as well as a dilute vitamin mix (Lynd *et al.* 1982).

Groundwaters were collected in new 20-L polypropylene containers after wells had been pumped for more than 4 well volumes. Water samples were filtered through methanol-washed 0.2-μm polycarbonate filters. The filters were frozen and stored until the lipids were analyzed. Aliquots of the water were diluted and plated by the pour-plate method onto vitamin- and mineral-supplemented media containing 2.0% Noble Agar (Difco, Detroit, MI) along with energy sources of yeast extract and trypticase (0.01–0.1%), methanol, methane, or glucose (5.0 mM).

Analytical Procedures

Acetate-incorporation experiments were conducted on sediments extracted by a modification (White *et al.* 1979) of the single-phase, chloroform–methanol method of Bligh and Dyer (1959). The lipid fraction was evaporated to dryness and portions were counted by liquid scintillation counting to determine the amount of radioactivity incorporated into microbial lipids.

The neutral lipids, glycolipids, and phospholipids were separated on silicic acid columns. Fractions were collected, subjected to methanolysis (White *et al.* 1979), and evaporated to dryness under a stream of N_2. The esters were analyzed directly by glass capillary gas–liquid chromatography (GC) as previously described (Bobbie and White, 1980). Gas chromatography–mass spectrometry verification of selected samples was performed on a Hewlett-Packard 5996A system fitted with a direct capillary inlet as previously described (Guckert *et al.* 1986; Nichols *et al.* 1987).

Thymidine incorporation experiments were treated with 2 mL of 0.3 M sodium hydroxide containing 1% sodium dodecyl sulfate, 10 mM thymidine, and 1% humic acid. After being heated to 100 °C for 4-h, the supernatants were collected and dialyzed in water where the radioactive thymidine incorporated into macromolecules was retained (Moriarty and Pollard 1982; Smith *et al.* 1986a). Aliquots were counted via liquid scintillation counting to determine the amount of radioactivity incorporated into DNA and macromolecules. Controls were conducted to insure that dialysis was sufficient and to verify that the radioactivity retained in the dialysis tubing (molecular weight cut off of 3500) was sensitive to DNAase and additional dialysis.

Chlorinated hydrocarbon analyses were performed on a Hewlett-Packard 5890 capillary gas chromatograph with a split–splitless injector, 50 m 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. A Tekmar Automatic Liquid Sampler and Liquid Sampler Concentrator equipped with a tenax/charcoal/silica gel trap provided autosampling. Liquid nitrogen was provided by a 25-L high-pressure Dewar flask (Cryofab Inc., Kenilworth, NJ) with ultrahigh-purity N_2 as the purge gas. Purge and trap analyses typically included N_2 purging at 44 mL/min for greater than 3 min, heating the trap to 180 °C, and desorbing for 0.9 min. The GC oven temperature was increased from approximately −70 to −30 °C at a rate of 20 °C per min, then to 10 °C at a rate of 2 °C per min, and then to 100 °C. Data were analyzed by the Hewlett-Packard 350 series laboratory data system.

Results and Discussion

Previous studies performed in this laboratory have quantified phospholipid fatty acids (PLFA) from a variety of natural waters, sediments, and soil columns (Smith *et al.* 1986a,b; White *et al.* 1979). Surprisingly, PLFA were not detected in the shallow groundwater from well MSB-3A near the M-Area seepage basin (Fig. 1). As shown in Table 1, groundwater from well MSB-3A exhibited a small number of colony-forming units (CFU) representing only four morphologies, more than 90% of which were fungal. Groundwater from MSB-4A, a well bordering an adjoining side of the seepage basin, exhibited more CFU, a smaller fraction of fungi, greater diversity, and measurable PLFA. Colony morphologies of microorganisms isolated from MSB-4A groundwaters resembled those from other groundwaters and those later observed from sediment materials. In other shallow aquifers (A wells) and deeper aquifers, abundant CFUs, more than 50 colony types, and PLFA were noted. Assuming 10.0 μmol PLFA/g (dry wt) of bacterial cell mass (White *et al.* 1979), the phospholipid concentration corresponded to microbial populations of 10^3/mL in all groundwaters examined except MSB-3A. Waters from MSB-3A possessed an odor resembling that of chlorinated hydrocarbons, leading to the hypothesis that MSB-3A was a toxic environment that had placed the microbial community under considerable stress.

Profiles of PLFA have often been used to evaluate community structure or nutritional status of microorganisms from environmental samples (Bobbie and White 1980; Nichols *et al.* 1987; Smith *et al.* 1986b; White *et al.* 1979, 1983). Phospholipid profiles from two groundwaters and three sediment cores obtained from the highly contaminated zones of well MSB-805 are shown in Table 2. Twenty-liter groundwater samples filtered through 0.2-μm polycarbonate filters exhibited more total PLFA and greater PLFA diversity than

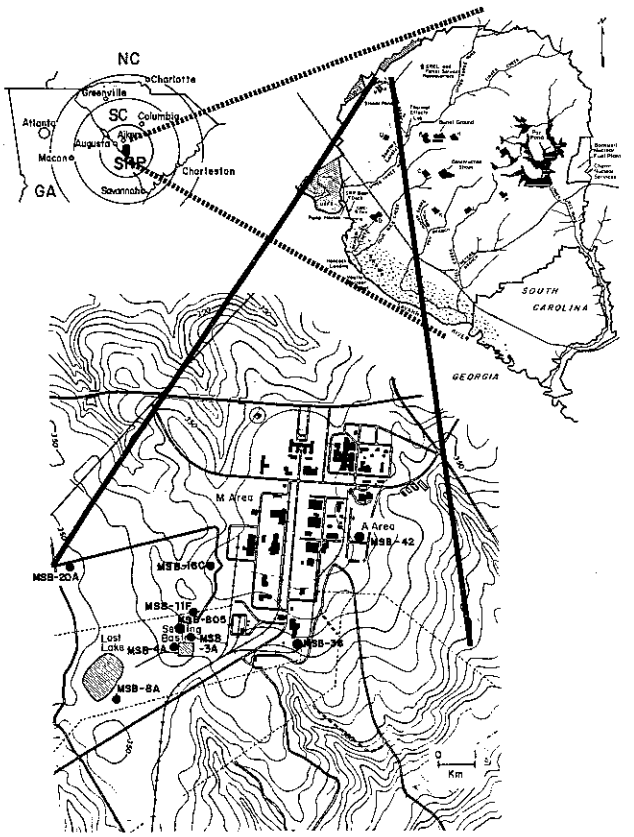


Figure 1. Map of MSB well clusters at the Savannah River Plant.

did the 50-g samples of lyophilized sediments. Nearly half of all PLFA could be accounted for by four fatty acids: 16:0, 18:0, 18:1w7cis, and 16:1w7cis. Although all four fatty acids are common to many microorganisms, only 16:0 and 16:1w7cis occurred in PLFA from well MSB-805 sediments. Sediments exhibited few PLFA with little diver-

Table 1
Microbial Colony-Forming Units, Diversity, and Phospholipid Biomass Measures from Groundwaters

Sample	Colony-forming units per mL	% Fungal	Diversity	PFLA (pmol/20 L)
MSB-3A	7.0×10^2	90	4 fungal types 2 were atypical	Less than 10
MSB-4A	2.3×10^3	22	10 types typical of cores	890
MSB-8A	n.d.	n.d.	n.d.	7200
MSB-11F	3.3×10^3	10	>50 types	n.d.
MSB-16C	1.5×10^3	1	>50 types	1100
MSB-20A	2.6×10^4	1	>50 types	n.d.

n.d., not determined.

sity, and no unique PLFA were observed. The levels of total PLFA corresponded to a resident bacterial population of approximately 10^6/g.

Phospholipid results obtained in this study appear atypical. Guckert *et al.* (1986) reported that in starved *Vibrio cholerae* the trans PLFA isomer assisted in survival by providing a nonfluid coating for microorganisms and by being resistant to metabolic turnover of cellular lipids. It was hypothesized that the increased trans/cis ratios could be indicative of stressed environments. Although a 16:1*w*7 fatty acid was present in significant amounts in M-Area sediments, only the cis isomer was observed.

Another atypical phospholipid result in this study was the detection of PLFA typical of eukaryotes. The aquifer sediments examined at 43-m depth contained a substantial concentration of a PLFA which was 23 carbons in length, suggesting the presence of microeukaryotes in these subsurface environments. Previous subsurface investigations did not detect significant PLFA greater than 22 carbons in length (Smith *et al.* 1986b; White *et al.* 1983). The role of eukaryotes in SRP sediments is currently being investigated and will be reported elsewhere (Ghiorse *et al.*, manuscript in preparation).

Table 2
Phospholipid Fatty Acid Profiles of M-Area Groundwaters and Sediments from MSB-805 Site[a]

	Phospholipid fatty acid (PLFA) pmol/10 L water or pmol/10 g sediment				
Type PLFA	MSB-4A Ground-water	MSB-8A Ground-water	MSB-805 Sandy Clay 3 m	MSB-805 Sandy Clay 43 m	MSB-805 Sand 57 m
i 14:0	52				
i 15:0	173	47			
a 15:0	100				
15:0	31				
i 16:0	36				
16:1*w*9c	77				
16:1*w*7c	302	1385			118
16:1*w*13t			104	147	
16:0	620	674	313	235	460
10*me* 16:0	65				
i 17:0	123				
a 17:0	42				
cyc 17:0	281	97			
17:0	22	44			
18:1*w*9	294		116	93	285
18:1*w*7c	823	1302			
18:0	151	86	116		
br 19:1	65				
c 19:0	767				
20:0	22				
22:0	27				
23:0			248	214	
Total	4030	3520	897	690	863

[a] Fatty acids are designated by the total number of carbon atoms, the number of double bonds, followed by the position of the unsaturation from the carboxyl end (*w*). Suffixes *c* and *t* indicate cis and trans geometry, while *i* represents isobranching.

In addition to ester-linked PLFA, ester-linked fatty acids of less polarity and free fatty acids were recovered from subsurface sediments. As shown in Table 3, ester-linked fatty acids less polar than phospholipids, likely neutral or glycolipids, accounted for approximately one-third of the total fatty acids recovered. Less than one-half of one percent of the fatty acids were ester linked to a polar group such as phosphate. Assuming 10.0 μmol PLFA/g (dry wt) of bacterial cell mass, the ester-linked phospholipid data corroborated the plate count results, which indicated a density of 10^3-10^6 microorganisms per gram of sediment. These results suggest that the free fatty acids and nonpolar lipids were detrital, or remnants of nonviable biomass. The low ratio of phospholipid to total lipid in these subsurface sediments highlights the importance of care needed during the extraction of the polar lipid fraction when employing lipids as a measure of viable biomass.

Radiotracer experiments have routinely been used to quantify metabolic activity and growth in aquatic and terrestrial environments, although the use of these techniques for subsurface environments requires incubation times of hours rather than minutes. Moriarty and Pollard (1982) recommended sampling intervals of seconds to minutes for thymidine incorporation experiments, while Smith *et al.* (1986a) found that 8-h was required in Antarctic sediments. Time course experiments ranging from 0.5 to 24-h in these studies enabled evaluation of activities spanning six orders of magnitude (data not shown).

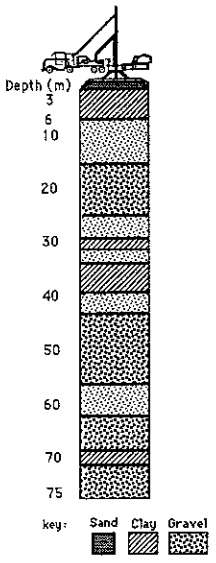
The results in Figure 2 represent activity profiles of the various subsurface stratigraphies at MSB-805. The incorporation of [1-^{14}C]acetate into microbial lipids was indicative of anabolism, while growth was estimated by [*Me*-^{3}H]thymidine incorporation into macromolecules such as DNA. Near-surface sediments exhibited moderate activities, while a zone between 30 and 43 m exhibited no measurable accumulation of acetate into lipids or thymidine into DNA. Thymidine incorporation was inhibited 7 m above and below the metabolically inhibited area. The zones of metabolic inhibition contained TCE concentrations greater than 200 mg/kg sediment. Aqueous samples from this and other contaminated zones which contained TCE concentrations greater than 300 mg/L appeared toxic. Above and below the TCE-laden zones were areas that exhibited greater metabolic activities than near surface soils. Water-bearing zones 60 m below the surface exhibited 100 times the activity of near surface sediments and 1000 times more activity than some of the clay layers.

Of the 68 subsurface sediment samples from five wells examined in this study, only nine exhibited nondetectable acetate incorporation activity, five of which were in the MSB-805 TCE plume. Five samples exhibited below detectable activity for both the

Table 3
Types of Fatty Acids Recovered from Subsurface Sediments from MSB-805

	pmol/g dry wt Sediment			
Sample	Free Fatty Acids[a]	Ester-Linked Neutral or Nonpolar Lipids[b]	Ester-Linked Glycolipids[c]	Phospholiplids[d]
805–812 m	442	163	49	2.6
% of total	67	25	7.4	0.4
805–824 m	1761	631	136	13
% of total	69	30	5.4	0.5

[a] Acids 14–24 carbons in length, no ester or ether linkages at carboxyl end.
[b] Ester-linked, nonpolar lipids which eluted with chloroform.
[c] Ester-linked, less polar lipids which eluted with acetone and characteristic of glycolipids.
[d] Ester linked to a polar group, typically phospholipids.



[^{14}C]Acetate incorporation into lipids a	[^{3}H]Thymidine incorporation into DNA b	TCE Enrichment % loss c	Trichloroethylene (µg/kg) d
147	1690	+99	–
815	0	+11	–
7560	0	N.D.	–
177	1930	N.D.	–
661	12,200	N.D.	670
675	4210	+51	320
2030	11,300	N.D.	190
13,100	0	N.D.	250
131,000	0	+7	16,300
0	0	N.D.	312,400
0	-	-20	41,800
0	0	N.D.	59,400
0	393	-37	361,000
0	0	N.D.	373,000
292	0	N.D.	38,000
82	0	+98	–
166,000	74,100	N.D.	–
30,400	1100	+14	–
212,000	35,900	N.D.	–
2230	3790	+98	–
125	0	N.D.	–
53,300	0	N.D.	–
2270	1540	+31	–
253	20,500	N.D.	–
0	1960	N.D.	–

Figure 2. Microbiological measurements in MSB-805 sediments. (a) Incorporation of [1-^{14}C]acetate into lipids expressed as dpm/day determined from duplicate time course experiments. Incubation tubes contained 2.0 g of sediment, 1.0 mL sterile distilled water and 5.0 µCi of [1-^{14}C]acetate. (b) Incorporation of [^{3}H]thymidine into macromolecules expressed as dpm/day determined from duplicate time course experiments. Tubes contained 1.0 g sediment, 1.0 mL sterile distilled water, 30 µCi of [*Me*-^{3}H]thymidine, and 2 nmol of nonradioactive thymidine. (c) Loss of 30 mg/L TCE in enrichment cultures utilizing glucose, methanol, propane, methane, yeast extract, or chlorinated hydrocarbons expressed as percentage of controls. (d) Nearby monitoring wells screened at 50–65 m depth contained TCE at concentrations exceeding 20 mg/L.

acetate and thymidine incorporation experiments. Four of these were in the TCE plume. Of the samples examined for acetate incorporation, only two incorporated more than three percent of the radioisotope into cellular lipids. Similarly, in only one thymidine incorporation experiment was there greater than one percent incorporation of the tritiated thymidine pool into DNA (data not shown). These results indicated that a 24-h incubation provided usable information, yet incorporated only a small fraction of the radiotracer pool into biomass.

Groundwater concentrations of TCE from this area (25–40 m) have approached 1000 mg/L. The TCE concentrations shown in Figure 2 demonstrate a depth profile of TCE in the subsurface sediments. In contrast, nearby wells screened in the aquifer at 50–65 m depth routinely contained TCE at concentrations exceeding 20 mg/L.

The data in Table 4 compare microbial activities in the sediments of three wells with common sedimentary formations. Within many formations the variability in measured activity was in part due to differences in water availability, hydrologic flow, sediment structure, and the associated biomass. Clays demonstrated much lower microbial activity than did sands. The Pee Dee variegated boundary clay exhibited little measurable microbial activity. Since clays required the greatest amount of handling in retrieving the material from the core liners, as well as being difficult to portion into the reaction tubes, the dramatically lower microbial activities in clay samples attested to aseptic processing. The range of microbial activities in subsurface sedimentary formations was dramatic and unexpected. Water-bearing sands contained considerably greater microbial activities with the highest activities occurring in the yellow-sand Congaree aquifer. Incorporation of

Table 4
Radioisotope Activity Measurements from M-Area Subsurface Formations

Formation and Depth	Description	[1-^{14}C]Acetate Incorporation into Lipids (dpm[a]); [*Me*-^{3}H]Thymidine[b] Incorporation into DNA		
		Well[a] MSB-36[b]	Well MSB-42	Well MSB-805
Upland	Surface sand	3550[c] (++[d])	310 (+)	147 (+)
8–15 m		4050 (++)	522 (+)	815 (−)
Tobacco Road	Upper tan clay	690 (−)	583 (+)	177 (+)
15–30 m	with sand	n.d.	n.d.	661 (++)
Dry Branch	Saturated	27,900 (+)	19,100 (+)	3,100[e] (−)
25–40 m	tan sand	n.d.	165,000 (+)	131,000 (+)
Congaree	Saturated	280,000 (+)	165,000 (++)	166,000 (++)
50–65 m	yellow sands	522,000 (++)	781,000 (++)	30,400 (+)
		n.d.	n.d.	212,000 (++)
Pee Dee	Variegated dry	283 (−)	95 (−)	253 (+)
77–100 m	boundary clay	n.d.	n.d.	0 (+)
Black Creek	Saturated	2,680 (+)	41,600 (−)	n.d.
>100 m	coarse sand	61,100 (+)	18,500 (+)	n.d.

[a] Multiple values in a set are for separate measurements in the same formation.
[b] Well MSB-36 was within 100 m of overhead discharge of waste water.
[c] 24-h incorporation rate of labeled acetate into lipids as described in text. In several instances additional samples were collected 3–7 m apart from the same lithological strata.
[d] 24-h incorporation of labeled thymidine; −, background; +, $<10^4$ dpm; ++, $>10^4$ dpm.
[e] From a TCE contaminated well near a severely contaminated zone.
n.d., not determined.

radiolabeled acetate varied three orders of magnitude between boundary clays and the yellow-sands aquifer in each well examined. The Black Creek aquifer, a formation used for potable and irrigation water, exhibited an order of magnitude lower microbial activity than the overlying yellow-sands aquifer. These data indicated that a large and active community of microorganisms exists in an aquifer 50–65 m beneath the soil surface which exhibits 100–1000 times more activity than near-surface soils.

Microcosms and microbiological enrichments were initiated under a variety of conditions with each sediment sample collected from MSB-805 and selected water/sediment samples collected from the other wells. Many enrichments and microcosms exhibited microbial growth or activities, but no successful enrichments were obtained from the sediments collected from the 30 to 40-m depths of MSB-805. Few successful enrichments were obtained from the 43-m-depth sediments whereas 25-m and 53- to 60-m-depth sediments yielded abundant and diverse microbial enrichments.

The data in Figure 3 show a depth profile for propane enrichments established with subsurface materials collected from MSB-805. Five-gram samples were added to 5.0 mL of basal medium plus 30 mg TCE per liter and incubated at room temperature for two months under an atmosphere containing 2% carbon dioxide, 8% propane, and 90% air. Near-surface sediments and those from water-bearing zones below the TCE plug removed TCE from the headspace. Three enrichments removed greater than 99.9% of the TCE as compared to the inhibited controls. Transfers from these enrichments demonstrated TCE degradation (Fliermans *et al.* 1988), while enrichments inoculated with sediment materials from zones with the highest TCE concentrations were not capable of removing TCE, nor was significant consumption of TCE noted in subsequent transfers.

The data in Figure 4 demonstrate the suitability of various enrichment conditions for the degradations of TCE. Inoculum was 10 g of sediment from 43- to 60-m depths of

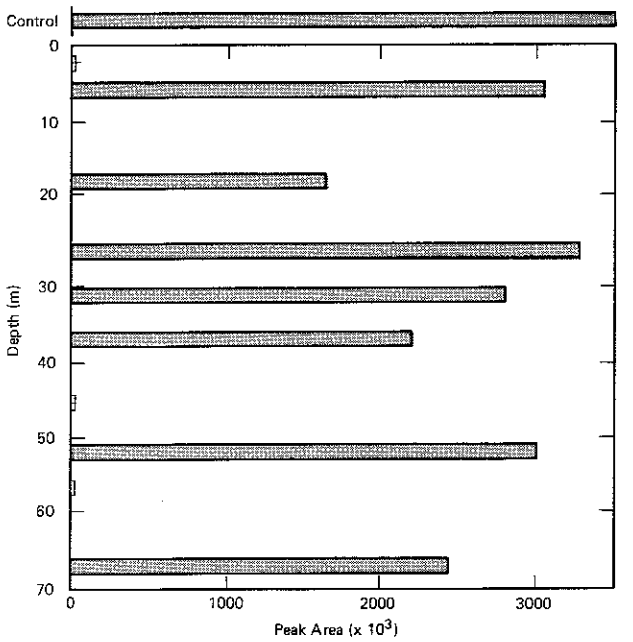


Figure 3. Loss of trichloroethylene in propane-amended soil enrichments. Five grams of sediment from propane-amended microcosms plus 5.0 mL of basal medium were incubated for 60 days under carbon dioxide, propane and air atmosphere (2%/8%/90%) containing 30 mg/L TCE. Bars indicate respective propane levels in samples.

MSB-805 to which 5.0 mL of basal medium was added. When compared to the controls, trichloroethylene was consumed both aerobically and anaerobically in the amended sediments. Although the addition of glucose led to a rapid loss of TCE, the TCE degradative activity was not transferable. In contrast, yeast extract plus tryptone, propane, and methane enrichments consumed significant amounts of TCE and their activity was transferable.

Several investigators have suggested a role for methane and methanotrophs in chlorinated hydrocarbon degradation. Methanotrophic biomarkers have been observed in methane-amended soil columns containing chlorinated hydrocarbons (Colby *et al.* 1977; Nichols *et al.* 1987; Wilson and Wilson 1985), but in unamended SRP sediments no evidence of methanotrophic biomarkers has been noted; however, data from the SRP sediments indicated that microorganisms have developed the ability to consume TCE at concentrations greater than 100 mg/L. Following months of microcosm and enrichment studies, a trend emerged showing that any aerobic sediment enriched with inocula from the 43- to 60-m-deep portions of MSB-805 which contained an electron donor that slowly stimulated a small fraction of the microbial community to consume greater than 90% of 50 mg/L exogenous TCE. Furthermore, the TCE-consuming ability was transferable.

This study demonstrated the presence of microbial activities in subsurface environments contaminated with short-chained chlorinated hydrocarbons. Zones containing TCE concentrations greater than 500 mg/L were devoid of microbial activity. As TCE concentrations decreased and water availability increased, microbial activities were greater than in near-surface sediments. Sediment enrichments capable of degrading TCE were readily obtained from the 50- to 60-m-deep portions of MSB-805, suggesting that the environment had selected microorganisms adapted to the in situ TCE concentrations.

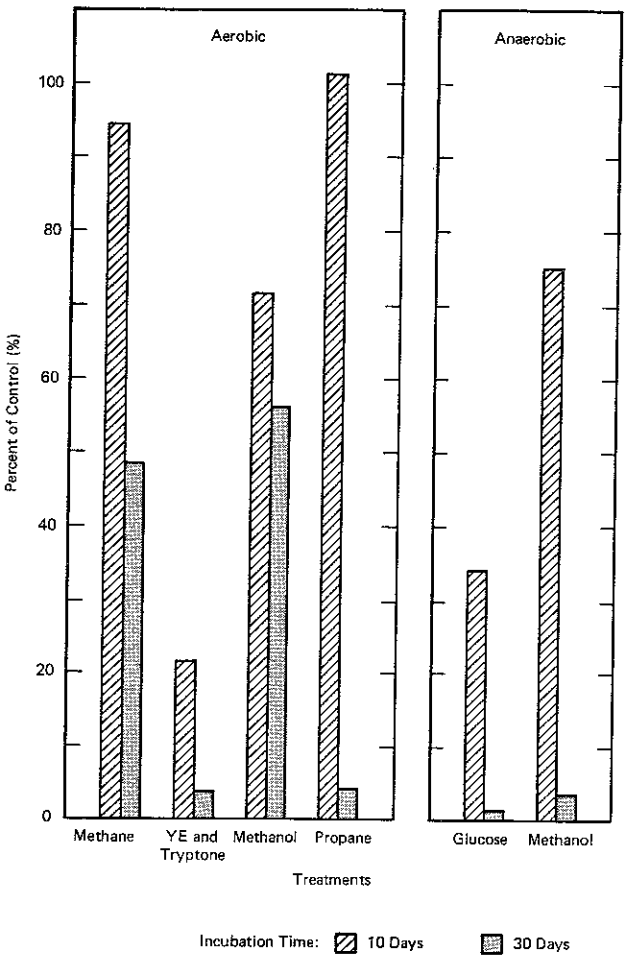


Figure 4. Loss of trichloroethylene from subsurface sediment enrichments. Duplicate serum vials containing 10 g of sediment, 5.0 mL of basal medium, 5 mM of respective substrates incubated aerobically and anaerobically under N_2/CO_2 (90%/10%) gas phase with 50 mg/L TCE. Controls were inhibited with 0.1% formalin at $t = 0$. Bars indicate levels of trichloroethylene in respective samples.

Activity measurements based upon acetate incorporation into lipids appeared to be a sensitive and quantitative method for evaluating the potential for biological metabolism and correlated well with thymidine incorporation experiments and the ability to enrich microorganisms. These findings suggest that it is possible to stimulate biological remediation using resident microorganisms; however, extremely toxic zones may not be suitable for direct biological treatment.

Acknowledgments

The information contained in the article was developed during the course of work under Contract DE-AC09-76SR00001 with the U.S. Department of Energy. This work was supported by a grant from Savannah River Laboratory, E. I. DuPont de Nemours and Co., Aiken, SC, Contract AX 681901, and the Office of Health and Environmental

Research of the Department of Energy. We thank J. J. Foreman for on-site technical support and the staff of the Institute for Applied Microbiology who participated in the field program. Hydrogeological, drilling and safety consultations were provided by E. I. duPont de Nemours and Co. Chlorinated hydrocarbon analyses of sediments obtained during the coring of wells were performed by Environmental Testing Services, Inc. (Edison, NJ).

References

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.

Bellar, T. A., and J. J. Litchenberg. 1974. Determining volatile organics at microgram-per-litre levels by gas chromatography. J. Am. Water Works Assoc. 66:739–744.

Bligh, E. G., and W. J. Dyer. 1959. A rapid method of lipid extraction and purification. Can. J. Biochem. Physiol. 35:911–917.

Bobbie, R. J., and D. C. White. 1980. Characterization of benthic microbial community structure by high resolution gas chromatography of fatty acid methyl esters. Appl. Environ. Microbiol. 39:1212–1222.

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.

Colby, J., D. F. Stirling, and H. Dalton. 1977. The soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath): Its ability to oxygenate *n*-alkanes, *n*-alkenes, ethers, and aliphatic, aromatic, and heterocyclic compounds. Biochem. J. 165:395–402.

Corapcioglu, M. Y., and A. Baehr. 1985. Immiscible contaminant transport in soils and groundwater with an emphasis on petroleum hydrocarbons: System of differential equations vs. single cell model. Environ. Sci. Tech. 17:23–37.

Council on Environmental Quality. 1981. Contamination of groundwater by toxic organic chemicals. U.S. Government Printing Office, Washington, DC.

Dockins, W. S., G. L. Olson, G. A. McFeters, and S. C. Turbak. 1980. Dissimilatory bacterial sulfate reduction in Montana groundwaters. Geomicrobiol. J. 2:83–98.

Fliermans, C. B., T. J. Phelps, D. Ringelberg, A. T. Mikell, and D. C. White. 1988. Mineralization of tetrachloroethylene by heterotrophic enrichment cultures. Appl. Environ. Microbiol. 54:1709–1714.

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.

Ghiorse, W. C., and D. L. Balkwill. 1983. Enumeration and characterization of bacteria indigenous to subsurface environments. Dev. Ind. Microbiol. 24:213–224.

Guckert, J. B., M. A. Hood, and D. C. White. 1986. Phospholipid, ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae:* Increase in the trans/cis ratio and proportions of cyclopropyl fatty acid. Appl. Environ. Microbiol. 52:794–801.

Kline, S. A., J. J. Solomon, and B. L. Van Duuren. 1987. Synthesis and reactions of chloroalkene epoxides. J. Org. Chem. 43:3596–3600.

Kobayashi, H., and B. E. Rittmann. 1982. Microbial removal of hazardous organic compounds. Environ. Sci. Technol. 16:170A–183A.

Lynd, L., R. Kerby, and J. G. Zeikus. 1982. Carbon monoxide metabolism of the methylotrophic acetogen, *Butyribacterium methylotrophicum*. J. Bact. 149:255–263.

Moriarty, D. J. W., and P. C. Pollard. 1982. Diel variation of bacterial productivity in seagrass (*Zostera capricorni*) beds measured by the rate of thymidine incorporation into DNA. Mar. Biol. 72:165–173.

Nelson, M. J. K., S. O. Montgomery, E. J. O'Neill, and P. H. Pritchard. 1986. Aerobic metabolism of trichloroethylene by a bacterial isolate. Appl. Environ. Microbiol. 52:383–384.

Nelson, M. J. K., S. O. Montgomery, W. R. Mahaffey, and P. H. Pritchard. 1987. Biodegrada-

tion of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Microbiol. 53:949–954.

Nichols, P. D., J. M. Henson, C. P. Antworth, J. Parsons, J. T. Wilson, and D. C. White. 1987. Detection of a microbial consortium, including type II methanotrophs, by use of phospholipid fatty acids in an aerobic halogenated hydrocarbon-degrading soil column enriched with natural gas. Environ. Toxicol. Chem. 6:89–97.

Okouchi, S. 1986. Volatilization coefficient for stripping trichloroethylene, 1,1,1,trichloroethane and tetrachloroethylene from water. Water Sci. Technol. 18:137–138.

Phelps, T. J., and J. G. Zeikus. 1985. Effect of fall turnover on terminal carbon metabolism in Lake Mendota sediments. Appl. Environ. Microbiol. 50:1285–1291.

Phelps, T. J., C. B. Fliermans, T. R. Garland, S. M. Pfiffner, and D. C. White. 1989. Methods for recovery of deep terrestrial subsurface sediments for microbiological studies. J. Microbiol. Methods (in press).

Phelps, T. J., D. B. Hedrick, D. Ringelberg, C. B. Fliermans, and D. C. White. 1988. Utility of radiotracer activity measurements for subsurface microbiology studies. J. Microbiol. Methods 9:15–27.

Schwarzenbach, R. P., and W. Giger. 1985. Behavior and fate of halogenated hydrocarbons in groundwater. In C. H. Ward, W. Giger, and P. L. McCarty (Eds.), Groundwater Quality. Wiley, New York, pp. 446–471.

Smith, G. A., P. D. Nichols, and D. C. White. 1986a. Fatty acid composition and microbial activity of benthic marine sediments from McMurdo Sound, Antarctica. FEMS Microbiol. Ecol. 38:219–231.

Smith, G. A., J. S. Nickels, B. D. Kerger, J. D. Davis, S. P. Collins, J. T. Wilson, J. F. McNabb, and D. C. White. 1986b. Quantitative characterization of microbial biomass and community structure in subsurface material: A procaryotic consortium responsive to organic contamination. Can. J. Microbiol. 32:3104–3111.

U.S. Environmental Protection Agency. 1982. National revised primary drinking water regulations, volatile synthetic organic chemicals in drinking water: Advanced notice of proposed rulemaking. Fed. Reg. 47:9349–9358.

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.

Westerick, J. J., J. W. Mello, and R. F. Thomas. 1984. The groundwater supply survey. J. Am. Water Works Assoc. 76:52–59.

White, D. C., R. J. Bobbie, J. D. King, J. Nickels, and P. Amoe. 1979. Lipid analysis of sediments for microbial biomass and community structure. In C. D. Litchfield and P. L. Seyfried (Eds.), Methodology for Biomass Determinations and Microbial Activities in Sediments, ASTM STP 673, American Society for Testing Materials, New York, pp. 87–103.

White, D. C., G. A. Smith, M. J. Gehron, J. H. Parker, R. H. Findlay, R. F. Martz, and H. L. Frederickson. 1983. The groundwater aquifer microbiota: Biomass, community structure and nutritional status. Dev. Industrial Microbiol. 24:201–211.

Wilson, J. T., and B. H. Wilson. 1985. Biotransformation of trichloroethylene in soil. Appl. Environ. Microbiol. 49:242–243.

Wilson, J. T., J. F. McNabb, D. L. Balkwill, and W. C. Ghiorse. 1983. Enumeration and characterization of bacteria indigenous to a shallow aquifer. Groundwater 21:134–142.