EVALUATING TRICHLOROETHENE BIODEGRADATION BY MEASURING THE IN SITU STATUS AND ACTIVITIES OF MICROBIAL POPULATIONS

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ABSTRACT

Trichloroethene (TCE) released from an unlined waste pond has contaminated the sediments and groundwater beneath and downgradient of a manufacturing facility. Characterization of the site microbiology revealed that dense and diverse microbial populations exist in the sediments and groundwater beneath the facility. The microbial biomass, diversity, growth, catabolism, and anabolism were greater in the sand units than in the silt or clay units. Microbial stress indicators were most prevalent in TCE-contaminated sediments within the unsaturated zone. Aerobic TCE biotransformation was confined mainly to sand units in the vicinity of the former waste pond. Radiolabeled carbon dioxide ($^{14}CO_2$) generation from [1,2-^{14}C]-TCE and [1,2-^{14}C]-vinyl chloride (VC) was also confined mainly to sand units in the vicinity of the former waste pond. A number of anaerobic dechlorination products (*cis*-1,2-dichloroethene, *trans*-1,2-dichloroethene, and VC) were detected during the biodegradation of [1,2-^{14}C]-TCE, but $^{14}CO_2$ was the dominant product in all cases. Overall, the water availability, the silt and clay content, and the distribution of TCE in the subsurface appear to be controlling microbial activity, growth, and TCE biodegradation.

INTRODUCTION

Trichloroethene (TCE) historically was used as a degreasing agent at a manufacturing facility in Merced, California, and was released to the subsurface through seepage from an unlined waste pond. Previous subsurface investigations found that TCE was present in sediments beneath the former waste pond, and in groundwater samples obtained from two of the three aquifers beneath the site, to a depth of approximately 60 m. TCE also was detected in groundwater samples obtained downgradient of the site (Bechtel Environmental Inc. 1988). Interestingly however, the breakdown products, i.e., *trans*- and *cis*-1,2-dichloroethene (*t* and *c*DCE,

respectively), and vinyl chloride (VC), which typically are associated with TCE-contaminated sediments and groundwater, were not detected. As a result, we conducted a subsurface investigation to determine if the requisite microorganisms capable of biodegrading TCE were absent or if a microbial population existed that could completely mineralize TCE without the production of VC or other intermediate products.

This paper presents the characterization of the microbial community and an assessment of microbial growth and activity (catabolic and anabolic) in sediment and groundwater samples from two exploratory boreholes (designated MB-1 and MB-2) and three existing extraction wells (designated MW-39, MW-40, and MW-44). The locations of the boreholes and wells, and TCE concentrations detected in sediments in the vicinity of the former waste pond are shown in the geologic cross section presented in Figure 1. TCE and VC biotransformation by the indigenous microorganisms in the sediment and groundwater samples is presented.

SAMPLING AND ANALYSIS

Summaries of sediment and groundwater collection, microbial community analysis, and TCE biotransformation are presented below. Details of the site geology and geochemistry are presented elsewhere (Major et al. in submission).

Sediment Collection

Two exploratory borings were cored using hollow-stem augers. No drilling fluids were used in this investigation. A decontaminated California-modified split-spoon sampler fitted with three sterile brass liners was driven into the ground in advance of the lead augers, and retrieved samples were aseptically processed in an anaerobic glove bag in the field laboratory. Samples were frozen on dry ice and were shipped by courier to the University of Tennessee for initiation of phospholipid fatty acid (PLFA) analysis.

Groundwater Collection

Temporary monitoring wells were installed in each of the exploratory borings. A sand pack was placed in the boring annulus around the well screen, and the well screen was isolated from the overlying formations. The monitoring wells were developed using a decontaminated submersible pump. Groundwater for microbial activity and mass loss experiments was collected using dedicated Teflon™ bailers attached to stainless-steel wires. Samples were transferred directly from the bailer into sample bottles through a Teflon™ stopcock. Sample bottles were filled without headspace and were shipped on ice by courier to the University of Tennessee. Groundwater samples collected for lipid analysis were filtered through 0.2 μm polycarbonate filters to trap the microorganisms. The filters were frozen on dry ice, shipped by courier to the University of Tennessee, and stored until the lipids were extracted and analyzed.

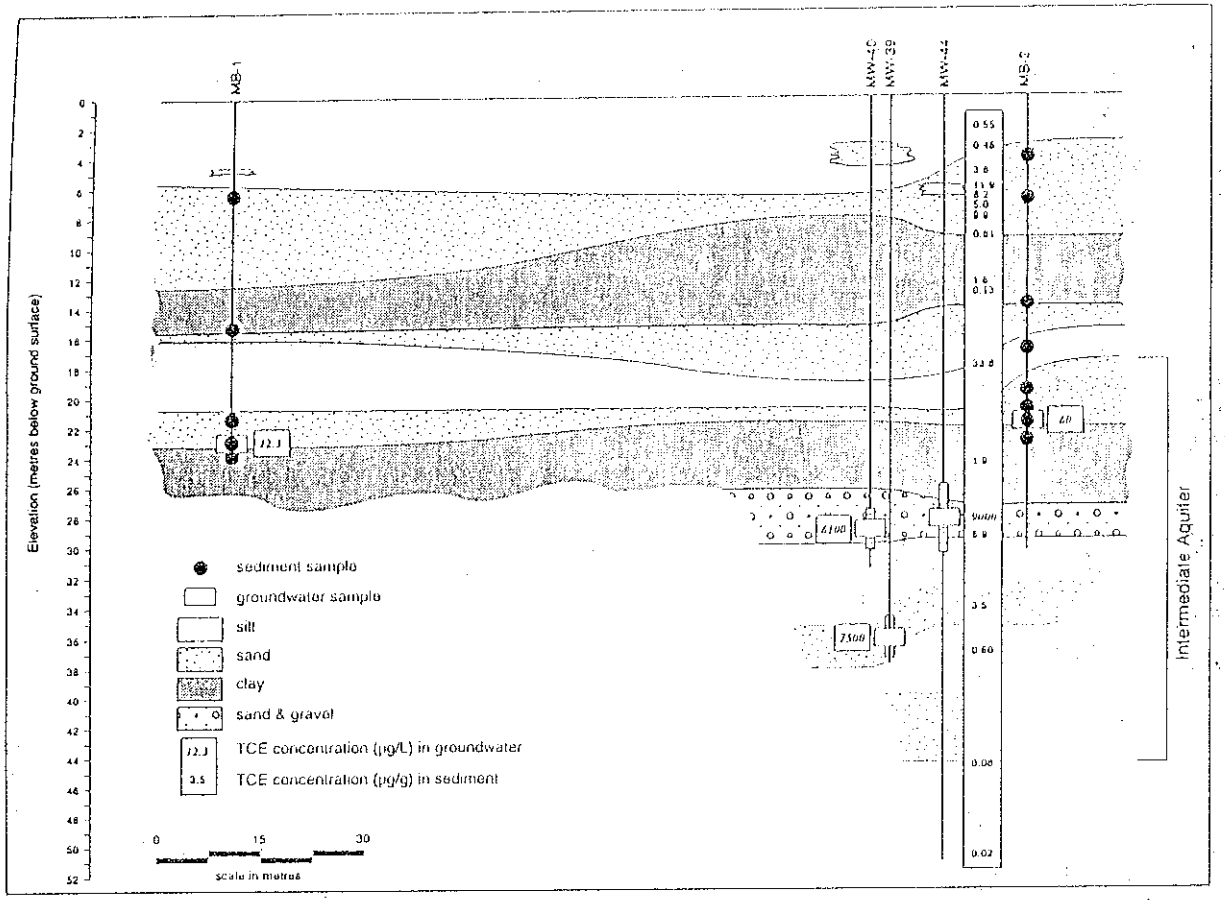

FIGURE 1. Geologic cross section showing extraction wells, exploratory borings, and TCE concentrations in sediments and groundwater in the vicinity of the former waste pond.

Phospholipid Analyses

PLFA analysis was used to measure the total viable biomass, the community composition, and the nutritional and physiological status of the microbial populations. Total phospholipids were extracted from 110 g (dry weight) of frozen and lyophilized (dehydrated) sediment samples or filters by a modification (White et al. 1979, 1983) of the single-phase chloroform-methanol method of Bligh and Dyer (1959). Fatty acids were designated by the total number of carbon atoms and by the number of double bonds followed by the position of the double bond closest to the ω (aliphatic) end. Geometry of the PLFA is designated by 'c' for *cis* or 't' for *trans*; 'i', 'a', and 'br' refer to iso-, anteiso-, and methyl-branching, respectively. Methyl-branching from the Δ end is indicated by its position followed by the designation 'Me' and the total number of carbon atoms. Cyclopropyl fatty acids are designated as 'cy'.

Assessment of Microbial Activity

Microbial growth was assessed by measuring the rate of [*methyl*-^{3}H]thymidine (76.0 mCi/mmole) incorporation into macromolecules of microorganisms in the sampled sediments. Catabolic potential was assessed by measuring the rate of [1,2-^{14}C]acetate (56 mCi/mmole) or [U-^{14}C]glucose (2.8 mCi/mmole) mineralization by the microorganisms in the sampled sediments and groundwater. Anabolic potential was assessed by measuring the rate of [1,2-^{14}C]acetate (56 mCi/mmole) or [^{3}H]acetate (3.3 mCi/mmole) incorporation by the microorganisms in the sampled sediments and groundwater.

All activity experiments were initiated within 72 hours of sediment and groundwater collection. Experiments were conducted under both aerobic and anaerobic conditions in either sterile polypropylene centrifuge tubes or crimp-top tubes containing sediment or groundwater and sterile distilled water. Radiolabeled substrate transfers were made using gastight syringes (Hamilton Co., Reno, Nevada). The amount of radiotracer added during activity experiments was small so that the electron donor pool size was not appreciably increased (i.e., from micromolar to millimolar concentrations). Time frames were minimized to ensure linearity of the results (Phelps et al. 1989). Additionally, radiotracers were carrier-free to ensure that there were no competing substrates (e.g., ethanol as a carrier would be a competing electron donor with the radiotracer acetate). Tubes were sealed with butyl septa and were incubated in the dark at ambient temperatures that were similar to the in situ temperature of 21 to 24°C. At selected time points, duplicate tubes were inhibited and the appropriate activity was analyzed. Results are expressed as disintegrations per minute/day/cell based on the corresponding PLFA biomass measurements.

Microcosm Biotransformation Experiments

Microcosm enrichment studies were conducted to examine the loss of TCE mass over time under various nutrient conditions. The microcosms consisted

of 58-mL serum vials containing either 10 g of sediment or 10 mL of groundwater, plus 5 mL of distilled water or medium (as indicated below). Serum vials were sealed with Teflon™ septa. Atmospheric gas made up the headspace in the microcosm, unless otherwise indicated in the treatments listed below. All experiments were performed in duplicate. Four experimental treatments were used:

Treatment 1: 5 mL distilled water (equivalent treatment with groundwater was not conducted).
Treatment 2: 5 mL mineral salts medium plus 5 mM phosphate.
Treatment 3: 5 mL distilled water plus 5% methane headspace (vol:vol).
Treatment 4: 5 mL of mineral salts medium plus 3% each of methane and propane headspace (vol:vol).

Treatment 1 examined the effect of water on releasing bound nutrients in the sediment. Treatment 2 attempted to determine if phosphate was limiting activity. Treatment 3 attempted to induce methanotrophic bacteria to degrade TCE, and Treatment 4 attempted to induce other alkane-alkene oxidizers to degrade TCE. The TCE was added to each vial at approximately 20 mg/L. Incubations were in the dark, at ambient temperature, and in an inverted position (to minimize TCE loss by volatilization). After 0, 2, 4, and 12 weeks of incubation, duplicate vials were sacrificed and the TCE mass loss was determined; 1 hour prior to analysis, each vial was acidified with 0.5 mL of 6 M HCl to stop microbial activity.

Microcosm controls consisted of (1) autoclaved site groundwater and sediment, (2) autoclaved site groundwater and sediment plus respective nutrient amendments, and (3) autoclaved site groundwater and sediment plus respective nutrient amendments, and 0.5 mL of 2 M NaOH (inhibitor).

Gas chromatography (GC) was used to measure the concentrations of TCE, *c*DCE, *t*DCE, VC, propane, methane, and CO_2 in the headspace of the microcosms. The limits of detection for sampled compounds were as follows: TCE, 0.1 µg/L; *c*DCE, 10 µg/L; *t*DCE, 10 µg/L; VC, 10 µg/L; and propane, methane, and CO_2, 0.05% (vol:vol). The detection limits of the volatile organic compounds (VOCs) are dissolved concentrations as determined by the Ideal Gas Law, Henry's law, and solubility coefficients.

Radiolabeled Mineralization Experiments

Aerobic and anaerobic mineralization experiments were conducted in 25-mL crimp top tubes containing either 2.0 mL of groundwater, or 2.0 g of sediment and 1.0 mL of sterile water, and carrier-free isotope. Either [1,2-^{14}C]VC (0.53 mCi/mmole), or [1,2-^{14}C]TCE (10 mCi/mmole) was added at 0.66 and 0.59 µCi per tube, respectively, using gastight syringes (Hamilton Co., Reno, Nevada). Time course experiments were performed in duplicate with multiple time points ranging from t_0 to 1 month with a minimum of 4 time points per isotope examined. All tubes had Teflon™ septa and were incubated in the dark at ambient temperatures similar to the *in situ* temperature of 21 to 24°C. At selected time points, duplicate tubes were inhibited with 0.5 mL of 2.0 M sodium

hydroxide. Each tube was acidified with 0.5 mL of 6M HCl 1 hour prior to analysis to inhibit microbial activity.

Radioactive $^{14}CO_2$, $^{14}CH_4$, and daughter products released during biodegradation of the radiolabeled parent compounds were analyzed by GC-gas proportional counting (Fliermans et al. 1988; Nelson & Zeikus 1974). Results were calculated based on the initial slope of product evolution and are reported as disintegrations per minute/day/cell based on the corresponding PLFA biomass measurements.

RESULTS AND DISCUSSION

Microbial Biomass

Table 1 presents the PLFA biomass results for the sampled sediments and groundwater. The total viable biomass was calculated assuming a conversion factor of $4x10^4$ colony-forming units (CFU) per pmole of PLFA (White et al. 1979). Microbial biomass was highest in the unsaturated sand units of both MB-1 and MB-2, and decreased with depth. In the saturated sediments, microbial biomass was generally an order of magnitude lower in MB-2 than in MB-1. The higher concentration of TCE in MB-2 may be inhibiting microbial growth. The smallest population densities were consistently found in the silt and clay units within the unsaturated and saturated sediments. Previous studies by White et al. (1983) confirm that decreased biomass generally is detected in sediments with high clay content. Microbial biomass estimates were 4 to 5 orders of magnitude higher for groundwater samples from MB-1 and MB-2 compared to groundwater from extraction wells MW-39, MW-40, and MW-44. The higher concentration of TCE in the extraction well groundwater may be inhibiting microbial growth.

Microbial Community Composition

Table 1 summarizes the PLFA profiles for the sampled sediments and groundwater. Significant microbial population densities and complex community structures were detected in the unsaturated sand units and groundwater of MB-1 and MB-2, as evidenced by the number of different PLFA detected in these samples. Community diversity and complexity generally decreased with depth, and was lower in silt and clay units than in sand units.

Uncontaminated subsurface sediments generally contain high proportions of Gram-positive microorganisms, however, shifts in the proportions of Gram-positive to Gram-negative microbiota have been observed in contaminated sediments (Smith et al. 1985). Samples collected from MB-1 and MB-2 consistently had higher percentages of monounsaturated PLFA (usually Gram-negative) than terminally branched, saturated PLFA (usually Gram-positive) (Table 1). The predominance of monounsaturated PLFA as compared to terminally branched, saturated PLFA in saturated sediment and groundwater samples suggests that there has been a shift of the microbial community in response to the TCE contamination.

TABLE 1. Summary of phospholipid fatty acid (PLFA) and polyhydroxyalkonate (PHA) profiles for sampled sediments and groundwater.

Sample Location	Unsaturated Sediments						Saturated Sediments							Groundwater				
	MB-1		MB-2				MB-1			MB-2				MB-1	MB-2	MW-39	MW-40	MW-44
Depth (m bgs)	6.7	15.2	4.0	6.7	13.7	16.8	21.3	22.9	23.8	19.5	20.7	21.6	22.9	21.6	21.6	45.1	40.8	46.0
Sample Description	sand	clay	sand	sand	clay	silt	sand	sand	silt/clay	sand	sand	sand	silt/clay					
Number PLFA detected	28	11	30	30	13	5	12	12	6	8	4	1	4	20	18	4	4	5
PLFA (mole %)																		
Saturates	20.2	81.7	31.8	40.6	33.4	63.2	64.8	32.5	61.7	38.0	67.8	100	55.8	23.7	19.4	40.5	40.4	26.7
Terminally branched saturates	24.3	5.42	27.4	16.9	2.67	-	7.68	1.10	-	6.71	-	-	-	2.55	2.16	-	-	28.2
Mid-chain branched saturates	6.60	-	9.35	5.70	-	-	-	4.07	-	9.26	-	-	-	-	-	-	-	-
monounsaturates	41.1	7.77	29.9	34.0	39.3	16.6	25.1	25.2	31.1	37.0	17.2	-	25.3	69.1	72.5	59.5	59.6	45.1
polyunsaturates	7.76	5.15	1.06	2.86	23.8	20.2	2.48	37.1	7.24	8.98	15.0	-	18.9	4.59	5.97	-	-	-
Total	100	100	100	100	99	100	100	100	100	100	100	100	100	100	100	100	100	100
pmole PLFA/g or mL	62.2	5.14	374	124	13.3	1.35	4.68	15.9	1.16	1.96	0.64	0.18	1.63	423	271	0.10	0.02	0.03
Biomass (CFU/g or mL)	2.5E+6	2.1E+5	1.5E+7	4.9E+6	5.3E+5	5.4E+4	1.9E+5	6.4E+5	4.6E+4	7.8E+4	2.6E+4	7.2E+3	6.5E+4	1.7E+7	1.1E+7	4.0E+3	8.0E+2	1.2E+3
PHA/PLFA ratio	2.09	1.95	45.0	19.9	15.8	-	38.5	-	-	-	-	-	-	0.14	-	-	-	-
Trans/cis ratio of 16:1w7	0.04	-	0.66	0.43	-	-	-	-	-	-	-	-	-	0.17	0.07	-	-	-
Trans/cis ratio of 18:1w7	-	-	0.23	0.17	-	-	-	-	-	-	-	-	-	0.13	0.10	-	-	-
Cyclo/mono ratio of cy17	0.28	-	0.57	0.76	-	-	0.99	-	-	**	-	-	-	-	-	-	-	-
Cyclo/mono ratio of cy19	0.17	-	0.49	0.30	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Notes:

bgs = below ground surface

- = Not Detected

Saturates = Sum of 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, and 22:0

Terminally branched saturates = Sum of i14:0, i15:0, a15:0, i16:0, i17:1w7c, a17:1w8c, i17:0, a17:0/1w8

Mid-chain branched saturates = Sum of Sat17:0, Sat18:0, 10Me16:0, 10Me18:0, Br16:1, Br18:1, Br19:1

Monounsaturates = Sum of 15:1w6, 16:1w5c, 16:1w7c, 16:1w7t, 116:1w9c, Cy17/17:1, 18:1w7c, 18:1w7t, 18:1w9c, Cy19, 19:1w12c

Polyunsaturates = Sum of 18:2w6, 18:3w3, 20:3w6

g or mL = gram dry weight of soil or milliliter of groundwater

Biomass calculated using a conversion factor of 4e+4 CFUs per pmole of PLFA

** = ratio approaches infinity

The PLFAs detected in groundwater samples from wells MW-39, MW-40, and MW-44 were considerably different than those obtained from the groundwater of MB-1 and MB-2 (Table 1). A maximum of 5 PLFAs were detected in MW-39, MW-40, and MW-44, whereas more than 18 PLFAs were detected in the groundwater samples from MB-1 and MB-2. The decreased community diversity in groundwater from MW-39, MW-40, and MW-44 may be attributable to the elevated TCE concentrations in the groundwater in the deeper sand units.

A large percentage (4.5 to 58.9 mole %) of the PLFAs detected in the sediment and groundwater samples was characteristic of eukaryotes (sum of the polyunsaturates, the saturates 20:0 and 22:0, and the monounsaturates i16:1w9c and 18:1w9c). Of particular note was the dominance of 18:2w6 in sediment and groundwater samples from MB-1 and MB-2. PLFA 18:2w6 is a product of aerobic desaturation activity (carried out by eukaryotic microorganisms such as protozoans) and is a known fungal fatty acid. The high percentage of this fatty acid and the presence of 18:1w9c (a precursor to polyunsaturate synthesis) suggest a strong presence of protozoans and fungi. Madsen et al. (1991) have shown that protozoan activity (e.g., grazing on bacteria) can be correlated with biodegradation activities of microorganisms and, as such, the high proportion of eukaryotic PLFAs should not be unexpected given the elevated microbial biomass of these borings.

Microbial Community Status

The polyhydroxyalkonate (PHA):PLFA ratio, the *trans:cis* ratio of 16:1w7 and 18:1w7, and the ratio of the cyclopropyl fatty acids (cy19 and cy17) to their precursor monounsaturates (18:1w7c and 16:1w7c) can be used to determine the nutritional and physiological status of microbial populations (Guckert et al. 1986). Nonideal growth conditions (ratio of cy17/16:1w7c and cy19/18:1w7c greater than 0.1) were evident in sand samples from the unsaturated zone in both MB-1 and MB-2, and in two sand samples from the saturated zone (MB-1, 21.3 m, and MB-2, 19.5 m). Physiological stress (starved) conditions (*trans/cis* ratio of 16:1w7 and 18:1w7 greater than 0.1) were evident in sand samples from the unsaturated zone and from the groundwater of both MB-1 and MB-2. PHA accumulation was evident in the unsaturated sediment samples from both wells, and in MB-1, 21.3 m, and MB-1, H_2O. The presence of PHA in these samples suggests that a carbon source (dissolved organic carbon or TCE) is available that the microorganisms cannot use for growth but can accumulate as storage lipid.

The highest stress levels were detected in microbial populations in MB-1, 4.0 m, and MB-2, 6.7 m. Limited water availability and residual TCE in the sediments and in the soil gas at these locations is likely affecting the microbial populations in these sediments.

Microbial Activity

Figure 2 presents the rates of thymidine incorporation and the rates of catabolic and anabolic activity in the sampled sediments and groundwater. Microbial growth, mineralization, and anabolism generally were higher in sa

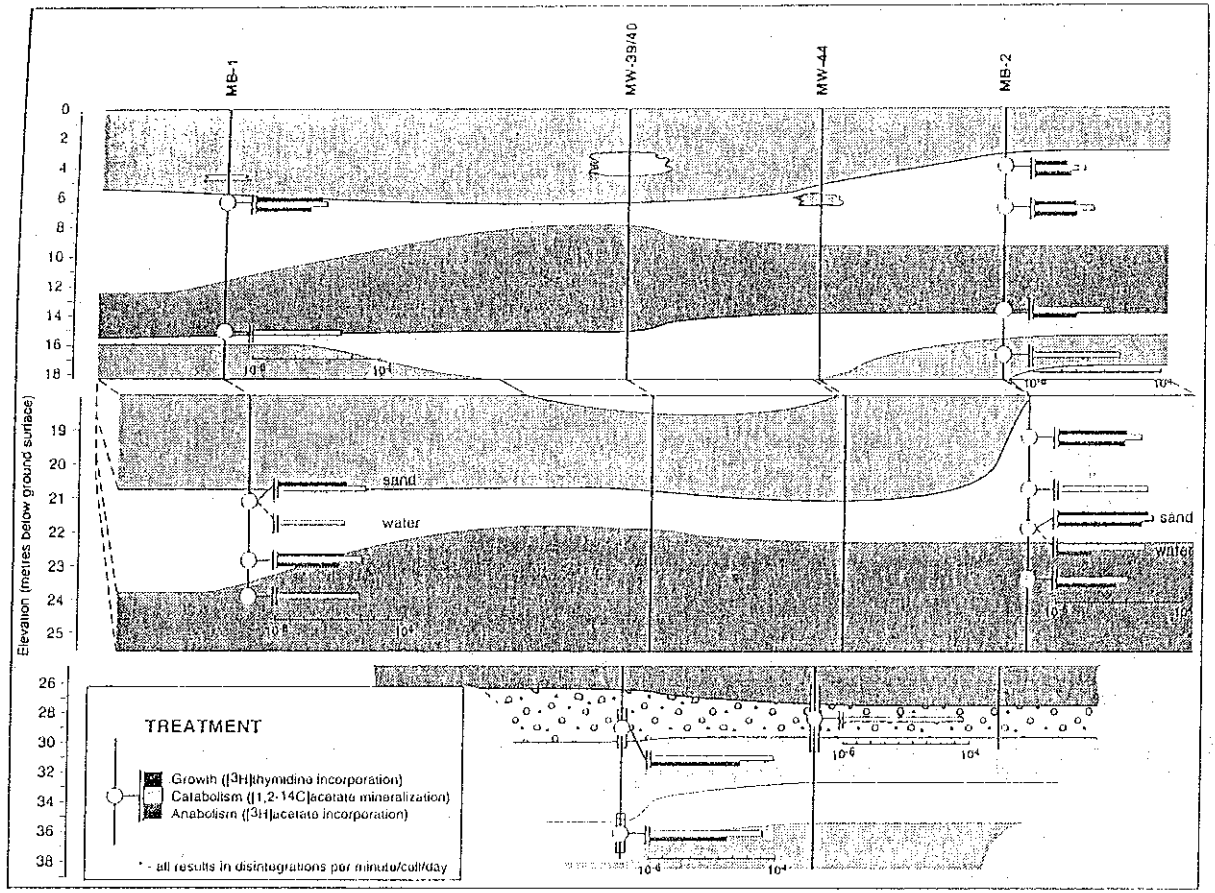

FIGURE 2. Microbial catabolism, anabolism, and growth in sampled sediments and groundwater.

samples than in silt and clay samples. These results are consistent with the lower population densities generally detected in the silt and clay units. The unsaturated sediment samples from MB-2 showed incorporation and mineralization rates several orders of magnitude lower than in comparable sediment samples from MB-1 and saturated sediment samples from both borings. Nutritional and physiological stress attributable to low water availability and exposure to elevated concentrations of TCE may be inhibiting the microbial populations in these samples.

Mineralization rates (glucose and acetate) and rates of acetate incorporation detected in the groundwater samples obtained from the extraction wells MW-39, MW-40, and MW-44 were 3 to 4 orders of magnitude higher than those detected in samples obtained from MB-1 and MB-2. However, population densities detected in groundwater samples from the extraction wells were 4 to 5 orders of magnitude lower than those observed in MB-1 and MB-2 (Table 1). The absence of detectable nutritional and physiological stress indicators (e.g., PHA accumulation) in the microbial communities in the extraction wells may explain the increased per cell activity observed.

The predominant production of CO_2 rather than CH_4 indicates that anaerobic metabolism at this site is not directed toward methanogenesis. The only samples in which methanogenesis was detected were MB-1, 21.3 m, and MB-1, 22.9 m. Had methanogenesis been significant, more than 85% of the acetate methyl groups would have formed methane, whereas less than 2% methane formation was observed in these two samples.

Enrichment with carbon-labeled glucose and acetate was detected during the activity experiments. Tenfold stimulations of aerobic catabolic activity were detected in a number of sediment samples and in groundwater samples from the extraction wells. In comparison, the [^{3}H]acetate experiments showed no increase in activity over time and thus provide a better snapshot of in situ activity.

TCE Biotransformation

Biotransformation of nonradiolabeled TCE was assessed under various aerobic nutrient conditions, due to the predominantly oxidizing geochemical conditions at the site. Figure 3 presents the percentage loss of TCE as compared to the sterile controls. TCE mass loss values less than 20% were considered to be experimental error. In samples that exhibited TCE mass loss, the percentage loss was similar between treatments. The similarity of the results from each treatment suggests that sufficient nutrients are present in the sediments but their accessibility may be limited under in situ conditions. Soil structure was probably disrupted during experimental setup, possibly leading to a release of previously inaccessible nutrients. This is particularly evident in the samples that received water. The addition of water to the disrupted sediments likely served to both relieve water stress in unsaturated sediment samples, and to release bound nutrients.

Sediment samples from MB-2 generally showed greater percentage decrease in TCE concentration than did equivalent samples from MB-1. Interestingly, the groundwater samples from MW-39, MW-40, and MW-44, all of which had low microbial population densities, showed TCE-biotransformation activity similar

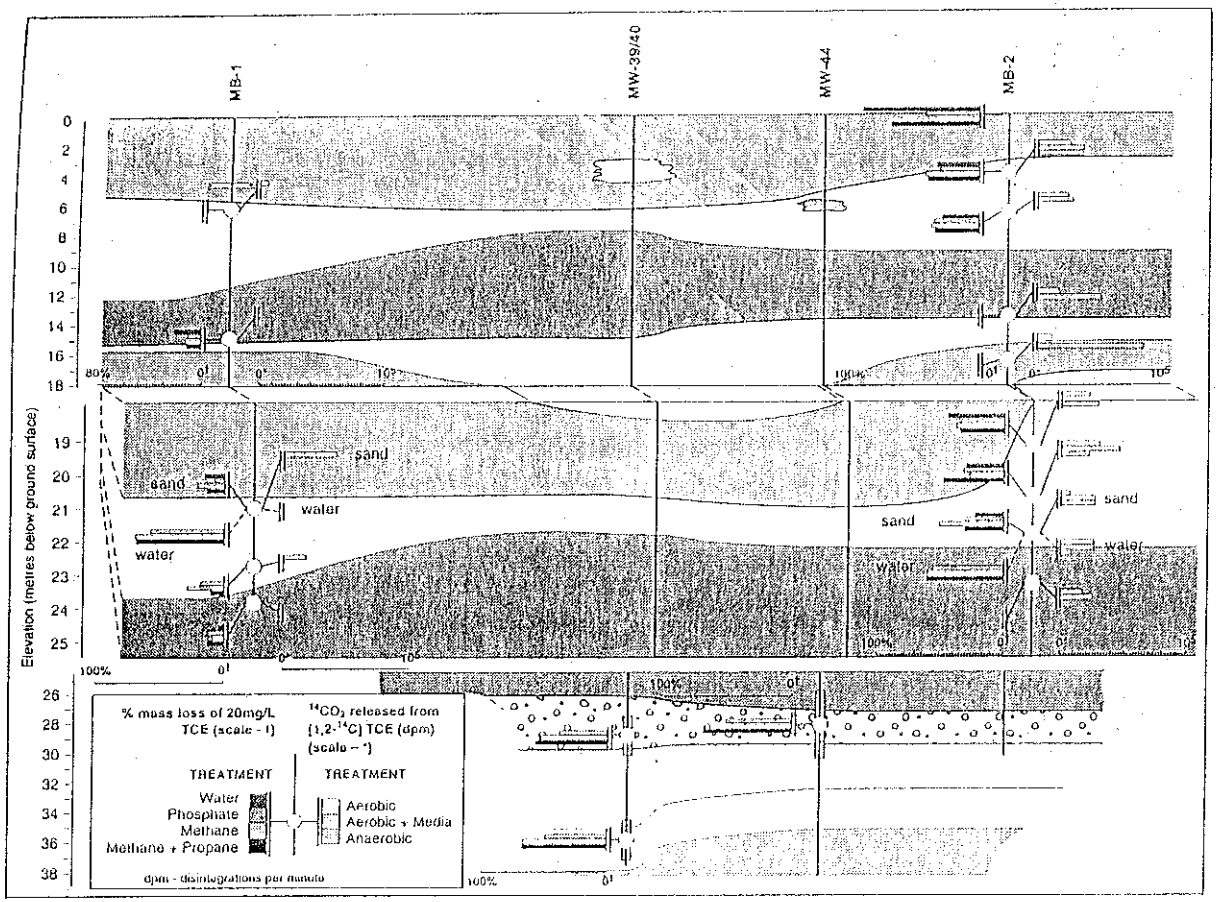

FIGURE 3. TCE biotransformation and $^{14}CO_2$ generation in sampled sediments and groundwater.

to the groundwater samples from MB-1 and MB-2. This activity indicates good biotransformation capacity by the existing microbial populations in the second and third sand units of the intermediate aquifer. No TCE biotransformation was observed in samples from the silt and/or clay units. Anaerobic dechlorination products *c*DCE and *t*DCE were detected at trace concentrations in several samples. Many of the groundwater samples that showed TCE mass loss became turbid with microbial growth, indicating good activity and growth potential in the presence of TCE.

Mineralization of Radiolabeled TCE and VC

Radiolabeled CO_2 was generated from at least one sediment or groundwater sample under all treatment conditions investigated (Figure 3). All sediment and groundwater samples from MB-2 showed mineralization of radiolabeled TCE to $^{14}CO_2$, whereas $^{14}CO_2$ production in MB-1 was confined to the sand samples. The production of $^{14}CO_2$ from radiolabeled VC was limited to the unsaturated sand samples in MB-2, and the saturated sand sample MB-2, 21.6 m. In addition to the production of $^{14}CO_2$, several anaerobic dechlorination products ([1,2-^{14}C]*c*DCE, [1,2-^{14}C]*t*DCE, and [1,2-^{14}C]VC) were detected during the biodegradation of [1,2-^{14}C]TCE. However, $^{14}CO_2$ was the dominant product in all cases.

The maximum extent of mineralization of TCE to CO_2 was 5%. This percentage would appear to be low in comparison to the amount of TCE mass loss observed in the nonradiolabeled experiments (average of 35%). However, typical catabolic activities of a carbon compound result in approximately one-third of the total carbon flow to cellular carbon, one-third to other water-soluble carbon compounds, and the remaining third to CO_2. Thus, a reasonable estimate of the expected amount of CO_2 over a 30-day incubation period would be 10% (based on the 35% average), which is in the range of the observed CO_2 levels. Given the amount of CO_2 produced, the time required for turnover of TCE would be months to years.

CONCLUSIONS

The collective PLFA data and the measured microbial activities (growth catabolism, and anabolism) indicate that significant microbial biomass exists in the subsurface beneath the facility that is capable of TCE mineralization to CO_2 both with and without the production of dechlorination intermediates (*c*DCE *t*DCE, and VC). The biomass and microbial activities are confined mainly to the sand units. The biomass is generally poised for aerobic activity as a result of the highly oxidizing conditions at the site. Anaerobic mineralization of TCE although not directed toward methanogenesis, also can be enhanced or induced. The water availability, the silt and clay content, the abundance of eukaryotic organisms, and the distribution of TCE in the subsurface appear to be the controlling factors of microbial activity, growth, and TCE biotransformation. The ability to biotransform TCE both aerobically and anaerobically in the

subsurface sediments at this site provides a promising and cost-effective remedial alternative to the current pump-and-treat remedial strategy.

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