THE EFFECTS OF TRICHLOROETHYLENE CONTAMINATION ON BACTERIAL COMMUNITY COMPOSITION ALONG FOUR MILE CREEK ON THE SAVANNAH RIVER SITE

by

ELIZABETH ANN RICHARDSON

(Under the direction of Charles H. Jagoe)

ABSTRACT

Trichloroethylene is a contaminant at C-area on the Savannah River Site, Aiken, SC. Indigenous soil bacteria are capable of metabolizing TCE present in the groundwater at C-area. TCE has a toxic effect on the bacteria resulting in selective changes in the bacterial community. This study endeavors to quantify those changes. Soil samples collected at the leading edge of the contaminant plume were analyzed for bacterial density, sole carbon source utilization, and the presence of methanotrophic bacteria. Geochemical data on the soil was obtained. Reference samples were similarly analyzed. The geochemical data revealed that the soils are not significantly different. The biological data revealed differences in the bacterial communities. The TCE contaminated soil had less diversity. The bacterial communities could also be distinguished by their carbohydrate substrate utilization. The data supports the hypothesis that the presence of TCE is affecting the bacterial community composition.

INDEX WORDS: Trichloroethylene, Methanotroph, PCR, DGGE, Natural attenuation, Co-metabolism, Community level physiological profile

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DEDICATION

This thesis is dedicated to God's glory and to my parents, Thomas and Mary Jane Murphy, who encouraged and supported my academic endeavors.

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INTRODUCTION

Trichloroethylene (TCE), a widely used organic solvent, is a common industrial pollutant. TCE is primarily anthropogenic, with the only known natural production by several species of marine algae (3). The Toxic Release Inventory (TRI) reported total releases for trichloroethylene in the United States of over 8.4 million pounds in 2001 (17). At least 861 of the 1,428 sites on the Environmental Protection Agency's National Priorities List reported trichloroethylene as a contaminant of concern (42). The International Agency for Research on Cancer identifies TCE as a probable human carcinogen and the Environmental Protection Agency, which previously listed TCE as class C-B2 or possible-probable human carcinogen, currently lists the status as "under review." Chronic ingestion of drinking water contaminated with TCE has been associated with increased incidence of childhood leukemia, and animal studies have shown carcinogenicity varies among sexes and species (43, 52). TCE metabolism produces toxic metabolites, which are capable of binding to macromolecules in the cell and disrupting normal cell activity.

Two common routes of exposure to TCE are inhalation and ingestion. Acute exposure to TCE is associated with depressed neurological function resulting in drowsiness, headache, and fatigue (52). Acute symptoms typically resolve after removal from the contaminated environment. Chronic exposure is associated with more severe effects such as damage to facial nerves, hearing and speech

impairment, and respiratory and eye defects which persist after removal from the contaminated environment (52). A precise dose-response relationship has been difficult to determine as most exposures are accidental and the exact concentration of TCE and duration of exposure is not available. Intentional exposure by inhalant abusers usually involves toxicant mixtures, effects cannot be solely attributed to TCE (43, 52).

TCE degrades by both biotic and abiotic processes. Various bacteria in the soil or sediment may degrade TCE either anaerobically by reductive dechlorination or aerobically with the aid of enzymes. TCE is highly volatile and readily evaporates from soil and water surfaces. Wind will act to disperse and dilute the TCE while reaction with atmospheric hydroxyl radicals breaks down the chemical (52).

This study was undertaken to examine the effects of a plume of TCE contaminated ground water on native bacterial communities present in vadose soils, especially mehanotrophic bacteria, which are capable of co-metabolizing the contaminant.

The presence of TCE and its metabolites could have toxic effects on the bacteria and thereby exert selective force that favors bacteria capable of metabolizing TCE or tolerating TCE exposure. To address this possibility, soil samples were collected from an area receiving TCE-contaminated ground water and a reference area. Soil chemistry, bacterial numbers, bacterial diversity, and bacteria substrate preferences were measured and compared between these areas.

METHODS

Site Description

The Savannah River Site (SRS) is an 800 km^2 U.S. Department of Energy (DOE) facility in South Carolina (Appendix 1, Figure 4) that was established in 1950 to produce nuclear materials for defense and other purposes. After the end of the Cold War, the mission of the SRS was redirected toward waste management and remediation.

One of the site's decommissioned reactors is located in C-area of the SRS. The area also contains a burning rubble pit (CBRP) originally used for waste reduction by open burning. Paper, plastics, rubber, oils, and organic solvents were discarded and burned in the pit, polluting soils and groundwater in the area. Monitoring wells in the area indicated that concentrations of several metals and organic solvents in ground water were above maximum Federal Drinking Water Standards in 2000. (1) A plume of contamination extends from the pit area toward Four Mile Creek and includes the Twin Lakes wetland area. Figure 1. Contamination in the vadose zone is estimated to cover an area of about 800 m^2 to a depth of about 18 m.

The highest concentration of TCE at the CBRP site was 286 µg/kg, measured at a depth of 9 meters (1). The maximum contamination level (MCL) for water permitted by EPA regulations is 5 ppb (52). Due to the mobility of TCE, the contamination in the vadose zone provides a continuing source of groundwater

contamination. The concentration of perchloroethylene (PCE), a metabolite of TCE, which is also seeping into the groundwater at levels exceeding regulatory requirements, indicates that some anaerobic bacterial degradation of TCE is taking place. The presence of cis- and trans- dichloroethane (DCE) indicates the occurrence of aerobic bacterial degradation (37). Well sampling in 2000 found that contaminated ground water containing TCE, PCE, and vinyl chloride was seeping into the surface water of Four Mile Creek (1). However, more recent studies by Mills and McArthur (37) and Rucker (47) indicated that these contaminants were not reaching the surface water of the creek. A multi-year drought, which began in 1999 and continued at least through 2001 may have influenced groundwater transport of these contaminants.

Field Collections

Field sampling sites were randomly selected in the riparian zone of Four Mile Creek. Previous samples (Appendix 1) had indicated the presence of methanotrophic bacteria within this area. Nine samples were collected from the east bank of the creek nearest the CBRP where the TCE plume intercepts the creek and 7 samples were collected from the west bank. Individual samples were collected at each site from a depth of 10-15cm using a shovel. Sampling dates were March 24 through April 1, 2003. Soil was removed from the shovel with sterile gloves, transferred to sterile plastic bags and stored on ice until analysis. The shovel was scraped of residual soil and rinsed with de-ionized water between samples to minimize cross-contamination.

Upon return to the lab, samples were manually homogenized and portioned for analysis. One 10 g aliquot was transferred to a sterile 500 ml flask, a second portion of at least 15 g was air dried for chemical analysis, a third portion of at least 10 g was used to determine percent moisture and a fourth portion was frozen at -80°C for later DNA extraction. The bulk soil remaining was used for soil texture characterization.

Bacterial Enumeration and Single Carbon Source Utilization

Bacterial enumeration was conducted according to the procedures of Wullom. (54) Ten milliliters of sterile saline (0.85%) was added to the flasks containing 10 g of fresh soil, which were then sonicated and serially diluted. Dilutions ranging from 0.1 to 0.001 were spread onto trypticase soy agar plates with a Nystatin fungicide, inverted and incubated at 25ºC. After 24 hours, colonies wer counted on plates showing 30-300 colonies, higher densities prohibited identification of individual colonies.

Single carbon source utilization assay was performed using Biolog Ecoplates. (Biolog, Inc. Hayward, CA) Each Ecoplate provides triplicate sets of 31 carbon sources and a control well, for a total of 96 wells per plate. Ecoplate wells were inoculated with 150 µL of diluted soil extract, sealed with parafilm and incubated at 25°C for 72 hours. Plates were read using a Biolog Plate Reader (Biolog).

Soil Characterization

Soils were dried at 50°C for 24 hours to determine moisture content. Soil samples were extracted with water to determine exchangeable ammonium and anions, (16) and with $BaCl_2$ to measure exchangeable cations (51). Major anions were measured by ion chromatography (Dionex). Major cations were measured by ICP-MS (Perkin Elmer Optima 4300 DV). Ammonium was analyzed using the nitrophenol method. Particle size was determined using wet soil and corrected for dry mass according to Gee (21). Soil pH was measured following the procedure of McLean (35).

Samples for carbon analysis were oven-dried, sieved (2mm) and analyzed at the UGA College of Agriculture & Environmental Science's Soil, Plant, and Water Laboratory using a combustion method.

DNA Extraction

Frozen samples were thawed under refrigeration prior to DNA extraction. DNA extraction followed the BIO 101 FastDNA Spin Kit for Soil (QBiogene, Inc.) protocol with modification for humic acid removal. The modification was brief centrifugation of the glassmilk silica-matrix bonded DNA solution, removal of the supernatant, washing the pellet with 5.5M guanidine thiocyanate and filtration. Eluted DNA was stored at -20°C.

DNA was quantified on a 1% agarose gel with at least three standards with varying concentrations of uncut lambda DNA, a Hi-Lo marker, and ethidium bromide stain. Gels were analyzed using the Stratagene Eagle Eye II imaging

system. Samples indicating the presence of DNA were then amplified by PCR with eubacterial primers to determine the presence of bacterial DNA. The primers used were 27F and 1387R. (53) See Appendix 1, Table 3 for primer details. Successful amplification of DNA of the expected size was taken as evidence of the presence of bacterial DNA. Non-specific amplification or no amplification was interpreted as the absence of bacterial DNA.

Screening of Methanotrophs by PCR

Purified environmental DNA was amplified by PCR using Ready-to-Go PCR beads (Amersham-Biosciences) according to the manufacturer's instructions, except that the protocol was modified for use in a hot-start procedure with AmpliWax PCR gems (Applied Biosystems). Thermocycler settings followed those of Wise et al. (53). The presence and size of amplification products was determined by agarose gel electrophoresis with ethidium bromide staining. To amplify methanotroph Type I 16S rDNA sequences present in the environmental DNA, the primers MethT1bR and MethT1dF (53), all primers were obtained from Integrated DNA Technologies, Inc., were used. Type II sequences were amplified with primers MethT2R and the bacteria-specific primer 27F (30, 53). The detection limit of the primers was established for Type I methanotrophs at a ratio of 1:100,000 and Type II methanotrophs at 1:50,000.

Nested PCR

A nested PCR approach was utilized to prepare the methanotroph-positive PCR products for DGGE. The primers were GC358F and 517R (40, 53). Hot start, touchdown PCR was used to prepare the product for DGGE. The thermocycling program followed the procedure used by Wise et al. (53).

DGGE Analysis of Methanotroph Type Communities

The nested PCR products obtained from a positive control (*Methylococcus capsulatus,* ATCC# 33009) and the samples testing positive for Type I methanotrophs were analyzed by DGGE using a Bio Rad D Gene System. DGGE gels (6.5% polyacrylamide with a denaturant gradient from 20-70%) were run for 3.5 hours at 130 V and 60°C. Gels were visualized by ethidium bromide staining on an Eagle Eye II system. Samples positive for Type II methanotrophs and a positive control (*Methylosinus trichosporium*, ATCC# 49242) were analyzed in the same manner as the Type I methanotrophs, except that the denaturing gradient was 30-60%.

DNA Sequencing

Representative bands were excised from the DGGE gel, placed in Tris-low EDTA (TLE) buffer solution and refrigerated for 24 hours. This solution was then used in a nested PCR reaction using the original methanotroph primers. The yield of DNA from the PCR was quantified on a 2% agarose gel. The PCR product was purified using ExoSAP, and sequenced using a BigDye v.3.1 kit, on a Perkin-Elmer ABI-Prism 377-96 (Applied Biosystems). The sequences were

analyzed with Sequencher 4.1 (Gene Codes) software and matched to known bacterial sequences through the National Center for Biotechnology Information website at http://www.ncbi.nlm.nih.gov/BLAST/ with nucleotide-nucleotide BLASTN. Methanotroph diversity was then compared between contaminated and reference locations. All statistical comparisons were made with SAS v. 8e (SAS Institute Inc., Cary, NC, USA)

RESULTS

Geochemical Analysis

Soil characteristics, including: ion content, percent moisture, particle size, pH, and carbon content are presented in Table 1. At all locations, soils had relatively low cation exchange capacity (CEC), with low concentrations of potassium, sodium, and iron. The percent moisture was variable and ranged from 16 to 65%. Soil texture was dominated by sand and clay. Soils were acidic with an average pH of 4.52. The percentage of carbon ranged from 0.3 to 7.6 with an average of 3.68. . There were no relationships among soil physical or chemical characteristics (by Pearson's Correlation, considered significant when $p < 0.05$).

Soil variables were compared among locations using Kruskal-Wallis tests, and differences were considered significant when $p < 0.05$. There were no significant differences between locations for any of the variables except iron, which was slightly higher in reference sites. Examination of the data showed that this difference was due to a single high value at one reference site. The similarities between the soils suggest that the bacterial communities present have the same set of resources and conditions for growth and could be expected to have similarities in community composition and physiological function.

Culture Dependent Analysis

Bacterial enumeration as colony forming units (CFUs) per gram of soil was made for each sampling location. The contaminated soil had a mean density of 3.70E+03 CFU/g and the reference soil had a mean density of 3.24E+03 CFU/g. Comparison of bacterial densities between contaminated and reference locations indicated no significant differences by either ANOVA (F=0.0909, p=0.7686) or Wilcoxon (X^2 =0.0952, p=0.7576) tests.

Most of the carbon substrates on the Ecoplates supported growth of bacteria from both contaminated and reference sites. The following carbon sources did not support growth at either site: 2-hydroxy benzoic acid, α-cyclodextrin, L-threonine, glycyl-L-glutamic acid, and α-ketobutyric acid. In addition, the contaminated samples did not utilize α-D-lactose.

Ecoplate profiles were analyzed using discriminant function analysis. The carbon sources were categorized into chemical groups: carboxylic acids, polymers, carbohydrates, amino acids, and amines and phenolic compounds. The average well color development was calculated for each carbon source and these values were compared. Each group was analyzed separately to determine if that group could be used to differentiate between the sampling locations. The resulting community level physiological profile revealed that the carbohydrate group could effectively discriminate between locations. (Figure 1) The carbohydrate carbon sources on the Ecoplate were: β-methyl-D-glucoside, D-galactonic acid γ-lactose, D-xylose, i-erythritol, D-mannitol, N-acetyl-D-glucosamine, D-cellobiose, glucose-1-phosphate, α-D-lactose, and D,L-α-

glycerol phosphate. The other carbon source groups did not discriminate between the samples.

Culture Independent Analysis

DNA was amplified in all samples processed with eubacterial primers indicating the presence of bacterial DNA. Subsequent amplification using methanotroph Type I primers resulted in non-specific amplifications which could not be used to support the presence of Type I methanotrophs in the soils. Therefore, further testing for Type I methanotrophs was not conducted. Studies by Eller and Frenzel (14) and Horz et al. (25) indicate that the Type I methanotrophs may be more closely associated with root structures and therefore may not have had a significant presence in the bulk soil examined in this study. However, amplification with Type II primers did result in production of the expected sized products for all sample locations. These products were further analyzed.

DGGE of the nested PCR product resulted in a gel image with multiple bands produced from each sample (Figure 2). Each lane of the gel represents an individual sampling location, with C = contaminated locations and R = reference locations. The DGGE gel had bands at 14 identifiable positions, labeled A-N on the image. . The reference and contaminated samples had 12 band positions in common and each had one unique band. The mean number of bands was 5.75±0.49 for the contaminated samples and 5.43±0.87 for the reference samples. This difference was not significant ($F=0.11$, $p=0.7444$, $\alpha=0.05$.)

The method of Leonard et al. (31) was used to compare the similarity indices for the two communities. Within contaminated samples, the mean similarity index was 0.58±0.08. Within reference samples, the similarity index was 0.39±0.14. This difference in similarity indices between the contaminated and reference communities was not significant ($t=1.164$, $p=0.2654$, $\alpha=0.05$.)

The bands separate on the gel based on their nucleotide composition and a single band may actually contain several very similarly composed, but different, sequences. Therefore, band count is an under-representation of the number of different species of methanotrophs present in the original sample (48).

Bands were excised and the DNA amplified for sequencing. Bands were selected from 11 positions with replicates representing both contaminated and reference samples. The resulting 37 sequences were matched to a potential of 16 different genera among the α-proteobacteria. Those genera were: *Chelatococcus, Methylocapsa, Methylocella, Blastochloris, Bradyrhizobium, Rhodopseudomonas, Ochrobactrum, Xanthrobacter, Methylocystis, Methylosinus, Methylobacterium, Roseomonas, Mesorhizobium, Rhizobium, Azospirillum, and Sphingomonas.*

The Coefficient of Jaccard was calculated at 0.625. The contaminated samples contained DNA matching 10 different genera whereas the reference samples matched the same 10 plus an additional six genera. The six unique genera identified constituted only 19% of the reference matches found. The reference soil showed greater diversity of methanotrophic bacteria. The six unique genera were only found at certain locations, whereas the 10 more

common genera were found in multiple sampling locations for both reference and contaminated soils. The lower abundance of these genera could make them more susceptible to elimination under adverse growth conditions.

DISCUSSION

The presence of TCE metabolites in groundwater and soil samples in the CBRP area indicates that some natural attenuation of TCE is occurring. This study supports the possibility that this natural attenuation is bacterially mediated. TCE co-metabolizing bacteria are present in the same soils as the TCE metabolites. Since TCE has toxic effects on bacteria, selection may result in differences in bacteria number, bacterial diversity, or community composition.

Despite the geochemical similarity of the reference and contaminated soils, differences were found in both the culturable and molecular aspects of the bacterial community. There was no significant difference in number of culturable bacteria between contaminated and reference sites. Culturable bacteria varied in the utilization of carbohydrates as a growth substrate. The methanotroph community varied in diversity of species.

The data in this study were obtained from samples collected over a period of about two weeks. Thus, the results represent a snapshot of bacterial communities during a brief time period. The stability of the bacterial community in the contaminated zone is unknown. Further and more drastic changes may occur if the concentration of TCE increases as the contaminant plume advances. Although the presence of methanotrophs and metabolites of TCE indicate natural attenuation is occurring, the rates of TCE degradation in the riparian zone have not yet been determined. Calculation of the degradation rates would be essential

for implementation of natural attenuation as an official remediation strategy at this site. In which case further bio-monitoring would be required and changes to the bacterial community could be tracked through time.

Øvreås and Torsvik (44) and Brodie et al. (8) found that bacterial communities were quantifiably different among soils with different physicochemical properties. In this study, there were no significant differences in soil structure or chemical properties between the reference and contaminated soils, so the bacterial communities should have been similar. Instead, there were differences in carbon source utilization and species diversity with the presence of TCE contamination.

Sequencing of the bacterial DNA revealed that the diversity of methanotrophic species varied between reference and contaminated soils. Species diversity was higher at the uncontaminated reference sites. Reduced species diversity may be due to stress associated with the presence of TCE. Exposure to TCE produces metabolic stress in bacteria. This stress is due to multiple factors (4, 15, 38, 45) including production of toxic metabolites, enzyme inhibition and differences in energy production from metabolism of TCE versus other potential substrates. Bacteria can break down TCE, but the process produces no net energy yield for the organism. When TCE is present some bacteria utilize this compound as a carbon source, which negatively impacts cellular energy production. Under these conditions, those bacteria that have a higher affinity for other substrates that are non-toxic are favored.

Bacteria that can induce higher oxygenase enzyme activities also have selective advantage under TCE stress (34). The induction of enzymes is related

to the concentration of substrate available. As TCE concentration increases, oxygenase enzyme activity is inhibited (57). Therefore, organisms with greater initial oxygenase activities may be better able to withstand TCE exposure. TCE stress also depletes available reducing energy, in the form of NADH (5, 57). Dissolved oxygen and primary substrate availability are also required for continued co-metabolism of TCE (39) since reducing power and dissolved oxygen are consumed without providing energy for cell growth.

TCE breakdown produces metabolites that can form DNA adducts (10, 55, 56,). Zhang et al. (57) showed that this type of DNA damage can be offset by repair mechanisms. However, bacteria may differ in their ability to repair DNA damage. Yeager et al. (56) found that some TCE sensitive strains had mutations in the loci that coded for DNA repair enzymes, specifically, gap repair and recombination repair enzymes.

Contaminant exposure will result in selective pressure favoring those organisms most capable of tolerating or eliminating the toxicant. The bacterial community exposed to stress will eventually be dominated by tolerant species and express less diversity than non-stressed communities (33). The results in this study concur in that the contaminated soil contained a less diverse community of methanotrophic bacteria when compared to the non-contaminated soil.

This study also differentiated the bacterial communities based on sole-carbon source utilization. The best discrimination among the contaminated and reference sites was obtained by measuring carbohydrate utilization. Staddon et

al. (50) used Ecoplates to measure carbon source utilization in forest soils after disturbance. Their results showed that carboxylic acid substrates provided the best discrimination. These results suggest that different subsets of carbon sources may provide better discrimination among bacterial populations under different types of chemical or physical stressors. Yeager et al. (56) found that some TCE sensitive strains have mutations in the loci determining carbon metabolism. In addition, Ayoubi (6) determined that the rate of TCE co-metabolism varied based on the carbon energy source provided. Several studies have found that augmenting a bacterial community's available carbon substrate can prolong the TCE co-metabolizing ability or increase the rate of co-metabolism (18, 19, 26, 32, 49).

Differences in carbon-source utilization appear to have strong influences on community structure and co-metabolizing potential. Selection of carbon source in a contaminated environment is crucial. The cell needs to avoid uptake of the contaminants to prevent damage to the cell and yet still needs to acquire a carbon source for nutrition. A bacterium that uptakes a broad range of carbon sources, but does not have the enzymes necessary to metabolize the contaminant or the repair mechanisms to correct contaminant mediated damage, is more likely to suffer the toxic effects of the contaminant and fail to survive.

The role of bacteria in remediation of hazardous wastes such as fuels, solvents, PCBs, and explosives has made the study and evaluation of in-situ bacterial populations essential. The bacteria present, their enzyme systems, and ability to tolerate and remediate toxic substances need to be determined and

monitored in order to demonstrate viable conditions for site bioremediation. PCR, DGGE and sole carbon source utilization using Biolog plates have become increasingly common methods of characterizing and differentiating bacterial communities (8, 9, 11-13, 20, 22-25, 27, 28, 36, 41, 46, 50, 53).

The benefits of bioremediation include less disruption of the natural environment, less expense, and less human exposure to the contaminant (26). At CBRP, the area affected by the contaminant plume is primarily woodland, wetland, and creek. A traditional method of remediation might include excavation of contaminated soil and removal to a treatment or storage site. Utilizing this method would require razing a large tract of land, worker exposure to a highly volatile solvent, and possible increased release into the nearby waterways. Bioremediation allows the woodlands to remain, the contaminant plume migrates at a more manageable pace, and the only people exposed to the contaminant are those collecting samples for monitoring. The techniques currently employed at CBRP are air sparging and soil vapor extraction wells, which are located directly over the pit to remediate “hot spots” (1, 47).

CONCLUSION

EPA requires that natural attenuation be monitored when used as a remediation strategy. (7) The locations used in this study are at the leading edge of the contaminant plume and are currently receiving low concentrations of contaminants. The bacteria present in the Twin Lakes seep zones and the vadose zone along Four Mile Branch are degrading the low level contamination at a rate that is preventing the TCE from reaching the open water of the creek. (37, 47) As the plume migrates and higher concentrations of TCE enter the vadose zone, the degradative activity of the methanotrophs will need to be reevaluated periodically to assure continued remediation. The data in this study may serve as a baseline for the future monitoring of TCE degradation in this area. In addition, other bacterial species known to degrade TCE may be present in the soil. Profiling the abundance and activity of these bacteria could contribute to the viability of natural attenuation at this site.

Location	Al^{+}	Ca^{2+}	K^{+}	Mg^{2+}	Na^{+}	Fe^{2+}	CEC	NH_4^{+}	Cl^{-}	NO_2^{-}	SO_3^{2-}
C1	0.524	0.052	0.042	0.03	0.0008	0.0004	0.644	0.014	0.008	0.03	0.083
C2	0.913	0.855	0.204	0.144	0.088	0.0023	1.666	0.012	0.009	0.03	0.051
C3	1.668	0.67	0.191	0.162	0.016	0.001	2.379	0.021	0.008	0.03	0.023
C4	0.663	0.48	0.081	0.055	0.0008	0.0007	1.043	0.014	0.01	0.001	0.04
C5	0.892	1.386	0.151	0.123	0.0008	0.0005	1.824	0.037	0.013	0.002	0.027
C6	1.3	0.304	0.178	0.079	0.025	0.0017	1.719	0.012	0.005	0.03	0.017
C7	1.01	0.0075	0.063	0.024	0.025	0.0053	1.171	0.009	0.005	0.03	0.033
C8	0.868	0.156	0.078	0.046	0.039	0.0006	1.118	0.004	0.008	0.03	0.021
C9	0.218	0.167	0.015	0.033	0.0008	0.0005	0.357	0.015	0.01	0.03	0.066
Mean	0.895	0.453	0.111	0.077	0.022	0.001	1.324	0.015	0.008	0.024	0.04
Standard Error	0.1407	0.1505	0.0234	0.0176	0.0095	0.0005	0.2101	0.003	0.0008	0.004	0.007
R1	1.005	0.0075	0.069	0.023	0.035	0.0033	1.169	0.003	0.003	0.03	0.04
R2	1.649	0.051	0.121	0.04	0.028	0.0079	1.936	0.009	0.004	0.03	0.02
R3	0.358	0.277	0.0078	0.071	0.0008	0.0006	0.615	0.014	0.009	0.03	0.085
R4	0.401	0.273	0.055	0.035	0.0008	0.0009	0.625	0.032	0.229	0.008	0.02
R5	0.9	0.536	0.142	0.083	0.0008	0.0023	1.372	0.037	0.151	0.03	0.035
R6	0.859	0.015	0.055	0.017	0.024	0.0027	1.002	0.002	0.054	0.03	0.004
R7	3.77	0.57	0.239	0.118	0.322	0.1218	4.778	0.012	0.106	0.03	0.053
Mean	1.277	0.247	0.098	0.055	0.059	0.02	1.642	0.016	0.079	0.027	0.037
Standard Error	0.446	0.090	0.029	0.0139	0.044	0.017	0.550	0.005	0.032	0.003	0.010
p-value	0.8738	0.3404	0.5602	0.3683	0.7822	0.0497	0.8738	0.5575	0.2873	0.5868	0.6715

Table 1: Ion Analysis Data for Sample Locations
Concentrations in ppm

Location	pH	%silt	%clay	%sand	%C	% moisture
C1	4.60	3.91	17.05	79.04	2.841	51.17
C2	4.66	1.87	15.86	82.27	3.891	37.19
C3	4.55	1.37	10.58	88.05	2.966	27.30
C4	4.63	2.01	31.65	66.34	5.545	56.84
C5	5.00	1.06	17.92	81.01	5.741	47.23
C6	4.68	1.73	3.72	94.55	1.833	19.30
C7	4.68	0.00	4.16	95.84	1.88	27.91
C8	4.56	1.02	5.92	93.06	0.7676	16.10
C9	4.55	10.84	29.05	60.11	7.632	64.29
Mean	4.66	2.65	15.10	82.25	3.68	38.59
Standard Error	0.046	1.083	3.405	4.153	0.743	5.711
R1	4.93	0.38	6.02	93.6	1.025	26.91
R2	4.42	0.24	2.59	97.17	0.8739	16.09
R3	4.77	6.92	27.67	64.41	6.77	56.98
R4	4.58	0.66	41.90	57.44	6.52	57.96
R5	4.49	6.38	44.01	49.61	7.429	65.18
R6	3.80	0.88	5.61	93.51	0.3773	18.43
R7	3.37	0.70	11.76	87.54	2.716	18.52
Mean	4.34	2.31	19.94	77.61	3.67	37.15
Standard Error	0.210	1.125	6.705	7.488	1.179	8.249
p-value	0.1815	0.2664	0.7913	0.7913	0.7913	0.6338

Table 2: Soil pH, Particle Size, Carbon and Moisture Contents for Sample Locations

Eubacterial	positive control environmental sample	expected size 900 bp
name	primer sequence	*E. coli* location
27F	5'- GAG TTT GAT CMT GGC TCAG –3'	27-45
1387R	5' - GGG CGG WGT GTA CAA GGC - 3'	1387-1404
Methanotroph Type I	positive control ATCC 33009 *Methylococcus capsulatus* (Bath)	expected size 920 bp
name	primer sequence*	*E. coli* location
MethT1dF	5' – CCT TCG GGM GCY GAC GAGT – 3'	84-102
MethT1bR	5' – GAT TCY MTG SAT GTC AAGG – 3'	988-1006
Methanotroph Type II	positive control ATCC 49242 *Methylosinus trichosporium* OB3b	expected size 950 bp
name	primer sequence*	*E. coli* location
27F	5'- GAG TTT GAT CMT GGC TCAG –3'	27-45
MethT2R	5' – CAT CTC TGR CSA YCA TAC CGG – 3'	997-1017
Nested PCR primers	positive control As specified above	expected size 190 bp
name	primer sequence	*E. coli* location
GC358F	5'- CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG –3'	341-358
517R	5' – ATT ACC GCG GCT GCT GG – 3'	517-534

*Y = C or T, R = A or G, M = A or C, S = C or G

Table 3: Primers used for Methanotroph Community Analysis

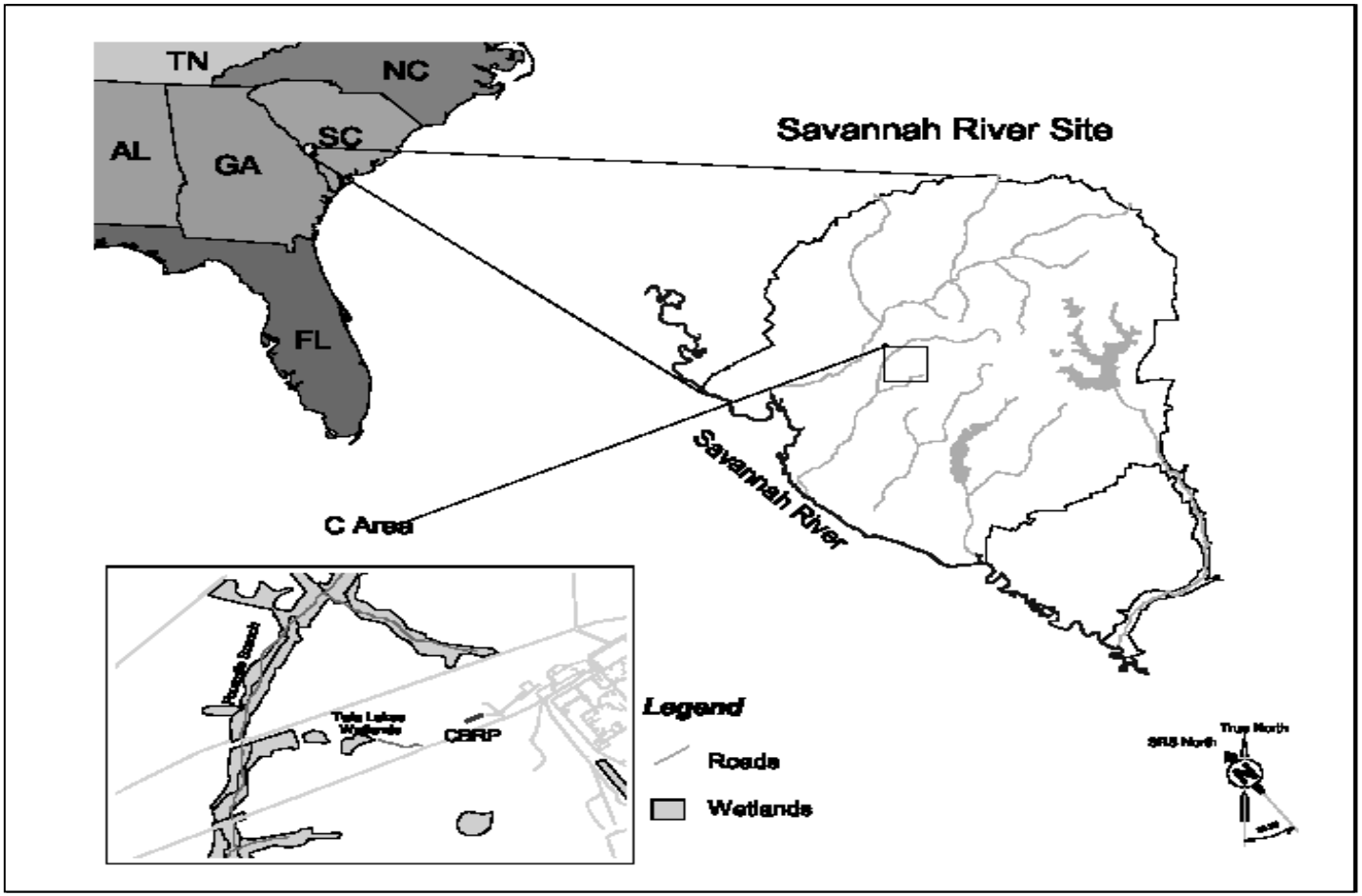

Figure 1: C-Area on the Savannah River Site, Aiken, South Carolina

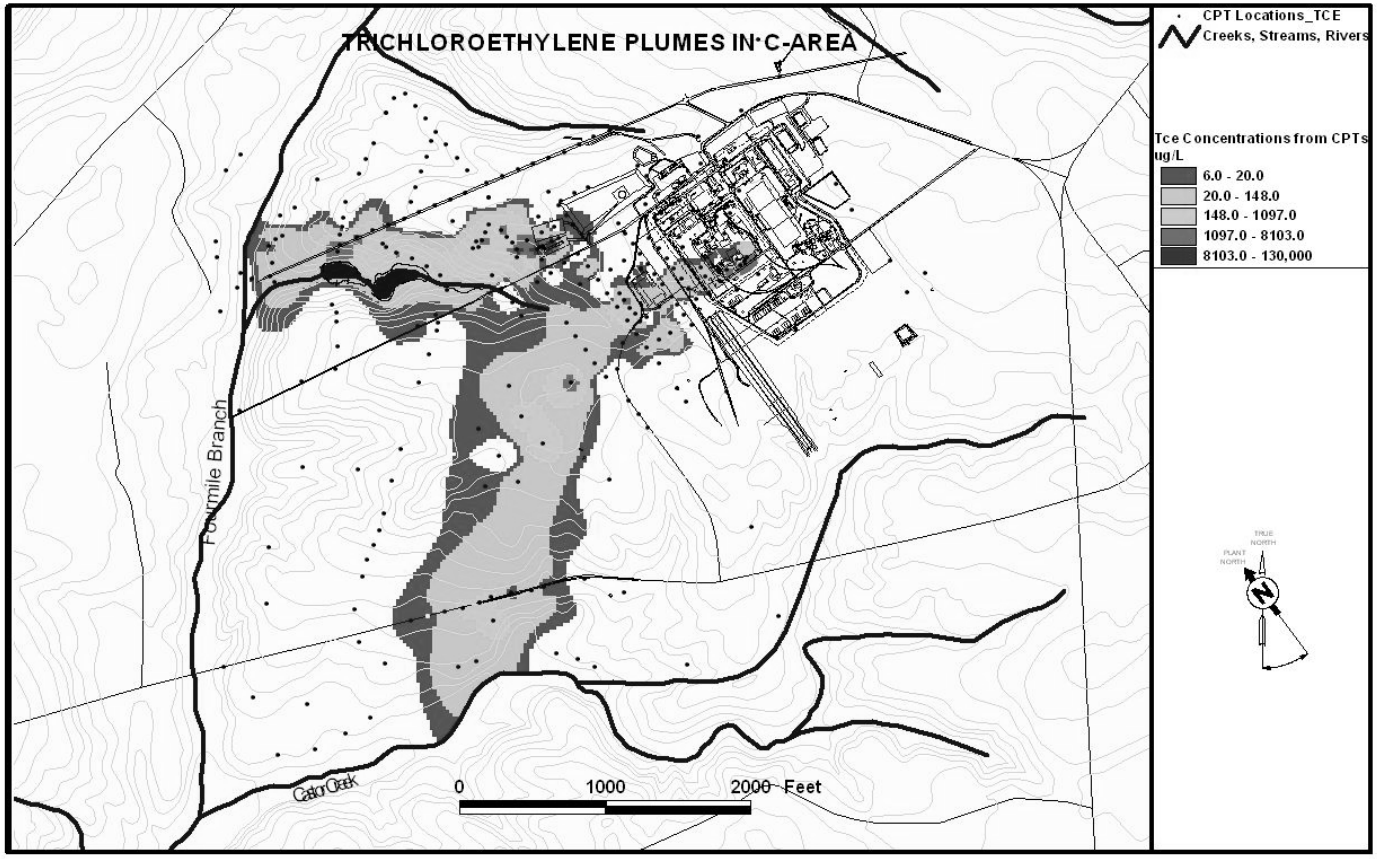

Figure 2: C-area TCE Contaminant Plumes

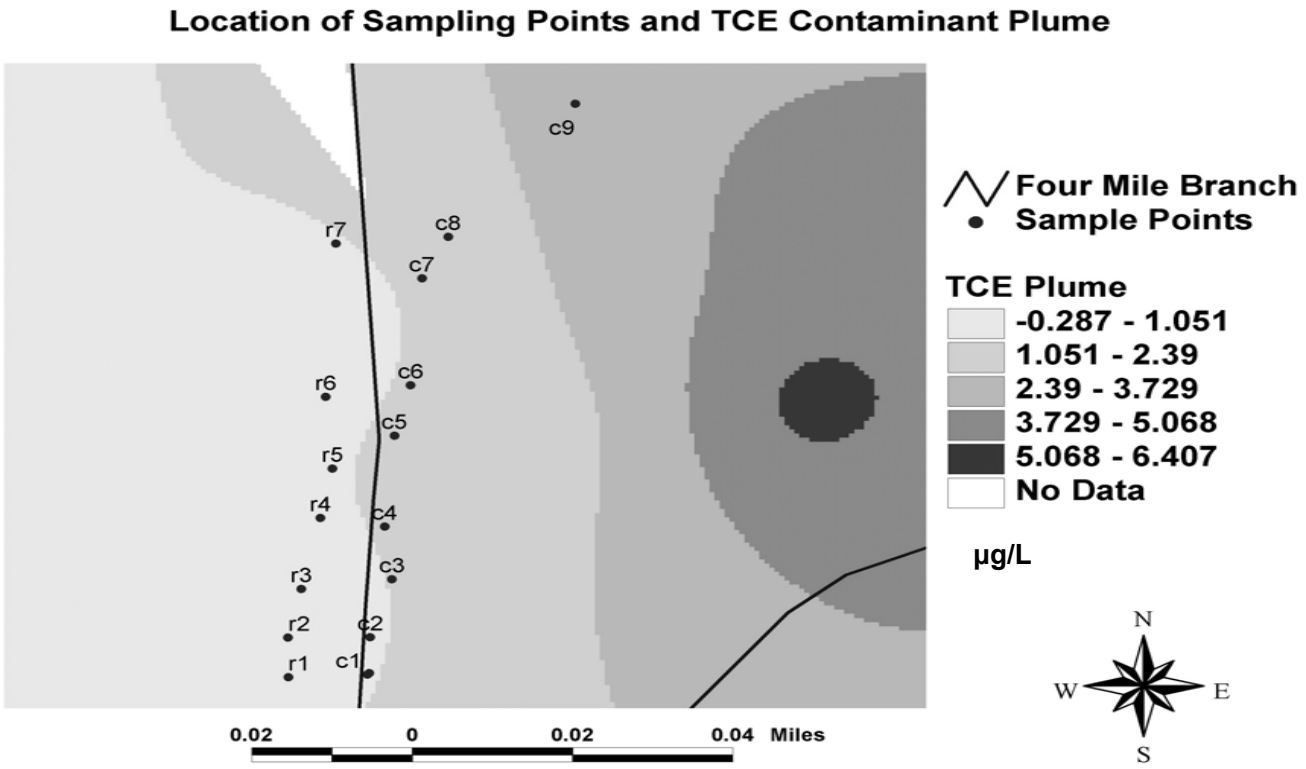

Figure 3. Sampling Locations along Four Mile Branch

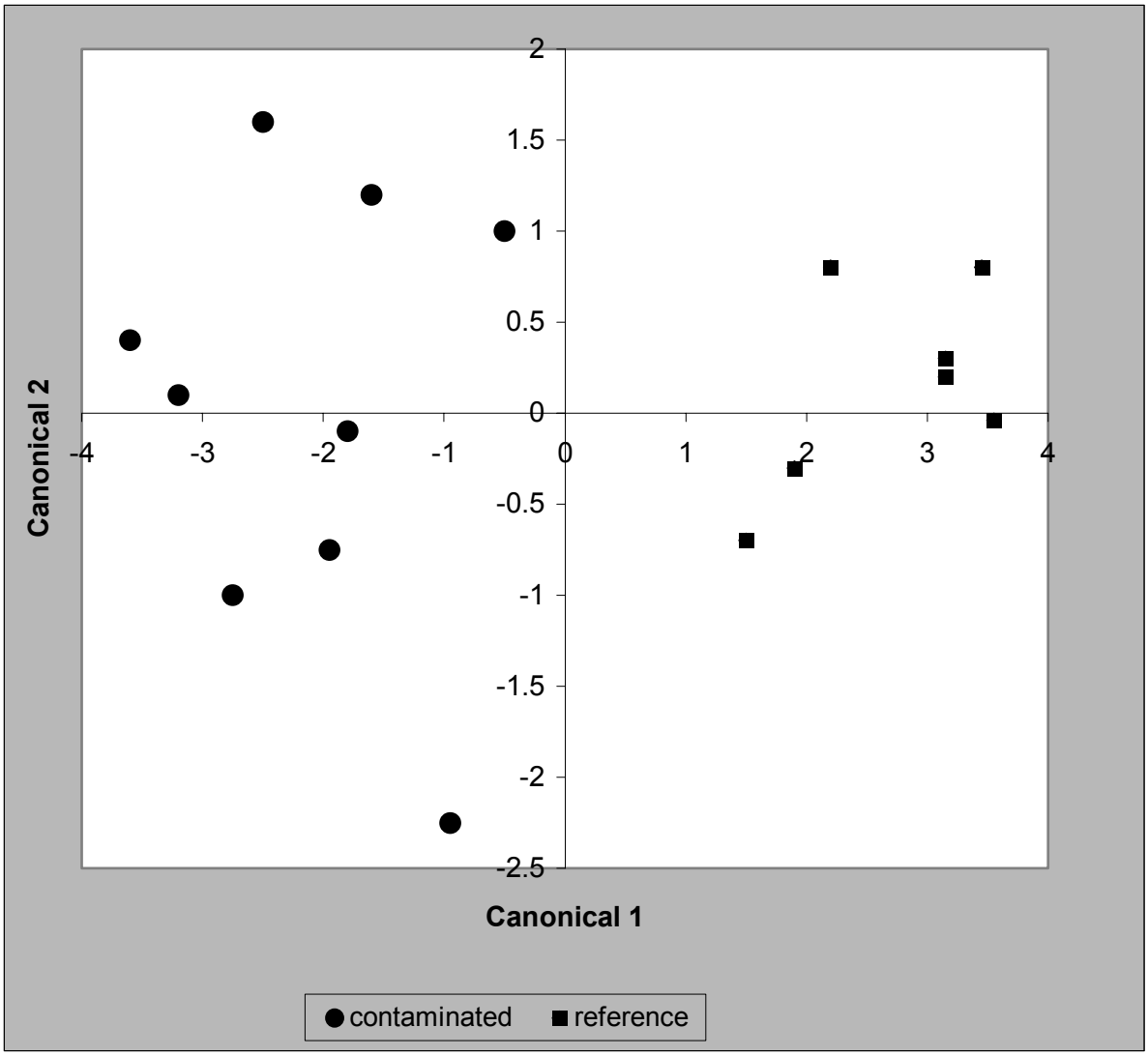

Figure 4: Discriminate Analysis of Carbohydrate Metabolism between Contaminated and Reference Soil Samples

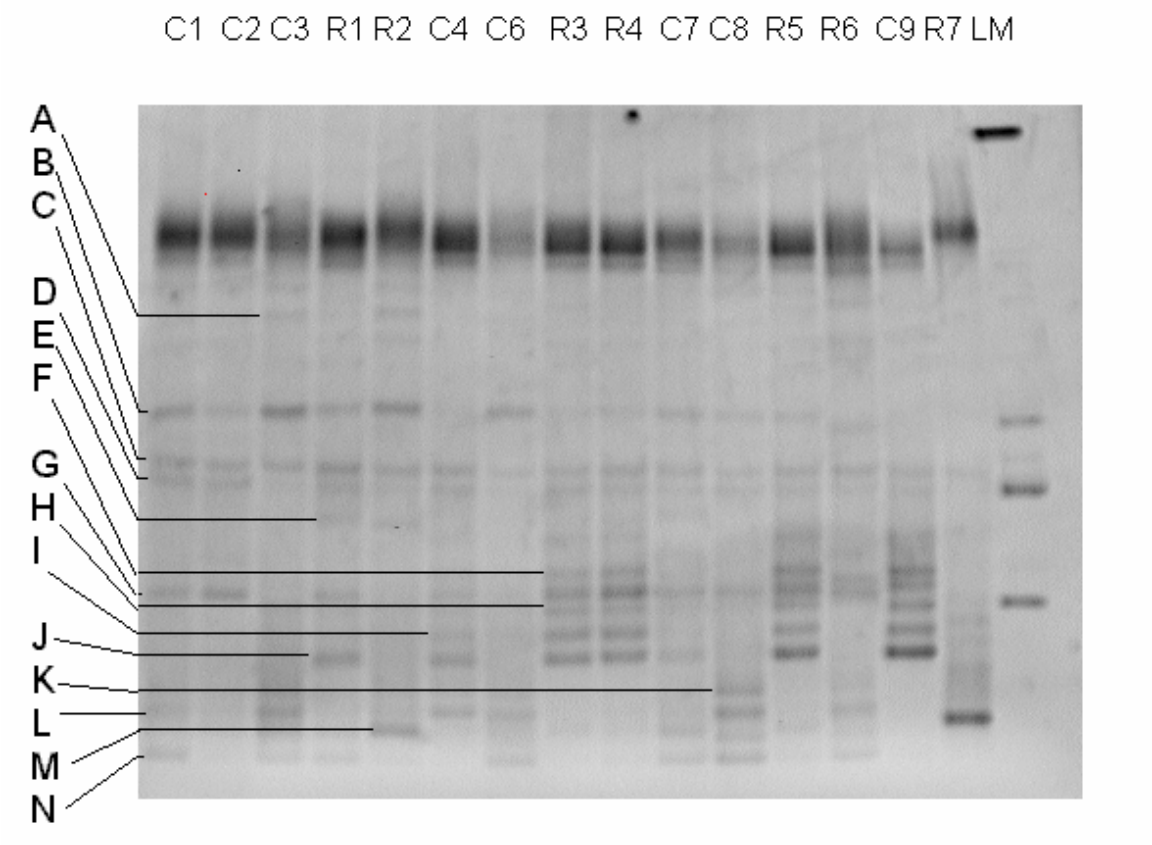

Figure 5: DGGE Gel of Methanotrophic Type II Bacteria
(C1-9) contaminated soils
(R1-7) reference soils
(LM) lane marker

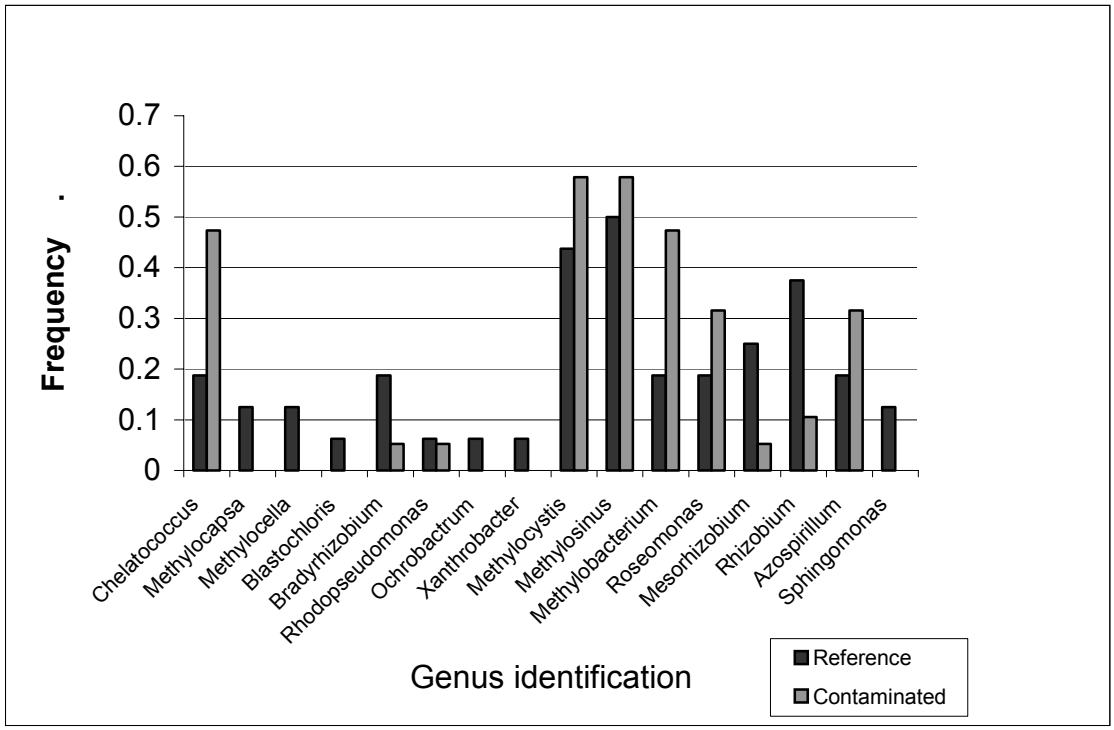

Figure 6: Frequency Distribution Showing Diversity of Methanotrophic Type II Bacteria

APPENDIX 1

INTRODUCTION

The samples described below were analyzed as part of a larger study investigating the degradation of TCE in the Twin Lakes wetland and adjacent Four Mile Branch riparian zone. The study involved both microbiological and geochemical aspects.

MATERIALS AND METHODS

Microcosm Soil Samples

Samples were collected from 3 random locations along the bank of Four Mile Branch Creek and 1 location along the zero order stream draining the Twin Lakes wetland using a flanged standpipe corer. Samples were portioned for determination of moisture content and particle size as outlined in the Methods section (p. 16). A portion from each location was frozen at –80C for DNA analysis. Nine microcosms (50 g of fresh soil with 100 ml of distilled water in a 250 ml bottle with Cheminert mininert sampling valve) were assembled for each location, 3 treatments with 3 replicates each. The treatments were: unamended soil, 2% methane amended soil, and autoclaved soil (abiotic control.) All microcosms were spiked with 200 µL of 0.7% TCE solution.

Microcosms were incubated for 15 days in an environmental chamber on a shaker table at 150 rpm, without light and at ambient temperature. Contents

were sampled by removing 0.50 ml aliquots with a 1.0 ml Hamilton syringe on days 0, 3, 6 ,9, and 15 for GC analysis. Following the sampling on day 15, all microcosms were sacrificed and the contents frozen at –80C for DNA analysis.

GC Analysis

- •Analysis was performed on a Hewlett-Packard 5890 Series II Plus gas chromatograph interfaced with a HP 5972 Mass Selective Detector.
- •Samples were introduced to the GC using a HP 7694 Headspace Sampler
- •Heated at 90C for 30 minutes
- •Helium was used as a carrier gas at a constant flow rate of 1.5 ml/min.
- •Analytical separation on a HP-PLOT Q capillary column (3m x 0.32mm ID, 20µm film thickness)
- •80C initial temperature for 2 min. followed by a linear increase of 10C/min to 200C, and held for 8.50 minutes
- •Injector temperature and GC/MS interface maintained at 250C
- •Amount of target compound was calculated by comparing compound target ion peak areas and peak heights obtained by 1.0 ml headspace sampler injections with that of standards made from dilutions of a Supelco (Belfonte, PA) VOC Custom Mix solution (light VOC's, 25 ppm each) using HPChem integration software.
- •Detection limits: 0.5ppb to 500ppb
- •VOC component values normalized to 9.75g (standard mass) of material

DNA Analysis

Analysis was performed following the procedures of Wise et al. (58) and as outlined in the Methods section (p. 11).

Vibracore Samples

Cores from 4 locations in the Twin Lakes wetland produced twenty-nine soil samples. Samples represented soils at intermittent depths to 13'. DNA extraction and methanotroph screens were performed as previously stated. In addition, all samples were screened for presence of sulfate-reducing bacteria (SRB) according to the procedures developed by Daly et al. (12)

RESULTS

Microcosm Results

The presence of Methanotroph Type I bacteria was indicated in soils "B" and "D." The Jaccard index shows that the two soil samples were more similar (42%) to each other than to the positive control (8%-20%.) Methanotroph Type II bacteria were indicated in soils "A", "B", and "D." Among the Type II bacteria, soils "B" and "D" had the greatest similarity at 71%. The "A" soil showed the least similarity to the positive control at 8% and only 11-12% similarity to the other two soil samples. Based on this information, a prediction might be made that microcosms with "B" and "D" soils would result in a similar degradation pattern and noticeably different from the "A" microcosms.

Vibracore Results

Eleven of the original 29 vibracore samples screened positive for eubacteria and of those eleven, one was positive for Type I methanotrophs and group 6 SRB. Three additional samples were positive for Type II methanotrophs. The seven remaining positive samples may still contain an unscreened group of bacteria, such as nitrate reducers, which may be capable of natural attenuation of TCE.

DISCUSSION

The GC/MS analysis of the microcosm samples revealed only 2 soil/treatment conditions that had significant TCE reduction. The third treatment (abiotic control) for soil "A" reduced TCE ($p=0.0114$.) This result was unexpected since the abiotic controls had been autoclaved to destroy microorganisms. In addition, each set of "A" microcosms revealed the presence of chloroform after 3 to 6 days of incubation. There were no MMO pathway degradation products identified in the samples. An abiotic pathway may be the explanation for this reduction in TCE concentration. Isolated measurements of chloroform also appeared in soil "B" treatment 2, day 6 and soil "D" treatment 2, day 9.

The first treatment (unamended soil) for soil "C" also indicated reduced TCE concentrations ($p=0.0034$.) However, no degradation products were measured and no methanotrophs were indicated in this soil. The "B" and "D" microcosms were similar in that they did not show significant amounts of TCE reduction and did not produce chloroform.

The methanotroph community profiles indicated similarities and differences between the soils sampled. The most similar soils, “B” and “D”, also demonstrated the most similar response to TCE. Whereas the more dissimilar soils, “A” and “C”, showed TCE patterns different from each other and from “B” and “D.”

The ability to predict the microbial response to TCE exposure will facilitate decision-making regarding contaminated sites and the most effective and cost-efficient remediation strategies to employ. Ongoing efforts to improve the microcosm procedures and accuracy of detecting degradation products may potentially refine the prediction process.

The vibracore samples that tested positive were all from the shallower core depths, less than 6’ deep. The deeper soil samples that did not test positive for bacteria all represented soil conditions that are not conducive to bacterial growth, i.e. low organic, C, and N contents. The presence of TCE degradation products was also stratified; TCE, DCE, and VC were all detected in the shallower soil sections. The presence of the degradation products in the same soil layers as the bacterial agents capable of degrading TCE supports the premise that bacterially mediated natural attenuation is actively occurring in these locations. At the time of these studies, TCE and its degradation products was not surfacing in Four Mile Creek. (37, 47)

CONCLUSIONS

The presence of volatile organic compounds such as DCE and VC in the soils of the wetlands and riparian zone along Four Mile Creek that are not contaminants attributed to the waste disposal practices at CBRP, but can be attributed to the natural attenuation of TCE, strongly suggest that the TCE contamination is undergoing intrinsic bioremediation.

It is important to note that, at the time of this study, the southeastern United States was experiencing a multi-year drought. (29) Ground water levels were depressed enough to make sampling at some wells in the CBRP area unreliable. Contaminant mobility was likely affected. This environmental condition illustrates the need to have monitoring of the natural attenuation process.

Primer name	Primer sequence*	ATCC control	Amplification target
27F	5’ GAG TTT GAT CMT GGC TCAG 3’		eubacterial
1387r	5’ GGG CGG WGT GTA CAA GGC 3’		eubacterial
DFM140	5’ TAG MCY GGG ATA ACR SYK G 3’	19998	SRB group 1
DFM842	5’ ATA CCC SCW WCW CCT AGC AC 3’		SRB group 1
DBB121	5’ CGG GTA GAT AAC CTG TCY TCA TG 3’	33891	SRB group 2
DBB1237	5’ GTA GKA CGT GTG TAG CCC TGG TC 3’		SRB group 2
DSB127	5’ GAT AAT CTG CCT TCA AGC CTG G 3’	43919	SRB group 4
DSB1273	5’ CYY YYY GCR RAG TCG STG CCC T 3’		SRB group 4
DCC305	5’ GAT CAG CCA CAC TGG RAC TGA CA 3’	33892	SRB group 5
DCC1165	5’ GGG GCA GTA TCT TYA GAG TYC 3’		SRB group 5
DSV230	5’ GRG YCY GCG TYY CAT TAG C 3’	13541	SRB group 6
DSV838	5’ SYC CGR CAY CTA GYR TCY ATC 3’		SRB group 6

Table 4: Eubacterial and Sulfate-Reducing Bacteria Primer Pairs

*Y = C or T, R = A or G, M = A or C, S = C or G

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