CHARACTERIZATION OF MICROBIAL COMMUNITIES IN TCE-CONTAMINATED SEEP ZONE SEDIMENTS

Brian A. Nevius[1], Christopher E. Bagwell[2], Robin L. Brigmon[2]

[1] Department of Biology and Geology, University of South Carolina Aiken
banevius@hotmail.com
[2] Westinghouse Savannah River Company, Aiken, South Carolina
christopher.bagwell@srs.gov

ABSTRACT

Hundreds of sites across the United States contain trichloroethene (TCE) contamination, including the Department of Energy's Savannah River Site (SRS) in Aiken, South Carolina. Previous studies have indicated that microorganisms are capable of efficiently degrading TCE to nonhazardous end products. In this project, molecular and growth based methods were used for microbial characterization of a TCE impacted seepzone where TCE degradation is naturally occurring. The results from this work provide clear evidence that the SRB may play a significant role in TCE degradation along the Twin Lakes seepline.

INTRODUCTION

Trichloroethene (TCE) was a common degreasing agent in dry-cleaning and machining industries, but the health hazards associated with this compound have largely restricted its use. At many locations TCE waste materials were simply dumped or buried in unlined pits or basins, and over time pollutants have seeped into subsurface groundwater supplies threatening downstream environments and public exposure (Hendrickson et al., 2002, Smidt, 2000). Flowing groundwater not only serves to transport TCE over large areas within the subsurface environment, but many contaminant plumes resurface through seepzones into streams, rivers, or wetland areas (Hendrickson et al., 2002). Hundreds of sites within the DOE complex have TCE contaminated groundwater, including the Savannah River Site in Aiken SC. A TCE plume originating from the C-Area Burning Rubble Pit (CBRP) on the Savannah River Site is now beginning to resurface at the Twin Lakes seep zone, a tributary of the Savannah River.

Bioremediation is an attractive, passive strategy for effective removal of TCE polluted sites. The success of bioremediation relies on the presence and activities of microorganisms that are capable of degrading TCE by catalyzing sequential dechlorination reactions, thereby transforming trichloroethene (TCE) → dichloroethene (DCE) → vinyl chloride (VC) → ethene (Hendrickson et al., 2002; Holliger et al., 1999; Smidt et al., 2000). *Dehalococcoides ethogenes* is the only known microorganism that can perform this entire process to completion. Other microbes, like the sulfate-reducing bacteria (SRB), can degrade TCE but incompletely to DCE or VC. Along the CBRP seepzone at the Savannah River Site TCE degradation is occurring naturally but this activity is inconsistent from site to site. TCE degradation is occurring at some sites but

not others, and where degradation is occurring, the process stops at DCE or VC. The microbiology of the CBRP seepzone has not been studied in detail. The goals of this study were to 1) determine why TCE degradation activity differs along the seepzone, 2) determine the role of SRB in TCE degradation, and 3) explore the use of DNA fingerprinting tools for rapid identification of bacteria capable of TCE degradation at different sites along the seepzone. To accomplish these goals classic microbiological growth based methods and DNA molecular based methods were used to examine the microbiology in CBRP seepzone sediments directly to explain TCE degradation.

MATERIALS AND METHODS

Field Site Description and Sample Collection. Sediment samples were collected in June 2003 along the flow path of a subsurface TCE plume originating at the C-Area Burning Rubble Pit (CBRP). Six groundwater sampling wells marked sediment collection sites in study. Furthest from the seepzone were sites 21 and 41. The saturated zone at these sites occurred at 6 – 8' depth and TCE degradation products had not been detected at these sites. The saturated zone at the intermediate sites 42 and 44 was at 5 – 6' depth and TCE degradation occurred at these sites but stopped at cis-1,2-dichloroethene (cDCE). Along the low-lying wetlands seepzone fringe, sites 48 and 50 were inundated and TCE degradation products cDCE and vinyl chloride (VC) had been detected at these locations. Sediment samples from these sites were collected within the 3 – 5' depth range. Sediment samples were collected using a hand auger. Upon collection, sediments were packed in ziplock bags and stored on ice during transport to the laboratory. Two grams of sediment per site were reserved for direct inoculation into SRB growth medium, and all remaining sediment was frozen at -80°C to await molecular analysis.

SRB Growth Medium. Postgate's Medium B with lactate as the sole carbon and energy source was used to select for the growth of SRB from field sediments (Flemming and Ingvorsen, 1998). One gram of sediment (2 replicates per site) was directly inoculated into 10ml (final volume) of Postgate's Medium B in an anaerobic chamber having an atmosphere of 95%N_2 and 5% H_2. Enrichment tubes were incubated in the dark at room temperature. Active sulfate reduction in Postgate's Medium B is evidenced by the production of FeS_2 that appears as a black precipitate in the growth medium.

Growth of SO_4 reducing enrichments on TCE. After 6 consecutive transfers on Postgate's Medium B, all positive sulfate reducing enrichment cultures were examined for their ability to degrade TCE in the absence of sulfate. Enrichment cultures were subcultured into a modified Postgate's Medium B with TCE (20µl of TCE saturated dH_2O, 2ppm) as the sole terminal electron acceptor. After 5 days incubation at room temperature in the dark, headspace gases were analyzed by gas chromatography – mass spectrometry (GC-MS) for analysis of TCE degradation products.

Genomic DNA extractions from sulfate reducing enrichment cultures. The protocol for DNA extraction used the Promega DNA Purification kit as recommended by the manufacturer (Promega, Madison, WI). Cell biomass from 3ml of each enrichment culture was collected by centrifugation at 10,000xg for 2min. Cell pellets were then re-suspended in 480µL of 50mM EDTA and 60µL of lysozyme (10mg/mL), and incubated at 37°C for 60 minutes. Cell lysis was performed using 600µL of Nuclei Lysis Solution and incubated at 80°C for 5 min. Cell lysates were treated with RNase-A (37°C for

30min) and protein removed by incubating on ice with the Protein Precipitation Solution. Genomic DNA was recovered by passage through an anion exchange spin column and stored at -20°C as an isopropanol precipitate.

Genomic DNA extraction from field sediments. The procedure used to extract genomic DNA from seep zone sediments was modified from Lovell and Piceno (1994) and Bond et al. (2000). First, 10g of sediment was re-suspended in 30mL of phosphate buffered saline (PBS) (pH 1.2) to remove contaminating metals, followed by a wash in 30mL 2X Buffer A (200mM Tris-HCl, pH 8.0, 15mM EDTA, 200mM NaCl, 2mM sodium citrate, 10mM $CaCl_2$, 1 part 50% glycerol) to remove humic acids. Washed sediments were then re-suspended in 13.5mL Buffer 1 (50mM Tris, pH 8.0, 100mM EDTA) and 2.5% (wt/vol) sodium lauryl sulfate (SDS). Cell lysis was performed by exposing sediments to 3 consecutive freeze-thaw cycles in liquid nitrogen and incubation at 75°C. The final thaw incubation was done for 30min with manual mixing every 5 min. Lysate was recovered by centrifugation at 10,000xg for 15min and sediments washed once more in 7mL of Buffer 2 (50mM Tris, pH 8.0, 100mM EDTA, 1% SDS) and incubated at 75°C for 15min. Combined supernatants were incubated with potassium acetate (0.05g / mL) at 4°C overnight. Precipitated humic acids were removed by centrifugation at 10,000xg at 4°C for 30 minutes, and supernatants were extracted with a half volume of phenol:chloroform:isoamyl alcohol (24:24:1). The aqueous phase was recovered and gDNA precipitated by adding 2 volumes of 100% ethanol and incubated at –20°C overnight. Genomic DNA was recovered by centrifugation at 10,000xg at 4°C for 30 minutes, and re-suspended in TE buffer. Final purification of genomic DNA used a combination of anion exchange chromatography and gel filtration.

PCR. Genomic DNA was quantified by spectrophotometry (260/280nm) and 25ng of DNA was used for amplification of the 'universal' bacterial 16S rRNA gene by the Polymerase Chain Reaction (PCR) using the HotStartTaq PCR kit (Qiagen, Valencia, Calif.) following the manufacturers instructions. PCR products were confirmed by electrophoresis on 1.5% agarose / TBE gels. PCR products were purified using the PCR Purification Kit (Qiagen) and re-suspended in TE buffer for T-RFLP analysis.

Terminal-Restriction Fragment Length Polymorphism (T-RFLP). T-RFLP is a DNA fingerprinting method that separates total genomic DNA into distinct bacterial populations by the use of DNA cutting enzymes. DNA from each bacterial population is cut differently with these enzymes yielding a distinct signature that can be resolved by gel electrophoresis. 16S rRNA gene products (4ul) were digested with 1ul restriction endonucleases *RsaI* and *Sau3A1* for 2h at 37°C. Digested products were then resolved on a DNA sequencing gel to distinguish bacterial populations present at each sampling location. Digital images were taken of the gel and analyzed by pattern recognition software developed at the University of Georgia.

RESULTS

DNA extraction from seepzone sediments. Genomic DNA was successfully extracted from sediments collected from sites 42, 44, 48, and 50, however repeated extractions from sites 21 and 41 sediments yielded no detectable bacterial DNA. This finding was consistent with the observation that sediments from sites 21 and 41 were composed of large grain sand with no indication of organic carbon. Previous studies have shown that these sites are quite oligotrophic with apparent low biomass. Since no genomic DNA

could be extracted from these sediments, we could not perform molecular analysis of these sites. Extractable DNA increased proportionally from sites 42 to 50, corresponding to an increased TCE degradation activity and decreasing distance to the seep zone. These findings would seemingly indicate that bacterial biomass is higher in the organic enriched seep zone sediments.

Enrichment of SRB and TCE degradation. Soils from all sampling sites were enriched under sulfate reducing conditions for growth dependent analysis of SRB. Following 4 days of incubation, the enrichment medium became noticeably more turbid as a result of cell growth in all tubes except those inoculated with sediments from sites 21 and 41 (no detectable TCE degradation at these sites). Even after 2 months of incubation, no apparent change in medium turbidity occurred from samples collected at these two sites. Conversely, within 6 days of incubation, sulfate reducing enrichments from all other sites were indicated by the production of black precipitated pyrite that precipitated out of solution. Sulfate reducing enrichment cultures obtained from the two sites showing TCE degradation to *cis*-DCE, 42 and 44, as well as the two sites showing nearly complete degradation of TCE to VC, 48 and 50. Sulfate reduction was stable for these enrichments for 6 consecutive transfers on Postgate's Medium B for SRB.

Following repeated growth of SRB under strict SO_4 reducing conditions, the enrichment cultures were examined for the ability to support cell growth by respiring TCE as the sole terminal electron acceptor. Following 100x fold dilution in modified Postgate's medium with TCE, a significant increase in medium turbidity was noted within 2 hours after inoculation. After 5 days, headspace gas analysis by GCMS failed to detect TCE degradation products. The only gas product observed was CO_2. TCE was not inhibitory to growth of the enrichment cultures or degraded.

DNA fingerprinting of seepzone bacterial communities. T-RFLP DNA fingerprinting revealed complex bacterial communities at each of the different seepzone sites. Numerous different bacterial populations were detected between sites 42 – 50. These community patterns were quite different between sites indicating that different types of bacteria inhabit each location. Web-based tools were used in attempt to identify the bacterial populations corresponding to each gel peak, but failed. The DNA cutting enzymes used in this study provided clearly profiles of different bacterial populations but insufficient data for identification.

DISCUSSION

This project is centered on natural attenuation to prevent TCE from further contaminating the environment. The purpose of this project was mainly to find out *why* TCE is degraded at some sites and not others, find out if sulfate-reducing bacteria are present at the sites where TCE-degradation is occurring, find out if they are responsible for the TCE-degradation occurring at these sites, and determine whether or not DNA-based methods can identify potential TCE-degraders at different sites.

One of the difficulties in this project was obtaining pure DNA from sediment samples that came from 3-8' underground. The extracted DNA from these samples was very dirty and was purified through a series of combined methods from Lovell and Piceno (1994) and Bond et al. (2000).

CONCLUSIONS

We know TCE is being degraded into other compounds such as *cis*-DCE and VC; however, it does not appear to be occurring at certain sites. These sites may have an insufficient biomass of the microorganisms capable of degrading TCE. We know sulfate-reducing bacteria are present at the *cis*-DCE and VC sites due to the degradation of sulfate into sulfide in the enrichment cultures. The presence of sulfide has been attributed to the black precipitate appearing in the enrichment tubes. Because the enrichment cultures showed no signs of TCE-degradation, we cannot conclude that the sulfate-reducing bacteria are directly involved in TCE-degradation at these sites; however, previous studies have indicated that SRB *do* play an important role. Future projects at these sites will require both similar and different methods to test for both SRB and other types of bacteria. Our DNA-fingerprinting methods are good for detecting differences among the sites; however, it is *not* helpful in detecting TCE-degraders, since we tested for *total* bacterial biomass.

ACKNOWLEDGEMENTS

We would like to thank Dr. William Pirkle and Dr. Garriet Smith at USC Aiken, Brian's mentors at USC Aiken who helped arrange this project through SCUREF. We would also like to thank SCUREF (South Carolina Universities Research and Education Foundation) that funded this project and William Bratt at Clemson University who helped with sample collection and is currently working on a project using some of the same samples.

REFERENCES

Bond, P. L., Druschel, G. K., Banfield, J. F. 2000. Comparison of Acid Mine Drainage Microbial Communities in Physically and Geochemically Distinct Ecosystems. Applied Environmental Microbiology 66:4962-4971.

Flemming, V. and Ingvorsen, K. 1998. Improved Most-Probable-Number Method To Detect Sulfate-Reducing Bacteria with Natural Media and a Radiotracer. Applied Environmental Microbiology 64:1700-1705.

Hendrickson, E. R., Payne, J. A., Young, R. M., Starr, M. G., Perry, M. P., Fahnestock, S., Ellis, D. E., Ebersole, R. C. 2002. Molecular Analysis of Dehalococcoides 16S Ribosomal DNA from Chloroethene-Contaminated Sites throughout North America and Europe. Applied Environmental Microbiology 68:485-495.

Holliger, C., Wohlfarth, G., and Diekert, G. 1999. Reductive dechlorination in the energy metabolism of anaerobic bacteria. FEMS Microbiology Reviews. 22:383-39.

Lovell, C. R., and Y. M. Piceno. 1994. Purification of DNA from estuarine sediments. Journal of Microbiology Methods. 20:161-174.

Smidt, H., Akkermans, A. D. L., van der Oost, J., de Vos, W. M. 2000. Halorespiring bacteria—molecular characterization and detection. Enzyme and Microbial Technology. 27:812-820.