REPORT DOCUMENTATION PAGE

Form Approved
OMB NO. 0704-0188

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	23 November 2004	Final Report: 1 April 2001 - 31 August 2004

4. TITLE AND SUBTITLE
Biodegradation of Chlorinated Ethenes in Mixed Waste Streams: Final Report.

5. FUNDING NUMBERS
G – DAAD 19-01-1-0445

6. AUTHOR(S)
Charles C. Somerville and Andrew N. Johnson

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Department of Biological Sciences
Marshall University
One John Marshall Drive, Huntington, WV 25755

8. PERFORMING ORGANIZATION REPORT NUMBER 200111-F

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U. S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER
P-42137.1-LS-DPS

11. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12 a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

12 b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

This report summarizes research conducted from 04/01/ 01 to 08/31/04 with support from the US Army Research Office DoD-EPSCoR program. The goal of the research was to study the biodegradation of chloroethenes under serial anaerobic/aerobic conditions. In the system used, water flowed through a contact chamber containing chloroethenes. Contaminated water was then pumped through a sediment column. From the sediment column the water flowed into an aerated chamber. Chloroethene concentrations were determined in samples from the contact chamber, the sediment column and the aerobic chamber. We tested means for establishing anaerobiosis in the sediment column, two sparging gas mixtures, addition of lactate as a growth substrate, control of conditions in the aerobic chamber, and the presence of a hydrocarbon fuel mixture as a co-contaminant. Results indicated that a N_2:H_2 gas mixture stimulated PCE degradation. We also found that the addition of exogenous lactic acid (2 mM), and the presence of petroleum hydrocarbons in reactor feed water resulted in 99.6% removal of PCE and 99.2% reduction in the total molar concentration of chloroethenes. In the last year of the research we established mixed microbial cultures in which vinyl chloride disappearance was concomitant with an increase in chloride concentration and biomass accumulation.

14. SUBJECT TERMS
Chlorinated Ethenes, PCE, TCE, DCE, Vinyl Chloride, Biodegradation

15. NUMBER OF PAGES
25

16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION ON THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	**UNCLASSIFIED**	**UNCLASSIFIED**	**UL**

NSN 7540-01-280-5500

Standard Form 298 (Rev.2-89)
Prescribed by ANSI Std. 239-18
298-102

REPORT DOCUMENTATION PAGE (SF298)
(Continuation Sheet)

(1) FORWARD (OPTIONAL) - NONE

(2) TABLE OF CONTENTS

(3) LIST OF APPENDIXES, ILLUSTRATIONS AND TABLES

(4) STATEMENT OF THE PROBLEM STUDIED

Chloroethenes are two-carbon alkenes with one or more chlorine substitutions. These compounds include perchloroethylene (PCE), trichloroethylene (TCE), 1,1-dichloroethylene (DCE), 1,2-*cis*-dichloroethylene (*cis*-DCE), 1,2-*trans*-dichloroethylene (*trans*-DCE), and vinyl chloride (VC). PCE and TCE have been used extensively in dry cleaning, degreasing of metals and as a solvents for fats, waxes, resins, oils, rubber, paints and varnishes (3). The common use of these solvents and their frequent improper disposal have caused widespread environmental contamination. In fact, PCE and TCE have been reported to be the top two volatile organic contaminants detected in groundwater in the United States (4). With the exception of VC, the chloroethenes are more dense than water and have very limited water solubility. Consequently, they form dense non-aqueous phase layers (DNAPLs) that persist in the subsurface and leach into groundwater over prolonged periods. Furthermore, the chloroethenes are toxic. The 12th Edition of the Merck Index (3) lists PCE as a substance that "may reasonably be anticipated to be a carcinogen", TCE as a "potential occupational carcinogen", and VC as "a known carcinogen".

Because of their pervasiveness in the environment, their toxicity, and their ability to form large and stable toxic plumes, cost-effective technologies to remove chloroethenes from groundwater are needed. Bioremediation is an attractive alternative because it takes advantage of the metabolic versatility of naturally-occurring microbes and can result in the transformation of toxic contaminants to harmless products. To date the biotic degradation of chloroethenes has been shown to occur in one of three ways: i) co-metabolic attack by non-specific oxygenases, ii) reductive dechlorination, and iii) aerobic mineralization of mono- and dichloroethenes. From the results of previous studies we hypothesized that ***the combination of reductive dechlorination and aerobic mineralization of mono- and dichloroethenes has the potential to form a pathway for the complete removal of chloroethenes from contaminated groundwater.*** We tested that hypothesis with support from the Army Research Office through the DoD-EPSCoR program (Grant No. DAAD19-00-R-0006; 4/1/01 to 8/31/04). The experimental design used and the results obtained are described below.

(5) SUMMARY OF IMPORTANT RESULTS

Year One - Construction of an Early Model Aquifer System. The concept behind the model aquifer system was to create a flow-through biodegradation system in which an anaerobic zone exists upgradient of an aerobic zone. The purpose of the anaerobic zone is to drive chemical and biological reduction of highly chlorinated ethenes (PCE, TCE) to mono- and dichloroethenes (DCEs, VC), which can then be biologically mineralized under aerobic conditions.

In initial experiments, water was gravity fed from a 15-liter glass reservoir to a 500-ml glass contact vessel containing water over a mixed PCE and TCE DNAPL. The contaminated water was then pumped from the contact vessel at approximately 10 ml/hr in an upward flow through a glass bioreactor column (Kontes Glass, Vineland, NJ) packed with sediment. Overflow water from the sediment column flowed into an aerated glass column with no column packing added (Kontes; see Fig. 1). The sediment used was rich in organic matter. Microbial respiration in the sediment column consumed the available oxygen and produced electrons for the reduction of chloroethenes with concomitant dechlorination. Air was bubbled into the aerobic chamber to encourage aerobic biodegradation of DCEs and VC. The rate of air delivery was kept very low in order to minimize air-stripping of chloroethenes. No attempt was made to remove oxygen from the inflow water or to add growth substrates to either the anaerobic or aerobic chambers.

Analytical Methods. Water samples (5 ml) were taken at three positions within the incubation system, as indicated in Fig. 1, and were extracted with 2 ml pentane. Extracts were sealed in glass crimp-top vials and stored at -70°C until analyzed. Samples were analyzed for chlorinated ethenes using a Buck Scientific (East Norwalk, CT) model 910 gas chromatograph (GC) equipped with a Valco Instruments (Houston, TX) model 140BN electron capture detector (ECD), and a Restek Corp. (Bellefonte, PA) MXT-VOL volatile organics capillary (0.53 mm x 30 m) GC column. Ultrapure nitrogen was used as both carrier and ECD makeup gas.

Year One Results. Fig. 2a shows the concentration of PCE in ppm at each of the three sampling ports during a 246 day period (3/6/01 through 11/6/01). Linear regression analyses of the data (Fig 2b) indicate a slight downward trend in PCE concentration in samples from port 1 during the course of the experiment, but upward trends in PCE concentrations in samples from ports 2 and 3 over the same period. Overall the data indicate a one to two log reduction in PCE concentration during passage through the incubation system.

Fig. 3a shows the concentration of TCE (ppm) in water samples taken at the three sampling ports during the experimental period. The regression analyses for these data (Fig. 3b) generate regression lines that are nearly overlapping, indicating that passage of contaminated water through the anaerobic/aerobic incubation system had no detectable impact on TCE concentration.

Fig.4a shows the concentration of DCEs (ppm) in water samples taken from sampling ports 1, 2 and 3. The data are similar to those for TCE in that regression analyses (Fig 4b) indicate no significant difference between the amount of DCEs entering the soil column and the concentration exiting the aerobic chamber.

The slopes of the regression lines for both TCE and DCEs are negative, indicating that less TCE and DCEs were entering the system over the course of the experiment. This may indicate some degradation of the DNAPL compounds within the contact bottle. However, the concentration of chlorinated ethenes in the system is also influenced by temperature. Since the later data points were generated from samples taken during winter months, the reduction in chlorinated ethene concentrations may be due, in part, to reduced solubility at lower ambient temperatures.

The data on chlorinated ethene concentrations in the anaerobic/aerobic incubation system indicate that PCE concentration was significantly reduced during transit through the soil column and further reduction in concentration occured in the aerobic chamber. There was no significant removal of TCE or DCEs in either the soil column or the aerobic chamber. Unfortunately, we were unable to report on the concentration of vinyl chloride in these samples. Vinyl chloride concentrations were below the limit of detection for the analytical method that was employed for these initial experiments.

These early experiments indicated that the simple model aquifer system was not sufficient for biodegradation of chlorinated ethenes, particularly TCE and DCEs. We predicted that degradation of the target compounds would be enhanced by the use of sparging gases to reduce the oxygen concentration in the feed water and the addition of exogenous carbon and energy sources to support the growth of dehalorespiring microbes. Those experiments were conducted during the second and third years of the project.

Year Two - Refinement of the Model Aquifer Design. During the first five months of the second project year, we continued to operate and sample the serial anaerobic/aerobic incubation system as described above. Most of our laboratory efforts during that period were dedicated to uncrating, building and testing the fermenter systems that were purchased to enhance our control of the aerobic stage of the process. On 19 June 2002, Mr. Andrew Johnson joined the research team. His efforts were dedicated to design and construction of the new anaerobic/aerobic system described below.

On 20 August 2002, the original incubation system was broken down and the sediment from the anaerobic column was mixed with sewage sludge from the Huntington, WV sewage treatment plant. This plant receives effluent from a local chemical plant, and the activated sludge is regarded as well adapted for industrial wastes. We also added river sediment taken near the outfall of a chemical manufacturing plant (38.368° N, 81.691° W) that was permitted to release PCE until 1989. Sand was added to the mixture to facilitate packing, and the material was used to fill two soil columns. Groundwater recovered from a PCE-contaminated site at Vienna, WV (with the cooperation of the West Virginia Department of Environmental Protection) was pumped up through the paired soil columns at a rate of approximately 3.3 ml per min. Pre-incubation of the soil columns continued through 9 September 2002.

The paired model aquifer system diagrammed in Fig. 5 was placed in operation on 9 September 2002. Feed water for the system was taken directly from the Ohio River at Huntington, WV. The water was boiled for 30 minutes to reduce dissolved oxygen, and then filtered through a 0.22 μm membrane to remove bacteria. The water was placed in two sterile glass carboys where it was continuously sparged with a mixture of 95% nitrogen and 5% hydrogen to maintain anaerobiosis. From these carboys the water was gravity fed to two 500-ml bottles containing dense non-aqueous phase layers (DNAPLs) of PCE. Water flowing into the bottles dissolves PCE and any other chlorinated ethenes in the DNAPL at the limit of their solubility. The solvent-contaminated water was then pumped upward through the anaerobic sediment columns at 3.3 ml/min. The anaerobic column in reactor 1 also received lactate delivered *via* a syringe pump at a rate equivalent to 2 mM in the soil column inflow. The anaerobic column in reactor 2 received no additional input. The outflows of both anaerobic columns were gravity fed into aerobic, continuously stirred tank reactors (CSTRs). Each reactor was controlled *via* a BioFlo 110 fermenter system (New Brunswick Scientific, Edison, NJ). Use of the fermenter allowed much greater control of

conditions and a longer residence time in the aerobic portion of the aquifer than in the earlier model aquifer design. The CSTRs were gently sparged with air to maintain high concentrations of dissolved oxygen while attempting to avoid air stripping of the chlorinated ethenes from the reactor fluids.

Analytical Methods. Water samples (5 ml) were taken at three positions within each model aquifer, as indicated in Figure 5, and extracted with 2 ml pentane. Extracts were sealed in glass crimp-top vials and stored at -70°C until analyzed. Samples were analyzed for chloroethenes using a Buck Scientific (East Norwalk, CT) model 910 gas chromatograph equipped with a Valco Instruments (Houston, TX) model 140BN electron capture detector and a Restek Corp. (Bellefonte, PA) MXT-VOL volatile organics capillary GC column (0.53 mm x 30 m). Ultra-pure nitrogen is used as both carrier and make-up gas.

Year Two Results. The data in Fig. 6 indicate that PCE was being removed, although not completely, from the reactor fluid from the beginning of the experiment. A small amount of PCE was detectable in samples at port 3 in reactor 1. This may indicate that either the anaerobic column was too small or the residence time in the anaerobic column was too short to remove all of the PCE from the reactor fluid. There was some detection of TCE in early samples from reactor 1, but TCE concentrations were very low from November of 2002 through the end of the sampling period. Small amounts of *cis*-DCE were detected throughout the experiment. *Trans*-DCE was rarely detected, and 1,1-DCE was not detected in reactor 1. The major product of PCE degradation was vinyl chloride. The detection of vinyl chloride in reactor one may indicate that the residence time in the aerobic vessel was insufficient for complete mineralization.

Fig. 7 shows the peak areas of PCE, TCE, *cis*-1,2-DCE, and vinyl chloride at each of the three sampling ports of reactor 2 from 12 September 2002 to 27 February 2003. The data in Fig 7. indicate that PCE was removed from reactor 2 fluid early in the experiment, as was seen in reactor 1. TCE, cis-1,2-DCE and vinyl chloride were produced, but in lower amounts than were detected in extracts of reactor 1 fluids. TCE peak areas seem to indicate a breakthrough from port 2 in November of 2002, but TCE was not detected in port 3 samples. This should probably not be taken to indicate that TCE was being degraded in the aerobic reactor, but it may indicate that TCE and other volatiles were being air-stripped from the CSTR. Reactor 2 showed some production of *cis*-DCE and vinyl chloride in later samples. No *trans*-DCE or 1,1-DCE were detected in reactor 2 samples. The lack of *trans*-DCE in these samples was interesting, but could not be clearly linked to the addition of lactate in reactor 1. The amount of vinyl chloride detected in reactor 2 samples was much less than that detected in reactor 1 samples. The difference may indicate that the addition of lactate to reactor 1 drives more PCE to reduced derivatives than is occurring in reactor 2.

These results indicates that chlorinated ethenes were being removed from reactor fluids in the model aquifer systems. Greater amounts of reduction products were detected in samples taken from the lactate supplemented reactor. Even though very low amounts of reduction products were detected coming from the aerobic reactor, we were not yet convinced that DCEs or vinyl chloride were being aerobically degraded.

Year Three – The Importance of Sparging Gases and Co-Contaminants. During the third project year (April 2003 through March 2004) we conducted three experiments to monitor the performance of the model aquifer system with and without sparging gases and in the presence of a light hydrocarbon co-contaminant. In the first experiment we compared the use of N_2:H_2 (95:5) sparged feed water to non-sparged feed water. In this experiment no lactate was added to either reactor. In the second experiment we compared N_2:CO_2 (70:30) sparged feed water to N_2:H_2 (95:5) sparged feed water. Again, no lactate was added to either reactor. In the third experiment we sparged the feed water of both reactors with N_2:H_2 (95:5) and added lactate at 2 mM in the feed water to both reactors. In addition, a model jet fuel mixture was added to the feed water of one reactor to determine the effect of a mixed waste condition on the fate of chloroethenes. The conditioning of the reactors, and the results of the three experiments are described below.

Reactor Conditioning (March – April 2003). During this period the lactate feed was removed from Reactor 1 (see Fig. 1), and both reactors continued to receive feed water sparged with N_2:H_2 (95:5) gas. Our intent during this conditioning period was to maintain strictly anaerobic conditions in the soil columns

and to establish similar microbial communities in both reactors by allowing time for the metabolism of lactate previously added to Reactor 1.

Effect of sparging gas vs no sparging gas *(May – July 2003).* The purpose of this experiment was to compare the biodegradation of chloroethenes in the model aquifer system with and without sparging gas. During a three month period the feed water to Reactor 1 received no sparging gas, and the feed water to Reactor 2 was sparged with a mixture of N_2:H_2 (95:5). No exogenous carbon source, except PCE, was added to either reactor. Water samples (5 ml) were taken at three positions in each model aquifer system and analyzed for chloroethenes as described above.

Results of this experiment are shown in Figures 8 and 9 and summarized in Table 1. In this experiment Reactor 1 received no sparging gas in the feed water and no exogenous carbon source. The removal of PCE in the system was 93.4% on average during the experimental period. Both DCE and *cis*-DCE were detected at ports 1, 2 and 3 indicating that reductive dechlorination was occurring both in the contact vessel and the soil column. *trans*-DCE was not detected. The degradation of dichloroethenes improved over time, and was nearly complete by the end of the experimental period. VC was also detected in all three ports. The amount of VC produced in the system was usually below 5 ppm but there were some transient spikes, suggesting that the degradation of VC was less stable than that of the other chloroethenes.

In this experiment Reactor 2 received feed water that had been sparged with an N_2:H_2 (95:5) gas mixture with no exogenous carbon source other than PCE. The removal of PCE in the system was 95.5% on average. Both DCE and *cis*-DCE were detected at ports 1, 2 and 3 indicating that reductive dechlorination was occurring both in the contact vessel and the soil column. *trans*-DCE was not detected. The degradation of dichloroethenes improved over time, and was nearly complete by the end of the experimental period. VC was also detected in all three ports. The amount of VC produced in the system was usually below 4 ppm and tended to decrease over the course of the experiment.

Comparison of the two reactors indicates a somewhat higher average percentage of PCE removal in the N_2:H_2 sparged reactor. Degradation of dichloroethenes was good in both reactors after an acclimation period of approximately 2 months. VC was not well degraded under either condition – the residual concentrations of 4 to 5 ppm were unacceptably high for an effective remediation technology.

Comparison of sparging gases *(August – October 2003).* The purpose of this experiment was to compare the effectiveness of two different sparging gases on the biodegradation of chloroethenes in the model aquifer system. During a 3 month period the feed water to Reactor 1 was sparged with a N_2:CO_2 (70:30) gas mixture, and the feed water to Reactor 2 was sparged with a N_2:H_2 (95:5) gas mixture. No exogenous carbon source, except PCE, was added to either reactor. Samples were collected, extracted, and analyzed as described above.

Results of Experiment 2 are shown in Figures 10 and 11 and summarized in Table 1. In this experiment Reactor 1 received feed water that had been sparged with an N_2:CO_2 (70:30) gas mixture with no exogenous carbon source other than PCE. The removal of PCE in the system was 92.3% on average. Both DCE and *cis*-DCE were detected at ports 1, 2 and 3 indicating that reductive dechlorination was occurring both in the contact vessel and the soil column. *trans*-DCE was not detected. The degradation of dichloroethenes improved over time, and was nearly complete by the end of the experimental period. VC was also detected in all three ports. The amount of VC produced in the system was usually below 5 ppm, but we did see transient spikes at all three sampling ports suggesting that the degradation of VC was less stable than that of the other chloroethenes.

In this experiment Reactor 2 received feed water that had been sparged with an N_2:H_2 (95:5) gas mixture with no exogenous carbon source other than PCE. The removal of PCE in the system was 95.4% on average. Both DCE and *cis*-DCE were detected at ports 1, 2 and 3 indicating that reductive dechlorination was occurring both in the contact vessel and the soil column. *trans*-DCE was not detected. The degradation of DCE improved over time, and was nearly complete by the end of the experimental period. Analysis for *cis*-DCE, however, indicated transient spikes in concentration even toward the end of the experimental period. VC was also detected in all three ports. The amount of VC produced in the system was usually below 5 ppm, but we saw a transient accumulation of VC in the aerobic chamber toward the end of the experimental period. The cause of the accumulation is unknown, but indicates that the removal of vinyl chloride in this system was relatively unstable.

Comparison of the two reactors under these experimental conditions indicates that N_2:H_2 (95:5) was a better sparging gas than N_2:CO_2 (70:30). Even though reductive dechlorination has been demonstrated to occur under methanogenic conditions, our observations support earlier reports that H_2 is a good electron donor for chloroethene metabolism. The conditions of this experiment still resulted in an unacceptable residual concentration of VC.

Effect of a model fuel co-contaminant *(October 2003 – March 2004).* Prior to this experiment both model aquifers received feed waters sparged with a N_2:H_2 (95:5) gas mixture. Also, the feed water to both reactors was supplemented with lactic acid (2 mM) as an exogenous carbon source. Furthermore, the outflow from the aerobic chamber of Reactor 1 was pumped into the aerobic chamber of Reactor 2 for a two month period. We had previously observed that sparging feed water with a N_2:H_2 (95:5) gas mixture provided more extensive degradation of PCE than using no sparging gas or using a N_2:CO_2 (70:30) gas mixture. We had also observed that the addition of lactic acid as an exogenous carbon source provided a notable increase in attached biomass in the aerobic chamber of Reactor 1. Our intent during this conditioning period was to establish optimum conditions for chloroethene degradation in both reactors and to, as nearly as possible, equalize the microbial communities in the two reactors.

The purpose of this experiment was to determine the effect of adding a complex mixture of organic chemicals on the degradation of chloroethenes in our model aquifer system. During this period the feed water to both Reactor 1 and Reactor 2 was sparged with a N_2:H_2 (95:5) gas mixture, and both reactors receive lactic acid (2 mM) as an exogenous carbon source. In addition, a model jet fuel mixture (Table 2) was added to the feed water of Reactor 1. Samples were collected, extracted, and analyzed as described above.

Results of Experiment 2 are shown in Figures 12 and 13 and summarized in Table 1. In this experiment Reactor 1 received feed water that had been sparged with an N_2:H_2 (95:5) gas mixture and lactic acid (2 mM) added as an exogenous carbon source. This reactor was also exposed to a model jet fuel mixture that was added to the feed water. The removal of PCE in the system was 99.6% on average. Of the dichloroethenes, only DCE was detected during the experiment, and the concentrations were near the detection limit except at one time point. VC was detected in all three ports. The amount of VC produced in the system increased over approximately 45 days, and then decreased to near non-detectable concentrations. Even at its peak, the concentration of vinyl chloride was always below 0.12 ppm, compared to 4 to 5 ppm detected during earlier experiments. The combination of N_2:H_2 (95:5) sparging gas, exogenous lactate, and the petroleum hydrocarbons present in the model jet fuel produced very promising conditions for the degradation of chloroethenes in groundwater.

In this experiment Reactor 2 received feed water that had been sparged with an N_2:H_2 (95:5) gas mixture, and with lactic acid (2 mM) added as an exogenous carbon source. This reactor was not exposed to the model jet fuel. The removal of PCE in the system was 98.7% on average. Of the dichloroethenes, only DCE was detected during the experiment. The concentrations of DCE were always below 60 ppb, and became even lower over the course of the experiment. VC was detected in all three ports. The amount of vinyl chloride produced in the system was relatively stable at around 70 ppb during most of the experimental period, and then decreased to near non-detectable levels toward the end of the experiment.

Comparison of the two reactors under these experimental conditions indicates that the addition of petroleum hydrocarbons not only did not inhibit the degradation of chloroethenes, but actually seemed to stimulate it. The conditions in Reactor 1 produced the best average removal of PCE that we have seen during any of our experiments. The removal of PCE was also very high in Reactor 2 indicating that sparging feed water with N_2:H_2 (95:5) and adding lactate as an exogenous carbon source can result in efficient degradation of chloroethenes even in the absence of a mixed waste stream in groundwater. Both reactors performed significantly better than previous experiments in the degradation of vinyl chloride, the most toxic of the chloroethenes. In fact, ***under these conditions Reactor 1 removed 99.2% and Reactor 2 removed 98.3% of the total molar amount of chloroethenes entering the system***. The maximum removal of total chloroethenes in all prior experiments was 91.7%

Year Three – Characterization of microbiota. During the final year of the project, we also began a more thorough study of the aerobic mineralization of vinyl chloride in the model aquifer system.

Minimal Salts Broth (MSB; 2) was prepared to a volume of 10 liters and stored at 4° C until use. Agarose (15 g/l) was added to make Minimial Salts Agar (MSA). Inoculant was taken from the aerobic CSTRs described above. Aliquots (100 μl) from the aerobic reactors were plated onto MSA plates - eight plates from each of the two aerobic reactors. The 16 plates were placed in a sealed 9 liter vessel. Vinyl chloride was injected into the vessel to make a 1% vol/vol atmosphere. Six inoculated MSA plates (3 from each aerobic reactor) were placed in a 3 liter sealed vessel without added vinyl chloride as a control. The sealed vessels were incubated at room temperature starting on January 7th 2003. Colonies growing on the MSM plates within the vinyl chloride atmosphere vessel were transferred to MSB microcosms. Microcosms were established in 70 ml serum vials sealed with butyl rubber stoppers by aluminum crimp tops. Sterile MSB (5 ml) was poured onto each MSM plate. A sterile swab was used to suspend the colonies. The swab was dipped into a 70 ml serum vial that contained 30 ml of MSB. The MSB on the MSA plates was aseptically transferred to the 70 ml microcosm making the final aqueous volume of the microcosm approximately 35 ml. Cultures were numbered and fed vinyl chloride. Microcosms were placed on a rotary shaker at room temperature on April 7, 2003. Subsequent experiments added controls with VC plus killed inoculum or no inoculum to indicate loss of VC from the sealed system.

The first generation microcosms received 0.35 ml of vinyl chloride as the sole carbon source at atmospheric pressure weekly until August 26, 2003. After August 26, 2003 microcosms were given large amounts of pure vinyl chloride (gas) until visual biomass had accumulated. Optical densities were monitored weekly by extracting 1 ml of shaken/vortexed aqueous phase of microcosm and recording the absorbance at 600 nm on an analytical spectrophotometer blanked with MSB.

Second generation microcosms were inoculated from the first generation microcosms described above. Aliquots (3 ml) were inoculated on August 27, 2003. All microcosm vials were autoclaved with appropriate amounts of MSB added. A no-inoculum control vial was setup to monitor loss of vinyl chloride other than by degradation. An autoclaved-inoculum control vial was also setup to account for inoculated biomass. The autoclaved inoculum vial was fed 10 ml of vinyl chloride sampled at atmospheric pressure.

Third generation cultures were attempts at growing pure isolates with the capability of degrading vinyl chloride. Vial ANJ2-8-3 which had been observed to accumulate biomass on the surface of the MSB, increase chloride concentration, and had yeilded a PCR amplification of the EaCoMT gene fragment (1) was used to isolate pure strains. An aliquot (100 μl) from the vial was plated onto a Tryticase Soy Agar (TSA) plate. Four distinct colony morphologies were noted, and a sample of each morphotype was streaked for isolation. Isolates of each morphotype were allowed to grow on TSA for 5 days. A sterile loop was used to collect colonies. The colonies were suspended in 10 ml of MSB. The suspension was vortexed and shaken. All 10 ml of the MSB suspension was used as initial inoculum of vials FHD2-007-3 through FHD2-007-6. TSA cultures were fed 10 ml of VC at atmospheric pressure (60 ppm VC). An additional set of pure cultures were established in Trypticase Soy Broth (TSB) and inoculated into microcosms. Due to the higher inoculum from the TSB isolates a higher initial VC feeding was used (10 ml at bottle pressure ~200 ppm). Initial chloride and VC headspace concentrations were recorded for all vials.

Culture ANJ2-46-11 was selected for vinyl chloride degradation kinetics study due to the high amount of biomass floating on the aqueous to gas headspace interface. An initial estimate vinyl chloride degradation curve showed ANJ2-46-11 had the ability to degrade vinyl chloride, but the resolution of the measurements were poor. The resolution problem was fixed by flushing the gas tight syringe between injections with solvent and ramping the GC oven to 150° to push off any vinyl chloride residuals left in the system.

A clean serum vial was inoculated with 10 ml of VC sampled at atmospheric pressure at time 0 and used as a control. Culture ANJ2-46-11 was assayed at time 0 to have 19 ppm of residual VC left in the headspace. ANJ2-46-11 was then fed 10 ml at bottle pressure and assayed to contain 193 ppm of VC in the headspace.

Characterization of microbiota - Results. Chloride analyses indicated large accumulations of chloride in VC first generation cultures after large feedings of vinyl chloride (Fig. 14). Increases in chloride concentration were observed as high as 3 fold increases (ANJ2-8-1) over a period of 42 days. Six of eight generation two experimental cultures showed significant increases in chloride accumulation over the 47 day period. Culture ANJ2-46-5 exhibited an accumulation rate of 4.4 ppm of chloride per day (Fig.

15). Culture ANJ2-46-11 showed a decrease of 87% of VC in head space gas after only 4.75 hours of incubation. In this microcosm VC was degraded at a rate of 42 ppm per hour. The concentration of VC in ANJ2-46-11 after 24 hours was observed to be 26 ppm of residual VC. The VC concentration in the control vial did not significantly change. The average concentration of the control vial was 59 ppm.

No generation three isolates (from ANJ2-8-3) accumulated any significant amount of chloride compared to the control cultures. Using chloride accumulation as a sign of metabolism of vinyl chloride, no pure cultures were isolated with the ability to degrade vinyl chloride.

A PCR amplicon of approximately 900 bases was amplified on December 2, 2003 using EaCoMT specific primers (1). A total of 47 reactions have produced the 900 base pair fragment from culture ANJ2-8-3. No PCR amplification using EaCoMT primers was observed using template from ANJ2-46-11 - a culture that has been observed to both accumulate chloride and degrade VC. Inoculum from ANJ2-8-11 was grown in TSB and inoculated back into MSB with VC added to the headspace. The culture ANJ2-75-1 was able to accumulate chloride but no amplicons from EaCoMT specific primers were observed.

Our inability to establish VC disappearance or chloride accumulation in pure cultures may indicate that VC degradation is being accomplished by mixed microbial consortia. Also, the lack of EaCoMT amplification from cultures that both consume VC and accumulate chloride may indicate that VC degradation in theses cultures is being accomplished by a novel degradative pathway. Further research will be required to determine if these preliminary findings hold true.

LITERATURE CITED

1. Coleman, N.V., T.E. Mattes, J.M. Gossett, and J.C. Spain. 2002. Appl. Environ. Microbiol. **68**:6162-6171.

2. Hartmans, S., and J.A.M. DE Bont. 1992. Appl. and Environ. Microbiol. **58**:1220-1226.

3. Budavari, S., M.J. O'Neil, A. Smith, P.E. Heckelman, and J.F. Kinneary (eds.). 1996. *The Merck Index* (12th edition). Merck & Co., Inc. Whitehouse Station, NJ.

4. Vogel, T.M., and P.L. McCarty. 1985. Appl. Environ. Microbiol. **49**:1080-1083.

Table 1. Summary of percent PCE removal from model aquifers under specified conditions.

Model Aquifer No. 1	% Reduction [PCE] No. 1	% Reduction [PCE] No. 2	Model Aquifer No. 2
N_2:H_2 (95:5) no added substrate no co-contaminant	**95.5**	**93.4**	no gas no added substrate no co-contaminant
N_2:H_2 (95:5) no added substrate no co-contaminant	**95.4**	**92.3**	N_2:CO_2 (70:30) no added substrate no co-contaminant
N_2:H_2 (95:5) lactate added (2 mM) model fuel added	**99.6**	**98.7**	N_2:H_2 (95:5) lactate added (2 mM) no co-contaminant

Table 2. Constituents of the model jet fuel.

Chemical component	Formula Weight (D)	Weight Added (g)	Amount Added (mmol)	Concentration (mM)
n-hexane	86.18	0.5	5.80	773.33
n-heptane	100.20	0.5	4.99	665.33
n-octane	114.23	0.5	4.38	584.00
tetradecane	198.39	0.5	2.52	336.00
cyclohexane	84.16	0.5	5.94	792.00
methylcyclohexane	98.19	0.5	5.09	678.67
ethylcyclohexane	112.21	0.5	4.46	594.67
toluene	92.14	0.5	5.43	724.00
p-xylene	106.17	0.5	4.71	628.00
mesitylene	120.19	0.5	4.16	554.67
cumene	120.19	0.5	4.16	554.67
indan	118.18	0.5	4.23	564.00
naphthalene	128.17	0.5	3.90	520.00
2-methylnapthalene	142.20	0.5	3.52	469.33
2,3-dimethylnapthalene	156.40	0.5	3.20	426.67

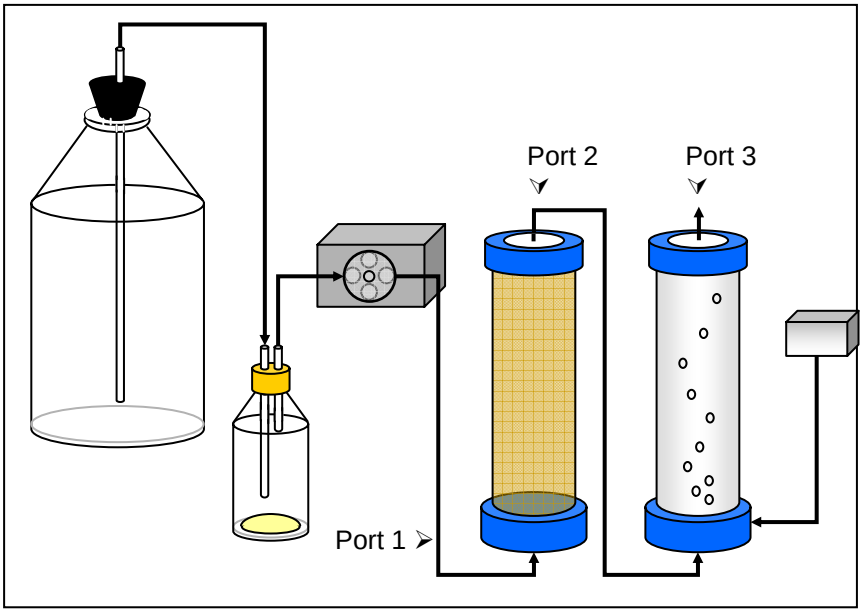


Figure 1. Diagram of the original model aquifer. Samples for determination of chlorinated ethenes were taken from sampling ports designated 1, 2 and 3. Samples from Port 1 indicate the type and concentration of chlorinated ethenes entering the anaerobic soil column. Samples from Port 2 indicate any removal of chlorinated ethenes due to passage through the soil column. Samples from Port 3 indicate removal during aerobic incubation.

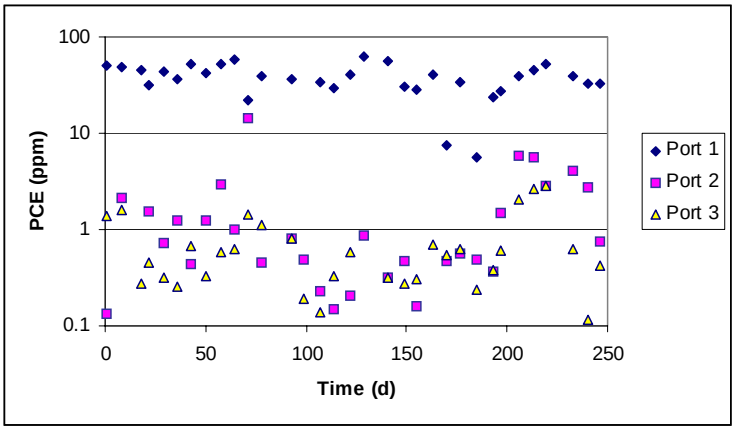


Figure 2a. Concentrations of PCE in ppm at sampling ports 1, 2, and 3. The position of the sampling ports is indicated in Fig. 1.

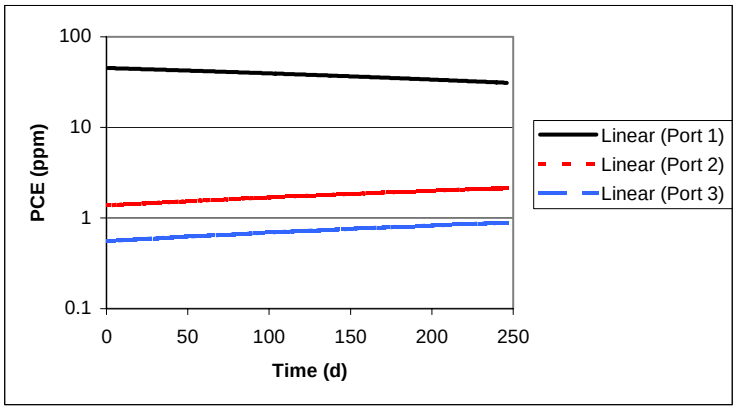


Figure 2b. Linear trendlines generated from the data shown in Fig 2a. Individual data points have been removed for clarity.

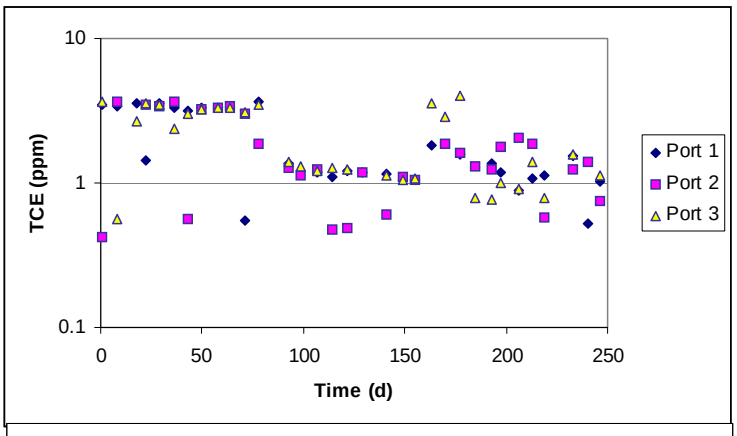


Figure 3a. Concentrations of TCE in ppm at sampling ports 1, 2 and 3. The position of the sampling ports is indicated in Fig. 1.

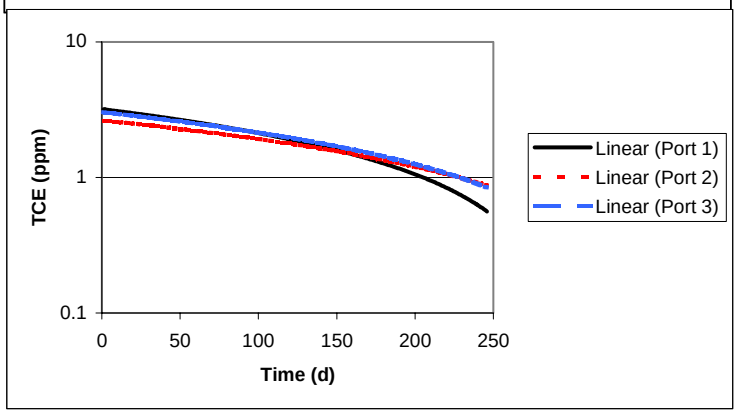


Figure 3b. Linear trendlines generated from the data shown in Fig 3a. Individual data points have been removed for clarity.

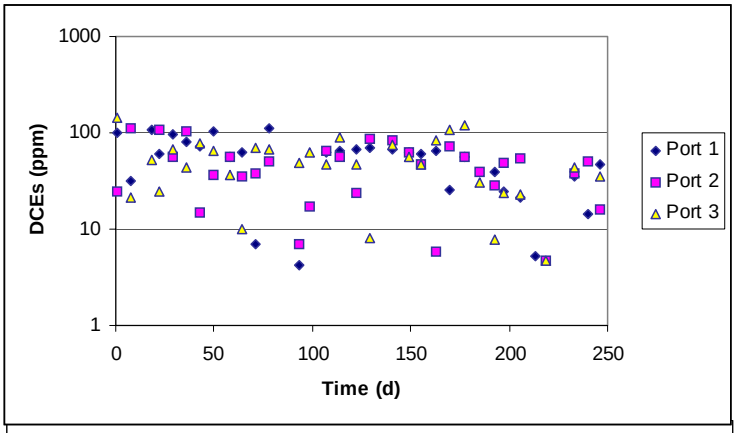


Figure 4a. Concentrations of DCEs in ppm at sampling ports 1, 2, and 3. The position of sampling ports is indicated in Fig. 1.

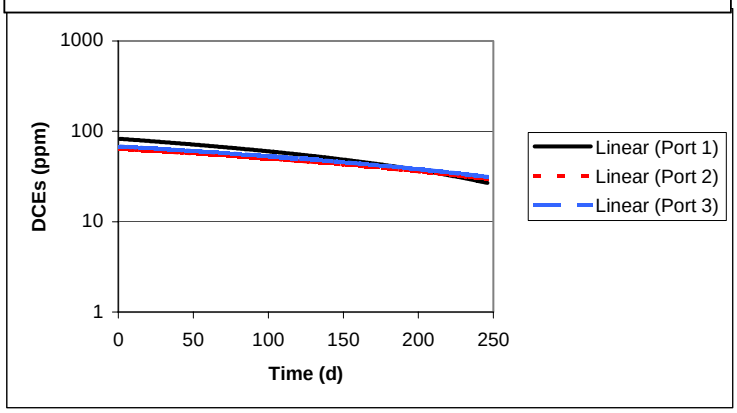


Figure 4b. Linear trendlines generated from the data shown in Fig. 4a. Individual data points have been removed for clarity.

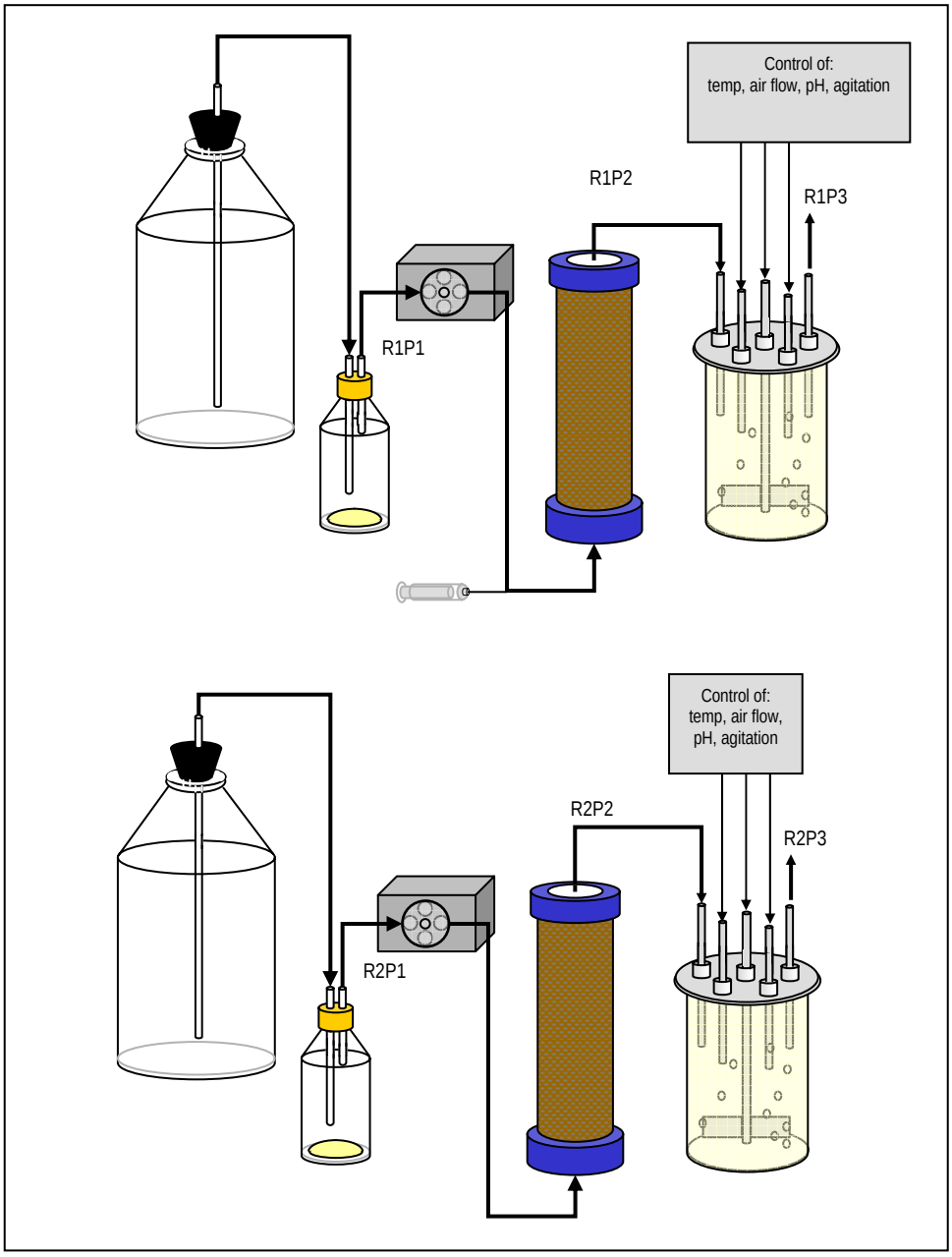


Figure 5. Diagram of the paired serial anaerobic/aerobic model aquifer system used to study the biodegradation of chlorinated ethenes. Details of the system are described in the text. The addition of lactate *via* a syringe pump is shown on Reactor 1. The positions of the sampling ports are indicated as: reactor 1, port 1 (R1P1); reactor 1, port 2 (P1P2); reactor 1, port 3 (R1P3); reactor 2, port 1 (R2P1); reactor 2 , port 2 (R2P2); and reactor 2, port 3 (R2P3).

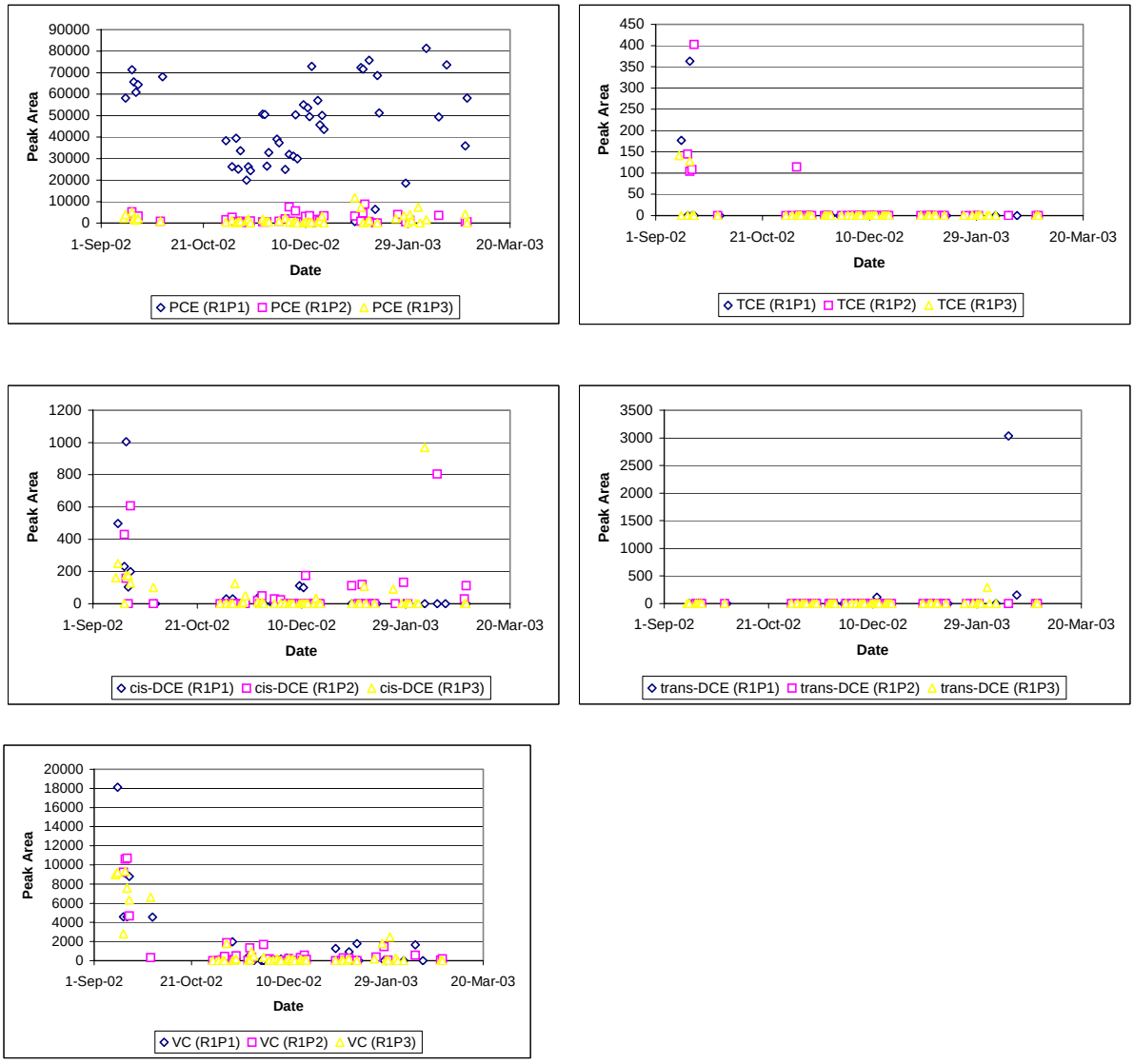


Figure 6. Peak areas of PCE, TCE, cis-1,2-DCE, trans-1,2-DCE, and vinyl chloride at each of the three sampling ports of reactor 1 from 12 September 2002 to 27 February 2003.

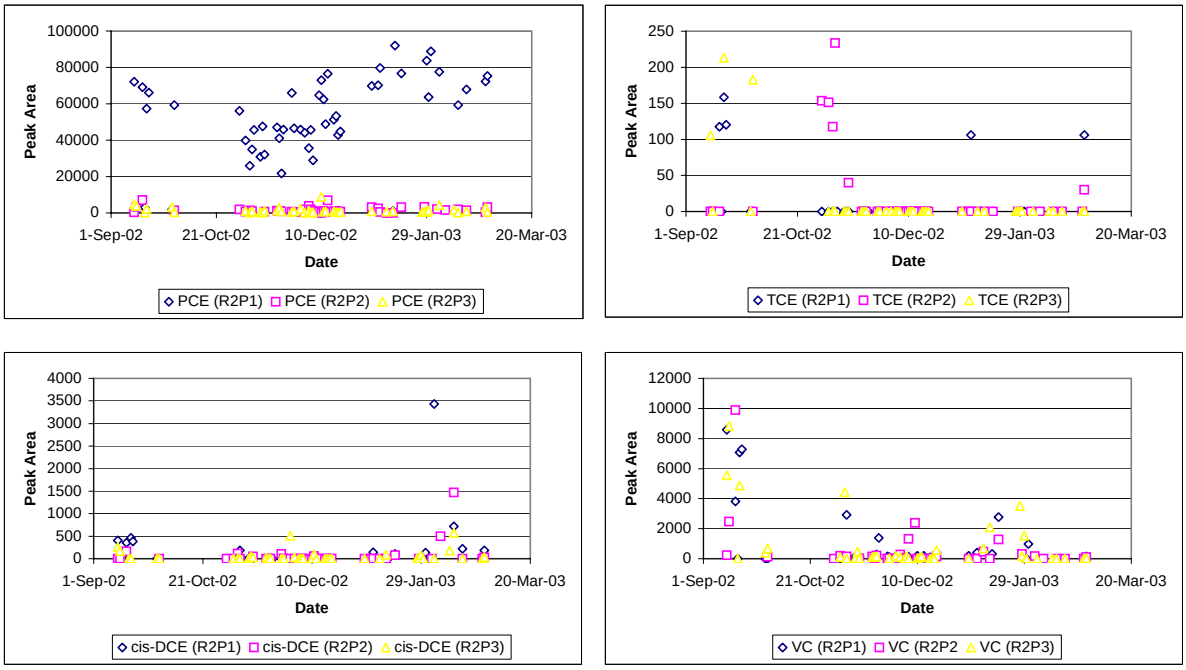


Figure 7. Peak areas of PCE, TCE, cis-1,2-DCE, and vinyl chloride at each of the three sampling ports of reactor 2 from 12 September 2002 to 27 February 2003.

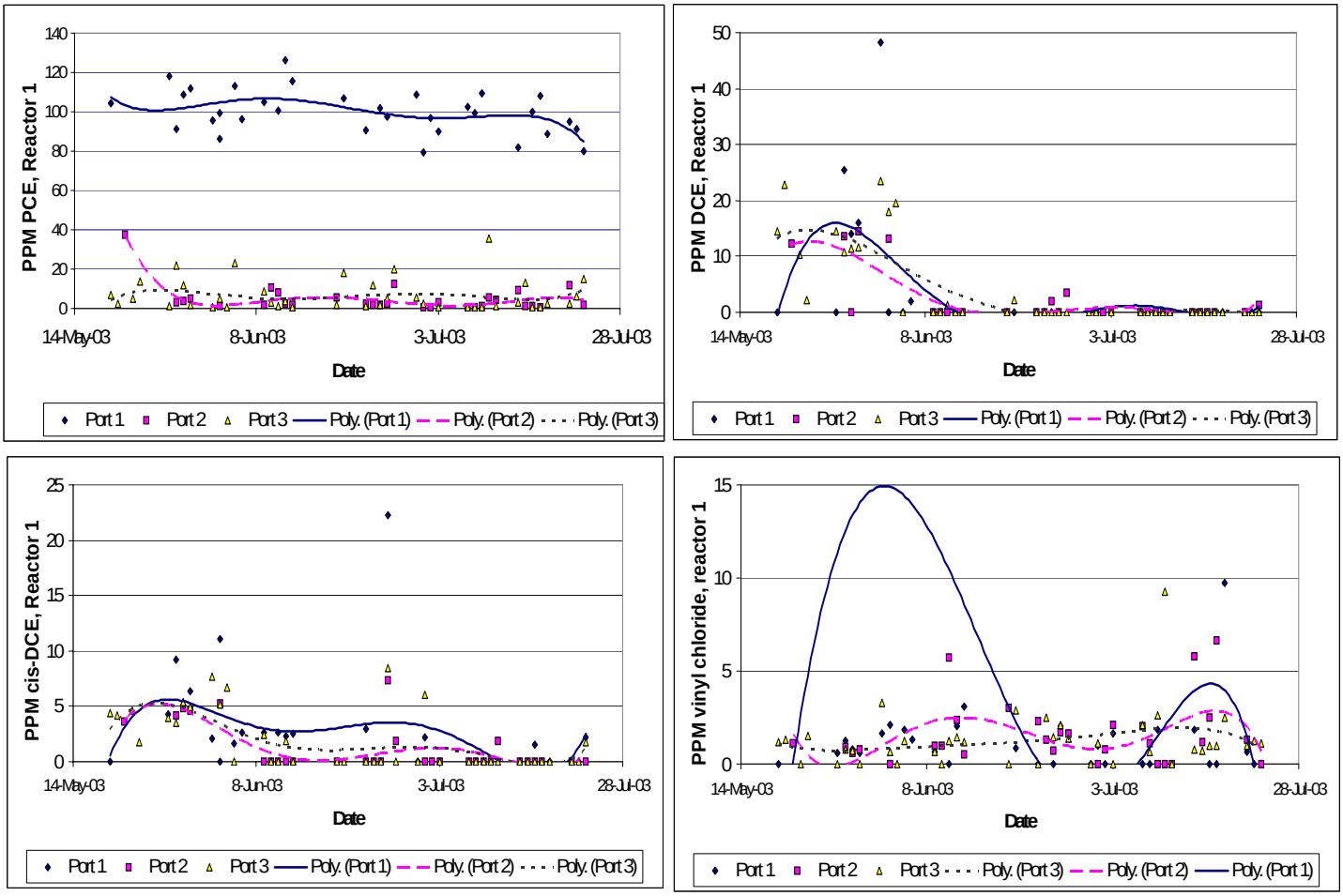


Figure 8. Effect of sparging gas *versus* no sparging gas – Reactor 1. This model aquifer received no sparging gas in the feed water and no exogenous carbon source other than PCE. Diamonds indicate samples taken at port 1. Squares indicate samples taken at port 2. Triangles indicate samples taken at port 3. The lines are 5th order polynomials intended only to emphasize trends in the data. The solid line indicates the trend at port 1, the long-dashed line indicates the trend at port two, and the short-dashed line indicates the trend at port 3 for PCE (top left), 1,1-DCE (top right), cis-1,2-DCE (bottom left), and VC (bottom right).

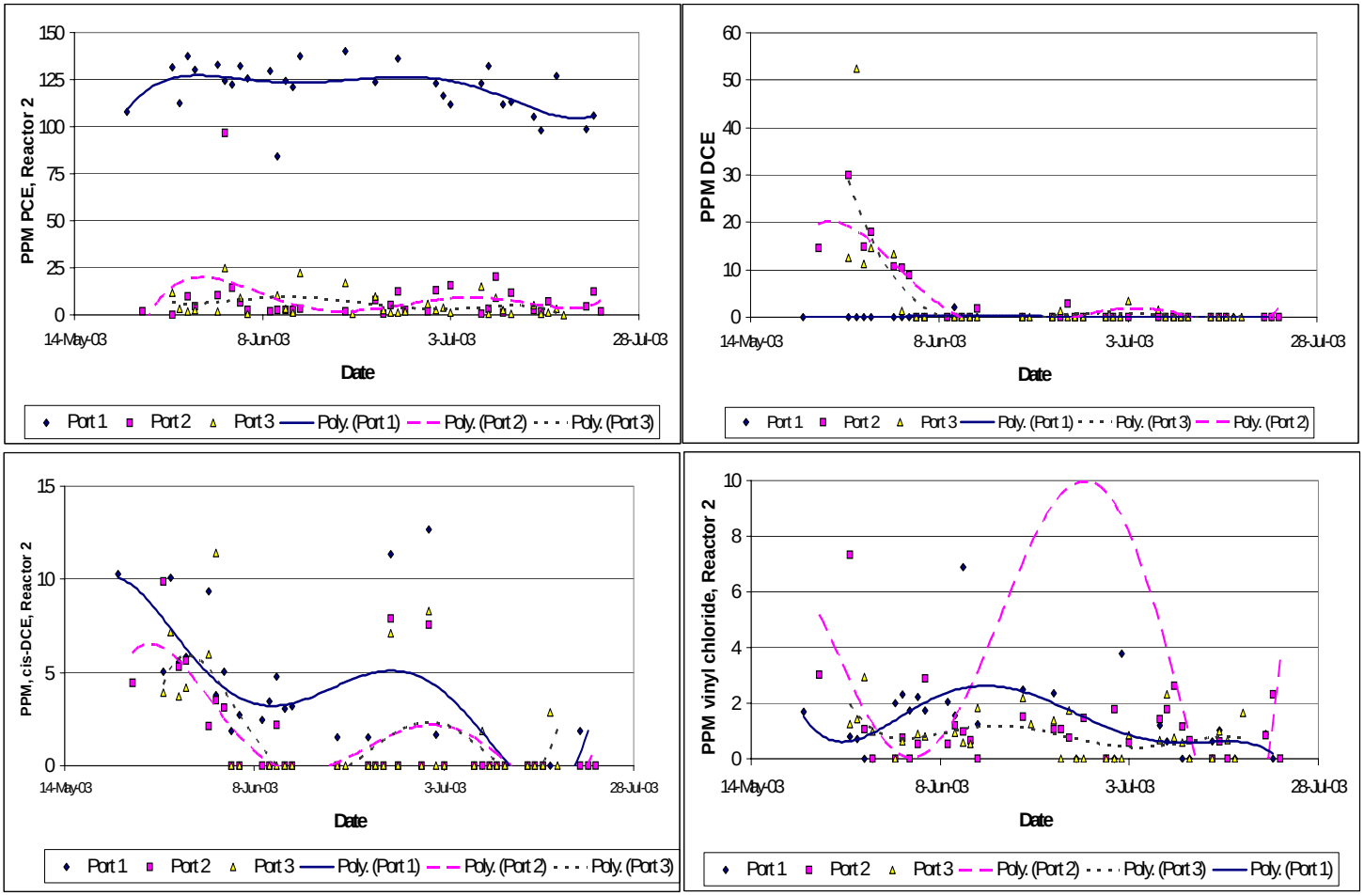


Figure 9. Effect of sparging gas *versus* no sparging gas – Reactor 2. This model aquifer received feed water that had been sparged with an N_2:H_2 (95:5) gas mixture and no exogenous carbon source other than PCE. Diamonds indicate samples taken at port 1. Squares indicate samples taken at port 2. Triangles indicate samples taken at port 3. The lines are 5th order polynomials intended only to emphasize trends in the data. The solid line indicates the trend at port 1, the long-dashed line indicates the trend at port two, and the short-dashed line indicates the trend at port 3 for PCE (top left), 1,1-DCE (top right), cis-1,2-DCE (bottom left), and VC (bottom right).

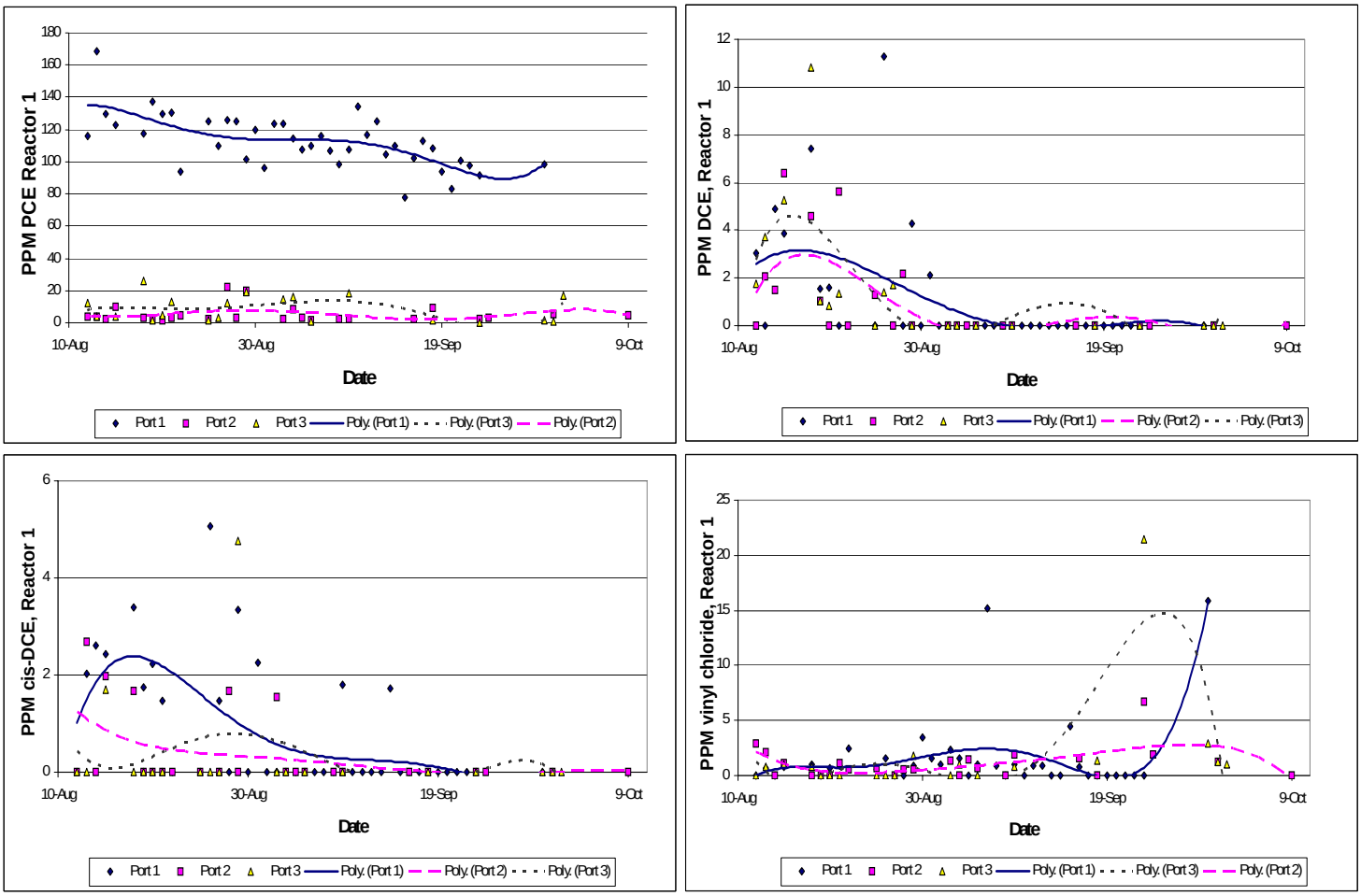


Figure 10. Comparison of sparging gases – Reactor 1. This model aquifer received feed water that had been sparged with an N_2:CO_2 (70:30) gas mixture and no exogenous carbon source other than PCE. Diamonds indicate samples taken at port 1. Squares indicate samples taken at port 2. Triangles indicate samples taken at port 3. The lines are 5th order polynomials intended only to emphasize trends in the data. The solid line indicates the trend at port 1, the long-dashed line indicates the trend at port two, and the short-dashed line indicates the trend at port 3 for PCE (top left), 1,1-DCE (top right), cis-1,2-DCE (bottom left), and VC (bottom right).

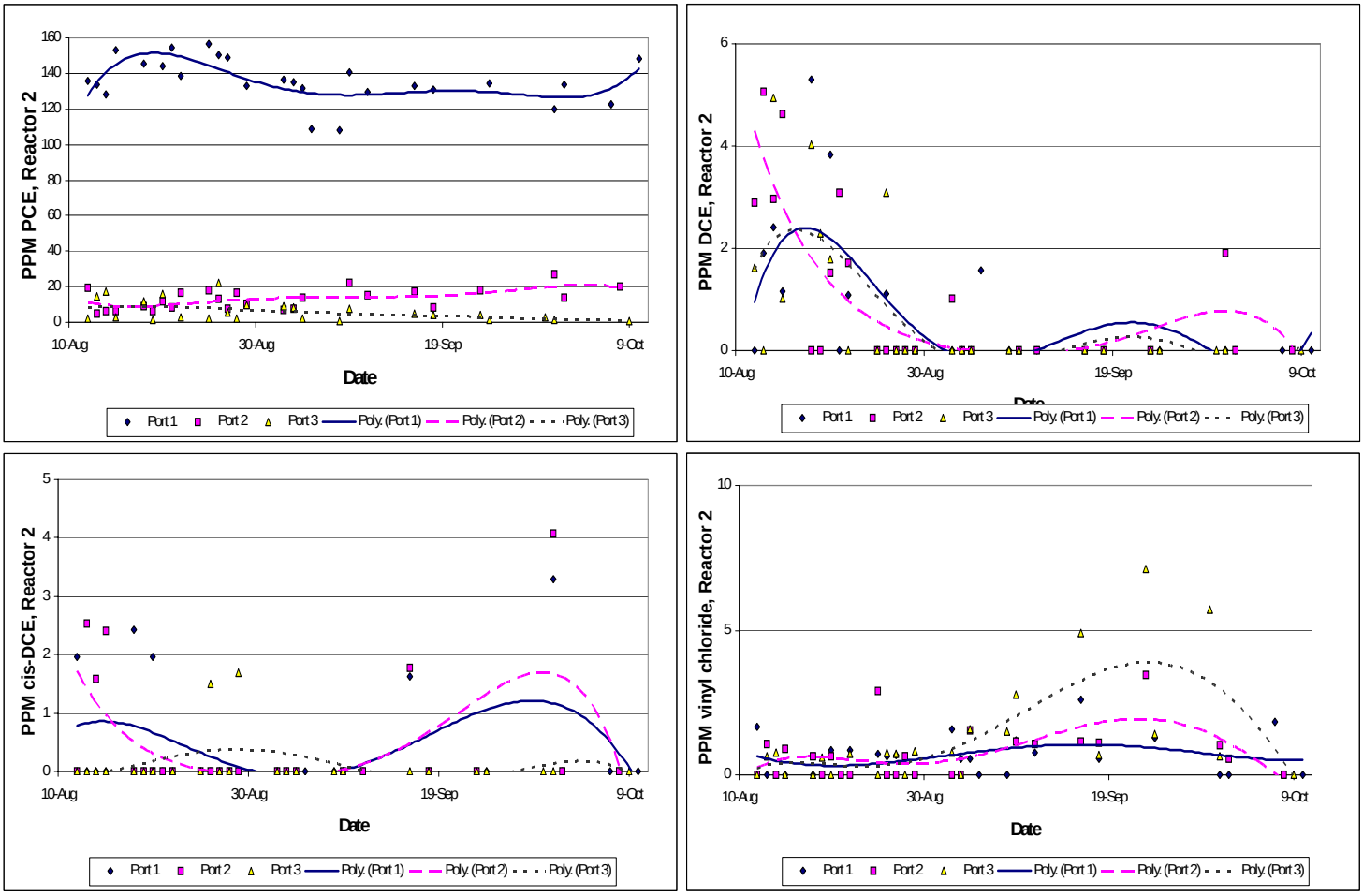
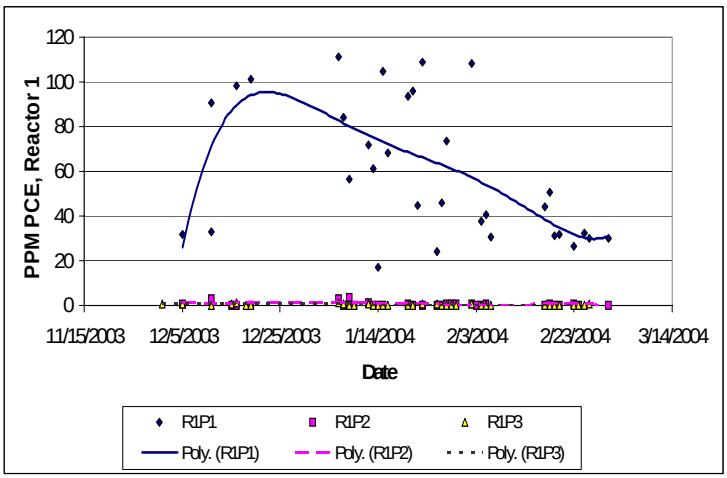
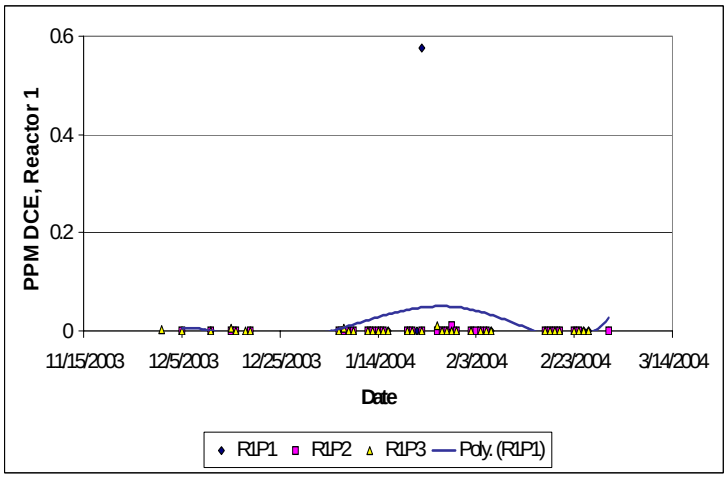


Figure 11. Comparison of sparging gases – Reactor 2. This model aquifer received feed water that had been sparged with an N_2:H_2 (95:5) gas mixture and no exogenous carbon source other than PCE. Diamonds indicate samples taken at port 1. Squares indicate samples taken at port 2. Triangles indicate samples taken at port 3. The lines are 5th order polynomials intended only to emphasize trends in the data. The solid line indicates the trend at port 1, the long-dashed line indicates the trend at port two, and the short-dashed line indicates the trend at port 3 for PCE (top left), 1,1-DCE (top right), *cis*-1,2-DCE (bottom left), and VC (bottom right).





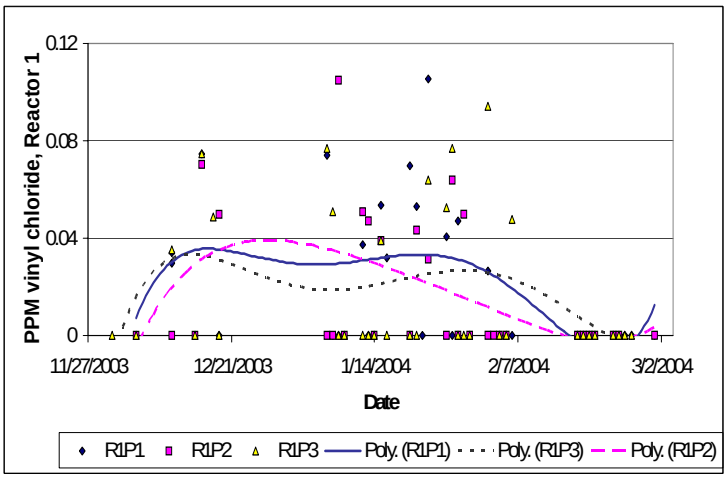
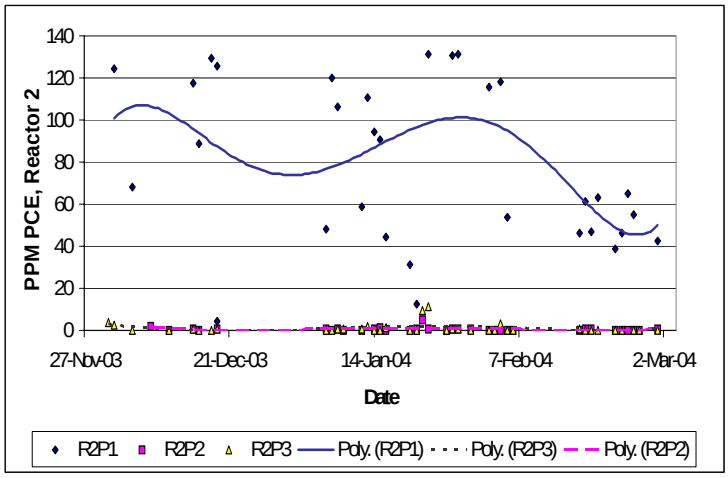
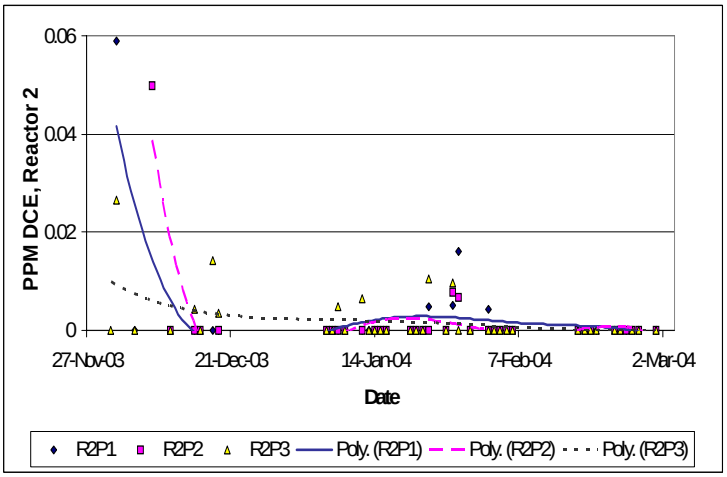


Figure 12. Effect of a model fuel co-contaminant – Reactor 1. This model aquifer received feed water that had been sparged with an N_2:H_2 (95:5) gas mixture and lactic acid (2 mM) added as an exogenous carbon source. This system was also exposed to a model jet fuel mixture that was added to the feed water. Diamonds indicate samples taken at port 1. Squares indicate samples taken at port 2. Triangles indicate samples taken at port 3. The lines are 5th order polynomials intended only to emphasize trends in the data. The solid line indicates the trend at port 1, the long-dashed line indicates the trend at port two, and the short-dashed line indicates the trend at port 3 for PCE (top left), 1,1-DCE (top right), and VC (bottom left). No *cis*-1,2-DCE was detected during this experiment.





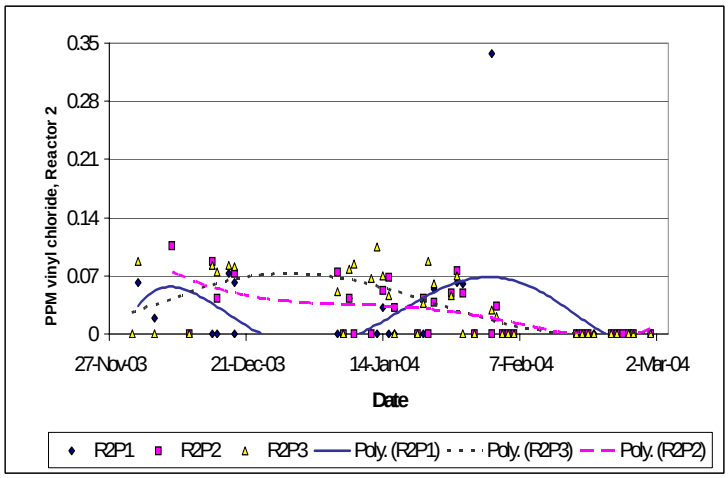


Figure 13. Effect of a model fuel co-contaminant – Reactor 2. This model aquifer received feed water that had been sparged with an N_2:H_2 (95:5) gas mixture and lactic acid (2 mM) added as an exogenous carbon source. This system was not exposed to the model jet fuel. Diamonds indicate samples taken at port 1. Squares indicate samples taken at port 2. Triangles indicate samples taken at port 3. The lines are 5th order polynomials intended only to emphasize trends in the data. The solid line indicates the trend at port 1, the long-dashed line indicates the trend at port two, and the short-dashed line indicates the trend at port 3 for PCE (top left), 1,1-DCE (top right), and VC (bottom left). No *cis*-1,2-DCE was detected during this experiment.

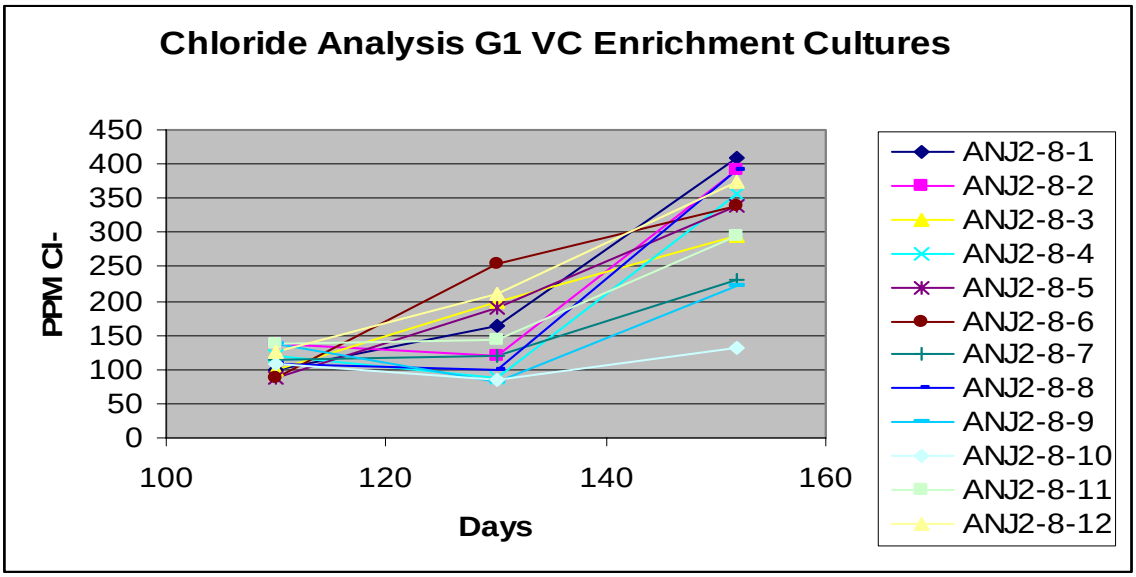


Figure 14. Chloride analysis of Generation 1 VC enrichment cultures.

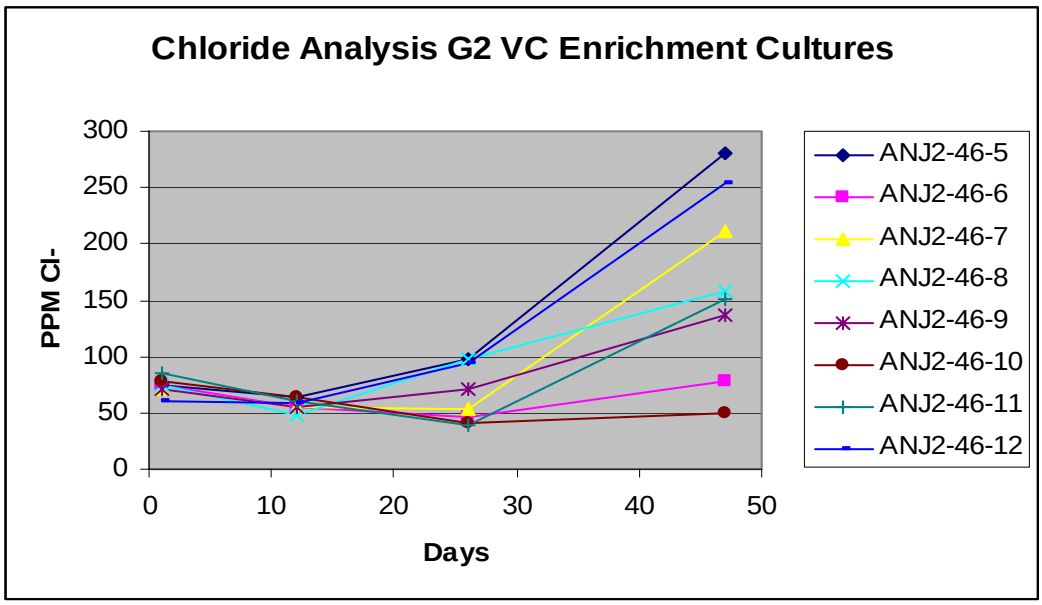


Figure 15. Chloride analysis of Generation 2 VC enrichment culture experimental vials. ANJ2-46-5 through ANJ2-46-12 are subcultures of VC generation 1

(6) List of papers submitted or published under ARO sponsorship during this reporting period.

(a) Manuscripts submitted, but not published - **none**

(b) Papers published in peer-reviewed journals - **none**

(c) Papers published in non-peer-reviewed journals or in conference proceedings - **none**

(d) Papers presented at meetings, but not published in conference proceedings -

Somerville, C.C. 2001. Biodegradation of Chlorinated Ethenes. First Annual Microbiology Mini-Symposium. United States Military Academy. 27 April, West Point, NY.

Somerville, C.C. 2001. Biodegradation of Chlorinated Ethenes in Mixed Waste Streams. Army Research Office Biosciences Workshop. 30 April - 2 May, Cashiers, NC.

Cayton, Jeremy W. and Charles C. Somerville. 2002. Biodegradation of chlorinated ethenes in a serial reactor system. ASB Bulletin 49:204-205.

Somerville, C.C. and A.N. Johnson. 2003. Biodegradation of Chlorinated Ethenes in Mixed Waste Streams. Army Research Office Biosciences Workshop. 27 - 30 April, Cashiers, NC.

Somerville, C.C. 2003. Opportunities in Environmental Microbiology. Presentation to the IDEA Investors Group. 29 August, Huntington, WV.

Somerville, C.C. and A.N. Johnson. 2004. Bioremediation of Groundwater Contaminants in Mixed Waste Streams. Presentation to Kemron Group. 19 March, Charleston, WV.

(7) "Scientific Personnel" supported by this project and honors/awards/degrees received.

(a) Charles C. Somerville, Ph.D.
Nominated for Gamma Beta Phi Outstanding Professor Award, 2001.
Nominated for Marshall Reynolds Outstanding Professor Award, 2001.
Phi Eta Sigma Fabulous Faculty Award, 2001.
Nominated for Gamma Beta Phi Outstanding Professor Award, 2002.
Researcher of the Year, Marshall University Chapter of Sigma Xi, 2001-2002.
Marshall and Shirley Reynolds Outstanding Teacher Award, 2004

(b) Mr. Jeremy W. Cayton, MA anticipated Spring 2005.
(c) Ms. Courtney Saunders, BS Fall 2002
(d) Mr. Andrew N. Johnson, M.S.
(e) Mr. Melvin Tyree, M.S. anticipated Spring 2005
(f) Ms. Lisa M. Smith, B.S. Fall 2003; M.S. anticipated Spring 2005
(g) Mr. Heath Damron, M.S. anticipated Spring 2005

(8) "Reports of Inventions" – none completed (paperwork submitted to Marshall University Research Corporation, 2 November 2004.

(9) Bibliography – none

(10) Appendixes - none