CONF-950483--11

Subsurface Microbial Communities and Degradative Capacities During Trichloroethylene Bioremediation†

Susan M. Pfiffner, David B. Ringelberg, David B. Hedrick,
Tommy J. Phelps, and Anthony V. Palumbo

Environmental Sciences Division[1]
Oak Ridge National Laboratory††
P. O. Box 2008
Oak Ridge, TN 37831-6038

Center for Environmental Biotechnology[2]
University of Tennessee
Knoxville, TN 37932

For: Proceedings of the Third International Symposium on
In Situ and On-Site Bioreclamation
San Diego, California

†Publication # of the Environmental Sciences Division, Oak Ridge National Laboratory.

††Oak Ridge National Laboratory is managed by Martin Marietta Energy Systems, Inc., for the U. S. Department of Energy under contract DE-AC05-84OR21400.

Subsurface Microbial Communities and Degradative Capacities During Trichloroethylene Bioremediation

Susan M. Pfiffner*, David B. Ringelberg, David B. Hedrick, Tommy J. Phelps, and Anthony V. Palumbo

ABSTRACT

Subsurface amendments of air, methane, and nutrients were investigated for the in situ stimulation of trichloroethylene-degrading microorganisms at the U. S. DOE, Savannah River Integrated Demonstration. Amendments were injected into a lower horizontal well coupled with vacuum extraction from the vadose zone horizontal well. The amendments were sequenced to give increasingly more aggressive treatments. Microbial populations and degradative capacities were monitored in groundwaters sampled bimonthly from 12 vertical wells. Data are presented from two representative wells.

The distance from the injection well clearly had an effect on the response of microbial populations in the monitoring wells. Major changes in microbial populations and activities occurred in response to 1% methane and the nutrient injections. Methanotrophs increased in and adjacent to the treatment zone during 1% methane injection, decreased during 4% methane injection, and increased with nutrient supplements. The addition of triethyl phosphate (TEP) and nitrous oxide (NO) to pulsed methane delivery resulted in dramatic stimulation of trichloroethylene (TCE) and tetrachloroethylene (PCE) degrading potentials observed from groundwater enrichments. In general, wells located between the horizontal wells (e.g., in MHT-6c) were affected more rapidly and to a greater extent than those outside the horizontal wells (e.g., MHT-10c).

INTRODUCTION

Diverse microbial communities with varied metabolic capabilities are present in subsurface environments (Balkwill 1989, Chapelle & Lovley 1990), including those capable of degrading chlorinated hydrocarbons such as TCE (Phelps et al. 1989a). Microbial biomass, community structure, and biodegradative activities are limited by properties of the subsurface environment such as moisture, pH, and the availability of carbon, nutrients, and electron donors/acceptors. Laboratory studies have shown that the addition of methane to bioreactors and cultures does enhance the degradation of TCE by methanotrophs (Wilson & Wilson 1985, Little et al. 1988, Niedzielski et al. 1990, Lackey et al. 1993). To determine if co-metabolic degradation of TCE could be enhanced by stimulation of the natural microbial community, DOE instituted an in situ remediation demonstration (Lombard et al. 1994) at the Westinghouse Savannah River Site (WSRS). The goal of this demonstration was to determine if in situ bioremediation can be enhanced by appropriate modifications of the environment.

Effective in situ bioremediation strategies require an understanding of the effects of pollutants and remediation techniques on subsurface microbial communities. Therefore, detailed characterization of the microbial community at a remediation site is important. The objective of this research was to apply microbiological and biochemical measurement techniques to monitor changes in the subsurface groundwater microbial communities and degradative capacities during in situ bioremediation. This paper examines some of the resulting changes in microbial populations near and away from the zone of maximum influence by comparing data from two representative wells. This comparison provides a preliminary evaluation of the effects of the injection regimes.

MATERIALS AND METHODS

The in situ bioremediation demonstration at the WSRS targeted an area of subsurface and groundwater contaminated with TCE and PCE from an abandoned process sewer line. A detailed description of the Integrated Demonstration is available elsewhere (Lombard et al. 1994). Briefly, a series of operational campaigns (Table 1) (Palumbo et al. 1995) was designed and implemented. Horizontal wells were used to deliver nutrients to the subsurface, and twelve vertical wells were used for monitoring. Data from two representative wells, MHT-6c (Fig. 1) located between the injection and extraction wells and well MHT-10c (Fig. 1) outside the injection and extraction wells, are the focus of this paper. Well MHT-6c was in the zone of maximum effect of added nutrients, while at MHT-10c methane concentrations were generally lower (Fig. 2). Groundwater was sampled twice monthly for microbiological studies according to documented WSRS well sampling protocols (Lombard et al. 1994). Measurements were initiated on site or samples were stored on dry ice until processed.

Biomass was assayed using ester-linked phospholipid fatty acids (PLFA) representative of the living biomass (White et al. 1979) and by most probable number (MPN) enumeration of the methanotrophs. PLFA was recovered from 1 to 1.5 L groundwater samples filtered through 0.2 μm pore size inorganic filters (Alltech). PLFA was quantitatively extracted from the frozen (-50°C) filters by a single phase chloroform-methanol-buffer method (White et al. 1979). The extract was fractionated into specific lipid classes by silicic acid column chromatography and trans-esterified into esters in a mild-alkaline methanolic solution (Guckert et al. 1985). MPN enumerations of the methanotrophs were based on

turbidity in a phosphate-buffered (pH 7.1) mineral salt medium (Palumbo et al. 1995). After sample inoculation, methane was added at 5% of headspace.

Biodegradative capacities were assessed by mineralization experiments that used 10 mL groundwater and 0.5 μCi of carrier-free [1,2-^{14}C]-TCE and -PCE as previously described (Palumbo et al. 1995, Phelps et al. 1989b). Double strength methanotrophic medium was used, except that phosphate was kept at 2 mM. All mineralizations were incubated for 30 days at room temperature, inhibited with 0.4 mL 2 M NaOH, and refrigerated until analyzed. Radioactive $^{14}CO_2$ was determined by gas chromatography-gas proportional counting (Phelps et al. 1989b) with a Shimadzu 8A gas chromatograph equipped with a thermal conductivity detector and Packard 894 gas proportional counter. Tubes were acidified with 0.5 mL of 6 M hydrochloric acid 1 hour before analysis. Data are expressed as percent mineralization to CO_2.

One-way analysis of variance (ANOVA) was performed on the data using the least significant difference method at a confidence interval of 95 percent. The statistical software used was Statgraphics® Plus by Manugistics, Inc.

RESULTS

The well (MHT-6c) between the horizontal wells clearly exhibited a more pronounced biomass response to the amendments than did the well farther away from the horizontal wells (Fig. 3). In well MHT-6c, total biomass, measured as PLFA, generally increased from the beginning of the methane additions through the nutrient addition treatment, and decreased to near background levels during the posttreatment phase (Fig. 3A). Total biomass was stimulated to a much lower extent in well MHT-10c and did not decrease to background levels during the posttreatment phase (Fig. 3A). The highest total biomass in well MHT-6c was approximately four times the highest biomass detected in well MHT-10c. ANOVA analysis for MHT-10c showed no statistical difference at 95 percent significance level, while MHT-6c showed the pulsed treatment to be significantly different from the 1% methane and the posttreatment periods.

As with total biomass, both wells exhibited increased numbers of methanotrophs during methane and methane plus nutrient injection (Fig. 3B). Methanotrophs in well MHT-6c greatly increased in the second half of the 1% methane treatment, dropped during the 4% methane treatment, rose again during the pulsed phase, and peaked during the nutrient treatment (Fig. 3B). Methanotroph numbers also increased in well MHT-10c, but at a much lower level than in MHT-6c. The highest methanotroph density detected in well MHT-6c was five times the maximum detected in well MHT-10c (Fig. 3B). ANOVA analysis of methanotroph enumerations on well MHT-6c showed statistically significant difference (95 percent confidence level) between the pre-methane treatments and treatments with methane additions. Furthermore, 1% methane addition differed significantly from pulsed and posttreatment regimes. Well MHT-10c showed a similar pattern to MHT-6c with the exception that air injection was not significantly different from 1% methane injection. The 1% methane treatment was significantly different from the pulsed methane in air treatment.

Changes in TCE and PCE degradation potentials (as measured by ^{14}C-contaminant mineralization) in wells MHT-6c and 10c were quite different during the various treatments (Fig. 4). During the post air stripping treatment, TCE and PCE degradation potentials were below detectable limits in well MHT-6c. In well MHT-6c, TCE degradation potential was highly variable over the entire course of treatments (Fig. 4A), while PCE degradation

potential increased during vacuum extraction and then decreased over the air and 1% methane treatments. PCE degradation potential was not detectable during the 4% methane and the first half of the pulsed treatments, then peaked again during the nutrient addition and posttreatment period (Fig. 4B). The pattern in well MHT-10c was different; there was PCE biodegradation during the post air stripping period, but not during the vacuum treatment (Fig. 4B). The 1% methane, 4% methane, nutrient addition, and posttreatment regimes each exhibited PCE degradation potentials. In well MHT-10c, these same regimes evidenced TCE degradation potentials. Overall, the TCE and PCE degradation potentials exhibited similar patterns in well MHT-10c, with the exception of the 4% methane treatment (Fig. 4). One-way analysis of variance on TCE mineralizations showed a significant difference between the pulsed and nutrient treatments for well MHT-10c. Statistical analysis of PCE mineralization for well MHT-6c indicated a significant difference between the nutrient treatment and all other treatments except air injection. Results of the nutrient treatment from well MHT-10c differed significantly from vacuum, air, 1% methane, 4% methane, and pulsed methane treatments. Importantly, the average and maximum TCE and PCE mineralization values for treatments increased with the addition of methane to the subsurface.

DISCUSSION

The addition of methane and nutrients to the subsurface environment at WSRS dramatically increased microbial biomass, methanotroph counts, and the toxicant biodegradative potential of the microbiota in the groundwaters from these two monitoring wells. An effect of the sampling well locations was seen in that much higher microbial biomass and methanotrophic counts were observed in well MHT-6c than MHT-10c. Well MHT-6c was located between the horizontal nutrient injection and extraction wells, while MHT-10c was outside this area and past the end of the injection well (Fig. 1). Higher densities of methanotrophs were expected in well MHT-6c due to its location in the zone of expected higher methane concentrations as was demonstrated in figure 2. This is significant because it shows that the addition of methane to the subsurface did stimulate the methanotrophs in the zone of effect. Decreased microbial activities during the 4% methane treatment may have resulted from insufficient nutrient and mineral availability. Fox example, phosphate concentrations were low and nitrate decreased during 1% methane injection (data not shown). Monitoring the microbial community and the degradative potentials provide indications of stress that may adversely affect toxicant degrading microorganisms. The impacts of the nutrient injection may have been more dramatic than evidenced by these two wells and are discussed elsewhere (Palumbo et al. 1995).

The highly heterogeneous nature of the subsurface environment can be seen in the values measured for TCE and PCE biodegradative potentials in well MHT-10c during the post air stripping treatment before methane addition, versus the below detectable limit values for MHT-6c. Significant amounts of biodegradation were observed in the early samples from MHT-10c, though the biomass values were low and methanotrophs were not detectable. Organisms capable of degrading chlorinated hydrocarbons have been found in subsurface environments (Wilson & Wilson 1985, Fliermans et al. 1988), including methanotrophs (Henry & Grbic-Galic 1990) at low population densities. The very high levels of biodegradative potential observed in MHT-10c during the nutrient and immediate posttreatment regimes may have been due to a preadapted population existing at this site. Apparently, due to the noncontinuous layers of clay at the site, the zone of influence of the

injection extended much farther than the original design anticipated. Thus, the biostimulation of methanotrophs and the increased degradation potentials seen in well MHT-10c. The extent of the biological effects was clearly detected by the various microbial monitoring techniques applied. These monitoring techniques provided essential information on spatial changes in microbial community structure and degradation potentials which was utilized to design the nutrient injection campaign and to improve the effectiveness of the bioremediation operations.

ACKNOWLEDGMENTS

This research was funded by the Office of Technology Development within the U.S. Department of Energy's Office of Environmental Management, under the Non-Arid Sites Integrated Demonstration at WSRS. We thank Terry Hazen, Carl Fliermans, Ken Lombard, Brian Looney, and WSRS staff for site management and sampling assistance. We also thank Mary Anne Bogle of ORNL for help with the site map.

REFERENCES

Balkwill, D. L. 1989. "Numbers, Diversity and Morphological Characteristics of Aerobic, Chemoheterotrophic Bacteria in Deep Subsurface Sediments from a Site in South Carolina." Geomicrobiol. J. 6(1):733-752.

Chapelle, F. H., and D. R. Lovley. 1990. "Rates of Microbial Metabolism in Deep Coastal Plain Aquifers." Appl. Environ. Microbiol. 56(6):1865-1874.

Fliermans, C. B., T. J. Phelps, D. Ringelberg, A. T. Mikell, and D. C. White. 1988. "Mineralization of Trichloroethylene by Heterotrophic Enrichment Cultures." Appl. Environ. Microbiol. 54(7):1709-1716.

Guckert, J. B., C. P. Antworth, P. D. Nichols, and D. C. White. 1985. "Phospholipid, Ester-linked Fatty Acid Profiles as Reproducible Assays for Changes in Prokaryotic Community Structure of Estuarine Sediments." FEMS Microbiology Ecology 31:147-158.

Henry, S., and D. Grbic-Galic. 1990. "Effect of Mineral Media on Trichloroethylene Oxidation by Aquifer Methanotrophs." Microb. Ecol. 20(2):151-169.

Lackey, L. W., T. J. Phelps, P. R. Bienkowski, and D. C. White. 1993. "Biodegradation of Chlorinated Aliphatic Hydrocarbon Mixtures in a Single-pass Packed-bed Reactor." Appl. Biochem. Bioeng. 39-40(1):701-713.

Little, C. D., A. V. Palumbo, S. E. Herbes, M. E. Lindstrom, R. L. Tyndall, and P. J. Gilmer. 1988. "Trichloroethylene Biodegradation by Pure Cultures of a Methane-Oxidizing Bacterium." Appl. Environ. Microbiol. 54(4):951-956.

Lombard, K. H., J. W. Borthen, and T. C. Hazen. 1994. "The Design and Management of System Components for In Situ Methanotrophic Bioremediation of Chlorinated Hydrocarbons at the Savannah River Site." In R. E. Hinchee (Ed.), Air Sparging for Site Remediation, pp.

81-96. Lewis Publishers, Boca Raton, FL.

Niedzielski, J. J., T. J. Phelps, R. M. Schram, S. E. Herbes, and D. C. White. 1990. "Biodegradation of Trichloroethylene in Continuous-Recycle Expanded-bed Bioreactors." Appl. Environ. Microbiol. 56(6):1702-1709.

Palumbo, A. V., S. P. Scarborough, S. M. Pfiffner, and T. J. Phelps. 1995. "Influence of Nitrogen and Phosphorous on the In-Situ Bioremediation of Trichloroethylene." Appl. Biochem. Biotech. In Press.

Phelps, T. J., D. Ringelberg, D. Hedrick, J. Davis, C. B. Fliermans, and D.C. White. 1989a. "Microbial Activities and Biomass Associated with Subsurface Environments Contaminated with Chlorinated Hydrocarbons." Geomicrobiology J. 6(1):157-170.

Phelps, T. J., E. G. Raione, D. C. White, and C. B. Fliermans. 1989b. "Microbial Activities in Deep Subsurface Environments." Geomicrobiol J. 6(1):79-91

White, D. C., W. M. Davis, J. S. Nickels, J. D. King, and R. J. Bobbie. 1979. "Determination of the Sedimentary Microbial Biomass by Extractable Lipid Phosphate." Oceologia 40(1):51-62.

Wilson, J. T., and B. H. Wilson. 1985. "Biotransformation of Trichloroethylene in Soil." Appl. Environ. Microbiol. 49(1):242-243.

CAPTION LIST

Figure 1. Map of demonstration site showing the horizontal wells (lines) and the monitoring wells (circles or stars). Coordinates are in feet and wells MHT-6c and MHT-10c are indicated by stars.

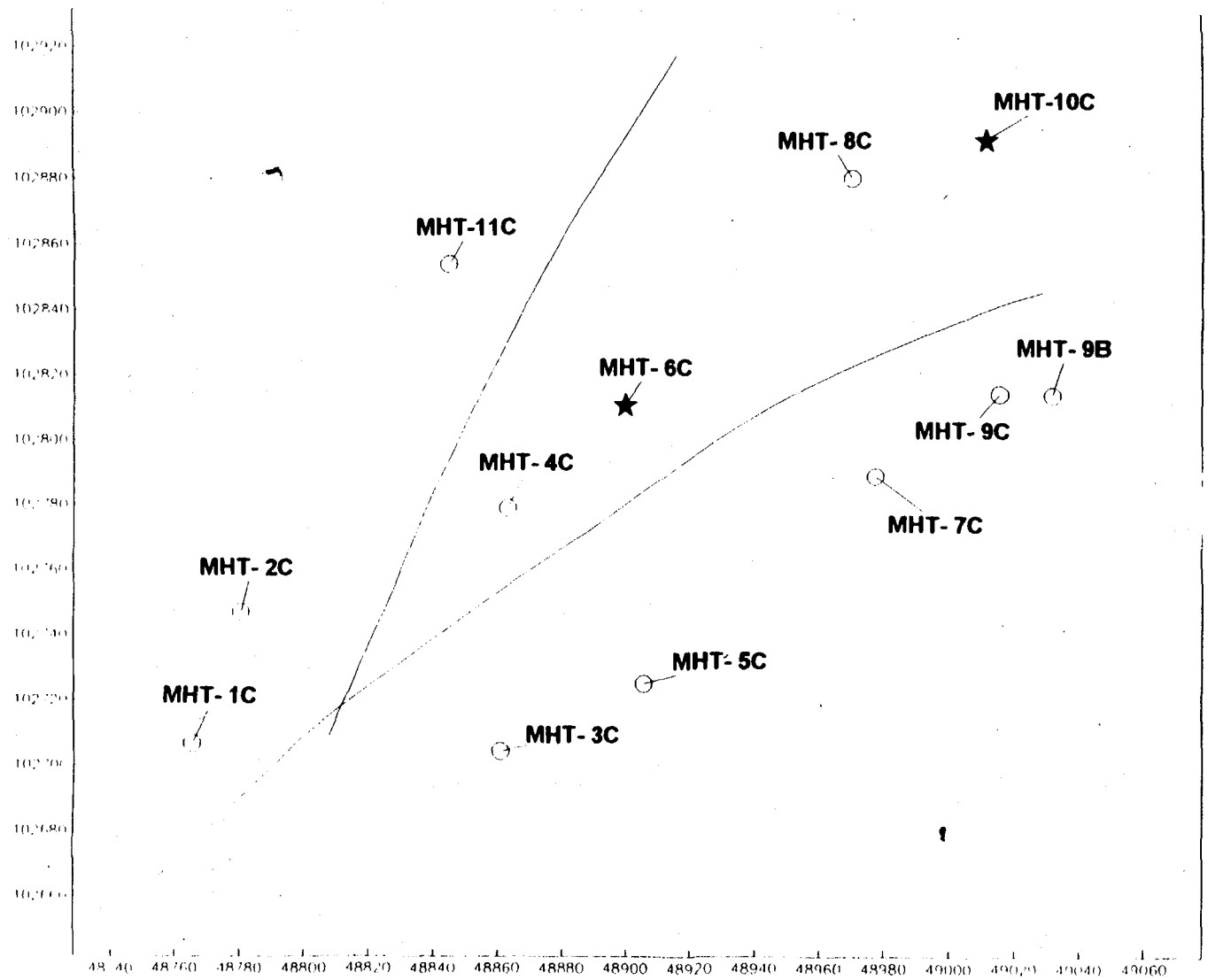
Figure 2. Dissolved methane concentrations in groundwater from wells MHT-6c and MHT-10c with operating conditions delineated by solid lines at the bottom of the figure. See Figure 3 for abbreviations.

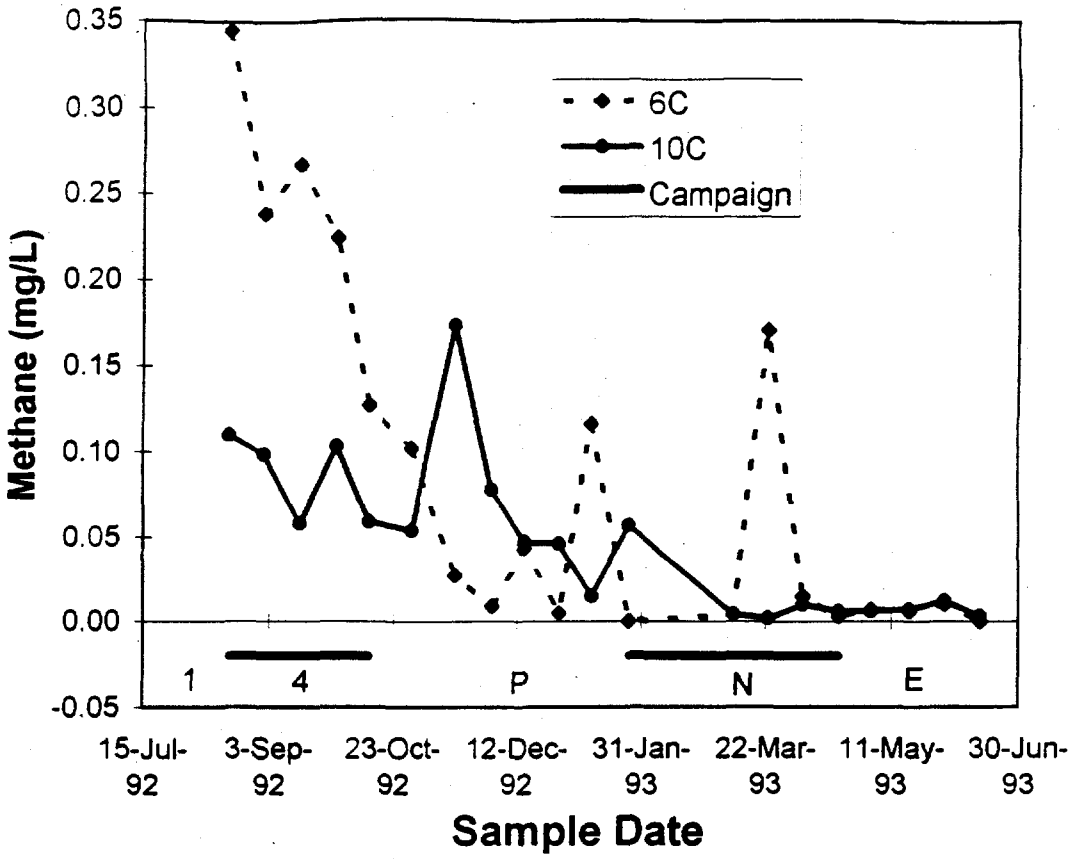
Figure 3. Changes in total biomass, as measured by PLFA (A) and total number of methanotrophs (B) during the of WSRS demonstration. The 95 percent confidence level for MPN enumeration is depicted by error bars on three representative values. Different operating campaigns are delineated by solid bars at the bottom of the figure and include post air-stripping (S), vacuum extraction (V), air injection (A), 1% methane injection (1), 4% methane injection (4), pulsed methane/air injection (P), nutrient addition (N) treatments, and period after end of treatment (E).

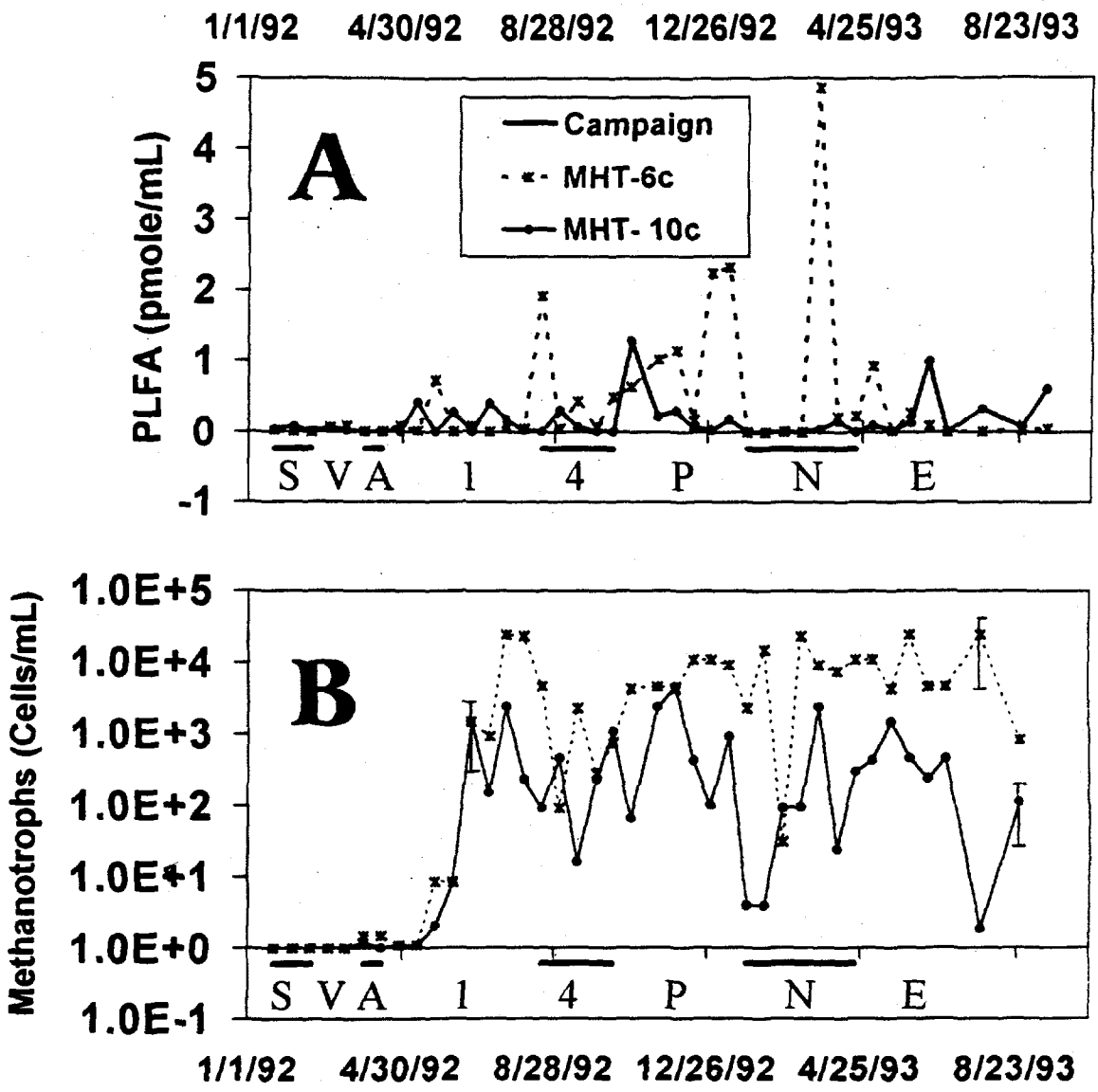
Figure 4. Mineralization potentials for TCE (A) and PCE (B) in wells MHT-6c and MHT-10c. Abbreviations are the same as for Figure 3.

KEY WORD LIST

biostimulation, trichloroethylene, methanotrophs (ic), subsurface amendments, groundwater monitoring, nutrient injection, microbial community structure


MHT-10C
MHT- 8C
MHT-11C
MHT- 6C
MHT- 9B
MHT- 9C
MHT- 4C
MHT- 7C
MHT- 2C
MHT- 5C
MHT- 1C
MHT- 3C
48840
48860
48880
48900
48920
48940
48960
48980
49000
49020
49040
49060


0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
Methane (mg/L)
6C
10C
Campaign
1
4
P
N
E
15-Jul-92
3-Sep-92
23-Oct-92
12-Dec-92
31-Jan-93
22-Mar-93
11-May-93
30-Jun-93
Sample Date


1/1/92
4/30/92
8/28/92
12/26/92
4/25/93
8/23/93
A
Campaign
MHT-6c
MHT- 10c
PLFA (pmole/mL)
5
4
3
2
1
0
-1
S V A 1 4 P N E
B
Methanotrophs (Cells/mL)
1.0E+5
1.0E+4
1.0E+3
1.0E+2
1.0E+1
1.0E+0
1.0E-1
S V A 1 4 P N E
1/1/92
4/30/92
8/28/92
12/26/92
4/25/93
8/23/93

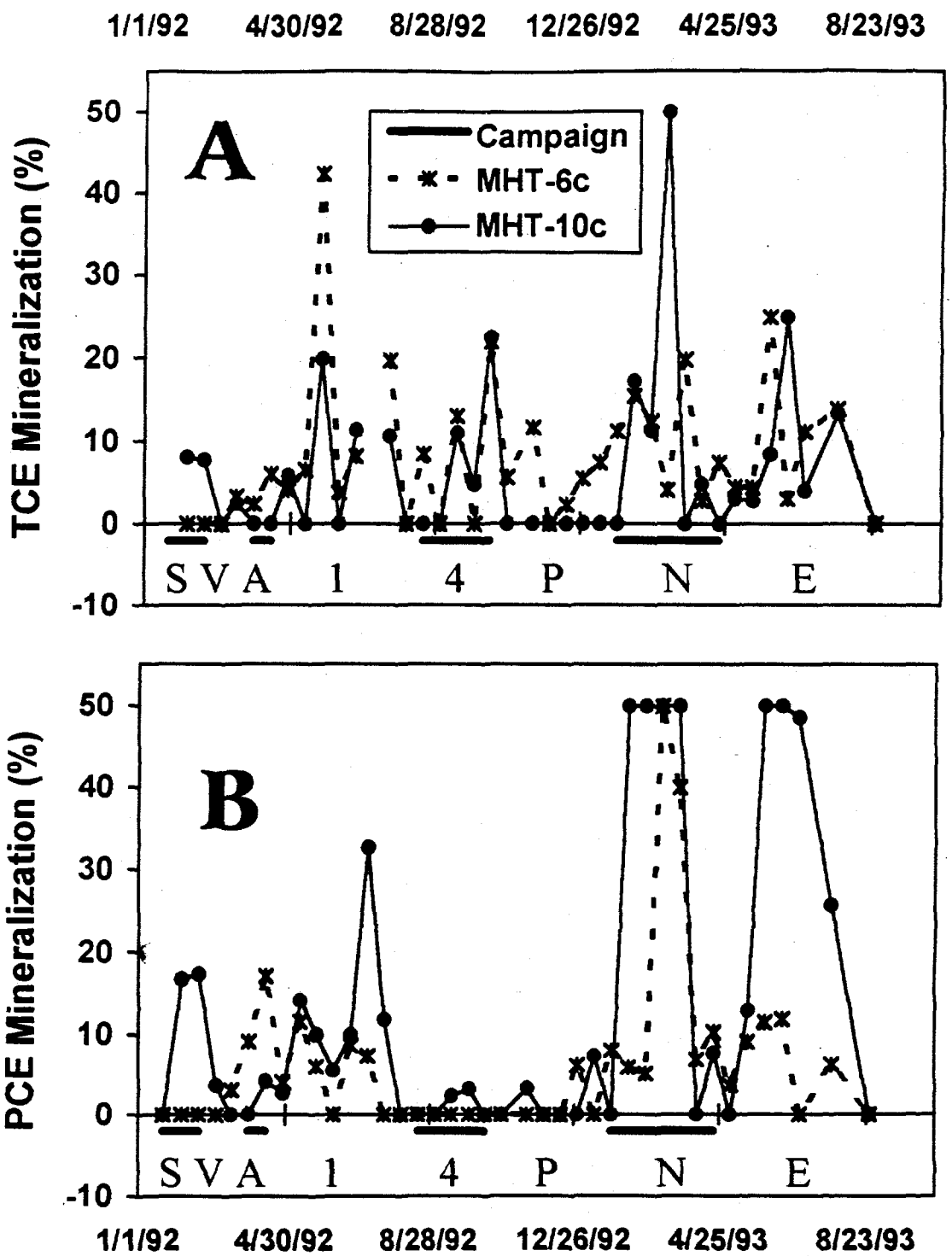

1/1/92
4/30/92
8/28/92
12/26/92
4/25/93
8/23/93
A
Campaign
MHT-6c
MHT-10c
TCE Mineralization (%)
50
40
30
20
10
0
-10
S V A 1 4 P N E
B
PCE Mineralization (%)
50
40
30
20
10
0
-10
S V A 1 4 P N E
1/1/92
4/30/92
8/28/92
12/26/92
4/25/93
8/23/93

Table 1. Operational Campaigns

Treatment	Date	Days [a]	Abbreviation
Post Air-Stripping			S
Vacuum Extraction	2\26\92	0	V
Air Injection	3\18\92	21	A
1% Methane Injection	4\20\92	54	1
4% Methane Injection	8\5\92	161	4
Pulsed Methane/Air	10\23\92	240	P
Nutrient Addition	1\18\93	327	N
Posttreatment	4\20\93	419	E

(a) Days are numbered sequentially from the initiation of bioremediation operations.