MONITORING OF SUBSURFACE BIOPROCESSES USING QUANTITATIVE BIOMARKER ANALYSES

M. E. Losi, J.S. Oslick, A. Eloskof (Tetra Tech FW, Inc., Santa Ana, California)
A. Peacock, D. C. White (University of Tennessee, Knoxville, Tennessee)
E. Dienzo, T. Macchiarella (Naval Facilities Engineering Command, San Diego, California)

ABSTRACT: This project addressed the hypothesis that the effectiveness of an implanted substrate in driving reductive dechlorination in groundwater could be monitored relatively simply and effectively through quantitative analysis of biomarkers found in microbiota accumulated on microbial "traps" suspended in monitoring wells at the site. This approach was assessed in conjunction with monitoring of traditional geochemical parameters, and qualitative assessments were made with respect to which approach better reflected the contaminant trends. Microbial biomarkers included phospholipid fatty acids, respiratory quinones, and targeted and community level gene sequences. Traditional geoparameters included a suite of electron acceptors, metabolic by-products, and other geochemical indicators of biological processes. The biomarker results yielded important insights into subsurface processes, including changes in community dynamics in response to substrate emplacement, confirmation of an anaerobic environment, a predominance of sulfate-reducing bacteria, and the presence of *Dehalococcoides ethenogenes*, a naturally-occurring dechlorinating organism. Traditional geoparameter data provided relatively limited insight regarding relationships between biological activity and contaminant behavior at the site. We conclude that the biomarker approach provides information regarding subsurface biological process that could be useful in supporting site remedial activities, and with continued development and testing, could supplement and/or eventually supplant select traditional groundwater geochemical analyses.

INTRODUCTION

Reductive dechlorination (enhanced or natural) is a major mechanism for biodegradation of chlorinated volatile organic compounds (CVOCs) in anoxic contaminated groundwater aquifers. As a central component of such strategies, a comprehensive groundwater monitoring program is implemented to provide lines of evidence that the desired bioprocesses are occurring, or are likely to occur. This monitoring program typically involves measurement of various geochemical parameters, including concentrations of electron acceptors, metabolic by-products, dissolved oxygen (DO) concentration, and oxidation/reduction potential (ORP). Data from these measurements are expected to elucidate subsurface conditions such that the prevalent bioprocesses can be deduced (such as aerobic versus anaerobic metabolism). These data are then used in conjunction with contaminant concentration trends to determine if the desired bioprocesses are occurring in accordance with a specified time frame. In practice, however, difficulties are often encountered when geochemical data are not consistent with contaminant plume data (or other site data), and therefore, may be of limited use. Complications in interpreting these data can, and often do, lead to situations where the insight gained from such monitoring is not commensurate with the substantial level of effort and costs involved.

This project addressed the hypothesis that the effectiveness of an implanted substrate [Hydrogen Release Compound® (HRC)] for enhancing reductive dechlorination in groundwater could be monitored more effectively by direct quantitative analysis of microbiota accumulated on "microbial traps" suspended in wells located within and near the substrate injection field. Indigenous microbes colonize the traps, and their biomarkers, reflect *in situ* environmental conditions in the wells. Recovered traps are analyzed for biomarkers that establish maintenance of effective *in situ* conditions supportive of reductive dechlorination (White, 1995). Since the microbes integrate their responses over time (White et al., 1998), this information may be a better indicator of *in situ* processes than monitoring of traditional parameters such as alternate electron acceptors and metabolic by-products, which generates "snapshot" data that may or may not represent prevailing overall conditions.

Objectives. The overall objective of this project was to assess quantitative and qualitative biomarkers from biofilms collected on traps suspended in strategic monitoring wells. This approach is expected to improve the efficiency, accuracy, and cost-effectiveness of monitoring *in situ* bioprocesses at this and similar sites undergoing engineered bioremediation or monitored natural attenuation (MNA). Specific objectives of the biomarker analyses were: 1) use ester-linked phospholipid fatty acids (PLFA) to measure viable microbial biomass, diversity, and community structure/dynamics in response to the substrate injection/migration; 2) obtain information regarding the redox status of the subsurface system through analysis of bacterial respiratory quinones; 3) use denatured gradient gel electrophoresis (DGGE) analysis to provide an indication of the dominant groups of organisms that are considered active in the subsurface; and 4) assess the presence of a known dechlorinating organism, *Dehaloccocoides ethenogenes* (DHE), through targeted DNA analysis.

The biomarker analyses were assessed concurrently with contaminant trend data and traditional groundwater geochemical analyses to determine if effective conditions to promote reductive dechlorination were present within the zone of HRC influence. A qualitative assessment was made as to which approach provided more useful information and better reflected contaminant trend data.

METHODS

The microbial traps were suspended in monitoring wells attached to a nylon line with a coated sinker. The biofilm trap samplers were constructed from Teflon® tubing cut into 4-cm lengths and loaded with Bio-Sep® beads. Bio-Sep beads consist of 2 to 3 millimeter spherical beads engineered from a composite of Nomex® and powdered activated carbon. Sterilized traps were deployed at the approximate middle-depth of groundwater within the screened interval in each of four wells. The test wells included one well within the HRC injection field [MW-14-S4 (S4)], two wells upgradient of the injection field [MW-14-S1 and MW-14-S2 (S1 and S2)], and one well downgradient of the injection field [MW-14-S5 (S5)]. Only wells S4 and S5 were within the CVOC plume.

The study encompassed four biomarker sampling events, generally coinciding with routine groundwater monitoring events. For each event, biofilm traps were deployed for durations ranging from approximately 4 to 6 weeks. Where required, data were normalized to account for time differences. Upon recovery from the wells, biofilm traps

were frozen and shipped on ice to the University of Tennessee, Center for Biomarker Analysis for processing.

Traps were analyzed for ester-linked PLFA (Zhang et al., 1997), respiratory quinones [ubiquinones (UQ) and menaquinones (MK)] (Lytle et al., 2001), and DNA (both targeted 16S rDNA and DGGE). The 16S rDNA in biomass accumulated on the biomarker traps was amplified through polymerase chain reaction (PCR) techniques and analyzed to assess the general microbial community structure, as well as the presence of DHE, a known dechlorinating organism. DGGE was performed using a D-Code 16/16 cm gel system (BioRad, Hercules, California) (Chang et al., 1999). Prominent bands were excised and subjected to sequencing. Sequence identification was performed by use of the BLASTN facility of the National Center for Biotechnology Information and "Sequence Match" facility of the Ribosomal Database Project (Maidak et al., 1999).

Table 1 summarizes the sampling that occurred as part of this project. Contaminant concentrations (CVOCs) and traditional groundwater geochemistry data were collected as part of the routine groundwater monitoring of the site. Analytes included dissolved hydrogen, ethane, methane, nitrate, sulfate, sulfide, total organic carbon (TOC) (this list includes key parameters discussed herein, others analytes were monitored, but were not detected and/or are not relevant to this discussion). In addition, lactate was measured, as were the major breakdown products of the lactate, including acetate, butyrate, propionate, and pyruvate. Finally, pH, DO, temperature, ORP, and Fe^{2+} were measured in the field using field instrumentation. Groundwater geochemistry parameters, contaminant concentrations, and substrate/by-product data are typically expected to provide information regarding the dominant bioprocesses at the site.

TABLE 1. Major events that occurred at the site relevant to this project.

Samples Collected (CVOCs and Geoparameter Analysis)	HRC Injection	Microbial Traps Collected for PLFA Analysis	Microbial Trap Collected for DHE Analysis
5/02, 8/02, 10/02, 1/03, 4/03, 6/03	6/02	8/02, 10/02, 1/03, 5/03	3/03, 6/03

RESULTS AND DISCUSSION

Contaminants of Concern Trends. Figure 1 presents the results of groundwater contaminant monitoring in the well closest to the source for six quarters at the site. The primary contaminant of concern is tetrachloroethene (PCE). Degradation products of PCE are also present, including trichloroethene (TCE), 1,2-dichloroethene (DCE) (cis- and trans-; cis- dominating), and vinyl chloride (VC). PCE concentrations increased markedly within the source area following the baseline groundwater monitoring in May 2002, likely as result of desorption of contaminants following the HRC injection in June 2002. Analyses for biomarkers were not conducted prior to the HRC injection. Following the third quarter of monitoring, PCE and TCE concentrations decreased. The reduction in PCE and TCE concentrations is attributed largely to biologically mediated reductive dechlorination. A subsequent increase in cis-1,2-DCE and detections of VC are associated with the reductive dechlorination of PCE and TCE. It is noted that the trends appear to suggest that 1,2-DCE is not being removed due to unfavorable reaction

conditions or kinetics; however, subsequent data and analysis (not shown) have confirmed CVOC mass removal, indicating that this is likely not the case.

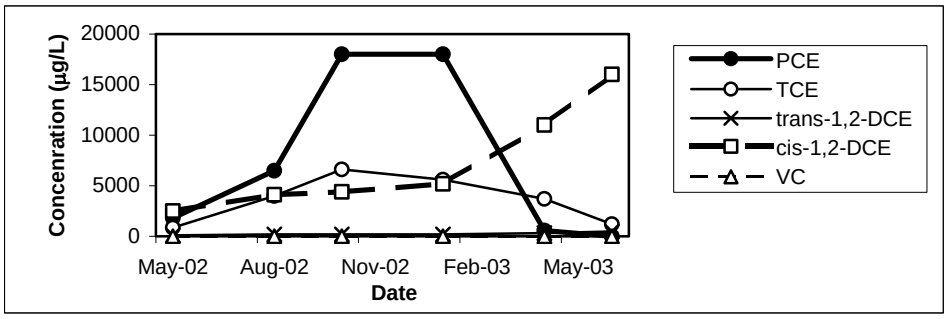

FIGURE 1. Contaminant trends observed in the source well during the study period.

Traditional Geoparameter Analyses. Laboratory results for the groundwater geochemistry parameters and substrate breakdown products (organic acids) are shown below for S4 in Table 2, along with site-wide mean and maximum values. Field measurements, including DO, ORP, and Fe^{2+} were also collected (not presented here), and indicated that before and after the substrate injection: (i) little or no DO was present across the entire site (≤0.5 mg/L), (ii) the entire site was under strongly reducing conditions with little spatial/temporal variation, and (iii) Fe^{2+} was detected infrequently and randomly across the site at concentrations near the detection limit (0.5 mg/L).

With respect to the laboratory data (Table 2), the majority of the analytes, including substrate breakdown products, hydrogen, TOC, ethane and nitrate, were either not detected or detected with relatively equal frequency and concentration across the site, with the exception of one slightly elevated detection of lactate in well S4.

Sulfate concentrations in the substrate injection area were lower than site-wide average levels, a possible indicator of biological activity. However, if depressed sulfate levels in the substrate injection area were related to microbial activity, correspondingly elevated levels of hydrogen sulfide should have been detected in S4, which was not the case (Table 2). The site is under marine (saltwater) influence, and therefore, elevated and variable sulfate concentrations are expected, and difficult to interpret with respect to microbial activity.

Methane was detected in all study wells. Substantially elevated and increasing methane levels were detected in the substrate-influenced well (S4) in the latter three sampling events. This indicates that methanogenesis was the dominant microbial process occurring, likely due to the emplaced substrate (HRC).

In summary, based on the groundwater geochemical parameter and substrate breakdown product data presented above, the study area (along with the entire site) appears to be characterized by strongly reducing conditions. This may be a result of naturally occurring organic matter that is present across the site. In addition, with the exception of increased methane production detected in well S4 in the latter half of the experiment, the geoparameter data provided little information to support substrate degradation or explain the observed decrease in contaminant concentrations in the area of the injection. Although the injection of substrate and contaminant trends suggest that a

dynamic subsurface environment should be observable, groundwater geochemistry data indicate relatively constant conditions with respect to biological activity. Therefore, the geoparameter data provides little insight regarding a potential relationship between biological activity and contaminant behavior at the site.

TABLE 2. Summary of laboratory results for geoparameter analyses.

Location	Sample Date	Acetate mg/L	Butyrate mg/L	Pyruvate mg/L	Lactate mg/L	Propionate mg/L	Dissolved Hydrogen nM/L
MW-14-S4 (Substrate injection area)	05/30/02	<1	<2.5	<2.5	1.7	<2.5	NA
	08/07/02	<0.5	<1.25	<1.25	6.9	<1.25	8
	10/22/02	2.3	<0.25	<0.25	<0.1	<0.25	1.5
	01/22/03	0.1	<0.25	<0.25	<0.1	<0.25	5.6
	04/23/03	1.4	<0.5	<0.5	<0.2	<0.5	2.1
	06/30/03	0.1	<0.25	<0.25	3.0	<0.25	2.8
No. detects/No. analyses (excludes MW-14-S4)		10/72	0/72	0/72	10/72	0/72	19/19
Site-wide mean (excludes NDs, MW-14-S4)		1.4	NA	NA	2.0	NA	2.5
Site-wide maximum (excludes MW-14-S4)		2.5	NA	NA	3.0	NA	6.4
Location	**Sample Date**	**TOC mg/L**	**Ethene µg/L**	**Methane µg/L**	**Nitrate-N mg/L**	**Sulfate mg/L**	**Sulfide mg/L**
MW-14-S4 (Substrate injection area	05/30/02	8.1	0.43	15	<0.1	1500	<1
	08/07/02	15	0.47	20	<0.1	1410	1.1
	10/22/02	11	0.28	31	<0.1	1400	<1
	01/22/03	10	<1.3	220	<0.1	1640	<1
	04/23/03	11	<1.3	950	0.113	1420	<1
	06/30/03	9.0	<1.3	2800	0.114	1410	<1
No. detects/No. analyses (excludes MW-14-S4)		67/72	15/72	70/72	10/72	72/72	6/72
Site-wide mean (excludes NDs, MW-14-S4)		8.8	1.0	21	0.29	2151	1.2
Site-wide maximum (excludes MW-14-S4)		12.5	0.9	42	0.81	2710	1.6

Biomarker Analyses. The total concentration of PLFA in a sample is considered an indicator of overall viable biomass. Because only viable bacteria produce PLFA, total PLFA is a reflection of only the viable, active biomass in a sample. As such, increases would be expected in substrate-influenced wells, indicating substrate-induced growth. Total PLFA data (not shown) appeared to indicate site-wide changes in biomass with time, including changes in upgradient wells. Therefore, increases in PLFA due to substrate injection could not be demonstrated. The reason for this is not clear, as a large amount of substrate injected into the subsurface would be expected to substantially enhance biomass. Analysis of the specific combination and diversity of PLFA measured in samples can also provide a picture of microbial community structure. Changes in community structure can directly indicate microbial responses to a subsurface disturbance (i.e. substrate injection, or oxygen sparging, etc.). “Cluster analysis” is a statistical technique used to determine similarity or difference between the microbial communities present in wells being compared, i.e. samples with similar community structure will have smaller linkage distances between them and will be grouped together most closely by the tree-structure of the cluster analysis plot. Sample data that are less similar plot at greater

linkage distances from each other. We first used this to determine that microbial communities in the two upgradient wells (S1 and S2) were very similar over the course of the study (not shown). Therefore, for simplification, the upgradient well, S2, was excluded from further analysis.

Cluster analysis of PLFA profiles comparing wells S1 (upgradient) and S4 (within the substrate injection area) showed distinctly different community structure was present in S4 relative to S1 (Figure 2). This is interpreted as evidence of substrate-induced response of the microbial community in well S4 and provides evidence that the substrate is influencing the subsurface environment, even though little to no evidence of substrate decomposition products (organic acids) was observed in S4. Cluster analysis of PLFA profiles comparing wells S5 (downgradient of substrate injection) and S4 (in the substrate injection area) showed that the community structures were distinctly different initially (quarters 1 and 2), but were more similar during the latter quarters. This is interpreted as providing evidence that the substrate was affecting microbial growth in S4 but not S5 initially (as would be expected), but over time as the substrate migrated downgradient, the community structures became more similar. Concurrently, no substantial evidence of substrate decomposition products was observed in organic acid data from well S5.

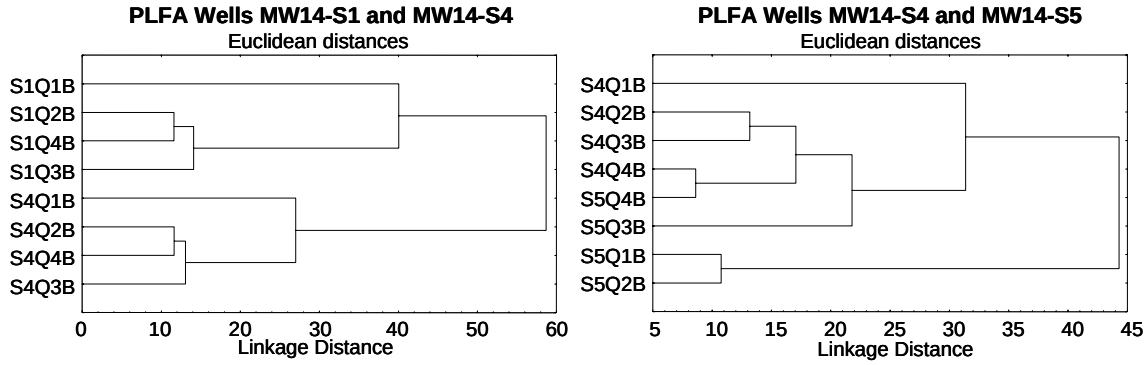

FIGURE 2. Cluster analysis showing comparison of microbial community structures between wells S1 and S4 (left) and between wells S4 and S5 (right). Q# indicates sampling quarter, and "B" indicates Bio-Sep bead media.

Quinones are compounds that transfer electrons within bacterial cells for energy generation. The types of quinones present can provide an indication of whether the cells are growing in the presence or absence of oxygen, which in turn, provides an indication of the redox state (aerobic versus anaerobic). The ratio of total quinones to PLFA [(UQ+MK)/PLFA)] was used to assess the activity of the microbial biomass. Quinone data were only successfully collected for all four quarters in well S4. The quinone/PLFA ratio increased markedly from the third to the fourth quarter in S4 (Figure 3), primarily influenced by a higher concentration of a single quinone, MK4. No comparable increase in quinone/PLFA was noted in the other wells from the third to the fourth quarter.

Menaquinones are indicators of an increase in anaerobic bioactivity and ubiquinones are indicators of aerobic bioactivity. A corresponding increase in PLFA was not noted. Because methanogenic bacteria are not detectable through the PLFA analysis used, this provides indirect evidence that substantial methanogenesis was occurring, indicating that the redox potential was in the range needed to support reductive

dechlorination, but that methanogenesis may be have been competing for reducing equivalents. This result was confirmed by increasing methane concentration in the well, the only "geoparameter" result that indicated any response to the injection.

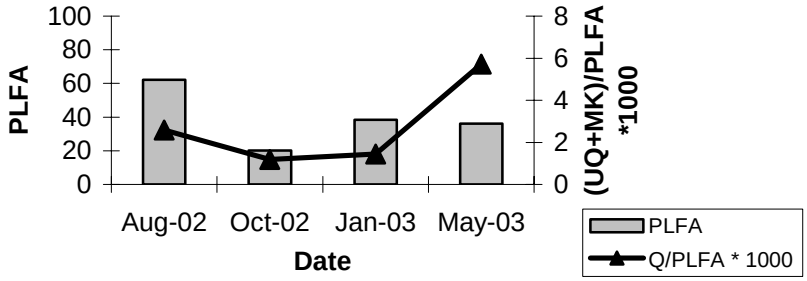

FIGURE 3. Total PLFA and (UQ+MK)/PLFA trends over time for well S4.

DNA analyses were conducted for both community level DNA and targeted DNA sequences for DHE. Quantitative DNA analysis confirmed the presence of the known reductive-dechlorinator DHE, and also indicated that the concentration of DHE in well S4, located within the injection field, may be elevated compared with the other wells (Table 3). Notwithstanding, an apparent buildup of cis-1,2-DCE was noted in this S4. DGGE results from all wells (not shown) indicated that the dominant populations present are sulfate-reducing bacteria, which is expected, given the relatively high sulfate concentrations at the site.

TABLE 3. Analytical Results, 16S rDNA.

Well	Date Sampled	*Dehaloccoides* spp Abundance (16S rDNA gene copies/bead)
S1	3/3/2003	1.84E+05
S2	3/3/2003	1.34E+05
S4	**3/3/2003**	**2.03E+05**
S5	3/3/2003	8.94E+04
S1	6/4/2003	8.52E+04
S2	6/4/2003	1.82E+05
S4	**6/4/2003**	**2.03E+05**
S5	6/4/2003	Not Detected

SUMMARY AND CONCLUSIONS

The results of this study indicate that biomarker data can potentially provide valuable insight into the biochemical processes related to bioremediation of groundwater impacted with chlorinated solvents. The interpretation of the results also reflects the specific nature of the contaminant trends and background conditions at this site.

DNA analyses performed for the site indicate that the indigenous microbial population supports the expected and nominally desirable bioprocesses. Detection of DHE suggests the potential for complete biological reductive dechlorination. The presence of DHE in well S4 provides evidence that complete reductive dechlorination can occur at this site, given the correct environmental conditions. DGGE data, however, imply that significant competition from sulfate-reducing bacteria is likely occurring. Low

concentrations of ethane and VC at the site, both before and after the HRC injection, also support the assumption that complete reductive dechlorination of contaminants at the site can ultimately occur.

Comparison of the biomarker results to the traditional groundwater geochemical parameter results used for assessing bioremediation processes indicates that assessing *in situ* bioprocesses using biomarkers at sites undergoing bioremediation can yield significant, valuable information that is not reliably discernable using traditional methods. Specifically, monitoring of PLFA and quinones yielded important evidence of microbial response to substrate emplacement and of competing bioprocesses and the oxidation/reduction status. Except for the evidence of increased methanogenic activity in the substrate injection area, little useful insight was obtained from the traditional groundwater geochemistry data. We conclude that the biomarker approach provides information regarding subsurface biological process that could be useful in supporting site remedial activities, including shifts in community dynamics and the presence of specific organisms and types of organisms. With continued development and testing, biomarker analyses could supplement and/or eventually supplant select traditional groundwater geochemical analyses.

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