Journal of Industrial Microbiology & Biotechnology (1997) 18, 177–188

Intrinsic bioremediation in a solvent-contaminated alluvial groundwater

RA Williams[1], KA Shuttle[1], JL Kunkler[1], EL Madsen[2] and SW Hooper[1]

[1]*Environmental Projects Laboratories, Merck & Co, Inc, PO Box 7, Elkton, VA 22827;* [2]*Section of Microbiology, Division of Biological Sciences, Cornell University, Wing Hall, Ithaca, NY 14853, USA*

An industrial site contaminated with a mixture of volatile organic compounds in its subsurface differed from previously reported locations in that the contamination consisted of a mixture of chlorinated, brominated, and nonhalogenated aromatic and aliphatic solvents in an alluvial aquifer. The source area was adjacent to a river. Of the contaminants present in the aquifer, benzene, toluene, and chlorobenzene (BTC) were of primary concern. Studies of the physical, chemical, and microbiological characteristics of site groundwater were conducted. The studies concentrated on BTC, but also addressed the fate of the other aquifer VOCs. Gas chromatographic analyses performed on laboratory microcosms demonstrated that subsurface microorganisms were capable of BTC degradation. Mineralization of BTC was demonstrated by the release of $^{14}CO_2$ from radiolabelled BTC. In the field, distribution patterns of nutrients and electron acceptors were consistent with expression of *in situ* microbial metabolic activity: methane, conductivity, salinity and *o*-phosphate concentrations were all positively correlated with contaminant concentration; while oxidation-reduction potential, nitrate, dissolved oxygen and sulfate concentrations were negatively correlated. Total aerobes, aerotolerant anaerobes, BTC-specific degraders, and acridine orange direct microscopic microorganism counts were strongly and positively correlated with field contaminant concentrations. The relative concentrations of benzene and toluene were lower away from the core of the plume compared to the less readily metabolized compound, chlorobenzene. Hydrodynamic modeling of electron-acceptor depletion conservatively estimated that 450 kg of contaminant have been removed from the subsurface yearly. Models lacking a biodegradation term predicted that 360 kg of contaminant would reach the river annually, which would result in measurable contaminant concentrations. River surveillance, however, has only rarely detected these compounds in the sediment and then only at trace concentrations. Thus, the combination of field modeling, laboratory studies, and site surveillance data confirm that significant *in situ* biodegradation of the contaminants has occurred. These studies establish the presence of intrinsic bioremediation of groundwater contaminants in this unusual industrial site subsurface habitat.

Keywords: bioremediation; biodegradation; groundwater; intrinsic bioremediation; aromatic hydrocarbons; *in situ*; aerobic; anaerobic; modeling

No single factor is considered sufficient to demonstrate the presence of intrinsic bioremediation. Instead, various types of data are required to show that natural attenuation occurs. As recommended for bioremediation investigations by the National Research Council [39], studies usually focus on three avenues of research: documenting the loss of contaminants from the site, showing experimentally that microorganisms in samples from the site have the potential to transform the contaminants, and demonstrating that this potential actually occurs in the field.

In situ bioremediation of aromatics from a number of subsurface environments has been reported in peer-reviewed literature. Compounds such as benzene [10]; benzene, toluene and xylene [8]; benzene, toluene, ethylbenzene, and xylene [6]; phenolics [15]; and phenolics and polycyclics [28] have all been shown to be naturally attenuated in subsurface environments.

Metabolism of BTC

Benzene, toluene and chlorobenzene are all microbiologically degradable. Benzene is readily degraded in the presence of oxygen [8,18,21,27,55]. Some studies propose that microorganisms are capable of linking benzene oxidation to nitrate reduction [4]; however, such reactions may not be universal [10,26]. Benzene is degradable under sulfate- [32] and iron-reducing [34] conditions. Methanogenic removal of benzene may or may not take place [10,19]. The difference of opinion as to whether benzene degrades under methanogenic conditions may be due to variations in the microbial populations examined by different investigators. Alternatively, leakage of even minute amounts of oxygen into a supposedly anaerobic experimental system can result in aerobic benzene degradation.

There is also extensive literature on the degradation of toluene. It can be metabolized aerobically [1,8,18,21,24, 27,55], during denitrification [4,12,16,24–26], by sulfate reduction [14,43], during methanogenesis [13,19,56], and with the reduction of Fe^{3+} [30,33].

Although chlorobenzene can be metabolized aerobically [41,44,46,53], it has not been reported to be degraded through the use of other electron-acceptors [7,41,44]. Molecular oxygen appears necessary for ring fission. Some removal through reductive dehalogenation may occur in conducive environments with excess chlorobenzene [38]. In general, intermediary metabolites of chlorobenzene detected during aerobic degradation appear similar to those documented for unhalogenated aromatic compounds.

Correspondence: SW Hooper, Environmental Projects Laboratories, Merck & Co, Inc, PO Box 7, Elkton, VA 22827, USA
Received 1 December 1995; accepted 27 July 1996

Potential for site remediation

Prior investigations of the manufacturing facility examined in this study established the presence of groundwater contaminated with chlorinated, brominated, and unhalogenated aromatic and aliphatic industrial solvents. The contamination was in an alluvial layer in very close proximity to a river receptor. The combination of solvent types, hydrogeology, and source/receptor proximity endow this site with a suite of characteristics unique among previously reported intrinsic remediation sites. Based on preliminary assessments of the physical and chemical conditions of the groundwater at this site (unpublished data), it was considered reasonable that intrinsic bioremediation of the groundwater might be occurring. Preliminary biodegradation studies using site soil and the manufacturing facility's waste treatment system also indicated that microorganisms at the site have the potential to degrade benzene, toluene and chlorobenzene (BTC) and other site VOCs. Therefore, a study documenting and characterizing natural attenuation of VOCs at the facility was undertaken. The present study focused on natural attenuation of BTC, because of their potential toxicological impact, but also addressed the fate of the other VOC contaminants.

Materials and methods

Site hydrogeology

The site is underlain with 5–9 m of highly permeable unconsolidated Pleistocene sediments over an eroded bedrock surface. The bedrock is significantly less permeable than the overlying alluvium. The water table lies between 1.5 and 8 m below the ground surface and has a seasonal fluctuation of up to 2.1 m. Most of the groundwater flow discharges to the river (unpublished data).

Sampling locations

Field sampling and data collection procedures were performed in spring (5/94), summer (8/94), and autumn (11/94). Figure 1 depicts the site, sampling well locations, and an isoconcentration map of the volatile organic carbon (VOC) found in the alluvium. Determinations of total, individual aromatic, and individual aliphatic VOC concentrations were by gas-chromatography/mass spectrometry. Isoconcentration lines were determined by interpolation from MT3D numerical modeling [57] of hydraulic flow data and soil and groundwater volatile organic carbon assays performed for the Resource Conservation and Recovery Act Facility Investigation (RFI) for the site. This contaminant database is composed of over 52 000 individual VOC observations taken over the course of 12 years. Numerous well and sampling locations not depicted in Figure 1 have also contributed to this database. Concentrations of benzene, toluene and chlorobenzene followed a similar distribution pattern but at lower concentrations (not shown in Figure 1). Within the limits of groundwater monitoring and modeling, it appears that the VOCs in general, and benzene, toluene, and chlorobenzene in particular, were introduced into the subsurface at a primary area located in the vicinity of well MW-12S. This assertion is consistent with site usage and site records (unpublished data). During periods of localized drought, the river is capable of reversing the groundwater hydrologic flow, smearing the contaminants in a southerly (or what is normally upgradient) direction (Figure 1). This is believed to account for the presence of a lengthy contaminant gradient running up the normal hydraulic gradient. Note that the isoconcentration lines in Figure 1 do not intersect the river. VOC monitoring of the river bed sediments only intermittently detected trace amounts of VOCs (unpublished RFI data) in spite of the fact that the alluvial groundwater flowed directly into the river.

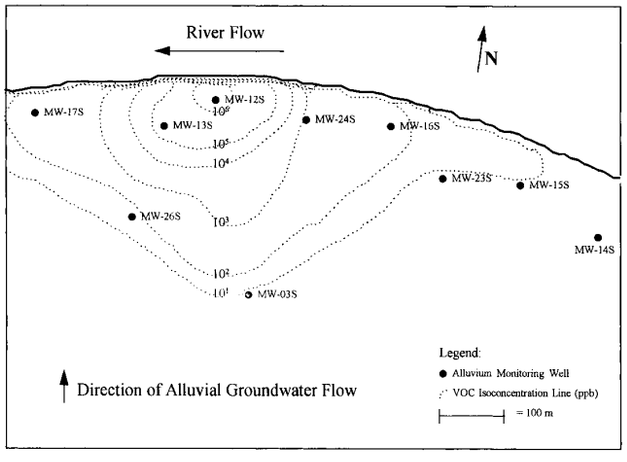

Figure 1 Site map of well locations and VOC concentration (WM = monitoring wells). The dotted lines indicate volatile organic carbon isoconcentration lines for the alluvial groundwaters expressed in $\mu g\ L^{-1}$. Isoconcentration lines were drawn via geographic and hydrologic interpolation of monitoring data through the use of the MT3D computer program [57].

Well sampling procedures

Wells were sampled by peristaltic pumping. For monitoring the chemical and physical characteristics of the pumped groundwater, flow-through probe cylinders were connected in series (Grant/YSI 3800, Yellow Springs Instrument Co, Yellow Springs, OH, USA and a Hydrolabs H20G/Scout 2 combination, Hydrolabs Corporation, Austin, TX, USA). These probes allowed the monitoring of temperature, pH, dissolved oxygen, oxidation-reduction potential, conductivity, and salinity. Wells were purged the longer of either three well volumes or until the probe readings became stable. Probe outputs were recorded once they had stabilized. The probes were then disconnected and groundwater was collected by filling sterile 4-L glass containers. The filled containers were sealed with a lid, placed on ice, and immediately transported to the laboratory. Samples intended for anaerobic studies were collected under a blanket of nitrogen by sparging the collected groundwater with nitrogen through a sterile aquarium airstone.

Four wells were selected for microbiological examination (MW-15S, MW16S, MW-24S, MW-12S; Figure 1). These wells represent conditions at the center of the plume (MW-12S), at moderate contamination (MW-16S, MW-24S), and background (MW-15S).

Water analyses

After well water samples were collected, all analytical procedures were typically performed within 4 h, and no later

than 12 h after collection. Quantitative tests for nitrate, nitrite, sulfate, sulfite, sulfide, free chlorine, ammonia, and ortho-phosphate were performed using a Hach DR/3000 spectrophotometer (Hach Company, Loveland, CO, USA).

Groundwater methane at the wells was measured by headspace analysis. Immediately after collection, well waters were fixed with formaldehyde (25 ml per 225-ml water sample) and sealed in glass jars. After transporting the samples to the laboratory, 5 ml of water was rapidly transferred to a 20-ml vial, sealed with a septum, and shaken to strip volatile gasses from the water. A 200-μl sample of headspace gas was injected into a Perkin Elmer model 8500 gas chromatograph fitted with a stainless-steel column (2.8 m $\times$ 0.3 cm) packed with Chromosorb 102 80/100, a flame ionization detector, and helium gas as a carrier gas. The temperatures of the injector and detector were 200 and 270°C, respectively. The oven temperature was a constant 50°C.

Microbiological enumeration

Total aerobic heterotrophs, aerotolerant anaerobes, and benzene-, toluene-, and chlorobenzene-degraders were enumerated by plate count methods. Aerobic heterotrophs and aerotolerant anaerobes were plated on dilute PTYG medium [3]. GasPak jar systems (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) were used to generate reduced conditions for anaerobe enumerations. Microorganisms able to use benzene, chlorobenzene, or toluene were enumerated on mineral medium [27], with the exception that Noble agar (Difco, Detroit, MI, USA) was used at 16.5 g L^{-1}. The plates were inverted and placed into humidified 10-gall glass aquaria that had been fitted with Teflon™ lids. Both water vapor and the organic solvent were supplied via the vapor phase. All plates were incubated at 25°C. Aerobes were counted after 7 and 14 days. All other plates were counted after 14 days.

Aerobic metabolism

Respirometry experiments were performed in Bioscience™ (Bethlehem, PA, USA) respirometers which were operated in accordance with manufacturer-specified procedures except that the bioreactors were modified to permit repeated sampling and to minimize volatilization losses by the addition of a side-arm sampling port capped with a Mininert™ valve (Supelco, Bellefonte, PA, USA). Three or more replicates were used in all experiments. Abiotic (killed) controls were treated by adding mercuric chloride (3.2 g L^{-1}) and acidifying the suspension to pH 2 with sulfuric acid.

Determinations of mineralization through the monitoring of $^{14}CO_2$ liberation from radiolabelled parent compounds were performed as previously described [35].

Purge and trap gas chromatography

Purge and trap gas chromatography was conducted to determine levels of benzene, toluene, and chlorobenzene in water samples derived from studies of aerobic metabolism. A Tekmar (Cincinnati, OH, USA) 2016/2032/3000 purge and trap autosampler complex was coupled to a Hewlett-Packard (Palo Alto, CA, USA) 5890 Series II Plus gas chromatograph. The chromatograph was fitted with a 30 m $\times$ 0.25 mm long HP-5 MS capillary column. Peak detection was by tandem photo- and flame-ionization detection (PID and FID, respectively; OI Analytical 4430 PID, College Station, TX, USA). The instrumentation was controlled by TekLink (Tekmar) and GC EnviroQuant (Hewlett-Packard) software.

Instrument parameters were as follows: helium was used as the carrier gas, at 0.6 ml min^{-1} and a split ratio of 23:1. A 3.8 ml min^{-1} septum purge was used. Air flow was 165 ml min^{-1} and the sweep gas was hydrogen at 35 ml min^{-1}. Make-up gas for the PID was 30 ml min^{-1} helium. The injector temperature was 200°C and the temperatures at the detectors were 250 and 200°C for the PID and the FID, respectively. Samples were purged for 8 min at 40°C and desorbed from the trap for 2 min at 180°C. The trap was then baked in preparation for the next sampling event. Oven temperature started at 35°C, held for 6 min, then rose at a rate of 10°C per min to a temperature of 110°C. The ramp was then increased to 50°C per min to a final temperature of 200°C. The total analysis time was 16.3 min.

Validation of the purge and trap gas chromatography procedures for quantifying benzene, toluene and chlorobenzene, including calibration curves, continuing calibration criteria, lab background and system integrity checks, spiked sample analysis criteria, minimum detection limits and method precision and accuracy, was carried out as per EPA Method 602 [52]. In all cases system performance exceeded these guidelines.

Modeling of contaminant fate through the alluvium

The MT3D-based hydrogeologic site model was used as the basis for contaminant fate modeling. The field was conceptually divided into six segments that spanned the plume perpendicular to the flow path, these were MW17S-MW13S; MW13S-MW12S; MW12S-MW24S; MW24S-MW16S; MW16S-MW23S; and MW23S-MW15S (Figure 1). Electron-acceptor depletions, determined during field testing events, were combined with hydrologic flow measurements to model electron-acceptor fluxes across the field segments. Electron acceptor losses due to abiotic mechanisms (which would be expected to affect all of this geologically homogeneous plume area equally) were automatically eliminated from the calculations since the relative differences in electron acceptor concentrations between wells were used as the measures of electron acceptor losses. The per line segment electron acceptor flux values were then combined to determine electron-acceptor flux values within the contaminant plume. The contaminant composition and concentrations, which were determined in the process of developing the site model (Appendix I), and the stoichiometry of electron-acceptor utilization (Appendix II) were used to convert electron-acceptor flux to the mass of contaminant metabolized across the field.

Results

Physical and chemical parameters

Alluvial groundwater and the adjacent river were sampled during each of three seasons. Each of 14 water chemistry parameters was measured twice each seasonal trip for each

Table 1 Concentrations of selected parameters: volatile organics, potential electron acceptors and methane

Well number	Log VOC (ppb)[a]	Dissolved O_2 (ppm)[b]	Nitrate (ppm)[b]	Sulfate (ppm)[b]	Log methane (ppm)[c]
MW-12S	6.1	0.2	0[d]	0	4.3
MW-13S	5.2	0.2	0	0	ND
MW-24S	3.3	0.2	0.6	0.8	4.4
MW-16S	2.7	0.2	0.8	42.0	3.0
MW-17S	2.0	0.3	2.0	64.5	ND
MW-26S	1.7	4.4	3.2	50.0	ND
MW-03S	1.5	1.6	4.2	39.5	ND
MW-15S	1.5	5.7	7.8	58.9	1.6
MW-23S	1.0	2.3	2.9	35.9	ND

[a]Determined by gas chromatography/mass spectrometry and taken from site RFI data. These data are the per well averages of over 13 000 VOC compound determinations made in the process of monitoring the site groundwater.
[b]Average of two observations on each of three sampling events.
[c]Determined in duplicate by gas chromatography of headspace gasses on each of three sampling events.
[d]A zero in the table indicates a reading of zero or a reading below the minimum detection limit.
ND = not determined.

of nine monitoring well waters and the river, producing a total of 840 observations (Table 1). Figure 2 shows results of a correlation analysis of the pooled and averaged conductivity data set for nine well water samples as plotted against the historical VOC concentrations of the individual wells. Conductivity ranged from 0.3 to 2.5 mS and salinity (one potential contributor) ranged from 0.1 to 1.4 parts per thousand. Except for the low VOC concentration wells, there is a nearly linear relationship between conductivity and VOC concentration. The Pearson product-moment coefficient [49] for the full data sets of log conductivity to log VOC concentration was 0.87. This positive relationship between conductivity and VOC concentration is a possible indicator of biodegradation of halogenated compounds known to be present in the site subsurface (see Appendix I).

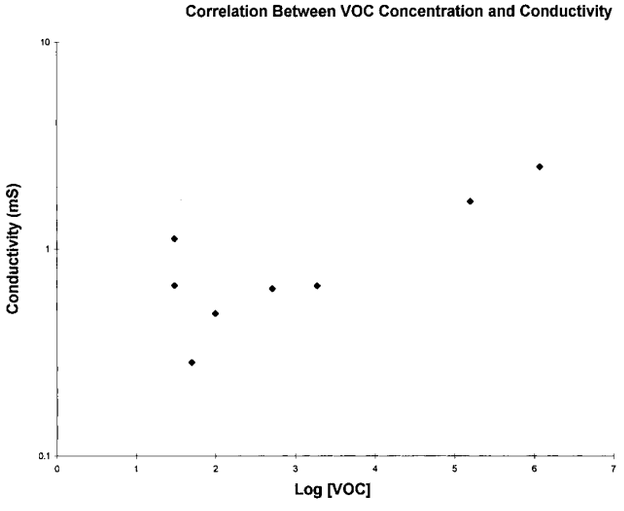

Figure 2 Relationship between conductivity of groundwaters and their volatile organic carbon (VOC) content. Each data point represents the per well average of duplicate conductivity observations on each of three sampling events for a total of six observations. Historical groundwater VOC concentrations are the per well averages of in excess of 13 000 individual VOC compound determinations taken over 4 years.

All water chemistry measurements were compared to VOC concentration in the manner shown for conductivity (Figure 2). Table 2 summarizes the correlations between VOC concentration and the measured parameters for the nine monitoring well waters. Correlation coefficients are given as Pearson product-moment correlations [49]. Since the parameters are believed to be biologically driven, Colton correlation rankings [9] are given. Commonly used in medicine and biology, the wide ranges for Colton correlations are a reflection of the inherent variability in biological systems [11].

Temperature and pH were relatively constant for the groundwaters. The range of average groundwater temperatures (pooled for each well) ranged from 12.1°C at MW-15S to 18.7°C at MW-03S. Moderate temporal variation over the three sampling time points was observed when the well temperature values were pooled (standard deviation of 2°C). Average pH (per well per sampling trip) ranged from 6.2 to 7.3. Standard deviations for the pH of each well water across all the three testing events were typically 0.07 pH units. This shows that aquifer pH varied little on the site, either temporally or spatially.

Dissolved oxygen ranged from 0.2 to 5.7 ppm (Table 1). These values represent upper boundaries for actual dissolved oxygen concentrations as some penetration of surface oxygen commonly occurs via the well casings. Oxidation-reduction potential ranged from a maximum of 221 to at least −193 mV. As with oxygen concentrations, the redox values are also upper boundary limits for the actual redox potentials in the site subsurface.

Nitrate concentrations ranged from undetected to 7.8 ppm (Table 1). Ammonium ion ranged from undetected to 24.7 ppm. Nitrate was only transiently noted at levels near the detection limit. Sulfate and sulfite concentrations correlated negatively with VOC concentrations. Sulfate

Table 2 Correlation of various parameters to VOC concentration

Parameter measured	Correlation coefficient[a] with VOC concentration	Proportionality of parameter to VOC concentration	Colton correlation[b] rating
Conductivity	0.87	Direct	Very Good to Excellent
Salinity	0.84	Direct	Very Good to Excellent
o-Phosphate	0.60	Direct	Moderate to Good
Redox	−0.84	Inverse	Very Good to Excellent
Nitrate	−0.77	Inverse	Very Good to Excellent
Dissolved oxygen	−0.67	Inverse	Moderate to Good
Sulfate	−0.54	Inverse	Moderate to Good

[a]Given as the Pearson product-moment correlation coefficient [49].
[b]Colton coefficients [9], a measure of relation between numerical measures in medical and biological statistics, use the following criteria for rating assignment (given on an absolute value basis): 0–0.25, little to no relationship; 0.25–0.50, fair degree of relationship; 0.50–0.75, moderate to good relationship; greater than 0.75, very good to excellent. The wide ranges of correlation that are accepted under the Colton criteria are a reflection of the inherent variability of biological systems [11].

Table 3 Chemical and microbiological characteristics of site groundwaters

Well number	Log VOC conc (ppm)[a]	Log conc B + T + C (ppm)[a]	Log AODC counts[b]	Log aerobic heterotrophs[c]	Log aerotolerant anaerobes[c]	Log benzene degraders[c]	Log toluene degraders[c]	Log chlorobenzene degraders[c]	Log methane concentration (ppm)[d]
MW-12S	6.1	5.6	6.8	5.3	3.7	2.8	3.2	1.8	4.3
MW-24S	3.3	3.0	5.6	3.2	1.4	0.8	0	1.0	4.4
MW-16S	2.7	2.8	4.3	2.8	1.0	0.7	0.7	0.9	3.0
MW-15S	1.5	0	4.8	2.8	1.8	0	1.2	0	1.6

[a]Determined by gas chromatography and taken from site RFI data. These data are the per well averages of over 13 000 VOC compound determinations made in the process of monitoring the site groundwater.
[b]AODC = acridine orange direct counts (cells ml^{-1}) as described by Balkwill and Ghiorse [3].
[c]Average of triplicate plate count observations (CFU ml^{-1}) made on each of three sampling events.
[d]Determined in duplicate by gas chromatography of headspace gasses on each of three sampling events.

concentrations ranged from a high of 64.5 ppm to undetected (Table 1). Sulfite concentrations ranged from 5.0 ppm to undetected. Like nitrite, sulfide was noted transiently at or near the detection limit. Ortho-phosphate concentrations ranged from 1.7 to 0.8 ppm. The 'moderate to good' correlation between *o*-phosphate and VOC concentration shown in Table 2 was unexpected. As biomass also correlates with VOC (Table 3) we speculate that the increased *o*-phosphate concentrations near the core of the plume may be due to the release of phosphate from the turn-over of microbial biomass. Nitrate, sulfate, and *o*-phosphate concentrations (8, 59, and 1 ppm, respectively) in the background waters indicated no dearth of these key inorganic nutrients in the waters initially contacting the contaminant plume.

Field electron acceptor distribution

Microbial metabolism of organic pollutants requires the presence of suitable electron acceptors. For a given electron donor, free energy yields for the acceptors are oxygen > nitrate > manganese > iron > organic > sulfate > inorganic carbon [50]. Analyses of well waters show that electron acceptors were depleted across the site as groundwater entered the contaminant plume. Figure 3 presents the acceptor concentration data, with notation of the 20% depletion of background concentration lines. The depletion lines were determined from the means of 90 site survey well water electron-acceptor concentration measurements (Table 1) and interpolation consistent with the site hydrogeological model and contaminant distribution patterns.

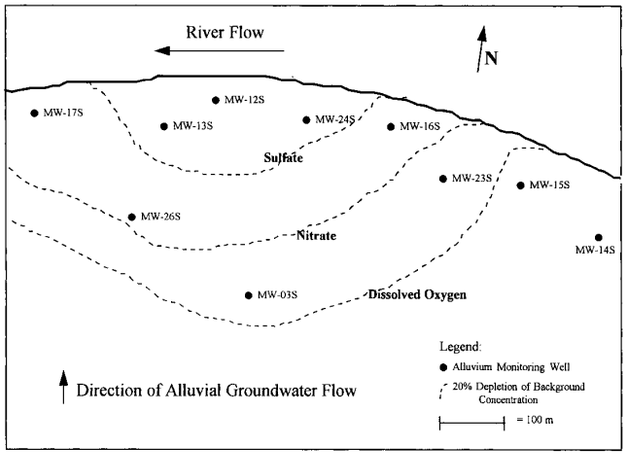

Figure 3 Map of electron-acceptor depletion. The plotted lines represent electron-acceptor concentration at 20% depletion of the background (MW-15) concentration. Depletion lines were determined from the means of site survey well water electron-acceptor concentration measurements and interpolation consistent with the site hydrogeological model and contaminant distribution patterns.

Electron acceptors were depleted along the hydrologic flow and contaminant concentration path (Table 1). Furthermore, electron acceptors were depleted in a manner consistent with the thermodynamic properties of the electron acceptors. The plot of the 20% depletion of background concentation lines in Figure 3 shows some spatial separation of electron acceptor depletion. This depletion pattern correlates with the dominant direction of groundwater flow and correlates inversely with the contaminant concentration gradient (Table 2). Limited site data also showed that dissolved iron and dissolved manganese, presumably the result of the use of iron and manganese as electron-acceptors, also had distribution patterns (not shown) that were consistent with *in situ* metabolic events. These patterns constitute evidence that microbial metabolism was occurring within the subsurface contaminant plume.

Field microbiological parameters

A linkage between VOC concentration and microbiological activity is also shown by the data presented in Table 3. As indicated by viable counts and acridine orange direct counts, population density increased with increasing VOC concentration. The Pearson correlation coefficients between the biomass parameters, the sum of the benzene, toluene and chlorobenzene concentrations and VOCs are given in Table 4. There is a strong direct correlation between biomass (in all measured forms) and VOC concentration.

The Pearson correlation coefficients also indicate that biomass was more closely correlated to the total VOC concentration than to BTC concentration (Table 4). The bulk of the volatiles in the groundwater at this site were identified as short chain aliphatics (See Appendix I). Since these compounds are more easily degraded than BTC, *in situ* microbial growth should be positively correlated to VOC concentration. Despite possible preferential VOC consumption, biomass correlated closely with BTC concentration. This suggests that BTC were also being used as growth substrates. Were BTC consumption minimal or absent, the

Table 4 Correlations of various measures of biomass and microbial activity with potential carbon source concentrations, ammonia concentration, and selected potential electron acceptors

	Pearson correlation coefficients[a]					
	Log VOC concentration	Log B+T+C concentration	Log dissolved oxygen	Log nitrate concentration	Log ammonia concentration	Log sulfate concentration
Acridine orange direct counts[b]	0.89	0.75	NS	NS	0.57	–0.84
Aerobic heterotrophs[c]	0.95	0.83	NS	NS	NS	–0.66
Aerotolerant anaerobes[c]	0.82	0.63	NS	NS	NS	–0.50
Benzene degraders[c]	0.99	0.94	–0.61	NS	0.50	–0.68
Toluene degraders[c]	0.74	0.58	NS	NS	NS	NS
Chlorobenzene degraders[c]	0.96	0.99	–0.85	–0.74	0.72	–0.76
Methane concentration	0.78	0.85	–0.90	–0.88	0.97	–0.92

[a]Given as the Pearson product-moment correlation coefficient [49]. NS = not significant (ie, correlation coefficient greater than –0.50 but less than 0.50).
[b]Acridine orange direct counts (cells ml^{-1}) performed as described by Balkwill and Ghiorse [3].
[c]Determined by plate count methods (CFU ml^{-1}) and represent the mean of triplicate observations from each of three sampling events.

correlation between BTC concentration and biomass would be expected to be weak.

The various measures of biomass also showed some correlations with electron acceptor and ammonia distribution (Table 4). There were not as many significant correlations with biomass for any particular electron acceptor or ammonia as there were for carbon-source measurements. This may be a result of a single aggregate source for carbon (ie VOCs, with BTC being a large fraction thereof), but also could reflect the simultaneous involvement of multiple electron-acceptors. The multiple potential electron-acceptors which could be used will, in effect, mask causal relationships between metabolism and electron acceptor depletion for any particular electron acceptor.

Direct field evidence for in situ metabolism

Specific evidence that benzene and toluene were metabolized *in situ* by site groundwater microorganisms is shown in Figure 4. Benzene, toluene, and chlorobenzene have similar physical and chemical properties and are therefore expected to have similar diffusivities and mobilities [42]. Because chlorobenzene is the most recalcitrant of the trio of BTC compounds, it was used as a relatively conserved tracer to reveal selective depletion of benzene and toluene in site waters. The ratios of benzene and toluene to chlorobenzene showed a clear and constant decline from the areas of highest to areas of lowest chlorobenzene concentration (Figure 4). This decrease in ratio would be consistent with benzene and toluene being removed faster than chlorobenzene *in situ*. Furthermore, comparisons of this figure with Table 1 and Figure 3 demonstrate that the removal occurred across regions of the plume demonstrating depletion of electron acceptors. This removal is consistent with known rates and mechanisms of benzene-, toluene-, and chlorobenzene-metabolism and provides evidence that at least B and T were biodegraded *in situ*.

Laboratory aerobic metabolism studies

Removal of benzene, toluene and chlorobenzene by the groundwater microorganisms followed the general pattern of a long initial linear phase followed by log removal (Figure 5). The inset on Figure 5 shows the removal data plotted in first order form (ln C/Co). Two phases of removal

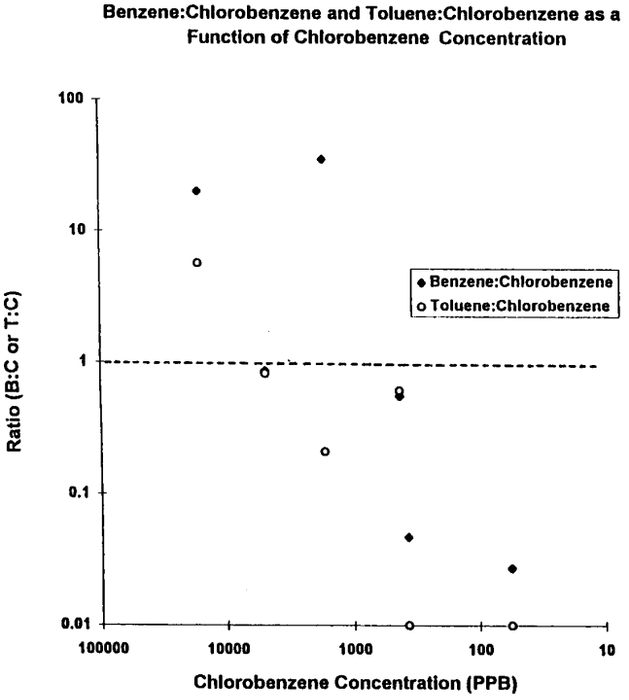

Figure 4 Ratios of B:C and T:C versus the concentration of chlorobenzene. The dashed line represents an invariant ratio between parameter pairs. Since the ratios between B and C and T and C are not constant, benzene and toluene must be preferentially removed from contaminated groundwater relative to chlorobenzene. Each point is the ratio of the per well averages of the noted compounds. Each well was measured for each of the indicated compounds at least 10 times.

are denoted by the regression lines. Both phases are statistically different from the killed controls and from each other ($P = 0.05$). The initial, slower phase of removal had a $k = -0.002$ while the second phase had a $k = -0.046$. The presence of two metabolic phases is probably an artifact of acclimation time for the microcosms, as noted by others [10,23].

The three compounds were removed at differing rates. At the end of the experiment, 95% of the initial 32 ppm of benzene had been removed, and 83% of each of the initial 32 ppm toluene and 16 ppm chlorobenzene had been

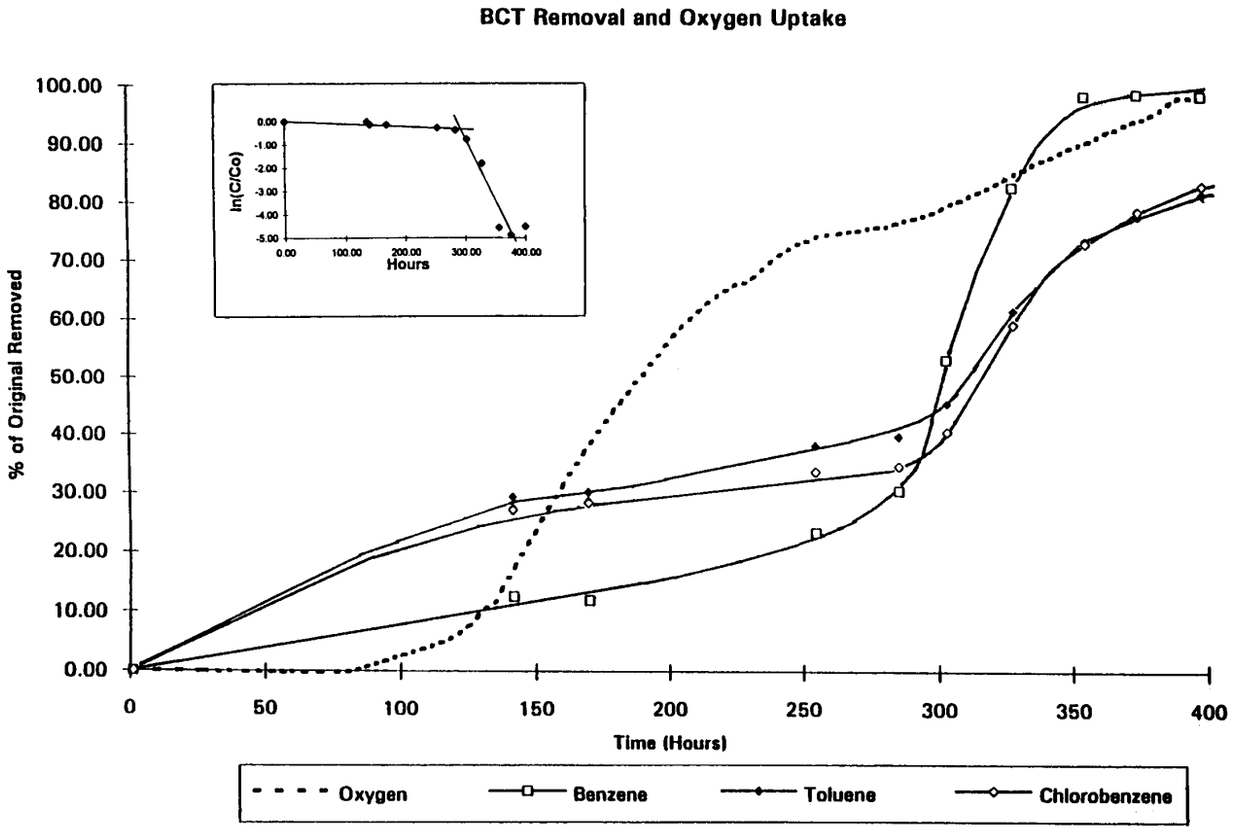

Figure 5 BTC depletion and oxygen uptake under aerobic conditions. For oxygen consumption, the *y*-axis represents % of total removed while for BTC the *y*-axis represents % of the starting concentration that was removed. The inset shows the first-order transformation of the benzene removal data. The benzene consumption from 0 to 300 h is statistically different from control losses (as shown by 95% confidence intervals). For both graphs, the data points are the average of triplicate observed data points minus the average of triplicate control sample values.

degraded. Oxygen uptake displayed an initial lag to approximately 80 h after the start of the experiment. The fastest rate of oxygen uptake occurred between 125 and 215 h. After that time, oxygen uptake continued at a slightly reduced rate.

Mineralization of benzene, toluene, and chlorobenzene

Aerobic incubations of well waters demonstrated mineralization of benzene, toluene and chlorobenzene. Experiments using radiolabelled contaminants evolved radiolabelled carbon dioxide over a 33-day period indicating that site microorganisms were capable of mineralizing the compounds of concern (Figure 6). Microorganisms from background well 15 (MW-15S) mineralized all three compounds. The rates of mineralization of benzene and toluene were virtually identical throughout the experiment. Chlorobenzene was mineralized at approximately half the rate of benzene and toluene. Mineralization of toluene was noted in waters from wells 16 and 24 (MW-16S, MW-24S). The largest quantity of compound mineralized was noted from well 12 (MW-12S). In that case, 40 ppm of benzene (13.0% of the total amount present) and 2 ppm of chlorobenzene (5.6% of the total) were mineralized. Less than 5% mineralization of the total amount of compound present was noted for benzene and chlorobenzene for wells 16 and 24 and for toluene for well 12. Reasons for the absence of mineralization activity in some wells is uncertain, however diauxie, toxicity, or microcosm nutrient limitations may have been involved.

Modeling of mass balances for contaminant flow

Conceptually, the plume was broken into six segments aligned from west to east. Electron-acceptor consumption data for these segments was obtained from the site hydrogeological model (developed by Nittany Geosciences, State College, PA, USA; [57]). Electron acceptor concentrations in these segments were taken to be the average of the concentrations at the two wells bounding each site line segment; the low contaminant-concentrations were taken at MW-15S. Concentration values represent an average of the values from the three site field trips. Table 5 summarizes the relevant information. Kilogram per year values were calculated by integrating the flux and concentration data over 12 months. As an example, for segment 17S-13S and dissolved oxygen (Table 5), a flux of 10.1 $m^3\ day^{-1}$ was multiplied by 1000 L m^{-3} and by 365 day y^{-1} to give L y^{-1} of groundwater flux within the segment. This quantity was then multiplied by the amount of dissolved oxygen depleted from background (MW-15S) to the line segment (5.7 ppm–0.2 ppm) and the result was divided by 10^6 to give kg y^{-1} of dissolved oxygen consumed.

The modeled consumption of electron acceptors was used to calculate masses of VOC and other accompanying hydrocarbons oxidized *in situ*. From historical contami-

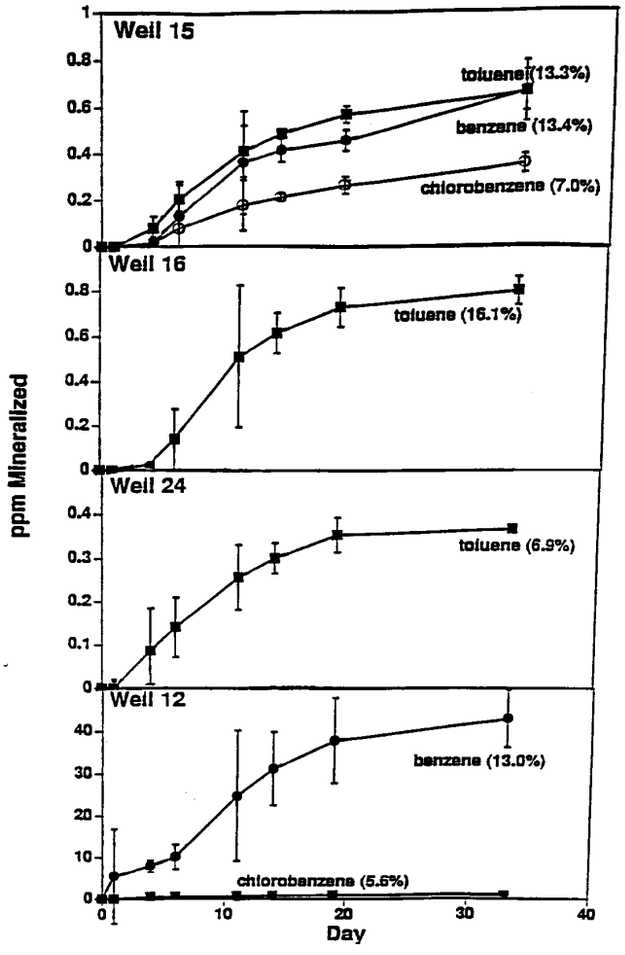

Figure 6 Mineralization of benzene, toluene, and chlorobenzene in site well waters by groundwater microorganisms over 33 days of incubation. Each point is the average of triplicate observations. Error bars indicate standard deviations. Plots show the treatments where the amount of radiolabelled CO_2 evolved exceeded more than 5% of the initial parent compound mixture. Parenthetical notations following the lines indicate the percent mineralization of the total amount of compound present in the reaction mixture.

Table 5 Consumption of electron acceptors across site segments

Segment	Flux (m^3 day^{-1})	Average measured concentrations			Calculated mass flows		
		DO (ppm)	NO3 (ppm)	SO4 (ppm)	DO (kg y^{-1})	NO3 (kg y^{-1})	SO4 (kg y^{-1})
17S–13S	10.1	0.2	1.0	32.2	20.3	25.1	98.4
13S–12S	7.3	0.2	0	0	14.7	20.8	156.9
12S–24S	8.8	0.2	0.3	0.4	17.7	24.1	187.9
24S–16S	40.7	0.2	0.7	21.4	81.7	105.5	557.1
16S–23S	6.8	1.2	1.9	38.9	11.2	14.6	49.6
23S–15S	15.6	4.0	5.4	47.4	9.7	13.7	65.5
15S	NC[a]	5.7	7.8	58.9	NC	NC	NC
Sum					155	204	1115

[a]NC = Not calculable. Alluvial groundwater flux data are from the Nittany Geosciences hydrodynamic model for the site. Averaged measured electron acceptor concentrations are from the present paper.

nation data an 'average' VOC composition ratio of $C_{19}H_{28}O_2$ was derived (Appendix I). Through stoichiometric analysis (Appendix II) the mass of electron acceptors consumed (155 kg y^{-1} DO, 204 kg y^{-1} nitrate, and 1115 kg y^{-1} sulfate) corresponds to 450 kg per year of VOC consumed ((155 kg y^{-1} DO/2.8 kg DO kg^{-1} VOC) + (204 kg y^{-1} nitrate/3.7 kg nitrate kg^{-1} VOC) + (1115 kg y^{-1} sulfate/3.3 kg sulfate kg^{-1} VOC) $\approx$ 450 kg y^{-1} VOC). The 450 kg per year consumption ignores contaminant removal through metabolic use of metals (notably iron) and carbon dioxide as electron acceptors. This figure also ignores the contribution west of MW-17S in which both oxygen and nitrate would be depleted (Figures 1 and 3). This estimated VOC consumption figure also ignores any microbial activity north of the site line segment wells. In addition, although lab data show that degradation in the river sediment would be substantial (not shown), contributions by this process are not accounted for in this analysis. All these factors would add significantly to electron acceptor, hence VOC and BTC, consumption. The removal estimate is therefore a conservative estimation of contaminant consumption.

The estimated mass of VOC and BTC consumption compared favorably with results from the site hydrogeologic model (Nittany Geosciences, State College, PA, USA), which suggests that in the absence of biodegradation 363 kg of VOC y^{-1} would enter the adjacent river. This 363 kg figure is inconsistent with previous field studies documenting no observable impact on the river nor on benthic, aquatic or riparian receptor species (unpublished data). If 363 kg of VOC actually entered the river annually, the effect would be measurable: high ppm levels of VOC would be present in the sediment. Monitoring of the river has only transiently noted low ppb levels of contaminants in the sediment and has not detected BTC in the river water (unpublished RFI data). As can be calculated from the flux data in Table 5, as little as 0.16 kg per year of any VOC compound flowing through the site, in the absence of biodegradation, would result in a sediment concentration at or near detection (5 ppb). Other forces must, therefore, be reducing the VOC burden in the groundwater from 363 kg y^{-1} to less than 0.2 kg y^{-1}. Comparison of the results from hydrogeological modeling with these biodegradation results (450 kg VOC consumed y^{-1}) demonstrates that natural attenuation removes a significant portion of the alluvial VOC before it can affect the river, thereby explaining the apparent lack of impact on the biota.

Discussion

The data presented in this paper provide evidence for intrinsic bioremediation of the site subsurface [39]:

(1) Established biodegradation of BTC. There is an extensive body of published literature establishing that benzene, toluene, and chlorobenzene are biodegradable. The physiological conditions for microbial metabolism are common in field sites, the biodegradative activities are widely distributed in nature, and, as a result of microbial activity BTC subsurface contamination can be expected to decrease over time.

(2) Site physical and chemical conditions. The physical-chemical conditions at the study site, like a variety of subsurface habitats, were conducive to biodegradation processes [2]. All major nutrients were present in concentrations high enough to support microbial growth.

(3) Laboratory studies of aerobic metabolism and mineralization of BTC. Aerobic metabolism of BTC by site microorganisms displayed biphasic kinetics (Figure 5). Although noted by other researchers and explained as acclimation time, it is not clear whether the two apparent phases of BTC metabolism are due to growth of the BTC degrader population, loss of a metabolic inhibitor, or preferential use of alternate carbon sources. It is unlikely that the first phase is competely explainable as a physiological acclimation time, as appreciable oxygen consumption occurred during this phase. Although an increase in the BTC degrader population is a possible reason for the change in rates, only a 1-log increase in total cell count was seen during the course of the experiment (data not shown). Furthermore, the presence of other potential carbon sources in the groundwater (Appendix I) makes it unlikely that the population expanded solely by metabolizing BTC. It is probable that the relative proportion of BTC degraders to other microorganisms changed during the course of the experiment. The loss of a metabolic inhibitor would seem unlikely as substantial oxygen uptake occurred throughout most of the initial phase of BTC degradation. The third possibility, preferential metabolism of alternative carbon sources in mixed cultures, has been previously noted by other bioremediation investigators. Swindoll *et al* [51] observed inhibition of aerobic metabolism of aromatics in subsurface sediment samples when more easily degradable carbon sources were present. A similar phenomenon has been noted under anaerobic conditions. In that case, degradation of acenaphthene and naphthalene was inhibited by naturally occurring organic carbon in soil [36,37]. A lag in removal of the compounds of concern with preferential use of other hydrocarbons has also been noted in groundwater-based experiments similar to these BTC removal studies [22] and in soil-based contaminant mineralization experiments [29]. Further testing is required to determine the exact cause of the apparent dual-phase BTC removal for this groundwater. Regardless of the reason for the apparent two phases of BTC removal, BTC was metabolized under aerobic conditions by the microorganisms in the groundwater from this site.

Microorganisms from the site subsurface were capable of converting BTC to their ultimate biodegradation end products, carbon dioxide and water (Figure 6). Due to imperfect mass balances in the tests, the rates of BTC mineralization shown in Figure 6 are conservative. Nonetheless, the data unequivocally demonstrate that microorganisms in site well waters were able to metabolize BTC and that this potential was most strongly expressed in aerobic waters on the fringe of the contaminant plume. As expected, benzene and toluene were more extensively aerobically mineralized than chlorobenzene.

(4) Field inorganic compound distribution patterns. The greatest limitation to bioremediation is usually the availability of electron acceptors [2]. Bacteria consume typical electron acceptors in the order oxygen > nitrate > manganese > iron > organics > sulfate > inorganic carbon [50]. Field data show that electron acceptor depletion occurs in the site subsurface (Table 1 and Figure 3). Furthermore, these depletions show partial spatial separation which is consistent with the thermodynamics of electron acceptor utilization by microorganisms. Although the field would be expected to have microniches in which utilization of different electron acceptors would occur in close proximity to each other, overall the field is expected to show gradients of electron acceptor usage. Conceptually these gradients would be distributed as overlapping, irregular bell-shaped curves whose relative positions are controlled by and reflect oxidation-reduction potentials and electron acceptor depletion. The approach taken here (Figure 3) examined electron acceptor usage as a percentage of background concentration and showed significant inverse correlations between electron acceptors and VOC concentration (Table 2). Thus electron acceptors are being consumed over the entire site. The geochemical gradients found at this site support the hypothesis and VOC contaminant compounds in the alluvium cause microorganisms to deplete final ambient electron acceptors in patterns predicted and found by others [31,45,54].

(5) Biomass distribution patterns. The size of every microbial population measured varied in proportion to the groundwater VOC and BTC concentrations across the plume (Table 4). The finding that aerobic biomass was most abundant in the most anaerobic region of the plume suggests widespread distribution of facultatively aerobic anaerobes and/or transient influx of dissolved oxygen from occasional heavy rain events. VOC and BTC concentrations also correlated with subsurface methane concentrations. These points indicate that metabolism was occurring within the plume and that the carbon source driving the metabolism was most likely the VOC and BTC contamination present within the site subsurface.

(6) Selective depletion of benzene and toluene in site waters. Contaminant concentrations in groundwater can decrease due to such factors as dilution, dispersion, adsorption and volatilization. Therefore, field studies typically use a tracer molecule (often fortuitously present in the mixtures) to monitor these factors; the concentrations of contaminants are then compared to the tracer in an effort to distinguish biotic from abiotic processes. The structural, chemical and physical similarities among benzene, toluene and chlorobenzene suggest that the three compounds will have similar mobilities [42]. Chlorobenzene, the more recalcitrant of the three compounds, also provided a conservative measure of microbiological removal for benzene and toluene, since at least some chlorobenzene was expected to be removed microbiologically. Monitoring the relative ratios of more and less degradable contaminants has previously been used for both flowing

[5,40,47,48] and non-flowing [20] systems. In the present study, the ratios B : C and T : C fell by several orders of magnitude as the chlorobenzene concentration was diminished (Figure 4). Therefore, field data suggests that benzene and toluene were removed at a faster rate than chlorobenzene. This is the pattern that would be expected if a selective process such as *in situ* bioremediation had occurred.

(7) Electron acceptor consumption and modeling. Hydrogeological models were used to calculate the mass of contaminants consumed in the field. By combining the alluvial groundwater flux with electron acceptor concentration data, the mass of electron acceptors reduced was determined. From this, and the contaminant mass and composition, the mass of contaminants oxidized was calculated. There was insufficient information on the concentration of CH_4 in this site's groundwater, therefore the impact of methanogenesis for this site could not be directly calculated this way. The model estimated that over 450 kg per year of VOC were consumed through natural attenuation. This figure ignores contaminant consumption through methanogenesis, consumption due to the use of metals as elecron acceptors, the mass removal north of the monitoring wells, and the mass removal west of MW-17S.

The information presented here adds to the growing body of case studies which demonstrate that naturally occurring microbial communities actively respond to and eliminate organic contaminants from field sites.

References

1 Allen-King RM, JF Barker, RW Gillham and BK Jensen. 1994. Substrate- and nutrient-limited toluene biotransformation in sandy soil. Environ Toxicol Chem 13: 693–705.
2 Baker KH and DS Herson. 1994. Bioremediation. McGraw-Hill, New York, NY.
3 Balkwill DL and WC Ghiorse. 1985. Characterization of subsurface bacteria associated with two shallow aquifers in Oklahoma. Appl Environ Microbiol 50: 580–588.
4 Barbaro JR, JF Barker, LA Lemon and CI Mayfield. 1992. Biotransformation of BTEX under anaerobic, denitrifying conditions: field and laboratory observations. J Contam Hydrol 11: 245–272.
5 Barker JF, JS Tessman, PE Plotz and M Reinhard. 1986. The organic geochemistry of a sanitary landfill leachate plume. J Contam Hydrol 1: 171–189.
6 Borden RC, CA Gomez and MT Becker. 1995. Geochemical indicators of intrinsic bioremediation. Ground Water 33: 180–189.
7 Bouwer EJ and PL McCarty. 1983. Transformations of halogenated organic compounds under denitrification conditions. Appl Environ Microbiol 45: 1295–1299.
8 Chiang CY, JP Salanitro, EY Chai, JD Colthart and CL Klein. 1989. Aerobic biodegradation of benzene, toluene, and xylene in a sandy aquifer—data analysis and computer modeling. Ground Water 27: 823–834.
9 Colton T. 1974. Statistics in Medicine. Little, Brown Inc, New York.
10 Davis JW, NJ Klier and CL Carpenter. 1994. Natural biological attenuation of benzene in ground water beneath a manufacturing facility. Ground Water 32: 215–226.
11 Dawson-Saunders B and RG Trapp. 1994. Basic and Clinical Biostatistics. pp 52–54, Appleton and Lange, Norwalk, CT.
12 Dolfing J, J Zeyer, P Binder-Eicher and RP Schwarzenbach. 1990. Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch Microbiol 154: 336–341.
13 Edwards EA and D Grbic′-Galic′. 1994. Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium. Appl Environ Microbiol 60: 313–322.
14 Edwards EA, LE Wills, M Reinhard and D Grbic′–Galic′. 1992. Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. Appl Environ Microbiol 58: 794–800.
15 Erlich GG, DF Goerlitz, EM Dodsy and MF Hult. 1982. Degradation of phenolic contaminants in ground water by anaerobic bacteria. Ground Water 20: 703–710.
16 Evans PJ, DT Mang, KS Kim and LY Young. 1991. Anaerobic degradation of toluene by a denitrifying bacterium. Appl Environ Microbiol 57: 1139–1145.
17 General Physics Corporation. 1992. Bioremediation Engineering Workshop Visual Aids handout.
18 Goldsmith CD and RK Balderson. 1988. Biodegradation and growth kinetics of enrichment isolates on benzene, toluene, and xylene. Water Sci Tech 20: 505–507.
19 Grbic′-Galic′ D and TM Vogel. 1987. Transformation of toluene and benzene by mixed methanogenic cultures. Appl Environ Microbiol 53: 254–260.
20 Harkness MR, JB McDermott, DA Abramowicz, JJ Salvo, WP Flanagan, ML Stephens, FJ Mondello, RJ May, JH Lobos, KM Carroll, MJ Brennan, AA Bracco, KM Fish, GL Warner, PR Wilson, DK Dietrich, DT Lin, CB Morgan and WL Gately. 1993. *In situ* stimulation of aerobic PCB biodegradation in Hudson River sediments. Science 259: 503–507.
21 Holm PE, PH Nielsen, HJ Albrechtsen and TH Christensen. 1992. Importance of unattached bacteria and bacteria attached to sediment in determining potentials for degradation of xenobiotic organic contaminants in an aerobic aquifer. Appl Environ Microbiol 58: 3020–3026.
22 Huesemann MH and KO Moore. 1994. The effects of soil type, crude oil type and loading, oxygen, and commercial bacteria on crude oil bioremediation kinetics as measured by soil respirometry. In: Hydrocarbon Remediation (Hinchee RE, BC Alleman, RE Hoeppel and RN Miller, eds), pp 58–71, CRC Press, Boca Raton, FL.
23 Hunt MJ, MA Beckman, MA Barlaz and RC Borden. 1995. Anaerobic BTEX biodegradation in laboratory microcosms and *in situ* columns. In: Intrinsic Bioremediation (Hinchee RE, JT Wilson and DC Downey, eds), pp 101–107, Battelle Press, Columbus, OH.
24 Hutchins SR, WC Downs, GB Smith, JT Wilson, DJ Hendrix, DD Fine, DA Kovacs, RH Douglass and FA Blaha. 1991. Nitrate for Biorestoration of an Aquifer Contaminated with Jet Fuel. EPA/600/S2-91/009.
25 Hutchins SR, SW Moolenaar and DE Rhodes. 1992. Column studies on BTEX biodegradation under microaerophilic and dentrifying conditions. J Haz Mat 32: 195–214.
26 Hutchins SR, GW Sewell, DA Kovacs and GA Smith. 1991. Biodegradation of aromatic hydrocarbons by aquifer microorganisms under dentrifying conditions. Environ Sci Tech 25: 68–76.
27 Karlson U and WT Frankenberger Jr. 1989. Microbial degradation of benzene and toluene in groundwater. Bull Environ Contam Toxicol 43: 505–510.
28 Klecka GM, JW Davis, DR Gray and SS Madsen. 1990. Natural bioremediation of organic contaminants in ground water: Cliffs-Dow Superfund site. Ground Water 28: 534–543.
29 Knaebel DB, TW Federle, DC McAvoy and JR Vestal. 1994. Effect of mineral and organic soil constituents on microbial mineralization of organic compounds in a natural soil. Appl Environ Microbiol 60: 4500–4508.
30 Lovley DR, MJ Baedecker, DJ Lonergan, IM Cozzarelli, EJP Phillips and DI Siegel. 1989. Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature 339: 297–298.
31 Lovley DR, FH Chapelle and JC Woodward. 1994. Use of dissolved H_2 concentrations to determine distribution of microbially catalyzed redox reactions in anoxic groundwater. Environ Sci Technol 28: 1205–1210.
32 Lovley DR, JD Coates, JC Woodward and EJP Phillips. 1995. Benzene oxidation coupled to sulfate reduction. Appl Environ Microbiol 61: 953–958.
33 Lovley DR and DJ Lonergan. 1990. Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. Appl Environ Microbiol 56: 1858–1864.
34 Lovley DR, JC Woodward and FH Chapelle. 1994. Stimulated anoxic biodegradation of aromatic hydrocarbons using iron (III) ligands. Nature 370: 128–131.

35 Madsen EL, JL Sinclair and WC Ghiorse. 1991. *In situ* biodegradation: microbiological patterns in a contaminated aquifer. Science 252: 830–833.
36 Mihelcic JR and RG Luthy. 1988. Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water systems. Appl Environ Microbiol 54: 1182–1187.
37 Mihelcic JR and RG Luthy. 1988. Microbial degradation of acenaphthene and naphthalene under denitrification conditions in soil-water systems. Appl Environ Microbiol 54: 1188–1198.
38 Montgomery L, N Assaf-Anid, L Nies, PJ Anid and TM Vogel. 1994. Anaerobic biodegradation of chlorinated organic compounds. In: Biological Degradation and Bioremediation of Toxic Chemicals (Chaudhry GR, ed), pp 256–276, Dioscorides Press, Portland, OR.
39 National Research Council. 1993. *In-Situ* Bioremediation: When Does It Work?, National Academy Press, Washington, DC.
40 Nelson CH, RJ Hicks and SD Andrews. 1994. An integrated system approach for *in situ* bioremediation of petroleum hydrocarbon-contaminated soil and groundwater. In: Bioremediation: Field Experience (Flathman PE, Jerger DE and JH Exner, eds), pp 429–456, Lewis Publishers, Boca Raton, FL.
41 Nishino SF, JC Spain, LA Belcher and CD Litchfield. 1992. Chlorobenzene degradation by bacteria isolated from contaminated groundwater. Appl Environ Microbiol 58: 1719–1726.
42 Perry RH, DW Green and JO Maloney. 1984. Perry's Chemical Engineers' Handbook, 6th edn. McGraw-Hill, New York.
43 Rabus R, R Nordhaus, W Ludwig and F Widdel. 1993. Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl Environ Microbiol 59: 1444–1451.
44 Reineke W and HJ Knackmuss. 1984. Microbial metabolism of haloaromatics: isolation and properties of a chlorobenzene-degrading bacterium. Appl Environ Microbiol 47: 395–402.
45 Rifai HS, RC Borden, JT Wilson and CH Ward. 1995. Intrinsic bioattenuation for subsurface restoration. In: Intrinsic Bioremediation (Hinchee RE, JT Wilson and DC Downey, eds), pp 1–29, Battelle Press, Columbus, OH.
46 Rochkind ML, JW Blackburn and GS Sayler. 1986. Microbial Decomposition of Chlorinated Aromatic Compounds. EPA/600/2-86/090.
47 Semprini L, GD Hopkins, PV Roberts and PL McCarty. 1991. *In situ* biotransformation of carbon tetrachloride, freon-113, freon-11, and 1,1,1-TCA under anoxic conditions. In: On-Site Bioreclamation, Processes for Xenobiotic and Hydrocarbon Treatment (Hinchee R, and R Olfenbuttel, eds), pp 41–58, Butterworth-Heinemann, Boston, MA.
48 Semprini L, PV Roberts, GD Hopkins and PL McCarty. 1990. A field evaluation of *in-situ* biodegradation of chlorinated ethenes. Ground Water 28: 715–727.
49 Sokal RR and FJ Rohlf. 1981. Biometry, 2nd edn. pp 565–572, WH Freeman and Company, San Francisco, CA.
50 Stumm W and JJ Morgan. 1996. Aquatic Chemistry, 3rd edn. John Wiley and Sons, New York, NY.
51 Swindoll CM, CM Aelion and FK Pfaender. 1988. Influence of inorganic and organic nutrients on aerobic biodegradation and on the adaptation response of subsurface microbial communities. Appl Environ Microbiol 54: 212–217.
52 US Environmental Protection Agency. 1984. Method 602—Purgeable Aromatics. 40 CFR Part 136, 43272; Federal Register 49, No. 209.
53 Van der Meer JR, TNP Bosma, WP de Bruin, H Harms, C Holliger, HHM Rijnaarts, ME Tros, G Schraa and AJB Zehnder. 1992. Versatility of soil column experiments to study biodegradation of halogenated compounds under environmental conditions. Biodegradation 3: 265–284.
54 Vroblesky DA and FH Chapelle. 1994. Temporal and spatial changes of terminal electron-accepting processes in a hydrocarbon-contaminated aquifer and the significance for contaminant bidegradation. Water Resources Res 30: 1561–1570.
55 Wilson BH. 1988. Biotransformation of chlorinated hydrocarbons and alkylbenzenes in aquifer material from the Picatinny Arsenal, New Jersey. In: Proceedings of the Technical Meeting: US Geological Survey Toxic Substances Hydrology Program, Phoenix, AZ, Sept 26–30, 1988. US Geological Survey Water Resources Investigations Report 88–4220.
56 Wilson JT, DH Kampbell and J Armstrong. 1993. Natural bioreclamation of alkylbenzenes (BTEX) from a gasoline spill in methanogenic groundwater. In: Hydrocarbon Remediation (Hinchee RE, BC Alleman, RE Hoeppel and RN Miller, eds), pp 201–218, CRC Press, Boca Raton, FL.
57 Zheng C. 1991. MT3D, A Modular Three-Dimensional Transport Model for Simulation of Advection, Dispersion, and Chemical Reactions of Contaminats in Groundwater systems. SS Papadopoulos and Associates, Rockville, MD, 173 pp.

Appendix I: Calculation of hydrocarbon utilization stoichiometry for site plume core groundwater

Compound presence and concentration data are from the July 1993 Resource Conservation and Recovery Act Facility Investigation (RFI) Report (Nittany Geosciences, unpublished).

Compound	PPM	Formula	% of Total	C(%)	H(%)	O(%)
Acetone	91	C_3H_6O	7.4	22.2	44.4	7.4
Benzene	330	C_6H_6	27.0	162.0	162.0	0.0
Bromobenzene	23	C_6H_5Br	1.9	11.4	9.5	0.0
Chlorobenzene	17	C_6H_5Cl	1.4	8.4	7.0	0.0
Chloroform	39	$CHCl_3$	3.2	3.2	3.2	0.0
1,2 Dichloroethane	41	$C_2H_4Cl_2$	3.4	6.8	10.2	0.0
Methanol	130	CH_4O	10.6	10.6	42.4	10.6
Methyl chloride	100	CH_3Cl	8.2	8.2	24.6	0.0
Methylene	100	CH_2Cl_2	8.2	8.2	16.4	0.0
Tetrahydrofuran	260	C_4H_8O	21.3	85.2	170.4	21.3
Toluene	91	C_7H_8	7.4	51.8	59.2	0.0
Total	1222		100.0	378.0	549.3	39.5

The aggregate stoichiometry is therefore:

$$\cong C_{380}\ H_{550}\ O_{40}/20 = C_{19}\ H_{28}\ O_2.$$

Assuming complete mineralization and no biomass proliferation,

$$C_{19}H_{28}O_2 + 25\ O_2 \rightarrow 19\ CO_2 + 14\ H_2O$$

Or following conversion for atomic weights:

2.8 g O_2/1g site hydrocarbon.

Appendix II: Electron acceptor/VOC consumption modeling

Conceptually, these calculations determined the mass of electron acceptors removed per year from the alluvial plume by microbiological activity as fresh groundwater is swept through the contaminated area. This number was then used to calculate the mass of organic materials removed by the metabolism implied by the amount of electron acceptor depletion. The removal of organic material was calculated using the following assumptions:

(1) The averaged field data from monitoring wells from this testing effort was an accurate representation of the field contaminant concentration over the course of a year.
(2) The field was essentially at steady-state: clean groundwater entered the plume at a constant rate, came into an equilibrium with the biota, and left at a fixed depletion.
(3) Linear interpolation between wells of VOC and electron acceptor concentrations in the groundwater is valid.
(4) Contaminants degraded were consumed for energy and biomass was assumed to remain constant.
(5) Background values of electron acceptor concentrations taken from a presumed clean well represented fresh groundwater.
(6) Fermentative degradation was neglected, as were degradation pathways using metal reduction; the only electron acceptors considered were O_2, NO_3^-, and SO_4^{-2}.

Overall stoichiometric equation [17]:

$$H_D + f_e H_A = \text{redox reaction}$$

where H_D is the half reaction for contaminant oxidation, H_A is the half reaction for electron acceptor reduction, and f_e is the fraction of organic material degraded to provide the biomass with energy. H_D was taken as $C_{19}H_{28}O_2$, as developed in Appendix I: although no molecule of this composition is expected, it is the aggregate stoichiometry of alluvial VOCs.

$$H_D = (1/100)\ C_{19}H_{28}O_2 + (36/100)\ H_2O \rightarrow (19/100)\ CO_2 + H^+ + e^-$$

Depending on which electron acceptor was used, H_A was:

Aerobic: $H_A = (1/4)\ O_2 + H^+ + e^- \rightarrow (1/2)\ H_2O$
Nitrate: $H_A = (1/6)\ NO_{3-} + H^+ + (5/6)e^- \rightarrow (1/12)N_2 + (1/2)H_2O$
Sulfate: $H_A = (1/8)\ SO_4^{-2} + H^+ + e^- \rightarrow S^{-2} + (1/2)H_2O$

Choosing an appropriate f_s and summing these equations allowed a stoichiometric relation between the amount of an electron acceptor consumed and the amount of one particular substrate consumed with the electron acceptor. For this site, the substrate composition ratio was $C_{19}H_{28}O_2$; the resulting stoichiometric equation for aerobic metabolism was approximately:

$$C_{19}H_{28}O_2 + 25\ O_2 \rightarrow 19\ CO_2 + 14\ H_2O$$

Similar equations were derived for nitrate reduction and sulfate reduction. The results are summarized in Table II.1:

Table II.1 Calculated mass relations between VOC and electron acceptors

Metabolic regime	Electron acceptor/VOC
Aerobic	2.8
Nitrate reduction	3.7
Sulfate reduction	3.3