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Stable Carbon Isotope Ratios and Biodegradation Rates of BTEX Compounds at the Tranguch Gasoline Site, Hazleton, Pennsylvania

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EXECUTIVE SUMMARY

In April 2002, NRL sampled groundwater at the Tranguch Gasoline Site (TGS), which is located in Hazleton, Pennsylvania. The goal of this study was to determine the rates and efficacy of on-site bacterial fuel hydrocarbon biodegradation; measure the overall metabolic activity and state of the on-site bacterial consortia; and determine fuel hydrocarbon stable isotope ratios. Biodegradation of benzene and toluene occurred in several wells, mostly associated with specific source areas (Tranguch Tire, Orlosky gas station, Sam's Amoco, and Hazleton Standard Oil). The mineralization rates were relatively high, and calculated turnover (removal of ambient pool) times for benzene and toluene were on the order of days. The exception was OSMW-02, which had a benzene turnover time of about 5 months. Bacterial production rates ranged from nondetectable to about $4 \mu\text{g C L}^{-1} \text{ d}^{-1}$. In wells with appreciable benzene, toluene, ethylbenzene, and xylene (BTEX), mineralization of benzene and toluene generally accounted for ~100% of the calculated production. In some cases, there was significantly more mineralization of benzene or toluene than the estimated production. This was probably due to a systematic error in the conversion factor used to calculate production. No empirical estimations of this conversion factor in groundwater are available. A series of experiments were conducted to determine whether nutrient additions enhanced microbial activity, either benzene/toluene mineralization or bacterial production. We found no corollary relationship between nutrient additions and bacterial activities. Nitrogen was added in an oxidized form (NO_3^-), but we observed no systematic increase in microbial activities in wells with low dissolved oxygen.

From compound-specific stable carbon isotope analysis, it was determined that the BTEX in each well with measurable contaminant levels was significantly different from BTEX of every other well ($P < 0.05$). Using only three components (in wells with incomplete BTEX profiles) for a multivariate significance analysis, BTEX was not always found to be different in different wells. Using principal components analysis, a relationship between BTEX in wells was used to cluster "like" BTEX sources. This analysis showed a close relationship between SAMW-01 and OSMW-02 (Sam's Amoco and Orlosky) and a close relationship between OSMW-04 and TTMW-07 (Orlosky and Tranguch Tire). A probability distribution was calculated to arrive at an "index" of relatedness among BTEX in wells. Again, SAMW-01 and OSMW-02 showed very close relation to one another, as did OSMW-04 to TTMW-07. Each of these clusters was in turn more distantly related to one another and to BTEX in wells downgradient. Results of the compound-specific stable carbon isotope analysis with limited sample distribution indicated that there are at least two likely sources on-site, one represented by SAMW-01 and OSMW-04 and another represented by OSMW-04 and TTMW-07. A more thorough sampling, including more wells between the putative sources, should help to more accurately define sources of on-site contamination.

STABLE CARBON ISOTOPE RATIOS AND BIODEGRADATION RATES OF BTEX COMPOUNDS AT THE TRANGUCH GASOLINE SITE, HAZLETON, PENNSYLVANIA

INTRODUCTION

In groundwater, there is complex cycling of natural organic matter (NOM), nutrients, and metabolites through food webs. In areas where hydrocarbon impacts occur, both NOM and contaminant hydrocarbons can be assimilated and respired by bacteria. Contaminant utilization is controlled by the same environmental factors that influence natural carbon and nutrient fluxes. Typical site evaluations focus on compound concentration surveys and/or biotreatability studies and do not give attention to determining the factors that control contaminant metabolism. As a result, remediation strategies may not achieve cleanup goals. Further understanding of the mechanisms controlling contaminant flux and transformation will enable managers to make scientifically sound and fiscally responsible decisions when choosing bioremediation strategies for site cleanup.

Fuel hydrocarbons are an excellent source of energy and carbon for microorganisms. However, other biogenic elements are needed in a relatively precise balance to sustain microbial metabolism. In contaminated groundwater, the major elements are nitrogen and phosphorous for building cellular constituents, and O_2 as a terminal electron acceptor. The typical balance between available carbon, nitrogen, and phosphorous in unimpacted groundwater sustains relatively even rates of microbial activity. In contrast, the large amount of carbon found in groundwater hydrocarbon plumes quickly creates an environment low in inorganic nutrients and dissolved O_2 . Many techniques have been used to understand the rate of hydrocarbon degradation. A common practice is to try to stimulate biodegradation of hydrocarbons by adding nutrients or O_2 . In most cases, these techniques have met with mixed results[1,2]. Disrupting the delicate balance of microbial activity at a contaminated site may ultimately decrease the biodegradation of hydrocarbon constituents[3]. It is generally agreed that a number of lines of evidence should be used to determine how and to what extent bioremediation of on-site groundwater contamination is occurring[2].

The most straightforward means of determining microbial activities is to measure their growth rates. Many techniques have been used over the years to do this, most requiring culturing or counting of microorganisms. These indirect methods have a number of limitations and cannot give information on the actual rates of microbial growth. In recent years, however, techniques have been developed that allow rapid (~1-2 h) and sensitive (to the $ng\ C\ L^{-1}$ level) measurements of microbial carbon demand, typically called bacterial production. These techniques measure the uptake of radiolabeled nucleotides or amino acids into microbial biomass and provide information on bacterial growth rates. A major benefit of these newer techniques is that they are nonspecific and measure the activity of the entire microbial assemblage[4,5]. These bacterial production assays provide an upper boundary for the amount of carbon fueling bacterial metabolism. In other words, the biodegradation rate of contaminant hydrocarbon cannot be greater than the measured bacterial production. Loss of contaminant in excess of the bacterial production value would then have to be attributed to physical or chemical changes.

In addition to measuring the total carbon demand of the microbial assemblage, some idea of the amount of specific contaminants being used by the bacteria should be known. A tracer study can be used to accomplish this. For this technique, several sentinel contaminants are acquired in their radiolabeled form (usually ^{14}C). For gasoline plumes, common sentinel tracers might be ^{14}C -benzene and ^{14}C -toluene. One adds a tracer

amount (i.e., much lower than the ambient amount) of the radiolabeled contaminant to an incubation containing groundwater from the study site. After a short incubation, typically ~24 h, the water sample is acidified and $^{14}\text{CO}_2$ is collected and radioassayed. The amount of $^{14}\text{CO}_2$ produced can then be related to the amount of contaminant degraded per unit time.

For many subsurface fuel-contaminated sites, multiple sources exist and complex mixing and transport result in uncertain assessment and organization of remedial action. Stable isotope analyses of carbon and nitrogen may help identify the individual sources and their ultimate fate in complex mixtures of NOM. Isotope analysis of carbon, nitrogen, and sulfur pools has provided a more thorough understanding of organic matter sources and cycling in a variety of ecosystems[6-8]. Development of stable isotope methods has been used to identify the cycling of carbon at a molecular level[9-12]. In addition, stable carbon isotope analysis ($\delta^{13}\text{C}$) has assisted in development and interpretation of bioremediation strategies for ecosystems that are contaminated with organic chemicals[13]. The recent development of gas chromatographic (GC) transfer of individual compounds, combusted inline, to an isotope ratio mass spectrometer (IRMS) provides the ability to identify individual contaminant sources[14]. Fractionation of carbon isotopes is related to the pool size of the substrate in question. Small pool sizes and anaerobic conditions favor isotope fractionation from source material to bio-products (biomass, CO_2). However, if the source material is the component being analyzed and its pool size is large, no significant fractionation will be observed[15]. If contaminant sources have a broad range in $\delta^{13}\text{C}$ in individual hydrocarbon compounds, it is possible to determine the contribution of a single source to the total loading. By using $\delta^{13}\text{C}$ for analysis of benzene, toluene, ethylbenzene, and xylene (BTEX), multiple petroleum sources were shown to be present in groundwater that was thought to be contaminated with one source[16]. Other recent research provides further support for the application of carbon isotope analysis to trace the contaminant sources. The approach has been applied in tracking nitroaromatic compounds, PCE and TCE[17], and jet fuels[18].

Another key step in environmental remediation is confirmation and measurement of contaminant degradation rates. Identification of the role of microorganisms in biogeochemical cycles will assist in monitoring contaminant turnover and developing of strategies for site cleanup. Over the past decade, basic research in microbial assemblage activities and carbon isotope chemistry has led to the development of technology that can identify sources of organic matter cycled through bacteria[3,9,19,20]. These techniques have been combined with $\delta^{13}\text{C}$ analysis of dissolved and particulate organic carbon (DOC and POC) and dissolved inorganic carbon (DIC) to examine the roles of bacterioplankton in aquatic carbon cycles[6,8,19,21]. The development of these new approaches in isotope biogeochemistry has provided the ability to determine carbon sources that support bacterial production from a variety of anthropogenic, autochthonous, and allochthonous sources. This technology has recently been applied to study petroleum hydrocarbons assimilated by bacteria[22]. However, in most terrestrial environments, the $\delta^{13}\text{C}$ of anthropogenic carbon sources is nearly identical to natural organic matter, resulting in uncertain identification of sources of carbon cycled through the microbial assemblage[23]. Cifuentes et al.[24] used $\delta^{13}\text{C}$ of CO_2 to monitor degradation of PAH and BTEX in groundwater and found that results of stable carbon isotope analyses indicated enhanced degradation. However, isotope ratios of the contaminant and indigenous carbon overlapped, thus inhibiting confirmation of contaminant degradation.

Significant cost savings will result from implementation of an appropriate cleanup strategy for specific site conditions or from a scientifically-based decision to not further impact the area. Additional cost savings could result from data documenting the extent to which the Navy is responsible when negotiating with other potentially responsible parties (PRPs). These savings could result from reduced sampling costs associated with integrated studies or reduced legal liability.

Site Description

The Tranguch Gasoline Site (TGS) is located in Hazleton, Pennsylvania (Fig. 1). Gasoline leaking from underground storage tanks (USTs) from several potentially responsible parties was discovered in early 1994.

The leak was found beneath the Tranguch Tire and Auto Center. It was estimated that 50,000 U.S. gallons of gasoline had leaked into the surrounding groundwater. In 1995, the USTs were removed and remedial investigation initiated. It was noted that an oily sheen could be seen on Black Creek. After initial surveys, an impacted area bound by Black Creek to the north, Hayes Street to the east, Vine Street to the west, and 17th Street to the south was designated. A number of follow-on studies have been initiated, and currently a soil vapor extraction system is installed and operational.



Fig. 1 — Tranguch Gasoline Site

Groundwater flows roughly east to west, with a slight northward trend. A series of groundwater monitoring wells has been installed on-site. For this sampling, a number were selected for their proximity to potential sources of contamination. A number were also selected to be in relatively unimpacted areas (Fig. 2).

Objectives

The objectives of this study were to evaluate in situ biodegradation of petroleum hydrocarbons in groundwater at the Tranguch Gasoline Site and to determine whether compound-specific stable isotope ratios of individual hydrocarbons can be used to distinguish different sources of contamination on-site.

Approach

The study of contaminant sources and biodegradative processes involves physical, chemical, and biological characterization of the study site. Data collected to date and in any future seasonal samples will be used to determine hydrocarbon source, transport, and intrinsic rates of biodegradation occurring in groundwater at the TGS.

Field Sampling and Sample Analysis

This section describes:

- Sampling objectives and locations,
- Sample size and sample frequency,
- Field methods, and
- Quality control sampling.

Sampling Objectives and Locations

Sampling objectives were to physically, chemically, and biologically characterize groundwaters spatially over the impacted site, maintain proper chain-of-custody and control of samples, and follow quality assurance/quality control procedures. Sampling was conducted in April 2002. The sample site is located in Hazleton, Pennsylvania. See Site Description on page 2 for sampling locations.

Sample Size and Frequency

Sample size is dependent on the type of sample analysis to be conducted. Groundwater samples were taken in acid-cleaned 500-mL amber glass bottles with Teflon-lined closures. Samples were stored at 4 °C until extracted and analyzed. Water samples used for biological analysis were immediately transferred to on-site laboratory facilities for processing within minutes of collection (Table 1).

Table 1 — Summary of Sample Collection, April 2002

Sampling Event	Sample Type	Analytical Parameter	Number of Field Samples and QC				Total Samples
			Field	Field Blanks	Trip Blanks	Total	
April 02	Water	BTEX*	16	1	1	18	18
		Stable carbon isotopes	16	1	1	18	18
		Nutrients*	16	1	1	18	18
		Bacterial production	16	16*	0	16	16
		Hydrocarbon mineralization	16	16*	0	16	16

*Analyses performed by Lockheed-Martin

Field Methods

This section describes the procedures for collection and preservation of samples.

Sample Collection

A peristaltic pump was used to pull in-well waters to the surface and into collection vessels. Aged Pharmed tubing was used during all samplings. Approximately three well volumes were purged within 12 h of sampling. Pier water samples were collected by using a weighted bottle suspended with nylon string.

Groundwater Samples

Groundwater samples were collected via a peristaltic pump through a Hydrolab DataSonde IV sensor system measuring conductivity, salinity, transmissivity, dissolved oxygen (DO), and density. Samples were

taken below the level of free product. The following brief protocol was used: Note time and date in field log. Collect water sample through a peristaltic pump. Note conductivity, salinity, transmissivity, and DO in field log. Transfer water to appropriate sample containers: (a) PAH samples to two 500-mL amber bottles; (b) microbiological samples to one 20-mL scintillation vial. Preserve PAH samples as appropriate with two pellets of NaOH. Label sample containers, fill in chain-of-custody documents and any other appropriate information in field logbook.

Analytical Methods

Analytical methods were chosen for applicability, sensitivity, and conformity. For maximum comparability with existing databases (NPDES discharges, water quality records, etc.), Environmental Protection Agency (EPA) methods (with described modifications) were chosen for most of the chemical analyses. Most microbiological methods were chosen from the scientific literature and have been subjected to extensive peer review before being published. No standard EPA or compliance analyses provide the microbiological information sought in this project. The Appendix summarizes analytical methods used in this project. Analyses are presented in operating procedure format. Data validation, reporting, and quality control are also covered.

RESULTS OF ANALYSES

Physical Analyses

Water Temperature

Groundwater temperature did not range more than 1°C and averaged 6.75 °C (Table 2).

Table 2 — Temperature Variation in Groundwater Samples

Sampling Event	Minimum Temperature (C)	Maximum Temperature (C)	Average Temperature (C)	Standard Error (C)
April 2002	6.67	6.84	6.75	0.25

Chemical Analyses

BTEX in Groundwater

BTEX concentrations were below minimum confidence detection limits in many samples. However, wells closest to PRPs had higher concentrations of BTEX (Table 3). The highest BTEX concentrations were found in well TTMW-07 located on the former Tranguch Tire property.

Alkane $\delta^{13}C$ Analysis

Groundwaters were extracted and analyzed for alkane $\delta^{13}C$. No alkanes were detected in concentrations sufficient to provide stable isotope values.

BTEX $\delta^{13}C$ Analysis

BTEX $\delta^{13}C$ was analyzed by purge and trap (P&T) extraction followed by GC separation and isotope ratio mass spectroscopy. The Appendix describes operating conditions. $\delta^{13}C$ Values were obtained for BTEX components in nine wells (Table 4). Significant variations in stable isotope signatures were observed.

Table 3 — BTEX Concentrations in Groundwater

Well	Benzene (μg^{-1})	Toluene (μg^{-1})	Ethyl- benzene (μg^{-1})	Xylenes (μg^{-1})	Total BTEX (μg^{-1})
BAG-55	U [†]	U	U	U	U
BAG-57	3.6	3.7	U	16.7	34
BS-01	U	U	U	U	U
EPA-MW47	19	U	U	U	19
HLSW-02	U	U	U	U	U
HLSW-07	N.D.*	N.D.	N.D.	N.D.	N.D.
HLSW-08	2.1	U	4.7	3.2	1U
HLSW-14	U	U	U	U	U
HLSW-17	U	U	U	U	U
HSMW-02	15	1.6	63	12.7	92.3
OSMW-02	34	2	20	2.2	58.2
OSMW-04	170	32	240	226	668
SAMW-01	1.7	5.2	16	12	34.9
SAMW-04	U	U	U	U	U
TTMW-06	2.7	U	U	U	2.7
TTMW-07	2500	2800	3600	11900	20800

†U Below limits of detection

*N.D. Not determined

Table 4 — Stable Carbon Isotope Ratios of Alkanes

Well	Benzene ($\delta^{13}\text{C}$)	Toluene ($\delta^{13}\text{C}$)	Ethyl- benzene ($\delta^{13}\text{C}$)	<i>o,m</i> - Xylenes ($\delta^{13}\text{C}$)	<i>p</i> -Xylenes ($\delta^{13}\text{C}$)
BAG-55	U [†]	U	U	U	U
BAG-57	-26.59	-25.56	-23.85	-28.61	-28.04
BS-01	U	U	U	U	U
EPA-MW47	-28.41	U	U	U	U
HLSW-02	U	U	U	U	U
HLSW-07	U	U	U	U	U
HLSW-08	-25.89	U	-26.29	-27.60	-25.94
HLSW-14	U	U	U	U	U
HLSW-17	U	U	U	U	U
HSMW-02	-26.88	U	-27.26	U	-26.92
OSMW-02	-26.76	-28.49	-24.88	-29.53	-26.07
OSMW-04	-28.41	-23.15	-26.16	-28.25	-26.98
SAMW-01	-25.56	-25.11	-25.56	-29.38	-26.81
SAMW-04	U	U	U	U	U
TTMW-06	-29.34	U	U	U	U
TTMW-07	-27.70	-25.49	-27.65	-27.54	-27.45

†U Below limits of detection

Dissolved Oxygen

Dissolved oxygen (DO) concentration ranged from 0.37 to 10.2 mg L⁻¹ in groundwater samples (Table 5).

Inorganic Nutrients

Nitrate concentrations were highest at BAG-55, BS-01, EAP-MW47, SAMW-04, and SAMW-04. Ammonium was highest at wells HSMW-02, OSMW-04, SAMW-01, and TTMW-07. Phosphate was not detected in any samples (Table 5).

Table 5 — Dissolved Oxygen and Nutrient Concentrations

Well	DO (mg L ⁻¹)	Nitrate (mg L ⁻¹)	Ammonium (mg L ⁻¹)	Phosphate (mg L ⁻¹)
BAG-55	9.40	5.03	U [†]	U
BAG-57	7.89	U	U	U
BS-01	9.90	2.42	U	U
EPA-MW47	8.50	U	U	U
HLSW-02	9.89	2.21	U	U
HLSW-07	10.16	U	U	U
HLSW-08	7.20	U	U	U
HLSW-14	6.33	1.82	U	U
HLSW-17	8.01	U	U	U
HSMW-02	0.37	U	0.13	U
OSMW-02	9.51	U	U	U
OSMW-04	5.49	U	0.51	U
SAMW-01	8.02	U	0.27	U
SAMW-04	8.61	2.96	U	U
TTMW-06	9.48	2.67	U	U
TTMW-07	8.75	U	0.47	U

[†]U Below limits of detection

Biological Analyses

Benzene and Toluene Mineralization in Groundwater

Benzene and toluene mineralization rates ranged from nondetection to over 37 µg L⁻¹ d⁻¹. Mineralization rates were highest in wells with significant BTEX contamination. Turnover times, the duration given the measured rate for the ambient pool of benzene or toluene to be degraded, ranged from roughly instantaneous (where no ambient BTEX was detected) to infinity (where ambient BTEX was detected, but no mineralization was). Realistic rates ranged from less than a day to several months (Table 6). Mineralization rates for several wells were measured with the addition of ~25 µM KNO₃ and ~1 µM K₂HPO₄. No predictable site-wide change in mineralization rates occurred after the addition of nutrient. However, there was a statistically significant increase in benzene and toluene mineralization rates in the furthest downgradient wells (BAG-57 and HLSW-14) after nutrient additions (Table 7).

Table 6 — Benzene and Toluene Mineralization Rates

Well	Benzene Mineralization ($\mu\text{g L}^{-1}\text{d}^{-1}$) $\pm 14\%$	Benzene Turnover Time (days) $\pm 14\%$	Toluene Mineralization ($\mu\text{g L}^{-1}\text{d}^{-1}$) $\pm 14\%$	Toluene Turnover Time (days) $\pm 14\%$
BAG-55	0.94	0*	U [†]	∞ [§]
BAG-57	3.14	0.05	0.05	3.17
BS-01	0.02	0	1.71	0
EPA-MW47	U	∞	0.55	0
HLSW-02	U	∞	U	∞
HLSW-07	U	∞	U	∞
HLSW-08	U	∞	0.08	0
HLSW-14	0.99	0	U	∞
HLSW-17	U	∞	8.88	0
HSMW-02	0.19	3.22	U	∞
OSMW-02	0.01	137	0.34	0.24
OSMW-04	37.5	0.19	2.46	5.40
SAMW-01	0.41	0.17	7.83	0.03
SAMW-04	0.71	0	U	∞
TTMW-06	0.31	1.01	0.63	0.18
TTMW-07	7.27	14.3	30.10	3.88

* Indicates no ambient pools so there is no turnover

†U Below limits of detection

§ Indicates no mineralization detected so infinite turnover time for ambient pool

Table 7 — Benzene and Toluene Mineralization Rates with Added Nutrients

Well	Benzene Mineralization ($\mu\text{g L}^{-1}\text{d}^{-1}$) $\pm 14\%$	Benzene Turnover Time (days) $\pm 14\%$	Toluene Mineralization ($\mu\text{g L}^{-1}\text{d}^{-1}$) $\pm 14\%$	Toluene Turnover Time (days) $\pm 14\%$
BAG-57	4.40	0.03	0.65	0.24
BS-01	U [†]	∞ [§]	U	∞
EPA-MW47	U	∞	U	∞
HLSW-07	0.06	0*	U	∞
HLSW-14	1.36	0	0.09	0
HLSW-17	2.63	2.69	0.66	2.04
OSMW-04	4.40	0.03	0.65	0.24

* Indicates no ambient pools so there is no turnover

†U Below limits of detection

§ Indicates no mineralization detected so infinite turnover time for ambient pool

Bacterial Production

Bacterial production was low for all wells sampled, most likely due to the low ambient groundwater temperature (~ 5 °C). It ranged from nondetection to $3.7 \mu\text{g C L}^{-1} \text{d}^{-1}$. The addition of $\sim 25 \mu\text{M KNO}_3$ and $\sim 1 \mu\text{M K}_2\text{HPO}_4$ to counteract any nutrient limitation yielded no predictable or significant change (Table 8).

Table 8 — Bacterial Production Rates

Well	Bacterial Production ($\mu\text{g C L}^{-1}\text{d}^{-1}$) $\pm 22\%$	Bacterial Production (nutrients added) ($\mu\text{g C L}^{-1}\text{d}^{-1}$) $\pm 22\%$
BAG-55	0.12	U [†]
BAG-57	0.21	0.14
BS-01	0.02	0.05
EPA MW47 COAL	U	U
HLSW-02	1.26	0.91
HLSW-03	0.17	0.36
HLSW-07	0.52	0.35
HLSW-08	0.20	0.32
HLSW-14	0.62	0.46
HLSW-17	U	U
HSMW-02	0.65	0.54
OSMW-02	0.11	0.23
OSMW-04	0.29	0.11
SAMW-01	3.72	4.00
SAMW-04	1.57	1.73
TTMW-06	0.40	0.33
TTMW-07	0.25	0.78

[†]U Below limits of detection

DISCUSSION

The main goals of this study were to determine the source of groundwater fuel hydrocarbons by chemical fingerprinting and to determine the rates of sentinel hydrocarbon biodegradation. We sampled several monitoring wells adjacent to the potentially responsible parties on-site. Variations in chemical and biological parameters indicated that several potential sources of hydrocarbons exist and that the majority of hydrocarbon contamination is likely degraded on-site, with very little off-site migration.

Stable Isotope Analysis

Stable carbon isotopes were for all BTEX compounds were obtainable for five of the nine wells containing sufficient analytical quantities of fuel hydrocarbons (Bag 57, OSMW-02, SAMW-01, TTMW-07, and OSMW-04). Partial analysis of at least three BTEX compounds was obtainable for two additional wells (HSMW-02 and HLSW-08), while only benzene was found in EPAMW-47 and TTMW-06. Three separate analyses of variance were conducted in Matlab® to determine if there were statistical differences in the stable carbon isotope profiles between wells. For samples with five BTEX compounds identified, a five-variable analysis was conducted. For samples with three to four BTEX compounds identified, a three-variable analysis was conducted.

Analysis of Variance

The first test conducted was a multiple analysis of variance (MANOVA). Five and three variable tests were performed. For samples with only benzene identified, a one-way analysis of variance (ANOVA) was conducted. Obviously, the greater the number of variables, the more confidence one has in interpreting the results. A probability value was obtained for each test, which indicates the probability that the two

sample means are the same. Generally, a probability > 0.05 indicates that there is no statistical difference between the sample means, whereas a probability < 0.05 indicates the sample means are different. By using a five-variable MANOVA, BTEX from each well was found to be different from BTEX in every other well (Table 9). By using only a three-variable MANOVA, we observed similarities between the BTEX profiles of several wells. For instance, there was no statistical difference between TTMW-07 (highest BTEX concentrations) and HSMW-02 (southeast) or HLSW-08 (east). Similarly, there was no statistical difference between BTEX compounds in HSMW-02 and HLSW-08 (Table 9). This test also showed no statistical difference between BTEX compounds in HSMW-02 and the far downgradient (northeast) well (Bag 57). It was also observed that BTEX in HLSW-08 (intermediate downgradient) was no different than BTEX in Bag 57 (farthest downgradient). By using a one-way ANOVA, benzene in EPAMW-47 was found to be no different from benzene in OSMW-04 and TTMW-06, and benzene in TTMW-06 was found to be no different than benzene in OSMW-04 (Table 9). From a cursory look at these tests, it appears that a relationship exists between OSMW-04, TTMW-07, TTMW-06, and HSMW-02. Downgradient wells HLSW-08 and Bag 57 appear to relate to several of the upgradient wells.

Table 9 — Probability Table
for MANOVA/ANOVA Analysis

Well	Compared to Well	Probability (<i>P</i>)
Five-compound tests		
Bag 57	OSMW-02	<0.001
Bag 57	SAMW-01	<0.001
Bag 57	OSMW-04	<0.001
OSMW-02	OSMW-04	<0.001
SAMW-01	OSMW-02	<0.001
SAMW-01	OSMW-04	<0.001
TTMW-07	OSMW-02	<0.001
TTMW-07	SAMW-01	<0.001
TTMW-07	Bag 57	<0.001
TTMW-07	OSMW-04	<0.001
Three-compound tests		
Bag 57	HLSW-08	0.105
HSMW-02	TTMW-07	0.096
HSMW-02	Bag 57	0.056
HSMW-02	SAMW-01	0.009
HSMW-02	OSMW-02	0.013
HSMW-02	OSMW-04	0.207
HSMW-02	HLSW-08	0.068
OSMW-02	HLSW-08	0.031
OSMW-04	HLSW-08	0.009
SAMW-01	HLSW-08	0.020
TTMW-07	HLSW-08	0.086
Benzene-only tests		
EPAMW-47	TTMW-07	0.009
EPAMW-47	HSMW-02	0.001
EPAMW-47	Bag 57	0.015

Table 9 (continued) — Probability Table
for MANOVA/ANOVA Analysis

Well	Compared to Well	Probability (<i>P</i>)
Benzene-only tests		
EPAMW-47	SAMW-01	0.000
EPAMW-47	TTMW-06	0.115
EPAMW-47	OSMW-02	<0.001
EPAMW-47	OSMW-04	0.326
EPAMW-47	HLSW-08	<0.001
TTMW-06	TTMW-07	0.005
TTMW-06	HSMW-02	0.001
TTMW-06	Bag 57	0.007
TTMW-06	SAMW-01	<0.001
TTMW-06	OSMW-02	<0.001
TTMW-06	OSMW-04	0.089
TTMW-06	HLSW-08	<0.001

Principal Components Analysis

In addition to determining the statistical differences among BTEX in the wells, it is of interest to know the relatedness between them. One means to accomplish this is to conduct a principal-components analysis (PCA). In data sets with multiple variables, groups of variables often behave similarly. More than one variable may, in fact, be describing the same principle of the system. PCA attempts to simplify a multivariate data set by replacing a group of variables with a single new variable, called a principal component. Each principal component is a linear combination of the original variables. The variance of each principal component is the maximum among all possible choices. The analysis provides information as to how much of the original variance is represented by each principal component. Therefore, when the primary components are graphed against one another, data sets that are highly similar will plot together, while dissimilar data sets will occupy different spaces on a graph.

A PCA analysis was conducted on the average carbon isotope values from wells with five BTEX components. About 90% of the variance was explained in the first two principal components (Fig. 3).

The first two principal components were then graphed relative to one another to determine how closely the BTEX in wells related (Fig. 4). TTMW-07 and OSMW-04 group together, or show similarity in the factors that cause variation. SAMW-01 and OSMW-02 also group together, perhaps indicating a shared source. HLSW-08 and Bag 57 (both downgradient) plot distinctly from the putative source wells, indicating either mixing or a separate source altogether. Because all BTEX components were not found in each well (c.f. Table 4), we conducted another PCA analysis using only three of the BTEX components. Again, about 90% of the variation could be explained within the first two principal components (Fig. 5). Results were similar with OSMW-04 and TTMW-07, and OSMW-02 and SAMW-01 both grouping together. Downgradient wells (HLSW-08, Bag 57) both plotted in different locations, and another putative source well (HSMW-02) plotted close to OSMW-04 and TTMW-07, indicating a possible shared source with these two wells (Fig. 6).

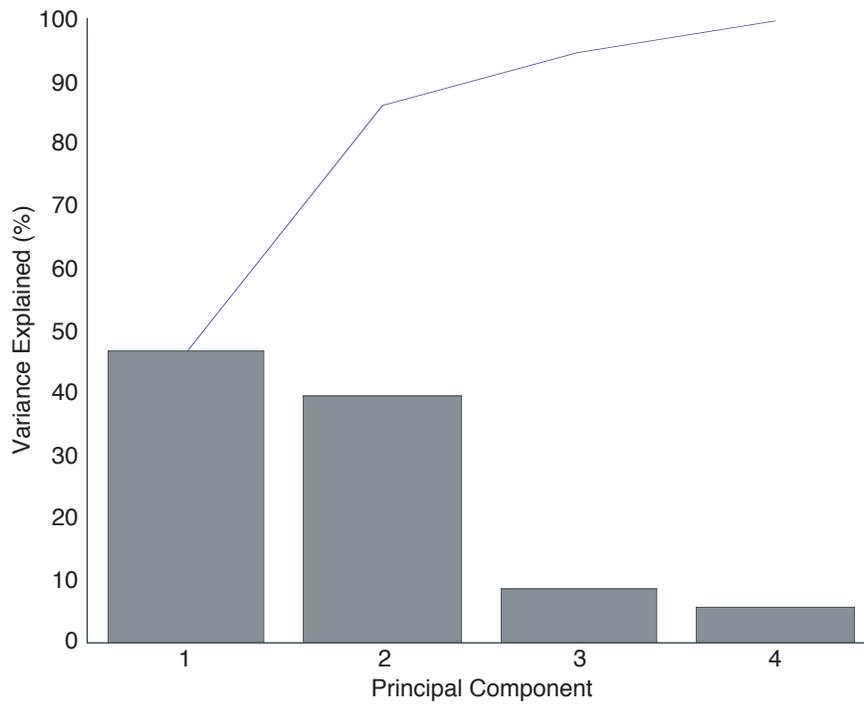


Fig. 3 — Variation explained by principal components

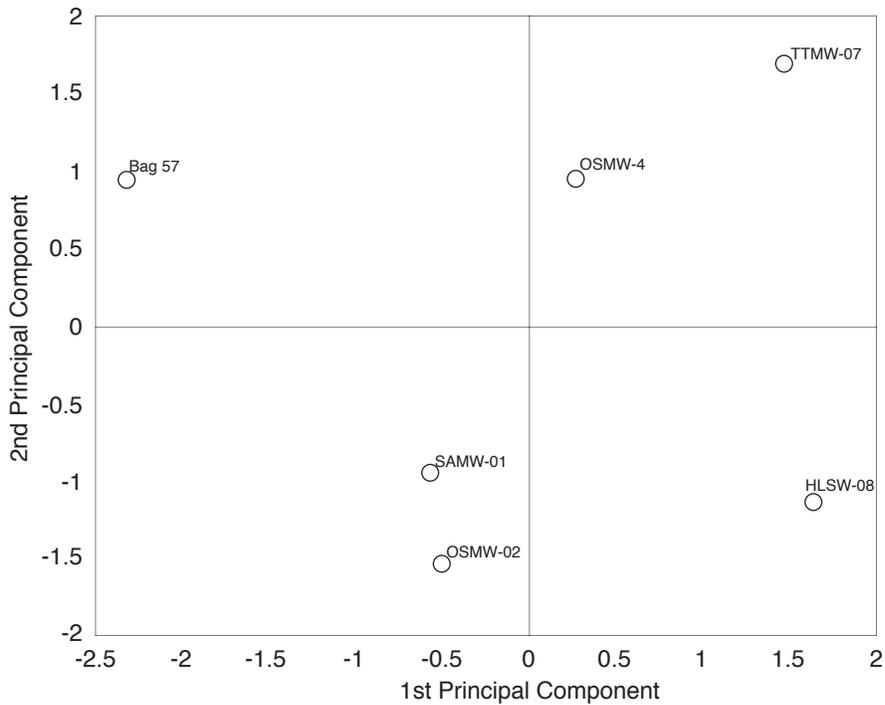


Fig. 4 — First vs second principal components (five-compound test)

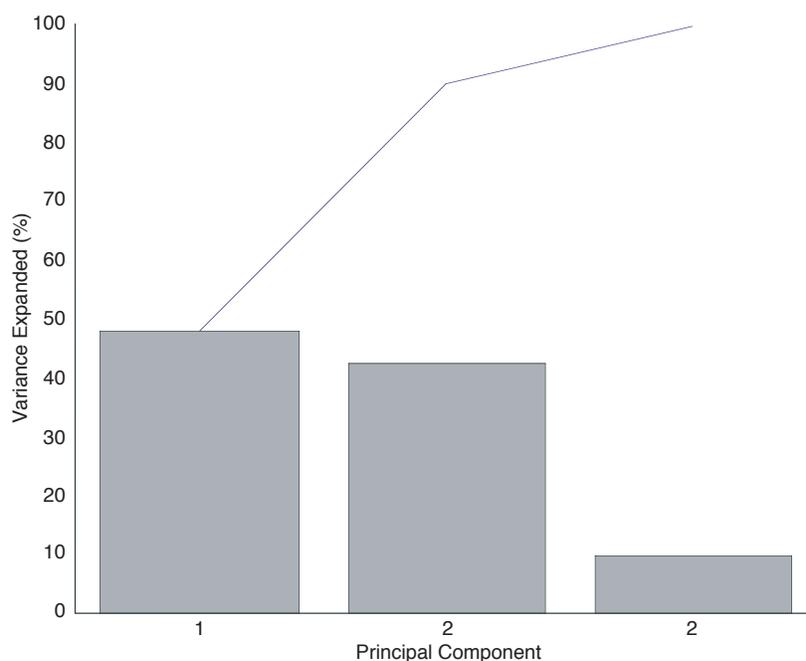


Fig. 5 — Variation explained by principal components

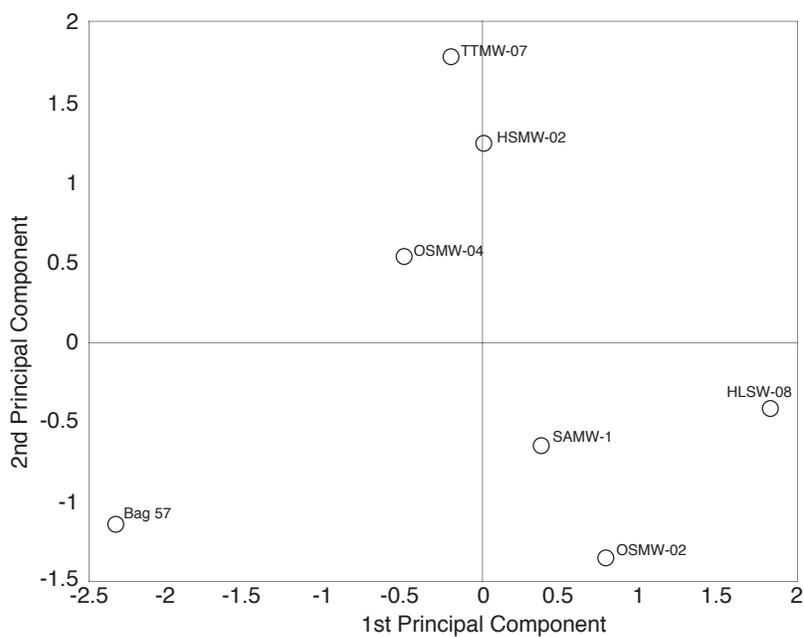


Fig. 6 — First vs second principal components (three-compound test)

Clustering Analysis

In addition to PCA analysis, clustering analysis can be used to determine a relative “distance” between relations in multivariate data. This would be analogous to plotting a family tree and using 1 inch to represent each generation of distance between progenitors and progeny. We used Matlab®’s clustering algorithms to determine the relatedness and similarity between stable isotope values of BTEX compounds. The length of

vertical lines in clusters is indicative of the “distance” of relatedness between wells. We superimposed the cluster dendrogram on the site map so that relatedness could be seen graphically. For the five-compound cluster analysis, results were similar to those seen in the PCA analysis: OSMW-02 and SAMW-01 were very closely related (black line), and OSMW-04 and TTMW-07 were very closely related (blue line). Both were related to HLSW-08 (although OSMW-02 and SAMW-01 were slightly closer). Finally, the furthest downgradient well (Bag 57) appeared to be related to all upgradient wells (Fig. 7).

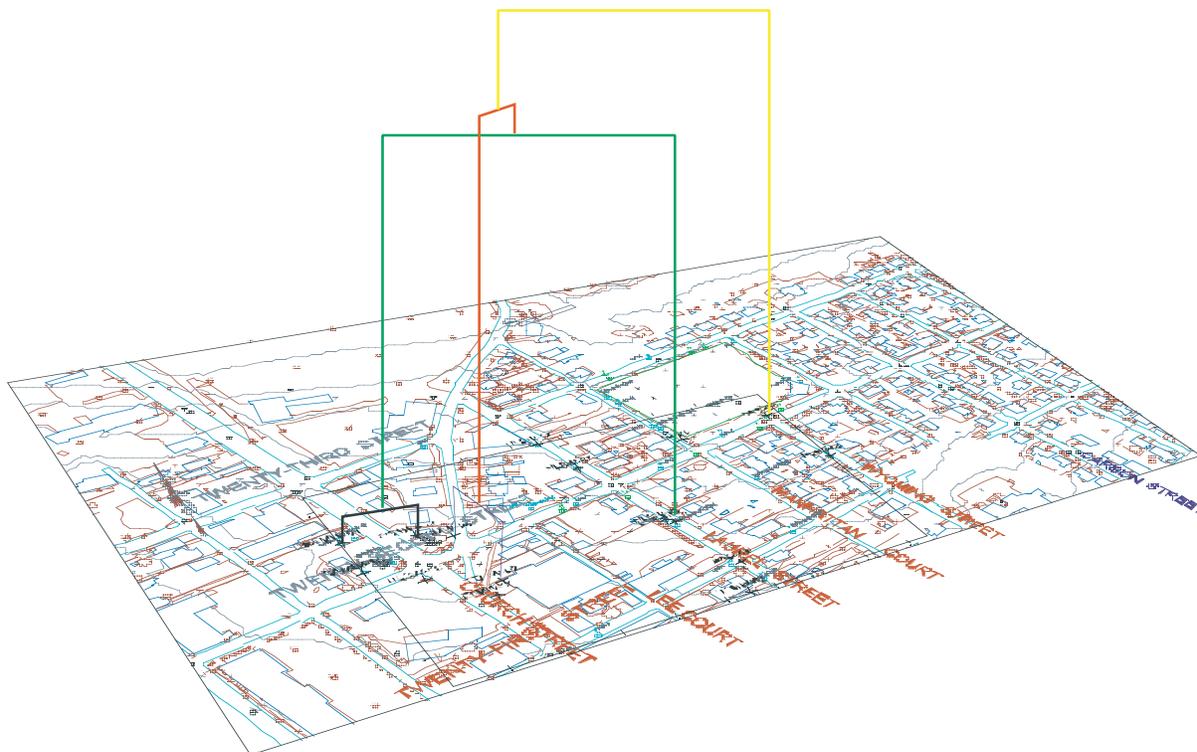


Fig. 7 — Cluster analysis dendrogram superimposed on site map (five-compound test)

Cluster analysis was also conducted to include those wells with only three or four BTEX components identified. In this analysis, OSMW-02 and SAMW-01 were less closely related to one another and more closely related to HLSW-08 (downgradient). OSMW-04 and TTMW-07 were also closely related, but HSMW-02 was most related to TTMW-07 using fewer BTEX components for the test. As with the five-compound test, all upgradient wells were distantly related to the most downgradient well, Bag 57 (Fig. 8). Based on these data and analyses, it seems most likely that there are at least two separate sources of BTEX compounds on-site. Because of the strong relationship seen in both PCA and clustering analysis, one source was in the vicinity of SAMW-01 and OSMW-02 (gas station), while another distinct source was in the vicinity of OSMW-04 and TTMW-07 (gas station, Tranguch Tire). Downgradient wells showed similarly to both of these putative source areas. HSMW-02 clustered with TTMW-02/OSMW-04 in the three-compound tests, indicating it may be part of the same source. However, the three-compound tests are not as robust as the five-compound tests, so intermediary sampling may be necessary to determine how closely related the fuel sources in these wells are. Because of the limited number of samples taken as part of this effort, we were not able to achieve the sampling resolution necessary to definitively identify all potential sources. It does appear highly likely that at least two separate BTEX sources are on-site.



Fig. 9 — Benzene concentrations interpolated over sampling area



Fig. 10 — Toluene concentrations interpolated over sampling area



Fig. 11 — Benzene concentrations interpolated over sampling area, Tranguch well removed

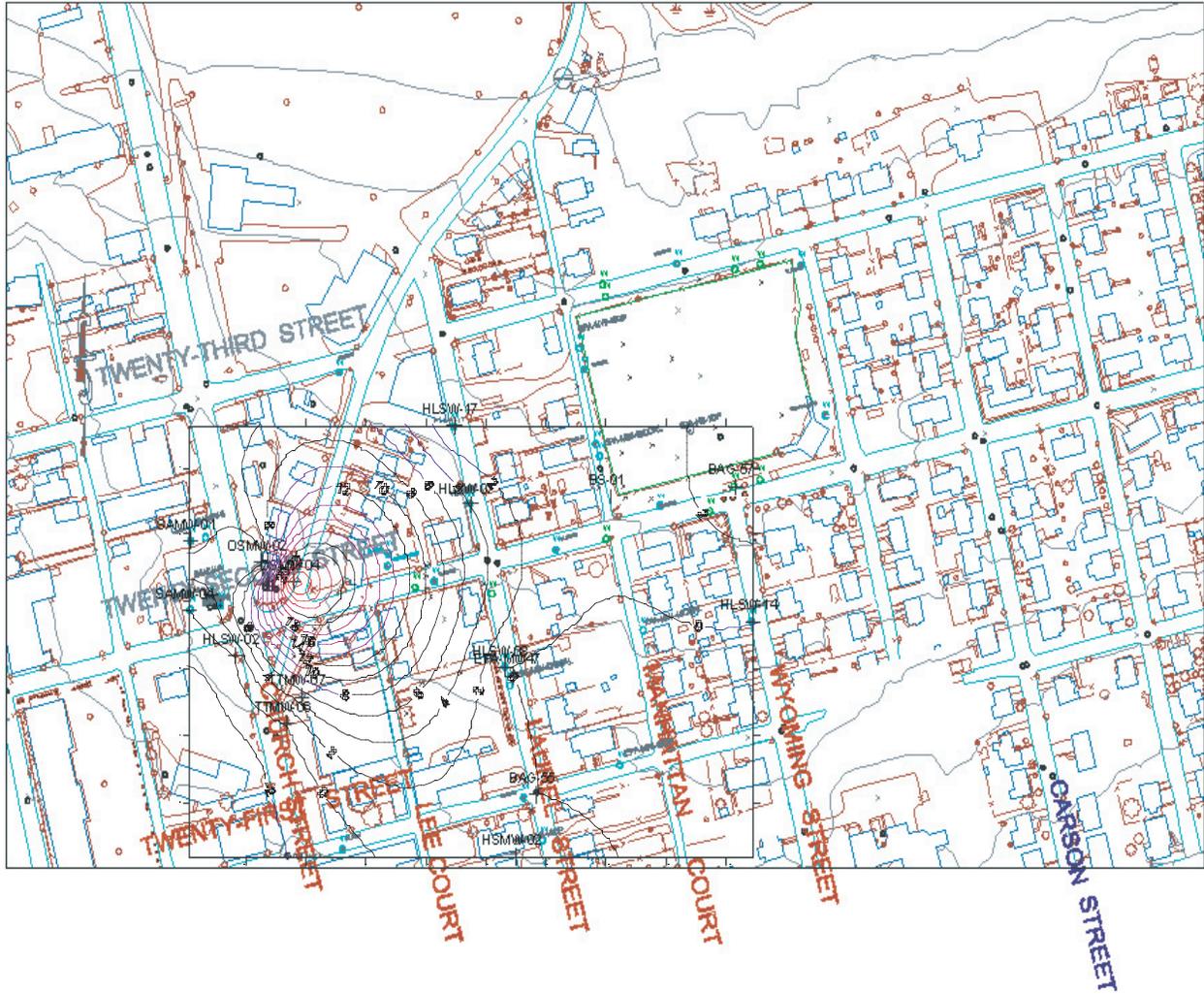


Fig. 12 — Toluene concentrations interpolated over sampling area, Tranguch well removed

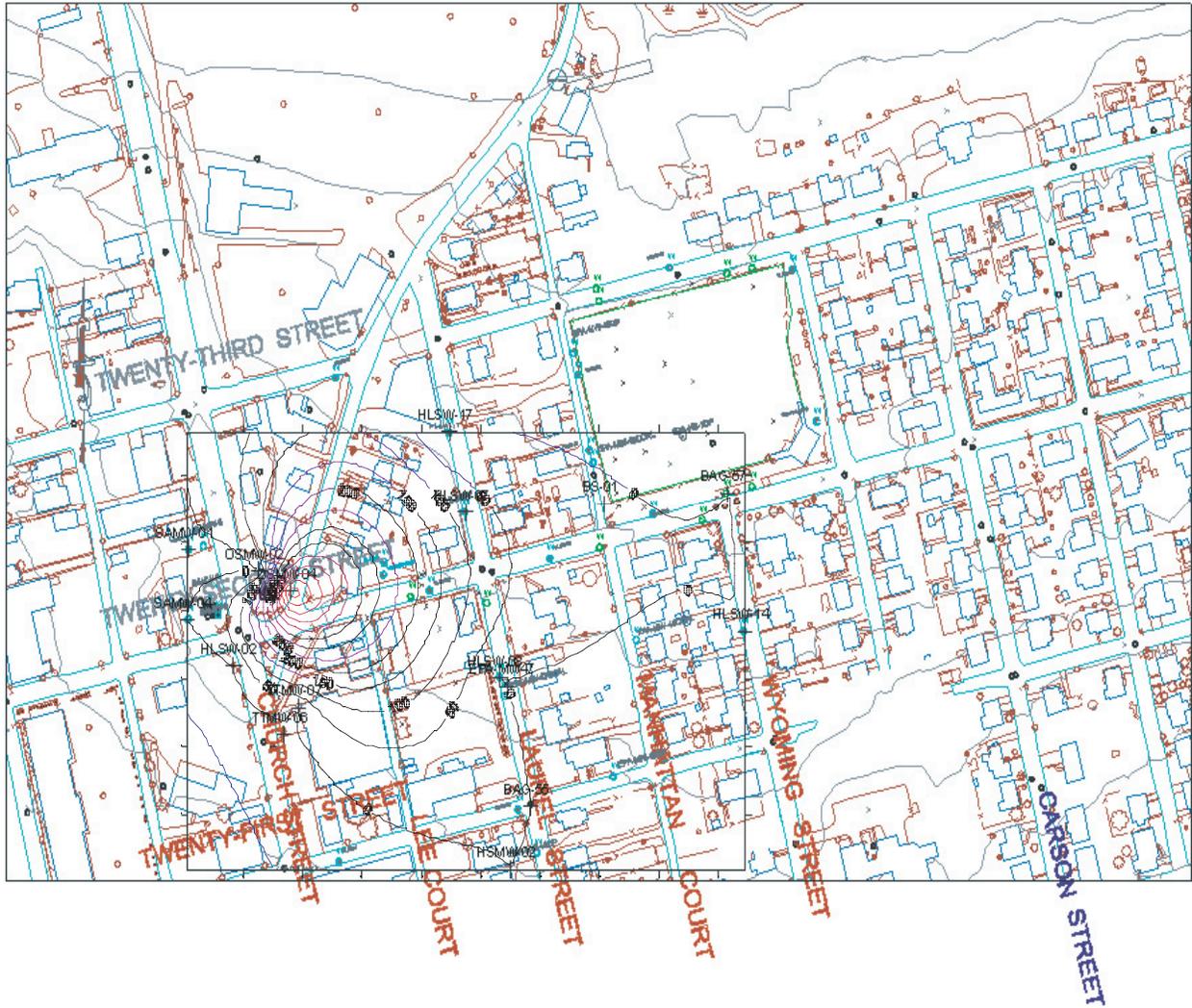


Fig. 13 — MTBE concentrations interpolated over sampling area

Dissolved Oxygen and Nutrients

Dissolved oxygen (DO) was measured during the sampling event using a field-deployed sensor system. Generally, DO concentrations are very low within groundwater hydrocarbon plumes[3,25,26]. It is unusual that DO concentrations were high in wells most adjacent to hydrocarbon source areas (Tables 3 and 5). Because O_2 makes up ~20% of the ambient atmosphere, it is possible that accurate field measurements of DO were not obtained. There was no correlation between DO and BTEX concentration ($P > 0.05$), further supporting the idea that perhaps the field measurements were incorrect. Dissolved nutrients (NO_3^- , NH_4^+ , and PO_4^{3-}) showed trends consistent with the historical literature[27,28] – very low NO_3^- and detectable NH_4^+ where BTEX literature[27,28] was prevalent, and the opposite where BTEX concentrations were low (Figs. 14 and 15). PO_4^{3-} was not detected in any of the samples.



Fig. 14 — NO_3^- concentrations interpolated over sampling area



Fig. 15 — NH_4^+ concentrations interpolated over sampling area

Ammonium ion concentration increased as a function of BTEX concentration, indicating that denitrifying conditions were prevalent at source areas (Fig. 16). Nitrate varied over the site but was consistently not detected where detectable levels of BTEX existed (Fig. 17). In all likelihood, BTEX biodegradation occurs close to source areas under denitrifying conditions. Interestingly, there was little stimulation of benzene and toluene mineralization with added NO_3^- . The addition of this electron acceptor should perhaps have stimulated benzene and toluene mineralization (Tables 6 and 7)[29]. In contrast, it has been shown that rapidly (i.e., less than 24 h) changing levels of electron acceptors may in fact decrease the biodegradation of organic contaminants in groundwater[3].

Benzene and Toluene Mineralization

Benzene and toluene mineralization were highest in wells with appreciable quantizes of BTEX (Tables 3 and 6). The high rates around source areas dominated the interpolation of benzene and toluene mineralization (Figs. 18 and 19). Significant benzene and toluene mineralization was measured at well BS-1 and BAG-57 on the downgradient portion of the plume. Benzene and toluene turnover times were relatively short (up to only 137 days). Generally, turnover times were less than 20 days. This indicates that BTEX compounds migrating toward the eastern portion of the impacted area are likely degraded before moving far off-site. Measurement of BTEX in downgradient wells (i.e., BS-1 and BAG-57) indicates that the biodegradation rate is lower than the BTEX delivery rate.

Bacterial Production

Bacterial production was low for all wells sampled. The general observation was high production upgradient of the plume, with lower production values at source areas and downgradient (Fig. 20). The addition of nutrients had no predictable effect on production (Table 8), and the interpolated levels over the entire site were very similar in trend to productions with no nutrients added (Fig. 21). Bacteria do not mineralize 100% of any compound to carbon dioxide. Generally, efficiencies for aromatic compounds are between 10-20%[3]. That is, 10-20% of the carbon is incorporated into bacterial biomass (and thus not measured in this assay), and 80-90% is respired as carbon dioxide. For wells SAMW-04, HLSW-08, and HSMW-02, benzene and toluene accounted for a very small amount of bacterial production, and thus bioremediation would not be occurring there relative to degradation of non-BTEX compounds (e.g., natural organic matter). For wells HLSW-14, SAMW-01, and TTMW-06, benzene and toluene mineralization accounted for about 30-50% of bacterial metabolism in the groundwater. This suggests that natural organic matter (or another VOC) accounted for the remaining production. In seven other wells (HLSW-17, EPA MW46 COAL, TTMW-07, OSMW-04, BS-01, BAG-57, and BAG-55), essentially all of the bacterial production was supported by the measured benzene and/or toluene mineralization rates, meaning that active bioremediation was going on at these sites or that the assemblage was adapted to degrade BTEX that would migrate onto the monitoring well station.

Phospholipid Fatty Acid (PFLA) Analysis

The bacterial assemblage was described by using three types of analyses, which are compared with other chemical analyses of groundwater from the same monitoring well. Two of the analyses involve rate measurements of bacterial growth (production) and BTEX metabolism (benzene and toluene mineralization) from samples removed from the subsurface environment. The third measurement involves identifying and quantifying the fatty acids specific to certain bacterial types (PLFA). This latter method does not provide rate information but does not involve ex situ incubations and thus may be a line of evidence that is more closely coupled to in situ conditions. This analysis will provide one of the first couplings of these measurements describing the bacterial assemblage in groundwater.

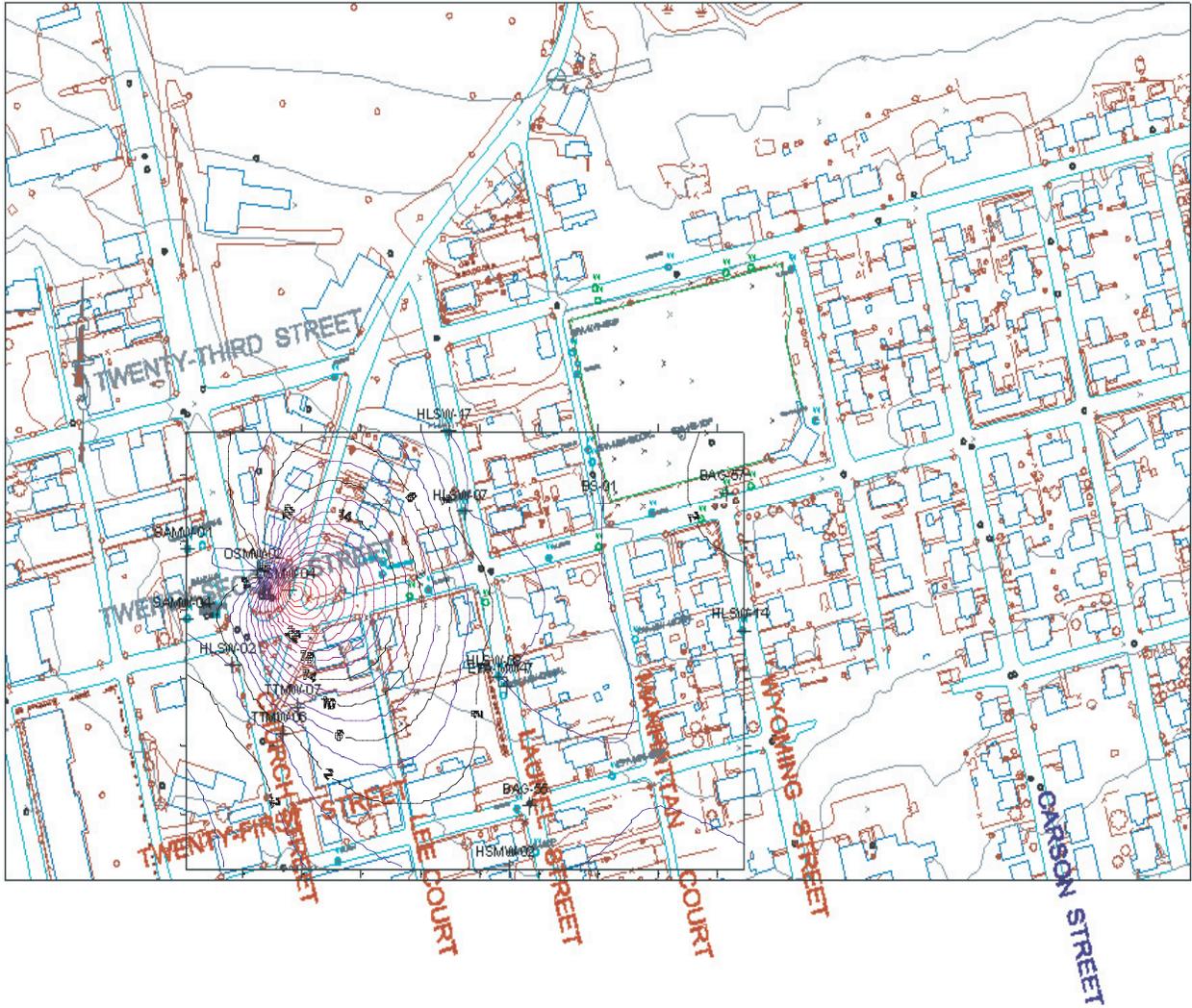


Fig. 18 — Benzene mineralization

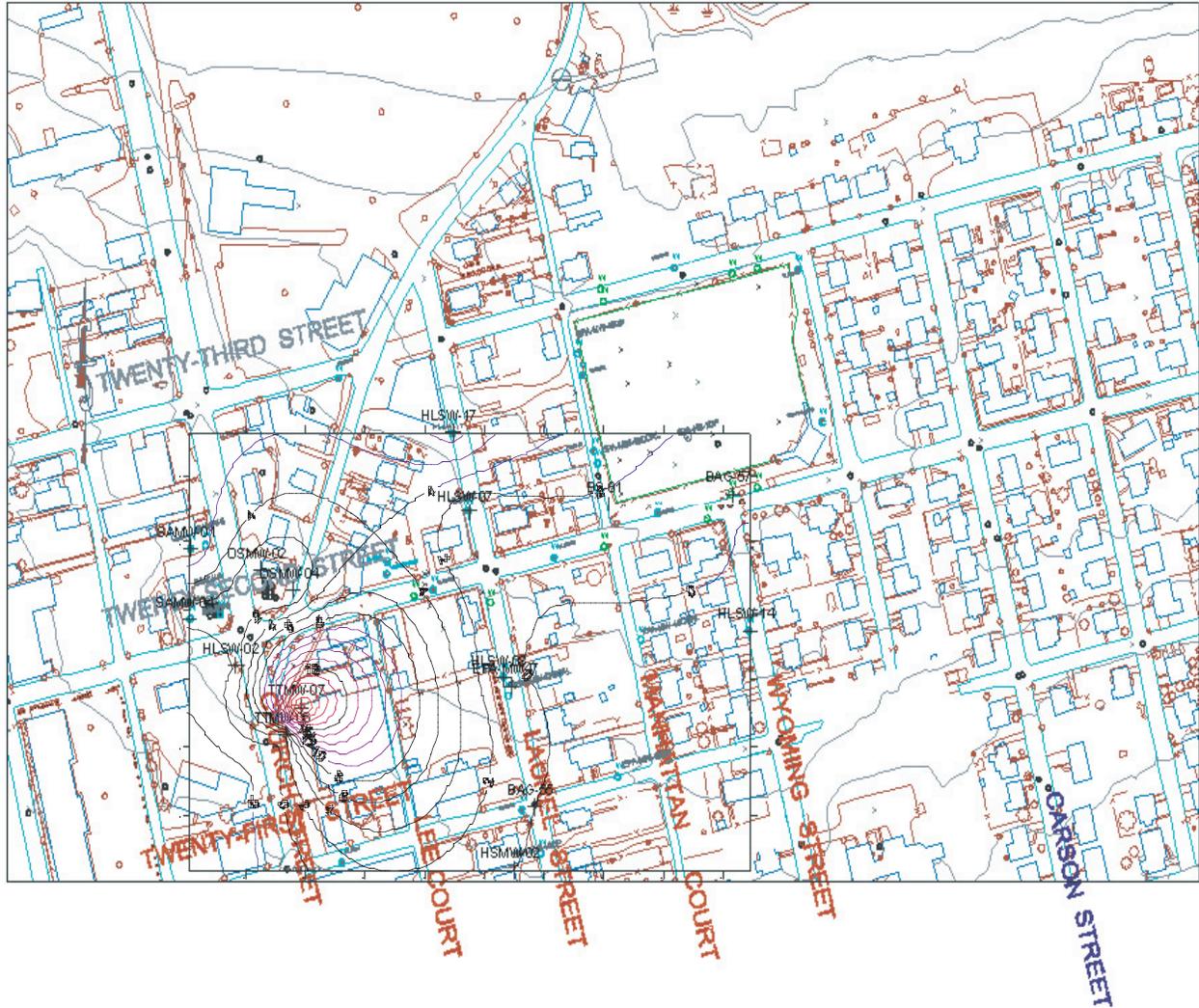


Fig. 19 — Toluene mineralization

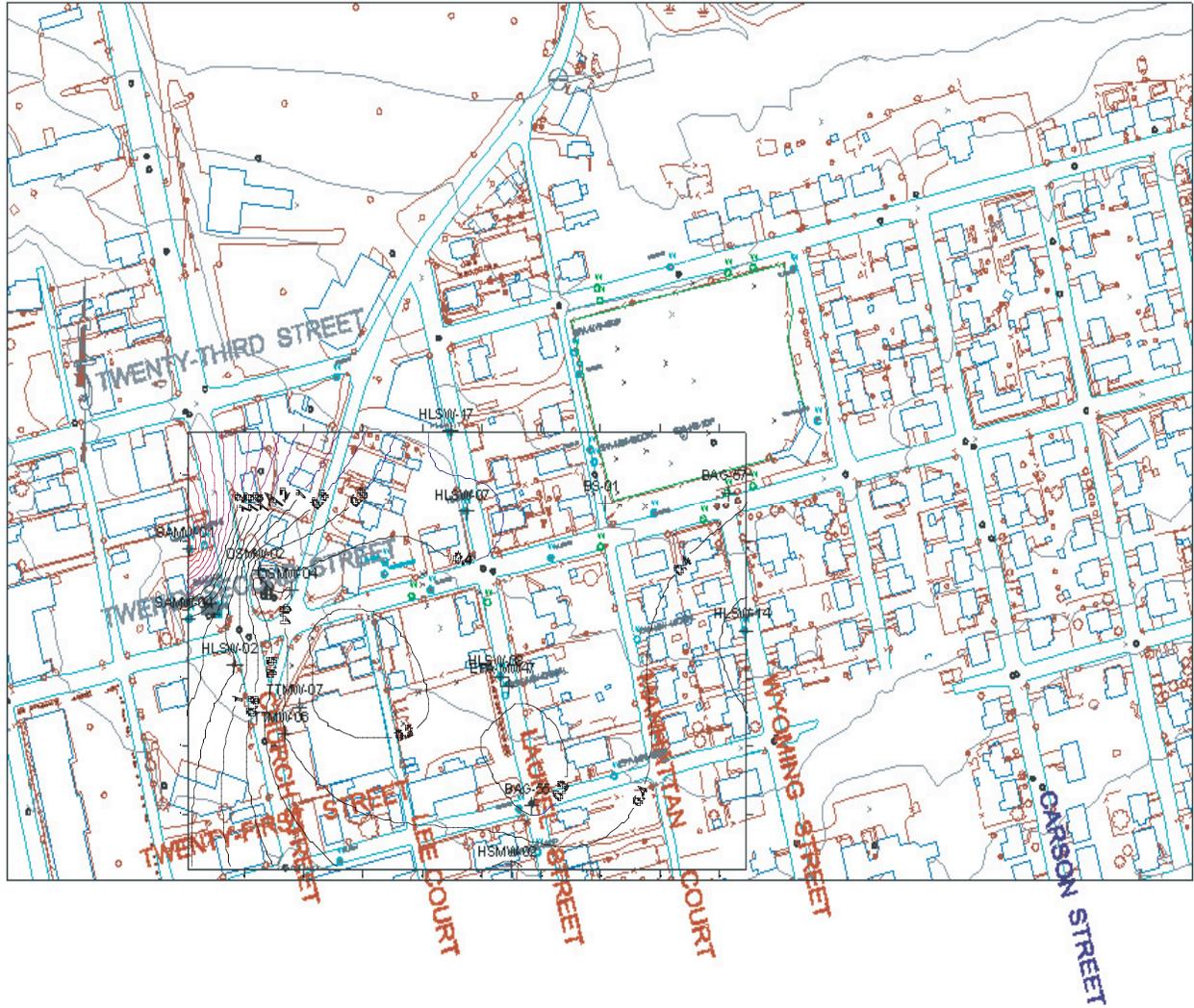


Fig. 20 — Bacterial production

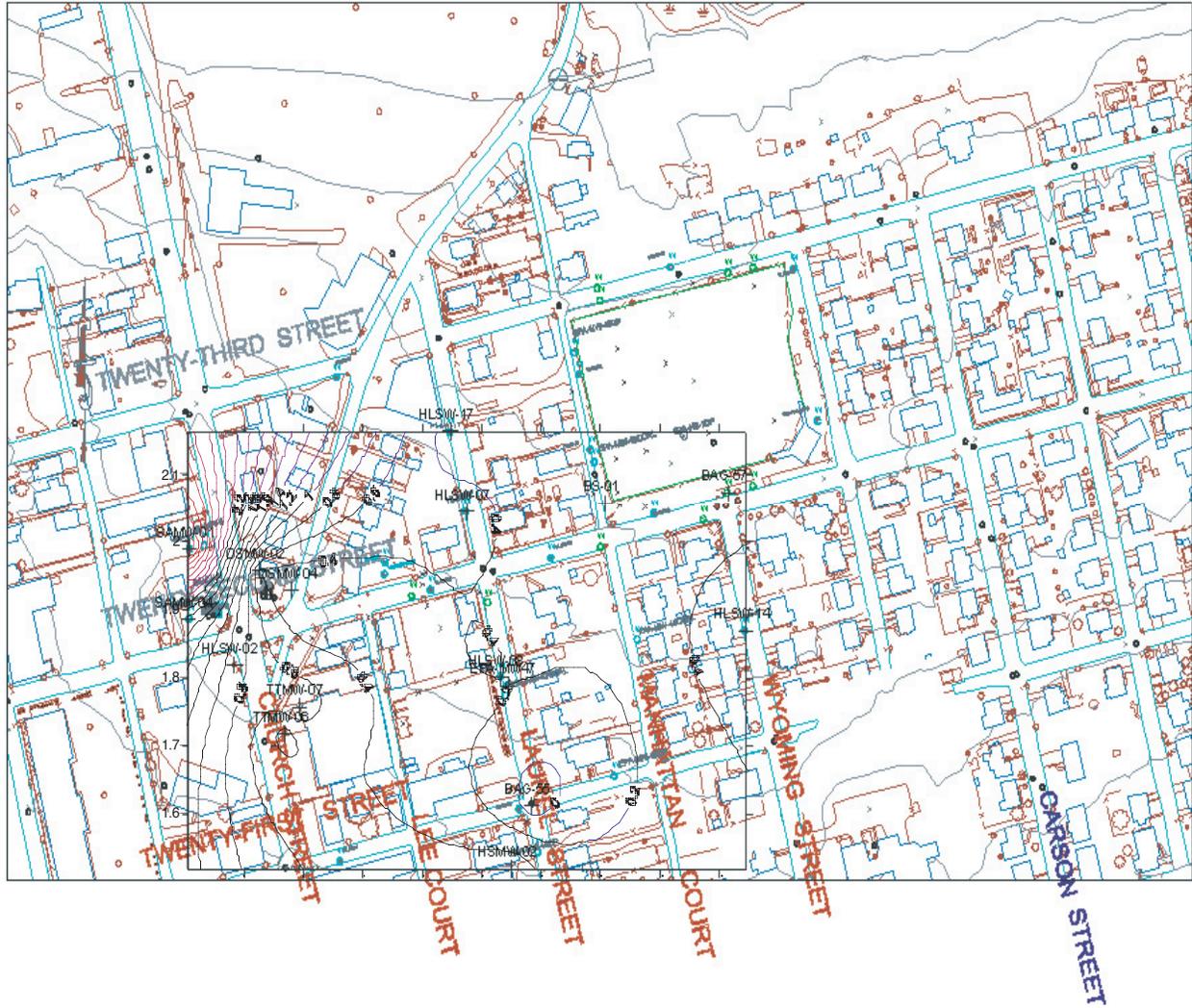


Fig. 21 — Bacterial production with added nutrients

Assemblage Profile

Of the 18 monitoring well samples evaluated for relative bacterial assemblage composition, 10 samples had a very similar composition. These 10 stations were in the source area of the plumes and downgradient so the primary environmental influence of the bacterial assemblage composition may have been the elevated fuel concentrations. Two other monitoring wells (SAMW-1, -4) harbored assemblages that were very similar to each other. The remaining six samples harbored assemblages that were different enough from these two described groups that they will be excluded from some of the subsequent analyses. These samples were generally near the fringe or outside of the plume and were probably more heavily influenced by other environmental factors like the presence of metals (e.g., HLSW-2), sulfate (e.g., HLSW-1), or alternate carbon sources (e.g., HLSW-7).

Organotolerance

Bacteria can alter their membrane structure in response to the presence of high concentrations of organic solvents, thus making them adapted for metabolizing carbon in contaminated environments (organotolerant). The relative degree of organotolerance is determined in the PLFA analyses with the ratio of *trans* to *cis* fatty acids. Ratios greater than 0.1 suggest that a large proportion of the bacteria present have recently been exposed to high organic solvent concentrations and are now organotolerant. Many of the bacterial assemblages surrounding the source area (e.g., SAMW-1, OSMW-2, -4, TTMW-1, -7, HLSW-3) were organotolerant, which is consistent with that expected for an area undergoing intrinsic bioremediation.

Biomass and Production

Of the 10 stations with similar bacterial assemblage compositions, the four stations nearest to the plume center had the highest amount of bacterial biomass (OSMW-2, -4, TTMW-7, HLSW-3) as determined with the PLFA analyses. Although the amount of biomass did not correlate with rate of bacterial production, these high standing stock values are probably reflective of reduced protozoan grazing rates resulting from BTEX toxicity. While these reduced grazing rates may result in higher bacterial abundance, they may reduce the overall rate of BTEX mineralization by reducing nutrient remineralization.

Potential for Source Removal

Benzene and toluene were able to be metabolized by the natural bacterial assemblage at many locations in the study site. The mineralization rates and the biomass concentrations were high in the middle of the source area, and this bacterial assemblage was found to be organotolerant. However, even with rates around $1 \mu\text{g BTEX carbon L}^{-1} \text{d}^{-1}$ being mineralized, natural attenuation may not be a likely means of source removal in areas over a $1000 \mu\text{g L}^{-1}$ of total BTEX. This is based on the biological rates measured at this one time point, which would be expected to be a low point seasonally.

Potential for Plume Migration Offsite

The capacity of the natural bacterial assemblage to adapt to BTEX input and metabolize the fuel in the fringes of the plume appears to be high when the ambient concentrations are under ca. $100 \mu\text{g L}^{-1}$ of total BTEX. The natural bacterial assemblage in much of the study site would likely be able to metabolize BTEX dissolved in the groundwater (over days to months) once the free product in the source area was removed or if the plume was sufficiently diluted with groundwater prior to migration downgradient.

CONCLUSIONS

Based on our analyses to date, we were able to determine the rates of groundwater bacterial production, benzene and toluene biodegradation, and the stable carbon isotope ratios of BTEX compounds. The following conclusions can be made:

- There was significant BTEX in samples around two of the PRPs (gas station and Tranguch Tire).
- Ammonium and nitrate concentrations indicated that reducing conditions existed in wells with significant BTEX contamination and that denitrification may be an important electron accepting process on-site.
- MTBE concentration data indicated that fuels from Tranguch Tire and the neighboring gas station were likely from different sources.
- Mineralization of benzene and toluene were highest in source areas, but it also was significant in wells with low-to-no BTEX contamination. Turnover times indicate that much of the benzene and toluene is utilized before having the opportunity to move off-site.
- Bacterial production was very low throughout the wells sampled. Highest production occurred upgradient of the plume, which presumably is not impacted. BTEX mineralization could account for almost all bacterial production at seven wells.
- Groundwater bacterial production was highest during warmer seasons, mimicking trends traditionally seen in adjacent estuarine water.
- PFLA analysis showed organotolerant populations in source areas and those downgradient. Biomass calculated from PFLA yields was highest downgradient of the source areas.
- Stable carbon isotope ratios were found to be significantly different among BTEX constituents when five components were analyzed. Statistical differences became less apparent with three- and one-compound tests.
- Stable carbon isotope analysis suggested that at least two sources of BTEX hydrocarbons exist on site – one located in the SAMW-01 and OSMW-02 region and one located in the OSMW-04 and TTMW-07 region. Clustering analysis showed a hierarchical relationship between the putative source wells and downgradient wells.

To better understand the processes described above and to determine the seasonal dynamics of chemical contaminants and biological activities, the following studies are suggested:

- A seasonal survey of wells should be conducted to determine the effect(s) of temperature on microbial activities.
- A site-specific empirical calculation of bacterial production conversion factor should be attempted.
- A careful measurement of Eh (potential) should be made at each monitoring well to determine what terminal electron accepting process predominates on-site.
- Natural abundance radiocarbon isotope analysis of inorganic carbon in the groundwater and vadose zone may be used as an in situ confirmation of bacterial processes measured with the radiotracer addition experiments.
- Stable isotope ratios should be measured in more wells near the potential source areas. Of the top 20 wells in BTEX concentration, isotope analyses were conducted only on three.

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Appendix

SUMMARY OF ANALYTICAL METHODS

STABLE CARBON ISOTOPE ANALYSIS

BTEX components were analyzed using a modification of U.S. EPA method 624. Depending on the ambient concentration, anywhere from 200 μ L to 20 mL of original sample was loaded on an OI model 4560 purge and trap concentrator fitted with a Supelco® BTEXTRAP trap. The sample was purged, BTEX trapped, and desorbed using the following parameters: Purge, 11 min at 25 °C; dry purge, 2 min at 25 °C; desorb, 6 min at 250 °C, followed by a post sample bake at 260 °C for 4 min. Following desorption, BTEX components were transferred to a Varian model 3000 gas chromatograph (GC), for separation on a 60 m, 250- μ m diameter Supleco® SPB-05 capillary column. To avoid any isotopic fractionation, the inlet was run in splitless mode. Column operating conditions were as follows: constant inlet pressure (25 psi), initial temperature, 35 °C, hold 5 min; ramp to 200 °C over 10 min; hold at 200 °C for 5 min; ramp to 300 °C over 2.5 min; hold at 300 °C for 3 min. The gas stream from the column was split with 80% of the flow directed through a Finnegan MAT isotope ratio mass spectrometer (IRMS) combustion interface to a Finnegan MAT Delta S IRMS. Known reference gas samples ($\delta^{13}\text{C}_{\text{PDB}} = -45.01$) were run in triplicate before and after sample introduction. Retention times were calculated based on known standards of BTEX compounds. Individual BTEX compound stable isotope ratios from triplicate runs were corrected by adding the difference between average final and initial reference gas standards (collected during each run). Another correction factor was applied to stable isotope values falling below 1.5 V signal intensity. Reference gas samples were run repeatedly at different concentrations providing deviation from linearity information for small signal voltages. Deviations were plotted against voltage ($n = 37$) over several days of instrument operation (Fig. A1). The resultant relationship was evaluated using the Matlab® curve-fitting tool. A Weibull distribution produced the best fit (goodness of fit, $r^2 = 0.86$). Based on the analysis, the following equation was applied to all voltages less than 1.5:

$$\text{offset} = 0.21 \times \text{volts}^{-0.8} \times e^{-0.36 \times \text{volts}^{0.58}}$$

Corrected $\delta^{13}\text{C}$ values for all detected BTEX compounds were processed using several multivariate statistical methods in Matlab® (see Discussion section).

BACTERIAL PRODUCTIVITY

^3H -Leucine was added to water and incubated to determine the amount of protein synthesis occurring within the bacterial population. Bacterial protein synthesis (via ^3H -leucine incorporation) is proportional to the increase of population biomass as described by Smith et al.[A1], as follows: Add a 1.0 mL water sample, or 50 mL sediment sample to a microfuge tube. Prepare tubes in triplicate with one killed control. Add 20 nM (final concentration) ^3H -leucine (ca. 1×10^6 DPM) to each tube and incubate at in situ temperature for 1 h. Add 100% (w/v) trichloroacetic acid to a final concentration of 5% (w/v) into each tube (to lyse bacteria and precipitate proteins). Perform this step at $t = 0$ for the killed controls. Centrifuge tubes for 7 min at

10,000 × g. Aspirate the supernatant (1.1 mL of waste) and wash the protein pellet with 1 mL of 5% (w/v) TCA, vortex and recentrifuge (7 min, 10,000 × g). Aspirate the supernatant (1.0 mL waste) and wash the pellet with 0.5 mL of 80% (v/v) ethanol, vortex, and recentrifuge (7 min, 10,000 × g). Aspirate the supernatant (0.5 mL of waste) and add 0.5 mL of scintillation cocktail, vortex, and radioassay for ^3H . Relate protein synthesis to bacterial productivity by using

$$g C mL^{-1} h^{-1} = \left(\frac{DPM_{sample}}{hr mL} \right) \left(\frac{mol leucine}{Y DPM} \right) \left(\frac{2 \times 10^{17} Cells produced}{mol leucine} \right),$$

$$where Y = (specific activity) \left(\frac{2.22 \times 10^6 DPM}{Ci} \right).$$

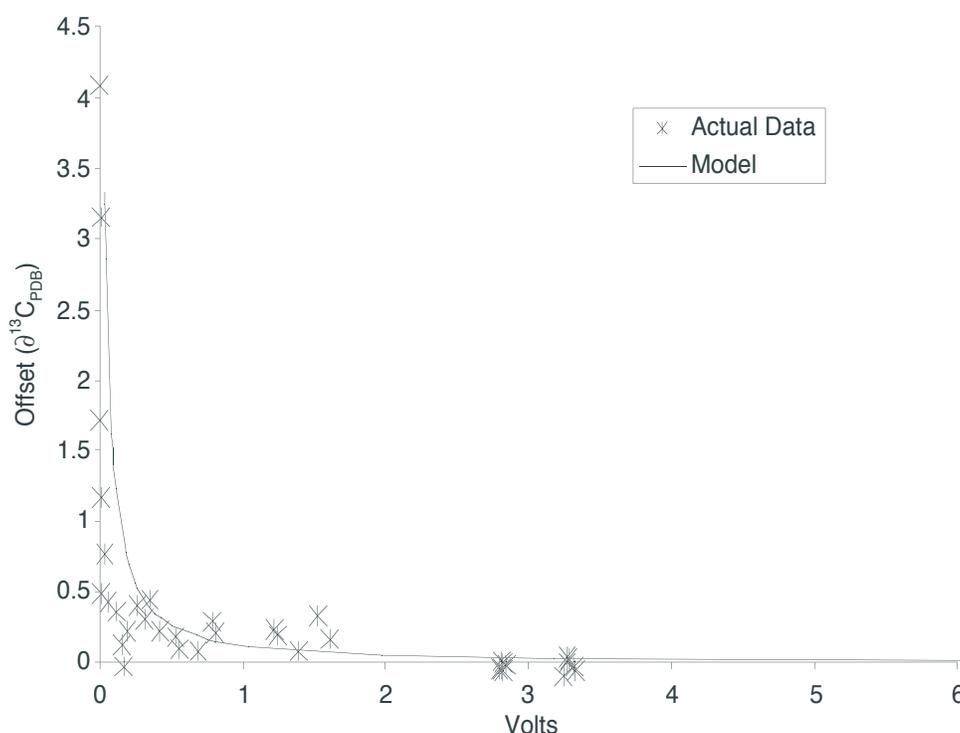


Fig. A1 — Offset ($\delta^{13}\text{C}_{\text{PDB}}$) vs IRMS signal voltage

CONTAMINANT MINERALIZATION

$^{14}\text{CO}_2$ production was monitored by using the following method: Transfer triplicate 1 mL water, or 1 mL sediment subsamples to 150 × 16 mm test tubes. Do likewise to one control. To the set of triplicates and control, suspend filter paper (soaked with 100 μL 1 N NaOH). This acts as a CO_2 trap. Add 1 mL 2 N H_2SO_4 to the control tube. Add ^{14}C -labeled substrate (benzene or toluene) to a final concentration of about 500 ng g^{-1} (depending on supplied specific activity). Incubate samples at collected water temperature for 12 to 24 h. To incubated samples, add 1 mL 2 N H_2SO_4 . Place tubes on rotary shaker for 4 to 6 h (preferably overnight). Remove and transfer NaOH soaked filter papers to 2 mL centrifuge tubes. Amend all scintillation vials with 1 mL scintillation cocktail and radioassay. Prepare triplicate tubes from one sample site with addition of 0.05 μCi $\text{NaH}^{14}\text{CO}_3$ to determine $^{14}\text{CO}_2$ recovery efficiency. Treat similarly from steps

6-8. Relate respiration to contaminant removal by using the following equation:

$$\text{Mineralization} \left(\frac{g}{L h} \right) = \left(\frac{DPM_{\text{sample}}}{L h} \right) \left(\frac{\text{mol substrate}}{Y DPM} \right) \left(\frac{\left(\frac{g \text{ substrate}}{\text{no. of labeled carbons}} \right)}{\text{mol substrate}} \right),$$

$$\text{where } Y = (\text{specific activity}) \left(\frac{2.22 \times 10^6 DPM}{Ci} \right).$$

QUALITY CONTROL SAMPLING

Various QC samples were collected during field operations to include field blanks and trip blanks.

Field Blanks

Field blanks were collected by rinsing sampling equipment with MilliQ water and collecting the rinse water. The rinse was analyzed for organics in a similar manner to water column samples. This analysis defines the contribution of equipment-borne contaminants to the samples.

Trip Blanks

A trip blank was prepared in the laboratory for shipment to the sample site. It consisted of a centrifuge tube filled with precombusted Ottawa sand. It was kept with the sampling equipment and handled the same as regular samples. The trip blank served to clarify the contribution of outside contamination to samples collected, shipped, and stored before analysis.

REFERENCES

- A1. D.C. Smith and F. Azam, "A Simple, Economical Method for Measuring Bacterial Protein Synthesis Rates in Seawater Using 3H-leucine," *Mar. Microb. Food Webs* **6**,107-114 (1992).