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SITE LOGIC Report

Stable Isotope Probing (SIP)

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Executive Summary

A Stable Isotope Probing (SIP) study was performed to determine whether biodegradation of benzene is occurring under existing site conditions. Bio-Trap® samplers baited with ^{13}C-labeled benzene were deployed in UWBZ26, UWBZ27, LSZ38 and LSZ39. Following a 52-day deployment period, the Bio-Traps were recovered to quantify ^{13}C incorporation into biomass and dissolved inorganic carbon (DIC). A complete summary of the SIP results is provided in Table 1 and Figures 1 through 5. Following are the key observations from the results obtained for the monitoring wells.

Stable Isotope Probing Results

- Quantification of ^{13}C-enriched PLFA conclusively demonstrated that benzene was metabolized under existing site conditions. The average PLFA $\delta^{13}C$ value in all wells was between 1,600‰ and 3,000‰, indicating a high level of incorporation of ^{13}C-labeled benzene into microbial biomass.
- The DIC $\delta^{13}C$ values in in UWBZ26, UWBZ27 and LSZ38 were greater than 1000‰, indicating that benzene was also mineralized during the deployment period. Results for LSZ39 were slightly lower at 924‰, falling within the moderate range.
- The total PLFA biomass concentration for all four wells was on the order of 10^5 cells/bead, which was within the moderate range.
- The PLFA community structures for all samples were primarily composed of indicators of proteobacteria followed by normal saturates and, to a lesser extent, firmicutes. Indicators of eukaryotes, actinomycetes, and anaerobic metal reducers were also detected in all the samples.

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Overview of Approach

Stable Isotope Probing (SIP)

Stable isotope probing (SIP) is an innovative approach to conclusively determine whether *in situ* biodegradation of a contaminant of concern is occurring.

With the SIP method, a Bio-Trap® is amended with a specially synthesized ^{13}C form of the contaminant of concern (e.g. ^{13}C-benzene). The ^{13}C essentially serves as a "label" to track biodegradation. For petroleum hydrocarbons and many other contaminants, biodegradation is a process whereby some microorganisms use the contaminant of concern as a carbon and energy source. When used as carbon source, contaminant carbon is incorporated into biomolecules such as phospholipids, DNA, and proteins supporting growth of new cells (biomass). When used as an energy source, contaminant carbon is oxidized to CO_2 as part of cellular metabolism. Thus, detection of the ^{13}C "label" in the end products of biodegradation (bacterial biomass and CO_2) at the end of the SIP study provides conclusive evidence of contaminant biodegradation.

To perform a SIP study, a Bio-Trap® is amended with the ^{13}C form of the contaminant of concern (e.g. ^{13}C-benzene) and deployed in an existing monitoring well for a period of 30 to 60 days. If present and active under the existing subsurface conditions, bacteria capable of utilizing the ^{13}C labeled contaminant of concern will colonize and grow in the Bio-Trap® over the course of the deployment period. Following recovery from the well, the Bio-Trap® is shipped to the laboratory and two approaches are used to conclusively evaluate contaminant biodegradation:

- Quantification of ^{13}C enriched phospholipid fatty acids (PLFA)
- Quantification of ^{13}C enriched dissolved inorganic carbon (DIC)

PLFA are a primary component of the membrane of bacterial cells and have long been used as a measure of microbial biomass. The detection of ^{13}C enriched PLFA during a SIP study indicates incorporation into microbial biomass and therefore conclusively demonstrates contaminant biodegradation.

Detection of ^{13}C enriched DIC which includes $^{13}CO_2$ conclusively indicates contaminant biodegradation and mineralization.

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Results

Table 1. Summary of the stable isotope probing results obtained from the Bio-Trap® Units.

Sample Name	UWBZ26 042020	UWBZ27 042020	LSZ38 042020	LSZ39 042020
Sample Date	4/20/2020	4/20/2020	4/20/2020	4/20/2020
MI ID	081RD1	081RD2	081RD3	081RD4
^{13}C Contaminant Loss				
^{13}C Benzene Pre-deployment (µg/bead)	177 ± 3	177 ± 3	177 ± 3	177 ± 3
^{13}C Benzene Post-deployment (µg/bead)	67 ± 5	88 ± 7	116 ± 16	90 ± 8
Biomass & ^{13}C Incorporation				
Total Biomass (Cells/bead)	2.89E+05	3.54E+05	4.18E+05	2.53E+05
^{13}C Enriched Biomass (Cells/bead)	1.08E+04	1.32E+04	2.21E+04	4.67E+03
Average PLFA Delta (‰)	1,628	2,750	2,940	1,853
Maximum PLFA Delta (‰)	6,337	5,559	13,101	5,228
^{13}C Mineralization				
DIC Delta (‰)	1,588	2,101	2,788	924
Community Structure (% total PLFA)				
Firmicutes (TerBrSats)	8.73	5.35	2.67	9.07
Proteobacteria (Monos)	60.71	73.73	79.19	62.63
Anaerobic metal reducers (BrMonos)	6.56	4.50	2.19	1.79
Actinomycetes (MidBrSats)	1.38	0.38	0.56	0.49
General (Nsats)	20.70	13.04	13.62	22.92
Eukaryotes (Polyenoics)	1.90	2.98	1.80	3.12
Physiological Status (Proteobacteria only)				
Slowed Growth	1.08	2.21	2.19	1.54
Decreased Permeability	0.03	0.01	0.21	0.04

Legend:
NA = Not analyzed NS = Not sampled J = Estimated result below PQL but above LQL I = Inhibited ND = Result not detected

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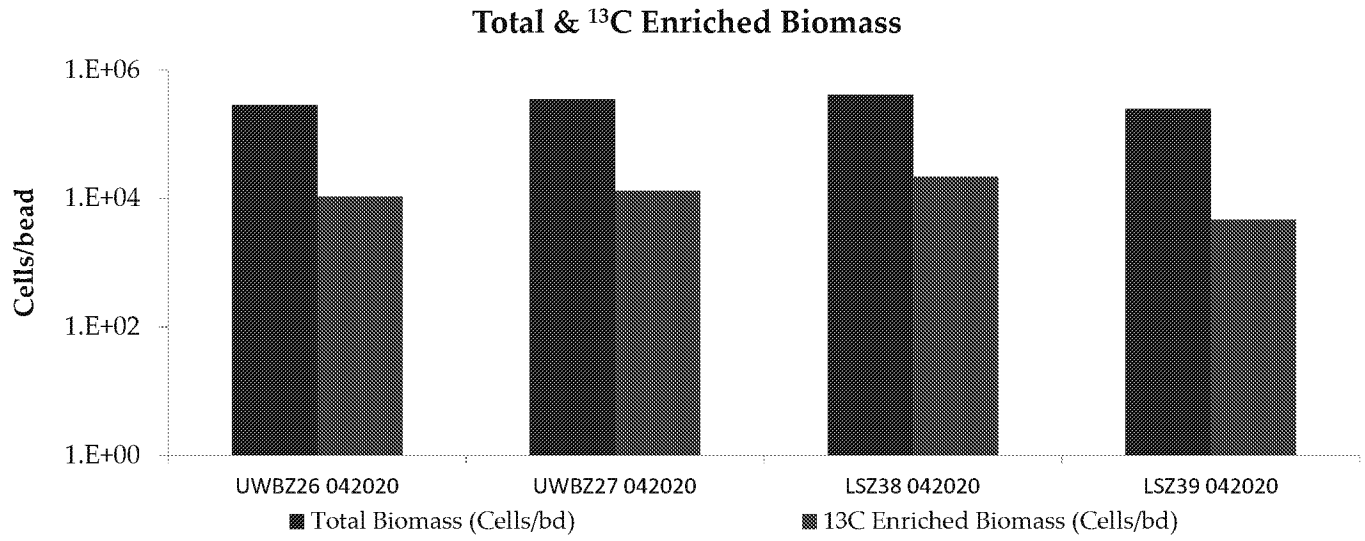

Figure 1. Biomass content is presented as a cell equivalent based on the total amount of phospholipid fatty acids (PLFA) extracted from a given sample. Total biomass is calculated based upon PLFA attributed to bacterial and eukaryotic biomass (associated with higher organisms).

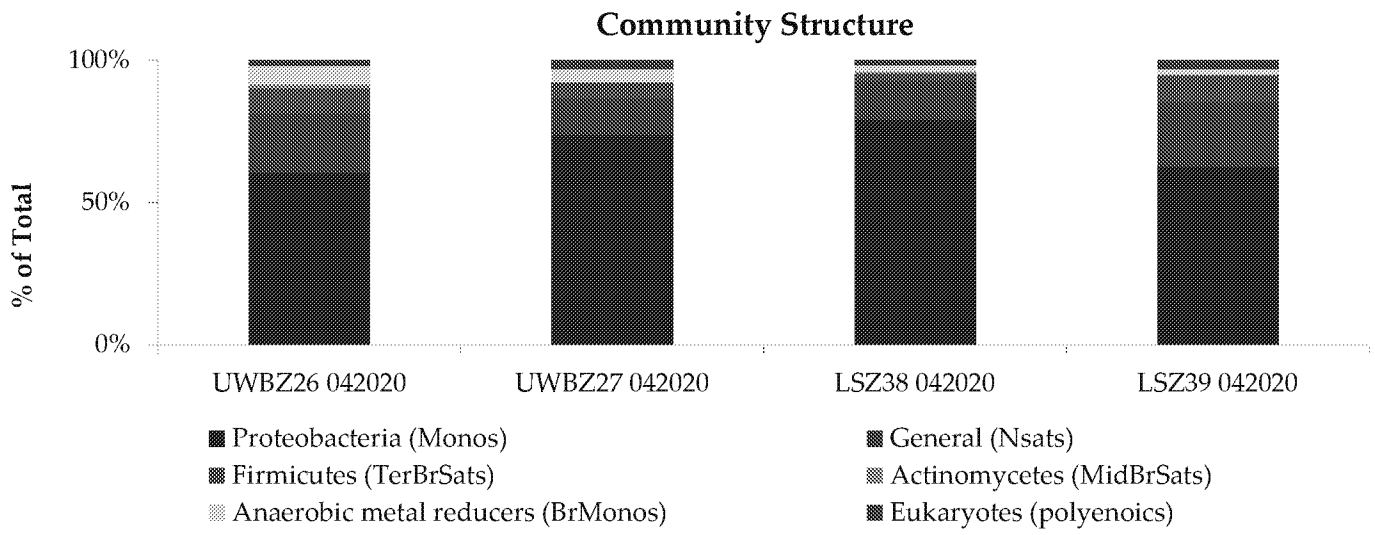

Figure 2. Relative percentages of total PLFA structural groups in the samples analyzed. Structural groups are assigned according to PLFA chemical structure, which is related to fatty acid biosynthesis. See the table in the interpretation section for detailed descriptions of the structural groups.

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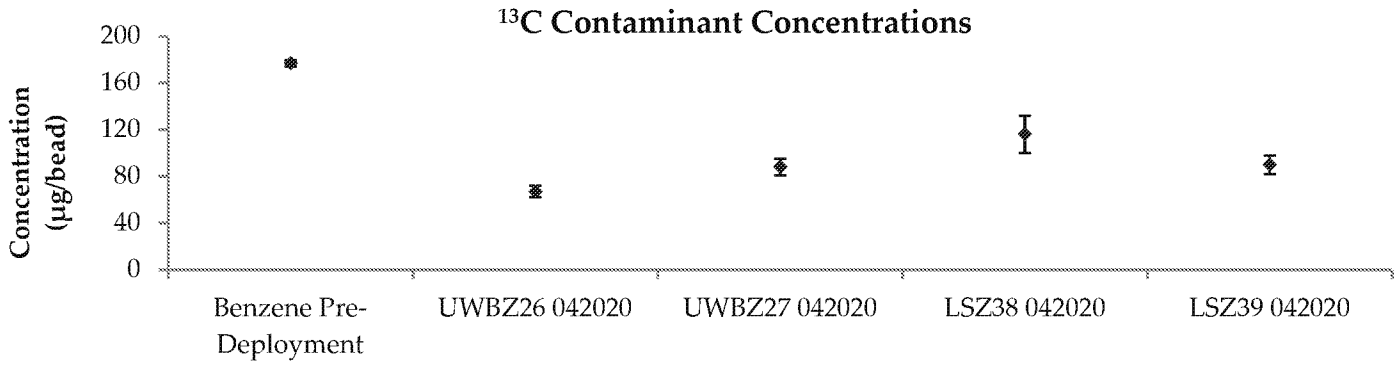

Figure 3. Comparison of Pre-deployment concentrations loaded on Bio-Sep beads to the concentrations detected after incubation.

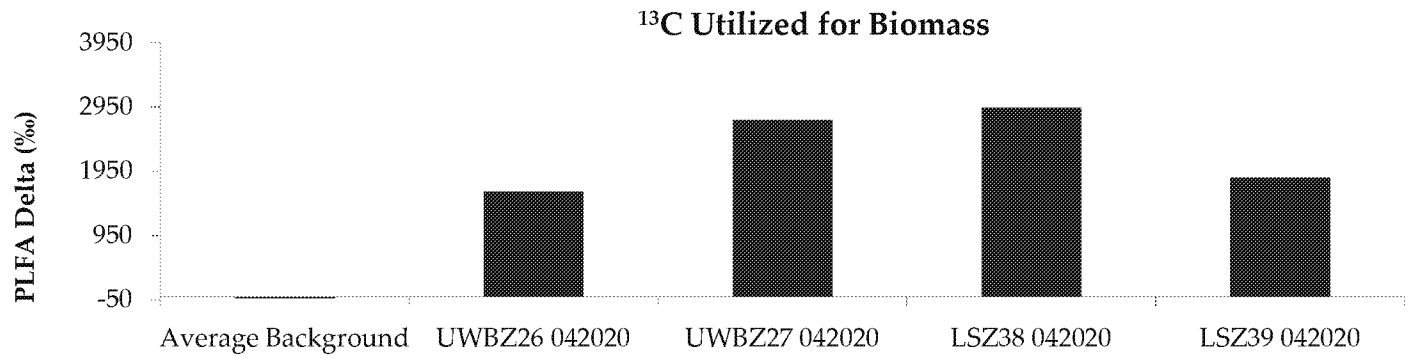

Figure 4. Comparison of the average Delta value obtained from PLFA biomarkers from each Bio-Trap® unit to the average background Delta observed in samples not exposed to ^{13}C enriched compounds.

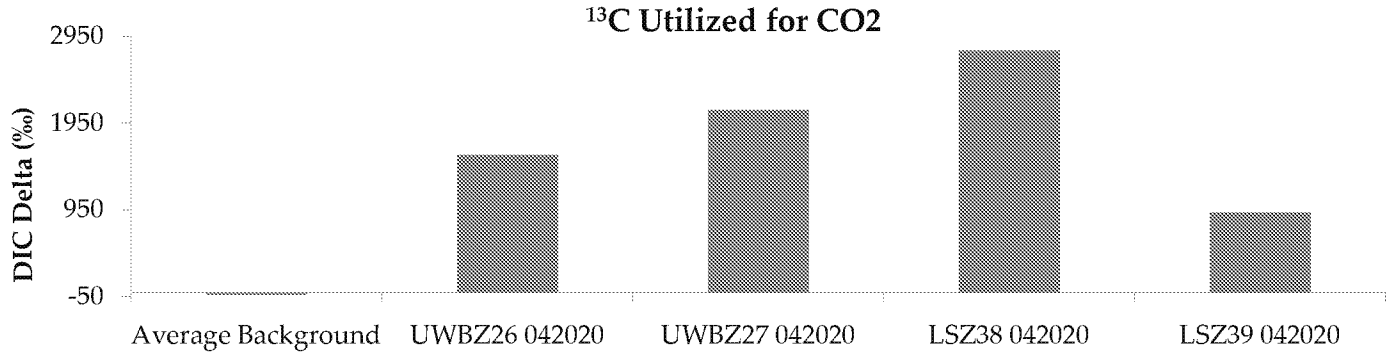

Figure 5. Comparison of the Delta value obtained from DIC from each Bio-Trap® unit to the average background Delta observed in samples not exposed to ^{13}C enriched compounds.

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Interpretation

Interpretation of the results of the SIP Bio-Trap® study must be performed with due consideration of site conditions, site activities, and the desired treatment mechanism. The following discussion describes interpretation of results in general terms and is meant to serve as a guide.

Contaminant Concentration: Bio-Traps® are baited with a ^{13}C labeled contaminant of concern and a pre-deployment concentration is determined prior to shipping. Following deployment, Bio-Traps® are recovered for analysis including measurement of the concentration of the ^{13}C labeled contaminant remaining. Pre- and post-deployment concentrations are used to calculate percent loss.

Biomass Concentrations: PLFA analysis is one of the most reliable and accurate methods available for the determination of viable (live) biomass. Phospholipids break down rapidly upon cell death (1,2), so biomass calculations based on PLFA content do not include "fossil" lipids from dead cells. Total biomass (cells/bead) is calculated from total PLFA using a conversion factor of 20,000 cells/pmole of PLFA. When making comparisons between wells, treatments, or over time, differences of one order of magnitude or more are considered significant.

Total Biomass		
Low	**Moderate**	**High**
10^3 to 10^4 cells	10^5 to 10^6 cells	10^7 to 10^8 cells

^{13}C Enriched Biomass: For SIP studies, ^{13}C enriched PLFA is determined to quantify ^{13}C incorporation into biomass as a line of evidence. The detection of ^{13}C enriched biomass provides conclusive evidence of contaminant biodegradation. However, biodegradation of a contaminant of concern is almost always performed by a small subset of the total microbial community. Therefore, the ^{13}C enriched biomass is typically several orders of magnitude lower than total biomass.

Average and Maximum PLFA Delta ^{13}C: Isotopic data is often reported as a delta value. The delta value is the difference between the isotopic ratio ($^{13}C/^{12}C$) of the sample (R_x) and a standard (R_{std}) normalized to the isotopic ratio of the standard (R_{std}) and multiplied by 1,000 (units are parts per thousand or "per mill" and denoted ‰). R_{std} is the international standard Vienna PeeDee Belemnite (VPDB) with an anomalously high $^{13}C/^{12}C$ ratio of 0.011237. Due to the high value of the R_{std}, computed delta ^{13}C values for most natural compounds are negative on a per mill basis.

Under natural conditions, the background delta ^{13}C value for PLFA is between -20 and -30‰. For a SIP Bio-Trap® study, biodegradation and incorporation of the ^{13}C labeled compound into PLFA results in a larger $^{13}C/^{12}C$ ratio (R_x) and thus delta values greater than under natural conditions.

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Typical PLFA delta values are provided below.

PLFA Delta (‰)		
Low	**Moderate**	**High**
0 to 100	100 to 1,000	>1,000

Dissolved Inorganic Carbon (DIC): Often, bacteria can utilize the ^{13}C labeled compound as both a carbon and energy source. The ^{13}C portion used as a carbon source for growth can be incorporated into PLFA as discussed above, while the ^{13}C used for energy is oxidized to $^{13}CO_2$ (mineralized).

^{13}C enriched CO_2 data is often reported as a delta value as described above for PLFA. Under natural conditions, the delta ^{13}C value for CO_2 is typically in the range of -25‰ to -10‰ (3). For an SIP Bio-Trap® study, mineralization of the ^{13}C labeled contaminant of concern (increased $^{13}CO_2$ production) would lead to a greater value of R_x and thus a positive delta value.

The detection of even low levels of ^{13}C enriched DIC provides conclusive evidence of contaminant biodegradation. However, delta values between 0 and 100‰ are generally considered relatively low, values between 100 and 1,000‰ are considered moderate, and values greater than 1,000‰ are considered high.

Dissolved Inorganic Carbon (DIC) Delta and %^{13}C		
Low	**Moderate**	**High**
0 to 100	100 to 1,000	>1,000

Community Structure (% total PLFA): Community structure data is presented as a percentage of PLFA structural groups normalized to the total PLFA biomass. The relative proportions of the PLFA structural groups provide a "fingerprint" of the types of microbial groups (e.g. anaerobes, sulfate reducers, etc.) present and therefore offer insight into the dominant metabolic processes occurring at the sample location. Thorough interpretation of the PLFA structural groups depends in part on an understanding of site conditions and the desired microbial biodegradation pathways. For example, an increase in mid chain branched saturated PLFA (MidBrSats), indicative of sulfate reducing bacteria (SRB) and Actinomycetes, may be desirable at a site where anaerobic BTEX biodegradation is the treatment mechanism, but would not be desirable for a corrective action promoting aerobic BTEX or MTBE biodegradation. The following table provides a brief summary of each PLFA structural group and its potential relevance to bioremediation.

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Description of PLFA structural groups.

PLFA Structural Group	General classification	Potential Relevance to Bioremediation Studies
Monoenoic (Monos)	Abundant in Proteobacteria (Gram negative bacteria), typically fast growing, utilize many carbon sources, and adapt quickly to a variety of environments.	Proteobacteria is one of the largest groups of bacteria and represents a wide variety of both aerobes and anaerobes. The majority of Hydrocarbon utilizing bacteria fall within the Proteobacteria
Terminally Branched Saturated (TerBrSats)	Characteristic of Firmicutes (Low G+C Gram-positive bacteria), and also found in Bacteriodes, and some Gram-negative bacteria (especially anaerobes).	Firmicutes are indicative of presence of anaerobic fermenting bacteria (mainly *Clostridia/Bacteriodes*-like), which produce the H_2 necessary for reductive dechlorination
Branched Monoenoic (BrMonos)	Found in the cell membranes of micro-aerophiles and anaerobes, such as sulfate- or iron-reducing bacteria	In contaminated environments high proportions are often associated with anaerobic sulfate and iron reducing bacteria
Mid-Chain Branched Saturated (MidBrSats)	Common in sulfate reducing bacteria and also Actinobacteria (High G+C Gram-positive bacteria).	In contaminated environments high proportions are often associated with anaerobic sulfate and iron reducing bacteria
Normal Saturated (Nsats)	Found in all organisms.	High proportions often indicate less diverse populations.
Polyenoic	Found in higher plants, and animals.	Eukaryotic scavengers will often prey on contaminant utilizing bacteria.

Physiological Status (Proteobacteria): Some Proteobacteria modify specific PLFA as a strategy to adapt to stressful environmental conditions (4, 5). For example, *cis* monounsaturated fatty acids may be modified to cyclopropyl fatty acids during periods of slowed growth or modified to *trans* monounsaturated fatty acids to decrease membrane permeability in response to environmental stress. The ratio of product to substrate fatty acid thus provides an index of their health and metabolic activity.

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Glossary

Delta (δ): A Delta value is the difference between the isotopic ratio ($^{13}C/^{12}C$) of the sample (R_x) and a standard (R_{std}) normalized to the isotopic ratio of the standard (R_{std}) and multiplied by 1,000 (units are parts per thousand denoted ‰).

$$\text{Delta} = (R_x - R_{std})/R_{std} \times 1000$$

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