Technical Memorandum
Bio-Trap Study Results Regarding PCP Degradation

(Addendum to PCP Degradation Tech Memo, TRC 2020)

Wauleco, Inc.
Wausau, Wisconsin

January 2022

BRRTS No. 02-37-000006

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1.0 Executive Summary

This Technical Memorandum (Tech Memo) summarizes the results of a Bio-Trap Study performed at the Wauleco site located in Wausau, Wisconsin conducted pursuant to the Site Investigation Work Plan for a Bio-Trap Study Field Work (Bio-Trap Work Plan, TRC, December 2020) approved by the Department in a letter dated February 29, 2021 (WDNR 2021). The Tech Memo serves as an addendum to the August 2020 Technical Memorandum, Lines of Evidence of PCP Degradation (2020 PCP Degradation Tech Memo, TRC 2020), and relies, to some extent, on the data and presentations in the 2020 PCP Degradation Tech Memo. Results presented in this Tech Memo conclusively demonstrate that PCP is biodegrading in groundwater within the residual phase light non-aqueous phase liquid (LNAPL) footprint, through the central portion of the groundwater plume, and in downgradient groundwater.

U.S. EPA's Natural Attenuation guidance (EPA, 1998) described three lines of evidence to demonstrate whether contaminant degradation is occurring. The 2020 PCP Degradation Tech Memo (TRC, 2020) provided a detailed summary of the literature demonstrating that PCP naturally biodegrades and demonstrated satisfaction of EPA's lines of evidence #1 and #2[1].

U.S. EPA's guidance (EPA, 1998) line of evidence #3 is a field or microcosm study to directly demonstrate that biodegradation is occurring. WDNR's October 30, 2020 letter agreed that PCP can be naturally degraded and that PCP concentrations have decreased across much of the site. However, WDNR requested/stated that "additional field studies are necessary to support the conclusion that biodegradation is the primary natural attenuation mechanism for the central portion of the groundwater plume." Therefore, this Bio-Trap Study was completed to further evaluate whether biodegradation of PCP is occurring throughout the Wauleco Site, including through the central portion of the groundwater plume.

The Bio-Trap Study used PCP labeled with carbon 13 (^{13}C), a stable carbon isotope found in nature at low concentrations, to demonstrate whether biodegradation was occurring. Bio-Traps baited with ^{13}C labeled PCP were placed in select wells for 90 days after which the laboratory analyzed for ^{13}C in bacterial biomass and dissolved inorganic carbon. The literature, included in the laboratory report by Microbial Insights (Appendix B), demonstrates that if the radio-labeled carbon present in the PCP is detected in the bacterial biomass or the dissolved inorganic carbon, then PCP is being biodegraded.

Analytical results (see Table 1) showed that:

- ^{13}C was detected in all eight sample locations, both within the residual phase LNAPL footprint, through the central portion of the groundwater plume, and in downgradient wells (see Figure 3). Seven of the eight samples had high concentrations of ^{13}C in the biomass, as those results are characterized by Microbial Insights.

[1] These lines of evidence are:
1. that a clear and meaningful trend of decreasing contaminant mass and/or concentration is occurring; and
2. that natural attenuation processes are active at the site at a rate which such processes will reduce contaminant concentrations.

- In addition, all eight samples showed the presence of ^{13}C in dissolved inorganic carbon, demonstrating that the PCP had been completely mineralized to carbon dioxide. Three were characterized as having high concentrations of ^{13}C, two moderate, and two low concentrations. Two of the three high concentration samples were from within the residual phase LNAPL footprint and one was in a downgradient location.

These Bio-Trap results conclusively demonstrate that biodegradation of PCP is occurring in groundwater throughout the Wauleco Site (i.e., within the residual phase LNAPL footprint, through the central portion of the groundwater plume, and in downgradient groundwater).

The moderate to high levels of biological degradation of PCP shown by the Bio-Trap Study, in combination with the concentration-distance analysis of biodegradation and dispersion in the 2020 PCP Degradation Tech Memo, demonstrates that this biodegradation is the primary natural attenuation mechanism occurring at the Site.

2.0 Background

2.1 Introduction

This Tech Memo is prepared as an addendum to the August 2020 Technical Memorandum, Lines of Evidence of PCP Degradation (2020 PCP Degradation Tech Memo, TRC 2020), and relies, to some extent, on the data and presentations in the 2020 PCP Degradation Tech Memo.

The Wauleco, Inc. (Wauleco), facility is located at 125 Rosecrans Street, Wausau, Wisconsin (Site; see Figure 1). The property is located in an area of mixed industrial and residential land use. The property is the location of a former window and patio door manufacturer from the early 1900s to the early 1990s. Manufacturing operations ceased in March 1991 and nearly all Site buildings were demolished by 1993.

As was common in the wood window manufacturing industry, surface coating on the exterior portions of wood windows manufactured at the site was performed using a wood preservative trade named Woodtox Preprime, manufactured by Kopper Chemical and Coating Company. Woodtox Preprime, commonly referred to as Penta, was a 5% solution of pentachlorophenol (PCP) dissolved in 85% mineral spirits, and 10% inerts. Penta was used at the Site from approximately 1944 until 1986.

2.2 Purpose and Background

The 2020 PCP Degradation Tech Memo presented information to demonstrate PCP biodegradation was occurring at the Wauleco Site, using the lines of evidence required by U.S. EPA's Natural Attenuation guidance (EPA, 1998). The data included:

- A comprehensive Conceptual Site Model (CSM) describing the Wauleco Site conditions, including the Light Non-Aqueous Phase Liquid (LNAPL) and groundwater.
- A comprehensive literature review demonstrating that PCP does biodegrade in both aerobic and anaerobic environments in soil and groundwater.
- A demonstration that PCP is biodegrading at the Wauleco Site using U.S. EPA's lines of evidence #1 and #2, as follows:
  - **Line of Evidence #1:** Data and analysis to satisfy EPA's first line of evidence for natural attenuation, by demonstrating a clear and meaningful trend of decreasing contaminant mass and/or concentration. This was demonstrated with both time-concentration and distance-concentration observations at Wauleco.
  - **Line of Evidence #2:** Data and analysis to satisfy U.S. EPA's second line of evidence for natural attenuation, through hydrogeologic and geochemical data that were used to demonstrate indirectly the types of natural attenuation processes active at the Site and the rate at which such processes will reduce contaminant concentrations.

U.S. EPA's guidance states that where lines of evidence 1 and 2 are inadequate or inconclusive then data from a field or microcosm study may be necessary.

A technical review of the 2020 PCP Degradation Tech Memo by the Wisconsin Department of Natural Resources (WDNR) was requested. The WDNR's technical review was presented in a WDNR letter dated October 30, 2020 (WDNR 2020) that stated in part:

- "Based on review of the technical memorandum, the Department agrees that PCP can be degraded naturally and that PCP concentrations in groundwater across much of site have decreased over time."
- "The Department conceptually agrees with the findings and conclusions of this technical memorandum, however; additional field studies are necessary to support the conclusion that biodegradation is the primary natural attenuation mechanism for the central portion of the groundwater plume."

To demonstrate whether PCP is degrading both outside and within the LNAPL footprint, TRC prepared the Site Investigation Work Plan for a Bio-Trap Study Field Work (Bio-Trap Work Plan, TRC, December 2020). As described in the Bio-Trap Work Plan, the purposes of this study were to:

- Respond to the WDNR's October 30, 2020 request for additional field studies.
- Determine whether bacteria capable of degrading PCP are present at the Wauleco site.
- Gather additional data to demonstrate that biodegradation of PCP is occurring within the central portion of the PCP groundwater plume. This data will be used to demonstrate whether PCP degradation is occurring throughout the PCP groundwater plume as well as beneath the area where residual phase LNAPL is present.
- Fulfill Wauleco's commitment in the 2020 PCP Degradation Tech Memo (refer to Section 1) to perform a study to assess the presence of necessary bacteria.

The Department approved of the Bio-Trap Work Plan sampling and analysis strategy in its letter dated February 29, 2021 (WDNR 2021).

As described in the Bio-Trap Study Work Plan, the study consisted of deploying bio-beads from Microbial Insights into eight wells across the residual phase LNAPL footprint and the downgradient PCP plume. The eight wells selected were presented in the Work Plan and approved by the Department. These bio-beads were spiked with PCP that contains the stable carbon isotope, carbon 13 (^{13}C). Following an approximate 90-day deployment period, the Bio-Traps were recovered to quantify whether ^{13}C contained in the PCP was incorporated into biomass and/or dissolved inorganic carbon (DIC).

When used as a 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 carbon dioxide (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 study provides conclusive evidence of contaminant biodegradation. Detection of ^{13}C enriched dissolved inorganic carbon (DIC) which includes $^{13}CO_2$ conclusively demonstrates contaminant biodegradation and mineralization. Example peer reviewed publications describing these techniques, referred to as stable isotope probing, include Geyer, et. al., 2005, and Madsen, 2006, as well as numerous others. Analyses for ^{13}C in the

biomass and in DIC were conducted by Microbial Insights on the eight Bio-Traps deployed at Wauleco.

2.3 Report Organization

This report is organized as follows:

- **Bio-Trap Study Results** – Section 3
- **Findings and Conclusions** – Section 4
- **References** – Section 5

3.0 Bio-Trap Study Results

3.1 Background and Methods

The Bio-Trap study consists of:

1. A group of "bio-beads" that were amended by Microbial Insights with ^{13}C labeled PCP were delivered to the Wauleco Site. These bio-beads were then installed in the eight wells described in the Bio-Trap Work Plan (refer to Figure 2) on July 14, 2021 and recovered on October 12, 2021, for a deployment of approximately 90 days. Methods used for storage, deployment, recovery and shipping followed Microbial Insight's Bio-Trap-Stable Isotope Probing Protocol (See Appendix A). During this time, groundwater flow through the bio-beads provides the conditions for bacterial growth on the beads, much like in the aquifer. The bacteria present in the aquifer seed the bio-beads, with subsequent growth in the bio-beads. The bio-beads are designed to retain the bacteria for subsequent analysis.

2. The bio-beads were then removed from the wells and analyzed for several properties. These properties, and a short description of their significance are presented below. Microbial Insights' report provides much more detail on these properties and the literature references supporting the interpretation of these results. Analytical data were provided for:

   - **Total Biomass (cells/bead)** – This is a measure of the number of bacteria per bead and reflects the amount of biological activity in the aquifer. Microbial Insights has conducted hundreds of these studies and developed ranges of low, medium, and high biomass. These ranges are shown in the summary results on Table 1 as low (red), medium (yellow), and high (green), consistent with Microbial Insights' report (see Appendix B).

   - **^{13}C enriched Biomass (cells/bead)** – This is a measure of the number of bacteria per bead that have incorporated ^{13}C originating from the ^{13}C labeled PCP present on the beads. As stated by Microbial Insights, the presence of ^{13}C within the biomass conclusively demonstrates whether degradation of PCP is occurring. Microbial Insights states that in their experience the ^{13}C presence is typically several orders of magnitude less than total biomass. Several orders of magnitude would be 0.1% of the total biomass containing ^{13}C. Therefore, over 1% of the total biomass is categorized by Microbial Insights as high, and are highlighted in green, on Table 1.

   - **Average and Maximum Enriched PLFA Delta (‰)** – These are measures of ^{13}C originating from the ^{13}C labeled PCP that ended up as a carbon molecule making up a phospholipid fatty acid (PLFA) in a bacterial body on the bio-beads. PLFA are a primary component of the bacterial cells, so that the PLFA Delta values greater than background conclusively demonstrates degradation of the ^{13}C labeled PCP. Microbial Insights' experience shows ranges of high, medium, and low values for these analyses. These ranges are also color coded on Table 1.

- **Dissolved Inorganic Carbon (DIC) Delta (‰)** – This is a measure of the amount of ^{13}C that has been mineralized (i.e., degradation of the PCP by the bacteria while using the PCP, or one of its degradation products, as a source of energy). DIC Delta (‰) naturally is in the range of -25‰ to -10‰. Microbial Insights states that ^{13}C enriched DIC provides conclusive evidence of contaminant biodegradation even at low levels of DIC Delta values. Microbial Insights provides ranges of DIC Delta for low, moderate, and high amounts of biodegradation. These ranges are color coded on Table 1.

The Bio-Traps were deployed in eight locations across the area to satisfy the plan's objectives of determining whether bacterial activity and PCP degradation are occurring at the Wauleco site, including within the central portion of the residual phase LNAPL footprint and throughout the PCP groundwater plume. As such, the locations for the eight Bio-Traps included:

Central Area of the Residual Phase LNAPL footprint:

- **W6R** – located within the residual phase LNAPL footprint, near the upgradient side, in a well that historically had mobile LNAPL;
- **W3A, W22, and W44** – Located in wells within the central portion of the residual phase LNAPL footprint. Downgradient of the former source area, but within the residual phase LNAPL footprint;
- **W10A** – Located in the furthest downgradient portion of the residual phase LNAPL footprint.

Northern Portion of the Residual Phase LNAPL footprint:

- **W2** – Located within the residual phase LNAPL footprint, on the north side, in a well that historically has had mobile phase LNAPL.

Southern Area of the Residual Phase LNAPL footprint:

- **W41** – Located adjacent to the southern portion of the residual phase LNAPL footprint, in a well that the WDNR has referred to as having a "recalcitrant" PCP concentration.
- **W11** – Located downgradient of the southern portion of the residual phase LNAPL footprint, in an area where PCP is shown to be degrading rapidly along the southeast concentration-distance profile (see discussion in the 2020 PCP Degradation Tech Memo).

3.2 Summary of Results

Microbial Insights laboratory report of the Bio-Trap results is included in Appendix B. Table 1 and Figure 3 summarize the results for the Bio-Trap analyses. Figure 3 illustrates the results for each Bio-Trap location, along with the extent of residual phase LNAPL.

As shown on Table 1 and Figure 3, all eight of the Bio-Trap locations conclusively demonstrated the degradation of PCP. In addition, all five Bio-Trap locations within the residual phase LNAPL footprint show high to medium levels of PCP degradation. The results show:

- Moderate levels of total biomass in all eight locations, indicating that there are moderate levels of bacterial activity present within the residual phase LNAPL footprint and in downgradient locations.

- All eight locations show the presence of enriched ^{13}C in the biomass and enriched in the PFLA. Microbial Insights and their list of peer reviewed journal articles state that this conclusively demonstrates that PCP is degrading within the residual phase LNAPL footprint and in downgradient locations.

- The presence of ^{13}C in mineralized forms of carbon (i.e., in dissolved inorganic carbon) conclusively demonstrates that PCP is being degraded while being used as an energy source for bacterial growth. As shown in Table 1, ^{13}C was detected at above background concentrations in all eight samples, and at moderate to high concentrations in five of the eight samples. Microbial Insights and their list of peer reviewed journal articles state that this conclusively demonstrates that PCP is degrading and is being mineralized to CO_2 within the residual phase LNAPL footprint and in downgradient locations.

- The ^{13}C was detected in dissolved inorganic carbon at high concentrations at locations W6R and W22 within the central portion of the residual phase LNAPL footprint and at location W11, downgradient of the southeast portion of the residual phase LNAPL footprint.

- Detection of ^{13}C in mineralized forms of carbon is a difficult form of ^{13}C to capture in the bio-beads because mineralized carbon occurs as gaseous carbon dioxide, which can be lost prior to being captured in the bio-beads or before analysis. Therefore, the amount of mineralization of PCP may be underestimated.

3.3 Census Analysis

Microbial Insights provided analysis to identify whether there were bacteria present that at least one research paper indicated as one set of bacteria that can biodegrade PCP. Analyses presented in Appendix B for the dechlorinating bacteria and the functional genes indicated low presence of these bacteria and genes. The conclusion from this analysis, in comparison with the Bio-Trap study is that the group of bacteria and genes targeted by these analyses are not the bacteria and genes that are the source of the conclusive demonstration of PCP biodegradation.

4.0 Findings and Conclusions

4.1 Findings

Key findings presented in this Technical Memorandum include the following:

- The Bio-Trap Study analytical results conclusively demonstrate that biodegradation of PCP is occurring in groundwater throughout the Wauleco Site (i.e., within the residual phase LNAPL footprint, through the central portion of the groundwater plume, and in downgradient groundwater).
- This demonstration that PCP is biodegrading is shown by the transfer of the stable isotope carbon 13 (^{13}C) that was built into the PCP that was amended to the bio-beads was transferred to biomass, as shown by ^{13}C being present in the biomass in all eight locations tested. In addition, the ^{13}C presence in dissolved inorganic carbon further demonstrates that the PCP, or its degradation products, are being used by the bacteria as an energy source.
- The comparison of the strength of the source of PCP to groundwater to the rate of PCP biodegradation qualitatively explains the changes in PCP in groundwater along each of the concentration-distance profiles. For example:
  - Where there is no overlying residual phase LNAPL, acting as a source of PCP to groundwater, the biodegradation rate results in decreasing PCP concentrations as groundwater migrates downgradient (e.g., near W11 along the Southeast Profile).
  - Where there is an overlying residual phase LNAPL, a strong source of PCP to groundwater can exceed the biodegradation rate, resulting in increases in PCP concentrations in groundwater, but a moderate to weak source of PCP to groundwater can be less than the biodegradation rate resulting in decreases in PCP concentrations in groundwater.
- The Centerline Profile has shown a change in the concentration profile from pre-2018 PCP trends (i.e., increasing PCP concentration between wells W03A and W10A) to progressively larger decreases in PCP concentration from 2019 to 2021.

4.2 Conclusions

The conclusions from this analysis are:

- Biodegradation of PCP is conclusively demonstrated throughout the Site through this Bio-Trap Study, particularly by detecting the radio-labeled ^{13}C in the PCP placed within the Bio-Traps showing up in the total biomass, the PLFA (i.e., bacterial biomass), and in some cases, within the dissolved inorganic carbon.
- This biodegradation of PCP was demonstrated throughout the Site, both within the residual phase LNAPL footprint and in downgradient areas.

This Bio-Trap Study responds to the WDNR's request in its October 30, 2020 review of the 2020 PCP Degradation Tech Memo, in particular, that PCP biodegradation not only can occur, but is conclusively demonstrated to be occurring throughout the Site and is the primary natural attenuation mechanism occurring that is resulting in the decline of PCP concentrations across much of the Site.

4.3 WDNR Review Request

Wauleco requests a technical meeting with WDNR to discuss the details of the Tech Memo.

5.0 References

Geyer, R. et. al. 2005. *In Site Assessment of Biodegradation Potential Using Biotraps Amended with ^{13}C-Labeled Benzene or Toluene*. Environmental Science and Technology, 2005, 39, 4983-4989.

Madsen, E. 2006. *The Use of Stable Isotope Probing Techniques in Bioreactor and Field Studies on Bioremediation*. Current Opinion in Biotechnology. 2006, 17:92-97.

TRC. 2020. Technical Memorandum Lines of Evidence of PCP Degradation. August 2020.

TRC. 2019. Technical Memorandum Residual Phase LNAPL Investigation. December 2019.

TRC. 2020. Bio-Trap Site Investigation Work Plan. December 2020.

U.S. EPA. 1998. Technical Protocol for Evaluating Natural Attenuation of Chlorinated Solvents in Ground Water. EPA/600/R-98/128.

WDNR. 2020. Department Review of Technical Memorandum Lines of Evidence of PCP Degradation Wauleco, Inc., 125 Rosecrans Street, Wausau, DNR BRRTS #02-37-000006. October 30, 2020.

WDNR. 2021. Department Review of Bio-Trap Site Investigation Work Plan. February 29, 2021.

Table 1
Bio-Trap Study Analytical Results
Wauleco Project Site
125 Rosecrans Street
Wausau, Wisconsin

Sample Name	Well W2	Well W3A	Well W6R	Well W10A	Well W11	Well W22	Well W41	Well W44
Biomass & ^{13}C Incorporation								
Total Biomass (Cells/bead)	**3.91E+05**	**5.95E+05**	**2.29E+05**	**9.01E+05**	**6.37E+05**	**1.25E+06**	**7.31E+05**	**1.10E+06**
^{13}CEnriched Biomass (Cells/bead)	**1.12E+04**	**2.46E+04**	**1.76E+04**	**8.27E+03**	**7.26E+04**	**8.15E+04**	**1.09E+04**	**1.37E+04**
^{13}C Enriched Biomass as Percent of Total Biomass	2.9%	4.1%	7.7%	0.9%	11.4%	6.5%	1.5%	1.2%
Average Enriched PLFA Delta (‰)	**1240**	**1312**	**7048**	72	**11680**	**8630**	**405**	**515**
Maximum Enriched PLFA Delta (‰)	**4740**	**6906**	**10027**	**256**	**20627**	**28360**	**1291**	**4711**
^{13}C Mineralization								
DIC Delta (‰)	-2	-3	3456	-8	7474	6803	2	4
Community Structure (% total PLFA)								
Firmicutes (TerBrSats)	0.00	**5.96**	**0.00**	**2.29**	**6.07**	**3.58**	**12.11**	**1.67**
Proteobacteria (Monos)	**76.05**	**73.78**	**80.83**	**67.89**	**67.76**	**70.27**	**65.23**	**68.73**
Anaerobic metal reducers (BrMonos)	**0.00**	**0.00**	**0.00**	**5.49**	**6.45**	**1.70**	**0.00**	**0.55**
Actinomycetes (MidBrSats)	**0.00**	**0.00**	**0.00**	**0.00**	**2.88**	**4.96**	**0.00**	**0.00**
General (Nsats)	**23.95**	**20.28**	**19.17**	**23.11**	**16.84**	**18.45**	**20.60**	**22.10**
Eukaryotes (Polyenoics)	**0.00**	**0.00**	**0.00**	**1.21**	**0.00**	**1.03**	**2.06**	**6.94**
Physiological Status (Proteobacteria only)								
Slowed Growth	0.28	**0.48**	**0.00**	**0.30**	**0.32**	**0.44**	**0.74**	**0.52**
Decreased Permeability	**0.00**	**0.13**	**0.00**	**0.36**	**0.00**	**0.06**	**0.00**	**0.05**

Total Biomass Values (cells/bead)	
Low	<1e5
Moderate	1e5 to 9e6
High	>1e7

Dissolved Inorganic Carbon (DIC) Delta (‰)	
Background	-30 to -20
Low	> -20 to 100
Moderate	100-1000
High	>1,000

Average and Maximum Enriched PLFA Delta (‰)	
Low	0-100
Moderate	100-1,000
High	>1,000

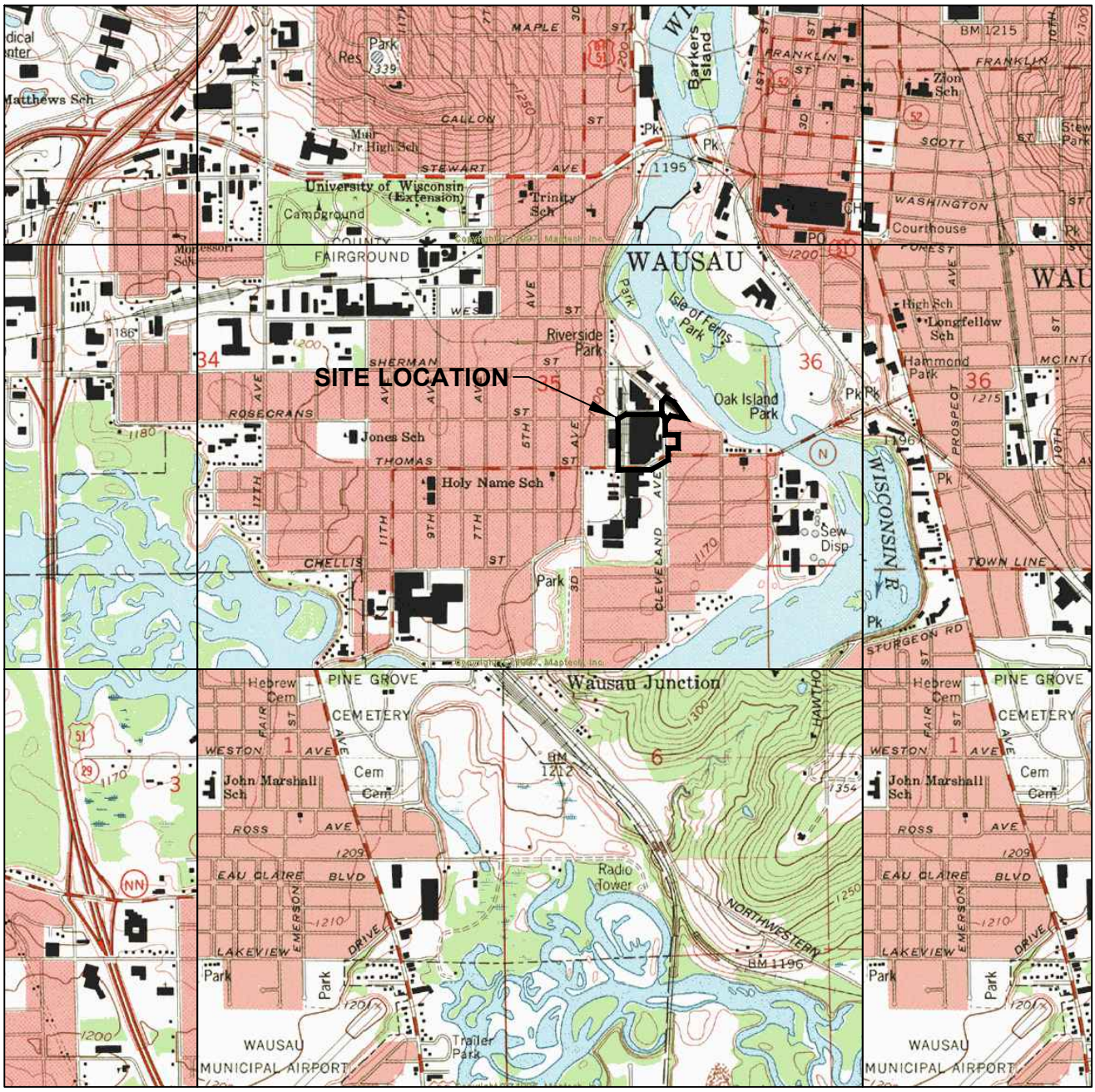
^{13}C is the stable isotope carbon 13 that was added to the biobeads as ^{13}C-labeled PCP.

PLFA - are phospholipid fatty acids. PLFA are a primary component of the membrane of bacterial cells.

DIC - is dissolved inorganic carbon, and would consist of carbon dioxide, carbonate, and bicarbonate dissolved in groundwater.

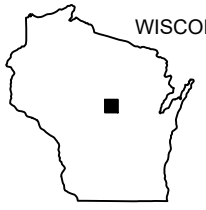
Enriched PLFA Delta (‰) is a measure of the ratio of ^{13}C to ^{12}C (the typical stable carbon isotope). Increases in the Delta value indicate a source of ^{13}C (i.e., from the ^{13}C-labeled PCP).

Prepared by K. Quinn, 12/9/2021
Checked by S. Sellwood, 12/10/2021



NOTE

BASE MAP DEVELOPED FROM THE WAUSAU WEST AND WAUSAU EAST, WISCONSIN 7.5 MINUTE U.S.G.S. TOPOGRAPHIC QUADRANGLE MAPS, DATED 1993. PART OF SECTION 35, T29N, R8E



QUADRANGLE LOCATION

TRC
708 Heartland Trail
Suite 3000
Madison, WI 53717
Phone: 608.826.3600

PROJECT:

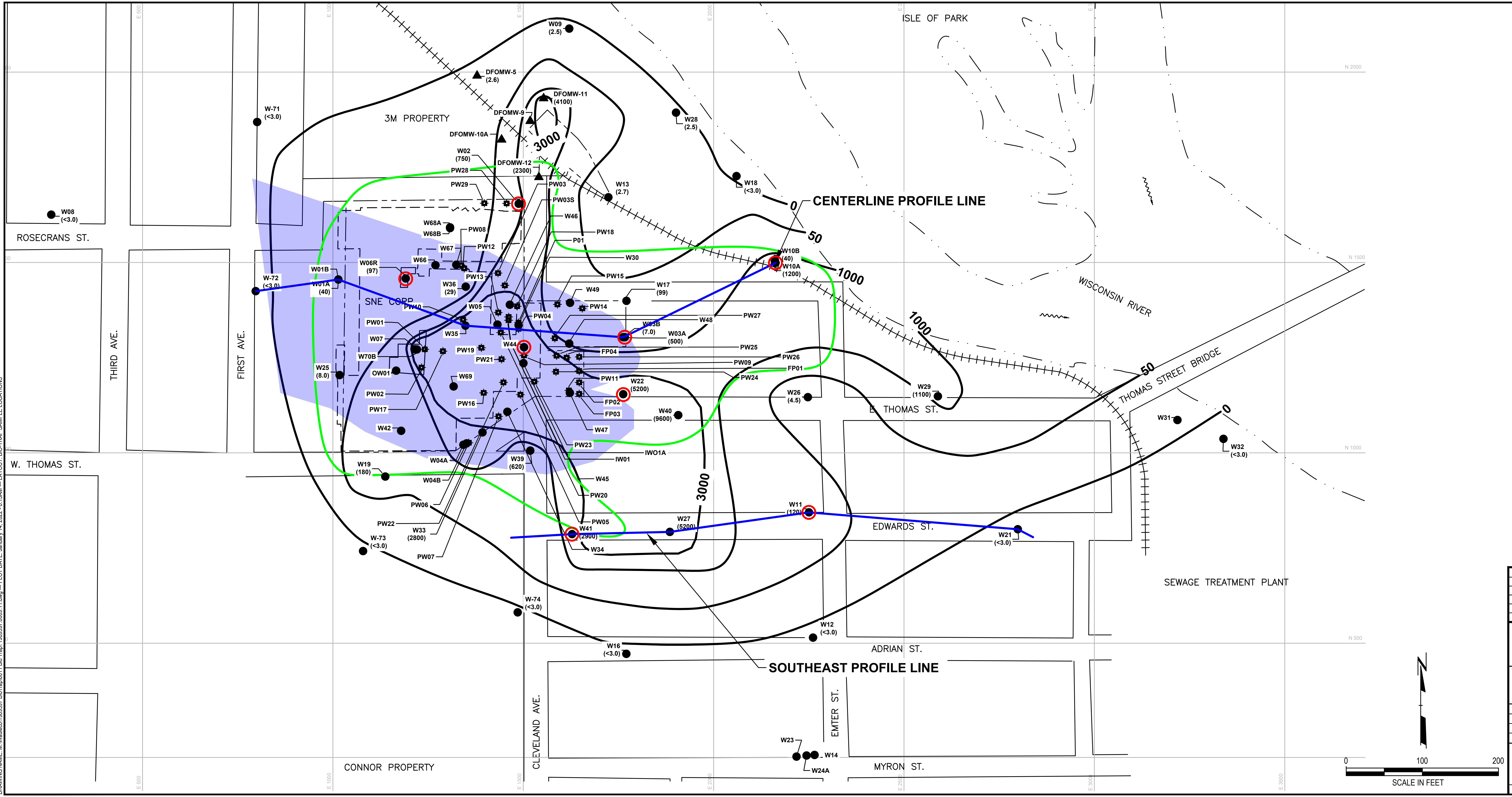
WAULECO, INC.
WAUSAU, WISCONSIN

TITLE:

SITE LOCATION MAP

DRAWN BY:	T.FIEBRANZ
CHECKED BY:	K. QUINN
APPROVED BY:	B. IVERSON
DATE:	JANUARY 2022
PROJ. NO.:	1989597.0011
FILE:	1989597.0011.01 SLM.dwg

FIGURE 1



LEGEND

- W17 (1.9) ● MONITORING WELL LOCATION AND PCP CONCENTRATION (ug/L)
- PW12 EXTRACTION WELL LOCATION AND NUMBER
- DFOMW-5 ▲ 3M GROUNDWATER MONITORING WELL
- APPROXIMATE PROPERTY LINE
- FORMER BUILDING OUTLINE
- 50 PCP ISOCONCENTRATION CONTOUR INTERVAL VARIES
- OUTLINE OF RESIDUAL PHASE LNAPL
- DISTANCE - CONCENTRATION PROFILE LINES
- BIO-TRAP DEPLOYMENT WELL

NOTES

1. BASE MAP DEVELOPED FROM DRAWING A107250-1 OF THE SEPTEMBER 1992 SEMI- ANNUAL GROUNDWATER MONITORING REPORT BY KEYSTONE ENVIRONMENTAL, MWH DRAWING 2082658.302160101-B1, AND 3M WELLS LOCATION BASED ON 3M MAPS.
2. GROUNDWATER SAMPLES OBTAINED BY TRC ON JULY 10-12, 16, 18, 19, 2018.
3. ANALYTE CONCENTRATIONS OBTAINED FROM LABORATORY DATA BY CT LABORATORIES, INC.
4. IN WELL CLUSTERS THE VALUE FROM THE SHALLOWEST WELL WAS USED TO DETERMINE ISOCONCENTRATIONS FOR THE ANALYTE.
5. THE NR140 ENFORCEMENT STANDARD (ES) FOR PCP IS 1.0 ug/L. THE NR140 PREVENTIVE ACTION LIMIT (PAL) FOR PCP IS 0.10 ug/L.
6. WAULECO WELLS PW02 AND W70B WERE ABANDONED ON 7/21/16 DURING SOIL MOUND REMOVAL ACTIVITIES BY TRC. 3M WELLS DFOMW9 AND DFOMW10A WERE ABANDONED BY 3M IN THE SUMMER OF 2015.

NO.	BY	DATE	REVISION	APP'D.

PROJECT:

WAULECO, INC.
WAUSAU, WISCONSIN

TITLE:

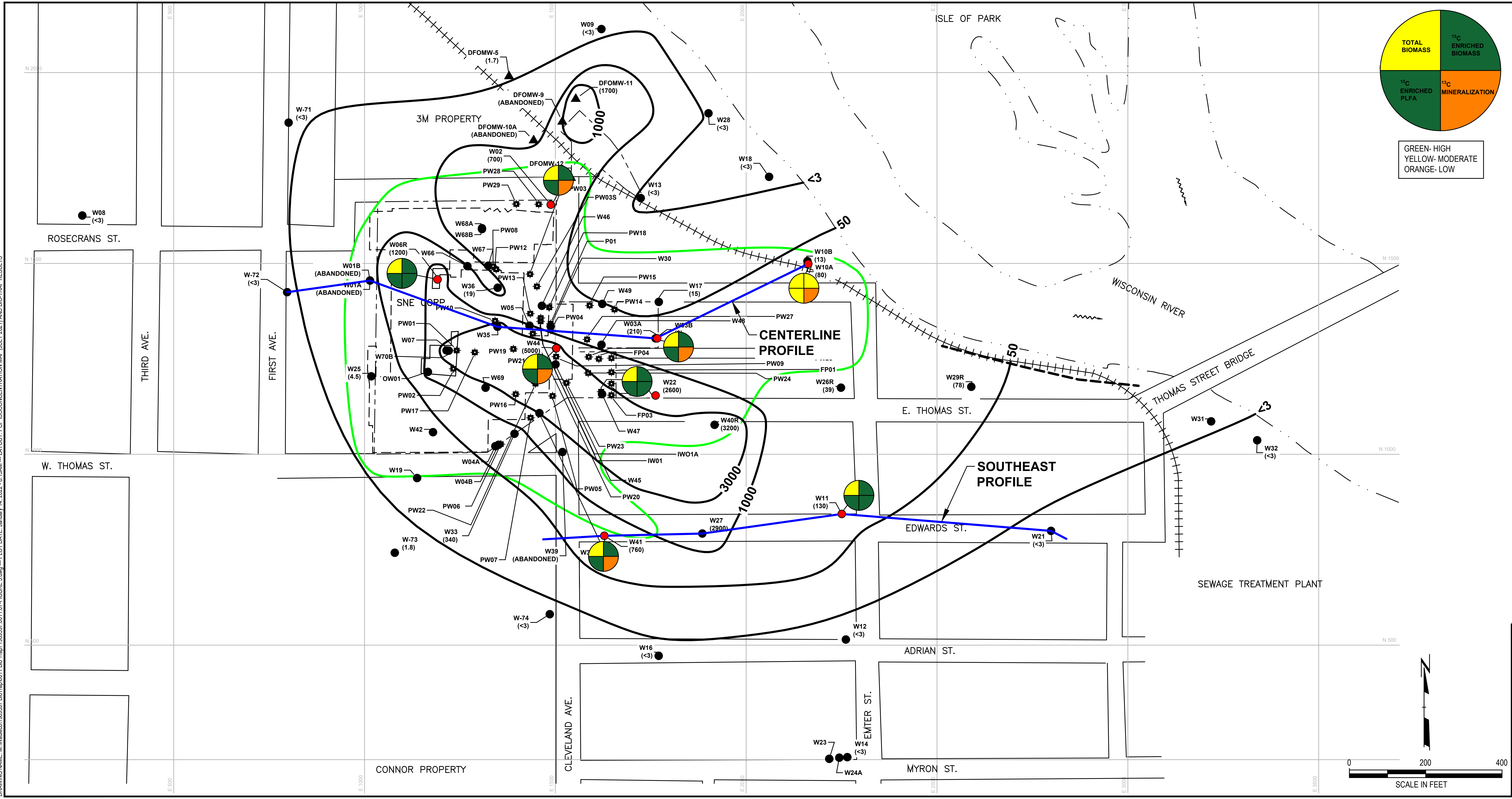
BIO-TRAP SAMPLE LOCATIONS

DRAWN BY:	T.FIEBRANZ	PROJ NO.:	1989597.0011
CHECKED BY:	K. QUINN		FIGURE 2
APPROVED BY:	B. IVERSON		
DATE:	JANUARY 2022		

TRC
708 Heartland Trail
Suite 3000
Madison, WI 53717
Phone: 608.826.3600

FILE NO.: 1989597.0009.11.dwg

DRAWING NAME: M:\Wauleco\1989597 BioTrap\0011 Bio Trap\1989597.0009.11.dwg — PLOT DATE: January 14, 2022 8:13AM — LAYOUT: BIO-TRAP SAMPLE LOCATIONS
Version: 2017-10-21



ISLE OF PARK
3M PROPERTY
ROSECRANS ST.
W. THOMAS ST.
THIRD AVE.
FIRST AVE.
SNE CORP
CENTERLINE PROFILE
SOUTHEAST PROFILE
WISCONSIN RIVER
THOMAS STREET BRIDGE
E. THOMAS ST.
EDWARDS ST.
ADRIAN ST.
MYRON ST.
EMTER ST.
CLEVELAND AVE.
CONNOR PROPERTY
SEWAGE TREATMENT PLANT
W09 (<3)
DFOMW-5 (1.7)
DFOMW-9 (ABANDONED)
DFOMW-11 (1700)
DFOMW-10A (ABANDONED)
DFOMW-12
W-71 (<3)
W28 (<3)
W18 (<3)
W02 (700)
PW28
PW29
W03
PW03S
W13 (<3)
W46
W08 (<3)
PW08
W68A
W68B
PW18
P01
W67
PW12
W06R (1200)
W66
W30
W10B (13)
W10A (80)
W01B (ABANDONED)
W01A (ABANDONED)
W-72 (<3)
PW13
W36 (19)
PW15
W17 (15)
W49
PW14
PW10
W05
PW04
W48
PW27
PW01
W35
W03A (210)
W03B
W07
W44 (5000)
PW19
PW21
FP04
W70B
W25 (4.5)
OW01
PW09
FP01
W69
PW24
W22 (2600)
W26R (39)
W29R (78)
PW02
PW16
FP03
PW17
W42
W47
W40R (3200)
PW23
IWO1A
IW01
W04A
W04B
W19
W45
PW05
PW20
PW06
PW22
W11 (130)
W27 (2900)
W33 (340)
W-73 (1.8)
W41 (760)
W39 (ABANDONED)
PW07
W21 (<3)
W31
W32 (<3)
W-74 (<3)
W12 (<3)
W16 (<3)
W23
W14 (<3)
W24A
1000
3000
50
<3
TOTAL BIOMASS
13C ENRICHED BIOMASS
13C ENRICHED PLFA
13C MINERALIZATION
GREEN- HIGH
YELLOW- MODERATE
ORANGE- LOW
0
200
400
SCALE IN FEET

LEGEND

W17 (15) ● MONITORING WELL LOCATION AND PCP CONCENTRATION (ug/L)

● WELL WITH BIO-TRAP SAMPLER

PW12 EXTRACTION WELL LOCATION AND NUMBER

DFOMW-5 ▲ 3M GROUNDWATER MONITORING WELL

APPROXIMATE PROPERTY LINE

FORMER BUILDING OUTLINE

50 PCP ISOCONCENTRATION CONTOUR INTERVAL VARIES

OUTLINE OF RESIDUAL PHASE LNAPL

APPROXIMATE LOCATION OF SHEET PILE WALL

NOTES

1. BASE MAP DEVELOPED FROM DRAWING A107250-1 OF THE SEPTEMBER 1992 SEMI- ANNUAL GROUNDWATER MONITORING REPORT BY KEYSTONE ENVIRONMENTAL, MWH DRAWING 2082658.302160101-B1, AND 3M WELLS LOCATION BASED ON 3M MAPS.
2. GROUNDWATER SAMPLES OBTAINED BY TRC ON JULY 6 ,7, 8, 12, 13, 14, 2021.
3. ANALYTE CONCENTRATIONS OBTAINED FROM LABORATORY DATA BY CT LABORATORIES, INC.
4. IN WELL CLUSTERS THE VALUE FROM THE SHALLOWEST WELL WAS USED TO DETERMINE ISOCONCENTRATIONS FOR THE ANALYTE.
5. THE NR140 ENFORCEMENT STANDARD (ES) FOR PCP IS 1.0 ug/L. THE NR140 PREVENTIVE ACTION LIMIT (PAL) FOR PCP IS 0.10 ug/L.
6. BIO-TRAP SAMPLERS WERE INSTALLED IN WELLS W02, W03A, W06R, W10A, W11, W22, W41, AND W44 FROM JULY 14 THROUGH OCTOBER 12, 2021.
7. THE CITY OF WAUSAU INSTALLED A STEEL SHEET PILING WALL IN 2020 TO REPLACE A ROCK WALL ON THE WISCONSIN RIVER BANK LOCATED WEST OF THE THOMAS STREET BRIDGE.

PROJECT:
WAULECO, INC.
WAUSAU, WISCONSIN

TITLE:
PCP ISOCONCENTRATION MAP (JULY 2021) AND BIO-TRAP RESULTS

DRAWN BY:	T.FIEBRANZ	PROJ NO.:	1989597.0011
CHECKED BY:	K. QUINN	FIGURE 3	
APPROVED BY:	B. IVERSON		
DATE:	JANUARY 2022		

TRC
708 Heartland Trail
Suite 3000
Madison, WI 53717
Phone: 608.826.3600

FILE NO.: 1989597.0011.07-FIGURE 3.dwg

DRAWING NAME: M:\Wauleco\1989597 BioTrap\0011 Bio Trap\1989597.0011.07-FIGURE 3.dwg — PLOT DATE: January 14, 2022 - 8:13AM — LAYOUT: PCP ISOCONCENTRATION MAP (JULY 2021) AND BIO-TRAP RESULTS

Version: 2017-10-21

Appendix A: Microbial Insights Bio-Trap – Stable Isotope Probing Protocol

Bio-Trap – Stable Isotope Probing Protocol

Sampling Instructions

Handling:

- Bio-Trap Samplers used for Stable Isotope Probing (SIP) are baited with ^{13}C-labeled contaminant of interest (e.g. benzene, MTBE, chlorobenzene) adsorbed onto the powder activated carbon (PAC). Controlled laboratory conditions show only minimal loss of contaminant due to volatilization. However, special considerations must be taken into account when handling SIP Bio-Trap Samplers in order to reduce the risk of volatilization.
- SIP Bio-Trap Samplers are shipped out chilled, on blue ice, and it is essential that they should be kept cool (not frozen) until deployment.
- When retrieving the Bio-Trap Samplers that have been deployed in the field, they should immediately be placed on ice and shipped on ice for next day delivery. These steps will ensure the most accurate results.
- Although the contaminant is absorbed onto the beads, caution should be used in handling these Bio-Trap Samplers because the contaminant compounds are associated with possible health and safety risks.

Note: Clean latex gloves (or similar) should be used at all times when handling the Bio-Trap Samplers.

Storage:

It is important to minimize the amount of time that Bio-Trap Samplers are stored prior to being installed in the field. The physical properties of the Bio-Trap Samplers that make them an ideal medium for collecting microbes also increase the chances of microbial or chemical contamination. Bio-Trap Samplers need to remain sealed and refrigerated (not frozen) until they can be installed in the field.

Installation:

- Prior to installing Bio-Trap Sampler, the monitoring well may need to be purged if it has not been sampled in a while. If purging is necessary, MI recommends that three well volumes be removed to ensure contact with formation water and reduce well bore effect.
- Attach the Bio-Trap Sampler's nylon loop (provided) to a nylon line (not provided) and suspend Bio-Trap Sampler at a depth where significant contaminant concentrations exist. If no data are available on the vertical distribution of contaminants, then suspend the Bio-Trap Sampler in the middle of the saturated screened interval.
- If large fluctuations in the water level are anticipated during the period of incubation, the Bio-Trap Sampler should be suspended from a float (contact MI for further details). Be sure not to suspend the bio-trap in the NAPL zone.
- Once installed, incubation times can vary depending upon the scope of the project. A typical Stable Isotope Probing (SIP) study incubation period is 30 days but is project dependant. Please contact us if you have questions regarding the optimum deployment period for your samples.

Retrieval:

- Open the monitoring well and pull up the Bio-Trap Sampler. Cut and remove the braided nylon line used to suspend the Bio-Trap Sampler.
- Transfer the recovered Bio-Trap Sampler to labeled (well number and date) zippered bags, seal and then double bag in a larger (one-gallon) zippered bag, immediately place on blue ice in a cooler.
- Repeat above for all the Bio-Trap Samplers from the site.
- A chain of custody (COC) form must be included with each shipment of samples.
- In order to minimize the potential effect of these samplers on the monitoring well, MI recommends purging three well volumes from the test well following the retrieval of the SIP Bio-Trap Samplers.

Hold time for this analysis is 24-48 hours.

Shipping Instructions

Packaging Samples:

1. Samples should be shipped in a cooler with ice or blue ice for next day delivery. If regular ice is used, the ice should be double bagged.
2. A chain of custody form must be included with each shipment of samples. Access our chain of custody at www.microbe.com.

Shipment for Weekday Delivery:

Samples for weekday delivery should be shipped to:

Sample Custodian
Microbial Insights, Inc.
10515 Research Drive
Knoxville, TN 37932
(865) 573-8188

Shipment for Saturday Delivery:

Coolers to be delivered on Saturday **must be shipped via FedEx** to our FedEx Drop Location (FedEx will not accept shipments from any other carriers). To ensure proper handling the following steps must be taken:

1. FedEx shipping label should be marked under (6) Special Handling, check Hold Saturday.
2. The cooler must be taped with FedEx SATURDAY tape.
3. The shipping label must be filled out with the Drop Location address below. Our laboratory name must be on the address label.

4. You MUST ***notify by email*** customerservice@microbe.com with the tracking number of the package on Friday (prior to 4pm Eastern Time) to arrange for Saturday pickup. Please make sure you write "Saturday Delivery" in the subject line of the message. Without proper labeling and the tracking number, there is no guarantee that the samples will be collected.

Samples for Saturday delivery should be shipped to:

Microbial Insights, Inc.
FedEx Drop Location
10601 Murdock Drive
Knoxville, TN 37932
(865) 573-8188

Notes:

- **Stable Isotope Probing (SIP) may preclude subsequent Compound Specific Isotope Analysis (CSIA) in the study well for a period of time. CSIA can be performed prior to SIP or at another location.**

Appendix B: Microbial Insights Bio-Trap Laboratory Reports

- Microbial Insights – SITE LOGIC Report Stable Isotope Probing (SIP)
- Microbial Insights – Census Analyses

Microbial Insights
SITE LOGIC Report Stable Isotope Probing (SIP)

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Knoxville, TN 37932
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Fax: 865.573.8133
Web: www.microbe.com

SITE LOGIC Report

Stable Isotope Probing (SIP)

Contact: Ken Quinn
Phone: 608-826-3653
Address: TRC Environmental Corporation
708 Heartland Trail
Madison, WI, 53707
Email: kquinn@trccompanies.com

MI Identifier: 037SJ
Report Date: November 18, 2021

Project: Wauleco

Comments: Report revised 11/30/21 to include QAQC information. Report revised 12/3/2021 to expand the executive summary. Report revised on 12/17/2021 to expand the executive summary and add color shading for the result ranges.

Executive Summary

A Stable Isotope Probing (SIP) study was performed to determine whether biodegradation of Pentachlorophenol (PCP) is occurring under existing site conditions. Bio-Trap® samplers baited with ^{13}C-labeled PCP were deployed in Wauleco W2, Wauleco W3A, Wauleco W6R, Wauleco W10A, Wauleco W11, Wauleco W22, Wauleco W41, Wauleco W44. Following a 90-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 Tables 1 and 2, and Figures 1 through 4. Following are the key observations from the results obtained for the monitoring wells.

Stable Isotope Probing (SIP) Results

- Quantification of ^{13}C-enriched PLFA conclusively demonstrated that PCP was metabolized under existing site conditions. The average PLFA $\delta^{13}C$ values for Wauleco W2, Wauleco W3A, Wauleco W6R, Wauleco W11, and Wauleco W22 were within the high range with values of 1240‰, 1312‰, 7048‰, 11680‰, and 8630‰, respectively. The average PLFA $\delta^{13}C$ values for Wauleco W41, and Wauleco W44 were within the moderate range with values of 405‰ and 515‰, respectively. The average PLFA $\delta^{13}C$ value for Wauleco W10A (72‰) fell within the low range.
- Quantification of ^{13}C-enriched DIC conclusively demonstrated that PCP was biologically degraded under existing site conditions. The average DIC $\delta^{13}C$ values for samples Wauleco W2, Wauleco W3A, Wauleco W10A, Wauleco W41, and Wauleco W44 were low with values of -2‰, -3‰, -8‰, 2‰ and 4‰, respectively. These results suggest that the mineralization of PCP was low at these locations.
- The average DIC $\delta^{13}C$ values for Wauleco W6R (3456‰), Wauleco W11 (7474‰), and Wauleco W22 (6803‰) fell within the high range. Detection of ^{13}C enriched DIC, which includes $^{13}CO_2$, conclusively indicates contaminant biodegradation and mineralization.
- 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.
- The total PLFA biomass concentrations in all monitoring wells were on the order of 10^5 to 10^6 cells/bead, which was within the moderate range. 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.
- 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.
    - The PLFA community structure for sample Wauleco W2 was comprised of monoenoics (76.05%) followed by normal saturates (23.95%).
    - The PLFA community structure for sample Wauleco W3A was primarily comprised of monoenoics (73.78%) followed by normal saturates (20.28%) and indicators of firmicutes (5.96%).
    - Similar to Wauleco W2, the PLFA community structure for sample Wauleco W6R was comprised of monoenoics (80.83%) followed by normal saturates (19.17%).
    - The PLFA community structure for sample Wauleco W10A was primarily comprised of monoenoics (67.89%) followed by normal saturates (23.11%), anaerobic metal reducers (5.49%) and indicators of firmicutes (2.29%) and eukaryotes (1.21%).

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- The PLFA community structure for sample Wauleco W11 was primarily comprised of monoenoics (67.76%) followed by normal saturates (16.84%), anaerobic metal reducers (6.45%), firmicutes (6.07%), and actinomycetes (2.88%).
- The PLFA community structure for sample Wauleco W22 was primarily comprised of monoenoics (70.27%) followed by normal saturates (18.45%), actinomycetes (4.96%), and firmicutes (3.58%). Less than 3% of the PLFA community structure was comprised of anaerobic metal reducers (1.70%), and eukaryotes (1.03%).
- The PLFA community structure for sample Wauleco W41 was primarily comprised of monoenoics (65.23%) followed by normal saturates (20.26%), firmicutes (12.11%) and indicators of eukaryotes (2.06%).
- The PLFA community structure for sample Wauleco W44 was primarily comprised of monoenoics (68.73%) followed by normal saturates (22.10%), and eukaryotes (6.94%). Less than 3% of the PLFA community structure was comprised of firmicutes (1.67%) and anaerobic metal reducers (0.55%).

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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	Wauleco W2	Wauleco W3A	Wauleco W6R	Wauleco W10A
Sample Date	10/12/2021	10/12/2021	10/12/2021	10/12/2021
MI ID	037SJ-1	037SJ-2	037SJ-3	037SJ-4
Biomass & ^{13}C Incorporation				
Total Biomass (Cells/bead)	**3.91E+05**	**5.95E+05**	**2.29E+05**	**9.01E+05**
^{13}C Enriched Biomass (Cells/bead)	**1.12E+04**	**2.46E+04**	**1.76E+04**	**8.27E+03**
^{13}C Enriched Biomass as Percent of Total Biomass	2.9%	4.1%	7.7%	0.9%
Average Enriched PLFA Delta (‰)	**1240**	**1312**	**7048**	**72**
Maximum Enriched PLFA Delta (‰)	**4740**	**6906**	**10027**	**256**
^{13}C Mineralization				
DIC Delta (‰)	**-2**	**-3**	**3456**	**-8**
Community Structure (% total PLFA)				
Firmicutes (TerBrSats)	0.00	**5.96**	0.00	**2.29**
Proteobacteria (Monos)	**76.05**	**73.78**	**80.83**	**67.89**
Anaerobic metal reducers (BrMonos)	0.00	0.00	0.00	**5.49**
Actinomycetes (MidBrSats)	0.00	0.00	0.00	0.00
General (Nsats)	**23.95**	**20.28**	**19.17**	**23.11**
Eukaryotes (Polyenoics)	0.00	0.00	0.00	**1.21**
Physiological Status (Proteobacteria only)				
Slowed Growth	**0.28**	**0.48**	0.00	**0.30**
Decreased Permeability	0.00	**0.13**	0.00	**0.36**

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

Total Biomass level (Cells/bd)	Low	E+03 to E+04
	Moderate	E+05 to E+06
	High	E+07 to E+08

Dissolved Inorganic Carbon (DIC) Delta (‰)	Background	(-25) to (-10)
	Low	> -10 to 100
	Moderate	100 to 1,000
	High	>1,000

Average and Maximum Enriched PLFA Delta (‰)	Low	0 to 100
	Moderate	100 to 1,000
	High	>1,000

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Table 2. Summary of the stable isotope probing results obtained from the Bio-Trap® Units.

Sample Name	Wauleco W11	Wauleco W22	Wauleco W41	Wauleco W44
Sample Date	10/12/2021	10/12/2021	10/12/2021	10/12/2021
MI ID	037SJ-5	037SJ-6	037SJ-7	037SJ-8
Biomass & ^{13}C Incorporation				
Total Biomass (Cells/bead)	**6.37E+05**	**1.25E+06**	**7.31E+05**	**1.10E+06**
^{13}C Enriched Biomass (Cells/bead)	**7.26E+04**	**8.15E+04**	**1.09E+04**	**1.37E+04**
^{13}C Enriched Biomass as Percent of Total Biomass	11.4%	6.5%	1.5%	1.2%
Average Enriched PLFA Delta (‰)	**11680**	**8630**	**405**	**515**
Maximum Enriched PLFA Delta (‰)	**20627**	**28360**	**1291**	**4711**
^{13}C Mineralization				
DIC Delta (‰)	**7474**	**6803**	**2**	**4**
Community Structure (% total PLFA)				
Firmicutes (TerBrSats)	**6.07**	**3.58**	**12.11**	**1.67**
Proteobacteria (Monos)	**67.76**	**70.27**	**65.23**	**68.73**
Anaerobic metal reducers (BrMonos)	**6.45**	**1.70**	0.00	**0.55**
Actinomycetes (MidBrSats)	**2.88**	**4.96**	0.00	0.00
General (Nsats)	**16.84**	**18.45**	**20.60**	**22.10**
Eukaryotes (Polyenoics)	0.00	**1.03**	**2.06**	**6.94**
Physiological Status (Proteobacteria only)				
Slowed Growth	**0.32**	**0.44**	**0.74**	**0.52**
Decreased Permeability	0.00	**0.06**	0.00	**0.05**

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

Total Biomass level (Cells/bd)	Low	E+03 to E+04
	Moderate	E+05 to E+06
	High	E+07 to E+08

Dissolved Inorganic Carbon (DIC) Delta (‰)	Background	(-25) to (-10)
	Low	> -10 to 100
	Moderate	100 to 1,000
	High	>1,000

Average and Maximum Enriched PLFA Delta (‰)	Low	0 to 100
	Moderate	100 to 1,000
	High	>1,000

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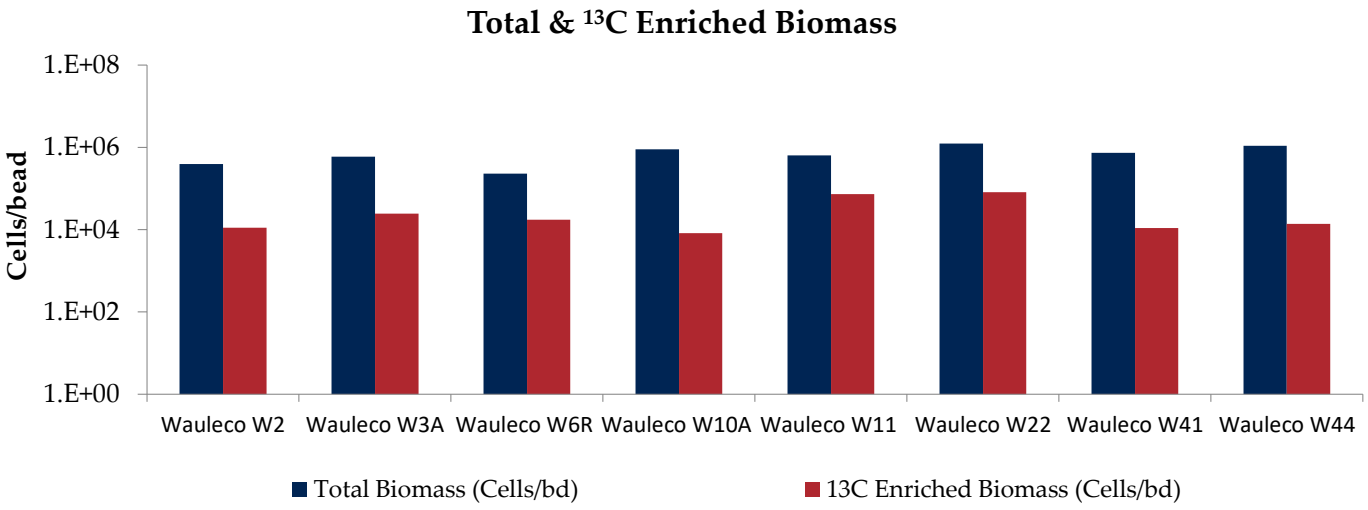

Figure 1. Biomass content is presented as a cell equivalent based on the total amount of phospholipid fatty acids (PLFA) extracted from a given sample. Total biomass is calculated based upon PLFA attributed to bacterial and eukaryotic biomass (associated with higher organisms).

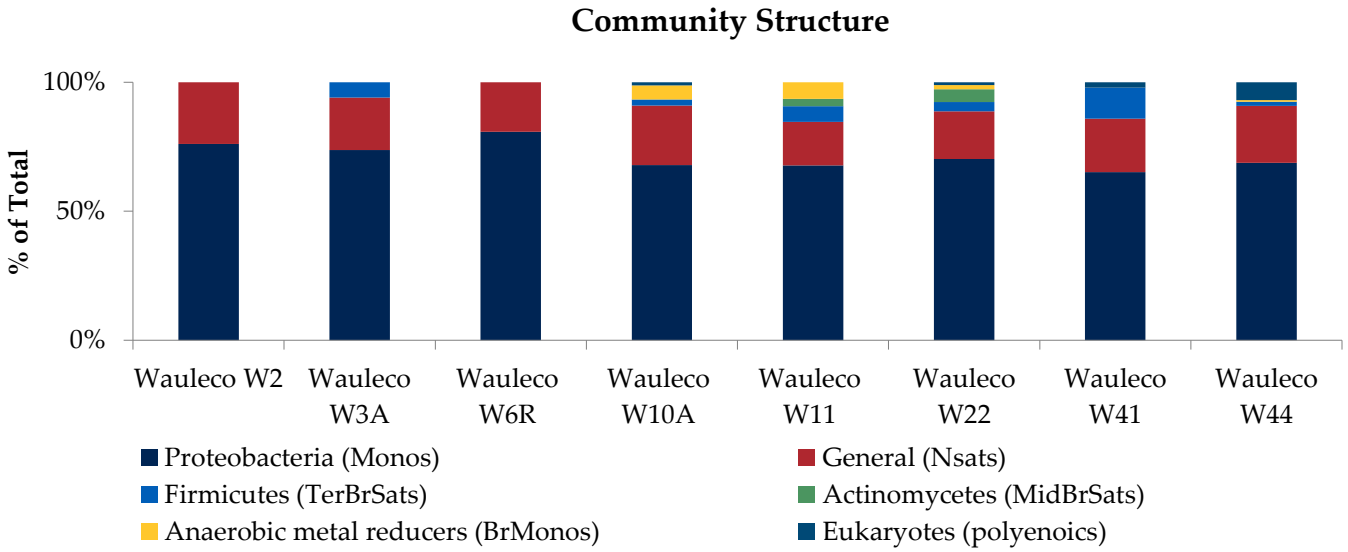

Figure 2. Relative percentages of total PLFA structural groups in the samples analyzed. Structural groups are assigned according to PLFA chemical structure, which is related to fatty acid biosynthesis. See the table in the interpretation section for detailed descriptions of the structural groups.

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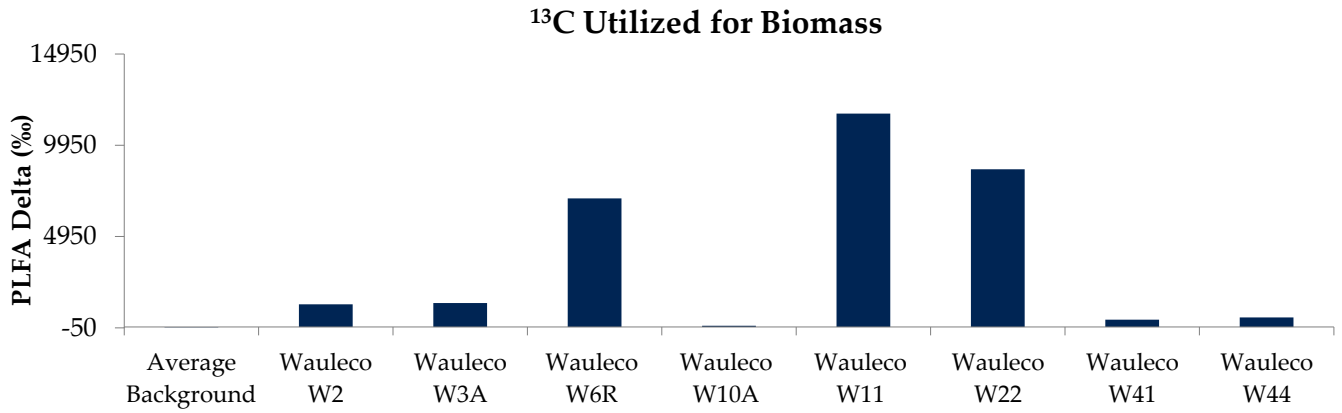

Figure 3. Comparison of the average Delta value obtained from PLFA biomarkers from each Bio-Trap® unit to the average background Delta observed in samples not exposed to ^{13}C enriched compounds.

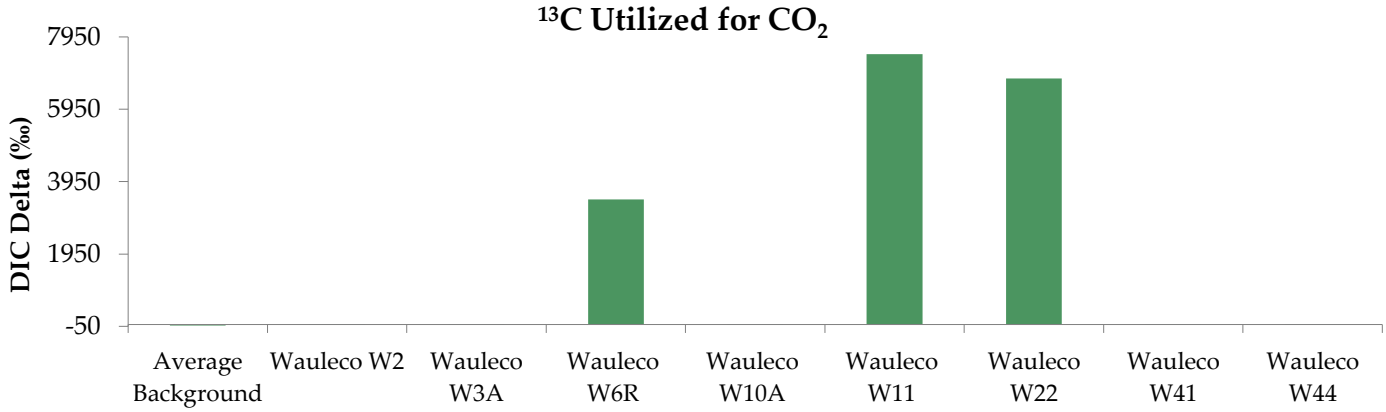

Figure 4. Comparison of the Delta value obtained from DIC from each Bio-Trap® unit to the average background Delta observed in samples not exposed to ^{13}C enriched compounds.

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Quality Assurance/Quality Control Data

Samples Received 10/13/2021

Component	Date Prepared	Date Analyzed	Arrival Temperature	Positive Control	Extraction Blank	Negative Control
PLFA	10/13/2021	10/29/2021	0 °C	112%	non-detect	non-detect
^{13}C-FAME	10/19/2021	11/1/2021	0 °C	Pass	not applicable	non-detect

Component	Date Prepared	Date Analyzed	Arrival Temperature	Mean Std. Deviation for Reference Material Replicates	Mean Absolute Accuracy for Calibrated Reference Materials
DIC	10/13/2021	11/15/2021	0 °C	±0.08 ‰	±0.15 ‰

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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.

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.

Typical PLFA delta values are provided below.

Enriched 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).

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^{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.

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

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response to environmental stress. The ratio of product to substrate fatty acid thus provides an index of their health and metabolic activity. In general, status ratios greater than 0.25 indicate a response to unfavorable environmental conditions.

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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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Microbial Insights
Census Analyses

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Client:	Ken Quinn TRC Environmental Corporation 708 Heartland Trail Madison, WI 53707	**Phone:** **Fax:**	608-826-3653 608-358-5193

Identifier: 037SJ **Date Rec:** 10/13/2021 **Report Date:** 10/25/2021

Client Project #: **Client Project Name:** Wauleco

Purchase Order #:

Test results provided for: CENSUS

Reviewed By:

MICROBIAL INSIGHTS, INC.

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CENSUS

Client: **TRC Environmental Corporation**
Project: Wauleco

MI Project Number: **037SJ**
Date Received: 10/13/2021

Sample Information

Client Sample ID:		**Wauleco W2**	**Wauleco W3A**	**Wauleco W6R**	**Wauleco W10A**	**Wauleco W11**
Sample Date:		10/12/2021	10/12/2021	10/12/2021	10/12/2021	10/12/2021
Units:		cells/bead	cells/bead	cells/bead	cells/bead	cells/bead
Analyst/Reviewer:		HT/CS	HT/CS	HT/CS	HT/CS	HT/CS
Dechlorinating Bacteria						
Dehalococcoides	*DHC*	<2.50E+01	<2.50E+01	<2.50E+01	<2.50E+01	<2.50E+01
Desulfitobacterium spp.	*DSB*	**2.33E+02 (J)**	**5.83E+04**	**1.91E+01 (J)**	**5.35E+03**	<2.50E+02
Functional Genes						
PCP Regulator Gene	pcpR	<2.50E+02	<2.50E+02	**6.92E+04**	<2.50E+02	<2.50E+02
Maleylacetate Reductase	pcpE	<2.50E+02	<2.50E+02	<2.50E+02	**3.84E+03**	<2.50E+02
PCP-4-Monooxygenase	pcpB	<2.50E+02	<2.50E+02	<2.50E+02	<2.50E+02	<2.50E+02

Legend:
NA = Not Analyzed NS = Not Sampled J = Estimated gene copies below PQL but above LQL I = Inhibited
< = Result not detected

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CENSUS

Client: **TRC Environmental Corporation**
Project: Wauleco

MI Project Number: **037SJ**
Date Received: 10/13/2021

Sample Information

Client Sample ID:		**Wauleco W22**	**Wauleco W41**	**Wauleco W44**
Sample Date:		10/12/2021	10/12/2021	10/12/2021
Units:		cells/bead	cells/bead	cells/bead
Analyst/Reviewer:		HT/CS	HT/CS	HT/CS
Dechlorinating Bacteria				
Dehalococcoides	*DHC*	<2.50E+01	<2.50E+01	<2.50E+01
Desulfitobacterium spp.	*DSB*	<2.50E+02	<2.50E+02	<2.50E+02
Functional Genes				
PCP Regulator Gene	pcpR	<2.50E+02	<2.50E+02	<2.50E+02
Maleylacetate Reductase	pcpE	<2.50E+02	<2.50E+02	<2.50E+02
PCP-4-Monooxygenase	pcpB	**9.93E+04**	<2.50E+02	<2.50E+02

Legend:
NA = Not Analyzed NS = Not Sampled J = Estimated gene copies below PQL but above LQL I = Inhibited
< = Result not detected

Quality Assurance/Quality Control Data

Samples Received **10/13/2021**

Component	Date Prepared	Date Analyzed	Arrival Temperature	Positive Control	Extraction Blank	Negative Control
DHC	10/13/2021	10/22/2021	0 °C	108%	non-detect	non-detect
pcpB	10/13/2021	10/22/2021	0 °C	100%	non-detect	non-detect
pcpR	10/13/2021	10/22/2021	0 °C	100%	non-detect	non-detect
pcpE	10/13/2021	10/22/2021	0 °C	81%	non-detect	non-detect
DSB	10/13/2021	10/22/2021	0 °C	99%	non-detect	non-detect