Extending The Applicability of Compound-Specific Isotope Analysis To Low Concentrations Of 1,4-Dioxane
Phase 2
SERDP Project ER-2535

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14. ABSTRACT
The objectives of this project are: to improve estimates of the long-term impact of natural attenuation processes on groundwater contaminants. Specific objectives of this research are:
1. Develop a reliable method for performing CSIA on low levels of 1,4-dioxane in groundwater (e.g., 1 to 10 micrograms per liter; µg/L);
2. Assess the fractionation of stable carbon and hydrogen isotope ratios during the aerobic co-metabolic biodegradation of 1,4-dioxane under laboratory and field conditions; and
3. Assess the use of stable carbon and hydrogen isotope ratios as tools to evaluate biodegradation of 1,4-dioxane at United States Department of Defense (DoD) sites with different groundwater conditions.

15. SUBJECT TERMS
1,4-dioxane, CSIA, dual-isotope plots, biodegradation, isotopic composition

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LIST OF ACRONYMS

1,1,1-TCA 1,1,1-trichloroethane
1,1-DCA 1,1-dichloroethane
1,1-DCE 1,1-dichloroethene
1,2-DCA 1,2-dichloroethane
1,2,3-TCP 1,2,3-trichloropropane
1,4-D 1,4-dioxane
A560 Ambersorb™ 560
ACB aerobic cometabolic biodegradation
AFB Air Force Base
AFCEC Air Force Civil Engineering Center
AFP Air Force Plant
ALDH aldehyde dehydrogenase
AOC Area of Concern
BAL below analytical limit of CSIA as reported by UWEIL
bgs below ground surface
C carbon, an element
^{12}C carbon-12, most abundant isotope of carbon with atomic mass of 12
^{13}C carbon-13, less abundant isotope of carbon with atomic mass of 13
^{14}C carbon-14 (radiocarbon), a radioactive isotope of carbon with atomic mass of 14
^{13}C/^{12}C Stable carbon isotope ratio
CCAFS Cape Canaveral Air Force Station
CDN Canadian currency
cis-1,2-DCE cis-1,2-dichloroethene
CSIA Compound-Specific Isotope Analysis
CVOC Chlorinated Volatile Organic Compound
$\delta^{13}C$ stable carbon isotope ratio as per mill (‰) difference from internationally accepted standard
DI direct injection
DO dissolved oxygen (in groundwater)
DoD Department of Defense, United States
DXMO dioxane monoxygenase
EA-IRMS elemental analyzer coupled with an isotope-ratio mass spectrometer
ERSON Statement of Need, Environmental Restoration Program Area
EISB engineered in situ bioremediation
ESTCP Environmental Security Technology Certification Program
FID Flame Ionization Detector
FTA Fire Training Area
g gram(s)
GAC granular activated carbon
GC Gas Chromatograph
GC-IRMS Gas Chromatography – Isotope-Ratio Mass Spectrometry
gpm gallons per minute
H hydrogen, an element
^{1}H protium, the most abundant isotope of hydrogen with atomic mass of 1


$^2\text{H}$ deuterium, less abundant isotope of hydrogen with atomic mass of 2
$^3\text{H}$ tritium, a radioactive isotope of hydrogen with atomic mass of 3
$^{2H}/^{1H}$ stable hydrogen isotope ratio
HWF Hazardous Waste Facility
ID internal diameter
IRMS isotope ratio mass spectrometer
ISBR In situ Bioreactor
ISCO In situ chemical oxidation
L liter(s)
JBCC Joint Base Cape Cod
mg milligram(s)
mg/L milligrams per liter
mg/g milligrams of 1,4-dioxane/gram of Ambersorb$^\text{TM}$560
min minutes
mL milliliter(s)
mL/min milliliters per minute
m meter
MCL Maximum Contaminant Level
mm millimeter
MNA Monitored Natural Attenuation
MTBE methyl tert-butyl ether
NA not applicable or not analyzed
ND not detected
NCSU North Carolina State University
NES UWEIL indicates there is not enough sample to perform CSIA
NPL National Priorities List
O oxygen atom
$^{16}\text{O}$ oxygen-16, the most abundant isotope of oxygen with atomic mass of 16
$^{18}\text{O}$ oxygen-18, less abundant isotope of oxygen with atomic mass of 18
OU Operable Unit
PCE tetrachloroethene
PLFA phospholipid fatty acid
qPCR quantitative polymerase chain reaction
ROD Record of Decision
RSL Regional Screening Level
SERDP Strategic Environmental Research and Development Program
SIM Selective Ion Monitoring
SLC-16 Space Launch Complex 16
SWMU Solid Waste Management Unit
TCE trichloroethene
TD thermal desorption
THF tetrahydrofuran
µg microgram(s)
µg/L micrograms per liter
US EPA United States Environmental Protection Agency
UWEIL University of Waterloo Environmental Isotope Laboratory
<table>
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<td>VAFB</td>
<td>Vandenberg Air Force Base</td>
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<tr>
<td>VC</td>
<td>vinyl chloride</td>
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<td>VOA</td>
<td>volatile organics analysis</td>
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<td>VPDB</td>
<td>Vienna Pee Dee Belemnite (internationally accepted standard for referencing carbon isotope ratios)</td>
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<td>VSMOW</td>
<td>Vienna Standard Mean Ocean Water (internationally accepted standard for referencing hydrogen isotope ratios)</td>
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<td>WWTP</td>
<td>wastewater treatment plant</td>
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ABSTRACT

Objective: The objectives of Project ER-2535 are aligned with the overall objective of the Statement of Need for the Environmental Restoration Program Area (ERSON-15-01): *to improve estimates of the long-term impact of natural attenuation processes on groundwater contaminants*. Specifically, this project sought to develop a low-cost, readily implementable method for assessing 1,4-dioxane degradation in groundwater by allowing for the application of compound-specific isotope analysis (CSIA) to low concentrations of 1,4-dioxane.

Technical Approach: The first phase of Project ER-2535 involved CSIA method development and application at four field sites and is reported in: *Final Report: Extending the Applicability of Compound-Specific Isotope Analysis to Low Concentrations of 1,4-Dioxane, SERDP Project ER-2535*. For this second phase of Project ER-2535, the CSIA method developed in Phase I was applied to 8 additional field sites. In addition, the isotopic compositions of ten different samples of manufactured 1,4-dioxane procured from chemical suppliers were characterized to help constrain the range in isotopic composition of 1,4-dioxane prior to degradation.

Results: For the 10 samples of manufactured 1,4-dioxane, the δ¹³C values ranged from -33.6‰ to -28.8‰ (median: -32.2‰) and δ²H ranged from -60‰ to -17‰ (median: -36‰). The majority of δ¹³C and δ²H values for 1,4-dioxane measured in groundwater samples were within the range determined for manufactured (i.e. undegraded source) 1,4-dioxane. Samples from some field sites had δ¹³C or δ²H values lower than the lowest values reported for neat 1,4-dioxane, indicating that the isotopic composition of manufactured 1,4-dioxane samples analyzed in this study does not represent the full range that can be expected at groundwater sites, and therefore conclusions regarding biodegradation of 1,4-dioxane based on the extent of enrichment beyond the “undegraded source” isotopic composition are subject to higher uncertainty. Dual isotope plots can provide more robust qualitative indications of biodegradation and can provide insights into the degradation conditions by comparing the dual isotope slope to those reported for different laboratory-controlled degradation reactions. Dual isotope trends consistent with biodegradation of 1,4-dioxane were found at four of the 12 sites studied over the course of this project; biostimulation of 1,4-dioxane degradation was occurring at two of these sites. The case studies show that CSIA can provide direct field-based evidence for 1,4-dioxane biodegradation in groundwater and, through the use of dual isotope plots, CSIA results can be compared to published enrichment trends to provide insights into the degradation conditions. On the other hand, at two of the sites where the dual isotope trend aligned with biodegradation, the magnitude of isotopic enrichment was smaller than observed in laboratory studies, perhaps due to blending of 1,4-dioxane from degradation zones with undegraded 1,4-dioxane in the screen interval of monitoring wells. Variability in isotopic composition of 1,4-dioxane at a site and/or increased analytical uncertainty at low concentrations of 1,4-dioxane can introduce uncertainty into the interpretation of field-based dual-isotope trends. Multiple lines of evidence are recommended for the interpretation of CSIA results.

Benefits: A relatively low-cost, commercially available method for assessing 1,4-dioxane degradation in groundwater was further developed in this work, and this may help to demonstrate the natural or enhanced biodegradation of 1,4-dioxane in groundwater at DoD sites.
KEYWORDS: 1,4-dioxane, CSIA, dual-isotope plots, biodegradation, isotopic composition
EXECUTIVE SUMMARY

1. Introduction

At many DoD sites, even the most costly and aggressive groundwater cleanup methods will not be able to achieve the low concentration cleanup goals set by the United States Environmental Protection Agency (US EPA) and/or state agencies for a variety of contaminants in a reasonable timeframe (NRC, 2013). A transition from active remediation to long-term management with monitored natural attenuation (MNA) is the most realistic outcome for many sites (NRC, 2013). ERSON-15-01, issued by the Strategic Environmental Research and Development Program (SERDP), calls for research that will lead to an improved understanding of long-term natural attenuation processes on contaminants in groundwater.

A critical process of natural attenuation is contaminant degradation. It is difficult to demonstrate degradation of 1,4-dioxane, which is often present at chlorinated solvent sites but is considered recalcitrant in the subsurface. This research project aimed to develop a method to provide direct evidence for the intrinsic degradation of 1,4-dioxane at low concentrations in groundwater. This limited-scope research project is important to the Department of Defense (DoD) because the expected outcome will allow for a cost-effective and readily implementable “field method” to assess the natural and enhanced biodegradation of 1,4-dioxane in groundwater. Specifically, this project sought to extend the applicability of compound-specific isotope analysis (CSIA) to low 1,4-dioxane concentrations in groundwater so that CSIA can be used to document in situ biodegradation of 1,4-dioxane at DoD sites.

CSIA is a powerful tool for providing evidence of degradation of many groundwater contaminants including fuel-related hydrocarbons, oxygenates, and chlorinated solvents (Hunkeler et al, 2009), where a change in the ratio of certain stable isotopes comprising the contaminant of interest (e.g. stable isotope ratios of carbon [13C/12C] and hydrogen [2H/1H]) typically occur with biodegradation. By convention, stable isotope ratios are reported in “delta” (δ) notation as the relationship between the isotopic ratio of a sample and an internationally defined standard, as defined in Equation 1. Here, the standard used for the 13C/12C ratio is the Vienna Pee Dee Belemnite (VPDB) and the standard for the 2H/1H ratio is Vienna Standard Mean Ocean Water (VSMOW). Each sample’s stable isotopic ratio is expressed relative to that of the standard in parts per thousand, or per mill (‰), using the following expression:

\[
\delta = \left( \frac{R_{sample}}{R_{standard}} - 1 \right) \times 1000 \text{ ‰}
\]  

Equation 1

Where \( R_{sample} \) is the 13C/12C or 2H/1H ratio measured in a 1,4-dioxane sample, and \( R_{standard} \) is the 13C/12C or 2H/1H ratio of VPDB or VSMOW, respectively. In delta notation, the 13C/12C ratio is expressed as “δ13C” and the 2H/1H ratio is expressed as “δ2H.” All isotope ratios reported herein use the delta notation, in units of per mill (‰).

Laboratory-controlled degradation reactions of 1,4-dioxane under various conditions were performed in connection with this project (ER-2535) and SERDP Project ER-2303. These studies found that the degree of enrichment in 13C and 2H varied among the reaction conditions, as shown by the slope of the dual isotope plot (\( \Delta \delta^{2H}/\Delta \delta^{13C} \)). These new findings demonstrate the unique
potential of CSIA for assessing 1,4-dioxane degradation at field sites. First, the dual-isotope slope may provide evidence for in situ biodegradation of 1,4-dioxane and insights on the activities of monooxygenases degrading 1,4-dioxane in groundwater. Second, the dual isotope slope facilitates the selection of enrichment factors for the quantification of the extent of 1,4-dioxane degradation using the Rayleigh equation.

2. Objectives
The objectives of Project ER-2535 are aligned with the overall objective of the Statement of Need for the Environmental Restoration Program Area (ERSON-15-01): to improve estimates of the long-term impact of natural attenuation processes on groundwater contaminants.

Specific objectives of this research are to:

1. Develop a reliable method for performing CSIA on low levels of 1,4-dioxane in groundwater (e.g. 1 to 10 micrograms per liter; µg/L);

2. Assess the fractionation of stable carbon and hydrogen isotope ratios during the aerobic co-metabolic biodegradation of 1,4-dioxane under laboratory and field conditions; and

3. Assess the use of stable carbon and hydrogen isotope ratios as tools to evaluate biodegradation of 1,4-dioxane at United States Department of Defense (DoD) sites with different groundwater conditions.

The project was implemented in two phases. Phase I completed the objectives listed above and the results are presented in the Final Report: Extending the Applicability of Compound-Specific Isotope Analysis to Low Concentrations of 1,4-Dioxane, SERDP Project ER-2535 (Haley & Aldrich, 2018). Phase II of Project ER-2535 sought to expand the results for the third objective by:

1. Increasing the database of stable isotopic composition of neat 1,4-dioxane sources by 10 in order to improve interpretation of field data; and

2. Increasing the number of field sites where CSIA is applied to low concentrations of 1,4-dioxane in groundwater at DoD sites.

This report documents the Phase II results.

3. Technical Approach
The overall technical scope of work was designed to achieve the Phase II objectives and included the following three tasks:

1. Assessment of 1,4-Dioxane Source Isotope Composition;
2. CSIA of 1,4-Dioxane at Field Sites; and
Under Task 1, the stable carbon and hydrogen isotope ratios of 1,4-dioxane from ten different commercially available samples were analyzed to expand the isotopic database. In addition to analyzing different 1,4-dioxane suppliers, a simple evaporation experiment was performed to assess whether the isotopic composition of 1,4-dioxane changes when neat 1,4-dioxane undergoes evaporation. As part of Task 2, six case studies were completed at DoD sites while two were completed at non-DoD sites. An average of six samples were collected from each site for CSIA of 1,4-dioxane. When combined with the four Phase I case studies, a total of 12 sites have been sampled for this project; 68 groundwater samples have been analyzed for δ¹³C, and 54 have been analyzed for δ²H, not including duplicate and replicate analyses and samples with insufficient mass for isotopic characterization. Task 3 included the management and coordination of research activities as well as routine project reports and technology transfer. This report comprises the final Task 3 deliverable.

4. Results and Discussion

4.1 Isotopic Composition of 1,4-Dioxane Obtained from Chemical Suppliers

As shown in Figure ES-1, the range and median δ¹³C values for undegraded “source” 1,4-dioxane are like those previously reported by Wang (2016) but higher than those reported by Pornwongthong (2014). The range in δ¹³C values of 1,4-dioxane for each of the three studies is relatively small, approximately 5‰, and when the three studies are combined, the 7.2‰ range (from -36.0‰ to -28.8‰) is relatively small compared to the typical range in source values for common organic pollutants. Hunkeler et al. (2009) recommends a minimum enrichment in δ¹³C of 2‰ beyond the undegraded “source” isotopic composition to demonstrate biodegradation, if such a conclusion were to be based solely on enrichment in δ¹³C. Based on this guidance and the maximum δ¹³C values for source 1,4-dioxane shown in Figure E1, it can be concluded that δ¹³C values greater than -26.8‰ may reflect degradation of 1,4-dioxane. This value may need to be adjusted if new source materials indicate higher δ¹³C values for undegraded 1,4-dioxane.

Figure ES-2 shows δ²H values for source 1,4-dioxane reported in this study are substantively different from those reported by Wang (2016), where the median value reported by Wang is nearly 100‰ “lighter” than 1,4-dioxane sources analyzed in this study. The δ²H values reported by Wang (2016) are substantially depleted compared to the Pornwongthong (2014) results, even for the same manufacturer; Pornwongthong (2014) δ²H values for 1,4-dioxane are closer to those reported in this study (Figure ES-2), but the median value is 30‰ lighter. The range in δ²H values shown in Figure ES-2 (-149‰ to -17‰) is large, but not beyond what has been reported for other groundwater contaminants. The relatively large range in δ²H values of 1,4-dioxane can complicate biodegradation assessments based solely on the enrichment in δ²H.
Ten manufacturers of 1,4-dioxane were present in the mid-1980s across the globe, and by 2000, only 5 were operating (Mohr, 2010). This indicates that sources of 1,4-dioxane procured today cannot represent the full range of manufacturers for historical releases which may have occurred several decades ago, and therefore it is possible that the full range in isotopic composition of 1,4-dioxane is not entirely represented in Figures ES-1 and ES-2. Industrial and municipal wastewater effluent can also be sources for 1,4-dioxane in groundwater, and in many cases, the 1,4-dioxane is not directly manufactured, it is instead a byproduct of manufacturing. The isotopic composition of byproduct 1,4-dioxane is not represented by the samples analyzed in this study.

No enrichment trend with evaporation of neat 1,4-dioxane was observed in passive evaporation experiments. The $\delta^{13}$C and $\delta^{2}$H values at 80% evaporation are within 0.2‰ and 3‰ of the initial values prior to evaporation (the analytical precision reported by the laboratory for $\delta^{13}$C and $\delta^{2}$H is ±0.5‰ and ±5‰, respectively). These results suggest that the isotopic composition of manufactured 1,4-dioxane may not be substantively affected by passive evaporative losses.

### 4.2 Isotopic Composition of 1,4-Dioxane in Groundwater Samples

The CSIA method developed in Phase I of this project was applied to 78 groundwater samples from 12 different field sites at concentrations ranging from non-detect to 17,200 micrograms per liter ($\mu$g/L) with a median concentration of 18 $\mu$g/L. Of these samples, 68 measurements of $\delta^{13}$C and 54 measurements of $\delta^{2}$H were reported. The $\delta^{13}$C and $\delta^{2}$H values for 1,4-dioxane measured at the 12 field sites are summarized in Figures ES-3 and ES-4, respectively.

As shown in Figure ES-3, most $\delta^{13}$C values for 1,4-dioxane in groundwater samples fall between -34‰ and -28‰, within the range of $\delta^{13}$C values for undegraded source 1,4-dioxane. The lowest
δ¹³C values between -38‰ and -36‰ indicate that “lighter” δ¹³C values for sources of 1,4-dioxane exist compared to those shown in Figure ES-2. Some of the “heavier” δ¹³C values between -26‰ and -22‰ reflect biodegradation due to biostimulation, others may be related to natural biodegradation and/or source variability and/or analytical uncertainty (for low concentrations).

Figure ES-3 (left) and ES-4 (right): Isotopic Composition of 1,4-Dioxane in Groundwater Samples from Field Sites. Figure ES-3 compares the frequency distribution for δ¹³C values of 1,4-dioxane in 68 groundwater samples from Phase I and Phase II field sites with the range in source isotopic composition shown in Figure ES-1. The thick red bar is the range in isotopic composition of the 10 samples analyzed in this study; the thin red bar extends the range to include other published values shown in Figure ES-1. Figure ES-4 compares the frequency distribution for δ²H values of 1,4-dioxane in 54 groundwater samples from Phase I and Phase II field sites with the range in isotopic composition of the 10 samples analyzed in this study (thick red bar) and other published values shown in Figure ES-2 (thin red bar).

As shown in Figure ES-4, the majority of δ²H values for 1,4-dioxane measured in groundwater samples fall between -50‰ and 0‰, overlapping the full range of δ²H values for undegraded source 1,4-dioxane, but with a proportion of samples being enriched by 10 to 20‰ relative to the maximum δ²H value for undegraded source 1,4-dioxane of -17‰. Based on the expected error for determination of δ²H values by CSIA, the δ²H values in Figure ES-4 between -17‰ and 0‰ cannot be attributed to biodegradation alone without considering other lines of evidence (e.g. concurrent enrichment in δ¹³C along a dual isotope enrichment trend). The values greater than 0‰ include samples from sites where other lines of evidence do not support biodegradation, as discussed Section 4.4.2. The δ²H values lower than -60‰ were reported in groundwater samples from three sites, indicating that some “lighter” δ²H values for sources of 1,4-dioxane exist compared to the sources of 1,4-dioxane analyzed in this study. Based on the large range in δ²H values for source 1,4-dioxane and given the larger uncertainty in analytical results, the use of δ²H values alone as an indicator for biodegradation is not recommended. Instead, comparison of δ¹³C and δ²H values along flowpaths can be useful, particularly when interpreted using a “dual isotope” approach on a case by case basis; this dual-isotope approach was applied to the case studies where paired δ¹³C and δ²H values were available.
4.3 Summary of Case Studies Applying CSIA to Groundwater Sites
Dual isotope trends consistent with biodegradation of 1,4-dioxane were found at 4 of the 12 sites evaluated under Phase I and Phase II of Project ER-2535:

1. McClellan Air Force Base (Section 4.4.1.1), only within the area where 1,4-dioxane was being treated in situ through biostimulation with propane and oxygen addition. The dual-isotope enrichment trend aligned with the trend observed in laboratory reactions of *Mycobacterium* 1A stimulated with gaseous alkanes (Figure ES-5).

2. Cape Cañaveral Air Force Station (CCAFS) Space Launch Complex 16 (SLC-16) (Section 4.4.1.3), one of the groundwater samples was strongly enriched relative to the other samples from this site, with δ\(^{13}\)C and δ\(^{2}\)H of -23.4‰ and -10‰, respectively, and plotting on a dual isotope trend consistent with that observed for laboratory degradation reactions with *R. rhodochrous ATCC 21198* grown on propane. A second sample had a smaller isotopic enrichment plotting on a dual-isotope enrichment trend similar to that determined for *P. dioxanivorans CB1190* (1,4-dioxane substrate, orange line; Figure ES-6).

3. Confidential DoD Site #2 (Section 4.4.2.5), where the dual isotope enrichment aligned with trends established for *Mycobacterium* 1A and *P. tetrahydrofuranoxidans K1* downgradient of the source zone (Figure ES-7).

4. Confidential Non-DoD Site #1 (Section 4.4.2.7), where the dual isotope enrichment aligned with the trend established for *P. tetrahydrofuranoxidans K1* in an area where a biostimulation pilot test (propane and oxygen addition) was underway, and farther downgradient, at the distal end of the plume where other lines of evidence suggest natural attenuation processes were occurring (Figure ES-8). Tetrahydrofuran (THF) is a co-contaminant with 1,4-dioxane in groundwater at this site, and THF had previously been implicated in the natural attenuation of 1,4-dioxane in earlier studies on this site using a multiple lines of evidence approach (Gedalanga et al., 2016).

CSIA was inconclusive with respect to biodegradation at 8 of the 12 sites, either because the enrichment trends associated with biodegradation of 1,4-dioxane were not observed, or because concentrations were too low for analysis.

Implications for Future Research and Benefits
The method developed under Project ER-2535 allows for applying CSIA to low concentrations of 1,4-dioxane in groundwater to demonstrate the natural or enhanced biodegradation of 1,4-dioxane at DoD sites. Through the use of dual isotope plots, CSIA results can be compared to published enrichment trends to provide insights into the degradation conditions by comparison with published dual-isotope trends for laboratory-controlled degradation reactions. Future research in laboratory and field-based degradation studies may strengthen the link between dual-isotope 1,4-dioxane degradation trends and in situ enzyme activity. The dual isotope plot can also be useful in selecting the appropriate enrichment factor for Rayleigh-type degradation calculations at groundwater sites.
Figure ES-5: Dual isotope plot for McClellan AFB samples showing paired $\delta^{13}C$ and $\delta^2H$ values (‰) for groundwater samples collected in June 2016 from biostimulation pilot test (red dots). MACB-1 and MACB-2 are in the biostimulation zone; IACB-1 is undegraded 1,4-dioxane. Laboratory-determined dual-isotope enrichment trends are shown for *R. rhodochrous* ATCC 21198 (propane substrate, blue line), *Mycobacterium* sp. 1A (propane substrate, magenta line) and *P. tetrahydrofuranoxidans* K1 (THF substrate, green line).

Figure ES-6: Dual isotope plot for CCAFS SLC-16 showing paired $\delta^{13}C$ and $\delta^2H$ values (‰). For reference, dual-isotope enrichment trends are shown for laboratory controlled degradation reactions of *P. dioxanivorans* CB1190 (1,4-dioxane substrate, orange line) and *R. rhodochrous* ATCC 21198 (propane substrate, blue line).

Figure ES-7: Dual Isotope Plot for Confidential DoD Site#2 showing paired $\delta^{13}C$ and $\delta^2H$ values (‰, red dots) for groundwater samples collected from locations in the 1,4-dioxane source area (MW87 and MW91) and further downgradient (MW88, MW89, MW90). Laboratory-determined dual-isotope enrichment trends are shown dual-isotope enrichment trends are shown for *R. rhodochrous* ATCC 21198 (propane substrate, blue line), *Mycobacterium* sp. 1A (propane substrate, magenta line) and *P. tetrahydrofuranoxidans* K1 (THF substrate, green line).

Figure ES-8. Dual Isotope Plot for Confidential Non-DoD Site#1 showing paired $\delta^{13}C$ and $\delta^2H$ values (‰, red dots) for groundwater samples collected along a mixed 1,4-dioxane/THF plume; duplicate samples are shown in red circles. IW1 was sampled after several months of biostimulation of 1,4-dioxane biodegradation as part of a pilot test. P78 is near the distal end of the plume. Laboratory-determined dual-isotope enrichment trends are shown for *R. rhodochrous* ATCC 21198 (propane substrate, blue line), *Mycobacterium* sp. 1A (propane substrate, magenta line) and *P. tetrahydrofuranoxidans* K1 (THF substrate, green line).
ACKNOWLEDGEMENTS

The authors thank Dr. Andrea Leeson of SERDP/ESTCP for guidance throughout this project and Dr. Hunter Anderson of AFCEC for assistance in selecting the field sites for this project. Humam El Mugammar performed the CSIA analysis at the University of Waterloo Environmental Isotope Laboratory in Waterloo, Ontario, Canada, and assisted with method development. Groundwater samples from six sites were generously provided by the team of ESTCP Project ER-201730; we thank Dr. Anthony Danko of NAVFAC, Dr. David Adamson of GSI Environmental Inc., and Dr. John Wilson of Scissortail Environmental, Inc. for providing samples and background information on these sites. Groundwater samples from AFP3 were provided by Rebecca Mora of AECOM. Groundwater samples from Hanscom AFB were provided by Kinshuk Shroff of Versar. We thank the University of Waterloo Environmental Isotope Laboratory staff for coordinating the shipping, receiving, and reporting of CSIA results. We thank Mike Nickelsen and Erica Schmitz of ECT2 in Rochester, New York for researching and procuring diverse 1,4-dioxane sources and conducting the evaporation experiment.
1. OBJECTIVE

The objectives of Project ER-2535 are aligned with the overall objective of the Statement of Need for the Environmental Restoration Program Area (ERSON-15-01): to improve estimates of the long-term impact of natural attenuation processes on groundwater contaminants. Specifically, this project sought to develop a low-cost, readily implementable method for assessing 1,4-dioxane degradation in groundwater by allowing for the application of compound-specific isotope analysis (CSIA) to low concentrations of 1,4-dioxane.

Specific objectives of this research are to:

1. Develop a reliable method for performing CSIA on low levels of 1,4-dioxane in groundwater (e.g. 1 to 10 micrograms per liter; µg/L);

2. Assess the fractionation of stable carbon and hydrogen isotope ratios during the aerobic co-metabolic biodegradation of 1,4-dioxane under laboratory and field conditions; and

3. Assess the use of stable carbon and hydrogen isotope ratios as tools to evaluate biodegradation of 1,4-dioxane at United States Department of Defense (DoD) sites with different groundwater conditions.

The project was implemented in two phases. Phase I completed the objectives listed above and the results are presented in the Final Report: Extending the Applicability of Compound-Specific Isotope Analysis to Low Concentrations of 1,4-Dioxane, SERDP Project ER-2535 (Haley & Aldrich, 2018). Phase II of Project ER-2535 sought to expand the results for the third objective by:

1. Increasing the database of stable isotopic composition of neat 1,4-dioxane sources by 10 in order to improve interpretation of field data; and

2. Increasing the number of field sites where CSIA is applied to low concentrations of 1,4-dioxane in groundwater at DoD sites.

This report addresses the Phase II results.


2. **BACKGROUND**

2.1 **Introduction**

At many DoD sites, even the most costly and aggressive groundwater cleanup methods will not be able to achieve the low concentration cleanup goals set by the United States Environmental Protection Agency (US EPA) and/or state agencies for a variety of contaminants in a reasonable timeframe (NRC, 2013). A transition from active remediation to long-term management with monitored natural attenuation (MNA) is the most realistic outcome for many sites (NRC, 2013). **ERSON-15-01**, issued by the Strategic Environmental Research and Development Program (SERDP), calls for research that will lead to an improved understanding of long-term natural attenuation processes on contaminants in groundwater.

A critical process of natural attenuation is contaminant degradation. It is difficult to demonstrate degradation of 1,4-dioxane, which is often present at chlorinated solvent sites but is considered recalcitrant in the subsurface. This research project aimed to develop a method to provide direct evidence for the intrinsic degradation of 1,4-dioxane at low concentrations in groundwater. This limited-scope research project is important to the DoD because the expected outcome will allow for a cost-effective and readily implementable “field method” to assess the natural and enhanced biodegradation of 1,4-dioxane in groundwater.

2.1.1 **Biodegradation of 1,4-Dioxane**

Many laboratory studies have documented the aerobic biodegradation of 1,4-dioxane by dozens of bacterial strains, including those with no previous 1,4-dioxane exposure, using various mono-oxygenase type enzyme systems (Mahendra & Cohen, 2006; He et al., 2018, Zhang et al., 2017). Recent laboratory studies have also shown that common co-contaminants of 1,4-dioxane (trichloroethene [TCE], cis-1,2-dichloroethene [cis-1,2-DCE] and 1,1-dichloroethene [1,1-DCE]) and certain metals, including hexavalent chromium (CrVI), may be inhibitory to its biodegradation (Zhang et al., 2017). The role of anaerobic processes towards the biodegradation of 1,4-dioxane is not well studied (Zhang et al., 2017).

Although numerous laboratory studies on aerobic biodegradation of 1,4-dioxane have contributed greatly towards understanding the biodegradation of 1,4-dioxane, most laboratory studies have involved high concentrations of 1,4-dioxane. Thus, less is known about the subsurface fate of 1,4-dioxane at low concentrations at sites where a transition to MNA is appropriate. Li et al. (2014) found evidence for the presence of 1,4-dioxane degrading microbes at five sites by quantifying catabolic biomarkers that correlate with 1,4-dioxane degradation activity found in microcosms. These results suggest that biomarkers could be an important tool for assessing 1,4-dioxane degradation potential at field sites; however, they do not provide direct evidence on the extent of 1,4-dioxane degradation that has occurred *in situ*. Chiang et al. (2012) used stable isotopic tools as well as enzyme and phospholipid fatty acid (PLFA) analysis to demonstrate the biodegradation of trichloroethene and 1,4-dioxane at a DoD site in Tucson, Arizona (Air Force Plant 44). Although their results demonstrated that microbes capable of degrading 1,4-dioxane were present and active at the site, the authors acknowledged the need to develop CSIA as a tool to directly demonstrate and monitor the in situ biodegradation of 1,4-dioxane. Adamson et al. (2015) reported that many
sites have dilute attenuating 1,4-dioxane plumes at low concentrations; this demonstrates the need to further develop CSIA for low concentrations of 1,4-dioxane.

### 2.1.2 Compound-Specific Isotope Analysis (CSIA)

CSIA is a powerful tool for providing evidence of degradation of many groundwater contaminants including fuel-related hydrocarbons, oxygenates, and chlorinated solvents (Hunkeler et al., 2009). CSIA has become an important tool to document biodegradation of an organic contaminant with similar chemical properties to 1,4-dioxane; methyl tert-butyl ether (MTBE) was thought to be resistant to biodegradation 20 years ago (Kolhatkar et al., 2002; Lesser et al., 2008). CSIA is now a primary technique used to show that MTBE degrades under both engineered and natural conditions (Wilson et al., 2005). The same could be true for 1,4-dioxane with the development of a reliable and practical method for performing CSIA on groundwater samples, which was achieved with the research presented herein.

Elements such as carbon (C) and hydrogen (H) are defined by the number of protons in their atomic nuclei; isotopes of any given element have the same number of protons but differ in the number of neutrons resulting in different atomic weights. Different isotopes of an element are otherwise equivalent (i.e. same chemical properties). Stable isotopes are those that do not decay radioactively. For example, the most common stable isotope of hydrogen, protium (\(^1\)H), contains one neutron in its nucleus, while the less common stable isotope, deuterium (\(^2\)H), contains two neutrons. Radioactive isotopes also exist for many elements (for example, \(^{14}\)C and \(^3\)H), but these are not measured with CSIA. CSIA measures the ratios of certain stable (i.e., not radioactive) isotopes within a molecule of interest; in this case, 1,4-dioxane (chemical formula C\(_4\)H\(_8\)O\(_2\)), is composed of four carbon (C) atoms, eight hydrogen (H) atoms, and two oxygen (O) atoms. The most common isotopes of C, H, and O are \(^{12}\)C, \(^1\)H, and \(^{16}\)O, respectively; the heavier, less abundant stable isotopes are \(^{13}\)C, \(^2\)H, and \(^{18}\)O. Stable isotope ratios are expressed as the relative abundance of the rare, heavier isotope to the more common, lighter isotope. For 1,4-dioxane, the stable isotope ratios relevant to this project are \(^{13}\)C/\(^{12}\)C, and \(^2\)H/\(^1\)H. These ratios comprise the isotopic composition (i.e., isotopic signature or fingerprint) of 1,4-dioxane that can be determined by CSIA within a groundwater sample.

By convention, stable isotopic compositions are reported in “delta” (\(\delta\)) notation as the relationship between the isotopic ratio of a sample and an internationally defined standard, as defined in Equation 1. Here, the standard used for the \(^{13}\)C/\(^{12}\)C ratio is the Vienna Pee Dee Belemnite (VPDB; derived from a marine carbonate) and the standard for the \(^2\)H/\(^1\)H ratio is Vienna Standard Mean Ocean Water (VSMOW; derived from seawater). Each sample’s stable isotopic ratio is expressed relative to that of the standard in parts per thousand, or per mill (‰), using the following expression:

\[
\delta = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000 \text{ ‰} \quad \text{Equation 1}
\]

Where \(R_{\text{sample}}\) is the \(^{13}\)C/\(^{12}\)C or \(^2\)H/\(^1\)H ratio measured in a 1,4-dioxane sample, and \(R_{\text{standard}}\) is the \(^{13}\)C/\(^{12}\)C or \(^2\)H/\(^1\)H ratio of VPDB or VSMOW, respectively. In delta notation, the \(^{13}\)C/\(^{12}\)C ratio is expressed as “\(\delta^{13}\)C” and the \(^2\)H/\(^1\)H ratio is expressed as “\(\delta^{2}\)H.” All isotope ratios reported herein use the delta notation, in ‰.
Degradation reactions often favor the breaking of bonds between the highly abundant lighter isotopes (e.g., $^{12}\text{C}^{-1}\text{H}$) compared to bonds involving the rarer heavier isotopes ($^{13}\text{C}^{-1}\text{H}$ or $^{12}\text{C}^{-2}\text{H}$), leading to isotopic fractionation of reactants and products. Because of isotopic fractionation, an increase (or enrichment) in $^{13}\text{C}^{12}\text{C}$ (increase in $\delta^{13}\text{C}$) and $^{2}\text{H}^{1}\text{H}$ (increase in $\delta^{2}\text{H}$) can be measured in the remaining contaminant using CSIA for a direct indication of degradation. Isotopic fractionation is typically negligible with other processes affecting contaminant concentrations such as dilution. By measuring the stable isotope ratio (e.g., $^{13}\text{C}^{12}\text{C}$) of a contaminant by CSIA, the extent of degradation can be calculated using the simplified Rayleigh isotopic enrichment model (Equation 2) if the $^{13}\text{C}^{12}\text{C}$ ratio of undegraded contaminant ($\delta^{13}\text{C}_o$) and carbon ($\varepsilon_c$) enrichment factor are known (Hunkeler et al., 2009):

$$\delta^{13}\text{C}_t = \delta^{13}\text{C}_o + \varepsilon_c ln f$$  \hspace{1cm} \text{Equation 2}

In Equation 2, $\delta^{13}\text{C}_t$ is the carbon isotope ratio of the contaminant of interest (e.g., 1,4-dioxane) at a point in time after degradation processes have started (i.e., in a monitoring well downgradient of the source area), $\delta^{13}\text{C}_o$ is the initial carbon isotope ratio of the contaminant of interest before the onset of degradation (or representative of the “source area” isotopic composition), and $f$ is the fraction of contaminant remaining ($[1-f] \times 100\%$ is equal to the percent of degraded contaminant). Similar calculations can be made for $\delta^{2}\text{H}$ values using hydrogen enrichment factors ($\varepsilon_H$). It is important to note that Equation 2 is based on the assumption of a closed system and well-mixed conditions, which may not always be applicable to degradation along a groundwater flowpath, for example, if dilution occurs (Thullner et al. 2012). Selection of an appropriate enrichment factor is critical for estimating the extent of degradation using the Rayleigh equation. For biodegradation reactions, enrichment factors are typically determined in laboratory-controlled degradation reactions. In general, due to analytical uncertainties, isotope shifts of $>2\%$ for $^{13}\text{C}$ and $>20\%$ to $40\%$ for $^{2}\text{H}$ are needed for proof of in situ degradation of contaminants, and, for many compounds, relatively minor extent of degradation ($<20$ to $50\%$ of initial concentration, depending on the compound) may not be detectable using CSIA (Braeckevelt et al., 2012).

Although the United States Environmental Protection Agency (US EPA) published a guidance manual on CSIA in 2009 (Hunkeler et al., 2009), there are no standard analytical methods promulgated by the US EPA or other agencies for performing CSIA, and no laboratory certification or audit process specific to determining the isotopic composition of common groundwater contaminants. For volatile organic compounds, including 1,4-dioxane, the isotopic ratios of carbon and hydrogen are often determined using a gas chromatograph (GC) coupled to an isotope ratio mass spectrometer (IRMS). The reporting limits for CSIA vary among laboratories, sample matrices, and extraction methods (Hunkeler et al., 2009). Commonly, an analyst will flag or omit a result if the concentration is below a certain threshold, thus calling the reliability of the result into question.

**2.1.3 Previous Studies on Isotopic Composition of 1,4-Dioxane**

The potential for different feedstocks and different processes used to manufacture 1,4-dioxane translates to the potential for differences in isotopic composition of 1,4-dioxane sources. The most common commercial process for 1,4-dioxane production involves the dehydration of ethylene...
glycol with heat and sulfuric acid (Mohr, 2010). Both alcohol groups of ethylene glycol lose a hydrogen atom to form the ether bond for ring closure, thereby producing 1,4-dioxane. Two other processes are used to produce 1,4-dioxane: one involves dimerization of ethylene oxide with a solid catalyst (acid ion exchange resin or zeolite), and the other involves heating bis(2-chloroethyl) ether and adding 20% sodium hydroxide (Mohr, 2010).

Pornwongthong (2014) and Wang (2016) reported on the stable carbon and hydrogen isotope composition of 1,4-dioxane samples purchased from different chemical suppliers (Table 1).

<table>
<thead>
<tr>
<th>Supplier</th>
<th>$\delta^{13}$C (%o, PDB)</th>
<th>$\delta^{2}$H (%o, SMOW)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acros Organics</td>
<td>-34.20 ± 0.32</td>
<td>-66.4 ± 2.4</td>
<td>Pornwongthong (2014)</td>
</tr>
<tr>
<td>Alfa Aesar</td>
<td>-34.80 ± 0.52</td>
<td>-64.3 ± 0.3</td>
<td>Pornwongthong (2014)</td>
</tr>
<tr>
<td>Fisher Scientific</td>
<td>-32.70 ± 0.32</td>
<td>-68.2 ± 2.4</td>
<td>Pornwongthong (2014)</td>
</tr>
<tr>
<td>Mallinckrodt</td>
<td>-31.98 ± 0.40</td>
<td>-66.6 ± 6.0</td>
<td>Pornwongthong (2014)</td>
</tr>
<tr>
<td>Sigma-Aldrich</td>
<td>-35.96 ± 0.65</td>
<td>-59.6 ± 4.4</td>
<td>Pornwongthong (2014)</td>
</tr>
</tbody>
</table>

Table 1: Isotopic Composition of Manufactured 1,4-Dioxane (prior to this study).

Pornwongthong (2014) reported $\delta^{13}$C values for 1,4-dioxane at the influent (-43.02 ± 0.18‰) and effluent (-41.59 ± 0.17‰) of a wastewater treatment plant (WWTP) at a polyester manufacturing facility, where 1,4-dioxane concentrations were 334 and 69 milligrams per liter (mg/L), respectively. The low $\delta^{13}$C values reported for 1,4-dioxane in the water samples from the WWTP are distinct from the manufactured 1,4-dioxane samples shown in Table 1, suggesting that different industrial processes may result in different isotopic signatures of 1,4-dioxane (Pornwongthong, 2014). Wang (2016) reported on a new method for performing CSIA on low concentrations of 1,4-dioxane in groundwater; that study reported on $\delta^{13}$C values of five groundwater samples (-35.89‰ to -31.21‰) ranging in concentration from 3.0 to 116 µg/L and $\delta^{2}$H values (-59.7‰ to -56.7‰) of three groundwater samples from 12.9 to 116 µg/L.

2.1.4 Isotopic Fractionation During Biodegradation of 1,4-Dioxane

The occurrence of isotopic fractionation during biodegradation of 1,4-dioxane was first documented in an aerobic microcosm experiment of 1,4-dioxane biodegradation by *Pseudonocardia dioxanivorans* CB1190; only $\delta^{13}$C values were reported in this study (Pornwongthong et al., 2011). In laboratory-controlled degradation reactions, Pornwongthong (2014) reported small enrichment in $^{13}$C (by 2.29 ‰) and strong enrichment in $^{2}$H (by 281 ‰) after 96% of 1,4-dioxane had been degraded by *Pseudonocardia dioxanivorans* CB1190. Enrichment factors of -1.38 ± 0.56‰ for $^{13}$C ($\epsilon_C$) and -45.1 ± 14.2‰ for $^{2}$H ($\epsilon_H$) were calculated from these reactions (Pornwongthong, 2014). Pornwongthong (2014) also reported $^{2}$H-enrichment with 1,4-
dioxane removal from aqueous solutions by aeration (εH=-28.6 ± 32.4‰) and sonolysis (εH=-55.1 ± 15.1‰) without observable enrichment in $^{13}$C.

Gedalanga et al. (2016) employed a multiple lines of evidence approach that included CSIA ($\delta^{13}$C only) of 1,4-dioxane in samples from 24 groundwater monitoring wells at a former landfill site located in the midwestern United States. Among these 24 samples, $\delta^{13}$C ranged from approximately -31.8‰ to -29.2‰, with the $\delta^{13}$C values above -30‰ coinciding with lower 1,4-dioxane concentrations, leading to the conclusion that isotopic enrichment provided evidence of degradation. Gedalanga et al. (2016) reported a detection limit of 100 µg/L for CSIA of 1,4-dioxane at that time.

As discussed in the Phase I Final Report for this project (Haley & Aldrich, 2018), degradation reactions of 1,4-dioxane by Mycobacterium sp.1A grown on gaseous alkanes were performed in a laboratory-controlled environment at North Carolina State University (NCSU). CSIA was performed by the University of Waterloo Environmental Isotope Laboratory (UWEIL) on residual 1,4-dioxane samples collected at various times during the degradation reactions for the purpose of determining enrichment factors. This work was expanded upon after the Phase I Final Report was submitted, where the following additional strains and substrates were assessed in similar laboratory-controlled degradation reactions at NCSU:

- *Rhodococcus rhodochrous* ATCC 21198 grown on propane;
- *Rhodococcus rhodochrous* ATCC 21198 grown on isobutane; and
- *Pseudonocardia tetrahydrofurans* K1 grown on tetrahydrofuran (THF).

Through collaboration with the UWEIL and Dr. Michael Hyman at NCSU, who is the Principal Investigator of SERDP Project ER-2303, enrichment factors were also determined for these three degradation reactions and reported in Bennett et al., 2018. This study confirmed the involvement of monooxygenase enzymes through the use of alkyne inhibitors in biologically active control experiments (Bennett et al., 2018). It was found that the degree of enrichment in $^{13}$C and $^2$H varied among the reaction conditions, as shown by the slope of the dual isotope plot ($\Delta\delta^2H/\Delta\delta^{13}C$) shown in Figure 1. These findings demonstrate the unique potential of CSIA for assessing 1,4-dioxane degradation at field sites. First, the dual-isotope slope may provide evidence for in situ biodegradation of 1,4-dioxane and insights on the activities of monooxygenases degrading 1,4-dioxane in groundwater. Second, the dual isotope slope facilitates the selection of enrichment factors for the quantification of the extent of 1,4-dioxane degradation using the Rayleigh equation.
Figure 1: Dual Isotope Enrichment Trends for Aerobic Cometabolic Degradation of 1,4-Dioxane. Enrichment in $^2$H relative to $^{13}$C during 1,4-dioxane degradation by strain 21198 grown on propane (blue dots) and isobutane (purple diamonds) and for strain K1 grown on THF (green squares). Solid lines represent the linear best fit with slope ($m$) and coefficient of determination ($R^2$) shown on the plot. Colored shaded areas represent 95% confidence intervals of the slope of the regression line.

A compilation of dual isotope slopes and enrichment factors from laboratory-controlled monooxygenase-mediated 1,4-dioxane biodegradation reactions is provided in Table 2. Similar to isotope fractionation during monooxygenase-mediated aerobic biodegradation of MTBE ($\varepsilon_C \sim -2.4\%$, $\varepsilon_H \sim -30\%$; Gray et al., 2002), stable hydrogen isotope fractionation is larger than carbon isotope fractionation during the monooxygenase-mediated aerobic biodegradation of 1,4-dioxane. With the exception of the THF-grown cultures, the small carbon isotope enrichment factors for 1,4-dioxane ($\varepsilon_C \sim 1.4$ to 2.7‰) require 52% to 77% degradation for sufficient isotopic enrichment in $^{13}$C (e.g. >2‰) to conclude biodegradation is occurring. Therefore, it may be difficult to observe biodegradation based solely on $\delta^{13}$C values. Based on the $\varepsilon_H$ values shown in Table 2, the $\delta^2$H values are more sensitive for detecting biodegradation during metabolic biodegradation of 1,4-dioxane with *P. dioxanivorans CB1190* and THF-grown *P. tetrahydrofuranoxidans K1*, and therefore should be included when assessing biodegradation of 1,4-dioxane.

While the reported values for $\Delta\delta^2$H/$\Delta\delta^{13}$C range from 7.5 to 37.2 for different experimental conditions, there are overlapping similarities among a subset of these results: the value for isobutane-grown *R. rhodochrous* ATCC 21198 is not substantially different from propane-grown *Mycobacterium* sp. 1A, and the value for metabolic biodegradation of 1,4-dioxane with *P. dioxanivorans CB1190* is similar to THF-grown *P. tetrahydrofuranoxidans K1*. Similarities in $\Delta\delta^2$H/$\Delta\delta^{13}$C may reflect the role of similar monooxygenases in degradation. It follows that determination of $\Delta\delta^2$H/$\Delta\delta^{13}$C at groundwater sites may provide site-specific evidence in support of
biodegradation of 1,4-dioxane if the field results follow one or more of the $\Delta \delta^2 \text{H}/\Delta \delta^{13} \text{C}$ enrichment trends listed in Table 2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth Substrate</th>
<th>$\Delta \delta^2 \text{H}/\Delta \delta^{13} \text{C}$</th>
<th>$\varepsilon \text{C (‰)}$</th>
<th>$\varepsilon \text{H (‰)}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. rhodochrous ATCC 21198</em></td>
<td>propane</td>
<td>7.5 ± 1.1</td>
<td>-2.7 ± 0.3</td>
<td>-21 ± 2</td>
<td>Bennett et al., 2018</td>
</tr>
<tr>
<td><em>R. rhodochrous ATCC 21198</em></td>
<td>isobutane</td>
<td>10.9 ± 2.2</td>
<td>-2.5 ± 0.3</td>
<td>-28 ± 6</td>
<td></td>
</tr>
<tr>
<td><em>P. tetrahydrofuran-oxidans K1</em></td>
<td>THF</td>
<td>37.2 ± 2.6</td>
<td>-4.8 ± 0.9</td>
<td>-150 ± 23</td>
<td></td>
</tr>
<tr>
<td><em>Mycobacterium sp. 1A</em></td>
<td>propane*</td>
<td>12.1 ± 5.8</td>
<td>-2.0 ± 0.4</td>
<td>-25 ± 20</td>
<td>Haley &amp; Aldrich, 2018</td>
</tr>
<tr>
<td><em>P. dioxanivorans CB1190</em></td>
<td>1,4-dioxane</td>
<td>31.4</td>
<td>-1.38 ± 0.56</td>
<td>-45.1 ± 14.2</td>
<td>Pornwongthong, 2014</td>
</tr>
</tbody>
</table>

Table 2: Dual-Isotope Trends and Enrichment Factors for 1,4-Dioxane Biodegradation

*Note: cells used to inoculate reaction bottles were grown on isobutane, but propane was used in reaction bottles as the growth substrate.

2.2 Technical Scope of Project ER-2535 Extension

The overall technical scope of work was designed to achieve the Phase II objectives and included the following three tasks:

1. Assessment of 1,4-Dioxane Source Isotope Composition;
2. CSIA of 1,4-Dioxane at Field Sites; and

2.2.1 Task 1 – Assessment of 1,4-Dioxane Source Isotopic Composition

A more comprehensive database for the isotopic composition of 1,4-dioxane allows for better constraints on the expected range in isotopic composition of “source” 1,4-dioxane, which is a useful reference for interpreting field results. Prior to completing Task 1, the published database on the stable isotopic composition of neat 1,4-dioxane (i.e., commercially available 1,4-dioxane) was small and highly variable, prompting the need for the isotopic characterization of a greater number of samples from different suppliers of 1,4-dioxane. Under Task 1, the stable carbon and hydrogen isotope ratios of 1,4-dioxane from ten different commercially available samples were analyzed to expand the isotopic database. In addition to analyzing different 1,4-dioxane suppliers, a simple evaporation experiment was performed to assess whether the isotopic composition of 1,4-dioxane changes when neat 1,4-dioxane undergoes evaporation.

2.2.2 Task 2 – CSIA of 1,4-Dioxane at Field Sites

Additional case studies were needed to further document the value of CSIA for demonstrating 1,4-dioxane degradation at DoD sites. As part of Phase II, six case studies were completed at DoD
sites while two were completed at non-DoD sites. An average of six samples were collected from each site for CSIA of 1,4-dioxane. When combined with the four Phase I case studies, a total of 12 sites have been sampled for this project; 68 groundwater samples have been analyzed for $\delta^{13}\text{C}$, and 54 have been analyzed for $\delta^{2}\text{H}$, not including duplicate and replicate analyses and samples with insufficient mass for isotopic characterization.

### 2.2.3 Task 3 – Final Report Addendum

Task 3 included the management and coordination of research activities as well as routine project reports and technology transfer. This report comprises the final Task 3 deliverable.
3. MATERIALS AND METHODS

Analytical methods for CSIA of 1,4-dioxane are substantively the same as those documented in the Phase I Final Report (Haley & Aldrich, 2018). The materials and methods for Phase II tasks are described below.

3.1 Task 1 – Assessment of 1,4-Dioxane Source Isotopic Composition

Samples of 1,4-dioxane procured from ten different chemical suppliers were submitted to the UWEIL for characterization of stable carbon and hydrogen isotope ratios. These sources were differentiated by their supplier as well as the lot number. The contents and 1,4-dioxane purity are listed for each sample in Table 3. It was not possible to determine where and/or how the 1,4-dioxane for each supplier was manufactured, and the possibility that different suppliers were obtaining 1,4-dioxane from the same manufacturer could not be ruled out. Nonetheless, the intent of Task 2 is to capture the variability in isotopic composition of 1,4-dioxane to serve as a baseline for comparison with 1,4-dioxane in groundwater.

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Supplier Lot Number</th>
<th>Contents</th>
<th>1,4-D Purity from Certificate of Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Dimensions</td>
<td>BN035</td>
<td>Neat 1,4-Dioxane</td>
<td>100.0%</td>
</tr>
<tr>
<td>J.T. Baker</td>
<td>#0000156618</td>
<td>Neat 1,4-Dioxane</td>
<td>99.9%</td>
</tr>
<tr>
<td>Alfa Aesar</td>
<td>P15E789</td>
<td>Neat 1,4-Dioxane</td>
<td>99.9%</td>
</tr>
<tr>
<td>ACROS Organics</td>
<td>B0534092</td>
<td>Neat 1,4-Dioxane</td>
<td>99.9%</td>
</tr>
<tr>
<td>ULTRA Scientific</td>
<td>NT060576</td>
<td>Neat 1,4-Dioxane</td>
<td>99.5 +/- 0.05% (w/w)</td>
</tr>
<tr>
<td>Honeywell</td>
<td>H239G</td>
<td>Neat 1,4-Dioxane</td>
<td>&gt;=99.9%</td>
</tr>
<tr>
<td>Sigma Aldrich Switzerland</td>
<td>BCBV5331</td>
<td>Neat 1,4-Dioxane</td>
<td>100.0%</td>
</tr>
<tr>
<td>Sigma Aldrich Wisconsin</td>
<td>SHBH3187V</td>
<td>Neat 1,4-Dioxane</td>
<td>99.93%</td>
</tr>
<tr>
<td>TCI America</td>
<td>YVP2C-AS</td>
<td>Neat 1,4-Dioxane</td>
<td>99.3%</td>
</tr>
<tr>
<td>Restek</td>
<td>A0134211</td>
<td>2,006 ug/mL in Methanol</td>
<td>99.0%</td>
</tr>
</tbody>
</table>

Table 3: Suppliers for Samples of Neat 1,4-Dioxane

Each of the above 1,4-dioxane samples was procured by Haley & Aldrich and shipped to the UWEIL for characterization of stable carbon and hydrogen isotope ratios. Each sample of 1,4-dioxane was analyzed in duplicate using the following two different methods:

1. Duplicate analyses by an elemental analyzer coupled with an isotope-ratio mass-spectrometer (EA-IRMS); and
2. Duplicate analyses by a gas chromatograph coupled with an isotope-ratio mass-spectrometer (GC-IRMS).

The methods employed and instruments used for determining the stable carbon and hydrogen isotope ratios by EA-IRMS and GC-IRMS are described in the Phase I Final Report (Haley & Aldrich, 2018). The Sigma Aldrich Wisconsin 1,4-dioxane had been used in the Haley & Aldrich
laboratory in Rochester, New York, and may have been contaminated with 1,1,1-TCA. All other sources were purchased new specifically for source isotopic composition analysis. The TCI America 1,4-dioxane was stabilized with butylated hydroxytoluene. No other impurities were listed for the above sources. Mohr (2010) lists the following common impurities in manufactured 1,4-dioxane: bis(2-chloroethyl) ether, water and 2-methyl-1,3-dioxane up to 0.1% by weight, and 2-ethyl-1,3-dioxane up to 0.03% by weight.

As a preliminary assessment as to whether the source isotope ratio of 1,4-dioxane could change due to evaporative losses, a bench-top experiment was conducted at the Haley & Aldrich laboratory in Rochester, New York. Four 5 mL glass vials were filled to the 5 mL mark with neat 1,4-dioxane from ACROS Organics and placed in the fume hood at room temperature. After 1,4-dioxane had evaporated to 4 mL in one of the vials, a 1 mL sample was removed for analysis, creating the 20% evaporation sample. A second bottle was allowed to evaporate to 3 mL, creating the 40% evaporation sample; a third bottle was allowed to evaporate to 2 mL, creating the 60% evaporation sample; a fourth bottle was allowed to evaporate to 1 mL, creating the 80% evaporation sample. These four samples were shipped to the UWEIL for analysis of $\delta^{13}\text{C}$ and $\delta^2\text{H}$ by GC-IRMS. Results from GC-IRMS analysis are presented in Section 4.2.

3.2 Task 2 – CSIA of 1,4-Dioxane at Field Sites

In Phase I of this project, the new method for CSIA of 1,4-dioxane was applied to groundwater samples from four DoD sites:

1. McClellan Air Force Base (AFB), Operable Unit (OU) D, in McClellan, near Sacramento, California;
2. Vandenberg Air Force Base, Site 24, near Lompoc, California;
3. Cape Canaveral Air Force Station, Facility 1381, Cape Canaveral, Florida; and
4. Cape Canaveral Air Force Station, Space Launch Complex 16 (SLC-16).

The CSIA results for these four sites are described in the Phase I Final Report but are also summarized herein. Summary information on these four sites is provided in Table 4A.

Eight additional sites were sampled as part of Phase II of Project ER-2535:

1. Air Force Plant 3, Tulsa, Oklahoma;
2. Hanscom AFB, Bedford, Massachusetts;
3. Joint Base Cape Cod (JBCC), Ashumet Valley, Buzzards Bay, Massachusetts;
4. Confidential DoD Site #1, located in California;
5. Confidential DoD Site #2, located in California;
6. Confidential DoD Site #3, located in California;
7. Confidential Non-DoD Site #1, a landfill site in the Midwest; and
8. Confidential Non-DoD Site #2, an industrial site in South Carolina.

Summary information on these eight sites is provided in Table 4B. Certain sites were kept confidential when requested by those providing samples for this study. At these sites, steps were
taken to maintain confidentiality, including the omission of site name and address, re-naming well IDs and removing the site name and address from references.

<table>
<thead>
<tr>
<th>Site Name, Location</th>
<th>Number of Samples (concentration of 1,4-dioxane in samples)</th>
<th>Co-contaminants</th>
<th>Background Redox Conditions</th>
<th>Other Relevant Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>McClellan AFB, OU-D McClellan, California.</td>
<td><strong>13</strong> (0.68 – 57 µg/L)</td>
<td>TCE, 1,2-DCA and 1,1-DCE at 1 to 10 µg/L.</td>
<td>aerobic; (DO≈ 4 mg/L)</td>
<td>Samples from pilot test of aerobic co-metabolic biodegradation described in Chu et al., (2018) funded under AFCEC Grant # FA8903-13-C-0002</td>
</tr>
<tr>
<td>Vandenberg AFB, Site 24 Lompoc, California</td>
<td><strong>2</strong> (78, 81 µg/L)</td>
<td>PCE, TCE and cis-1,2-DCE up to 14 µg/L</td>
<td>aerobic; (DO ≈ 1.5 to 3.8 mg/L);</td>
<td>Samples near area of prior biosparge pilot test of aerobic co-metabolic biodegradation (Lippincott, 2015) supported by AFCEC contract number FA8903-11-C-8101.</td>
</tr>
<tr>
<td>Cape Cañaveral Facility 1381. Cape Cañaveral, Florida</td>
<td><strong>7</strong> (&lt;3 µg/L)</td>
<td>trace levels of CVOCs (3 µg/L or less)</td>
<td>moderately reducing</td>
<td>Steam- and iron-enhanced soil mixing, in situ bioremediation, air sparging, phyto remediation, groundwater extraction, and recirculation deployed at this site. Site is included in SERDP Project ER-2307 (Adamson et al., 2017).</td>
</tr>
<tr>
<td>Cape Cañaveral Space Launch Complex 16. Cape Canaveral, Florida</td>
<td><strong>10</strong> (1,730 J to 17,200 µg/L)*</td>
<td>high concentrations of CVOCs (&gt; 100 mg/L)</td>
<td>moderately reducing</td>
<td>No remediation prior to collection of samples for CSIA. Site is included in SERDP Project ER-2307 (Adamson et al., 2017). *1,4-dioxane concentration was biased high due to high CVOCs in sample.</td>
</tr>
</tbody>
</table>

**Table 4A: Summary of Phase I ER-2535 Sampling Sites**

**Abbreviations:** AFB (Air Force Base); OU (Operable Unit); µg/L (micrograms per liter); mg/L (milligrams per liter); TCE (trichloroethene); 1,2-DCA (1,2-dichloroethane); 1,1-DCE (1,1-dichloroethene); DO (dissolved oxygen); AFCEC (Air Force Civil Engineering Center); cis-1,2-DCE (cis-1,2-dichloroethene); CVOCs (chlorinated volatile organic compounds).
<table>
<thead>
<tr>
<th>Site Name, Location</th>
<th>Number of samples (concentration of 1,4-dioxane in samples)</th>
<th>Co-contaminants</th>
<th>Background Redox Conditions</th>
<th>Other Relevant Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air Force Plant 3 Tulsa, Oklahoma</td>
<td>10 (90 – 250 µg/L)</td>
<td>TCE and 1,1-DCE at 100 - &gt;1,000 µg/L</td>
<td>Mixed (DO from &lt;0.5 to &gt;2 mg/L)</td>
<td>6 wells sampled, 1 sampled 5 different times. AFCEC funding pilot test of in-well bioreactors. (AECOM, 2018)</td>
</tr>
<tr>
<td>Hanscom AFB, Bedford, MA</td>
<td>10 (0.076J – 19 µg/L)</td>
<td>Low CVOC concentrations to &gt;1,000 µg/L in some wells</td>
<td>Aerobic</td>
<td>Bio-Trap® samplers will be deployed in the 10 wells sampled for CSIA for qPCR analysis (Versar, 2018).</td>
</tr>
<tr>
<td>Joint Base Cape Cod Ashumet Valley, MA</td>
<td>4 (&lt;0.25 µg/L)</td>
<td>PCE &amp; TCE &lt;100 µg/L</td>
<td>Aerobic</td>
<td>Concentrations of 1,4-dioxane &lt; 1 µg/L. Site included in ESTCP Project ER201730.</td>
</tr>
<tr>
<td>Confidential DoD Site #1</td>
<td>5 (ND&lt;1 – &gt;1,000 µg/L*)</td>
<td>CVOCs from ND to &gt;1,000 µg/L</td>
<td>mixed (DO from 0.3 to 3.4 mg/L)</td>
<td>Site included in ESTCP Project ER201730. *1,4-D concentration inadvertently not analyzed in one of the 5 wells for this study; 2015 result was 8,275 µg/L.</td>
</tr>
<tr>
<td>Confidential DoD Site #2</td>
<td>5 (1.4 J – 970 µg/L)</td>
<td>TCE and 1,1-DCE at 100 to &gt;1,000 µg/L</td>
<td>aerobic, DO from 0.3 to 3.4 mg/L</td>
<td>Groundwater temperatures up to 39.4 degrees Celsius due to steam-injection for remediation. Site included in ESTCP Project ER201730.</td>
</tr>
<tr>
<td>Confidential DoD Site #3</td>
<td>4 (0.88 J – 16 µg/L)</td>
<td>CVOCs (10 µg/L range)</td>
<td>anaerobic (negative ORP)</td>
<td>Site included in ESTCP Project ER201730.</td>
</tr>
<tr>
<td>Confidential Non-DOD Site #1 Midwest USA</td>
<td>5 (8.3 – 310 µg/L)</td>
<td>THF to &gt;300 µg/L</td>
<td>Primarily anaerobic except for pilot test area.</td>
<td>1,4-dioxane biodegradation suspected based on other lines of evidence (Gedalanga et al., 2016). Site included in ESTCP Project ER201730. Oxygen/propane infusion pilot test near well IW-1 (sampled in this study).</td>
</tr>
<tr>
<td>Confidential Non-DOD Site #2 South Carolina</td>
<td>4 (130 – 10,000 µg/L)</td>
<td>VC and 1,1-DCE (&lt; 13 µg/L)</td>
<td>anaerobic (based on presence of VC and low DO)</td>
<td>Phytoremediation implemented at site. Site included in ESTCP Project ER201730.</td>
</tr>
</tbody>
</table>

**Table 4B: Summary of Phase II ER-2535 Sampling Sites**

**Abbreviations:** 1,4-D (1,4-dioxane); DO (dissolved oxygen) EISB (engineered in situ bioremediation); ND (not detected); PCE (tetrachloroethene); THF (tetrahydrofuran); VC (vinyl chloride); qPCR (quantitative polymerase chain reaction for quantification of functional genes implicated in the metabolism and co-metabolism of 1,4-dioxane).
3.2.1 Groundwater Sample Collection and Isotopic Characterization of 1,4-Dioxane in Groundwater

Groundwater samples were collected as part of groundwater monitoring for each site by field sampling crews using the same methods specified in the sampling plan for each site. Each groundwater sample for Air Force Plant 3 was collected in six 40 milliliter (mL) glass volatile organic analysis (VOA) vials with Teflon-lined septa and preserved with hydrochloric acid. For all other Phase II sites, samples were collected in 1 liter (L) amber glass jars without preservative. All samples were placed in ice-filled coolers and shipped to UWEIL for CSIA of 1,4-dioxane. Concentrations of 1,4-dioxane were analyzed at contract laboratories used for routine groundwater sampling at each site. The 1,4-dioxane concentrations were then provided to the UWEIL prior to performing CSIA on the 1,4-dioxane samples.

For the groundwater samples in VOAs, a sequential extraction of 1,4-dioxane was performed on the six VOA vials following the methods described in the Phase I Final Report (Haley & Aldrich, 2018), where approximately 0.3 grams of granular synthetic sorbent (Ambersorb™ 560; A560) were added to the first VOA to adsorb 1,4-dioxane for 24 hours on a rotary shaker. After 24 hours, the sorbent was filtered from the sample and added to the second VOA, for another 24 hours on the shaker, and so forth until all six VOAs were extracted onto the same small quantity of A560. For the 1 liter samples, the extraction method for 1,4-dioxane was adapted by UWEIL from the column experiments described in the Phase I Final Report to increase efficiency and improve sensitivity. Here, the 1 liter aqueous sample was slowly pumped through a column of about 0.3 grams of A560 at a rate of 0.2 mL/minute using a multi-head peristaltic pump. After approximately 3.5 days, the entire sample was pumped through the A560.

After loading 1,4-dioxane from the aqueous phase to the A560, the sorbent sample was dried and transferred to a stainless-steel thermal desorption tube. The 1,4-dioxane was then thermally desorbed from the sorbent into a gas chromatograph connected to a catalytic interface and isotope ratio mass spectrometer, as discussed in the Phase I Final Report.

3.2.2 Case Studies

The background for sites sampled in Phase I is included in the Phase I Final Report (Haley & Aldrich, 2018). These data are included for comparison to the eight sites sampled under this second phase of the project. Additional information on the eight sites described in Table 4B is provided in Section 4 of this report, along with the results for each case study.
4. RESULTS AND DISCUSSION

The results and discussion of this research are presented below and organized as follows:

- Section 4.1 includes a summary of the results for 1,4-dioxane source isotope composition; and

- Section 4.2 provides an assessment of the isotopic composition of 1,4-dioxane at field sites.

All laboratory reports for CSIA received from UWEIL are included as Appendix A of this report. These results have been shared with all who contributed samples to this project.

4.1 Isotopic Composition of 1,4-Dioxane Obtained from Chemical Suppliers

The isotopic compositions of 1,4-dioxane purchased from ten different suppliers are summarized in Table 5, including duplicate samples for both methods of analysis (EA-IRMS and GC-IRMS). EA-IRMS provides a “bulk” isotopic composition of all combustible carbon and hydrogen-based molecules present in the sample because, unlike CSIA, there is no GC column to separate different organic compounds present in the sample prior to combustion. Thus, for the two samples that are 100% pure 1,4-dioxane (Molecular Dimensions and Sigma Aldrich Switzerland), the reported values for $\delta^{13}C$ and $\delta^2H$ are representative of 1,4-dioxane and should be comparable to the values obtained by GC-IRMS. On the other hand, the $\delta^{13}C$ and $\delta^2H$ values reported for 1,4-dioxane for the other eight samples of lower purity could be influenced by other hydrocarbons in the mixture, in addition to 1,4-dioxane.

The average difference (taking the mean of duplicates) between $\delta^{13}C$ and $\delta^2H$ measured by EA-IRMS versus GC-IRMS is less than 0.5 ‰ for $\delta^{13}C$ and less than 5 ‰ for $\delta^2H$ for most of the high purity samples. These are within the analytical precision of CSIA and indicates that the two methods are comparable and reproducible, with the following exceptions:

- The $\delta^2H$ measured by EA-IRMS for the Honeywell sample shown in red font in Table 5 was approximately 12 ‰ higher than the GC-IRMS result, which may be indicative of contribution from impurities.

- Large differences between EA-IRMS and GC-IRMS were observed for the samples from TCI America and Restek, which are consistent with the contribution of carbon and hydrogen isotopes from butylated hydroxy toluene (BHT) in the TCI America sample and from methanol in the Restek sample.

For the purposes of source characterization of 1,4-dioxane, the mean of the primary and duplicate results analyzed by GC-IRMS are considered most representative for the isotopic composition of each 1,4-dioxane sample and these values are used to represent the range in manufactured “undegraded” 1,4-dioxane in Figures 2 and 3.
<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Sample ID</th>
<th>EA-IRMS $\delta^{13}$C</th>
<th>GC-IRMS $\delta^{13}$C</th>
<th>EA-IRMS $\delta^2$H</th>
<th>GC-IRMS $\delta^2$H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Dimensions</td>
<td>14D-1</td>
<td>-31.3</td>
<td>-31.2</td>
<td>-50</td>
<td>-51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-31.3</td>
<td>-31.3</td>
<td>-49</td>
<td>-45</td>
</tr>
<tr>
<td>J.T. Baker</td>
<td>14D-2</td>
<td>-31.4</td>
<td>-31.5</td>
<td>-47</td>
<td>-48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-31.6</td>
<td>-31.6</td>
<td>-47</td>
<td>-48</td>
</tr>
<tr>
<td>Alfa Aesar</td>
<td>14D-3</td>
<td>-33.5</td>
<td>-33.5</td>
<td>-33</td>
<td>-31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-33.5</td>
<td>-33.7</td>
<td>-33</td>
<td>-32</td>
</tr>
<tr>
<td>ACROS Organics</td>
<td>14D-4</td>
<td>-32.9</td>
<td>-33.1</td>
<td>-38</td>
<td>-36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-33.0</td>
<td>-33.0</td>
<td>-37</td>
<td>-30</td>
</tr>
<tr>
<td>ULTRA Scientific</td>
<td>14D-5</td>
<td>-29.1</td>
<td>-28.6</td>
<td>-31</td>
<td>-30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-29.2</td>
<td>-28.9</td>
<td>-32</td>
<td>-29</td>
</tr>
<tr>
<td>Honeywell</td>
<td>14D-6</td>
<td>-31.5</td>
<td>-32.0</td>
<td>-48</td>
<td>-60</td>
</tr>
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<td></td>
<td>-31.6</td>
<td>-31.8</td>
<td>-47</td>
<td>-55</td>
</tr>
<tr>
<td>Sigma Aldrich Switzerland</td>
<td>14D-7</td>
<td>-32.4</td>
<td>-32.3</td>
<td>-57</td>
<td>-60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-32.4</td>
<td>-32.6</td>
<td>-56</td>
<td>-60</td>
</tr>
<tr>
<td>Sigma Aldrich Wisconsin</td>
<td>14D-8</td>
<td>-33.4</td>
<td>-33.5</td>
<td>-39</td>
<td>-40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-33.2</td>
<td>-33.3</td>
<td>-37</td>
<td>-37</td>
</tr>
<tr>
<td>TCI America (stabilized with BHT)</td>
<td>14D-9</td>
<td>-29.3</td>
<td>-29.6</td>
<td>-22</td>
<td>-16</td>
</tr>
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<td></td>
<td></td>
<td>-29.3</td>
<td>-29.5</td>
<td>-23</td>
<td>-17</td>
</tr>
<tr>
<td>Restek (1,4-dioxane at 2 mg/L in methanol)</td>
<td>14D-10</td>
<td>-25.7</td>
<td>-32.6</td>
<td>-120</td>
<td>-29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-25.9</td>
<td>-32.6</td>
<td>-119</td>
<td>-29</td>
</tr>
</tbody>
</table>

**Table 5**: Isotopic Composition of 1,4-Dioxane Obtained from Chemical Suppliers

Note: all results in per mill notation. Samples in red font indicate the mean value of the primary and duplicate sample measured by EA-IRMS differs from the GC-IRMS result by more than 0.5 ‰ for $\delta^{13}$C or 5 ‰ for $\delta^2$H.
Figure 2: Carbon isotopic composition of manufactured (undegraded) 1,4-dioxane samples from this study (ER-2535; n=10), Pornwongthong, 2014 (P, 2014; n=5), Wang 2016 (W, 2016; n=7) and all three datasets combined (All; n=22). Range and median values for each are shown.

Figure 3: Hydrogen isotopic composition of manufactured (undegraded) 1,4-dioxane samples from this study (ER-2535; n=10), Pornwongthong, 2014 (P, 2014; n=5), Wang 2016 (W, 2016; n=7) and all three datasets combined (All; n=22). Range and median values for each are shown.

As shown in Figure 2, the range and median $\delta^{13}C$ values for undegraded “source” 1,4-dioxane reported in Table 5 (this study) are like those reported by Wang (2016) but higher than those
reported by Pornwongthong (2014). The discrepancy between Pornwongthong and this study may simply be related to different batches being analyzed, although the studies have some overlap in 1,4-dioxane suppliers, the lot numbers in Pornwongthong (2014) were different from those used in this study. The range in δ¹³C values of 1,4-dioxane for each of the three studies is relatively small, approximately 5‰, and when the three studies are combined, the 7.2‰ range (from -36.0‰ to -28.8‰) is small compared to the typical range in source values for common organic pollutants. For example, Thullner et al. (2012) compiled a range of source δ¹³C values for hydrocarbons and CVOCs from -22‰ to -35‰. The relatively small range in δ¹³C values of 1,4-dioxane may be beneficial for demonstrating biodegradation. Hunkeler et al. (2009) recommends a minimum enrichment in δ¹³C of 2‰ beyond the undegraded “source” isotopic composition to demonstrate biodegradation, if such a conclusion were to be based solely on enrichment in δ¹³C. Based on this guidance and the maximum δ¹³C values for source 1,4-dioxane shown in Figure 2, it can be concluded that δ¹³C values greater than -26.8‰ may reflect degradation of 1,4-dioxane. This value may need to be adjusted if new source materials indicate higher δ¹³C values for undegraded 1,4-dioxane.

Figure 3 shows δ²H values for source 1,4-dioxane reported in Table 5 (this study) are substantively different from those reported by Wang (2016), where the median value reported by Wang is nearly 100‰ “lighter” than 1,4-dioxane sources analyzed in this study. Although lot numbers are not reported in Wang (2016), it is likely that they are different from those sampled in this project. Nonetheless, the discrepancy is so large and systematic, that it is difficult to attribute solely to different lot numbers. The δ²H values reported by Wang (2016) are substantially depleted compared to the Pornwongthong (2014) results, even for the same manufacturer (Table 1); Pornwongthong (2014) δ²H values for 1,4-dioxane are closer to those reported in this study (Figure 3), but the median value is 30‰ lighter. For reference, source δ²H values of fuel hydrocarbons and MTBE range from -125‰ to -30‰ (Thullner et al., 2012). Accordingly, the range in δ²H values shown in Figure 3 (-149‰ to -17‰) is large, but not far beyond what has been reported for other groundwater contaminants. The relatively large range in δ²H values of 1,4-dioxane can complicate biodegradation assessments based solely on the enrichment in δ²H at sites where δ²H values of source (undegraded) 1,4-dioxane are near the lighter end of the reported range for manufactured 1,4-dioxane.

Ten manufacturers of 1,4-dioxane were present in the mid-1980s across the globe, and by 2000, only 5 were operating (Mohr, 2010). This indicates that sources of 1,4-dioxane procured today cannot represent the full range of manufacturers for historical releases which may have occurred several decades ago, and therefore it is possible that the full range in isotopic composition of 1,4-dioxane is not entirely represented in Figures 2 and 3. Industrial and municipal wastewater effluent can also be sources for 1,4-dioxane in groundwater, and in many cases, the 1,4-dioxane is not directly manufactured, it is instead a byproduct of manufacturing. The isotopic composition of byproduct 1,4-dioxane is not represented by the samples analyzed in this study.

4.2 Isotopic Composition of Neat 1,4-Dioxane During Passive Evaporation

The results of the passive evaporation experiment for neat 1,4-dioxane are shown in Table 6 for 1,4-dioxane produced by ACROS Organics (99.9% purity). No enrichment trend with evaporation is apparent and the δ¹³C and δ²H values at 80% evaporation are within 0.2‰ and 3‰ of the initial
values prior to evaporation (the analytical precision reported by the laboratory for $\delta^{13}$C and $\delta^2$H is $\pm0.5\%$ and $\pm5\%$, respectively). These results suggest that the isotopic composition of manufactured 1,4-dioxane may not be substantively affected by passive evaporative losses. This is consistent with findings for other volatile organic compounds. For example, Hunkeler et al. (2009) concluded that isotope fractionation during volatilization, dissolution, diffusion and sorption is typically not discernible outside of the typical analytical uncertainty of CSIA ($\pm0.5\%$ and $\pm5\%$, for $\delta^{13}$C and $\delta^2$H, respectively). On the other hand, Kuder et al. (2009) measured small hydrogen isotope effects during evaporation of MTBE dissolved in gasoline and in water, using column experiments with different air flow rates, and found that the effect of evaporation on the isotopic composition of MTBE could be readily discerned from biodegradation using dual isotope trends. Thus, for evaporation of 1,4-dioxane, passive volatilization from neat 1,4-dioxane did not result in measurable fractionation, but the possibility of a measurable isotope shift during more extreme conditions of evaporation or evaporation from aqueous phases cannot yet be ruled out because conditions at higher air flow rates were not tested as part of this work.

<table>
<thead>
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<th>Sample</th>
<th>$\delta^{13}$C (% VPDB)</th>
<th>$\delta^2$H (% VSMOW)</th>
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<tr>
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Table 6: Isotopic Composition of 1,4-Dioxane During Evaporation

### 4.3 Isotopic Composition of 1,4-Dioxane in Groundwater Samples

The CSIA method developed in Phase I of this project was applied to 78 groundwater samples from 12 different field sites at concentrations ranging from non-detect to 17,200 µg/L with a median concentration of 18 µg/L. Of these samples, 68 measurements of $\delta^{13}$C and 54 measurements of $\delta^2$H were successfully performed. The laboratory reports received from UWEIL for all samples are included as Appendix A to this report.

The $\delta^{13}$C and $\delta^2$H values for 1,4-dioxane measured at the 12 field sites are summarized in Figures 4A and 4B. As shown in Figure 4A, the majority of $\delta^{13}$C values for 1,4-dioxane measured in groundwater samples fall between -34‰ and -28‰, like the range of $\delta^{13}$C values for undegraded source 1,4-dioxane shown in Figure 2. The lowest $\delta^{13}$C values between -38‰ and -36‰ are for the Confidential Non-DOD Site #2 described in greater detail in Section 4.4.2. These results indicate that “lighter” $\delta^{13}$C values for sources of 1,4-dioxane exist compared to those shown in Figure 2. Some of the “heavier” $\delta^{13}$C values between -26‰ and -22‰ reflect biodegradation due to biostimulation (2 samples from McClellan AFB Site), others may be related to natural biodegradation and/or source variability (1 sample from SLC-16) and/or analytical uncertainty (for low 1,4-dioxane concentrations [$< 3$ µg/L] at Cape Cañaveral Site 1381).
As shown in Figure 4B, the majority of δ²H values for 1,4-dioxane measured in groundwater samples fall between -50‰ and 0‰, overlapping the full range of δ²H values for undegraded source 1,4-dioxane, but with a proportion of samples being enriched by 10 to 20‰ relative to the maximum δ²H value for undegraded source 1,4-dioxane of -17‰. Based on the expected error for determination of δ²H values by CSIA, Braeckevelt et al. (2012) noted that an enrichment of 10 to 20‰ may be an indication of biodegradation but could also be analytical variability, and that 20 to 40‰ enrichment in δ²H between two samples along a flowpath is conclusive of biodegradation provided that other sources can be ruled out. Thus, the δ²H values in Figure 4B between -17‰ and 0‰ cannot be attributed to biodegradation alone without considering other lines of evidence. The values greater than 0‰ include samples from Air Force Plant 3 and Hanscom AFB sites, where other lines of evidence do not support biodegradation, as discussed Section 4.4.2.

The δ²H values lower than -60‰ were reported at McClellan AFB (n=3), Cape Cañaveral Site SLC-16 (n=2), and Hanscom AFB (n=1), indicating that some “lighter” δ²H values for sources of 1,4-dioxane exist compared to the sources of 1,4-dioxane analyzed in this study. Based on the large range in δ²H values for source 1,4-dioxane and given the larger uncertainty in analytical results, the use of δ²H values alone as an indicator for biodegradation is not recommended.

It is common practice in CSIA field studies to use the highest concentration sample(s) from a contaminant source area as a reference value for the initial isotopic composition for assessing isotopic enrichment due to biodegradation. While this approach works well for most organic contaminants that are present at high concentrations and may have originated as non-aqueous...
phase liquids, it may be more difficult for 1,4-dioxane since it is often present at lower concentrations in groundwater. In cases where there is no clear “source” isotope composition of 1,4-dioxane, comparison of $\delta^{13}C$ and $\delta^2H$ values along flowpaths can be useful, particularly when interpreted using a “dual isotope” approach on a case by case basis, as described in Section 2.1.4.

4.4 CSIA of 1,4-Dioxane at Field Sites (Case Studies)

The 12 sites evaluated under Phase I and II of this project can be thought of as expedited case studies on the application of CSIA at field sites under a variety of conditions. Since the four sites listed in Table 4A were discussed in greater detail in the Phase I report, the primary conclusions for each Phase I site are re-visited in Section 4.4.1. Section 4.4.2 summarizes the site conditions and results for each of the eight sites sampled under Phase II.

4.4.1 Phase I Sites

The results for sites sampled in Phase I listed in Table 4A are included in the Final Report (Haley & Aldrich, 2018) and are summarized herein.

4.4.1.1 McClellan Air Force Base

At McClellan Air Force Base, a pilot test successfully used propane and oxygen to promote the biodegradation of 1,4-dioxane by an aerobic co-metabolic process as described in Chu et al. (2018). The pilot test layout is shown in Figure 5A. Based on analysis of three target gene abundances in groundwater samples using qPCR methods, Chu et al. (2018) demonstrated an increase in propane monooxygenase activity following propane and oxygen addition and documented the biodegradation of low concentrations of 1,4-dioxane (i.e. from ~50 µg/L to < 1 µg/L). For these reasons, this biostimulation pilot test made for an excellent case study for demonstrating the ability of CSIA to detect biodegradation of 1,4-dioxane in groundwater.

As described in the Phase I Final Report, CSIA was performed on 1,4-dioxane in 13 groundwater samples collected over the course of approximately 10 months prior to and during the pilot test; 10 of these samples have paired $\delta^{13}C$ and $\delta^2H$ values. The CSIA results from samples collected prior to biostimulation, and/or from outside of the biostimulation zone, have shown a large range in the isotopic composition of 1,4-dioxane, as follows:

- $\delta^{13}C$: from -33.7 to -28.3‰ (a 5.4‰ range)
- $\delta^2H$: from -89‰ to -38‰ (a 51‰ range)

In Figure 5B, the CSIA results from the June 2016 groundwater monitoring event are plotted with the recently published dual isotope trends shown in Figure 1 and Table 2; other CSIA results are provided in the Phase I Final Report. Key findings are provided from Figure 5B:

1. The samples affected by biodegradation (MACB-1 and MACB-2) have $\delta^{13}C$ values of -25.4‰ and -24.1‰, which are up to 4.0‰ higher than any of the other measured $\delta^{13}C$ values for 1,4-dioxane at the site, and up to 4.8‰ higher than the $\delta^2H$ values of 1,4-dioxane
in recirculated groundwater introduced three to seven feet upgradient along with propane and oxygen at IACB-1, consistent with biodegradation of 1,4-dioxane.

2. Large enrichment in $\delta^{2}H$ values, from -89‰ at IACB-1 to -6‰ and -23‰, at MACB-1 and MACB-2, respectively, up to 30‰ higher than any of the other $\delta^{2}H$ values for 1,4-dioxane at the site, and up to 83‰ higher than the $\delta^{2}H$ values of 1,4-dioxane in recirculated groundwater at IACB-1, consistent with biodegradation of 1,4-dioxane.

3. The extent of enrichment in $\delta^{13}C$ and $\delta^{2}H$ at MACB-2 aligns well with the enrichment trend determined for Mycobacterium sp. 1A (magenta line of Figure 5B), which is consistent with other lines of evidence:

   a. Chu et al. (2018) reported on the biostimulation with propane and the detection of this Mycobacterium strains in treatment zone groundwater; and

   b. The 1,4-dioxane concentration decrease from 24 µg/L at IACB-1 to 0.86 µg/L at MACB-2 amounts to a 97% decrease. Using the Rayleigh model (Equation 2), the 4.8‰ enrichment in $\delta^{13}C$, and the enrichment factor for Mycobacterium sp. 1A grown on gaseous alkanes ($\varepsilon = -2.0 \pm 0.4‰$; Table 2), the calculated decrease in 1,4-dioxane due to degradation is from 86% to 95%, which compares well with the measured decrease in concentration. In other words, the extent of isotopic enrichment at MACB-2 is consistent with that reported for Mycobacterium sp. 1A grown on gaseous alkanes.

4. The values for $\delta^{13}C$ and $\delta^{2}H$ for MACB-1 plot between the enrichment trends for Mycobacterium sp. 1A and P. tetrahydrofuranoxidans K1 (green line of Figure 5B). Given that the extent of 1,4-dioxane concentration decrease and corresponding $\delta^{13}C$-enrichment are similar at MACB-1 and MACB-2, and other lines of evidence align with the involvement of Mycobacterium sp. 1A grown on gaseous alkanes, there is no reason to believe 1,4-dioxane degradation conditions were different at MACB-1 compared to MACB-2. Therefore, although the sample for MACB-1 plots farther from the enrichment trend established for Mycobacterium sp. 1A grown on gaseous alkanes, this may be an artifact of analytical variability of CSIA at low concentrations of 1,4-dioxane.

5. The $\delta^{13}C$ and $\delta^{2}H$ values for MW-10 plot near the $\delta^{13}C$ and $\delta^{2}H$ enrichment trend for P. tetrahydrofuranoxidans K1 (green line of Figure 5B), which could be interpreted as a different degradation pathway than observed at MACB-1 and MACB-2. However, unlike MACB-1 and MACB-2, MW-10 is not directly along the flow path from the injection well (Figure 5A) and, given the established variability in the isotopic composition of 1,4-dioxane at this site, and that the 1,4-dioxane concentration at MW-10 is higher (44 µg/L) than at IACB-1 (24 µg/L), this result may simply be variability in isotopic composition of 1,4-dioxane in site groundwater.

Based on the findings above, it has been shown that CSIA can provide direct field-based evidence for 1,4-dioxane biodegradation in groundwater and, through the use of dual isotope plots, CSIA results can be compared to published enrichment trends to provide insights into the degradation
conditions. The dual isotope plot can be useful in selecting the appropriate enrichment factor for Rayleigh-type degradation calculations. On the other hand, variability in isotopic composition of 1,4-dioxane within a given site, and/or increased analytical uncertainty at low concentrations of 1,4-dioxane, can introduce uncertainty into field-based dual-isotope trends. Multiple lines of evidence are recommended for the interpretation of CSIA results.

Figure 5. Pilot Test Layout and Dual Isotope Plot – McClellan Air Force Base. Figure 5A (left): Pilot test layout from Chu et al. (2018) which involved extracting groundwater at MACB-3 and re-injecting it at IACB-1 to form a groundwater circulation loop. Intermittent propane and oxygen addition by infusion into extracted groundwater from MACB-3 prior to re-injection at IACB-1 promoted the aerobic co-metabolic biodegradation of 1,4-dioxane at treatment efficiencies greater than 98% removal. Figure 5B (right): Dual isotope plot showing paired $\delta^{13}C$ and $\delta^2H$ values (‰) for groundwater samples collected in June 2016. The three samples shown as red dots in Figure 5B span a decrease in 1,4-dioxane concentration from 24 µg/L at injection well (IACB-1) to <1 µg/L\textsuperscript{1} at monitoring wells MACB-1 and MACB-2, located three and seven feet downgradient of IACB, respectively (MACB-3 is extracted groundwater that is re-injected at IACB-1; MW-10 is a nearby monitoring well). For reference, dual-isotope enrichment trends are shown for *R. rhodochrous ATCC 21198* (propane substrate, blue line), *Mycobacterium* sp. 1A (propane substrate, magenta line) and *P. tetrahydrofuranoxidans KI* (THF substrate, green line) based on $\Delta\delta^2H/\Delta\delta^{13}C$ values shown in Table 2 and the observed enrichment in $\delta^{13}C$ in MACB-1 and MACB-2, relative to IACB-1.

\textsuperscript{1} 1,4-dioxane concentrations for CSIA were from different samples collected on 2 June 2016 and therefore <1 µg/L may not represent the concentration of the actual samples analyzed by CSIA. Groundwater samples collected on 27 May and 9 June 2016 (the closest sampling dates bracketing 2 June) ranged from 1.3 to 1.8 µg/L; it is possible that the 1,4-dioxane concentrations in MACB-1 and MACB-2 samples were in the 1 to 2 µg/L range since UWEIL was able to analyze the samples from MACB-1 and MACB-2 with a peak area greater than 0.5 volts. MW-10 sampled on 9 June 2016 for CSIA; concentration = 44 µg/L.)
4.4.1.2 Vandenberg Air Force Base Site 24

As discussed in the Phase I Final Report, only two monitoring wells were sampled at Site 24 for CSIA of 1,4-dioxane: 24-PMW-01R (81 µg/L) and 24-MW-34B (78 µg/L). The δ¹³C value for 24-MW-34B (-27.3‰), reportedly influenced by a biosparge pilot test, was enriched in ¹³C by 2.4‰ relative to the other sample from 24-PMW-01R (-29.7‰); the δ²H values were similar (-42‰ and -44‰, respectively). It is possible that the higher δ¹³C for 24-MW-34B reflects enrichment in ¹³C due to biodegradation, but without knowledge of the starting isotopic composition of 1,4-dioxane, and given the lack of enrichment in δ²H, it is difficult to conclude that the δ¹³C and δ²H values indicate degradation because the different results could also reflect variation in isotopic composition of 1,4-dioxane sources. Additional lines of evidence and more CSIA data are needed to interpret the results for this site.

4.4.1.3 Cape Cañaveral Air Force Station Facility 1381 and Space Launch Complex 16

Both sites are part of the SERDP Project ER-2307 and are described in greater detail in Adamson et al., 2017. Of the seven samples collected of CCAFS Facility 1381 groundwater for analysis of the δ¹³C and δ²H values, δ²H could not be analyzed in any of the samples due to low concentrations (< 3 µg/L). Stable carbon isotope ratios were determined for five of the seven samples, and four of the five samples had δ¹³C values from -25.5‰ to -22.4‰. Given that the current range of δ¹³C values does not extend higher than -28.8‰ (Figure 2), it is possible that these more ¹³C-enriched samples are representative of biodegradation. This conclusion is supported by the presence of biomarkers for 1,4-dioxane (detection of Pseudonocardia strains and target functional genes dioxane monooxygenase (DXMO) and aldehyde dehydrogenase (ALDH); Adamson et al., 2017).

Figure 6: Dual isotope plot for CCAFS SLC-16. Paired δ¹³C and δ²H values (‰) are shown. The four samples shown as red dots have 1,4-dioxane concentrations of 1,820 µg/L at SLC16-SB2 (48-50°), 1,750 µg/L at SLC16-SB2 (40-42°), 17,200 µg/L at SLC16-SB1 (40-42°) and 9,000 µg/L at SLC16-SB1 (48-50°). For reference, dual-isotope enrichment trends are shown for P. dioxanivorans CB1190 (1,4-dioxane substrate, orange line) and R. rhodochrous ATCC 21198 (propane substrate, blue line) based on Δδ²H/Δδ¹³C values shown in Table 2 and the observed enrichment in δ¹³C.
Ten groundwater samples were collected at CCAFS SLC-16 for CSIA of 1,4-dioxane; however, due to low concentrations, $\delta^{13}$C and $\delta^2$H values were reported for only five and four samples, respectively (Haley & Aldrich, 2018); the four samples with paired $\delta^{13}$C and $\delta^2$H values are shown in Figure 6. The $\delta^{13}$C and $\delta^2$H values in the two samples from SLC16-SB2 are lower than the samples from SLC16-SB1; the sample SLC16-SB1 (48-50’) was the most strongly enriched in the heavier isotopes, with $\delta^{13}$C and $\delta^2$H of -23.4‰ and -10‰, respectively (Figure 6). As shown in Figure 6, the enrichment at SLC16-SB1 (48-50’) extends beyond the expected range in $\delta^{13}$C for undegraded 1,4-dioxane shown in Figure 2 and is consistent with the dual isotope trend determined for *R. rhodochrous ATCC 21198* (propane substrate; Table 2). The smaller apparent enrichment at SLC16-SB1 (40-42’) aligns with the dual isotope enrichment trend for *P. dioxanivorans CB1190*. Assuming the initial undegraded isotopic composition of 1,4-dioxane is represented by the samples from SLC16-SB2, then enrichment in $\delta^{13}$C and $\delta^2$H along dual isotope trends is evidence for biodegradation of 1,4-dioxane. This is also supported by the presence of biomarkers for 1,4-dioxane (detection of *Pseudonocardia* strains and target functional genes DXMO and ALDH; Adamson et al., 2017) in soil and groundwater samples from the site. However, the higher 1,4-dioxane concentrations at SLC16-SB1 versus SLC16-SB2 complicates the assumption that the SLC16-SB2 samples represent the undegraded 1,4-dioxane signature. Additional information to support a reference value or range for the initial isotopic composition of undegraded 1,4-dioxane would help clarify whether the enriched values at SLC16-SB1 represent biodegradation of 1,4-dioxane or variability in the isotopic composition of 1,4-dioxane at the site.

4.4.2 Phase II Sites

The results of the Phase II Sites are summarized herein.

4.4.2.1 Air Force Plant 3

AFP3 is in northeastern Tulsa, Oklahoma, and has been used for aircraft manufacturing and other industrial operations since 1942 (AECOM, 2013). The location from which samples were collected for this project are Area of Concern 1 (AOC1), which is associated with two former vapor degreasers that utilized TCE and 1,1,1-TCA, and OT010, also known as Solid Waste Management Unit (SWMU) 8, which is associated with the Building 3 former floor drain system. In 1969, the drain system was installed to collect paint, stripping waste, and contaminated water from aircraft component stripping, blasting, and painting activities. The drain system was found to be leaking in 1987; CVOC and 1,4-dioxane plumes are associated with the leaks. CVOCs detected include 1,1-DCA, 1,1-DCE, 1,2-DCA, cis-1,2-DCE, TCE, and vinyl chloride. The plumes were found to have multiple source areas but AOC1 and OT010 have been found to be distinct and not co-mingled. The distribution of 1,4-dioxane is consistent with that of 1,1-DCE and 1,1-DCA (AECOM, 2013; AECOM, 2018).

A groundwater treatment system comprised of two interceptor trenches and a shallow-tray aerator was installed in 1995 and shut down in 1999; semi-annual or annual sampling has been conducted since that time (AECOM, 2013).
Groundwater samples collected from AFP 3 were completed in connection with an Air Force Civil Engineering Center (AFCEC)-funded research project to test *in situ* bioreactors (ISBRs) designed to accelerate 1,4-dioxane biodegradation through bioaugmentation. The ISBRs were specially designed and loaded with Bio-Sep® beads cultured with CB1190 to enhance 1,4-dioxane degradation. Air sparging at the base of the ISBR created circulation of groundwater through the unit, flowing from bottom to top, and nutrients were introduced into the middle of the unit. The screened interval of monitoring wells with ISBRs ranged from 7 to 23 ft bgs. Ten samples from six locations were collected by AECOM between January and September 2018 for analysis of $\delta^{13}C$ and $\delta^2H$.

The groundwater samples collected at Air Force Plant 3 are from a small area near the source zone. Table 7 shows the results from six monitoring wells sampled in January 2018; one of these wells (81W01) was sampled four more times between March and September 2018. The results are also shown in a dual isotope plot shown in Figure 7. The concentrations of 1,4-dioxane are generally consistent among the different locations (Table 7). None of the $\delta^{13}C$ values are sufficiently enriched to indicate biodegradation; all of the $\delta^2H$ values are near the high end of the expected range for undegraded 1,4-dioxane (Figure 3). The sample from 8MNW59 has a $\delta^2H$ value of +12‰, which is beyond the range for undegraded 1,4-dioxane (Figure 3) and 21‰ to 42‰ higher than the other samples. This could be evidence for biodegradation if not for the lack of enrichment in $\delta^{13}C$ and that the 1,4-dioxane concentration is consistent with the other results. The replicate analyses of samples from well 81W01 yield a consistent range in $\delta^{13}C$ (within 2‰) and $\delta^2H$ (within 21‰) and do not exhibit a linear dual isotope trend along published enrichment trend lines (Figure 7). Therefore, these results are not conclusive with respect to biodegradation of 1,4-dioxane at the site based on the limited number of wells sampled herein.

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<th>Date</th>
<th>Sample</th>
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*Table 7: CSIA Results for Air Force Plant 3*
Figure 7: Dual isotope plot for Air Force Plant 3. Paired $\delta^{13}$C and $\delta^{2}$H values (‰) are shown. Samples collected in January 2018 (blue dots) and replicate analyses of 81W01B (red dots); the data do not follow a linear trend.

4.4.2.2 Hanscom Air Force Base

Hanscom AFB is in Middlesex County, near Bedford, Massachusetts, and was primarily used for aircraft maintenance and research and development support (US EPA, 2007). OU-1 includes three sites: Fire Training Area II (Site 1), Paint Waste Disposal Area (Site 2), and Jet Fuel Residue/Tank Sludge Disposal Area (Site 3). Contamination stems from activities starting in the 1960s, including burning wastes in unlined pits, disposing of waste solvents and metal plating wastes, and burial of drums with waste airplane fuel and paints (US EPA, 2007). Hanscom AFB was listed on the National Priorities List (NPL) in 1994. Site 2 is located approximately 2,000 feet southeast of Site 1; Site 3 is located approximately 4,000 feet southwest of Site 1.

Soil excavation was conducted at all sites within OU-1; drum excavation was also completed at Sites 2 and 3. A groundwater extraction, treatment, and recharge system comprised of trenches, conventional interceptor wells, and vacuum-enhanced recovery wells has been in operation at OU-1 since April 1991 (US EPA, 2007). At Site 1, both permanganate and molasses have periodically been injected to treat CVOCs. CVOCs detected above their respective criteria include 1,1,1-TCA, TCE, cis-1,2-DCE, and vinyl chloride. TCE has decreased to 15 µg/L from a maximum concentration of 1,000 µg/L.

Hanscom AFB is underlain by Andover Granite, which includes north-northeast trending fault lines. Overlying the bedrock are glacial lacustrine deposits and discontinuous glacial till. Outwash sediments consisting of silts and fine to coarse sands form the uppermost unit (US EPA, 2007). Groundwater flows northeastward through both the bedrock and overlying unconsolidated sediments, which has two transmissive zones separated by a semi-confining unit of lacustrine silt and clay (US EPA, 2007). Depth to groundwater ranges from 5 to 10 ft bgs.
Groundwater samples for this study were collected from Hanscom AFB by Versar, Inc between 10 February and 19 February 2019. Ten samples were collected for CSIA of 1,4-dioxane: two from the surface aquifer, four from the upper/till aquifer, and four from the bedrock aquifer (Versar, 2018). The CSIA results are summarized in Table 8.

Based on Table 8:

- All of the $\delta^{13}$C values are within the typical range of undegraded “source” 1,4-dioxane;
- All of the $\delta^2$H values are within the typical range of undegraded “source” 1,4-dioxane except for RAP2-4T (+58‰);
- The groundwater samples from surficial aquifer wells OW2-6 (Site 2) and OW3-14 (Site 3) have similar $\delta^{13}$C values within analytical precision. The higher $\delta^2$H value at OW3-14 (+2 ‰) versus OW2-6 (-31‰) likely reflects difficulties with analytical precision at the low concentration at OW3-14 (0.076 J µg/L); and
- The groundwater sample from Lower Till well B245 (Site 1) has highest $\delta^{13}$C value (-28.0‰) compared to all other samples but a $\delta^2$H value could not be obtained due to analytical limitations. While enrichment in $\delta^{13}$C due to biodegradation is a possible explanation, it is difficult to interpret this result conclusively because there is no paired $\delta^2$H value.

A dual isotope plot is shown in Figure 8 and includes published enrichment trends associated with the biodegradation of 1,4-dioxane. While the CSIA results shown in Table 8 appear to loosely follow an enrichment trend in $\delta^2$H, the enrichment in $\delta^{13}$C is smaller than would be expected for biodegradation. A linear regression through the Site 1 datapoints yields a slope consistent with biodegradation ($\Delta\delta^2$H/$\Delta\delta^{13}$C = 30; Table 2), but the regression statistics show a weak correlation ($R^2 = 0.24$; p-value = 0.19). Although some datapoints appear to fall along the enrichment trends shown in Figure 8, beginning at RAP1-6R, the samples are not along a flowpath that would indicate concentration attenuation with distance. The high $\delta^2$H value at RAP2-4T (+58‰) is difficult to explain; the lack of enrichment in $\delta^{13}$C (-31.5‰) is inconsistent with biodegradation, therefore this may be related to an analytical artifact at low concentration (3.3 µg/L), although UWEIL did not report any difficulties with this sample that would indicate a problem. In summary, the CSIA results are not conclusive with respect to biodegradation of 1,4-dioxane. Other lines of evidence may be helpful to further assess whether 1,4-dioxane biodegradation is occurring, particularly near B245 and RAP2-4T where CSIA results differed from others. Versar (2018) states that Bio-Trap® samplers will be deployed in these wells to quantify functional genes that have been linked to 1,4-dioxane biodegradation. These results may provide additional lines of evidence to support biodegradation of 1,4-dioxane and may also provide insights into the CSIA results.
Table 8: CSIA Results for Hanscom Air Force Base
(Note: BAL = below analytical limit;)

<table>
<thead>
<tr>
<th>Date</th>
<th>Sample</th>
<th>Site Number</th>
<th>Aquifer</th>
<th>1,4-dioxane (µg/L)</th>
<th>δ¹³C (‰ VPDB)</th>
<th>δ²H (‰ VSMOW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/19/2019</td>
<td>RAP2-4T</td>
<td>Site 1</td>
<td>Lower Till</td>
<td>3.3</td>
<td>-31.5</td>
<td>+58</td>
</tr>
<tr>
<td>2/10/2019</td>
<td>B245</td>
<td>Site 1</td>
<td>Lower Till</td>
<td>1.3</td>
<td>-28.0</td>
<td>BAL</td>
</tr>
<tr>
<td>2/10/2019</td>
<td>RAP1-6R</td>
<td>Site 1</td>
<td>Bedrock</td>
<td>17</td>
<td>-33.1</td>
<td>-67</td>
</tr>
<tr>
<td>2/10/2019</td>
<td>B244A</td>
<td>Site 1</td>
<td>Bedrock</td>
<td>3.1</td>
<td>-32.2</td>
<td>-10</td>
</tr>
<tr>
<td>2/10/2019</td>
<td>B240</td>
<td>Site 1</td>
<td>Bedrock</td>
<td>2.8</td>
<td>-32.4</td>
<td>-54</td>
</tr>
<tr>
<td>2/11/2019</td>
<td>OW2-6</td>
<td>Site 2</td>
<td>Surface</td>
<td>6.2</td>
<td>-32.2</td>
<td>-31</td>
</tr>
<tr>
<td>2/11/2019</td>
<td>B114-MW</td>
<td>Site 2</td>
<td>Lower Till</td>
<td>19</td>
<td>-32.0</td>
<td>-47</td>
</tr>
<tr>
<td>2/10/2019</td>
<td>B248</td>
<td>Site 2</td>
<td>Lower Till</td>
<td>0.87</td>
<td>-31.4</td>
<td>-22</td>
</tr>
<tr>
<td>2/10/2019</td>
<td>B252</td>
<td>Site 2</td>
<td>Bedrock</td>
<td>4.5</td>
<td>-32.8</td>
<td>-17</td>
</tr>
<tr>
<td>2/10/2019</td>
<td>OW3-14</td>
<td>Site 3</td>
<td>Surface</td>
<td>0.076 J</td>
<td>-32.8</td>
<td>2</td>
</tr>
</tbody>
</table>

Figure 8: Dual Isotope Plot for Hanscom Air Force Base. Paired δ¹³C and δ²H values (‰) are shown for samples from Site 1 (red dots) and Sites 2 and 3 (blue dots). For reference, dual-isotope enrichment trends are shown for *R. rhodochrous ATCC 21198* (propane substrate, blue line), *Mycobacterium* sp. 1A (propane substrate, magenta line) and *P. tetrahydrofuranoxidans K1* (THF substrate, green line) based on Δδ²H/Δδ¹³C values shown in Table 2, with an origin at RAP1-6R (lowest δ¹³C and δ²H values in Table 8).

4.4.2.3 Joint Base Cape Cod, Ashumet Valley

Joint Base Cape Cod (JBCC) is located in Buzzards Bay, Massachusetts. The Ashumet Valley plume is located immediately south of JBCC and consists of three disconnected plumes. These
plumes are downgradient of a former fire training area (FTA-1), used from 1958 to 1985, and former Massachusetts Military Reservation sewage treatment plant, used from 1936 to 1995 (CH2M, 2017). Treated wastewater was routed to sand infiltration beds located just south of FTA-1.

The Ashumet Valley plumes were first identified in 1979. An interim remedy, a groundwater extraction and treatment system, was implemented in 1999 for treatment of PCE and TCE as well as naturally occurring thallium and manganese that were mobilized by reducing conditions induced by sewage disposal. Approximately 49,000 tons of soil were excavated at FTA-1 for onsite thermal treatment between 1995 and 1997. Maximum historical PCE and TCE concentrations were 109 µg/L in 1998 and 83 µg/L in 1997, respectively (CH2M, 2017). During a five-year review of the treatment system, it was recommended that 1,4-dioxane and PFAS be investigated further (CH2M, 2017). Other co-contaminants include 1,1,1-TCA and 1,1-DCE. Concentrations of 1,4-dioxane are low and not widespread, ranging up to 1 µg/L in 2017 at well 30MW0585A (CH2M, 2017).

The Ashumet Valley plume lies within the Mashpee Pitted Plain, a poorly-graded glacial outwash feature (CH2M, 2017). The plain is underlain by silty glaciolacustrine sediments and basal till; in some areas, the plain immediately overlies bedrock. The glacial sediments are approximately 300 to 400 feet thick in the Ashumet Valley area. Groundwater is unconfined and depth to groundwater ranges from a few feet to 60 ft bgs (CH2M, 2017). Groundwater flow is either to the south-southeast or south-southwest at a gradient of 0.001 to 0.002 ft/ft. Flow paths are complicated by the presence of two kettle ponds and an abandoned cranberry bog; hydraulic connection between groundwater and these surface water features generate localized upward and downward vertical gradients (CH2M, 2017).

Groundwater samples were collected by Scissortail Environmental, Inc. from four monitoring wells along the Ashumet Valley 1,4-dioxane plume centerline for analysis of $\delta^{13}C$ and $\delta^{2}H$ between 20 and 23 August 2018. The results are summarized in Table 9, listed in order of increasing distance downgradient of FTA-1 and the sewage treatment plant. Concentrations of 1,4-dioxane were quantified by US EPA Method 8270 with Selective Ion Monitoring (EPA Method 8270 SIM); all results were reported as less than the reporting limit of 0.25 µg/L. These 1,4-dioxane concentrations are below the lowest limits tested for CSIA in Phase 1; however, in some instances, UWEIL has been able to report results below 1 µg/L. Results at concentrations this low may be subject to analytical error and should be interpreted with caution.

Monitoring well 30MW0585A has historically had the highest (albeit very low) concentration of 1,4-dioxane at the site. As shown in Table 9, $\delta^{13}C$ and $\delta^{2}H$ values of -32.1‰ and +0.5‰, respectively, were reported for 30MW0585A; the $\delta^{13}C$ value is consistent with undegraded source 1,4-dioxane and the $\delta^{2}H$ value may not be accurate, given that no $\delta^{2}H$ values are reported for the other three samples due to analytical limitations. The other $\delta^{13}C$ values are also within the range of undegraded source 1,4-dioxane. Because of the low concentrations of 1,4-dioxane, these results may be subject to higher analytical uncertainty than normal. Further application of CSIA for assessing the attenuation of 1,4-dioxane in groundwater at JBCC is not recommended due to the low concentrations for 1,4-dioxane.
<table>
<thead>
<tr>
<th>Date</th>
<th>Sample</th>
<th>1,4-dioxane (µg/L)</th>
<th>δ(^{13})C (% VPDB)</th>
<th>δ(^2)H (% VSMOW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8/8/2018</td>
<td>30MW0585A</td>
<td>&lt;0.25</td>
<td>-32.1</td>
<td>+0.5</td>
</tr>
<tr>
<td>8/8/2018</td>
<td>95MW1171A</td>
<td>&lt;0.25</td>
<td>-31.8</td>
<td>BAL</td>
</tr>
<tr>
<td>8/20/2018</td>
<td>95MW1174A</td>
<td>&lt;0.25</td>
<td>-32.6</td>
<td>BAL</td>
</tr>
<tr>
<td>8/7/2018</td>
<td>95MW1173A</td>
<td>&lt;0.25</td>
<td>-32.2</td>
<td>BAL</td>
</tr>
</tbody>
</table>

Table 9: CSIA Results for Joint Base Cape Cod, Ashumet Valley
(Note: BAL = below analytical limit)

4.4.2.4  Confidential DoD Site #1

Confidential DoD Site #1 is in California and has been used for aviation activities by the Army and Navy since 1917 (Resolution Consultants, 2014). DoD Site #1 includes a former Hazardous Waste Facility (HWF), a former Industrial Waste Treatment Plant built in 1972 to treat rinse water contaminated by plating, stripping, and other industrial processes, and an Oily Waste Treatment Plant, built in 1978 to primarily treat ship bilge water (Resolution Consultants, 2014). Treated waste was routed to 20 surface impoundments, which were taken out of service between 1985 and 1988. DoD operations are ongoing and active HWF units continue to be used, including a current Industrial Waste Treatment Plant, an oil recovery plant, and two collection, storage, and transfer facilities (Battelle and Accord Engineering Inc., 2019).

Groundwater at DoD Site #1 has been monitored since 1984. In addition to 1,4-dioxane, co-contaminants include PCE, TCE, cis-1,2-DCE, 1,2-DCA, 1,1-DCE, 1,1-DCA, 1,1,1-TCA, vinyl chloride, and a variety of metals (Battelle and Accord Engineering Inc., 2019). Recent CVOC concentrations are less than 4 µg/L for each compound. Historical 1,4-dioxane concentrations were up to 14,000 µg/L.

The site geology is characterized by fine- to medium-grained sandstone, overlain by sandy beach deposits and artificial fill. Unconfined groundwater occurs in the A zone silt and A zone monitoring wells are screened above the fresh water-salt water interface (Battelle and Accord Engineering Inc., 2019). Depth to groundwater varies from approximately 28 ft bgs to 7 ft bgs near the shoreline. Groundwater in the A zone silt flows northwest towards the Pacific Ocean at a shallow gradient approximately 0.0003 ft/ft (Battelle and Accord Engineering Inc., 2019). Upward vertical gradients are present across the site.

Groundwater samples were collected from five monitoring wells in the A zone by Scissortail Environmental, Inc. between 6 and 9 August 2018 and shipped to UWEIL for analysis of δ\(^{13}\)C and δ\(^2\)H. The concentrations of 1,4-dioxane reported in separate samples from these wells ranged from non-detect (<1 µg/L) to 140 µg/L. A sample from MW20 was not collected for quantification of 1,4-dioxane due to a miscommunication; however, the last time MW20 was sampled in 2015, the concentration was 8,275 µg/L; therefore, a high concentration of 1,4-dioxane was expected to be present in the sample from MW20. Although there should have been a sufficient quantity of 1,4-
dioxane to perform CSIA on all samples except for MW24, UWEIL could only report a result for MW21 ($\delta^{13}$C only).

There are two possible explanations for the lack of results in these samples:

1. The 1,4-dioxane concentrations were decreasing as the wells were purged, leading to lower actual concentrations for the CSIA samples, compared to the samples collected for quantification earlier in the purging process; or

2. High concentrations of other unknown solute(s) in the matrix may have preferentially sorbed to the A560 sorbent, thereby reducing the amount of 1,4-dioxane available for analysis. Unfortunately, no conclusions can be drawn from this dataset except that the $\delta^{13}$C value for MW21 is within the range of manufactured (undegraded) source 1,4-dioxane.

Follow-up sampling to determine whether 1,4-dioxane concentrations change during purging is recommended. If the concentrations do not change, it can be concluded that matrix interference may be the cause, in which case, another method for CSIA of 1,4-dioxane may be considered for this site, and/or additional sample preparation steps may be undertaken.

<table>
<thead>
<tr>
<th>Date</th>
<th>Sample</th>
<th>1,4-dioxane (µg/L)</th>
<th>$\delta^{13}$C (‰ VPDB)</th>
<th>$\delta^{2}$H (‰ VSMOW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8/9/2018</td>
<td>MW20</td>
<td>--</td>
<td>BAL</td>
<td>NES</td>
</tr>
<tr>
<td>8/8/2018</td>
<td>MW21</td>
<td>140</td>
<td>-31.5</td>
<td>NES</td>
</tr>
<tr>
<td>8/8/2018</td>
<td>MW22</td>
<td>110</td>
<td>BAL</td>
<td>NES</td>
</tr>
<tr>
<td>8/7/2018</td>
<td>MW23</td>
<td>39</td>
<td>BAL</td>
<td>NES</td>
</tr>
<tr>
<td>8/6/2018</td>
<td>MW24</td>
<td>ND &lt;1</td>
<td>BAL</td>
<td>NES</td>
</tr>
</tbody>
</table>

Table 10: CSIA Results for DoD Site #1
(Note: BAL = below analytical limit; NES = note enough sample; -- = not analyzed)

4.4.2.5 Confidential DoD Site #2

Confidential DoD Site #2 includes a dissolved CVOC plume that has been associated with historical releases from aircraft testing and maintenance shops as well as chemical storage tanks and pipelines. Groundwater is unconfined within sandy hydraulic fill sediments and occurs at depths from 4 to 25 feet bgs. Recharge occurs primarily by infiltration of irrigation water. The CVOC plume predominantly occurs between 30 to 60 ft bgs and terminates approximately 3,000 feet downgradient at the shoreline with the Pacific Ocean (NOREAS Inc., 2013). The CVOC plume includes TCE, cis-1,2-DCE, 1,1-DCE and 1,4-dioxane (Trevet Inc. and Geosyntec, 2017).

Groundwater samples were collected from five monitoring wells screened near the water table by Scissortail Environmental, Inc. between 6 and 9 August 2018 and shipped to UWEIL in an ice-filled cooler for analysis of $\delta^{13}$C and $\delta^{2}$H. The concentrations and CSIA results for 1,4-dioxane in groundwater samples from these five wells are summarized below in Table 11, along with field
parameters measured during sample collection. Monitoring well MW91 is considered to be within the 1,4-dioxane source area where 1,4-dioxane concentrations have generally been greater than 1,000 µg/L (Figure 9). MW87 is also located in an area of historically higher 1,4-dioxane concentrations (historically > 100 µg/L; Figure 9), although the August 2018 sample was reported at 86 µg/L. The other three monitoring wells sampled were in an area farther downgradient from the source area. Dissolved oxygen (DO) concentrations and positive ORP values indicate generally aerobic conditions at the water table where these wells are screened. Higher than normal groundwater temperatures (e.g., MW89; 39.4 °C; Table 11) indicate the influence of a steam injection remediation test being performed nearby (Dr. John Wilson, personal communication).

Assuming the CSIA results for MW87 and MW91 represent undegraded source 1,4-dioxane, the samples from wells farther downgradient (MW88, MW89 and MW90) plot along dual-isotope enrichment trends (Figure 10). Specifically, the regression line with origin at MW91 through MW87, MW88 and MW89 has a $\Delta \delta^2H/\Delta \delta^{13}C$ of 38.8 ($R^2=0.98$), which compares well with that of THF-grown strain K1 ($\Delta \delta^2H/\Delta \delta^{13}C=37.2$; Table 2 and Figure 10). The concentration of 1,4-dioxane in the sample from MW88 was relatively low (1.4 µg/L), such that an increased uncertainty in the results can be expected; however, given that the MW88 result plots along the same enrichment line as MW89 (130 µg/L), the MW88 result is consistent with the biodegradation trend.

On the other hand, a regression from the same origin (MW91) but through MW90 results in a $\Delta \delta^2H/\Delta \delta^{13}C$ of 11.9 ($R^2=0.72$), which compares well with that of alkane-grown strain Mycobacterium IA ($\Delta \delta^2H/\Delta \delta^{13}C=12.1$; Table 2 and Figure 10). The different enrichment trend for MW90 compared to MW88 and MW89 may be indicative of differences in microbial communities and/or enzyme activities in groundwater near MW91 at the distal end of the plume compared to the mid-plume area (MW88 and MW89). Because MW90 is the only result on the enrichment line for Mycobacterium IA, this interpretation is subject to confirmation with additional CSIA samples and/or other lines of evidence which may help to strengthen this conclusion.

Both dual-isotope enrichment trends shown in Figure 10 provide lines of evidence in support of the aerobic biodegradation of 1,4-dioxane in groundwater at the site. The Rayleigh equation (Equation 2) was used to calculate the extent of degradation (Table 12). The enrichment at MW88 and MW89 is consistent with approximately 20% to 43% and 10% to 14% degradation of 1,4-dioxane, respectively, using enrichment factors associated with the THF-grown culture of P. tetrahydrofuranoxidans K1 (Table 12). This is substantially less than is inferred from the concentration decrease between MW91 and MW87 and MW89 (about 99.9% and 87%, respectively; Table 12), suggesting that dilution of 1,4-dioxane is also contributing to concentration attenuation. On the other hand, the 2.2‰ enrichment in $\delta^{13}C$ at MW-90 aligns with the dual-isotope trend for alkane-grown Mycobacterium IA, which has smaller enrichment factors for $^2H$ and $^{13}C$ (Table 2). Although the overall enrichment in $\delta^2H$ is lower for MW90 than MW88 (Figure 10), the calculated extent of degradation is greater (60 to 75 %), due to the smaller enrichment factors and greater enrichment in $\delta^{13}C$. This demonstrates the utility of using the slope of the dual isotope plot ($\Delta \delta^2H/\Delta \delta^{13}C$) to select an appropriate enrichment factor for Rayleigh-model degradation estimates, since the conclusion of greater degradation near the end of the plume at MW90 would not be realized if the enrichment factors based on the THF-grown culture were substituted in Equation 2, and/or the extent of degradation were based on 1,4-dioxane concentrations alone. Although Rayleigh-model degradation estimates are rarely accurate at field
sites because the model conditions of a well-mixed closed-system are often not met, the dual isotope slope is unaffected by dilution and/or masking. Unless substantial variation in isotopic composition of undegraded 1,4-dioxane exists at the site, the dual isotope plot will provide a more robust, albeit somewhat qualitative, field indicator of biodegradation.

<table>
<thead>
<tr>
<th>Date</th>
<th>Sample</th>
<th>1,4-dioxane (µg/L)</th>
<th>δ¹³C (‰ VPDB)</th>
<th>δ²H (‰ VSMOW)</th>
<th>DO (mg/L)</th>
<th>ORP (mV)</th>
<th>Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8/8/2018</td>
<td>MW91</td>
<td>970</td>
<td>-32.5</td>
<td>-39</td>
<td>1.65</td>
<td>361</td>
<td>29.3</td>
</tr>
<tr>
<td>8/9/2018</td>
<td>MW87</td>
<td>86</td>
<td>-32.7</td>
<td>-35</td>
<td>0.76</td>
<td>318</td>
<td>26.4</td>
</tr>
<tr>
<td>8/8/2018</td>
<td>MW89</td>
<td>130</td>
<td>-32.0</td>
<td>-17</td>
<td>0.4</td>
<td>360</td>
<td>39.4</td>
</tr>
<tr>
<td>8/6/2018</td>
<td>MW88</td>
<td>1.4 J</td>
<td>-31.3</td>
<td>+14</td>
<td>3.38</td>
<td>198</td>
<td>25.8</td>
</tr>
<tr>
<td>8/7/2018</td>
<td>MW90</td>
<td>14</td>
<td>-30.4</td>
<td>-9</td>
<td>1.38</td>
<td>314</td>
<td>23.8</td>
</tr>
</tbody>
</table>

**Table 11:** CSIA Results and Groundwater Parameters for Confidential DoD Site #2

**Figure 9:** Conceptualization of 1,4-dioxane plume at Confidential DoD Site#2. Approximate locations of monitoring wells sampled for CSIA of 1,4-dioxane are shown in red circles.
Figure 10: Dual Isotope Plot for Confidential DoD Site#2. Dual isotope plot showing paired $\delta^{13}$C and $\delta^2$H values (‰, red dots) for groundwater samples summarized in Table 11 collected from locations shown in Figure 9. For reference, dual-isotope enrichment trends are shown for *R. rhodochrous ATCC 21198* (propane substrate, blue line), *Mycobacterium* sp. 1A (propane substrate, magenta line) and *P. tetrahydrofuranoxidans K1* (THF substrate, green line) based on $\Delta\delta^2$H/$\Delta\delta^{13}$C values shown in Table 2, with an origin at MW-91 (most upgradient well shown in Figure 9).

<table>
<thead>
<tr>
<th>Well ID</th>
<th>$\delta^{13}$C$_o$ (%)</th>
<th>$\delta^{13}$C$_t$ (%)</th>
<th>$\Delta\delta^2$H/$\Delta\delta^{13}$C</th>
<th>$\varepsilon_C$ (%)</th>
<th>$f$</th>
<th>(1-$f$), % Degraded</th>
<th>Concentration Decrease (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW88</td>
<td>-32.6</td>
<td>-31.3</td>
<td>38.8</td>
<td>-4.8±0.9</td>
<td>0.57 – 0.80</td>
<td>20 - 43%</td>
<td>99.9%</td>
</tr>
<tr>
<td>MW89</td>
<td>-32.6</td>
<td>-32.0</td>
<td>38.8</td>
<td>-4.8±0.9</td>
<td>0.86 – 0.90</td>
<td>10 – 14%</td>
<td>87%</td>
</tr>
<tr>
<td>MW90</td>
<td>-32.6</td>
<td>-30.4</td>
<td>11.9</td>
<td>-2.0±0.4</td>
<td>0.25 – 0.40</td>
<td>60 - 75%</td>
<td>99%</td>
</tr>
</tbody>
</table>

Table 12: Summary of Degradation Calculations with Rayleigh Equation for DoD Site #2

4.4.2.6 Confidential DoD Site #3

Confidential DoD Site#3, located in California, was previously used as an airfield and for a variety of industrial operations (Parsons, 2011). Historical operations and facilities include lined and unlined drainage ditches used for flight operation liquid wastes, a firefighter training area, and a former scrap yard. Groundwater contamination includes TCE, cis-1,2-DCE, 1,1-DCA, 1,1-DCE, vinyl chloride, and 1,2,3-TCP (Parsons, 2011) in addition to 1,4-dioxane, which was detected in 2007 and 2008 along with cis-1,2-DCE (12 µg/L or less), 1,1-DCE (15 µg/L or less), and vinyl chloride (less than 1 µg/L) (Parsons, 2011). A relatively shallow 1,4-dioxane plume, generally from 15 to 45 feet bgs, and less than ¼-mile in length, and with 1,4-dioxane concentrations up to 240 µg/L, has been mapped using depth-discrete groundwater sampling.

The site subsurface comprises stream-deposited alluvium, up to 190 feet in thickness, over bedrock (sandstone, siltstone, and mudstone; Parsons, 2011). The water table occurs in the alluvial deposits at depths between 5 to 15 ft bgs and slopes generally to the southwest at a gradient of 0.002 ft/ft (Tidewater Inc., 2017). Shallow groundwater is characterized by generally low DO concentrations (~0.2 mg/L) and negative ORP values, consistent with anaerobic processes. Groundwater samples were collected from four monitoring wells by Scissortail Environmental, Inc. between 28 and 31
January 2019 and shipped to UWEIL in an ice-filled cooler for analysis of $\delta^{13}$C and $\delta^2$H. The 1,4-dioxane concentrations and CSIA results are organized by increasing distance downgradient are shown in Table 13.

<table>
<thead>
<tr>
<th>Date</th>
<th>Sample</th>
<th>1,4-dioxane (µg/L)</th>
<th>$\delta^{13}$C (‰ VPDB)</th>
<th>$\delta^2$H (‰ VSMOW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01/31/2019</td>
<td>MW2A</td>
<td>16</td>
<td>-30.6</td>
<td>-46</td>
</tr>
<tr>
<td>01/30/2019</td>
<td>MW4A</td>
<td>3.2</td>
<td>-31.3</td>
<td>-0.2</td>
</tr>
<tr>
<td>01/29/2019</td>
<td>MW1A</td>
<td>0.88 J</td>
<td>-31.3</td>
<td>-19</td>
</tr>
<tr>
<td>01/28/2019</td>
<td>MW3A</td>
<td>4.5</td>
<td>-31.8</td>
<td>-36</td>
</tr>
</tbody>
</table>

**Table 13: CSIA Results for Confidential DoD Site #3**

![Dual Isotope Plot](image)

**Figure 11.** Dual Isotope Plot for Confidential DoD Site#2. Paired $\delta^{13}$C and $\delta^2$H values (‰, red dots) are shown for groundwater samples summarized in Table 13. For reference, dual-isotope enrichment trends are shown for *R. rhodochrous ATCC 21198* (propane substrate, blue line), *Mycobacterium* sp. 1A (propane substrate, magenta line) and *P. tetrahydrofuranoxidans K1* (THF substrate, green line) based on $\Delta\delta^2$H/$\Delta\delta^{13}$C values shown in Table 2, with an origin at MW3A (lowest $\delta^{13}$C value in Table 13).

The result from MW2A, the most upgradient location, has the highest $\delta^{13}$C and lowest $\delta^2$H value of the four samples, so a dual-isotope trend beginning at MW2A will not fit the remaining three samples. A dual-isotope plot is provided as Figure 11, assuming that MW3A is the starting isotope composition. CSIA results for MW1A and MW4A align with the degradation line for *P. tetrahydrofuranoxidans K1*, but this apparent enrichment trend does not follow the groundwater flowpath, the 1,4-dioxane concentrations at MW1A and MW4A are low (higher uncertainty), and based on the result for MW2A, there may be variability in the isotopic composition of 1,4-dioxane. For these reasons, the CSIA results are not conclusive on their own. If other lines of evidence (e.g. biomarkers and concentration trends) point to the potential for biodegradation of 1,4-dioxane, additional CSIA sampling may help to identify areas of 1,4-dioxane biodegradation.
4.4.2.7 Confidential Non-DoD Site #1

Confidential Non-DoD Site #1 in the midwestern United States operated as an industrial waste landfill from 1968 to 1979 (Gedalanga et al., 2016). A large, dilute plume of 1,4-dioxane (up to 420 µg/L) and THF (up to 340 µg/L) extends approximately two miles from the former landfill (Gedalanga et al., 2016). The site is underlain by approximately 400 ft of glacial deposits comprised of outwash sands and gravel with interbedded layers of clay-rich till and lacustrine clays (Gedalanga et al., 2016). Groundwater exists in both unconfined and semiconfined conditions at depths ranging from 70 to 100 ft with a seepage velocity of up to one foot per day to the west and northwest. Several shallow surface water bodies redirect the plume approximately 1,700 ft downgradient of the former landfill (Gedalanga et al., 2016).

Gedalanga et al. (2016) used multiple lines of evidence to infer that natural degradation of 1,4-dioxane was occurring at this site, including biomarkers and CSIA. Biomarkers for 1,4-dioxane biodegradation, including DXMO and ALDH were found in high abundance in groundwater samples collected at several locations along the entire length of the 1,4-dioxane plume. The CSIA results showed a range in $\delta^{13}C$ from -31.8‰ to -29.2‰, with higher values in areas where concentration attenuation had occurred, consistent with enrichment in $^{13}C$ due to biodegradation of 1,4-dioxane. Stable hydrogen isotope ratios of 1,4-dioxane ($\delta^2H$) were not analyzed in the Gedalanga study. Based on these multiple lines of evidence and others, including concentration decreases with time and fate and transport modeling, Gedalanga et al. concluded that biodegradation of 1,4-dioxane is occurring naturally at the site and that the co-contaminant, THF, may have a role in the degradation of 1,4-dioxane.

A biostimulation pilot test was performed in the vicinity of IW1 and P70 (Figure 12) between mid-April 2018 and 5 October 2018. The test involved weekly injection events to stimulate aerobic co-metabolic biodegradation of 1,4-dioxane. Each event involved extracting 8,000 to 9,000 gallons of groundwater from an extraction well located 30 feet south (cross-gradient) of IW1, infusing the extracted groundwater with oxygen (60 mg/L) and propane (20 mg/L), and re-injecting the amended water at IW1.

Groundwater samples were collected between 1 and 4 October 2018 at the locations shown in Figure 12 by Scissortail Environmental, Inc., and submitted to UWEIL in an ice-filled cooler for CSIA of 1,4-dioxane. Duplicate samples were collected at each location; the duplicate samples had been placed on hold and stored in the laboratory refrigerator for several months after analysis of the primary samples had been performed. For the first sample set, the 1,4-dioxane was loaded onto A560 on 22 October 2018, within two weeks of receiving the samples. For the 2nd set of samples, the loading onto the A560 was performed about five months later, in late March 2019. The duplicate samples were analyzed to provide an empirical assessment of long hold times on CSIA samples. The results for both sets of analyses are shown in Table 14.

The range in $\delta^{13}C$ values of 1,4-dioxane is consistent with the range previously reported in Gedalanga et al. (2016). Higher $\delta^{13}C$ and $\delta^2H$ values of 1,4-dioxane were reported in the sample from IW1 (pilot test injection well) and the farthest downgradient well P78. Figure 13 includes the dual-isotope enrichment trend ($\Delta\delta^2H/\Delta\delta^{13}C$) determined previously for THF-grown culture of P. tetrahydrofuranoxidans K1. Since the source of 1,4-dioxane is a landfill, and given the length of
the plume, the isotopic composition of 1,4-dioxane may vary over the plume length, and therefore
the isotopic composition of 1,4-dioxane at P28 and P66 may not be as representative of the source
“undegraded” isotopic composition in the distal end of the plume. Since monitoring well P70 is
located only 15 feet cross-gradient of IW1, the CSIA results for P70 may better represent
undegraded 1,4-dioxane prior to degradation in the pilot test area and distal plume area. The
samples from IW1 and P78 plot along the dual-isotope enrichment trend of THF-grown culture of
*P. tetrahydrofuranoxidans K1*. This finding is consistent with previous studies at the site
implicating the presence of THF co-contaminant in the natural attenuation of 1,4-dioxane
(Gedalanga et al., 2016). Monitoring well P78, near the distal end of the plume, is too far away to
have been influenced by the pilot test; therefore, the isotopic enrichment appears to be natural,
whereas the isotopic enrichment at IW1 is most likely related to the pilot test, since the sample
from nearby well P70 does not show any degradation relative to P28 and P66. This is an interesting
result since propane was being used as the substrate to promote 1,4-dioxane biodegradation at
IW1, but the enrichment trend is consistent with that of a THF-grown culture. Since THF is present
in site groundwater, this suggests that organisms grown on THF may also be most actively
involved in biodegradation of 1,4-dioxane in the pilot test area.

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**Figure 12:** Conceptualization of 1,4-Dioxane plume at Confidential Non-DoD Site#1.
Schematic of mixed 1,4-dioxane/THF plume shown in solid grey outline as emanating from
closed landfill. Locations of monitoring wells sampled for CSIA of 1,4-dioxane are shown as red
dots. The injection well for the biostimulation pilot test of 1,4-dioxane biodegradation (IW1) is
shown as the open red circle. The blue dashed line shows the general flow direction of
groundwater during plume evolution.
<table>
<thead>
<tr>
<th>Date of Groundwater Sample Collection</th>
<th>Well ID</th>
<th>1,4-dioxane concentration (µg/L)</th>
<th>δ^{13}C (% VPDB)</th>
<th>δ^{2}H (% VSMOW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt; 2 weeks hold time</td>
<td>~5 months hold time</td>
</tr>
<tr>
<td>10/3/18 P28</td>
<td>310</td>
<td>-31.5</td>
<td>-31.6</td>
<td>-51</td>
</tr>
<tr>
<td>10/2/18 P66</td>
<td>100</td>
<td>-31.6</td>
<td>-30.6</td>
<td>-44</td>
</tr>
<tr>
<td>10/4/18 P70</td>
<td>41</td>
<td>-30.9</td>
<td>-30.1</td>
<td>-57</td>
</tr>
<tr>
<td>10/1/18 P78</td>
<td>10</td>
<td>-29.9</td>
<td>-29.7</td>
<td>-11</td>
</tr>
<tr>
<td>10/4/18 IWI</td>
<td>8.3</td>
<td>-29.8</td>
<td>-28.8</td>
<td>-3</td>
</tr>
</tbody>
</table>

Table 14: CSIA Results for Confidential Non-DOD Site #1

<table>
<thead>
<tr>
<th>Well ID</th>
<th>δ^{13}C₀ (%‰)</th>
<th>δ^{13}C₁ (%‰)</th>
<th>ε₃C (%‰)</th>
<th>f</th>
<th>(1-f), % Degraded</th>
<th>Concentration Decrease (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P78</td>
<td>-30.9</td>
<td>-29.9</td>
<td>-4.8±0.9</td>
<td>0.77 – 0.83</td>
<td>17 - 23%</td>
<td>76%</td>
</tr>
<tr>
<td>IW1</td>
<td>-30.9</td>
<td>-29.8</td>
<td>-4.8±0.9</td>
<td>0.75 – 0.82</td>
<td>18 – 25%</td>
<td>80%</td>
</tr>
</tbody>
</table>

Table 15: Summary of Degradation Calculations for Confidential Non-DoD Site #1

The Rayleigh equation (Equation 2) was used to calculate the extent of degradation based on the small enrichment in δ^{13}C and the enrichment factor associated with the THF-grown culture of *P. tetrahydrofuranoxidans K1* (Table 15). The isotopic enrichment at P78 and IW1 is consistent with approximately 17 to 25 percent degradation of 1,4-dioxane (Table 15); 1,4-dioxane concentrations are 76% and 80% lower at P78 and IW1, respectively, compared to P70 (Table 14 and 15). Similar to the findings at Confidential DoD Site #2, dilution of 1,4-dioxane likely contributes to concentration attenuation and/or mixing with “undegraded” 1,4-dioxane may be decreasing the extent of enrichment.

The longer hold times did not affect the CSIA results for P28 and P78, as the δ^{13}C and δ^{2}H values for the duplicate samples are essentially the same as for the primary samples. For P66, the δ^{13}C value was enriched by 1.0 ‰, but δ^{2}H was unchanged. Small enrichment in δ^{13}C (0.8 and 1‰) and δ^{2}H (16 and 36 ‰) was observed in duplicate samples relative to primary samples at P70 and IW1, respectively. Notwithstanding the minor differences, the dual isotope trend observed in the primary dataset is reproduced in the duplicate dataset (Figure 13). Taken as a whole, the entire duplicate dataset leads to the same conclusion as the primary dataset; i.e., that enrichment in δ^{13}C and δ^{2}H is due to biostimulation near IW1 and P70, and natural attenuation at the distal end of the plume at P78 is occurring. This also suggests that if samples require longer storage times, in most cases, only minor enrichment, if any, will occur. However, for samples from biostimulation zones, it may be prudent to preserve the samples by acidification to pH<2 with hydrochloric acid, or by raising the pH with 1 percent by weight trisodium phosphate, consistent with EPA guidance (Hunkeler et al., 2009).
Figure 13. Dual Isotope Plot for Confidential Non-DoD Site#1. Paired $\delta^{13}C$ and $\delta^2H$ values (‰, red dots) are for groundwater samples at locations shown in Figure 12. Dual-isotope enrichment trends are shown for \textit{R. rhodochrous} \textit{ATCC 21198} (propane substrate, blue line), \textit{Mycobacterium} sp. 1A (propane substrate, magenta line) and \textit{P. tetrahydrofuranoxidans K1} (THF substrate, green line) based on $\Delta \delta^2H/\Delta \delta^{13}C$ values shown in Table 2, with an origin at P70 (closest well to IW1).

4.4.2.8 Confidential Non-DoD Site #2

This non-DoD site in South Carolina formerly cleaned parts from a fiber manufacturing process (Parsons, 2015). Industrial activities at the site began in 1975 and included immersing parts in a triethylene glycol bath. Rinse water was routed to an onsite septic system and leach field via a sump until 1982, after which time it was transported offsite for treatment. Site investigation was initiated in 1987 and operations ceased in 1991. A phytoremediation system was constructed in 2007 to address lingering, low-level 1,4-dioxane concentrations in groundwater.

Groundwater occurs between 2 to 4 ft bgs and occurs within an approximately 20-ft thick layer of silt (Parsons, 2015). Groundwater within this shallow aquifer flows to the northwest at an approximate gradient of 0.04 ft/ft (Parsons, 2012). A deep aquifer is also present at the site but lacks significant contamination (Parsons, 2015).

Sources of 1,4-dioxane include heating triethylene glycol, which produces 1,4-dioxane, as well as the potential release of 1,1,1-TCA. 1,4-dioxane is the most widely detected contaminant at the site, with concentrations ranging from 3 to 10,000 µg/L, and concentrations remaining stable or decreasing slightly since 2002. Historic co-contaminants include CVOCs; vinyl chloride and 1,1-DCE are the only two remaining at concentrations less than 13 µg/L (Parsons, 2015).

Groundwater samples were collected from the South Carolina Site between 12 and 15 November 2018 by Scissortail Environmental, Inc. Four samples were collected for analysis of $\delta^{13}C$ and $\delta^2H$: two from the source area (MW-5 and MW-6) and two downgradient (MW-26 and MW-21R). As shown in Table 16, the $\delta^{13}C$ values for 1,4-dioxane are lighter than any of the source values reported in Section 4.1, but the $\delta^2H$ values are similar to those reported at other sites. The different isotopic composition of 1,4-dioxane at this site may reflect a different type of source, perhaps as a byproduct of the manufacturing process.
A linear regression through the four datapoints yields a slope of 26.9, similar to aerobic biodegradation with CB1190 ($\Delta \delta^2H/\Delta \delta^{13}C = 31.4$; Table 2), but the regression statistics do not lend confidence to the enrichment trend ($R^2 = 0.29$; p-value = 0.46). The dual isotope plot shown in Figure 14 shows an enrichment in $\delta^2H$ values that seems to align similar to the biodegradation trend but the differences in isotopic composition of 1,4-dioxane shown in Table 14 are small and may reflect analytical variability and/or variability in the isotopic composition of 1,4-dioxane in site groundwater. Therefore, these results are inconclusive with respect to the biodegradation of 1,4-dioxane at this site.

<table>
<thead>
<tr>
<th>Date</th>
<th>Sample</th>
<th>1,4-dioxane (µg/L)</th>
<th>$\delta^{13}C$ (% VPDB)</th>
<th>$\delta^2H$ (% VSMOW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11/14/2018</td>
<td>MW-5</td>
<td>10000</td>
<td>-37.2</td>
<td>-32</td>
</tr>
<tr>
<td>11/15/2018</td>
<td>MW-6</td>
<td>280</td>
<td>-37.5</td>
<td>-9</td>
</tr>
<tr>
<td>11/13/2018</td>
<td>MW-26</td>
<td>130</td>
<td>-37.0</td>
<td>-7</td>
</tr>
<tr>
<td>11/12/2018</td>
<td>MW-21R</td>
<td>290</td>
<td>-36.8</td>
<td>+6</td>
</tr>
</tbody>
</table>

**Table 16**: CSIA Results for Non-DoD South Carolina Site

**Figure 14**: Dual isotope plot showing paired $\delta^{13}C$ and $\delta^2H$ values (%o, red dots) for groundwater samples summarized in Table 16. For reference, the dual-isotope enrichment trend for *P. dioxanivorans CB1190* (red line) is shown, based on $\Delta \delta^2H/\Delta \delta^{13}C$ values shown in Table 2, with an origin at MW-5 (highest concentration of 1,4-dioxane; Table 14). The length of the red line represents the maximum enrichment in $\delta^2H$ expected based on the difference between maximum and minimum observed $\delta^{13}C$ values ($\Delta \delta^{13}C = 0.7‰$) and $\Delta \delta^2H/\Delta \delta^{13}C = 31.4$.

**4.5 Summary**

In Section 4.1, the stable isotopic composition of neat 1,4-dioxane was assessed through the analysis of 10 samples of 1,4-dioxane procured through chemical suppliers. The $\delta^{13}C$ values ranged from -33.6 to -28.8‰ (median: -32.2‰) and $\delta^2H$ ranged from -60 to -17‰ (median: -36‰). In Section 4.2, bench testing results lead to the conclusion that the isotopic composition of
manufactured 1,4-dioxane would not be substantively changed due to passive evaporative losses. The majority of $\delta^{13}C$ values for 1,4-dioxane measured in groundwater samples fall between -34‰ and -28‰, similar to the range of $\delta^{13}C$ values for undegraded source 1,4-dioxane shown in Figure 2; the majority of $\delta^{2}H$ values for 1,4-dioxane measured in groundwater samples fall between -50‰ and 0‰, overlapping much of the range of $\delta^{2}H$ values for undegraded source 1,4-dioxane, but with a proportion of samples being enriched by 10 to 20‰ relative to the maximum $\delta^{2}H$ value for undegraded source 1,4-dioxane of -17‰.

Samples from some field sites had $\delta^{13}C$ or $\delta^{2}H$ values lower than the lowest values reported for neat 1,4-dioxane, indicating that the isotopic composition of neat 1,4-dioxane samples analyzed in Section 4.1 do not represent the full range that can be expected at groundwater sites. Since the samples of 1,4-dioxane characterized in Section 4.1 may not fully represent the range of 1,4-dioxane for historical releases that may have occurred several decades ago, conclusions regarding biodegradation of 1,4-dioxane based on the extent of enrichment beyond the “undegraded source” isotopic composition are subject to higher uncertainty. Instead, $\delta^{13}C$ values are most useful when coupled with $\delta^{2}H$ values (a “dual isotope” approach) and interpreted on a site-specific basis. Dual isotope plots can provide robust qualitative indications of biodegradation and can provide insights into the degradation conditions by comparing the dual isotope slope to those reported for different laboratory-controlled degradation reactions.

Dual isotope trends consistent with biodegradation of 1,4-dioxane were found at the following sites:

1. McClellan Air Force Base (Section 4.4.1.1), only within the area where 1,4-dioxane was being treated in situ through biostimulation with propane and oxygen addition. The dual-isotope enrichment trend aligned with the trend observed in laboratory reactions of *Mycobacterium* 1A stimulated with gaseous alkanes.

2. Cape Canaveral Air Force Station (CCAFS) Space Launch Complex 16 (SLC-16) (Section 4.4.1.3), one of the groundwater samples was strongly enriched relative to the other samples from this site, with $\delta^{13}C$ and $\delta^{2}H$ of -23.4‰ and -10‰, respectively, and plotting on a dual isotope trend consistent with that observed for laboratory degradation reactions with *R. rhodochrous ATCC 21198* grown on propane. A second sample had a smaller isotopic enrichment plotting on a dual-isotope enrichment trend similar to that determined for *P. dioxanivorans CB1190*.

3. Confidential DoD Site #2 (Section 4.4.2.5), where the dual isotope enrichment aligned with trends established for *Mycobacterium* 1A and *P. tetrahydrofuranoxidans K1* downgradient of the source zone.

4. Confidential Non-DoD Site #1 (Section 4.4.2.7), where the dual isotope enrichment aligned with the trend established for *P. tetrahydrofuranoxidans K1* in an area where a biostimulation pilot test (propane and oxygen addition) was underway, and farther downgradient, at the distal end of the plume where other lines of evidence suggest natural attenuation processes were occurring.
CSIA was inconclusive with respect to biodegradation at 8 of the 12 sites, either because the enrichment trends associated with biodegradation of 1,4-dioxane were not observed, or because concentrations were too low for analysis.

4.6 Lessons Learned

The following lessons were learned over the course of this project:

- At Confidential DoD Site#2 and Confidential Non-DOD Site #1, where the dual isotope trends aligned with biodegradation, the magnitude of isotopic enrichment was much smaller than observed in laboratory studies. This is a common observation when applying CSIA at many sites for most types of contaminants where mixing of “undegraded” 1,4-dioxane can often obscure degradation signals at groundwater sites. The degree of isotopic enrichment from degradation can be greatly attenuated if a monitoring well draws water from zones of active biodegradation and zones where biodegradation is not occurring. Under this common scenario, the overall isotope signature in the blended sample will be dominated by the less-degraded fractions (Braeckvelt et al., 2012; Hunkeler et al., 2009). Collection of depth-discrete samples with shorter sampling intervals can mitigate this effect.

- Since there is a wide variation in the isotopic composition of 1,4-dioxane, it will be difficult to prove biodegradation of 1,4-dioxane based solely on the enrichment in $^{13}$C or $^2$H. The importance of considering multiple lines of evidence and interpreting CSIA results on a site-specific basis cannot be understated, particularly when interpreting small isotopic enrichments (e.g. enrichment in $\delta^{13}$C less than 2‰ and less than 20 to 40‰ for $\delta^2$H; Braeckvelt et al., 2012). At some sites, it may be possible to decrease uncertainty related to the isotopic composition of initial undegraded 1,4-dioxane by collecting more samples in the higher concentration zones.

- As shown for the Confidential Non-DoD Site #2 (Section 4.4.2.8), it is possible for paired data to fall along established dual isotope trends due to analytical variability or variability in the source 1,4-dioxane isotope composition; this can lead to “false positives.” For this reason, strong correlation in the dual isotope trend and multiple lines of evidence are useful for assessing CSIA results.

- In some cases, CSIA results could not be reported by the laboratory although concentrations of 1,4-dioxane should have been sufficient (e.g., Confidential DoD Site #1, Section 4.4.2.4), and in other cases, CSIA results were reported for groundwater samples where 1,4-dioxane was not detected (JBCC, Section 4.4.2.3). No issues were reported by the laboratory and therefore it is difficult to explain these results without further investigation. It is possible that the sorption of 1,4-dioxane on A560 will be different in different sample matrices, particularly if high concentrations of other organics are present. On the other hand, difficulties in quantification of 1,4-dioxane at low concentrations may underpredict the 1,4-dioxane concentration, such that CSIA results may be reported for “ND” results. Therefore, before submitting a larger number of samples for CSIA, it may be prudent to “test” CSIA on a limited number of samples to assess whether site specific conditions are amenable to performing CSIA, and whether any steps can be undertaken to improve data quality.
• At low concentrations, increased uncertainty in the results are expected, and this can complicate the interpretation of the results. It can be difficult to assess whether a result is “real” or an artifact of lower precision or normalizing errors. For this reason, replicate analyses of “anomalous” results are recommended. Increased sample volumes for loading onto A560 may also help reduce uncertainty at low concentrations. Analysis and normalization to multiple 1,4-dioxane standards of known isotopic composition may also increase precision and accuracy, if such standards can be obtained by the isotope laboratory.

• Industrial and municipal wastewater effluent can also be sources for “byproduct” 1,4-dioxane to groundwater (where 1,4-dioxane is inadvertently formed during an industrial process). The isotopic composition of byproduct 1,4-dioxane has not been thoroughly characterized which can complicate CSIA at sites where byproduct 1,4-dioxane is present.
5. CONCLUSIONS

This project sought to develop a low-cost, readily implementable method for assessing 1,4-dioxane degradation in groundwater by allowing for the application of CSIA to low concentrations of 1,4-dioxane. This objective has been achieved, as the CSIA method developed in Phase I of this project was applied to 78 groundwater samples from 12 different field sites at concentrations ranging from non-detect to 17,200 µg/L with a median concentration of 18 µg/L. Of these samples, 68 measurements of δ¹³C and 54 measurements of δ²H were reported. It has been shown that CSIA can provide direct field-based evidence for 1,4-dioxane biodegradation in groundwater and, through the use of dual isotope plots, CSIA results can be compared to published enrichment trends to provide insights into the degradation conditions. The dual isotope plot can be useful in selecting the appropriate enrichment factor for Rayleigh-type degradation calculations. On the other hand, variability in isotopic composition of 1,4-dioxane within a given site, and/or increased analytical uncertainty at low concentrations of 1,4-dioxane, can introduce uncertainty into field-based dual-isotope trends. Application of CSIA to assess biodegradation of 1,4-dioxane at field sites is more likely to be successful at sites where bio-stimulation is occurring, because the location of the degradation zone is known before sampling is undertaken, and the degradation zone is typically well-monitored.

For assessing the natural biodegradation of 1,4-dioxane with CSIA, a sound site conceptual model coupled with high-resolution, depth-discrete groundwater sampling may help to focus CSIA sampling on zones where biodegradation is most likely to occur (e.g. near the water table where oxygen concentrations may be higher) and thereby increase the likelihood of a conclusive outcome. Replicate analyses of δ¹³C and δ²H can increase confidence in the CSIA results for 1,4-dioxane, particularly in low concentration samples, and may help to identify analytical difficulties. Collection of larger sample volumes (e.g. several liters) may allow for the laboratory to achieve lower reporting limits for critical low concentration samples and increased sensitivity for complex matrices. Although analysis of both δ¹³C and δ²H is recommended so the dual isotope analysis can be performed, it may be possible to conduct preliminary screening using only δ¹³C values, to identify a subset of locations for follow-up analysis of both δ¹³C and δ²H.

CSIA provides direct evidence that a certain amount of 1,4-dioxane biodegradation has occurred over time at some location in the source or plume. Because subsurface conditions can change over time, isotopic evidence of 1,4-dioxane degradation is not necessarily indicative of future degradation potential unless it can be established that current conditions support degradation, and that these conditions will likely persist into the future. Therefore, other lines of evidence in addition to CSIA are important for MNA assessments. Determination of biomarker concentrations for certain monooxygenase enzymes implicated in 1,4-dioxane degradation in groundwater samples may demonstrate the ability for continued degradation of 1,4-dioxane in situ. Laboratory-controlled degradation reactions with carefully collected samples of site soil and/or groundwater may be used to estimate 1,4-dioxane degradation rates for use in fate and transport models. The development of CSIA for 1,4-dioxane as described herein is complimentary to other ongoing DoD-funded applied research for developing a quantitative framework for evaluating the natural attenuation of 1,4-dioxane and CVOCs in groundwater (Project ER-201730).
5.1 Technology Transfer

The CSIA method developed as part of Phase I is offered by the UWEIL and available for use at groundwater sites. Based on the UWEIL website accessed in January 2020, the cost for low level extraction pretreatment (1 L sample volume, minimum 1,4-dioxane concentration of 0.5 µg/L) is $75 (Canadian currency; CDN), in addition to the cost for GC-IRMS ($130 for $\delta^{13}\text{C}$ and $150$ for $\delta^2\text{H}$) for a total cost of $355$ CDN. For applying CSIA to low concentrations of 1,4-dioxane at your site, please contact UWEIL to discuss logistics and timing. Contact information is provided below, and information is also found on the UWEIL website (uwaterloo.ca/environmental-isotope-laboratory):

Reem El Mugammar  
EIL Client Services  
Phone: 519.888.4732  
Email: eilab-2@uwaterloo.ca

Shipping Address  
University of Waterloo  
Environmental Isotope Laboratory (EIT 5025)  
263 Phillip Street  
Waterloo, ON N2L 3W8  
Canada
6. LITERATURE CITED


NOREAS Inc., 2013. Draft Feasibility Study Work Plan, Confidential DoD Site #2, California.


Pornwongthong, P., 2014. Stable isotopic and molecular biological tools to validate biodegradation of 1,4-dioxane. PhD Dissertation, Civil Engineering Department, University of California, Los Angeles.


Trevet Inc. and Geosyntec, 2017. *In Situ* Chemical Oxidation (ISCO) Treatability Study Technical Memorandum, Confidential DoD Site #2, California.


APPENDIX A:  Supporting Data: CSIA Results from UWEIL
**TABLE A-1: Summary of Laboratory Report File Numbers for CSIA Results**

<table>
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<tr>
<th>Site Name, Location</th>
<th>No. of samples</th>
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<th>Preservative</th>
<th>File Date</th>
<th>File #</th>
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<td>6 x 40 mL glass</td>
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<td>2018260</td>
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<td>1/31/2019</td>
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<tr>
<td>Confidential DoD Site#2</td>
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<td>Hanscom AFB</td>
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</tr>
</tbody>
</table>

**"A" primary samples extracted 22-Oct-2018**

**"B" duplicate samples extracted 22-Mar-2019**
APPENDIX B: List of Scientific/Technical Publications
List of Scientific/Technical Publications for Project ER-2535

1. Articles in peer-reviewed journals


2. Technical Reports

Haley & Aldrich, Inc., 2018. Final Report, Extending the Applicability of Compound-Specific Isotope Analysis to Low Concentrations of 1,4-Dioxane, SERDP Project ER-2535. January 2018

3. Conference or symposium proceedings


Chu, M.Y. and Bennett, P., *Stable Carbon and Hydrogen Isotope Ratios for Assessing the Fate and Transport of 1,4-Dioxane*, Fifth International Symposium on Bioremediation and Sustainable Environmental Technologies, April 15-18, 2019, Baltimore, Maryland.

APPENDIX C: FACT SHEET
FACT SHEET: PROJECT ER-2535
EXTENDING THE APPLICABILITY OF COMPOUND-SPECIFIC ISOTOPE ANALYSIS TO LOW CONCENTRATIONS OF 1,4-DIOXANE
U.S. Department of Defense (DoD) Strategic Environmental Research and Development Program (SERDP) Research Project

NATURE OF THE PROBLEM
1,4-Dioxane (1,4-D) is a persistent emerging contaminant that is found at military and industrial sites around the world. 1,4-D was primarily used as a solvent stabilizer and has recently been shown to biodegrade by some natural subsurface microbial communities. CSIA is an analytical method that quantifies the ratios of different stable isotopes of certain atoms comprising a target chemical. Stable isotope ratios change during biodegradation of most chemicals, making CSIA a powerful diagnostic tool for demonstrating natural biodegradation of many contaminants. Unfortunately, the concentration of 1,4-D historically needed to perform CSIA was relatively high (>100 micrograms per liter; µg/L). Thus, at sites where 1,4-D concentrations in groundwater are below 100 µg/L, it has not been possible to use CSIA to demonstrate that natural or enhanced in situ biodegradation of 1,4-D is occurring.

TECHNICAL APPROACH
• Based on laboratory bench tests, a synthetic hydrophobic resin was selected to extract 1,4-D from groundwater samples and concentrate it to levels where CSIA could be performed.
• CSIA was performed on samples from laboratory-controlled reactions to determine isotopic enrichment factors during cometabolic biodegradation of 1,4-D with different pure cultures and substrates. Diagnostic dual-isotope enrichment-trends for 1,4-D degradation with different cultures and substrates were determined. These results were published in a scientific journal (see sidebar).
• Using this new concentration technique, CSIA was successfully performed on groundwater samples from 12 groundwater sites to concentrations lower than 1 µg/L. At four sites, isotopic enrichment trends in field data aligned with those determined in the laboratory.

APPLICATION OF CSIA FOR ASSESSING DEGRADATION OF 1,4-DIOXANE
As an outcome of Project ER-2535, CSIA can now be applied to low concentrations of 1,4-D at groundwater sites. Through analysis of stable carbon and hydrogen isotope ratios of 1,4-dioxane, a dual-isotope enrichment trend can provide insights into organisms and/or enzymes active in 1,4-D degradation on a site-specific basis. This method is likely to be most effective when applied to field applications of in situ 1,4-D bioremediation, because sampling can target specific biodegradation zones. The method can be applied to assess natural biodegradation of 1,4-D in groundwater if the isotopic composition of source of 1,4-D prior to the onset of biodegradation can be established by collecting samples along a flowpath and/or known source areas. Replicate samples should be collected to allow the laboratory to re-analyze samples if necessary. This CSIA method is available commercially at the University of Waterloo Environmental Isotope Laboratory (uwaterloo.ca/environmental-isotope-laboratory).

• Academic collaborators included the University of Waterloo, Canada, and North Carolina State University
• Peer-reviewed paper titled “Enrichment with Carbon-13 and Deuterium during Monooxygenase-Mediated Biodegradation of 1,4-Dioxane” published in Environmental Science and Technology Letters, 2018; Vol. 5, Issue 3.