Development of a Quantitative Framework for Evaluating Natural Attenuation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-Dioxane in Groundwater

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13. SUPPLEMENTARY NOTES

Monitored natural attenuation (MNA) has emerged as a preferred remedial option at many sites contaminated with chlorinated solvents because it offers a cost-effective and practical approach for cleanup of solutes in groundwater. However, existing MNA protocols do not include 1,4-dioxane and commonly co-occurring chlorinated solvents like 1,1,1-TCA, 1,1-DCA, and 1,1-DCE. The objectives of this project were to: 1) develop a modified model and framework for evaluating natural attenuation of these compounds; 2) develop and validate a protocol to directly measure rate constants for natural biodegradation of 1,4-dioxane using ^{14}C-labeled 1,4-dioxane and groundwater from 10 different field sites; 3) use the field and lab data to establish if there is consistency between various lines of evidence for 1,4-dioxane attenuation. An evaluation of MNA relies on establishing various lines of evidence, including secondary and tertiary lines of evidence that help demonstrate degradation processes and associated rates that are responsible for the primary line of evidence (decreasing concentrations of the target compound(s)). This project developed a new fate and transport model to easily evaluate historical monitoring data to predict biodegradation rate constants as well as new decision matrices (flowcharts) that serve as a guided tour on how to interpret potential lines of evidence for MNA. These were then integrated into an existing software platform (BioPIC) that allows users to access both the model and the decision matrices. Several approaches were also used to generate input data to support and validate the model and framework. First, rate coefficients and lines of evidence for attenuation were calculated and/or measured at multiple sites using a focused sampling program at 10 field sites. Second, degradation and the associated rate constants for 1,4-dioxane at these same sites were determined using a ^{14}C-labeled assay developed for this project.

The ^{14}C assay was successfully validated and used to establish 1,4-dioxane natural attenuation capacity at 9 of the 10 field sampling sites. Similarly, the model was able to extract rate constants for 1,4-dioxane biodegradation at 8 of 9 sites that had sufficient data to evaluate. Several project performance objectives focused on ensuring that the decision framework was functional and captures all relevant processes for the targeted compounds. The success criteria for all these objectives were successfully achieved, in part because the model provides quantitative support for the decision framework. Multiple performance objectives related to the sensitivity and robustness of the ^{14}C assay in quantifying 1,4-dioxane rate constants were also achieved. Performance objectives that were partially achieved or not achieved were related to: i) consistency between the model-predicted rate constants and the ^{14}C-based rate constants (achieved at 3 of 9 field sites with sufficient data); and ii) correlations between biomarker abundance and rate constants (no clear relationship could be established). Collecting the necessary data and performing a comprehensive MNA evaluation at a single site using the project deliverables was estimated to cost approximately $30,000. This assessment could result in substantial savings if the use of MNA is justified vs. more aggressive remedies. No regulatory or procurement issues are anticipated during implementation, and end-user concerns are minimal since data can be collected during a single mobilization without disruption to site activities.

15. SUBJECT TERMS

1,4-dioxane, attenuation, model, MNA, biodegradation, chlorinated solvents, 1,1-DCE, 1,1,1-TCA, 1,1-DCA

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ACRONYM LIST

AFCEC	Air Force Civil Engineering Center
ALDH	Aldehyde Dehydrogenase
BioPIC	Biological Pathway Identification Criteria
BRL	Below Reporting Limit
BSM	Basal Salts Medium
cis-DCE	cis-1,2-Dichloroethene
CCL4	Candidate Contaminant List-4
CO_2	Carbon dioxide
COC	Chemical of Concern
CSIA	Compound-Specific Isotope Analyses
CVOC	Chlorinated Volatile Organic Compound
1,1-DCA	1,1-Dichloroethane
DCA	1,1-DCA Reductase
1,1-DCE	1,1-Dichloroethene
DHB	*Dehalobacter*
DHC	*Dehalococcoides*
DNAPL	Dense Non-Aqueous Phase Liquid
DO	Dissolved Oxygen
DoD	Department of Defense
DoN	Department of Navy
DXMO	Dioxane Monooxygenase
DoN	Department of the Navy
EPA UCMR3	US Environmental Protection Agency's Unregulated Contaminant Monitoring List 3
ER	Environmental Restoration
ESTCP	Environmental Security Technology Certification Program
FSGW	Filter Sterilized Groundwater
HASP	Health and Safety Plan
HEAA	2-Hydroxyethoxyacetic Acid
HPLC	High Performance Liquid Chromatography
ID	Identification
IDW	Investigation Derived Waste
ISCO	In Situ Chemical Oxidation
LNAPL	Light Non-Aqueous Phase Liquid
MCL	Maximum Contaminant Level
MNA	Monitored Natural Attenuation
NAVFAC	Naval Facilities Engineering Command
N/A	Not Available
ND	Non-detect/not detected
N_2	Nitrogen
O&M	Operations and Maintenance
ORP	Oxidation-Reduction Potential
PCE	Tetrachloroethene
PHE	Phenol Hydroxlyase
PI	Principal Investigator

PRM/PrMO	Propane Monooxygenase
PTFE	Polytetrafluoroethylene
QA/QC	Quality Assurance/Quality Control
qPCR	Quantitative Polymerase Chain Reaction
RMO/RDEG	Ring Hydroxylating Toluene Monooxygenase
RMSE	Root Mean Square Error
RNA	Ribonucleic Acid
RPM	Remedial Project Manager
SCAM	Short Chain Alkane Monooxygenase
SDIMO	Soluble Di-Iron Monooxygenase
SERDP	Strategic Environmental Research and Development Program
sMMO	Soluble Methane Monooxygenase
SOP	Standard Operating Protocol
TBA	Tert-Butyl Alcohol
1,1,1-TCA	1,1,1-Trichloroethane
TCE	Trichloroethene
TCFM	Trichlorofluoromethane
THF	Tetrahydrofuran
THM/THFMO	Tetrahydrofuran Monooxygenase
TOC	Total Organic Carbon
USEPA	United States Environmental Protection Agency
VC	Vinyl Chloride
VOC	Volatile Organic Compound

µg/L	Microgram per Liter
ppm	Part per Million
mL	Milliliter
bgs	Below Ground Surface
dpm	Decays Per Minute
ft	Feet
yr	Year

TABLE OF CONTENTS

ACRONYM LIST i
TABLE OF CONTENTS iii
LIST OF FIGURES vii
LIST OF TABLES xi
ACKNOWLEDGEMENTS xiii
ABSTRACT xiv
EXECUTIVE SUMMARY 1
1.0 INTRODUCTION 1
1.1. BACKGROUND 1
1.2. OBJECTIVE OF THE DEMONSTRATION 4
1.3. REGULATORY DRIVERS 4
2.0 TECHNOLOGY 6
2.1. TECHNOLOGY DESCRIPTION 6
2.2. ADVANTAGES AND LIMITATIONS OF THE TECHNOLOGY 10
3.0 PERFORMANCE OBJECTIVES 12
3.1 Performance Objective #1: Modify BIOCHLOR (or develop new but similar fate and transport model) to Account for Biological Degradation of 1,4-Dioxane 16
3.1.1 Data Requirements 16
3.1.2 Success Criteria 16
3.1.3 Outcome 16
3.2 Performance Objective #2: Modify BIOCHLOR (or develop new but similar fate and transport model) to Account for Biological and Abiotic Degradation of 1,1,1-TCA and its Transformation Products 17
3.2.1 Data Requirements 17
3.2.2 Success Criteria 17
3.2.3 Outcome 17
3.3 Performance Objective #3: Development of a quantitative decision matrix to elucidate degradation pathways and select the most efficacious remediation approach for 1,4-Dioxane 18
3.3.1 Data Requirements 18
3.3.2 Success Criteria 19
3.3.3 Outcome 19

3.4 Performance Objective #4: Development of a quantitative decision matrix to elucidate degradation pathways and select the most efficacious remediation approach for 1,1,1-TCA, 1,1-DCA, and 1,1-DCE 20
3.4.1 Data Requirements 21
3.4.2 Success Criteria 21
3.4.3 Outcome 21
3.5 Performance Objective #5: Ease of Use of New Version of BioPIC 22
3.5.1 Data Requirements 22
3.5.2 Success Criteria 23
3.5.2 Outcome 23
3.6 Performance Objective #6: Validate Rate Constants for 1,4-Dioxane Degradation using a ^{14}C Assay Conducted with Groundwater from the Study Sites 23
3.6.1 Data Requirements 23
3.6.2 Success Criteria 24
3.6.3 Outcome 24
3.7 Performance Objective #7: Determine whether Aerobic Biological Cometabolism or Biological Degradation of 1,4-Dioxane Explains the Degradation Rate Constant for Attenuation of 1,4-Dioxane at Field Scale 26
3.7.1 Data Requirements 26
3.7.2 Success Criteria 26
3.7.3 Outcome 26
4.0 SITE DESCRIPTION 28
4.1. SITE LOCATION AND HISTORY 28
4.2. SITE GEOLOGY/HYDROGEOLOGY 28
4.3. CONTAMINANT DISTRIBUTION 28
5.0 TEST DESIGN 45
5.1 CONCEPTUAL EXPERIMENTAL DESIGN 45
5.2 BASELINE CHARACTERIZATION ACTIVITIES 45
5.3 DESIGN AND LAYOUT OF TECHNOLOGY COMPONENTS 45
5.3.1 Design of Technology Components 46
5.3.2 Layout of Technology Components 46
5.4 FIELD TESTING 48
5.5 SAMPLING PLAN 50
5.5.1 Overview 50
5.5.2 Quality Assurance Sampling Program 50
5.5.3 Calibration of Analytical Equipment 55

5.5.4 Decontamination Procedures 55
5.5.5 Sample Documentation 56
5.6 LAB STUDY RESULTS 58
5.6.1 Materials and Methods 58
5.6.2 Results 67
5.6.3 Discussion 72
5.6.4 Conclusions 73
5.7 SAMPLING RESULTS 95
5.7.1 Site 1 (DoD) 95
5.7.2. Site 2 (DoD) 107
5.7.3. Site 3 (Industrial) 114
5.7.4. Site 4 (Industrial) 122
5.7.5. Site 5 (DoD) 129
5.7.6. Site 6 (DoD) 141
5.7.7. Site 7 (DoD) 146
5.7.8. Site 8 (DoD) 154
5.7.9. Site 9 (DoD) 158
5.7.10. Site 10 (DoD) 163
5.8 COMPILATION AND COMPARISON OF ALL LAB AND FIELD DATA 168
5.8.1. Comparison of 1,4-Dioxane Biodegradation Rates 170
5.8.2. Influence of Geochemical Conditions of Observed 1,4-Dioxane Biodegradation Rates 171
5.8.3. Influence of Initial 1,4-Dioxane Concentrations on Observed 1,4-Dioxane Biodegradation Rates 174
5.8.4. Influence of Site CVOC Concentrations on Observed 1,4-Dioxane Biodegradation Rates 175
5.8.5. Influence of Biomarker Abundance on Observed 1,4-Dioxane Biodegradation Rates 177
5.9 DECISION FRAMEWORK 183
6.0 PERFORMANCE ASSESSMENT 187
6.1 Modify BIOCHLOR (or develop new but similar fate and transport model) to Account for Biological Degradation of 1,4-Dioxane 187
6.2 Modify BIOCHLOR (or develop new but similar fate and transport model) to Account for Biological and Abiotic Degradation of 1,1,1-TCA 189
6.3 Development of a Quantitative Decision Matrix to Elucidate Degradation Pathways and Select the Most Efficacious Remediation Approach for 1,4-Dioxane 191

6.4 Development of a Quantitative Decision Matrix to Elucidate Degradation Pathways and Select the Most Efficacious Remediation Approach for 1,1,1-TCA. 1,1-DCA, and 1,1-DCE 193
6.5 Ease of Use of New Version of BioPIC 194
6.6 Validate Rate Constants for 1,4-Dioxane Degradation Using a ^{14}C Assay Conducted with Groundwater from the Study Sites 194
6.7 Determine Whether Aerobic Biological Cometabolism or Biological Degradation of 1,4-Dioxane Explains the Degradation Rate Constant for Attenuation of 1,4-Dioxane at Field Scale 194
7.0 COST ASSESSMENT 197
7.1 COST MODEL 197
7.2 COST DRIVERS 199
7.3 COST ANALYSIS 199
8.0 IMPLEMENTATION ISSUES/LESSONS LEARNED 202
8.1 IMPLEMENTATION ISSUES 202
8.2 LESSONS LEARNED 202
9.0 REFERENCES 206
APPENDICES 210

APPENDIX A Results Summary Tables
APPENDIX B User's Guide for BioPIC (Decision Framework)
APPENDIX C User's Guide for MNA Rate Constant Estimator
APPENDIX D Derivation of Correlations Between Biomarker Abundance and Biodegradation Rate Constants
APPENDIX E Results of Beta Testing of Decision Framework

LIST OF FIGURES

Figure ES.1. Comparison of 1,4-Dioxane Biodegradation Rate Constants Obtained From ^{14}C Assays (Blue) and Model Predictions (Red). ES-8

Figure ES.2. Comparison of Abundance of DNA Biomarkers for 1,4-Dioxane Cometabolism (Functional Genes) and 1,4-Dioxane Rate Constants Obtained Using ^{14}C Assay. ES-9

Figure 4.1. Site Locations by Region. 29

Figure 4.2. Site 1 (DoD) 35

Figure 4.3. Site 2 (DoD) 36

Figure 4.4. Site 3 (Industrial). 37

Figure 4.5. Site 4 (Industrial) 38

Figure 4.6. Site 5 (DoD) 39

Figure 4.7. Site 6 (DoD) 40

Figure 4.8. Site 7 (DoD) 41

Figure 4.9. Site 8 (DoD) 42

Figure 4.10. Site 9 (DoD) 43

Figure 4.11. Site 10 (DoD) 44

Figure 5.1. Overview of Technical Approach 45

Figure 5.2. Gantt Chart for Field Program. 49

Figure 5.6.1. Schematic of the ^{14}C-assay. 78

Figure 5.6.2. Schematic of the apparatus used to separate $^{14}CO_2$. 78

Figure 5.6.3. ^{14}C activity from 5 mL samples that were passed through the SPE cartridges repeated times. 79

Figure 5.6.4. Analysis of the purity of the ^{14}C-1,4-dioxane stock solution. 80

Figure 5.6.5. Evaluation of the ^{14}C assay using the CB1190 culture grown on 1,4-dioxane at varying dilution levels. 81

Figure 5.6.6. Evaluation of the ^{14}C assay using the ENV487culture grown on propane at varying dilution levels. 82

Figure 5.6.7. Inhibition test using acetylene for the CB1190 culture in AMSM. 83

Figure 5.6.8. ^{14}C product accumulation from ^{14}C-1,4-dioxane by the CB1190 culture in AMSM under aerobic conditions (blue), under aerobic conditions with acetylne present (red), and under anaerobic conditions (green). 84

Figure 5.6.9. Inhibition test using acetylene for ENV487 culture in BSM. 85

Figure 5.6.10. ^{14}C product accumulation from ^{14}C-1,4-dioxane by the ENV487 culture in BSM under aerobic conditions (blue), under aerobic conditions with acetylene present (red), and under anaerobic conditions (green). 86

Figure 5.6.11. Effect of sample handling on biodegradation of 1,4-dioxane by the CB1190 culture at an initial concentration of 163 µg/L in groundwater (Site 6, Well 2). 87

Figure 5.6.12. Effect of sample handling on biodegradation of 1,4-dioxane by the CB1190 culture at an initial concentration of 10.2 mg/L in groundwater (Site 6, Well 2). 88

Figure 5.6.13. Effect of sample handling on biodegradation of 1,4-dioxane by the ENV487 culture at an initial concentration of 163 µg/L in groundwater (Site 6, Well 3). 89

Figure 5.6.14. ^{14}C-product accumulation for Site 3, Well 4; a lower rate constant for Site 3, Well 5; and a non-significant result for Site 5, Well 3. 90

Figure 5.6.15. Correlation between first order degradation rate coefficients and the concentration of A) 1,1-DCE; and B) total VOCs. 91

Figure 5.6.16. Effect of nutrient addition in microcosms (auxiliary industrial site) bioaugmented with CB1190. 92

Figure 5.6.17. Effect of nutrient addition in microcosms (Site 6, Well 2) bioaugmented with CB1190. 93

Figure 5.6.18. Effect of nutrient addition in microcosms (Site 6, Well 3) bioaugmented with ENV487. 94

Figure 5.7.1.1. Site 1 (DoD) Distribution of Concentrations of 1,4-Dioxane. 102

Figure 5.7.1.2. Site 1 (DoD) Summary of 1,4-Dioxane/CVOC Concentrations and Attenuation of 1,4-Dioxane with Distance from the Source..... 103

Figure 5.7.1.3. Site 1 (DoD) Summary of Biomarker and Isotope Data for 1,4-Dioxane. 104

Figure 5.7.1.4. Site 1 (DoD) Summary of Biomarkers for CVOCs and Concentration Trends and Rate Constants for 1,1,1-TCA and 1,1-DCA and 1,1-DCE. 105

Figure 5.7.1.5. Site 1 (DoD) Summary of Concentration Trends and Rate Constants for PCE, TCE, *cis*-DCE and Vinyl Chloride. 106

Figure 5.7.2.1. Site 2 (Industrial) Distribution of Concentrations of 1,4-Dioxane. 111

Figure 5.7.2.2. Site 4 (Industrial) Summary of 1,4-Dioxane/CVOC Concentrations and Attenuation of 1,4-Dioxane with Distance from the Source..... 112

Figure 5.7.2.3. Site 1 (DoD) Summary of Biomarker Data For 1,4-Dioxane and CVOCs. 113

Figure 5.7.3.1. Site 3 (Industrial) Distribution of Concentrations of 1,4-Dioxane. 119

Figure 5.7.3.2. Site 3 (Industrial) Summary of 1,4-Dioxane/CVOC Concentrations and Attenuation of 1,4-Dioxane with Distance from the Source..... 120

Figure 5.7.3.3. Site 3 (DoD) Summary of Biomarker Data for 1,4-Dioxane and CVOCs. 121
Figure 5.7.4.1. Site 4 (Industrial) Distribution of Concentrations of 1,4-Dioxane 126
Figure 5.7.4.2. Site 4 (Industrial) Summary of 1,4-Dioxane/CVOC Concentrations and Attenuation of 1,4-Dioxane with Distance from the Source 127
Figure 5.7.4.3. Site 4 (DoD) Summary of Biomarker Data for 1,4-Dioxane and CVOCs. 128
Figure 5.7.5.1. Site 1 (DoD) Changes in Concentrations of 1,4-Dioxane and CVOCs over time. 136
Figure 5.7.5.2. Site 5 (DoD) Summary of 1,4-Dioxane/CVOC Concentrations and Attenuation of 1,4-Dioxane with Distance from the Source 137
Figure 5.7.5.3. Site 5 (DoD) Summary of Biomarker Data and Isotope Data for 1,4-Dioxane. 138
Figure 5.7.5.4. Site 5 (DoD) Summary of 1,4-Dioxane/CVOC Concentrations and Attenuation of PCE, TCE, *cis*-DCE and Vinyl Chloride with Distance from the Source 139
Figure 5.7.5.5. Site 5 (DoD) Summary of 1,4-Dioxane/CVOC Concentrations and Attenuation of PCE, TCE, *cis*-DCE and Vinyl Chloride with Distance from the Source 140
Figure 5.7.6.1. Site 6 (DoD) Summary of 1,4-Dioxane/CVOC Concentration Trends and Rate Constants 144
Figure 5.7.6.2. Site 6 (DoD) Summary of Biomarker and Isotope Data. 145
Figure 5.7.7.1. Site 7 (DoD) Distributions of 1,4-Dioxane/CVOCs in 2017 and 2019 151
Figure 5.7.7.2. Changes in Concentrations of 1,4-Dioxane and CVOC over time. 152
Figure 5.7.7.3. Site 7 (DoD) Summary of Biomarker Data and Isotope Data For 1,4-Dioxane. 153
Figure 5.7.8.1. Site 8 (DoD) Summary of 1,4-Dioxane/CVOC Concentration Trends and Rate Constants 156
Figure 5.7.8.2. Site 8 (DoD) Summary of Biomarker and Isotope Data. 157
Figure 5.7.9.1. Site 9 (DoD) Summary of 1,4-Dioxane/CVOC Concentration Trends and Rate Constants 161
Figure 5.7.9.2. Site 9 (DoD) Summary of Biomarker and Isotope Data. 162
Figure 5.7.10.1. Site 10 (DoD) Summary of 1,4-Dioxane/CVOC Concentration Trends and Rate Constants 166
Figure 5.7.10.2. Site 10 (DoD) Summary of Biomarker and Isotope Data. 167
Figure 5.8.1. Comparison of 1,4-Dioxane Biodegradation Rate Constants Obtained From ^{14}C Assays (Blue) and Model Predictions (Red). 171

Figure 5.8.2. Influence of Geochemical Indicator Parameters on Observed 1,4-Dioxane Biodegradation Rates Constant from ^{14}C Assays. 173

Figure 5.8.3. Influence of Initial 1,4-Dioxane Concentration on Observed 1,4-Dioxane Biodegradation Rates Constant from ^{14}C Assays. 174

Figure 5.8.4. Influence of Initial CVOC Concentrations on Observed 1,4-Dioxane Biodegradation Rates Constant from ^{14}C Assays. 176

Figure 5.8.5. Influence of Ratio of 1,4-Dioxane/1,1-DCE Concentrations on Observed 1,4-Dioxane Biodegradation Rates Constant from ^{14}C Assays. 177

Figure 5.8.6. Influence of Potential Biomarkers for 1,4-Dioxane Cometabolism on Observed 1,4-Dioxane Biodegradation Rates Constant from ^{14}C Assays. 179

Figure 5.8.7. Comparison of DNA Biomarkers for 1,4-Dioxane Cometabolism and 1,4-Dioxane Rate Constants Obtained Using ^{14}C Assay..... 180

Figure 5.8.8. Influence of Potential Biomarkers for Chlorinated Solvent Degradation on Observed 1,4-Dioxane Biodegradation Rates Constant from ^{14}C Assays. 181

Figure 5.8.9. Comparison of DNA Based and RNA Based Biomarkers for Chlorinated Solvent Degradation..... 182

Figure 5.9.1. Decision Framework for 1,4-Dioxane. 184

Figure 5.9.2. Decision Framework for Chlorinated Ethanes 185

Figure 5.9.3. Decision Framework for Chlorinated Ethanes. 186

Figure 6.1. Interface for the Simple 1,4-Dioxane Model in the “MNA Rate Constant Estimator”. 188

Figure 6.2 Interface for the Simple Chlorinated Ethane Model in the “MNA Rate Constant Estimator”. 190

Figure 6.3. Example Simulation Showing 1,4-Dioxane Plume Extent Over Time (Rate Constant = 0.32 per year). 192

LIST OF TABLES

Table ES.1. Summary of statistically significant net rate coefficients for 1,4-dioxane from ^{14}C assay. [a] ES-5

Table 2.1. Advantages and Potential Limitations of the Technology 11

Table 3.1. Summary of Performance Objectives 13

Table 4.1. Summary of Relevant Location and History Information by Site 30

Table 4.2. Summary of Relevant Hydrogeologic Characteristics by Site 31

Table 4.2. Summary of Relevant Hydrogeologic Characteristics by Site *(continued)* 32

Table 4.3. 1,4-Dioxane and CVOC Concentrations at Monitoring Wells Included in Field Demonstration 33

Table 4.3. 1,4-Dioxane and CVOC Concentrations at Monitoring Wells Included in Field Demonstration *(continued)* 34

Table 5.1. Summary of Sampling Plan per Site 51

Table 5.2. Part 1. Summary of Analytical Methods for Samples of Groundwater Collected per Site. Groundwater is the only matrix sampled. 52

Table 5.2. Part 2. Summary of Analytical Methods for Samples of Groundwater Collected per Site. Groundwater is the only matrix sampled 53

Table 5.3. Summary of QA/QC Sampling Program 54

Table 5.6.1. Rate coefficients determined with CB1190 and ENV487. 75

Table 5.6.2. Summary of statistically significant net rate coefficients. [a] 76

Table 5.6.3. 1,4-Dioxane GC measurements for groundwater samples that exhibited a statistically significant decrease during the incubation period. [a] 77

Table 5.7.1.2 Geochemistry of water samples from Site 1. 101

Table 5.7.2.1. Rate constants for 1,4-dioxane degradation provided by ^{14}C assay for Site 2. 110

Table 5.7.2.2. Geochemistry of water samples from Site 2. 110

Table 5.7.3.1. Rate constants for 1,4-dioxane degradation provided by ^{14}C assay at Site 3 118

Table 5.7.3.2. Geochemistry of water samples from Site 3. 118

Table 5.7.4.1. Rate constants for 1,4-dioxane degradation provided by ^{14}C assay at Site 4 125

Table 5.7.4.2. Geochemistry of water samples from Site 4. 125

Table 5.7.5.1. Rate constants for 1,4-dioxane degradation provided by ^{14}C assay for Site 5. 135

Table 5.7.5.2. Geochemistry of water samples from Site 5. 135

Table 5.7.6.1. Rate constants for 1,4-dioxane degradation provided by ^{14}C assay for Site 6. 143
Table 5.7.6.1. Geochemistry of water samples from Site 6. 143
Table 5.7.7.1. Biodegradation rate constants predicted from the abundance of biomarkers in groundwater from monitoring wells. 150
Table 5.7.7.2. Geochemistry of water samples from Site 7. 150
Table 5.7.8.1. Geochemistry of water samples from Site 8. 155
Table 5.7.9.1. Geochemistry of water samples from Site 9. 160
Table 5.7.10.1. Geochemistry of water samples from Site 10. 165
Table 5.8. Summary of Site-Specific Lines of Evidence for 1,4-Dioxane Attenuation at Project Field Sites 169
Table 6.1. Summary of Model-Predicted 1,4-Dioxane Rate Constants vs. ^{14}C-Based 1,4-Dioxane Rate Constants 196
Table 7.1. Summary of Elements Considered for the Project Cost Model 198
Table 7.2. Results of Cost Modeling 201

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ABSTRACT

Introduction and Objectives: Monitored natural attenuation (MNA) has emerged as a preferred remedial option at many sites contaminated with chlorinated solvents because it offers a cost-effective and practical approach for cleanup of solutes in groundwater. However, existing MNA protocols do not include 1,4-dioxane and commonly co-occurring chlorinated solvents like 1,1,1-TCA, 1,1-DCA, and 1,1-DCE. The objectives of this project were to: 1) develop a modified model and framework for evaluating natural attenuation of these compounds; 2) develop and validate a protocol to directly measure rate constants for natural biodegradation of 1,4-dioxane using ^{14}C-labeled 1,4-dioxane and groundwater from 10 different field sites; 3) use the field and lab data to establish if there is consistency between various lines of evidence for 1,4-dioxane attenuation.

Technology Description: An evaluation of MNA relies on establishing various lines of evidence, including secondary and tertiary lines of evidence that help demonstrate degradation processes and associated rates that are responsible for the primary line of evidence (decreasing concentrations of the target compound(s)). This project developed a new fate and transport model to easily evaluate historical monitoring data to predict biodegradation rate constants as well as new decision matrices (flowcharts) that serve as a guided tour on how to interpret potential lines of evidence for MNA. These were then integrated into an existing software platform (BioPIC) that allows users to access both the model and the decision matrices. Several approaches were also used to generate input data to support and validate the model and framework. First, rate coefficients and lines of evidence for attenuation were calculated and/or measured at multiple sites using a focused sampling program at 10 field sites. Second, degradation and the associated rate constants for 1,4-dioxane at these same sites were determined using a ^{14}C-labeled 1,4-dioxane assay developed for this project.

Performance and Cost Assessment: The ^{14}C assay was successfully validated and used to establish 1,4-dioxane natural attenuation capacity at 9 of the 10 field sampling sites. Similarly, the model was able to extract rate constants for 1,4-dioxane biodegradation at 8 of 9 sites that had sufficient data to evaluate. Several project performance objectives focused on ensuring that the decision framework was functional and captures all relevant processes for the targeted compounds. The success criteria for all these objectives were successfully achieved, in part because the model provides quantitative support for the decision framework. Multiple performance objectives related to the sensitivity and robustness of the ^{14}C assay in quantifying 1,4-dioxane rate constants were also achieved. Performance objectives that were partially achieved or not achieved were related to: i) consistency between the model-predicted rate constants and the ^{14}C-based rate constants (achieved at 3 of 9 field sites with sufficient data); and ii) correlations between biomarker abundance and rate constants (no clear relationship could be established). A cost model was developed that included collecting the necessary data and performing a comprehensive MNA evaluation using the project deliverables. This resulted in an estimated total cost of approximately $30,000 for assessing MNA at a single site. While not incorporated into the model, this assessment could result in substantial savings if the use of MNA is justified vs. more aggressive remedies.

Implementation Issues: No regulatory or procurement issues are anticipated. Implementation involves standard equipment and no special permitting. End-user concerns are minimal since data can be collected during a single mobilization without disruption to site activities.

Publications: Three manuscripts in preparation: 1) development and validation of the ^{14}C assay for 1,4-dioxane; 2) multi-site comparison of lines of evidence for 1,4-dioxane biodegradation based on field, lab, and modeling results; 3) description of new MNA decision framework. One published article: 1) Adamson et al. (2021) on trends in 1,4-dioxane analyses.

EXECUTIVE SUMMARY

INTRODUCTION

Monitored natural attenuation (MNA) has emerged as a preferred remediation technology at many sites contaminated with chlorinated solvents because it offers a cost-effective and practical approach for cleanup of solutes in groundwater. Some of the advantages of MNA include lower costs and minimal additional environmental impacts (such as greenhouse gas emissions caused by active remediation), while still providing effective site cleanup for protection of human health and the environment. Establishing the appropriateness of MNA at most sites involves evaluating several different co-contaminants and/or degradation by-products. For example, at many sites with a release of 1,1,1-Trichloroethane (1,1,1-TCA), biological reductive dechlorination of 1,1,1-TCA produces 1,1-dichoroethane (1,1-DCA), and abiotic degradation of 1,1,1-TCA produces 1,1-dichloroethene (1,1-DCE). 1,4-Dioxane is also a concern at many chlorinated solvent sites because its primary use was to stabilize 1,1,1-TCA. There is considerable empirical data on the co-occurrence of 1,4-dioxane at chlorinated solvents sites (Anderson et al., 2012; Adamson et al., 2014), which has implications for site management decisions.

For sites that are considering MNA as part of their remedial strategy, existing protocols can be used to support evaluations for many chlorinated solvents (e.g., USEPA, 1998; USEPA, 1999). Although still valid, many of these documents are dated, and significant research on the degradation of chlorinated solvents has been completed since they were published and many new analytical tools for quantifying degradation have been developed in recent years (see Adamson and Newell, 2014; Lebron et al., 2015; and Wiedimeier et al., 2017 for additional guidance). Importantly, the existing protocols emphasize the chlorinated alkenes such as trichloroethene (TCE), and little guidance is provided for 1,1,1-TCA and 1,1-DCA.

Similarly, no guidance documents are currently available to assist Remedial Project Managers (RPMs) in effectively evaluating natural attenuation of 1,4-dioxane. Several studies have provided evidence of 1,4-dioxane attenuation at field sites that is correlated with known aerobic biodegradation processes (Li et al., 2015; Adamson et al., 2015; Gedalanga et al., 2016; da Silva et al., 2017). At the same time, there have been significant advances in identifying naturally occurring microbes and functional genes associated with 1,4-dioxane biodegradation. Another documented line of evidence for 1,4-dioxane attenuation is the site-specific analysis of samples for stable isotopes of carbon and hydrogen (Bennett et al., 2018).

Collectively, these studies help demonstrate the potential applicability of MNA for 1,4-dioxane. However, the lack of a quantitative framework to evaluate the contribution of natural attenuation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-dioxane in groundwater makes it difficult to include natural attenuation as part of the remedy at a site that contains these contaminants. This represents a critical gap in our ability to effectively cleanup groundwater sites since incorporation of natural attenuation processes into the comprehensive remedy at a site should substantially reduce the cost to complete the remedy by reducing the extent of an active treatment that is required.

OBJECTIVES

The overarching objectives of this project were to:

1) Develop a new decision tool for evaluating MNA for missing constituents that is based on:

a. A fate and transport model (similar to USEPA's BIOCHLOR model) that can analyze historical monitoring data to extract rate constants for natural degradation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-dioxane so that RPMs can use the rate constants to support MNA remedy evaluations.
b. Decision matrices (flowcharts) that can be used by RPMs to walk through how biodegradation rates can be estimated, as well as how to interpret other potential lines of evidence for MNA.
c. An integrated platform that allows users to access both the model and the decision matrices, specifically by updating the existing BioPIC software.

2) Develop and validate a protocol to directly measure rate constants for natural biodegradation of 1,4-dioxane using ^{14}C-labeled 1,4-dioxane and groundwater from multiple different field sites;

3) Evaluate existing *q*PCR assays for organisms and enzymes involved in the biodegradation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-dioxane by comparing the density of gene copies to the rate constants for removal of the contaminants at field scale as extracted from monitoring data or the rate constants for 1,4-dioxane degradation as determined by the ^{14}C assay; and

4) Use data collected from multiple field sites to establish if there is consistency between various lines of evidence for 1,4-dioxane attenuation.

TECHNOLOGY DESCRIPTION

To build a quantitative case for MNA of these compounds, it is necessary to develop lines of evidence for attenuation of the target compounds, including demonstrating that: 1) plumes are stable and/or decreasing; 2) the mechanisms for attenuation can be identified; and 3) the attenuation rates can be estimated. As described below, methodologies for this project are patterned after several ESTCP projects, including the decision matrices used to develop BioPIC, the ^{14}C-labeled TCE assay used to confirm degradation of TCE, identification of *q*PCR targets that can serve as biomarkers for degradation, and the development of CSIA methods for 1,4-dioxane.

Collection and Analysis of Field Data: A focused field program was implemented to collect data at 10 sites where 1,4-dioxane and chlorinated solvents are present. The work involved the coordination with site managers and may benefit their efforts to obtain site closure for these sites. Groundwater samples were collected from a minimum of 4 monitoring wells per site and analyzed in the field for standard geochemical parameters (dissolved oxygen, ORP, pH, temperature, specific conductance, and Fe(II). Groundwater samples from these locations were also sent to Clemson University for the ^{14}C-labelled 1,4-Dioxane assay and to various labs for analysis of concentrations of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, 1,4-dioxane, dissolved gases, *q*PCR markers, and specific isotopic ratios (^{2}H and ^{13}C).

Rate constants for degradation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and/or 1,4-dioxane were also estimated using data at field sites. These rates were extracted by calibrating a fate and transport model (described below) to field data and empirically determining the values of the rate constants that provide the best fit of the model projection to the actual field data (i.e., concentration vs. distance in monitoring wells located in the direction of groundwater flow).

Bench-Scale ^{14}C Assays: To provide an independent estimate of degradation rates for 1,4-dioxane, water samples from field sites were incubated with ^{14}C-labeled 1,4-dioxane as part of a method specifically developed by Clemson University for this project. They have extensive experience with use of ^{14}C-labeled compounds, including purification of commercially synthesized ^{14}C-TCE. The ^{14}C method for 1,4-dioxane was adapted from another high performance liquid chromatography (HPLC) protocol for ^{14}C-TCE that was successfully validated as part of ESTCP Project ER-201584 (Wiedemeier et al., 2017). A similar approach was employed for this project using ^{14}C-1,4-dioxane but with different separation techniques to account for 1,4-dioxane's non-volatility. The total amount of ^{14}C products measured in samples collected at predetermined time intervals was used to ascertain pseudo first-order rates of 1,4-dioxane transformation (after correcting for losses in filter-sterilized controls).

Decision Framework and Software Development: Under this task, a simple Microsoft Excel spreadsheet model was developed that can be used to simulate the transport and degradation of TCA and its degradation products (including 1,1-DCA, chloroethane, 1,1-DCE, and VC), and 1,4-Dioxane. These compounds are included because they are missing from existing, similar modeling tools (e.g., BIOCHLOR). However, for completeness, this model also includes a module for chlorinated ethenes. This model allows users to estimate a first-order biodegradation rate constant based on curve fitting to existing field data along the flow path and centerline of the constituent plume. Data required for this curve fitting include historical concentration data for the target constituents along the flow path and aquifer hydrogeologic data including hydraulic conductivity, porosity, hydraulic gradient, bulk density, and fraction of organic carbon.

In addition, separate decision matrices were developed for the degradation pathways for 1,4-Dioxane and 1,1,1-TCA and its degradation products including 1,1-DCA and 1,1-DCE. Once completed, an easy-to-use software package that automates the use of the decision matrices was developed. This was accomplished by updating an existing Microsoft Excel spreadsheet application for evaluating chlorinated ethenes, the Biological Pathway Identification Criteria (BioPIC), so that it includes these new decision matrices for 1,4-dioxane and the chlorinated ethanes.

Parameters that were used to develop the new decision matrices include degradation rates, stable isotope data (i.e., CSIA) for qualitative evidence that degradation has occurred, potential *q*PCR markers for 1,4-dioxane degradation, potential *q*PCR targets for chlorinated solvent degradation, and the biogeochemical parameters including dissolved oxygen, Fe(II), and methane.

Finally, the field data included in this project served as benchmark sites to try to establish the relationship between the rate constants for 1,4-dioxane degradation and various lines of evidence, including the abundance of possible biomarkers of biodegradation. This was accomplished using two different approaches. One focused on developing simple analytical expressions that predicted the expected rate constant using lab-derived kinetic parameters for the functional genes associated with these specific markers, and then comparing these to the degradation rates measured from field samples. The second used the biomarker data from sites where these were detected. Specifically, the abundance of specific biomarkers (e.g., genes that encode monooxygenases that are known or suspected to degrade 1,4-dioxane) was compared to the first-order rate constants for degradation of 1,4-dioxane in the ^{14}C assays to see if a statistically significant correlation could be developed and used to predict the expected rate constant. In cases where the association is acceptable, this serves as a secondary line of evidence for MNA, and it would support MNA evaluations at other

sites by helping predict and/or explain biodegradation rates. The goal was to build these approaches into the decision tool (BioPIC) as part of this project.

PERFORMANCE ASSESSMENT

Validation of Bench-Scale ^{14}C Assay for 1,4-Dioxane

The ^{14}C assay for 1,4-dioxane was successfully validated. The test demonstrated the sensitivity to quantify 1,4-dioxane degradation half-lives in excess of 100 years, which met the project performance objective. The validation process was supported by experiments using multiple known 1,4-dioxane degraders to confirm the role of monooxygenases in the observed degradation. The ^{14}C assay was deployed to test groundwater samples from the 10 project field sites. Statistically significant rate coefficients were determined for approximately 44% of the samples, but 90% of the sites had at least one location with a significant rate constant. For Site 3, which had the three highest rate coefficients, propane biosparging had occurred in the recent past, so the significant activity in those samples is likely related. For the other samples with statistically significant rate coefficients, most of the corresponding half-lives are close to or above 100 years (see **Table ES.1**). This suggests that biodegradation is occurring, but at a rate that may be slow in terms of supporting natural attenuation.

Alternatively, the ^{14}C assay may be underestimating the *in situ* degradation rates, as suggested by results with nutrient amendments. A key advantage for a groundwater-only assay is the lower cost and complexity associated with collecting groundwater alone versus soil cores. However, the soil likely harbors many of the nutrients needed for metabolic or cometabolic degradation of 1,4-dioxane. On this basis, the groundwater ^{14}C assay may be viewed as a screening tool, i.e., in the event that a statistically significant rate is detected, it may be worthwhile to construct microcosms with soil and groundwater. The presence of the soil should allay any concerns about a lack of nutrients, as well concerns regarding a low level of microbes. The ^{14}C assay is very sensitive, so the likelihood of missing the fact that 1,4-dioxane biodegradation is occurring seems remote.

Table ES.1. Summary of statistically significant net rate coefficients for 1,4-dioxane from ^{14}C assay. [a]

Site No.	Well No. [b]	Groundwater *k* (yr^{-1})	FSGW *k* (yr^{-1})	Net *k* (yr^{-1})	Net $t_{1/2}$ (yr)
2	1	0.0041 ± 0.0018	0.0020 ± 0.0012	0.0021 ± 0.0021	328 ± (165, 27514)
3	3	0.0156 ± 0.0033	0.0042 ± 0.0019	0.0114 ± 0.0033	61 ± (46, 90)
	4	0.109 ± 0.0153	0.0137 ± 0.0019	0.0957 ± 0.0149	7.2 ± (6.3, 8.6)
	5	0.0286 ± 0.0048	0.0077 ± 0.0024	0.0209 ± 0.0052	33 ± (27, 44)
4	1[ii]	0.0250 ± 0.0098	0.0091 ± 0.0056	0.0159 ± 0.0110	44 ± (26, 141)
	2	0.0142 ± 0.0043	0.0081 ± 0.003	0.0061 ± 0.0051	113 ± (62, 651)
	2[ii]	0.0234 ± 0.0107	0.0091 ± 0.0056	0.0143 ± 0.0117	49 ± (27, 271)
	2[iii]	0.362 ± 0.0574	0.0643± 0.0172	0.297± 0.0580	2.3 ± (2.0, 2.9)
	4	0.0127 ± 0.0025	0.0054 ± 0.0015	0.0073 ± 0.0028	95 ± (69, 153)
	5[c]	0.181 ± 0.0515	0.0643 ± 0.0172	0.117 ± 0.0526	5.9 ± (4.1, 11)
	6[c]	0.431 ± 0.0512	0.0643± 0.0172	0.367 ± 0.0484	1.9 ± (1.7, 2.2)
5	1	0.0070 ± 0.0023	0.0033 ± 0.0023	0.0037 ± 0.0023	189 ± (102, 1254)
	2	0.0086 ± 0.0029	0.0044 ± 0.0014	0.0042 ± 0.0032	164 ± (94, 650)
6	3	0.0081 ± 0.0018	0.0054 ± 0.0016	0.0026 ± 0.0023	262 ± (140, 2001)
7	4	0.0076 ± 0.0029	0.0026 ± 0.0014	0.0050 ± 0.0031	140 ± (86, 376)
8	2[c]	0.0169 ± 0.0069	0.0078 ± 0.0039	0.0091 ± 0.0077	76 ± (41, 510)
	3[c]	0.0206 ± 0.0030	0.0096 ± 0.0029	0.0110 ± 0.0040	63 ± (46, 99)
9	1[c]	0.0446 ± 0.0072	0.0191 ± 0.0044	0.0255 ± 0.0082	27 ± (21, 40)
	2[c]	0.0210 ± 0.0046	0.0078 ± 0.0039	0.0132 ± 0.0058	53 ± (37, 94)
	3 [c]	0.0103 ± 0.0020	0.0045 ± 0.0019	0.0058 ± 0.0027	120 ± (82, 222)
10	2	0.0768 ± 0.0170	0.0081 ± 0.0034	0.0688 ± 0.0141	10 ± (8.4, 13)
	2[c]	0.0333 ± 0.0097	0.0081 ± 0.0034	0.0252 ± 0.0099	27 ± (20, 45)
	4	0.0948 ± 0.0136	0.0081 ± 0.0034	0.0868 ± 0.0136	8.0 ± (6.9, 9.5)
	4[c]	0.203 ± 0.0378	0.0081 ± 0.0034	0.195 ± 0.0368	3.6 ± (3.0, 4.4)

[a] ± represents the 95% confidence limit.
[b] First sampling event unless followed by [ii] = 2nd sampling event; [iii] = 3rd sampling event.
[c] Soil or sediment present along with the groundwater.

Decision Framework and Software Development

For the decision framework objective, the first step was to develop a new software tool (titled "**MNA Rate Constant Estimator**") to serve as a simple fate and transport model for determining 1,4-dioxane and chlorinated ethane degradation rate constants and predicting source and plume

behavior over time. These serve as critical first and second lines of evidence during typical MNA evaluations. The new software represents an improvement over the original platform that was going to be used (BIOCHLOR) because it is more compatible with newer versions of Microsoft Excel. This modeling tool met the project success criteria, which were based on incorporating the relevant processes for 1,4-dioxane and chlorinated ethane natural attenuation into the model. This was accomplished using an analytical solution for solute transport equations based on advection, dispersion, linear equilibrium adsorption, and the relevant biotic and abiotic degradation processes for these compounds. The root mean square error (RMSE) between the field-measured and simulated data is displayed and can be used to calibrate the model. The model can also support the following key elements of MNA remedy selection: (1) it can make projections that will indicate whether the site-specific goals (e.g., a concentration threshold at a point of compliance) can be attained now and/or in the future; and (2) for cases where goals are not currently met, it can determine how much source decay or treatment is needed to achieve downgradient goals.

Next, a decision framework was developed for evaluating MNA for 1,4-dioxane and chlorinated ethanes, using information from literature and project-specific data to identify the relevant lines of evidence. While these lines of evidence differ slightly depending on the class of compounds, they generally included the following:

- Is the compound being degraded based on model predictions?
- Does biomarker abundance explain the model-predicted degradation rate?
- Do isotope data suggest that the compound is being degraded?
- Is there confirmatory evidence of degradation based on lab-based studies (e.g., ^{14}C assays)?
- Are geochemical conditions supportive of the targeted degradation pathways?
- Are other biomarkers for biodegradation present?
- Are other compounds present that would be expected to inhibit the targeted degradation pathway?

For each class of compounds, these elements were then incorporated into a Decision Framework that can be visualized as a flowchart. These flowcharts were then converted into a "guided tour" within the Microsoft Excel-based BioPIC tool. This updated version of BioPIC was another deliverable for this project, and detailed explanations of the decision criteria as well as other help text are provided in the associated User's Guide.

Comparison of Lines of Evidence for Attenuation Based on Field and Lab Data

The field program was completed at 10 sites, and in addition to providing samples for the ^{14}C assay (described above), it generated a large dataset for evaluating other lines of evidence for attenuation. For the purposes of site evaluation and this project, these data obtained from the field sites can be placed into three broad categories:

1. Site-specific confirmation that the capacity for 1,4-dioxane biodegradation is present and an estimate of the biodegradation rate
   a. Statistically significant 1,4-dioxane biodegradation rate constant using the ^{14}C assay – *successfully obtained at 9 of the 10 sites in this study.*
2. Site-specific evidence that 1,4-dioxane attenuation is occurring
   a. Biodegradation rate constant based on model calibration of concentration vs. distance data – *successfully obtained at 8 of the 9 sites in this study with sufficient data to model*

   b. Isotope fractionation along the groundwater flow path – *successfully obtained at 7 of 10 sites in this study*

3. Site-specific data that are supportive of the proposed attenuation mechanism (i.e., 1,4-dioxane degradation)
   a. Consistency between ^{14}C-based rate constant and the model-predicted rate constants – *successfully obtained at 5 of 9 sites where positive ^{14}C activity was observed*
   b. Consistency between biomarker abundance and model-predicted rate constants – *successfully obtained at 1 of 8 sites with model-predicted rate constants*
   c. Presence of favorable geochemical conditions – *included 7 of 10 sites in this study (and 6 of 9 sites where ^{14}C-based degradation was observed*
   d. Low levels of potentially inhibitory CVOCs – *included 7 of 10 sites in this study (and 7 of 9 sites where ^{14}C-based degradation was observed)*

The key lessons learned from this dataset on 1,4-dioxane attenuation include the following:

Widespread prevalence of 1,4-dioxane degradation capacity. 1,4-dioxane degradation capacity was established at 9 of 10 sites based on the ^{14}C assays. Evidence for 1,4-dioxane degradation and/or degradation capacity was observed at several sites where the apparent dissolved oxygen levels were relatively limited. While the reaction requires oxygen to proceed, field-measured dissolved oxygen levels may not reflect the actual availability of oxygen. This may be due to performance issues for dissolved oxygen probes (particularly at low levels), but it also suggests that the long screens typically used in monitoring wells (10-ft) yield mixed groundwater samples from several sub-intervals with different redox conditions. This mixing may lower the bulk dissolved oxygen measured in a field sample. It should be noted that this type of mixing may also mask the isotope fractionation signal. In addition, the oxygen demand of dilute concentrations of 1,4-dioxane is relatively low; 1 mg/L of DO could theoretically oxidize more than 0.5 mg/L of 1,4-dioxane to CO_2. For the decision framework, a dissolved oxygen level of 0.1 mg/L is considered the screening-level threshold for establishing whether geochemical conditions are favorable for 1,4-dioxane degradation. Installing shorter-screened monitoring wells, particularly in shallow portions of the aquifer where infiltration may enhance oxygen availability, is recommended for evaluating 1,4-dioxane degradation.

Variability within sites was observed: At the sites where the ^{14}C assay generated positive results, a statistically significant rate constant was obtained at 25% to 75% of sampled locations within the same site. This highlights that: 1) 1,4-dioxane degradation capacity at a particular site may be localized; and 2) collecting data from multiple locations is critical. The finding that 1,4-dioxane degradation capacity was variable within a site is perhaps not surprising, given that this is an aerobic process that is reliant on oxygen availability, which is expected to vary due to infiltration and other redox-driven processes. Similarly, sampling multiple locations (including within the source and the plume) is important not only for increasing representativeness of the data, but for making sure that evaluations based on spatial patterns (specifically isotope fractionation patterns and calibration of the fate and transport model) can be performed.

Prevalence of in situ 1,4-dioxane degradation based on model predictions/isotope fractionation. 1,4-dioxane biodegradation rate constants could be established using model predictions for 8 of the 10 study sites. These site-wide, first-order rate constants ranged from 0 to 0.32 per year, with the upper end equivalent to a half-life of 2.2 year. A total of 6 sites had model-predicted rate constants that were greater than 0.06 per year (equivalent half-life of < 12 year). Evidence for

isotope fractionation was obtained at 7 of 10 sites. These data provide solid evidence that in situ biodegradation of 1,4-dioxane is actually occurring at a significant percentage of sites.

Lines of evidence for 1,4-dioxane natural attenuation did not always converge. While some sites had many converging lines of evidence for natural attenuation, others had lines of evidence that diverged. This included the data associated with potential biomarkers for 1,4-dioxane cometabolism, which were not particularly illustrative of 1,4-dioxane attenuation trends suggested by the other datasets. At 7 of the 10 study sites, a biodegradation rate constant was obtained with both the ^{14}C rate assay and the model calibration. This is an important convergence because the ^{14}C rate assay suggests that the capacity exists for biodegradation (with some indication of the relative rate by location), while the model calibration suggests that in situ biodegradation is occurring (with an estimate of the site-wide rate). These two analyses, along with the isotope fractionation patterns, are likely to be the strongest lines of evidence for natural attenuation. **Figure ES.1** shows a comparison of the ^{14}C-derived rate constants with those generated during model calibration, which was part of a performance objective for this task that was based on similarity between these two types of rate constants. The success criteria were met at 3 of the 9 sites with sufficient data, while data from 2 other sites showed moderate consistency. At sites where they were not consistent, the model-calibrated rate was typically much higher. The 1,4-dioxane biodegradation rate constants obtained from the ^{14}C assay spanned a wide range but were typically slow. This may explain why wells at some sites did not exhibit a statistically significant rate constant; rates for slower reactions are generally more challenging to quantify. As noted previously, the ^{14}C assays are typically performed with groundwater that contains little or no solids or supplemental nutrients, such that the 1,4-dioxane rate constants likely underestimate in situ biodegradation rates.

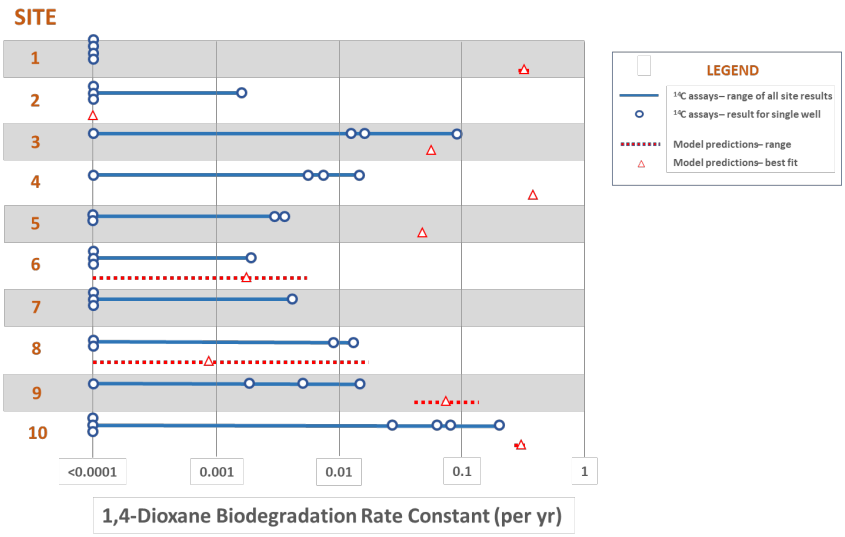


Figure ES.1. Comparison of 1,4-Dioxane Biodegradation Rate Constants Obtained From ^{14}C Assays (Blue) and Model Predictions (Red).
Note that no model-predicted rate constant could be obtained for Site 7, and ranges for model-predicted rate constants were not obtained for some other sites.

Given that each of the individual analyses has some limitations, the data from this study highlight the importance of not relying on a single line of evidence for documenting natural attenuation. Within the decision framework, this is accomplished by making the user go through all possible lines of evidence (and documenting which ones are supportive) as opposed to prescriptive outcomes based on each individual line of evidence.

Lack of prevalence of direct biomarkers for 1,4-dioxane. Possible biomarkers for direct metabolism of 1,4-dioxane (THFMO/DXMO, ALDH) were only detected at one of the ten study sites (Site 3). These data suggest that either: 1) these biomarkers are not sufficiently prevalent to

serve as a valuable line of evidence for 1,4-dioxane biodegradation; and/or 2) organisms capable of direct metabolism are relatively rare when compared to organisms capable of cometabolizing 1,4-dioxane. It should be noted that the one site where the biomarkers for direct oxidation were detected also had several other positive lines of evidence for natural attenuation. This indicates that the THFMO/DXMO and ALDH biomarkers may have some value as corroborating evidence.

Lack of predictive power for biomarkers: As noted above, the biomarkers for direct metabolism of 1,4-dioxane were infrequently detected and therefore did not provide much help in developing predictive relationships for rate constants. Similarly, no clear relationship could be established between rate constants obtained using the ^{14}C assay and the abundance of possible biomarkers for 1,4-dioxane cometabolism (**Figure ES.3**). These *q*PCR tests targeted several monooxygenases genes that may degrade 1,4-dioxane, but the abundance of the gene or gene transcripts were not well correlated with observed activity. A parallel approach for developing a correlation (based on lab-derived kinetic parameters) generally underpredicted the model-calibrated rate constant. At this stage, it appears that sampling groundwater for the current set of functional gene targets has limited ability to predict rate constants, either because other (unidentified) enzymes are responsible for the observed activity and/or because of the limitations of assaying groundwater samples (e.g., less biomass to establish abundance or rates). Consequently, other methods (e.g., models) are likely to serve as better secondary lines of evidence for evaluating MNA.

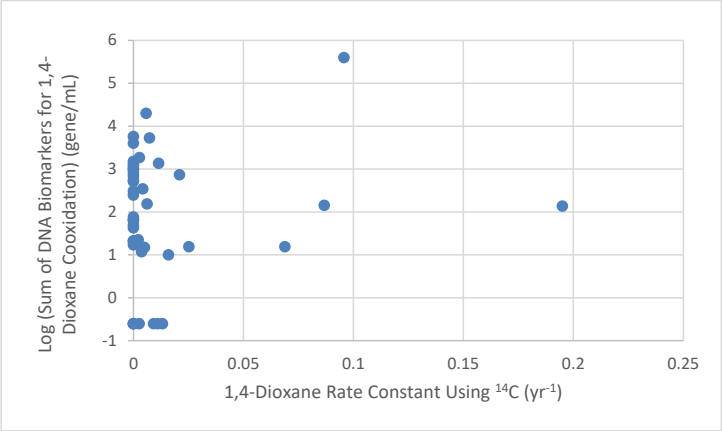


Figure ES.2. Comparison of Abundance of DNA Biomarkers for 1,4-Dioxane Cometabolism (Functional Genes) and 1,4-Dioxane Rate Constants Obtained Using ^{14}C Assay.
No clear relationship could be established.

Slow 1,4-dioxane rates are common but may be an artifact of experimental protocol. The rate constants based on concentration vs. distance data described above are derived from a model that accounts for the effects of non-destructive processes. However, they are still calibrated parameters that may be influenced by uncertainty in other input parameters. In contrast, the rates obtained using the ^{14}C assay (which is designed to control for all other processes) were much slower, with a median value of 0.0137 per year (equivalent half-life of 51 year) at the locations where a significant rate constant could be established. Furthermore, no rate constant could be established at 56% of the well locations where groundwater was tested using this assay. However, the use of groundwater likely limits the initial biomass (including attached-growth organisms that would be associated with soil) and nutrient availability within the microcosms. These factors would not only lower the observed degradation rates but make it more challenging to establish a statistically significant rate constant (i.e., increases the chance for a false negative). It may be worthwhile to use groundwater-based assays as a screening step and/or construct microcosms with soil and groundwater to provide more refined rate data.

COST ASSESSMENT

The framework developed as part of this project requires an assessment of site-specific data. A cost model was developed that includes collecting and evaluating data that are relatively unique to this approach. The model was then applied for a single site with a reasonably well-delineated 1,4-dioxane plume and chlorinated solvent plume, where the source and a downgradient point of compliance have been established. In addition, it was assumed that relevant groundwater transport parameters are known or could be easily estimated. Application requires collecting data from at least four existing monitoring wells along the groundwater flow path that exhibit a decreasing trend of concentration vs. distance, and then using the decision framework to go through the lines of evidence for attenuation of both 1,4-dioxane and the applicable chlorinated solvents.

Based on these assumptions, the total cost for the assessment of MNA of a single site using this tool was approximately $30,000. The primary cost drivers are collecting data on specific parameters that may not normally be collected as part of a standard assessment (e.g., ^{14}C assay, CSIA and/or biomarker data) and the labor cost associated with learning and using the software platform for the decision frameworks (BioPIC). The cost model did not attempt to project the cost savings if the tool justifies the selection of MNA vs. implementing more aggressive remedial options (e.g., biostimulation). However, MNA is generally a cost-effective technology in terms of capital and O&M costs, and it can also reduce the environmental impact at these cleanup sites.

IMPLEMENTATION ISSUES

Implementation issues for the MNA decision framework are expected to be minimal. No permits are required. Obtaining approval from applicable regulatory bodies to collect the required data is likely to be straightforward; it involves collecting groundwater samples using conventional protocols that would follow standard work plans. The approach can be implemented using standard equipment in short mobilizations that should not disrupt site activities. Data can be generated using analyses that are either currently offered by commercial labs (1,4-dioxane and CVOC analysis, geochemical parameters, stable isotopes, hydrogeologic parameters, and possible biomarkers for biodegradation) or expected to be offered through commercial/academic lab partnering agreements (the ^{14}C assay for 1,4-dioxane degradation).

The primary regulatory issue will be educating RPMs and regulators on the protocol, which helps addresses a gap in the existing MNA guidance (i.e., lack of protocols for evaluating 1,4-dioxane and chlorinated ethanes). The goal was to develop a decision framework that builds on previous frameworks for chlorinated ethenes, relies on a similar “lines of evidence” approach, and has a strong quantitative basis for establishing secondary and tertiary lines of evidence (e.g., fate and transport model to help estimate biodegradation rates). These elements were all successfully incorporated into the resulting decision framework and should facilitate regulatory acceptance.

This decision framework and the associated data are designed to provide a site with the technical basis for evaluating MNA as a viable remedy. As a result, the decision to collect these data should be made in context with the timeline for remedial investigations/feasibility studies (or similar site assessments). For a compound of emerging concern like 1,4-dioxane, there may be some reluctance from site managers to generate such a robust dataset if the site-specific regulatory drivers have yet to be established. At some sites—especially those where insufficient data are available to fully evaluate the primary line of evidence for natural attenuation—a screening-level approach may be warranted. This would involve initially collecting limited data on the secondary lines of evidence (focusing on one or two types of data), and then expanding efforts as more concentration vs. time/distance data become available.

1.0 INTRODUCTION

1.1. BACKGROUND

Monitored natural attenuation (MNA) has emerged as a preferred remediation technology at many sites contaminated with chlorinated solvents because it offers a cost-effective and practical approach for cleanup of solutes in groundwater. Some of the advantages of MNA include lower costs and minimal additional environmental impacts such as greenhouse gas emissions caused by active remediation, while still affording protection of human health and the environment and effecting site cleanup. Establishing the appropriateness of MNA at most sites involves evaluating several different co-contaminants and/or degradation by-products. For example, at many sites with a release of 1,1,1-Trichloroethane (1,1,1-TCA), biological reductive dechlorination of 1,1,1-TCA produces 1,1-Dichoroethane (1,1-DCA), and abiotic degradation of 1,1,1-TCA produces 1,1-Dichloroethene (1,1-DCE). 1,4-Dioxane is also a concern at many chlorinated solvent sites because its primary use was to stabilize 1,1,1-TCA. There are considerable empirical data on the co-occurrence of 1,4-dioxane at chlorinated solvents sites (Anderson et al., 2012; Adamson et al., 2014).

Protocols exist for the evaluation and implementation of MNA for chlorinated solvents (e.g., USEPA, 1998; USEPA, 1999; Wiedemeier et al., 1999; US DoN, 1998). Although still valid, many of these documents are dated and significant research on the degradation of chlorinated solvents has been completed since they were published and many new analytical tools for quantifying degradation have been developed in recent years (see Adamson and Newell, 2014; Lebron et al., 2015; and Wiedimeier et al., 2017 for additional guidance). Also, the existing protocols emphasize the chlorinated alkenes such as trichloroethene (TCE), and little guidance is provided for 1,1,1-TCA and 1,1-DCA. To-date, no guidance documents are available to assist Remedial Project Managers (RPMs) in effectively evaluating the natural attenuation of 1,4-dioxane.

Several studies have provided evidence of 1,4-dioxane attenuation at field sites that is correlated with known aerobic biodegradation processes (Li et al., 2015; Adamson et al., 2015; Gedalanga et al., 2016; da Silva et al., 2017). At the same time, there have been significant advances in identifying naturally occurring microbes and functional genes associated with 1,4-dioxane biodegradation (Gedalanga, et al., 2014; Hatzinger et al., 2017; Deng et al., 2018; Rolston et al., 2019; Ramalingam and Cupples, 2020). This includes cultures capable of metabolic, growth-supporting biodegradation (e.g., *Pseudonocardia dioxanivorans* CB1190, *Mycobacterium dioxanotrophicus* PH-06) as well as cultures that degrade 1,4-dioxane cometabolically after growing on primary substrates such as propane or isobutane (e.g., *Rhodococcus ruber* ENV425, *Mycobacterium vaccae* JOB5). Presently, the only bacteria in pure culture that can grow on 1,4-dioxane require molecular oxygen to initiate the metabolism of 1,4-dioxane (Gedalanga, et al., 2014). In the available pure cultures, tetrahydrofuran monooxygenase (THFMO, also referred to as DXMO or by the gene name *thmA*), some propane monooxygenases (identified by the gene name *prmA* or PrMO) (Deng et al., 2018) or similar monooxygenases transforms 1,4-Dioxane to 2-hydroxyethoxyacetaldehyde (Sales et al., 2013; Deng et al., 2017). An aldehyde dehydrogenase (ALDH) then transforms the 2-hydroxyethoxyacetaldehyde to 2-hydroxyethoxyacetic acid (HEAA). Cultures that can grow on 1,4-dioxane further degrade the HEAA.

However, most cultures with the THF monooxygenase cannot grow on 1,4-dioxane. Similarly, not all propane monoxygenases have been shown to metabolically degrade 1,4-dioxane (Deng et al., 2018). In these other cultures, 1,4-dioxane is only cometabolized to HEAA or some other product.

Other monooxygenases investigated as possible or likely to be able to cometabolize 1,4-dioxane include soluble methane monooxygenase (sMMO), ring hydroxylating toluene monooxygenase (RMO and RDEG), phenol hydroxlyase (PHE), and short-chain alkane monooxygenases (SCAM) (Bennett et al., 2018). Recently, a toluene-oxidizing monooxygenase has been described that can oxidize low concentrations of 1,4-dioxane and can also oxidize propane at sufficiently high rates that its activity can support the growth of the host bacterium using propane as a sole source of carbon and energy (Deng et al. 2020). The majority of these have been classified as soluble di-iron monooxygenases (SDIMO) (He et al., 2017; Deng et al., 2018). For example, the SCAM enzyme is frequently found in bacteria that can grow on a broad range of gaseous and short chain alkanes (C_2-C_6). SCAM is thought to catalyze the terminal oxidation of alkanes to primary alcohol products. Bacteria that express SCAM can cometabolically degrade 1,4-dioxane at low, environmentally relevant concentrations (≤100 ppb) and have also been shown to oxidize a wide variety of chlorinated 1,4-dioxane-associated co-contaminants. It has been shown that model strains expressing other monooxygenases such as sMMO or one of several toluene-oxidizing monooxygenases can degrade high (≥50 ppm) concentrations of 1,4-dioxane. However, the activity of sMMO towards 1,4-dioxane has not been reproduced, even at the level of the purified enzyme (Hatzinger et al., 2017). The activity of the model toluene-oxidizing monooxygenases towards lower concentrations of 1,4-dioxane (≤100 ppb) also has not been confirmed.

A *q*PCR assay has been developed for many of the genes that encode the enzymes described above, and in most cases these assays are now commercially available (e.g., Microbial Insights) or can be completed by academic labs (e.g., SCAM at North Carolina State University in Michael Hyman's research lab).

In the case of 1,4-dioxane, collecting data on multiple gene targets may be useful. For example, the term propane monooxygenase has been widely used in the literature and was historically used to generically describe any undefined propane-oxidizing monooxygenase. More recently, two distinctly different enzymes have been referred to as propane monooxygenase. One of these enzymes is SCAM. The second enzyme is found in a wide diversity of hydrocarbon-oxidizing bacteria including organisms that can grow on substrates including methane, non-methane alkanes, alkenes (e.g., propene or isoprene), MTBE, and even 1,4-dioxane. Unlike SCAM, this enzyme (PrMO) has a restricted substrate range and is thought to sub-terminally oxidize propane to 2-propanol. Although expression of PrMO can enable some bacteria to grow on propane (and potentially ethane and *n*-butane), the only contaminants unequivocally known to be degraded by this enzyme are NDMA and phenol. This enzyme is encoded by the *prm*ABCD gene cluster and can be quantified qPCR using the PPO assay. The dramatically different catalytic capabilities of PrMO and SCAM justifies a nomenclature that distinguishes these two enzymes, especially as genome analyses now indicate that many gaseous alkane-oxidizing bacteria possess genes that encode both PrMO and SCAM. Consequently, qPCR-based analyses demonstrating changes in the abundance of one of these genes can potentially also exhibit quantitatively equivalent changes in the other. This issue of multiple monooxygenases within a single organism also extends to bacteria such as *P. dioxanivorans* CB1190 that also possess genes encoding PrMO in addition to genes encoding THFMO/DXMO.

In addition to the enzymes discussed above, there are a number of other biomarkers that are potentially useful for understanding the environmental fate of 14D. For example, an aldehyde dehydrogenase (Aldh) has been identified as a secondary biomarker 14D biodegradation by THFMO-expressing strains such as *P. dioxanivorans* CB1190.

These gene assays have proven useful in establishing correlations between biomarkers for 1,4-dioxane degradation and observed degradation rates. Li et al. (2013) found that the *thmA* gene (which encodes the THFMO enzyme) and similar genes were widespread in groundwater at a site in Alaska that was contaminated with 1,4-Dioxane. Li et al. (2015) studied 1,4-dioxane degradation in water samples from three sites in California that were contaminated with 1,4-dioxane. Biodegradation was observed in 12 of 16 microcosms. Li et al. (2013) found that there was a significant correlation between the abundance of *thmA* genes that developed in microcosm studies and the zero-order rate of degradation of 1,4-dioxane in the microcosms. These studies show that bacteria in aquifers that are contaminated with 1,4-dioxane can degrade 1,4-dioxane, and that the rate of degradation can be associated with the abundance of bacteria that have the gene for *thmA*.

Collectively, these studies help demonstrate the potential applicability of MNA for 1,4-dioxane. However, the lack of a quantitative framework to evaluate the contribution of natural attenuation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-dioxane in groundwater makes it difficult to include natural attenuation processes as part of the comprehensive remedy at a site that contains these contaminants. This represents a critical gap in our ability to effectively cleanup contaminated groundwater sites since incorporation of natural attenuation processes into the comprehensive remedy at a site should substantially reduce the cost to complete the remedy by reducing the extent of an active treatment that is required.

To build a quantitative case for MNA of these compounds, it is necessary to develop lines of evidence for attenuation of the target compounds, including demonstrating that: 1) plumes are stable and/or decreasing; 2) the mechanisms for attenuation can be identified; and 3) the attenuation rates can be estimated. This requires the use of a fate and migration model to not only estimate rates but to help differentiate between processes that contribute to reductions in contaminant concentrations. BIOCHLOR is one such model and has proven to be a popular and effective tool for evaluating rates at chlorinated solvents sites where MNA is being considered (Aziz et al., 2000). The advantages of this model are that it is available at no cost, is USEPA-approved, and simulates advection, dispersion, sorption, and biodegradation. Unfortunately, the original BIOCHLOR model does not include a module for the abiotic dehydrochlorination of 1,1,1-TCA to 1,1-DCE and the simultaneous degradation, biological or abiotic, of 1,1,1-TCA, 1,1-DCE, 1,1-DCA, vinyl chloride (VC), and chloroethane. Similarly, 1,4-dioxane degradation is not considered in the available MNA guidance, in part because it its widespread occurrence at groundwater sites was not demonstrated until relatively recently (Anderson et al., 2012; Adamson et al., 2013).

This project was aimed at developing a modified model and framework for evaluating natural attenuation of these compounds. Several approaches were used to generate input data that can support and validate the model and framework. First, rate coefficients and lines of evidence for attenuation were calculated and/or measured at multiple sites using a focused field program. Second, degradation and the associated rate constants for 1,4-dioxane at many of these same sites were definitively determined using a ^{14}C-labeled 1,4-dioxane assay developed as part of this project.

1.2. OBJECTIVE OF THE DEMONSTRATION

The overarching objectives of this project were to:

1) Develop a new decision tool for evaluating MNA for these key missing constituents that is based on:
    a. A fate and transport model (similar to USEPA's BIOCHLOR model) that can analyze historical monitoring data to extract rate constants for natural degradation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-Dioxane so that RPMs can use the rate constants to support MNA remedy evaluations.
    b. Decision matrices (flowcharts) that can be used by RPMs to walk through how biodegradation rates can be estimated, as well as how to interpret other potential lines of evidence for MNA.
    c. An integrated platform that allows users to access both the model and the decision matrices, specifically by updating the existing BioPIC software.
2) Develop and validate a protocol to directly measure rate constants for natural biodegradation of 1,4-Dioxane using ^{14}C-labeled 1,4-Dioxane and groundwater from multiple different field sites;
3) Evaluate existing *q*PCR assays for organisms and enzymes involved in the biodegradation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-Dioxane by comparing the density of gene copies to the rate constants for removal of the contaminants at field scale as extracted from monitoring data or the rate constants for 1,4-Dioxane degradation as determined by the ^{14}C assay; and
4) Use data collected from multiple field sites to establish if there is consistency between various lines of evidence for 1,4-dioxane attenuation.

In addition, the project team completed an evaluation of a historical 1,4-dioxane monitoring dataset to better understand how analytical method selection and sensitivity have influenced trends in 1,4-dioxane detection frequency and the implications for site identification.

1.3. REGULATORY DRIVERS

The chlorinated solvents that were investigated as part of this project are heavily regulated at the state and federal levels. As a result, site managers have considerable motivation to address groundwater contaminated with compounds such as 1,1,1-TCA and several of its by-products (including federal MCLs for 1,1,1-TCA, 1,1-DCE, cis-DCE, and VC).

In contrast, the regulatory status for 1,4-dioxane is still evolving. USEPA has yet to establish maximum contaminant levels (MCLs) for 1,4-dioxane in drinking water. It is considered a probable human carcinogen, but the occurrence data was relatively limited until 1,4-dioxane was included in the third round of USEPA's Unregulated Contaminant Monitoring Rule (UCMR3). This involved testing of drinking water from approximately 5,000 public water systems in the U.S. between 2013 and 2015. The results indicated that 1,4-dioxane was detected in at least one sample from 21% of these systems, and that 6% of these systems had at least one sample that was above the USEPA health-based reference level of 0.35 µg/L (Adamson et al., 2017).

USEPA has been using this occurrence data to support regulatory determinations for 1,4-dioxane in drinking water. In early 2020 as part of a preliminary regulation determination on several compounds on the Candidate Contaminant List-4 (CCL4), USEPA decided against making a preliminary determination on 1,4-dioxane. Despite this, several states have begun to issue health-based advisory and/or cleanup levels for 1,4-dioxane in water. For example, New York set an MCL of 1 μg/L for 1,4-dioxane in July 2020. Other states have enforceable standards for groundwater and/or drinking water, with Massachusetts currently the strictest of any states (0.3 μg/L).

In light of these regulatory drivers, the DoD has been active in understanding potential liabilities associated with 1,4-dioxane and its common co-contaminants at their facilities. This includes characterization and remediation efforts at many sites. These efforts have been supported by a number of research projects funded through SERDP and ESTCP over the past 5 years, as well as projects funded directly by the branches (e.g., AFCEC, NAVFAC). Because of this investment, the DoD is now well-positioned to benefit from the decision matrices and other products of this project. Application will increase the number of sites where MNA is implemented, and as a result, will reduce capital and O&M expenses and minimize impacts associated with invasive remediation options.

2.0 TECHNOLOGY

2.1. TECHNOLOGY DESCRIPTION

The methodologies for this project are patterned after several ESTCP projects, including the decision matrix used to develop BioPIC, the ^{14}C-labeled TCE assay used to confirm degradation of TCE, identification of *q*PCR targets that can serve biomarkers for degradation, and the development of CSIA methods for 1,4-dioxane. These are discussed in more detail below. To facilitate regulatory approval, the logic was built around a "Lines of Evidence for MNA" approach (USEPA, 1999). This included three main approaches:

Collection and Analysis of Field Data: A focused field program was used to collect data at several sites. The work involved the coordination with site managers and may benefit their efforts to obtain site closure for these sites. At each site, field work was generally scheduled to coincide with routine groundwater sampling events completed by on-site contractors. Groundwater samples were collected from a minimum of 4 monitoring wells per site and analyzed in the field for standard geochemical parameters (dissolved oxygen, ORP, pH, temperature, specific conductance, and Fe(II)). Groundwater samples were also collected and sent to Clemson University for the ^{14}C-labelled 1,4-dioxane assay and for analysis of concentrations of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-dioxane, and to Microbial Insights (http://www.microbe.com) for analysis of *q*PCR markers, and to the University of Waterloo for quantification of specific isotopic ratios (^{2}H and ^{13}C).

Rate constants for degradation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and/or 1,4-dioxane were also estimated using data at field sites. These rates were extracted by calibrating a fate and transport model to field data and empirically determining the values of the rate constants that provide the best fit of the model projection to the actual field data (i.e., concentration vs. distance in monitoring wells located in the direction of groundwater flow). The field data will include historical data and data collected as part of the work completed specifically for this project.

A full description of the sampling and analysis program is provided in Section 5.

Bench-Scale ^{14}C Assays: To provide an independent estimate of degradation rates for 1,4-Dioxane, water samples from field sites were incubated with ^{14}C-labeled 1,4-dioxane as part of a method specifically developed by Clemson University for this project. The Clemson research group has extensive experience with use of ^{14}C-labeled compounds, including purification of commercially synthesized 1,4-dioxane by high performance liquid chromatography (HPLC).

The ^{14}C method for 1,4-dioxane was adapted from a protocol for ^{14}C-TCE that was successfully validated as part of ESTCP Project ER-201584 (Wiedemeier et al., 2017). That method entails adding purified ^{14}C-TCE to sealed serum bottles (160 mL) containing groundwater (100 mL). Aqueous samples are removed over time to analyze for the presence of degradation products. The sample pH is raised above 10 to retain CO_2 and then sparged with N_2 to remove residual TCE. In addition to retaining CO_2, soluble degradation products that are non-volatile at an alkaline pH level are also quantified. This method has been applied to different TCE degradation processes (Mills et al., 2018; Yu et al., 2018) and proven to be sensitive enough to detect pseudo first-order rates as low as $7x10^{-4}$ year (equivalent half-life of approximately 1,000 year).

A similar approach was employed for this project using ^{14}C-1,4-Dioxane. However, since 1,4-Dioxane is essentially non-volatile, it cannot be separated from degradation products by stripping samples with N_2. Separation of the unreacted 1,4-Dioxane from its degradation products was accomplished by a combination of acidic stripping with N_2 and HPLC. The extent of 1,4-Dioxane degradation was then monitored as the accumulation of degradation products by collecting samples at predetermined intervals (several hours to several days) and analyzing the distribution of ^{14}C soluble products and $^{14}CO_2$. The total amount of ^{14}C products formed was calculated and used to ascertain pseudo first-order rates of 1,4-Dioxane transformation. A full description of these methods can be found in Section 5.6 of this report.

Decision Framework and Software Development: Under this task, a simple Microsoft Excel spreadsheet model was developed that can be used to simulate the transport and degradation of TCA and its degradation products (including 1,1-DCA, 1,1-DCE, and VC), and 1,4-dioxane. These compounds are included because they are missing from existing, similar modeling tools (e.g., BIOCHLOR). However, for completeness, this model also includes a module for chlorinated ethenes. This model allows users to estimate a first-order biodegradation rate constant based on curve fitting to existing field data along the flow path and centerline of the constituent plume. Data required for this curve fitting include historical concentration data for the target constituents along the flow path and aquifer hydrogeologic data including hydraulic conductivity, porosity, hydraulic gradient, bulk density, and fraction of organic carbon. Note that this approach is identical to that used to estimate degradation rates for ESTCP Project ER-201129, *Selection of Bioremediation Approaches: Development and Validation of a Quantitative Framework and Management Expectation Tool for the Selection of Bioremediation Approaches (Monitored Natural Attenuation [MNA], Biostimulation and/or Bioaugmentation) at Chlorinated Ethene Sites*.

In addition, separate decision matrices were developed for the degradation pathways for 1,4-Dioxane and 1,1,1-TCA and its degradation products including 1,1-DCA and 1,1-DCE. Once completed, an easy-to-use software package that automates the use of the decision matrices was developed. This was accomplished by updating an existing Microsoft Excel spreadsheet application for evaluating chlorinated ethenes, the Biological Pathway Identification Criteria (BioPIC), so that it includes these new decision matrices for 1,4-dioxane and the chlorinated ethanes.

Parameters that were used to develop the new decision matrices include degradation rates, stable isotope data (i.e., CSIA) for qualitative evidence that degradation has occurred, potential *q*PCR markers for 1,4-dioxane degradation (THFXO/DXMO, ALDH, sMMO, RMO, RDEG, PHE, and others), potential qPCR targets for chlorinated solvent degradation (*Dehalococcoides* (DHC), and *Dehalobacter* (DHBt) abundance, 1,1-DCA reductase (DCA) abundance), and the biogeochemical parameters including dissolved oxygen, Fe(II), and methane. A short rationale for each is provided below:

- ***1,4-dioxane biodegradation rates:*** Two primary methods were used to estimate rates constants. The first was the bench-scale ^{14}C assay described above, which was developed as part of this project to well-specific first-order biodegradation rate constants. The second was applying a groundwater fate and transport model developed specifically for this project to predict the biodegradation rate from site data. Specifically, this model simulates

advection, dispersion, and sorption and then allows the user to fit the data to obtain an estimate of the site-wide biodegradation rate constant.

- ***CSIA data:*** A proposed line of evidence for 1,4-dioxane attenuation is the site-specific analysis of samples for stable isotopes of carbon and hydrogen (Bennett et al., 2018). This is based on the assumption that the isotopic ratios for these two elements (e.g., the ratio of $^{13}C/^{12}C$, typically expressed as $\delta^{13}C$ in units of per mil) will increase due to preferential degradation of the lighter isotope. Data from a single sample is unlikely to provide evidence for 1,4-D biodegradation. This is because there is significant variability in the known isotopic composition of undegraded 1,4-dioxane sources, as well as data from a recently completed SERDP project (ER-2535: PI Bennett) that suggests that these known source compositions do not represent the full range that might be encountered at contaminated sites. As a result, any attempt to establish biodegradation by comparing the isotopic composition of a groundwater sample to known source compositions (similar to the CSIA approach described for chlorinated ethenes) is subject to considerable uncertainty for 1,4-D and unlikely to serve as a convincing line of evidence at this time. Collecting multiple samples along the groundwater flow path is a more appropriate approach because it relies on site-specific isotopic data to document 1,4-D degradation. Note that the reported hydrogen isotope enrichment factors for various 1,4-dioxane degradation processes are higher than those for the carbon isotope (Bennett et al., 2018), but the $\delta^{2}H$ range measured in product samples (as well as environmental samples) also vary over a wider range than those for $\delta^{13}C$ (SERDP ER-2535 Phase II report).
- ***Biomarkers for 1,4-dioxane metabolism:*** As described in Section 1.1, genes that encode for a tetrahydrofuran monooxygenase (referred to as THMXO or DXMO depending on the source) and aldehyde dehydrogenase (ALDH) have been shown to metabolize 1,4-dioxane as part of a growth supporting biodegradation reaction. As such, these biomarkers serve as a possible line of evidence for the presence of metabolic 1,4-dioxane degraders. Positive relationships between 1,4-dioxane biodegradation capacity and abundance of these genes have been established using field samples from 1,4-dioxane sites (e.g., Li et al., 2013; Li et al., 2014; Gedalanga et al., 2016). As a result, qPCR assays that can quantify the abundance of these genes (i.e., DNA-based) are commercially available, as well as qPCR assays that can quantify the abundance of the gene transcript (RNA-based) as a more definitive indicator that these enzymes are active. However, it is important to note that genes encoding these monooxygenases are not necessarily confined to those that can metabolize 1,4-dioxane in a growth-supporting reaction.
- ***Biomarkers for 1,4-dioxane cometabolism (co-oxidation):*** As described in Section 1.1, this includes a variety of genes that encode monooxygenases that have been implicated as potentially able to degrade 1,4-dioxane as part of cometabolic process (also known as co-oxidation, where 1,4-dioxane is a fortuitously degraded cosubstrate by organisms that use another compound as a primary growth substrate). For this project, the focus was on those biomarkers that could be quantified at commercial labs (RMO, RDEG, SMMO, PHE) using qPCR assays that focus on the functional gene as well as the RNA transcript. However, there is some debate about the role that these non-specific enzymes play in 1,4-dioxane degradation (e.g., Hatzinger et al., 2017), particularly in situ under natural conditions. To our knowledge, attempts at correlating the presence of these genes with in situ 1,4-dioxane biodegradation rates have been limited (Sadeghi et al., 2016). Two

additional gene targets were quantified at academic labs (SCAM at North Carolina State University; *prmA* gene at New Jersey Institute of Technology). Note that *prmA* encodes for propane monooxygenases that may be capable of cometabolism and/or direct metabolism of 1,4-dioxane.

- ***Biomarkers for chlorinated solvent metabolism:*** A variety of biomarkers for chlorinated solvent degradation were included (DHC, DHBt, and DCA) to: 1) serve as evidence for whether or not these compounds were expected to degrade; 2) to help establish if anoxic conditions were present. While these may not be normally seen as positive lines of evidence for 1,4-dioxane biodegradation, biodegradation of chlorinated solvents may help alleviate any inhibitory effects.
- ***Geochemical parameters (dissolved oxygen, methane, ethene/ethane, dissolved iron):*** Dissolved oxygen (and the oxidation-reduction potential) are bulk field measurements that would be expected to correlate with 1,4-dioxane activity (Adamson et al., 2015; da Silva et al., 2017). The other dissolved gases (methane, ethene, and ethane) would be more associated with anaerobic activity, but they would also suggest the presence of robust microbial population and lessens concerns about other factors (e.g., pH, metals) that might otherwise inhibit multiple types of microbial activity. Similarly, dissolved iron (Fe(II)) is formed through microbial iron reduction under anaerobic conditions, but Fenton-type reactions that rely on Fe(II) have also been shown to degrade 1,4-dioxane (Sekar and DiChristina, 2014).

Finally, the field sites included in this project served as benchmark sites to try to establish the relationship between the rate constants for 1,4-Dioxane degradation and the abundance of *q*PCR markers. A similar approach was described in Wilson et al. (2019) for cometabolism of TCE. For this project, two different methods were investigated. One focused on developing simple analytical expressions that predicted the expected rate constant using lab-derived kinetic parameters for the functional genes associated with these specific markers, and then comparing these to the degradation rates measured from field samples. The second used the biomarker data from sites where these were detected. Specifically, the abundance of specific biomarkers (e.g., genes that encode THFXO/DXMO and ALDH) was compared to the first-order rates of degradation of 1,4-Dioxane in the ^{14}C assays to see if a statistically significant correlation could be developed and use to predict the expected rate constant. In cases where the association is acceptable, this serves as a secondary line of evidence for MNA, and it would support MNA evaluations at other sites by helping predict and/or explain biodegradation rates. The goal was to build these approaches into the decision tool (BioPIC) as part of this project.

In a parallel effort, an evaluation of trends in 1,4-dioxane analytical method selection and sensitivity was performed to better understand if the data quality is sufficient for identifying and evaluating sites where this compound is present. A summary of key findings is provided here since the results have already been published (Adamson et al., 2021) and it did not rely on the data from the project field program:

- The dataset consisted of public sampling records from 2000 to 2019 that included > 106,000 analyses of 1,4-dioxane from 822 different U.S. sites.

- A clear shift towards using Method 8270 and Method 8270 SIM for 1,4-dioxane analysis was observed. This is despite the biases that can accompany the use of these semi-volatile methods due to poor extraction efficiencies. Method 8270 and Method 827 SIM exhibited better sensitivity based on significantly lower reporting limits relative to those observed for Method 8260 in particular.
- 1,4-Dioxane concentrations at contaminated sites are typically low, with a median detected concentration of 10 μg/L at the set of sites that were evaluated in this effort. This resulted in higher detection frequencies for the methods with lower reporting limits, and it has significant implications when these methods are used to identify 1,4-dioxane contaminated sites.
- The results indicate that 1,4-dioxane is unlikely to be present at the majority of sites where it was analyzed but not detected, but that these sites have typically relied on methods with less sensitivity, such that there is the potential for false negatives.
- Even when focusing only on data collected in the past 2 years, the reporting limits achieved by the existing methods are still generally higher than recently issued regulatory standards. This means that efforts to identify and characterize 1,4-dioxane in the environment will continue to face challenges unless improved methods become available.

2.2. ADVANTAGES AND LIMITATIONS OF THE TECHNOLOGY

The proposed technology couples a series of detailed site assessments with an improved decision framework for managing sites with chlorinated solvents and 1,4-dioxane. The data and the framework will be applicable to the field sites that are part of this demonstration, as well as other sites with similar co-contaminant issues. The advantages and limitations of this approach can be evaluated in terms of its ease of use, applicability, and the knowledge gained following implementation. **Table 2.1** summarizes the advantages and disadvantages within this context.

Table 2.1. Advantages and Potential Limitations of the Technology

Advantages	Limitations
Radiolabeled ^{14}C assay provides unequivocal line of evidence of 1,4-dioxane attenuation and preliminary estimate of rate	Collection of non-conventional data (e.g., biomarkers, isotopes) may be difficult and/or cost-prohibitive for some sites *(note that project-generated products can still be used without collecting these types of data)*
Using the decision framework requires no permanent installations or long-term monitoring obligations for a site	Provides support for MNA remedy selection but will not decrease remediation timeframe unless alternative remedy is selected
Using the decision framework requires data collected using conventional methods (i.e., groundwater sampling from monitoring wells)	
Products are publicly available, free, and use platforms that are familiar to most users (Excel)	
Provides guidance on remedy selection	
Improvement over existing methods that omit chlorinated ethanes and 1,4-dioxane	

3.0 PERFORMANCE OBJECTIVES

For the purposes of evaluating the performance of the field demonstration, the following performance objectives for this phase of the project were developed (**Table 3.1**). Task numbers listed in **Table 3.1** refer to the tasks in the original proposal. Note that slight modifications to the Performance Objectives listed in the project Demonstration Plan have been made to reflect changes in the scope.

Table 3.1. Summary of Performance Objectives

Performance Objective	Data Requirements	Success Criteria	Performance Objective Met?
Modify **BIOCHLOR** *(or develop new but similar fate and transport model)* to account for the biological degradation of 1,4-dioxane (Task 6).	None	The model is able to mathematically describe the physical migration (advection, dispersion, and sorption) and first-order biodegradation of 1,4-dioxane.	Yes
Modify **BIOCHLOR** *(or develop new but similar fate and transport model)* to account for the biological and abiotic degradation of 1,1,1-TCA and its transformation products (Task 6).	None	The model is able to mathematically describe the physical migration (advection, dispersion, and sorption) and the biological degradation of 1,1,1-TCA to 1,1-DCA and then the subsequent biodegradation of 1,1-DCA to chloroethane and ethane, as well as the abiotic degradation of 1,1,1-TCA to acetic acid and 1,1-DCE, and then the subsequent biological degradation of 1,1-DCE to vinyl chloride, and the subsequent biological degradation of vinyl chloride to ethene.	Yes
Development of a quantitative decision matrix to elucidate degradation pathways and select the most efficacious remediation approach for 1,4-dioxane (Task 6)	Historical data on 1,4-dioxane concentrations. Historical biogeochemical data (e.g., dissolved oxygen, Fe[II], methane, ORP, etc.). Site-specific hydrogeologic parameter values. Rate constants developed under Task 2. *q*PCR data obtained from field sites.	Use of the decision matrix leads to a transparent and credible evaluation of MNA based on the site-specific data that are available. If MNA is not feasible, the decision matrix will identify a concentration of 1,4-dioxane that must be attained in the source for MNA to be feasible.	Partially

Table 3.1. Summary of Performance Objectives (continued)

Performance Objective	Data Requirements	Success Criteria	Performance Objective Met?
Development of a quantitative decision matrix to elucidate degradation pathways and select the most efficacious remediation approach for 1,1,1-TCA, 1,1-DCA, and 1,1-DCE (Task 6)	Historical data on 1,1,1-TCA, 1,1-DCA, and 1,1-DCE concentrations. Historical biogeochemical data (e.g., dissolved oxygen, Fe[II], methane, ORP, etc.). Site-specific hydrogeologic parameter values. Rate constants developed under Task 2. *q*PCR data obtained from field sites.	Use of the decision matrix leads to a transparent and credible evaluation of MNA based on the site-specific data that are available. If MNA is not feasible, the decision matrix will identify a concentration of 1,1,1-TCA, 1,1-DCE and 1,1-DCA that must be attained in the source for MNA to be feasible.	Yes
Ease of use of new version of BioPIC (Task 6)	Experience with RPMs in beta testing	RPMs can successfully apply the method to their own sites.	Incomplete*

Table 3.1. Summary of Performance Objectives (continued)

Performance Objective	Data Requirements	Success Criteria	Performance Objective Met?
Validate rate constants for 1,4-dioxane degradation at up to 10 study sites, using a ^{14}C-assay conducted with groundwater from the sites (Task 4 and 5).	Quantity of ^{14}C label in carbon dioxide and other polar degradation products after various periods of incubation	If 0.01% of the 1,4-dioxane is oxidized in the assay, it will be detected at 95% certainty.	No
		Impurities accounted for < 0.1% of total dpm added to microcosms and did not influence rate determination	Yes
		Rates meaningful for MNA (e.g., long half-lives > 10 yr) could be quantified	Yes
		Two different methods for quantifying rate constants yielded statistically similar results	Yes
		80% confidence interval on the rate constant for cometabolism of 1,4-dioxane is ≤ 50% of the rate constant.	Yes (18 of 24 cases)
Determine whether aerobic biological cometabolism or biological degradation of 1,4-dioxane explains the bulk rate constant for attenuation of 1,4-dioxane at field scale.	Model-predicted rate constants for attenuation of 1,4-dioxane extracted from field data and rate constants for cometabolism of 1,4-dioxane determined by the ^{14}C-assay.	Aerobic biological cometabolism will be considered to explain the bulk rate constant if the model-predicted rate constant is contained within the 95% confidence interval of the rate constant obtained from the ^{14}C assay for cometabolism ***OR*** if ^{14}C rate constant is larger than model-predicted rate constant	Partially (3 of 9 sites met)

3.1 PERFORMANCE OBJECTIVE #1: Modify BIOCHLOR (or develop new but similar fate and transport model) to Account for Biological Degradation of 1,4-Dioxane

The original objective was to update the existing modeling package **BIOCHLOR** so that it would include 1,4-dioxane. Due to limitations in the existing BIOCHLOR code, the project team later decided to develop a new software (titled the "**MNA Rate Constant Estimato**r") to perform the same functions using a slightly different code and an improved interface.

3.1.1 Data Requirements

There are no specific data requirements. The software can be updated and checked using default and/or dummy values for the various input parameters.

3.1.2 Success Criteria

The objective was considered achieved if the new model (MNA Rate Constant Estimator) performs the operations and calculations and provides output as described above in a manner that is adequate to screen sites to understand natural attenuation of 1,4-dioxane. In addition, relevant processes for 1,4-dioxane attenuation must be incorporated into the model.

3.1.3 Outcome

The success criteria for this performance objective were achieved.

The model contains input parameters for the relevant hydrogeologic characteristics and chemical concentrations, with default parameters that are appropriate for 1,4-dioxane. The model will predict the concentration of 1,4-dioxane with distance along the flow path in the aquifer (the prediction line). The model can compute the root mean square error (RMSE) between the prediction line and the concentrations of 1,4-dioxane in the monitoring wells at the site being simulated. The user uses trial and error to determine if there are other values for the rate constant that provide a better fit based on a visual comparison of a chart that compares the prediction line to the field data and by minimizing the RMSE. This will allow the user to put more weight on data points that in their professional judgement deserve more weight. Examples would include wells that are closer to sentry wells, or wells that are closer to the centerline of the plume, or wells with a screened interval that is more representative of the depth interval occupied by the plume in the aquifer.

The code was upgraded to work effectively in the most recent versions of the Microsoft Excel application. BIOCHLOR has some compatibility issues with recent versions of Excel, and these are generally minimized in the new software. Other new features include a source decay term (k_{point}) to provide more realistic simulations, and a button that allows users to choose when calculations are performed to reduce wait times during model calibration.

3.2 PERFORMANCE OBJECTIVE #2: Modify BIOCHLOR (or develop new but similar fate and transport model) to Account for Biological and Abiotic Degradation of 1,1,1-TCA and its Transformation Products

As described in the previous performance objective, the project team decided to develop a new software (titled "MNA Rate Constant Estimator") to model a larger suite of compounds. This included chlorinated ethanes such as 1,1,1-TCA. BIOCHLOR can only model the sequential degradation of organic compounds along a single pathway. In the case of BIOCHLOR this is sequential reductive dechlorination of chlorinated ethenes. The new software will simultaneously model the degradation of 1,1,1-TCA by two pathways.

3.2.1 Data Requirements

There are no data requirements. The software can be updated and checked using default and/or dummy values for the various input parameters.

3.2.2 Success Criteria

The new model (MNA Rate Constant Estimator) performs the operations and calculations and provides output as described above in a manner that is adequate to screen sites to understand natural attenuation of key constituents using the relevant pathways. 1,1,1-TCA. The model is able to mathematically describe the physical migration (advection, dispersion, and sorption) and relevant attenuation mechanisms.

3.2.3 Outcome

The success criteria for this performance objective were achieved.

Both of the relevant pathways for 1,1,1-TCA attenuation were incorporated into the new model. In the first pathway, 1,1,1-TCA will degrade to 1,1-DCA and 1,1-DCA will degrade to chloroethane by sequential reductive dechlorination, and chloroethane will degrade to ethane by reductive dechlorination. In the second pathway, 1,1,1-TCA will degrade to 1,1-DCE by an abiotic elimination reaction and to acetic acid by an abiotic hydrolysis reaction. 1,1-DCE will also degrade by two pathways, biological reductive dechlorination to vinyl chloride. Finally, vinyl chloride will degrade via one primary pathway, specifically biological reductive dechlorination to ethene. Relevant abiotic pathways mediated by reactive minerals were not included because a rate correlation for this pathway could not be developed.

To simulate attenuation via these two pathways, the model will accept rate constants for (1) the biological transformation of 1,1,1-TCA to 1,1-DCA, (2) the biological transformation of 1,1-DCA to chloroethane, (3) the biological transformation of 1,1-DCE to vinyl chloride, and (4) the biological transformation of vinyl chloride to ethene. The model will accept the temperature of the groundwater to estimate the rate constant for abiotic transformation of 1,1,1-TCA to acetic acid and the rate constant for abiotic transformation of 1,1,1-TCA to 1,1-DCE.

The model will accept initial concentrations of 1,1,1-TCA at the source and the rate constants for attenuation of 1,1,1-TCA in the source over time (k_{point}).

The model output will provide projected concentrations of 1,1,1-TCA, 1,1-DCE, 1,1-DCA, chloroethane, vinyl chloride, and ethene at each well along the flow path. As described above for 1,4-dioxane, the model can then be used to fit values for the rate constants for each of the relevant reactions by visual inspection and minimizing the root mean square error (RMSE) between the prediction lines and the corresponding concentrations of (1) 1,1,1-TCA, (2) 1,1-DCA, (3) 1,1-DCE, and (4) vinyl chloride in the monitoring wells at the site being simulated.

The code will work in the most recent versions of the Excel spreadsheet, as described above for 1,4-dioxane.

3.3 PERFORMANCE OBJECTIVE #3: Development of a quantitative decision matrix to elucidate degradation pathways and select the most efficacious remediation approach for 1,4-Dioxane

The decision matrix is designed to determine if Monitored Natural Attenuation (MNA) can provide a remedy at the site. If MNA is not appropriate, it may be necessary to implement an active remedy. In that case, the MNA Rate Constant Estimator should be able to predict the reduction in concentrations of 1,4-dioxane at the source that are necessary to ensure that the maximum concentrations of contaminants at the point of compliance are less than the regulatory standards.

3.3.1 Data Requirements

Data are required to appropriately calibrate the model includes the hydraulic gradient at the site, the hydraulic conductivity, the distribution of wells in space downgradient of the source, and the concentrations of 1,4-dioxane in groundwater from the wells. There are other calibration parameters that are usually assumed instead of being measured. These include the effective porosity of the aquifer matrix and the dispersion coefficient. Sorption can be estimated from an assumed value for the fraction of organic carbon in the aquifer matrix, but often the fraction of organic carbon is determined on core samples from the aquifer.

Only aerobic organisms have been consistently documented to be able to degrade 1,4-dioxane. Data on the geochemistry of the site, including concentrations of dissolved oxygen, Fe(II), methane, and the oxidation reduction potential (ORP) will be used to evaluate the supply of dissolved oxygen necessary to support aerobic biodegradation of 1,4-Dioxane.

The bulk field scale rate constants extracted from the field data using the fate and transport model was verified and supported by comparison to the rate constants for aerobic biological cometabolism of 1,4-Dioxane produced from the ^{14}C assays of 1,4-dioxane biodegradation.

In addition, biomarker data were compiled. This includes qPCR biomarkers for direct biodegradation of 1,4-Dioxane, such as dioxane monooxygenase (DXMO) and aldehyde dehydrogenase (ALDH). The qPCR markers for cometabolism of 1,4-Dioxane included soluble methane monooxygenase (sMMO), ring hydroxylating toluene monooxygenase (RMO and RDEG), phenol hydroxlyase (PHE), and short-chain alkane monooxygenase (SCAM). In addition, samples were analyzed for the *prmA* gene that encodes for propane monooxygenase that are

capable of direct metabolism and/or cometabolism of 1,4-dioxane. Both sets of biomarkers were used to determine if correlations exist with model and/or ^{14}C derived 1,4-dioxane rate constants.

3.3.2 Success Criteria

The success criteria for the projections of the model are defined by the goals for MNA that apply to the particular site under consideration. As a result, the ability of a specific site to demonstrate the necessary lines of evidence for MNA to be acceptable are beyond the control of this project team.

Instead, an alternative approach for evaluating model performance in supporting MNA remedy evaluation was developed. First, the user must have to ability to make projections from the MNA Rate Constant Estimator that will indicate whether the site-specific goals will be attained. If so, then MNA is a plausible remedy for 1,4-dioxane contamination at the site. If not, then the user must have the means to estimate: (1) if the plume is stable, such that increased source decay may eventually decrease concentrations below necessary thresholds at the point of compliance; and/or (2) the extent of source remediation that would be necessary to ensure that MNA could be used for (post-treatment) long-term management of the site.

A parallel criterion was also used to evaluate these data. This involved creating scatter plots to compare the first-order rates of degradation of 1,4-dioxane in the ^{14}C assays to the abundance of the DXMO and ALDH markers in groundwater from the benchmark sites. If the association is acceptable, these plots were included in the decision matrix. At a new site under evaluation, if the abundance of the qPCR markers and the bulk rate constant for degradation plots within the same area occupied by the benchmark sites, then the scatter plots will be used to provide an additional third line of evidence to support the bulk rate constant for 1,4-dioxane degradation at the site under evaluation.

3.3.3 Outcome

The success criteria for this performance objective were partially achieved.

The general logic in the decision matrices follows the logic developed for PCE, TCE, cis-DCE and vinyl chloride in ESTCP project ER-201129 (Lebrón et al., 2015). The new elements developed as part of this project includes decision matrices for different compounds classes (chlorinated ethanes and 1,4-dioxane) as well as the fate and transport model which can be used to support the MNA assessment. These elements were all successful incorporated into the project deliverables.

In the case of 1,4-dioxane, the decision matrix and the model include the targeted lines of evidence, the relevant input parameters, and the ability to calibrate field data to obtain a rate constant and make projections about plume trends. During calibration, the user enters historical data on concentrations of 1,4-dioxane, and a rate constant for degradation of 1,4-dioxane can be estimated using the model. This is accomplished by entering an initial guess of the rate constant (using the results of a ^{14}C biodegradation assay, if available) and then using the mean square error of the fit to the projection line to evaluate the rate constant that provides the best fit. The user can then apply a succession of smaller values for the rate constant in the model to determine the minimum value of the rate constant that will still meet the objectives for MNA at the site. (i.e., achieving a goal

concentration by the point of compliance). The calibrated model can be run forward in time to determine whether concentrations of 1,4-dioxane will consistently meet the goals established for the site and MNA can appropriately be considered for a remedy.

If the calibration shows that MNA will not achieve these goals, then the model can still be used to directly support additional remedial decision-making. In these cases, the user can simply incorporate source decay and/or reduce the source concentration in the model and then see how these impact the predicted plume concentrations. This can be a simple iterative process, whereby the user lowers the source concentration until there is confidence that the concentration goal at the downgradient point of compliance will be achieved. This source concentration is then compared to the current source concentration to determine the percent source reduction needed to achieve the remedial goal. The user than must evaluate possible source treatment options based on an assumption that that percent reduction is the minimum that must be achieved.

Two other lines of evidence for 1,4-dioxane attenuation are also incorporated into the project deliverables. Compound Specific Isotope Analysis (CSIA) can be used to determine if the isotopes of hydrogen and carbon have been fractionated in 1,4-dioxane in the more downgradient portions of the plume. Fractionation is expected from biodegradation of 1,4-dioxane. The results of the CSIA analysis of 1,4-dioxane for H and C can successfully support the evaluation of MNA at sites where the values for $\delta^{2}H$ or $\delta^{13}C$ in 1,4-dioxane down-gradient of the source exceed the values for 1,4-dioxane in the source by more than the uncertainty in the determination of the values. Secondly, the groundwater will be assayed for the abundance of DNA that codes for known enzymes that degrade 1,4-dioxane. The presence of these functional genes indicates that organisms are present in the groundwater that have the capacity to degrade 1,4-dioxane if the enzymes are expressed.

However, the attempts at correlating biomarker abundance to the observed 1,4-dioxane biodegradation rate constants were unsuccessful because: (1) biomarkers for direct oxidation were only detected at one of ten sites and thus did not provide sufficient data quality; and (2) biomarker correlations using lab-derived kinetic parameters generally underpredicted the rate constants that were generated from the model calibrations of field data (see Section 5.8).

3.4 PERFORMANCE OBJECTIVE #4: Development of a quantitative decision matrix to elucidate degradation pathways and select the most efficacious remediation approach for 1,1,1-TCA, 1,1-DCA, and 1,1-DCE

The decision logic for 1,1,1-TCA, 1,1-DCA and 1,1-DCE is similar to that used for 1,4-Dioxane, except that it involves slightly different lines of evidence and all three compounds must meet the goals for the site. The decision matrix is designed to determine if Monitored Natural Attenuation is a viable remedial option at the site. If MNA is not appropriate, it may be necessary to implement an active remedy. In that case, the MNA Rate Constant Estimator should be able to predict the reduction in concentrations of these compounds at the source that are necessary to ensure that the maximum concentrations of contaminants at the point of compliance are less than the regulatory standards.

3.4.1 Data Requirements

The data requirements for calibrating the model for these compounds are the same as described for 1,4-dioxane model, except the model will require concentrations of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and vinyl chloride. The model will require the same geochemical data, but the interpretation will be different. Dissolved oxygen is detrimental to the bacteria that carry out biological reductive dechlorination. The geochemical data can be used to establish that dissolved oxygen is not present in the groundwater and reductive dechlorination is favorable.

Rate constants for biological degradation of 1,1,1-TCA to 1,1-DCA and 1,1-DCA to chloroethane can be extracted by calibration of the model to the field data. This sequence of degradation is carried out by certain strains of bacteria in the genus *Dehalobacter*. Data on the abundance of DNA amplified by qPCR primers for *Dehalobacter* (DHBt) and for the 1,1-DCA reductase enzyme (DCA) were also compiled.

3.4.2 Success Criteria

The success criteria for the projections of the model are defined by the goals for MNA that apply to the particular site under consideration. As noted above, the ability of a specific site to demonstrate the necessary lines of evidence for MNA to be acceptable are beyond the control of this project team.

Instead, an alternative approach for evaluating model performance in supporting MNA remedy evaluation was developed. First, the user must have to ability to make projections from the MNA Rate Constant Estimator that will indicate whether the site-specific goals will be attained. If so, then MNA is a plausible remedy for 1,1,1-TCA, 1,1-DCA, and 1,1-DCE contamination at the site. If not, then the user must have the means to estimate: (1) if all plumes are stable, such that increased source decay may eventually decrease concentrations below necessary thresholds at the point of compliance; and/or (2) the extent of source remediation that would be necessary to ensure that MNA could be used for (post-treatment) long-term management of the site.

3.4.3 Outcome

The success criteria for this performance objective were achieved.

The general logic in the decision matrices follows the logic developed for PCE, TCE, DCE and vinyl chloride in ESTCP project ER-201129 (Lebrón et al., 2015). This project successfully expanded the scope and added value by developing decision matrices for different compounds classes (chlorinated ethanes and 1,4-dioane) and the fate and transport model which can be used to support the MNA assessment.

In the case of 1,1,1-TCA, 1,1-DCA, and 1,1-DCE, the decision matrix and the model include the targeted lines of evidence, the relevant input parameters, and the ability to calibrate field data to obtain a rate constant and make projections about plume trends. During calibration, the user enters historical data on concentrations of each compound, and the individual rate constants can be estimated using the model. The goal is to use mean square error of the fit to the concentration vs. distance data to evaluate the rate constants that provides the best fit. The user can then apply a

succession of smaller values for the rate constant in the model to determine the minimum value of the rate constant that will still meet the objectives for MNA at the site. (i.e., achieving a goal concentration by the point of compliance). Source decay may be required, particularly for 1,1,1-TCA since it is readily degradable by both abiotic and biotic pathways. The MNA Rate Constant Estimator provide a source decay parameter that can be further adjusted to improve fit. Projection over time can be performed following calibration to establish if concentrations of all three CVOCs will consistently meet the goals established for the site and MNA can appropriately be considered for a remedy.

Like 1,4-dixoane, the model can still be used to directly support additional remedial decision-making for these compounds even if the calibration shows that MNA will not achieve the concentration goals. In these cases, the user estimates the amount of source reduction required to ensure that the downgradient concentrations are below the required thresholds. This is done through an iterative process where the user decreasing the source concentration until the downgradient concentration goals are reached. The user can use this information to help select an active treatment method that would be expected to achieve this degree of source reduction.

CSIA and biomarker data are also incorporated into the modules for 1,1,1-TCA, 1,1-DCA, and 1,1-DCE to support MNA remedy evaluations. Fractionation during groundwater transport can serve as a secondary line of evidence for MNA, and the presence of functional genes and gene transcripts indicates that organisms are present and/or active in the groundwater that have the capacity to degrade the target compounds.

Note that predictions of the rate constant for 1,1,1-TCA, 1,1-DCA, and 1,1-DCE biodegradation based on potential biomarkers and lab-derived kinetic parameters were also developed and incorporated into the MNA Rate Constant Estimator (see **Appendix D**). However, a comprehensive comparison between these rate constants and the model-calibrated rate constants that were derived from concentration vs. distance data were not attempted (see Section 6.4).

3.5 PERFORMANCE OBJECTIVE #5: Ease of Use of New Version of BioPIC

A decision matrix is useful if the decisions are based on clear distinctions that are derived from objective criteria as opposed to subjective evaluations. As an example, the matrix will select between options based on whether the groundwater is aerobic. The objective criteria for "aerobic groundwater" will be as follows: concentrations of dissolved oxygen ≥ 0.1 mg/L, Fe(II) ≤ 0.5 mg/L and methane ≤ 0.005 mg/L. All the decisions in the matrix were based on measurements that are supported with published operating procedures and data quality objectives. The logic in the decision matrix was organized into a spreadsheet-based computer application (BioPIC) that steps to the next relevant criteria as each decision is made until the conclusion is reached.

3.5.1 Data Requirements

Every criterion should be based on a measurement or assay that is commercially available. To the extent possible, the criteria will be data that are routinely collected as part of a remedial investigation.

A detailed User's Guide will be developed to help users apply the decision logic.

3.5.2 Success Criteria

The primary deliverable (BioPIC, including the new MNA Rate Constant Estimator) is considered successful if each step in the chain of logic is so intuitive and self-explanatory that an RPM or a design engineer working as a consultant to an RPM can immediately implement BioPIC without reading the entire Final Report.

3.5.2 Outcome

An evaluation of the success criteria for this performance objective still needs to be completed.

Beta testing with a list of qualified environmental professionals (mix of DoD personnel, regulators, academics, and consultants) is scheduled to occur during live training webinars on 27 April 2021 and 29 April 2021. The beta testers will be provided with the software, user's guide, and a brief list of questions that will be used to solicit feedback. The results of this testing will help the project team refine the deliverables for public release, and it is envisioned that the feedback will be summarized in an appendix of the Final Report.

3.6 PERFORMANCE OBJECTIVE #6: Validate Rate Constants for 1,4-Dioxane Degradation using a ^{14}C Assay Conducted with Groundwater from the Study Sites

The following protocol was used to evaluate a set of performance objectives that focused on validating the rates obtained from the ^{14}C assay. Note that the performance objectives for this task have been expanded since the Demonstration Plan was submitted.

First, groundwater from the study sites was sampled and shipped to Clemson University to conduct the required microcosm study. ^{14}C-labelled 1,4-dioxane was presented to naturally occurring bacteria in the groundwater. If the bacteria cometabolize the 1,4-dioxane, the main expected product was carbon dioxide; soluble products that are incompletely oxidized may also accumulate. The rate of cometabolism was measured by taking a subsample of the water in the microcosms, acidifying it, sparging it with nitrogen gas, and then passing the gas stream through an alkaline solution to trap the $^{14}CO_2$. ^{14}C-labelled products left in the acidic solution were then separated from unreacted ^{14}C-1,4-dioxane by passage through a solid phase extraction cartridge that selectively adsorbs 1,4-dioxane. If a sufficient level of soluble non-strippable products was obtained, they were further characterized by separation on a high-performance liquid chromatograph. The ^{14}C-labelled degradation products were quantified using liquid scintillation counting.

3.6.1 Data Requirements

The microcosms were amended with a purified aqueous solution of ^{14}C-labelled 1,4-dioxane. An aliquot of the stock solution were added to liquid scintillation medium (cocktail) to determine the disintegrations per minute (dpm) in the 1,4-dioxane added to the microcosms. At approximately weekly time intervals, water in the microcosms were subsampled and processed as described above to determine the dpm associated with degradation products.

3.6.2 Success Criteria

The success criteria specified in the Demonstration Plan states that the ^{14}C-assay protocol will be considered a success if the radio-label that was originally present in 0.01% of the ^{14}C-labelled 1,4-dioxane added to the microcosm can be detected as radio-labelled products. To calculate the dpm in the subsample that was originally associated with the ^{14}C-labelled 1,4-dioxane, the total dpm added to a microcosm (based on the known level of ^{14}C in the stock solution added) was divided by the volume of water in the serum bottle and then multiplied by the volume in the subsample (typically 5.1 mL). This value is multiplied by 0.0001 to determine a dpm goal.

The protocol to remove the ^{14}C-labelled 1,4-dioxane from the water in the microcosms was tested by adding the ^{14}C-labelled 1,4-dioxane to bicarbonate buffered water, then subjecting a subsample to the protocol for measuring ^{14}C degradation products. The protocol will be considered adequate if the dpm in the processed samples is less than the goal of 0.01% impurities.

The accumulation of ^{14}C-products over time was used to determine the pseudo first-order rate coefficients (k) by fitting experimental data to a mass balance model for ^{14}C in the microcosms. Rate coefficients were determined using the optimization solver in MATLAB by minimizing the sum of squared errors between the prediction and the ^{14}C product accumulation data over time. Triplicate bottles were fit simultaneously to obtain a single value for k (Mills et al., 2018). MATLAB rate coefficients were compared to ones obtained through a spreadsheet in Excel to validate calculations performed in the script. Overall, the fitting procedure was to be considered successful if the difference between the two methods is not statistically significant.

k values and 95% confidence intervals were determined for the groundwater microcosms and microcosms that contained only filter sterilized groundwater (FSGW). The energy in the beta decay of the ^{14}C-labelled 1,4-dioxane was expected to cause abiotic degradation of the ^{14}C-labelled 1,4-dioxane. This process is called radiolysis. FSGW controls were run with the same groundwater to measure the rate constant for radiolysis. When the rate coefficients for both were statistically significant, a net rate coefficient for the groundwater (k_{net}) was calculated by subtraction and the 95% confidence interval (CI_{net}) for k_{net} was calculated by propagation of error. The ^{14}C assay was tested with two cultures that are known to aerobically biodegrade 1,4-dioxane: *Pseudonocardia dioxivorans* CB1190, which grows on 1,4-dioxane as a sole source of carbon and energy, and ENV497, an enrichment culture that cometabolize 1,4-dioxane following growth on propane. The ^{14}C assay was considered successful if k_{net} values of at least 0.0231 per year are obtainable, corresponding to a half-life of 30 years.

Another performance objective from the Demonstration Plan stated that the 80% confidence interval on the rate constant for cometabolism of 1,4-dioxane was less than 50% of the rate coefficient.

3.6.3 Outcome

The success criteria for this performance objective were partially achieved, with four of the five sub-objectives meeting the specified criteria.

Addition of the purified ^{14}C-1,4-dioxane to the microcosms (0.5 mL per bottle) increased the initial concentration of 1,4-dioxane by ~165 µg/L. The average initial dpm added per bottle was 548,333 ± 25,807, with no significant change during the period of use (Ramos-Garcia, 2020). Using this average amount added, 0.01% corresponds to 55 dpm per bottle (i.e., 0.0001 x 548,333), which contained an average of 100 mL. Based on a subsample volume of 5.1 mL, the 0.01% target was 2.8 dpm of ^{14}C degradation products. The blanks that were evaluated containing only liquid scintillation cocktail typically had ~30 dpm. It was not possible to find a statistically significant difference between vials with 30 dpm and ones with 32.8 dpm. Consequently, this criterion was not met. However, as described below, a more critical metric was met.

The initial purity of the ^{14}C-1,4-dioxane was assessed by adding 0.5 mL to triplicate bottles containing 1 mM HCO_3^- in DDI water. Another set received no ^{14}C-1,4-dioxane. Aqueous and headspace samples were then subjected to analysis of ^{14}C degradation products. The sum of the $^{14}CO_2$ and ^{14}C-nonstripable residue (NSR) was 0.064% of the total ^{14}C added to the bottles. This is above the success criterion of having less than 0.01% impurities. Nevertheless, the impurities (409 dpm/bottle, after subtracting activity in the bottles with no ^{14}C added) were minor in relation to the total ^{14}C added to the bottles (634,653 dpm/bottle).

Each time an experiment was started, the purity of the stock added was assessed in the same manner. Based on 16 samples analyzed, the average level of impurities at time zero for samples with growth media (AMSM and BSM) that was not inoculated with CB1190 or ENV487 was 0.023 ± 0.015%. Based on 246 of the microcosms analyzed, the average level of impurities at time zero was 0.038 ± 0.031%. Although higher than the initial target of 0.01%, this was considered to be sufficiently low to allow for detection of biodegradation activity over six weeks of incubation.

The assay was evaluated with metabolic and cometabolic cultures (i.e., CB1190 and ENV487). Following six weeks of incubation, the increase in ^{14}C products for CB1190 was sufficient to determine a k_{net} at least as low as 0.016 per year, corresponding to a half-life of 43 years. For ENV487, the lowest rate coefficient measured was 0.021 per year, corresponding to a half-life of 33 years (Section 5.6). These results confirmed that the assay is adequately sensitive to detect rate coefficients that are meaningful in the context of assessing *in situ* remediation. As will be shown in Section 5.6 for groundwater samples, even lower statistically significant rate coefficients were detectable, corresponding to half lives in excess of 100 years.

MATLAB determined rate coefficients were compared to ones obtained through a spreadsheet in Excel to validate calculations performed in the script. Overall, the difference between the two methods was not statistically significant (0.04 ± 0.06%), indicating the MATLAB script worked as expected.

The performance objective for the magnitude of uncertainty associated with the rate coefficients was met for a majority of the well samples that had statistically significant k_{net} values. For 18 of the 24 wells that had statistically significant k_{net} values (Section 5.6), the 80% confidence interval was less than 50% of the rate coefficient. The ratio for six of the statistically significant k_{net} values exceeded this objective, with five of these between 53 and 69%.

3.7 PERFORMANCE OBJECTIVE #7: Determine whether Aerobic Biological Cometabolism or Biological Degradation of 1,4-Dioxane Explains the Degradation Rate Constant for Attenuation of 1,4-Dioxane at Field Scale

Based on the present state of knowledge, 1,4-dioxane is only known to degrade under aerobic conditions. If this is the case at the field sites in this study, the value of the bulk rate constant extracted using the fate and transport modeling tool developed for this project (the MNA Rate Constant Estimator) and the value of the rate constant obtained from the ^{14}C assay should be the same. Because the rate constants are obtained from difference measurements using different methods, the rate constant provided by the ^{14}C assay provides an independent validation of the bulk (site-wide) biodegradation rate constant extracted from the field data. If values of the rate constants are in acceptable agreement, it is likely that the value for the bulk biodegradation rate constant is correct.

3.7.1 Data Requirements

This performance objective requires information provided by previous performance objectives: the bulk biodegradation rate constant for 1,4-dioxane degradation as extracted from the field data using the software model and the estimate of the rate constant for aerobic biological cometabolism or biological degradation of 1,4-dioxane provided by the ^{14}C assay.

3.7.2 Success Criteria

The ^{14}C assay provides a rate constant for biological degradation of 1,4-dioxane and a 95% confidence interval on the rate constant. These are well-specific values.

The primary success criterion was based on determining if the rate constant for degradation of 1,4-Dioxane at field scale (based on model predictions) is contained within the 95% confidence interval of one or more of the biological degradation rate constants provided by the ^{14}C assay. If so, then the biodegradation of 1,4-dioxane will be accepted as a plausible explanation for the bulk rate constant for degradation of 1,4-dioxane seen at field sites.

A secondary (less stringent) success criterion was based on simply determining if the rates provided by one or more of the ^{14}C assay is greater than the model-predicted rate constant for 1,4-dioxane.

3.7.3 Outcome

The success criteria for this performance objective were partially achieved.

The ^{14}C assay provided rates for each well that was sampled, and the results showed that there was considerable variability between rate constants obtained from different locations at the same site (see Section 5.6 and Section 6.6). The model is calibrated to provide a site-wide rate constant, and the calibration is based on an assumption that any variability across the site is captured by the bulk rate constant generated by the model. Therefore, it is not expected that the model-predicted rate constants would fall within the confidence intervals of all of the ^{14}C rate constants, but rather that

some consistency would be apparent between the model-predicted rate constant and one or more of the location-specific ^{14}C rate constants.

One or more of the criteria for success were achieved at well(s) at 3 of the 9 sites where significant ^{14}C rate constants had been established (Sites 3, 4, and 6). At 2 other sites, all ^{14}C based rate constants were within an order of magnitude of the site-wide model-predicted rate constant. In general, the 1,4-dioxane degradation rate constants predicted through model calibration were much larger than the rate constants from the ^{14}C assay. As discussed in Section 5.6, the rate constants from the ^{14}C assay may be underestimating in situ degradation rates because the microcosms are constructed from groundwater, which may introduce biomass and/or nutrient limitations.

4.0 SITE DESCRIPTION

This section focuses on the selection of sites where field data were collected by the project team (Task 4 in the proposal). Existing data from several other sites was also evaluated, but those sites are not discussed here. Additional information on conditions at each of the field demonstration sites is included in Section 5 along with the field sampling results.

Ten different plumes from 8 different installations or properties were selected for field sampling. Several of these sites were identified at the proposal phase. The sites were primarily DoD facilities but also included two commercial/industrial sites. For confidentiality reasons, the specific identities of these sites are not listed. For the purposes of this report:

- Each plume was considered a separate "site", even if it was located at the same facility as another plume. This applied to one DoD facility where three different plumes were sampled; these three plumes were spatially distinct with no co-mingling.

- Each site was assigned a number that was based on the order in which it was sampled (1 through 10). These numbers were then used when describing site conditions and results throughout the remainder of this report (e.g., Site 1 (DoD), Site 4 (Industrial), etc.).

4.1. SITE LOCATION AND HISTORY

The ten sites were all in the continental United States and represented a reasonable amount of geographic diversity (**Figure 4.1**). A summary of relevant geographic information and site history is included in **Table 4.1**.

Note that the information is based on what was made available to the project team at the start of the project and do not necessarily reflect recent changes.

4.2. SITE GEOLOGY/HYDROGEOLOGY

A summary of relevant geologic and hydrogeologic characteristics for the demonstration sites is provided in **Table 4.2**. For these sites, the focus is on the groundwater-bearing units where project samples were collected.

4.3. CONTAMINANT DISTRIBUTION

Table 4.3 provides a summary of contaminant distribution by site. For the purposes of this project, contaminants of interest included 1,4-dioxane and associated chlorinated solvents. At each site, the 1,4-dioxane was generally co-mingled with a chlorinated solvent plume (i.e., co-released from one or more areas that were contributing the observed plume).

A graphic representing known plume conditions is also provided for each site in **Figures 4.2 – 4.11**. The goal is to generally reflect conditions that were known to the project team at the start of the demonstration, with a focus on the monitoring wells (and associated groundwater-bearing unit)

where project samples were also collected. Note that the concentration and contour lines shown in these graphics are based on conditions from historical site reports and do not necessarily represent more recent data.

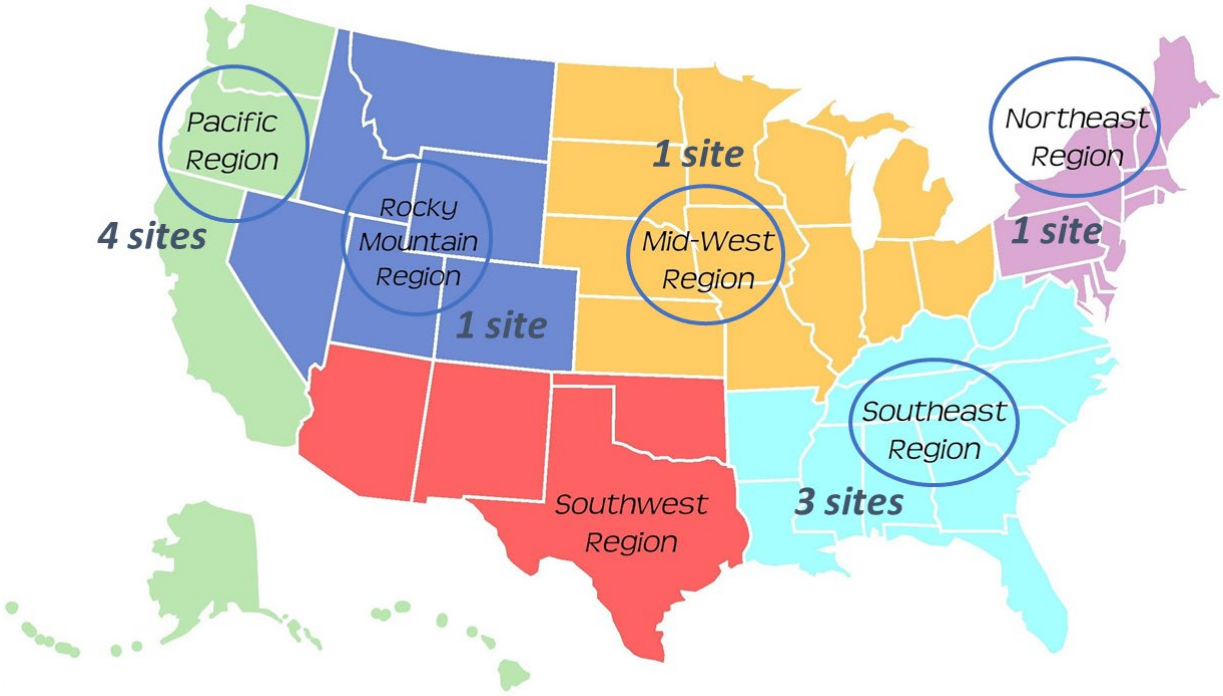


Figure 4.1. Site Locations by Region

Table 4.1. Summary of Relevant Location and History Information by Site

Parameter	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Site 9	Site 10
Location by U.S. Region (see Figure 4.1)	Pacific	Northeast	Midwest	Southeast	Pacific	Pacific	Pacific	Southeast	Rocky Mountain	Southeast
DoD or Industrial Facility?	DoD	DoD	Industrial	Industrial	DoD	DoD	DoD	DoD	DoD	DoD
History of Operations	• Industrial area with historic aircraft testing/maintenance shops, tanks, and pipelines • Multiple releases in different areas (including one reportedly in early 1990s) contributed to LNAPL/DNAPL impacted area and chlorinated ethene plume	• Former sewage treatment plant and fire training areas serve as source for organics in groundwater • Re-infiltration of treated groundwater likely contributing 1,4-dioxane within plume	• Former landfill • Accepted industrial waste in late 1960s to late 1970s	• Manufacturing facility with cleaning processes that utilized chlorinated solvents and triethylene glycol (both containing 1,4-dioxane) • Used until early 1990s • Suspected releases of rinse water from septic system and sump	• Former Hazardous Waste Facility that included Industrial Waste Treatment Plant and Oily Waste Treatment Plant • Includes both inactive and active permitted waste disposal units (including 20 surface impoundments)	• Industrial operations area including warehouses and airfield complex • Includes both inactive and active permitted waste disposal units (including 20 surface impoundments)	• Former Chemical Waste Disposal Area • Estimated 8 to 32 million gallons of chemical wastes from industrial operations, including paints, solvents, caustics, acids, and oils, were disposed between 1940s and mid-1970s	• Area of concern within current test area due to releases from former underground storage tank for fuels, solvents, and other organics/inorganics • Tank used in 1970s and 1980s	• Solid waste management unit that is part of larger landfill area • Also includes former grit/oil pit	• Solid waste management unit with former vapor degreasing facility and leach pit • Area also includes former lab facility • Chlorinated solvents used from 1950s to 1970s
Remediation Status	• Focused removal actions including ISB in downgradient plume • Remedial alternatives still being screened • 1,4-dioxane is potential COC but no remedy selected or in place	• Comingled chlorinated solvent plume is managed using groundwater extraction • 1,4-dioxane is potential COC with ongoing feasibility study; currently managed by existing groundwater extraction system	• Low-permeability cap in place with landfill gas collection system • Extensive monitoring network and long-term monitoring program that include 1,4-dioxane and THF	• Cleaning system dismantled and building razed • Site regulated under Voluntary Cleanup program • Ongoing feasibility study • 1,4-dioxane is COC and phytoremediation implemented as interim in source area as interim remedy	• On-going monitoring requirements • Pilot scale remedies tested include air sparging, SVE, and ISB • Remedial alternatives still being screened • 1,4-dioxane is potential COC but no remedy selected or in place	• Full-scale ISB and zero-valent iron barrier implemented for chlorinated solvents, along with natural attenuation as long-term remedy • 1,4-dioxane is potential COC with ongoing feasibility studies, but no remedy selected	• Multiple Areas of Concern • Remedial alternatives still being screened • 1,4-dioxane is potential COC but no remedy selected or in place	• Partial removal of tank • Ongoing RI work for CVOCs and 1,4-dioxane	• In situ chemical oxidation (persulfate) as source area treatment • Several interceptor trenches located mid plume to capture and treat groundwater • 1,4-dioxane is COC; ISCO used to treat source (as well as CVOCs)	• Groundwater extraction system and thermal treatment of source area to address chlorinated solvents • 1,4-dioxane is potential COC with on-going remedial investigation/feasibility studies

Table 4.2. Summary of Relevant Hydrogeologic Characteristics by Site

Parameter	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Site 9	Site 10
Depth to water	4 to 25 ft bgs (shallow unconfined)	60 ft bgs (unconfined)	25 ft bgs (shallow confined to semi-confined)	2 to 4 ft bgs (shallow unconfined)	20 to 25 ft bgs (shallow unconfined)	5 to 15 ft (shallow unconfined)	4 to 10 ft bgs (shallow unconfined)	10 to 35 ft bgs (confined)	10 to 20 ft bgs (shallow unconfined)	15 to 20 ft bgs (shallow and intermediate unconfined)
Predominant soil type in relevant groundwater-bearing unit	Sand/silty sand	Sand, medium to coarse grained with some gravel	Sand and gravel	Interbedded silt and clay with isolated permeable sands	Sand/silty sand	Sand/silty sand	Sand/silty sand	Fine-grained to coarse grained sand with silt	Sand, silty sand	Sand with silt and gravel
Thickness of groundwater-bearing unit	Upper portion of shallow aquifer is up to 20 ft thick and underlain by silt (A silt); multiple additional zones extend to > 100 ft bgs	150 to 250 ft	Up to 200 ft thick with multiple zones separated by silt confining layers; near source wells screened below 100 ft bgs (confined) and downgradient wells screen above 50 ft bgs (unconfined)	Low-permeability water table aquifer is approx. 20 ft thick; underlain by consolidated unit (marl)	Upper portion of shallow aquifer is approx. 20 ft thick and underlain by silt (A silt); multiple additional zones extend to > 100 ft bgs	Upper portion of shallow aquifer is approx.. 10 to 20 ft thick and underlain by silt; multiple additional zones extend to > 100 ft bgs	Upper portion of shallow aquifer is approx. 20 ft thick and underlain by silt (A silt); multiple additional zones extend to > 100 ft bgs	Aquifer is 20 to 40 ft thick and is overlain and underlain by distinct clay confining units	Aquifer is approx. 30 ft thick but varies seasonally; underlain by bedrock	Shallow/interm. aquifer zones approx..40 ft thick; two zones are separated by discontinuous silty clay; shallow/interm. zones underlain by deeper zone and then bedrock
Flow characteristics	• Flow direction = northeast • Transmissivity = 0.62 – 1.58 ft2/min • Horizontal gradient = 0.001 – 0.002	• Flow direction = south/southeast • Hydraulic conductivity = 2.5 – 380 ft/day • Horizontal gradient = 0.001 – 0.002 • Groundwater seepage velocity 1 - 4 ft/d	• Flow direction = west/northwest • Groundwater seepage velocity reported between 0.7 – 1.0 ft/d	• Flow direction = northwest • Horizontal hydraulic gradient = 0.034 – 0.04	• Flow direction = west/northwest • Horizontal groundwater gradients = 3.4 to 4.4×10^{-4} • Groundwater seepage velocities: A layer was 0.057 ft/day, 0.069-0.133 ft/day for B layer, 0.012-0.105 for C layer and 0.184 for D layer.	• Flow direction = west/southwest • Horizontal gradient = 0.001 – 0.003 • Groundwater seepage velocity 100-200 ft/yr	• Flow direction = west • Flow data not provided but assumed to be similar to Site 1 and Site 5 due to proximity and similar regional hydrogeology	• Flow direction = north/northeast • Hydraulic gradient = 0.01 • Hydraulic conductivity ranged from 0.58 to 5.1 ft/d • Groundwater seepage velocity estimated between 0.019 and 0.17 ft/d	• Flow direction = east/southeast • Hydraulic gradient = 0.01 to 0.05 • Groundwater seepage velocity reported between 0.75 to 150 ft/yr	• Flow direction = north/northeast (towards extraction well) • Hydraulic conductivity = 0.0007 cm/s (shallow) to 0.14 cm/s (interm.) • Groundwater seepage velocity reported between 145 ft/yr (shallow) to 2600 ft/yr (intermed.)

Table 4.2. Summary of Relevant Hydrogeologic Characteristics by Site *(continued)*

Parameter	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Site 9	Site 10
Geochemical conditions	• Generally neutral pH (range of 6-7.4). • DO typically low (range of 0.4-3 mg/L) • ORP readings suggest mixed redox conditions (range of -73 – 208 mV) • Elevated specific conductivity	• Slight acidic (pH range of 5-6. • DO between 5-10 mg/L at most locations except where organic releases suspected • Low turbidity and specific conductivity	• Generally neutral pH (range of 6-7). • DO typically low (range of 0.05-2.4 mg/L within plume • TOC > 1 mg/L in majority of wells • Measurable iron and methane in most wells; typically > 1 mg/L	• Generally neutral pH (range of 6-7). • DO typically low (range of 0.3-2 mg/L in shallow wells) • ORP readings suggest mixed redox conditions (no data on other NA parameters)	• Generally neutral pH (range of 6-7). • DO typically low (range of 0.3-2 mg/L). • ORP varied by location and season • Elevated specific conductivity	• Generally neutral pH (range of 6-7). • DO typically low (range of 0.5-3 mg/L). • ORP varied by location and season • Specific conductivity 1 – 5 mS/cm • Trace detections of methane under natural conditions	• pH typically slightly above neutral (6.4 – 10.2) • DO typically < 1.0 mg/L with some exceptions • Evidence of elevated iron, DOC, and methane in several wells suggests mixed redox conditions • Elevated specific conductivity	• DO above 4 mg/L in many wells • pH near neutral to slightly acidic • Low specific conductivity (< 1 mS/cm) but some elevated DOC/turbidity	• DO above 5 mg/L in many wells, • Near neutral pH • Little or no methane detected • Generally high specific conductivity in all wells (> 5 mS/cm) • Elevated turbidity and organic carbon (> 10 mg/L) note in several wells	• DO above 4 mg/L in many wells, particularly in shallow source wells and extraction wells • ORP measurements suggest conditions are oxidizing to mildly reducing • Slightly acidic pH (range of 5 – 7) • Low specific conductivity
Other relevant conditions	• Groundwater discharges to surface water within 3000 ft of source area • Freshwater-salt water interface (encountered within approximately 25 to 55 ft bgs) may reduce permeability and limit migration	• Groundwater flow below pond and eventually discharges to another pond > 2 miles downgradient of primary source area	• THF and TBA present in majority of wells (occasionally > 100 mg/L)	--	• Freshwater-salt water interface (encountered within approximately 35 to 65 ft bgs but below the shallow aquifer) may reduce permeability and limit migration	--	• Groundwater discharges to surface water within 2000 ft of source area • Freshwater-salt water interface (encountered at within approximately 40 ft bgs) may reduce permeability and limit migration	--	• Interceptor trenches may cause localized effects on flow and redox conditions	• Extraction well located approx. 1000 ft downgradient of source area limits further migration

Table 4.3. 1,4-Dioxane and CVOC Concentrations at Monitoring Wells Included in Field Demonstration

Well ID	Well Depth (ft bgs)	Maximum 1,4-Dioxane Concentration (µg/L) (Date)	CVOCs present	Representative CVOC Concentration (µg/L) (Date)
Site 1				
1	N/A	160 (3 Dec 2013)	TCE, cis-DCE, 1,1-DCA, 1,1-DCE	TCE = 67 (2015) cis-DCE = 0.1 (2015) 1,1-DCA = 0.9 (2015) 1,1-DCE = 1.8 (2015)
2	N/A	29 (26 Mar 2014)	TCE, 1,1-DCE	TCE = 11 (2015) 1,1-DCE = 1.8 (2015)
3	N/A	73 (27 Mar 2014)	TCE, cis-DCE, 1,1-DCA, VC, 1,1-DCE	TCE = 880 (2015) cis-DCE = 1.4 (2015) VC = 94 (2015) 1,1-DCA = 18 (2015) 1,1-DCE = 18 (2015)
4	N/A	170 (4 Nov 2015)	TCE, 1,1-DCA, 1,1-DCE	TCE = 1600 (2015) 1,1-DCA = 58 (2015) 1,1-DCE = 150 (2015)
Site 2				
1	153	0.39 (20 Jul 2015)	BRL	--
2	192	0.37 (16 Jul 2015)	PCE, TCE	PCE = 31 (30 Mar 2000) TCE = 8.6 (30 Mar 2000)
3	186	0.37 (9 Oct 2015)	ND	--
4	124	0.993 (28 Feb 2014)	PCE, TCE	PCE = 4.8 (4 Aug 2016) TCE = 18.5 (4 Aug 2016)
Site 3				
1	25	6.7 (2 Nov 2017)	ND	--
2	95	140 (2017)	ND	--
3	196	290 (2017)	1,1-DCA	1,1-DCA = 4
4	?	?	?	?
5	126	30 (8 Nov 2017)	ND	--
Site 4				
1	10	62000 (24 Apr 2002)	1,1,1-TCA, 1,1-DCA, 1,1-DCE, VC	1,1,1-TCA = 7 (29 Oct 2003) 1,1-DCA = 78 (24 Apr 2002) 1,1-DCE = 39 (14 Apr 2004)
2	10	20000 (14 Apr 2004)	1,1-DCA, 1,1-DCE, VC	1,1-DCA = 41 (14 Apr 2004) 1,1-DCE = 52 (23 Apr 2002)
3	12	560 J (18 May 2011)	1,1-DCA, 1,1-DCE	1,1-DCA = 9 (20 Sep 2006) 1,1-DCE = 3 J (11 Sep 2007)
4	13	9500 (13 May 2003)	1,1-DCA, 1,1-DCE, VC	1,1-DCA = 130 (09 Oct 2009) 1,1-DCE = 16 (13 Sep 2007)
Site 5				
1	43	66 J (2015)	TCE, 1,2-DCE, 1,1-DCE	TCE = 342.5 (2015) 1,2-DCE = 17.5 (2015) 1,1-DCE = 180 (2015)
2	43	36.3 J (2015)	1,2-DCE	1,2-DCE = 23.8 (2015)
3	44	22J (2015)	TCE, 1,2-DCE, 1,1-DCE	TCE = 157 (2015) 1,2-DCE = 18.3 (2015) 1,1-DCE = 70.8 (2015)
4	44	40 UJ (2015)	BRL	--
Site 6				
1	26	160 J (2 Feb 2016)	1,1-DCE, cis-DCE, TCE, VC, 1,1-DCA, trans-1,2-DCE	1,1-DCE = 50 (24 Feb 1993) cis-DCE = 12 (10 Oct 2007) TCE = 0.54 (10 Oct 2007) VC = 0.9 (18 Dec 1996) 1,1-DCA = 6 (24 Feb 1993) trans-1,2-DCE = 0.31 J (5 May 2015)
2	22	9.8 (18 Mar 2014)	1,2-DCA, 1,1-DCE, cis-DCE, VC, 1,1-DCA,	1,2-DCA = 2.6 (3 Jul 1996) 1,1-DCE = 18 (3 Jul 1996) cis-DCE = 1.5 (31 Jul 2015) VC = 3.3 (3 Jul 1996) 1,1-DCA = 9 (13 Sep 1993)
3	27	3.8 J (8 Jul 2016)	cis-DCE	cis-DCE = 1.2 (8 Jul 2016)
4	24	28 (9 Oct 2007)	1,1-DCE, cis-DCE, VC, 1,1-DCA	1,1-DCE = 10 (8 Jul 1996) cis-DCE = 0.82 (8 Jul 2016) VC = 2.1 (11 Nov 1997) 1,1-DCA = (3 Nov 1992)
Site 7				
1	23	36 (2017)	BRL	--
2	21	37 (2017)	BRL	--
3	20	13 J (2017)	BRL	--
4	N/A	380 (2017)	cis-DCE, 1,1-DCA, VC, 1,1-DCE	cis-DCE = 370 (2011) 1,1-DCA = (160) VC = 26 (2011) 1,1-DCE = 92 (2011)

Table 4.3. 1,4-Dioxane and CVOC Concentrations at Monitoring Wells Included in Field Demonstration *(continued)*

Well ID	Well Depth (ft bgs)	Maximum 1,4-Dioxane Concentration (µg/L) (Date)	CVOCs present	Representative CVOC Concentration (µg/L) (Date)
Site 8				
1	N/A	4.38 (May 2019)	1,1-DCE	1,1-DCE = 7.03
2	N/A	19.1 (May 2019)	1,1,1-TCA, 1,1-DCE, 1,1-DCA	1,1-DCE = 19.4 (2019) 1,1,1-TCA = 0.803 (2019) 1,1-DCA = 7.1 (2015)
3	N/A	40.6 (May 2019)	1,1,1-TCA, 1,1-DCE, 1,1-DCA	1,1-DCE = 35.2 (2019) 1,1,1-TCA = 4.44 (2019) 1,1-DCA = 9.6 (2015)
4	N/A	19.1 (May 2019)	1,1-DCE, CF	1,1-DCE =3.67 (2019) CF = 1.7 (2015)
Site 9				
1	N/A	784 (June 2019)	1,1-DCA, cis-DCE, TCE, VC	1,1-DCA = 57.3 (June 2019) cis-DCE = 1180 (June 2019) TCE = 110 (June 2019) VC = 67.1(June 2019)
2	N/A	116 (June 2019)	1,1-DCA, cis-DCE, VC	1,1-DCA = 18.7 (June 2019) cis-DCE = 93.5 (June 2019) VC = 6.4 (June 2019)
3	N/A	45.4 (June 2019)	1,1-DCA, cis-DCE, TCE, VC	1,1-DCA = 13 (June 2019) cis-DCE = 33.7 (June 2019) TCE = 7.4 (June 2019) VC = 0.55 (June 2019)
4	N/A	1.9 (June 2019)	1,1-DCA, cis-DCE, VC	1,1-DCA = 1.4 (June 2019) cis-DCE = 0.4 (June 2019) TCE = 0.45 (June 2019)
Site 10				
1	N/A (interm.)	110 (2015/2016)	1,1-DCA, 1,1-DCE, cis-DCE, TCE, PCE, TCFM	1,1-DCA = 23 1,1-DCE = 887 cis-DCE = 3180 TCE = 387 PCE = 3600 TCFM = 3
2 (extraction well)	N/A (interm.)	21 (2015/2016)	1,1-DCA, 1,1-DCE, cis-DCE, TCE, PCE, TCFM	1,1-DCA = 5 1,1-DCE = 91 cis-DCE = 156 TCE = 20 PCE = 114 TCFM = 11
3	N/A (shallow)	700 (2015/2016)	N/A	--
4	N/A (interm.)	150 (2015/2016)	N/A	--

Notes: (1) ND = Not detected, BRL = Below the reporting limit, N/A = Not available, -- = Not applicable
(2) J = Estimated value
(3) *1,4-Dioxane and CVOCs concentrations not based on historical data

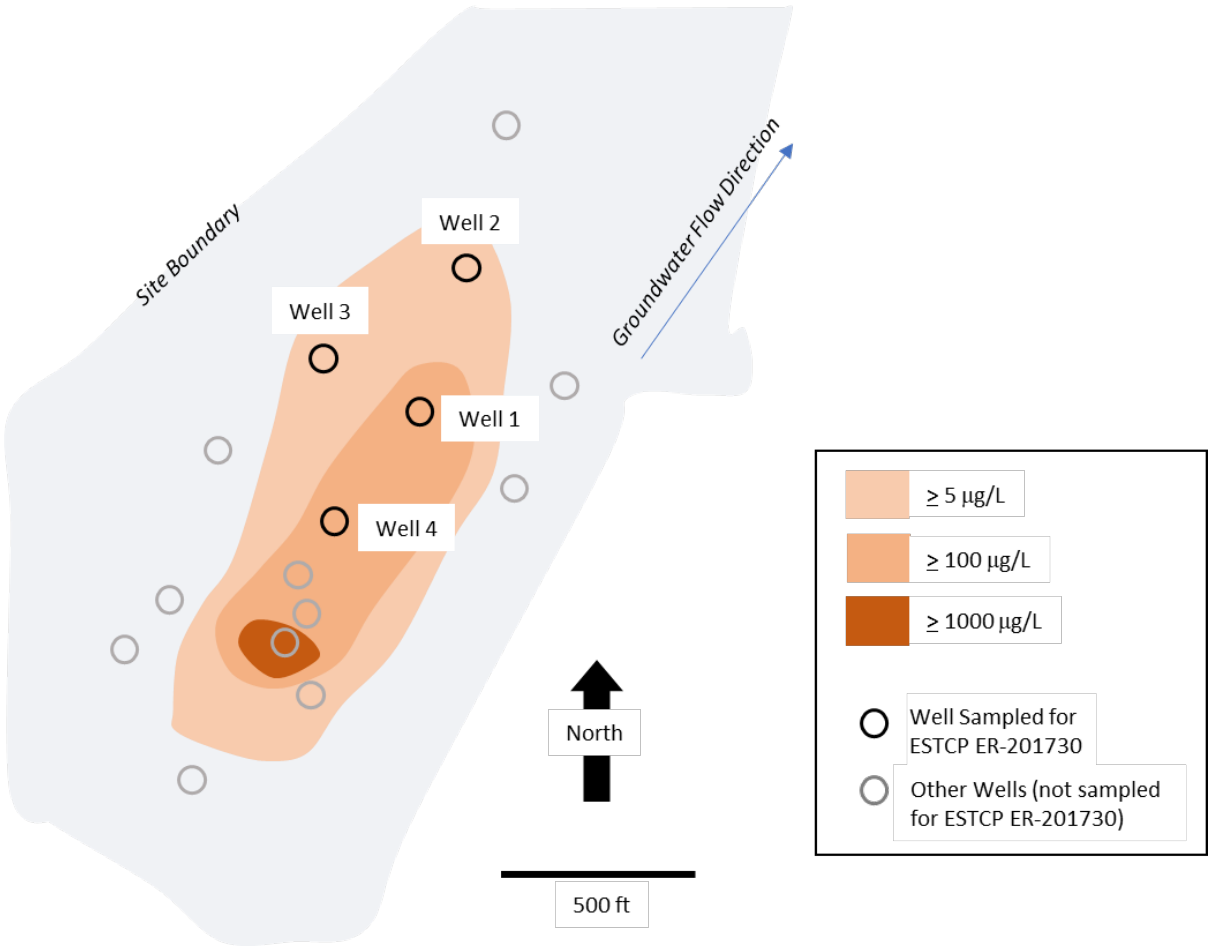


Figure 4.2. Site 1 (DoD).
Colors represent 1,4-dioxane concentrations based on data collected prior to the project demonstration.

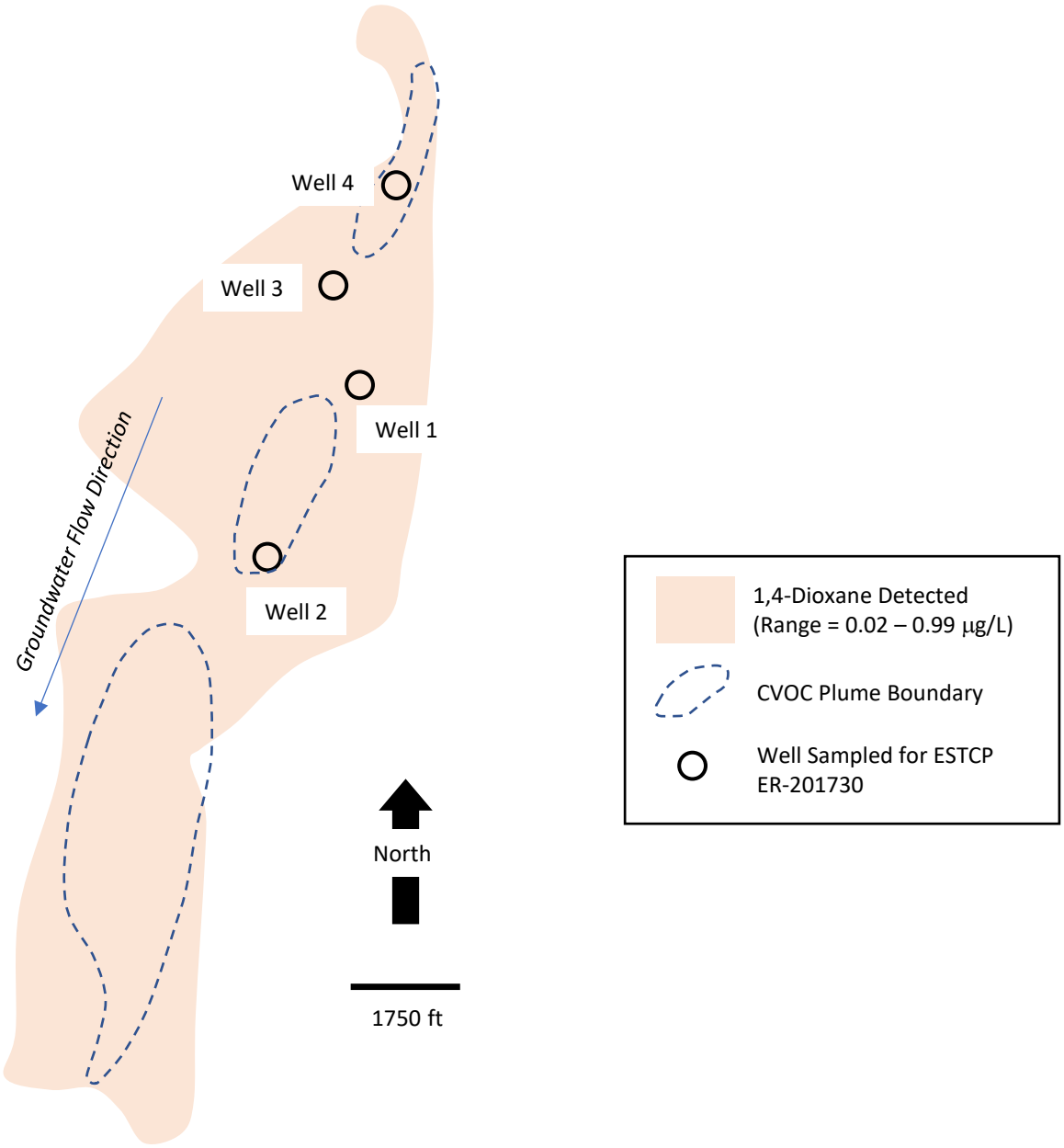


Figure 4.3. Site 2 (DoD).
Colors represent 1,4-dioxane concentrations based on data collected prior to the project demonstration.

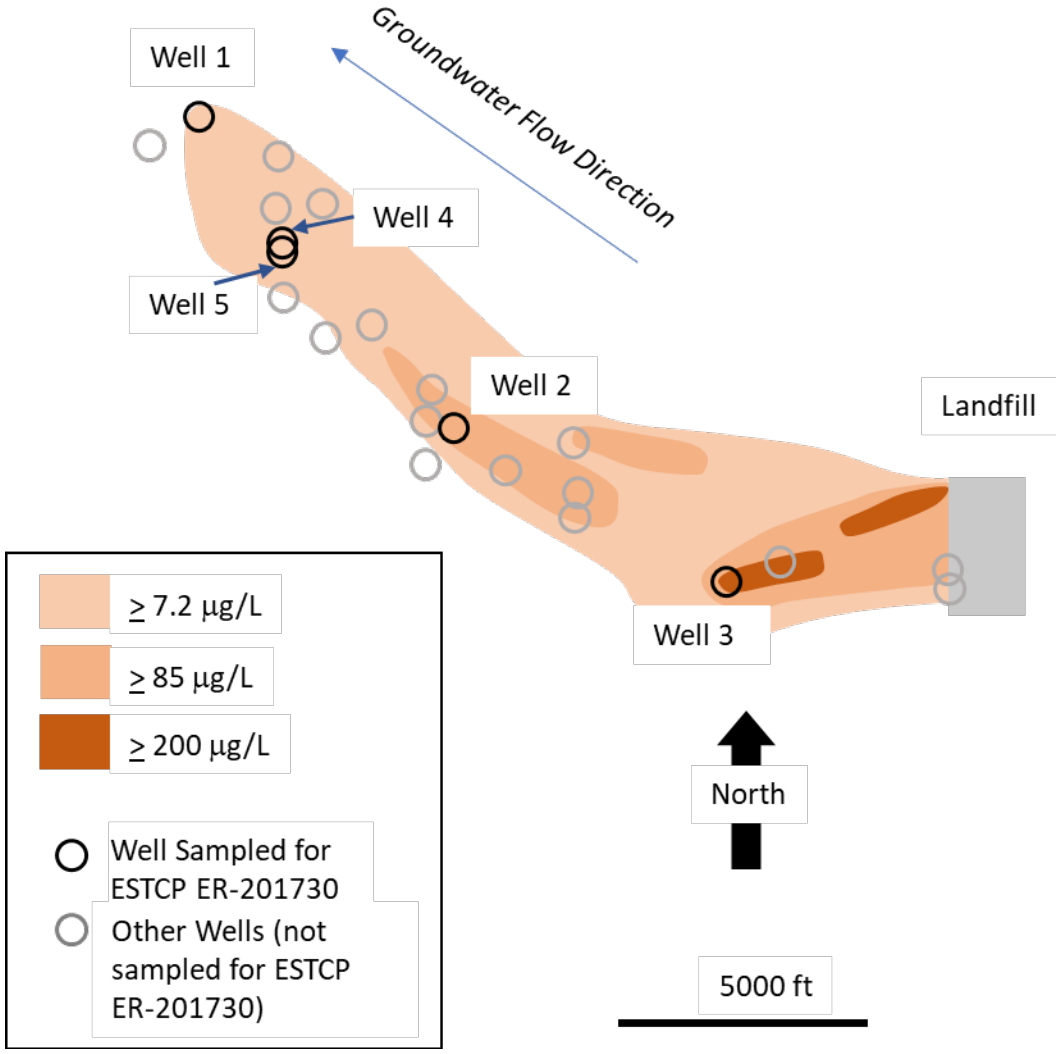


Figure 4.4. Site 3 (Industrial).
Colors represent 1,4-dioxane concentrations based on data collected prior to the project demonstration.

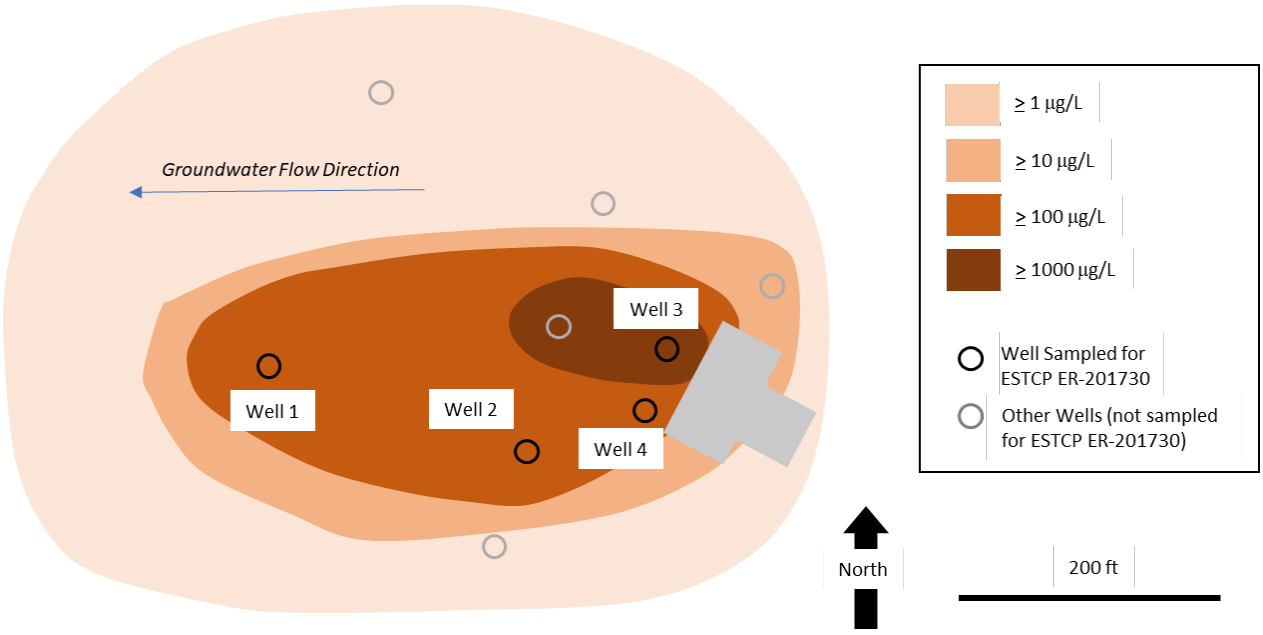


Figure 4.5. Site 4 (Industrial).
Colors represent 1,4-dioxane concentrations based on data collected prior to the project demonstration.

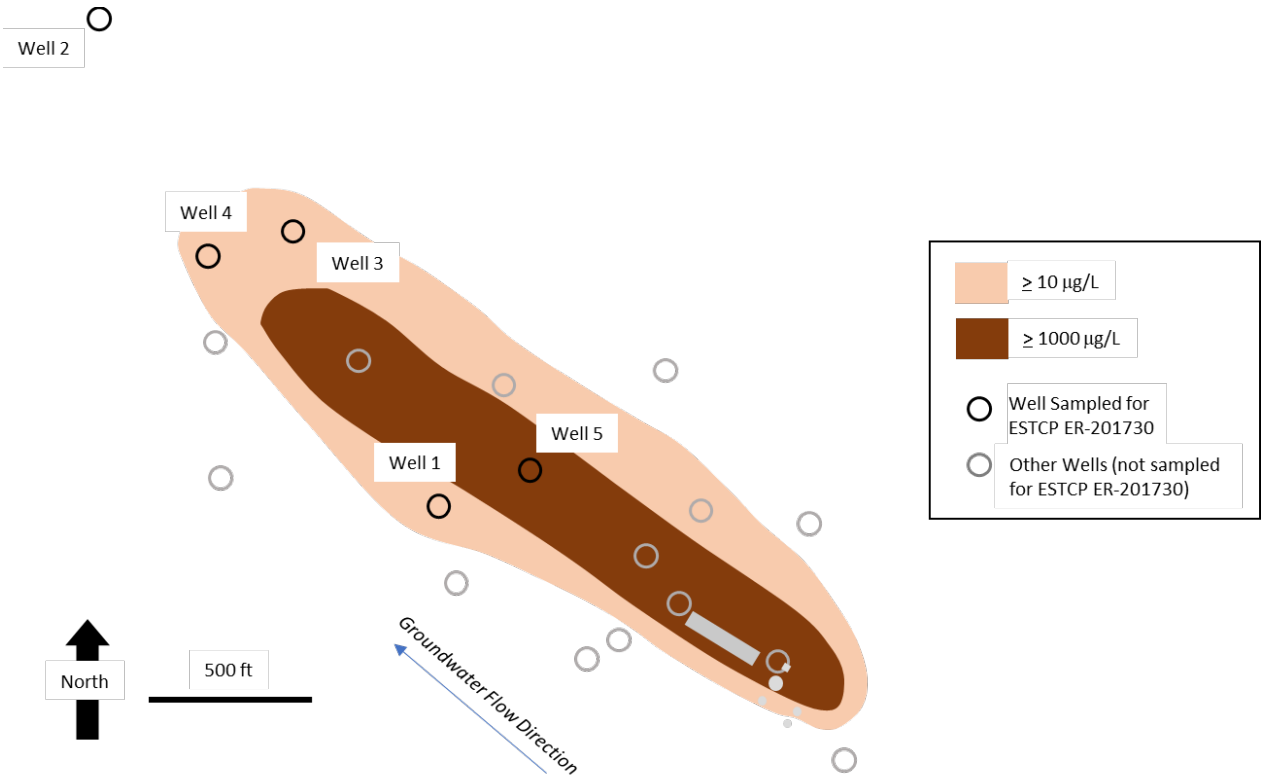


Figure 4.6. Site 5 (DoD).
Colors represent 1,4-dioxane concentrations based on data collected prior to the project demonstration.

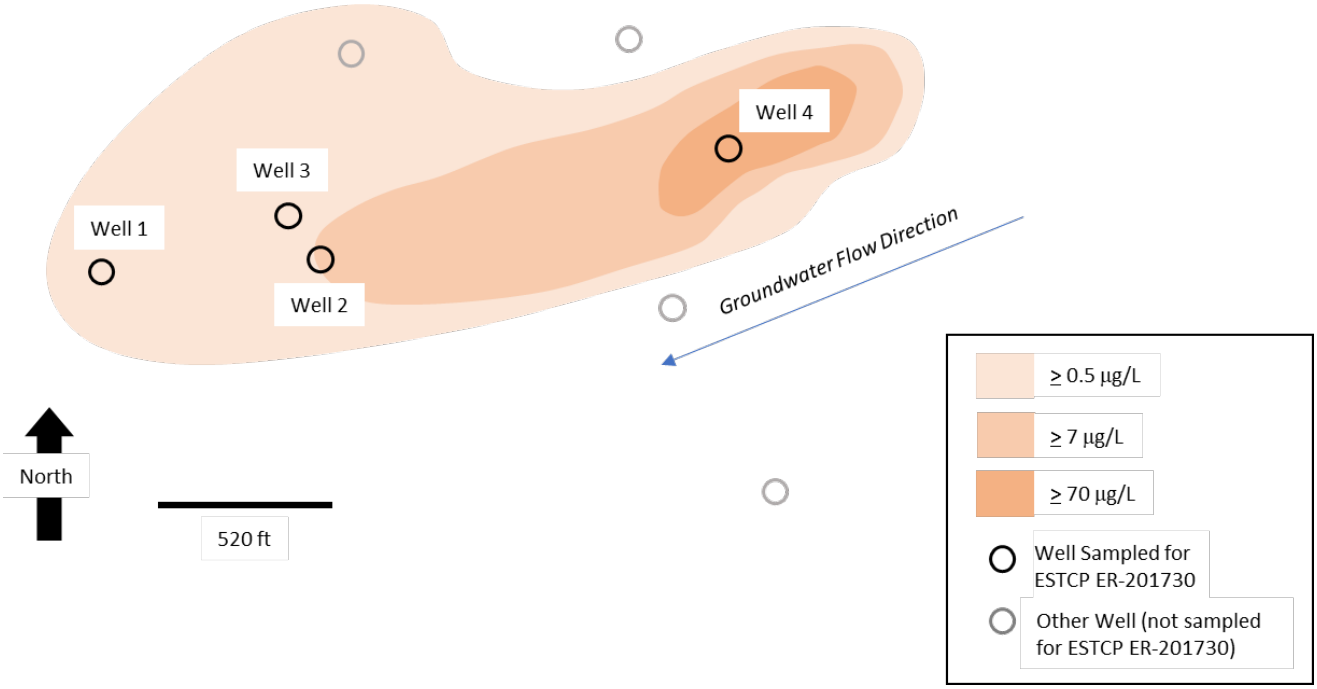


Figure 4.7. Site 6 (DoD).
Colors represent 1,4-dioxane concentrations based on data collected prior to the project demonstration.

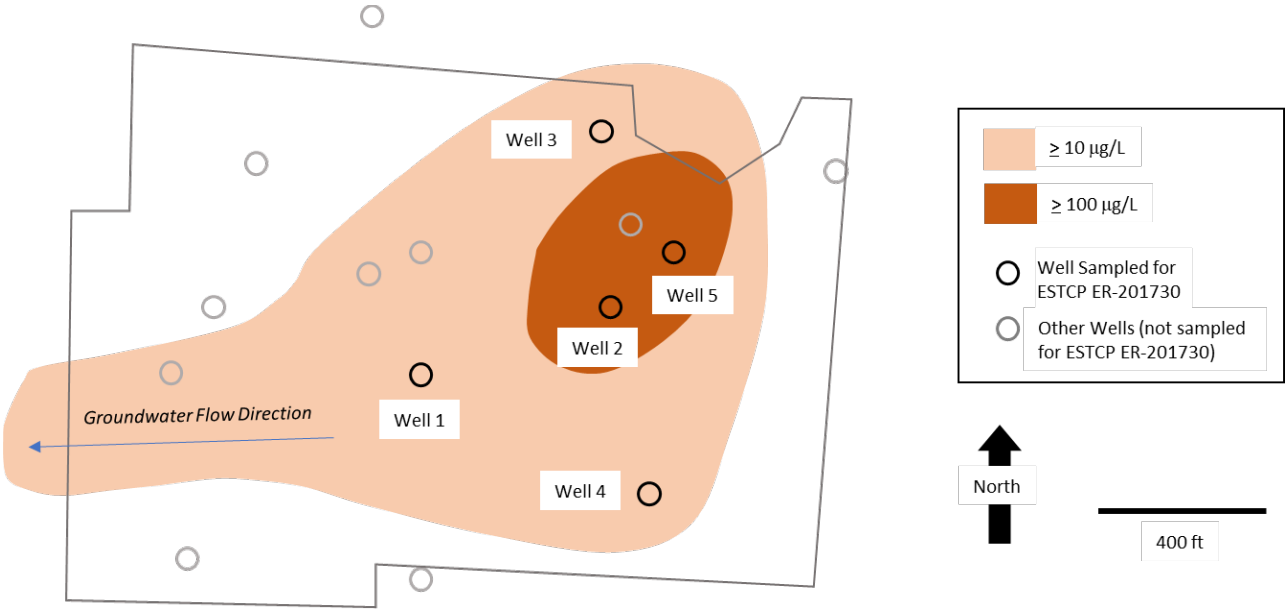


Figure 4.8. Site 7 (DoD).
Colors represent 1,4-dioxane concentrations based on data collected prior to the project demonstration.

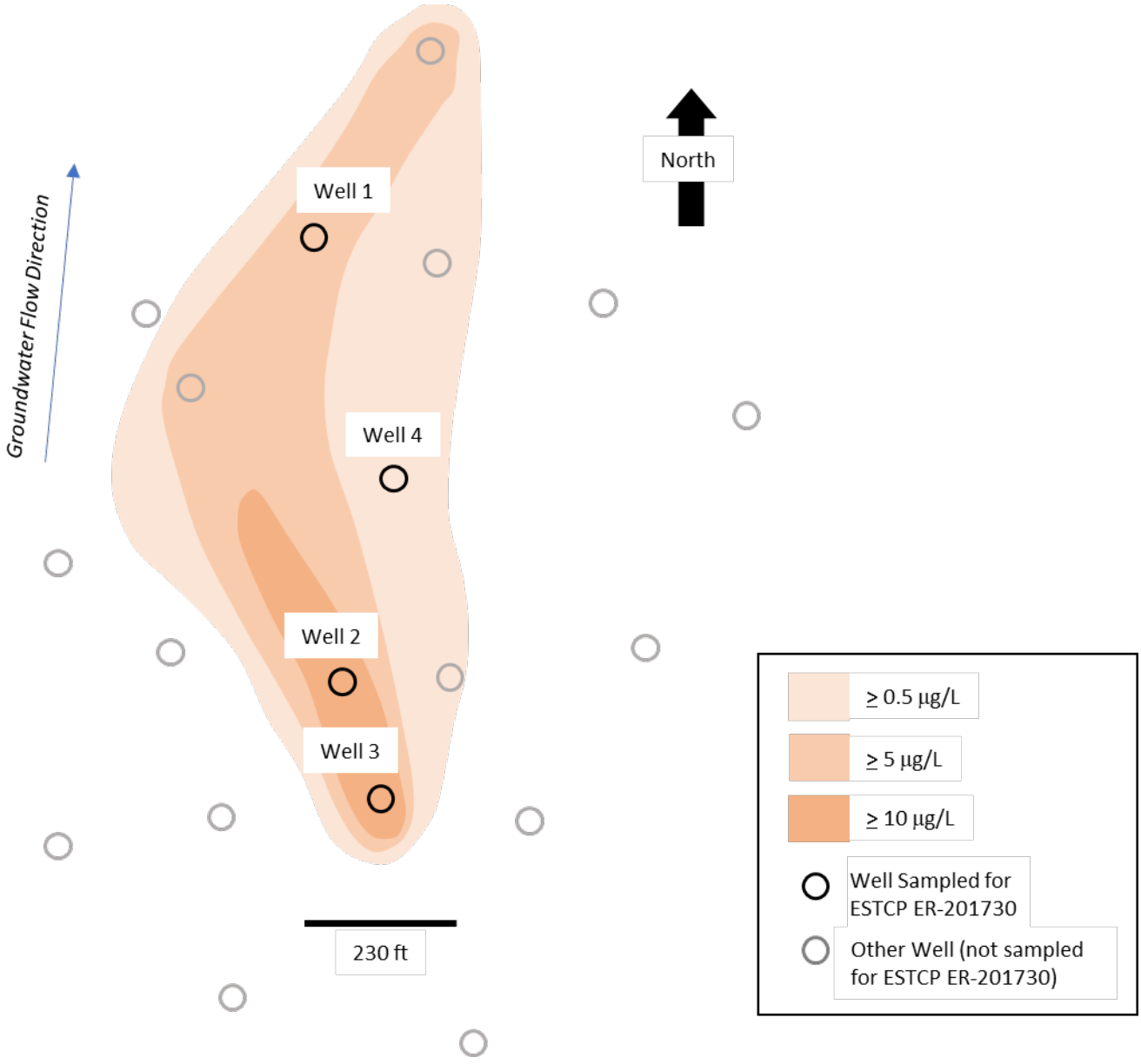


Figure 4.9. Site 8 (DoD).
Colors represent 1,4-dioxane concentrations based on data collected prior to the project demonstration.

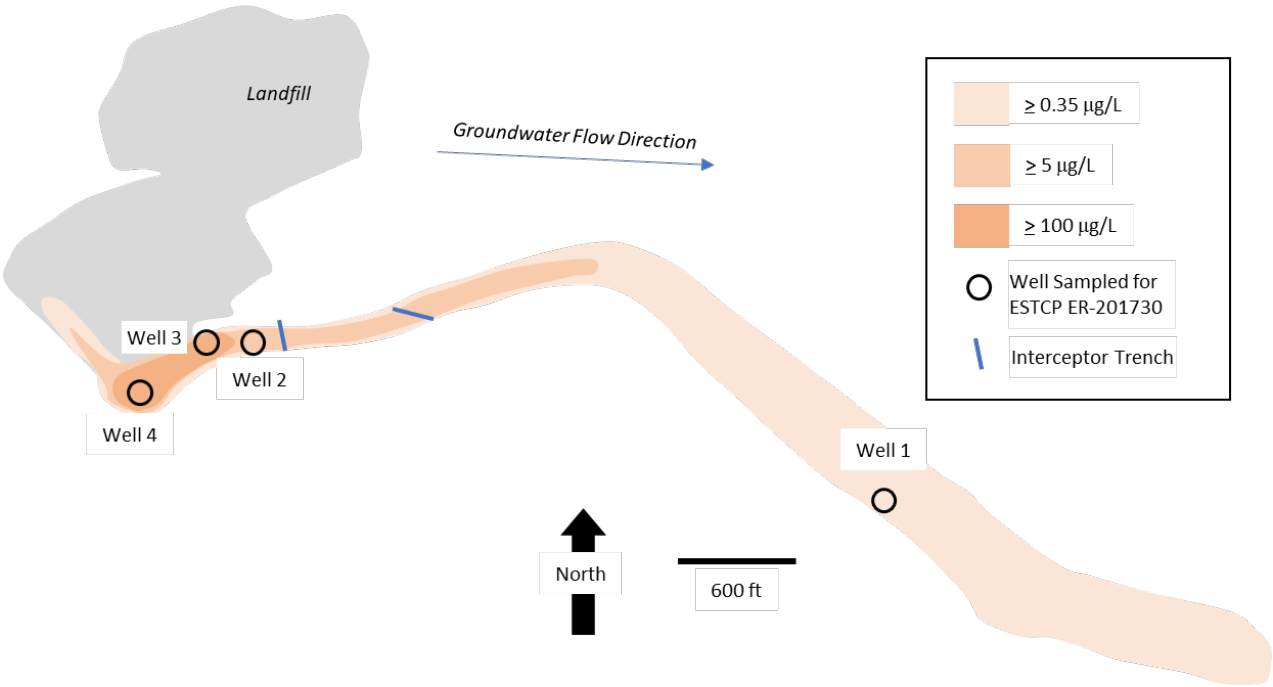


Figure 4.10. Site 9 (DoD).
Colors represent 1,4-dioxane concentrations based on data collected prior to the project demonstration.

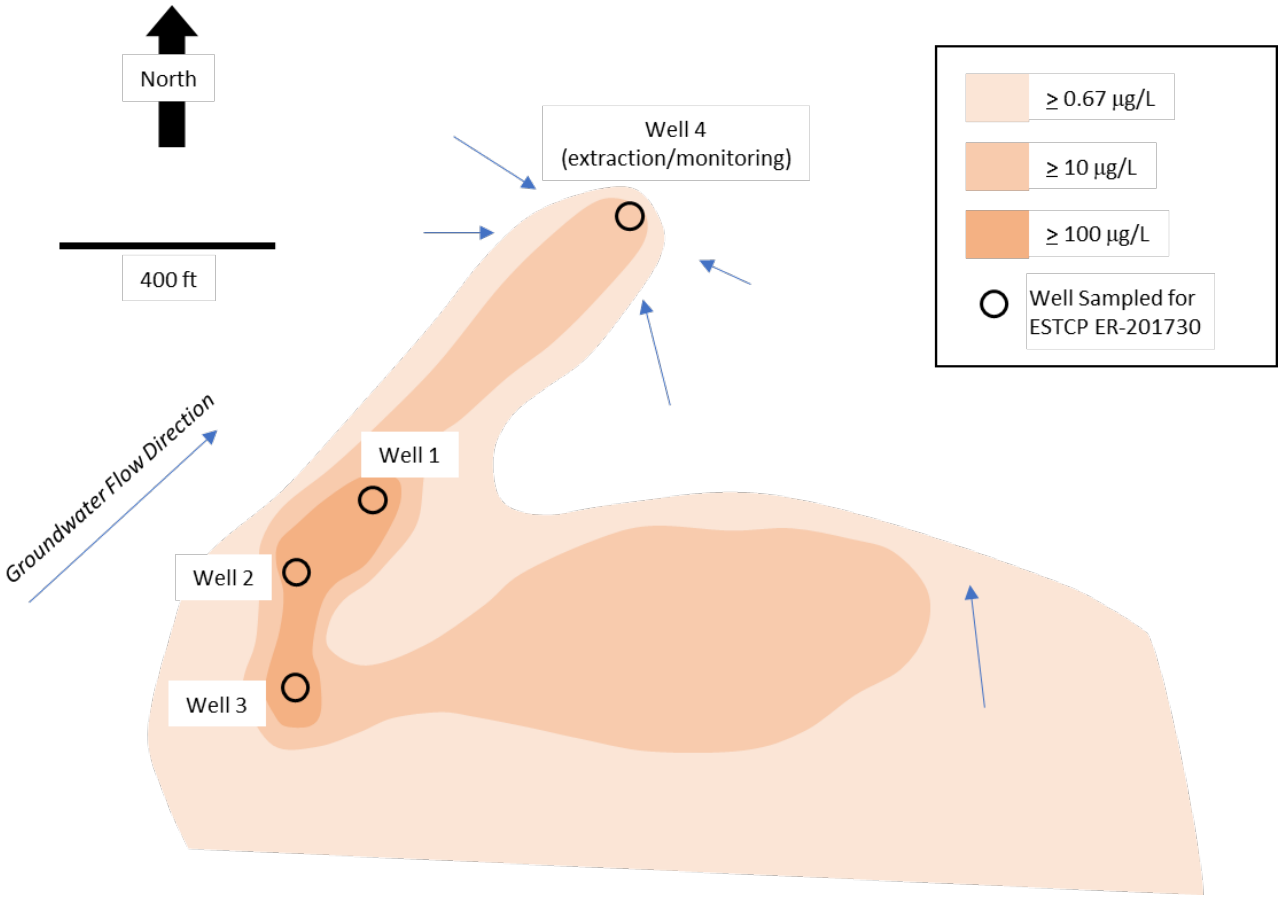


Figure 4.11. Site 10 (DoD).
Colors represent 1,4-dioxane concentrations based on data collected prior to the project demonstration.

5.0 TEST DESIGN

5.1 CONCEPTUAL EXPERIMENTAL DESIGN

The technical approach for this project involved a combination of lab and field studies to support development of a decision framework and associated guidance tools. **Figure 5.1** shows the major elements of this approach and how they complement each other.

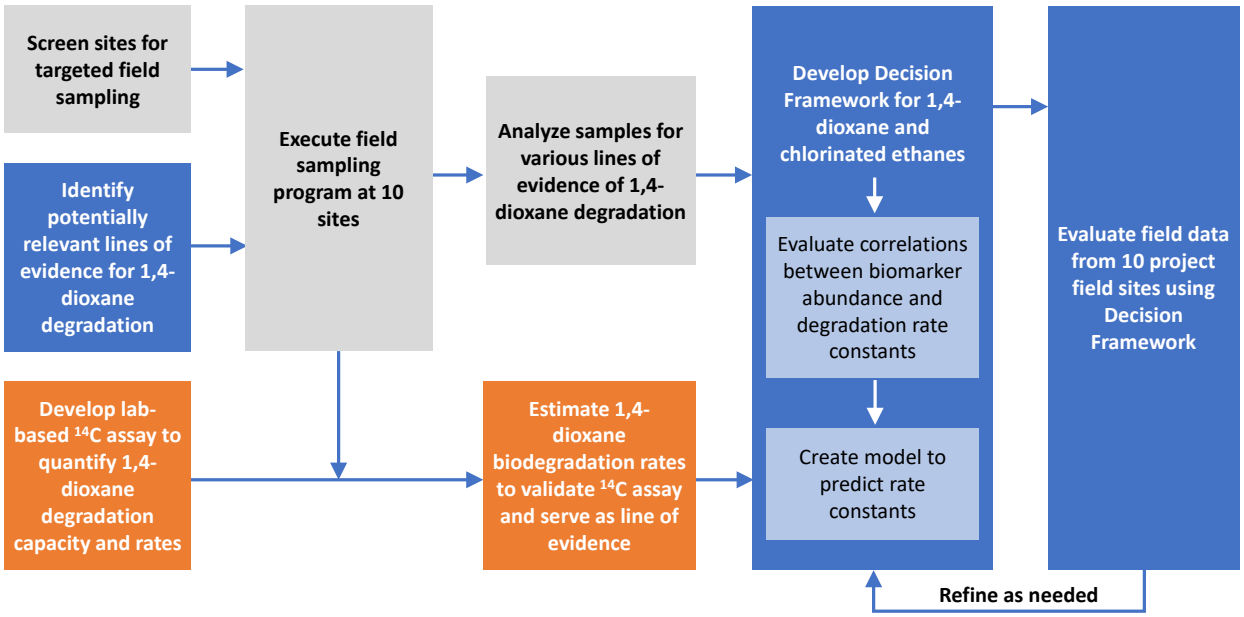


Figure 5.1. Overview of Technical Approach

The specific methodology for collecting data to support project objectives relied on sampling groundwater from existing monitoring wells at multiple sites. A similar approach was followed at each site after accounting for site-specific requirements. Data collected during field sampling were then used to support a lines of evidence approach for 1,4-dioxane attenuation and to build the natural attenuation decision framework and associated modeling tool.

5.2 BASELINE CHARACTERIZATION ACTIVITIES

The field work involved a single mobilization to each site to collect groundwater samples. There were no baseline characterization activities required beyond reviewing existing characterization data to support well selection.

5.3 DESIGN AND LAYOUT OF TECHNOLOGY COMPONENTS

The following subsections describe the primary components of the field sampling and the locations at each area where these procedures were utilized. As noted above, locations were based on a review of existing site data.

Components associated with the other project tasks are described in other sections of this report, specifically Section 5.6 which describes the development and validation of the lab-based ^{14}C assay for 1,4-dioxane and Section 5.9/Appendix B which describe the development of the decision framework.

5.3.1 Design of Technology Components

The only technology components relevant to this demonstration are groundwater monitoring wells. At each site, samples were collected only from existing wells. Therefore, there were no additional design considerations for these components.

Specifications for the wells that were sampled differed depending on the site. A complete list of the wells, along with relevant details were provided in the Section 4.3 in **Table 4.3.**

Samples were collected from wells based on the protocols described in Section 5.4.

5.3.2 Layout of Technology Components

Graphical representations of each of the locations that were sampled at each site are included as **Figures 4.2 – 4.11**. Versions of these maps with data collected as part of this project are also presented in **Section 5.7**.

At each site, the objective was to sample at different points within a plume along the direction of groundwater flow. Because these are existing monitoring wells, spacing between locations varies and was outside of the project team's direct control. However, care was taken to avoid wells that were heavily influenced by current or recent remediation activities (with one exception at Site 10).

A short rationale for sampling locations is provided below for each site (see also **Tables 4.2 – 4.3** for additional details on site conditions).

Site 1 (DoD): All four wells are screened within the shallow aquifer along the groundwater flow path with generally decreasing 1,4-dioxane concentrations along this path (from 170 µg/L to 29 µg/L). The source area with the highest 1,4-dioxane concentration (> 1000 µg/L) was not selected in part due to the presence of high concentrations of several CVOCs (including 1,1-DCE at > 1000 µg/L). Measured dissolved oxygen levels are low throughout the site, suggesting that any 1,4-dioxane degradation that exists may be localized.

Site 2 (DoD): All four wells lie along the general groundwater flow direction with gradual increased distances from the presumed source area. One of these wells is located within the north portion of the CVOC plume, and one within the center plume that is separated from north portion of the plume. The other two wells are located outside of the current plume footprints, specifically in between the north and center plumes. All four wells are screened in the saturated zone below the water table. From the sampling results of DO and ORP in wells on this site in 2016, aerobic and oxidizing environment are likely present in these selected four wells. 1,4-Dioxane was detected in these wells, with the northmost well having the highest concentration equal to 0.99

µg/L. Given that the concentrations of 1,4-dioxane detected previously in these wells were relatively low, cometabolic biodegradation of 1,4-dioxane would likely be favored over 1,4-dioxane metabolic activity.

Site 3 (Industrial): A total of five wells were sampled due to recent biostimulation efforts with propane and existing evidence of 1,4-dioxane biodegradation capacity at this site. This includes measurable DXMO gene abundance in two wells nearer the source with relatively high concentrations of 1,4-dioxane (> 100 µg/L). A non-specific oxygenase (sMMO) is also present at many wells, including the downgradient, lower 1,4-dioxane wells that were also included in current project. Note that methane and low dissolved oxygen levels are also common at this site, despite the other lines of evidence for 1,4-dioxane degradation. This suggests that: 1) the wells are screened across vertical intervals where different redox conditions may exist; and 2) bulk indicators may not provide enough information to screen for 1,4-dioxane activity. Also of note at this site are the low CVOC concentrations (i.e., reducing the possibility that these compounds will inhibit 1,4-dioxane degradation), as well as the presence of THF (a known primary substrate for supporting 1,4-dioxane co-metabolism).

Site 4 (Industrial): Two of the wells are close to the source of contamination (> 1000 µg/L of 1,4-dioxane), while the other two are further down the flow path in the aquifer (historically 200 to 300 µg/L of 1,4-dioxane). For all the wells, the screened interval includes the water table, or is within a few feet of the water table, such that exchange of oxygen in the capillary fringe may be able supply oxygen for aerobic biodegradation of 1,4-dioxane. The lower concentrations may not support acclimation and may only degrade 1,4-dioxane through cometabolism. In three of the wells, the concentrations of dissolved oxygen based on historical data ranged between 0.26 to 0.98 mg/L, but the ORP readings were all negative. This indicates that some portion of the water collected by the well contains reduced iron or sulfide and is devoid of oxygen. However, one of the wells had a positive ORP value, indicating that the water collected into the well may be oxygenated.

Site 5 (DoD): Four wells along the groundwater flow path were selected for sampling and full characterization. Based on existing monitoring data**,** these wells have relatively low but adequate concentrations of dissolved oxygen to support degradation or cometabolism of 1,4-dioxane, and little or no iron or sulfide have been observed. These wells also have low to moderate concentrations of 1,1-DCE, which might inhibit degradation or cometabolism of 1,4-dioxane. Well 2 has low levels of 1,4-dioxane but has been impacted by the CVOC plume; it was included to evaluate the capacity of the unimpacted microbial community to cometabolize 1,4-dioxane. This plume also has low levels of aromatic hydrocarbons which may support biological growth and/or expression of monooxygenases that may benefit 1,4-dioxane degradation.

Site 6 (DoD): All the wells that were included in the sampling program are generally located along the flow path of the site groundwater, with one lying in the center of the 1,4-dioxane plume and the other three outside the plume. All four are shallow wells that are screened near the water table. The well located in the center of the plume had the highest concentration of 1,4-dioxane detected at about 160 µg/L, while the other three wells were reported to have concentrations at around 4 – 28 µg/L. Concentrations of all CVOCs were low; none were above 100 µg/L (and most were below 10 µg/L) during the most recent monitoring event. Dissolved oxygen measurements from these

wells indicated that the environment was highly reducing, though ORP readings suggest that redox conditions are likely to vary considerably based on depth, location, and season.

Site 7 (DoD): The wells that were selected for sampling have measurable 1,4-dioxane concentrations (38 to 170 µg/L) that decrease in the direction of groundwater flow. They also have low but potentially adequate concentrations of dissolved oxygen to support degradation or cometabolism of 1,4-dioxane and have low concentrations of methane and iron. Within the exception of the near source well, CVOC concentrations are below reporting limits, minimizing the potential for inhibition of 1,4-dioxane biodegradation.

Site 8 (DoD): All four wells lie along the general groundwater flow direction with gradually decreasing 1,4-dioxane concentrations (from 41 to 4 µg/L) that are more likely to support co-metabolism than direct metabolism of 1,4-dioxane, even within the presumed source area. CVOCs are generally not detected in any of the wells, except for 1,1-DCE (maximum = 35 µg/L). Geochemical parameter data suggest that the shallow aquifer where these wells are screened is oxidizing, including significant dissolved oxygen (> 4 mg/L in most wells).

Site 9 (DoD): The four wells were all along the narrow groundwater flow path and were screened in the shallowest aquifer. 1,4-dioxane decreased significantly along this path, in part because of interceptor trench is located downgradient of the third well in this path. As a result, the fourth well measures (in part) attenuation under slightly different conditions. However, in all cases, significant dissolved oxygen is present within the entire aquifer. Both TCE and cis-DCE are present at high concentrations within the source area wells (> 1000 µg/L) but decrease significantly moving downgradient, and 1,1-DCE is present either at low (< 10 µg/L) or non-detectable levels throughout the source area and downgradient plume.

Site 10 (DoD): A well with the highest 1,4-dioxane concentration (700 µg/L) measured at the site was selected to represent the presumed source area, and an extraction well located farther downgradient was selected to represent low-concentration conditions at the edge of the plume. Two other wells with intermediate 1,4-dioxane concentrations that are located between the source well and extraction well were also selected. The groundwater flow direction and potential for 1,4-dioxane migration is largely dictated by the extraction well. The extraction well is screened slightly deeper than the source area well but has significant dissolved oxygen due to pumping; the source area well has high DO due to its shallow screen. CVOC concentrations are generally high throughout this site, including several wells with hundreds to thousands of µg/L of 1,1-DCE, PCE, TCE, and cis-DCE. This suggests the potential for inhibition but also confirms that reductive dechlorination is occurring either on-site or upgradient.

5.4 FIELD TESTING

The implementation schedule for the ten demonstration sites is presented as a Gantt chart (**Figure 5.2)**.

Sampling Location	Aug – Dec 2018					Jan – Dec 2019											Jan – Nov 2020										
Site 1								*								*											
Site 2																											
Site 3																											
Site 4														*													
Site 5																*											
Site 6																											
Site 7																											
Site 8																	Contracting period for sampling additional sites, plus extra coordination due to COVID19 concerns										
Site 9																											
Site 10																											

Notes: Month 1 = August 2018. Month 28 = November 2020. Cells denoted by * reflect dates when selected wells were resampled for one or more parameters.

Figure 5.2. Gantt Chart for Field Program.

The only field testing done under this project (ESTCP Project ER-201730) was to sample groundwater from established monitoring wells. The relevant tasks included:

- Approval from site manager(s)
- Coordination with site contractors (HASP, work plan, equipment, staging area, etc.):
- Finalization of start date and travel arrangements. Note that the field program for the final three sites overlapped with site-specific travel and access restrictions related to COVID19. This slowed project implementation slightly but did not prevent the program from being completed at any site.
- Completion of all access requirements (badging, training, etc.). Site access typically required pre-approval of designated personnel.
- Groundwater sampling. Method-specific sampling kits were procured from designated labs and brought to the site by project personnel. The program was implemented based on the following process:
  - At the first seven sites, sampling was coordinated with sampling done by contractors to the site owner, who were generally sampling the site as part of normal compliance monitoring. At these sites, the site contractors opened and sampled each well for their purposes, then provided water to our project team for further processing. All sampling kits were provided by our project team.
  - At the final three sites, sampling was completed by our project personnel. At these sites, all necessary equipment was procured by the project team, but site contractors typically provided assistance in accessing wells and other coordination steps.
- Sample packaging and shipment.
- Receipt of sample shipment at labs.

At each site, the entire program was completed within 2 to 4 days. Given that the focus was sampling, there was no specific system start-up, no extended system operation, and no system shutdown. The project will not result in permanent installations at the demonstration sites. As a result, there are no specific decommissioning considerations. Demobilization activities were relatively limited but typically focused on handling of investigation-derived waste (IDW). At the first seven sites, disposal of IDW was the responsibility of site contractors because our sampling was done in conjunction with the existing monitoring program. At the final three sites, our project team was responsible for collecting and staging IDW in containers and locations specified by site personnel. At two of these final three sites, our team collected a sample of the IDW for waste characterization purposes, and then relayed the characterization data to site personnel who coordinated disposal.

5.5 SAMPLING PLAN

5.5.1 Overview

A summary of the sampling plan for this demonstration project is provided in **Table 5.1**, and the analytical program for these samples is summarized in **Table 5.2** (Part 1 and Part 2).

Collectively, the sampling and analysis plan generate groundwater-based data to help evaluate contaminant concentrations and other lines of evidence for attenuation along a plume at each site, as well as providing samples for ^{14}C lab-based assays at Clemson University. A separate description of the sampling and analysis plan for the ^{14}C assays is provided in Section 5.6. A description of the rationale for including the analyte/parameter list for the field samples was included in Section 2.1. Another important consideration was that all these analyses are offered by commercial labs and thus could be applied by other users:

5.5.2 Quality Assurance Sampling Program

Laboratory analyses were completed using the methods and protocols outlined in **Table 5.2**. In all cases, method-specific containers were obtained from the respective laboratories prior to sampling. Labs were consulted on the necessary lab QA/QC samples to ensure that appropriate sample containers for these samples were provided to field personnel as well. A summary of the QA/QC sampling program is provided in **Table 5.3**.

Table 5.1. Summary of Sampling Plan per Site

Project Component	Matrix	Collection Method	Number of Samples	Analyte(s)	Location
Groundwater Sampling of Existing Monitoring Wells	Groundwater	Sampling from wells using existing protocols (typically low-flow sampling using peristaltic or bladder pump; bailer used in some instances)	At 4 or 5 existing wells per site	Biomarkers for 1,4-Dioxane and CVOC degradation, groundwater for ^{14}C-Assay of 1,4-Dioxane biodegradation, DO, Fe(II), pH, Temperature, Specific Conductance, ORP, Stable Isotopes (^{13}C, ^{2}H) in 1,4-Dioxane	Existing Monitoring Wells at Site (see **Figures 4.2 – 4.11** for specific locations)
				CVOC and 1,4-Dioxane concentrations (if not part of previously planned monitoring event)	

Table 5.2. Part 1. Summary of Analytical Methods for Samples of Groundwater Collected per Site. Groundwater is the only matrix sampled.

Analyte	Method	Range	Accuracy	Resolution
Dissolved Oxygen (0-1.0 mg/L)	CHEMetrics RhodazineD Immediately after well purging	0 to 1.000 ppm	± 10% error at 0.750 ppm, ± 20% error at 0.250 ppm, ± 30% error at 0.1 ppm	MDL: 0.025 ppm
Dissolved Oxygen (0-15 mg/L)	CHEMetrics Indigo Carmine Immediately after well purging **OR** YSI Multimeter with flow cell	0 to 15.0 ppm	± 0.6 at 2.0 ppm, ± 0.8 at 4.0 ppm, ± 1.1 at 11.0 ppm	N/A
Fe(II)	CHEMetrics Phenanthroline Immediately after well purging	0 to 6.00 ppm (mg/L)	≤ 0.08 ppm at 0 ppm, ± 0.30 ppm at 1.50 ppm, ± 0.45 at 4.50 ppm	N/A
pH	Direct Reading Hach Pocket Pro, Static container **OR** YSI Multimeter with flow cell	0.00 to 14.00 pH	± - 0.02 pH	0.01 pH
Temperature		-15º to 170º C	1º C	0.1º C
Specific Conductance		0.0 µS/cm to 19.99 mS/cm	± 1% FS	0.1 µS/cm, 1 µS/cm, 0.01 mS/cm
Oxidation reduction potential		-999 to 999 mV	± 2 mV	1 mV
^{14}C-Assay for 1,4-dioxane biodegradation	Developed as part of this study	See Section 5.6 of this report		
DNA biomarkers DHBt, DCA, DXMO, ALDH, sMMO, RMO, RDEG, PHE	qPCR	N/A	N/A	5 gene copies/mL.
RNA biomarkers DHBt, DCA, DXMO, ALDH, sMMO, RMO, RDEG, PHE	qPCR	N/A	N/A	5 gene copies/mL
SCAM alkane Biomarker for 1,4 dioxane degradation	Affinity assay, qPCR	N/A	N/A	5 gene copies/mL
prmA gene biomarker for 1,4-dioxane degradation	qPCR	N/A	N/A	10 gene copies/mL
Stable isotope analysis for 1,4 dioxane, C and H	CSIA	N/A	± 1 ‰ for C ± 20 ‰ for H	H: 200 µg/L, C: 100 µg/L

Note: N/A = Not available or not applicable

Table 5.2. Part 2. Summary of Analytical Methods for Samples of Groundwater Collected per Site. Groundwater is the only matrix sampled

Analyte	Method	Container	Preserved/ Chilled	Analytical Lab
Dissolved Oxygen (0-1.0 mg/L)	CHEMetrics RhodazineD	Analysis at well head	No	N/A (field measurement)
Dissolved Oxygen (0-15 mg/L)	CHEMetrics Indigo Carmine **OR** YSI Multimeter w/ flow cell	Analysis at well head	No	
Fe(II)	CHEMetrics Phenanthroline	Analysis at well head	No	
pH	Direct Reading Hach Pocket Pro	Analysis at well head	No	
Temperature		Analysis at well head	No	
Specific Conductance		Analysis at well head	No	
Oxidation reduction potential		Analysis at well head	No	
^{14}C-Assay for 1,4-dioxane biodegradation	Developed as part of this study	Two 1-liter glass media bottles + four 260 mL glass serum bottles with a butyl rubber septum and a crimp cap.	Keep and Ship at ≤4ºC	Clemson University
DNA biomarkers DHBt, DCA, DXMO, ALDH, sMMO, RMO, RDEG, PHE	qPCR	One-liter water filtered onto as Sterivex filter.	Keep and Ship at ≤4ºC	Microbial Insights
RNA biomarkers DHBt, DCA, DXMO, ALDH, sMMO, RMO, RDEG, PHE	qPCR	One-liter water filtered onto as Sterivex™ filter.	Preserve after collection, Keep and Ship at ≤4ºC	Microbial Insights
SCAM alkane Biomarker for 1,4 dioxane degradation	Affinity assay, qPCR	One-liter water filtered onto as Sterivex™ filter.	Keep and Ship at ≤4ºC	North Carolina State University
prmA gene biomarker for 1,4-dioxane degradation	qPCR	One-liter water filtered onto as Sterivex™ filter.	Keep and Ship at ≤4ºC	New Jersey Institute of Technology
Stable isotope analysis for 1,4 dioxane, C and H	CSIA	For each isotope, four 40-mL VOA vials, HCL preserved	Keep and Ship at ≤4ºC	University of Waterloo

Table 5.3. Summary of QA/QC Sampling Program

QA/QC Sample Category	Sampling Frequency	Analytes
Equipment Rinsate	N/A	N/A
Rinsate Water Sample	N/A	N/A
Trip Blanks	1 each day	CVOCs 1,4-dioxane
Field Duplicates	Approximately one for every ten samples	CVOCs 1,4-dioxane ^{14}C-Assay done in triplicate on water from each well
Field Calibration Or Field Calibration Check	1 each day	DO pH Specific Conductance ORP
Field Blank	N/A	Filter-sterilized control using water from each well was conducted as part of ^{14}C-Assay

Note: N/A = Not applicable

5.5.2.1 Field Quality Assurance Procedures

Project personnel collected field QA/QC samples to i) evaluate field precision and accuracy, and ii) facilitate validation of sample results. Field sampling precision and accuracy were assessed through the collection and laboratory analysis of field replicates and field blanks.

Data from field QC samples were examined to determine if any problems were evident for specific media (in this case groundwater) or with laboratory procedures. The individual laboratories were responsible for reviewing the QA/QC data and to develop and inform the team on appropriate corrective actions in the event of problems.

- Trip Blanks: The effectiveness of sample handling techniques was evaluated by submitting preserved trip blank samples for laboratory analysis. Proper labeling and documentation were completed for trip blanks. Trip blanks were prepared and analyzed with other samples being analyzed for COCs at a minimum frequency of one per day when sampling water.

- Field Duplicate Samples: The precision of field sample collection techniques was evaluated by collecting and analyzing field duplicates. Duplicate samples were defined as those samples collected simultaneously from the same source under identical conditions into separate but identical containers, and preserved, stored, transported and analyzed in the same manner. Thus, to prepare a duplicate, an aliquot was collected from a sample source

and divided equally into two separate but identical sample containers. Each duplicate was identically preserved, stored, transported and analyzed. Field duplicates were given a different identification number to disguise the source of the sample from the laboratory. Field replicates were analyzed by the same laboratory analyzing investigative samples. During the course of the demonstration, duplicates were collected at a minimum frequency of one duplicate for every 10 samples (10%).

5.5.2.2 Laboratory Quality Assurance Procedures

The off-site laboratories (Microbial Insights, North Carolina State University, and Clemson University, New Jersey Institute of Technology) implemented lab-specific QA/QC programs to ensure the reliability and validity of analyses performed in the laboratory. Analytical procedures were documented in writing as SOPs, each including a section addressing minimum QC requirements for the procedure. Internal quality control checks differ slightly for individual procedures, but in general QC requirements included the following:

- Method blanks
- Instrument blanks
- Matrix spikes/matrix spike duplicates
- Surrogate spikes
- Laboratory duplicates
- Laboratory control standards
- Internal standard spikes
- Mass spectral tuning

Laboratories were provided with extra sample volume for preparation and analysis of matrix spike and matrix spike duplicates. Field personnel labeled all samples to facilitate MS/MSD analysis. All matrix spikes were completed by lab personnel.

QC sample results were properly recorded and included in the analytical data packages. The data package contained sufficient QC information to allow reconstruction and evaluation of the laboratory QC process by an independent data reviewer. Data generated in the laboratory were properly recorded and compiled into a deliverable package containing sufficient QC information for comparison to relevant criteria.

5.5.3 Calibration of Analytical Equipment

The majority of analyses were completed at the analytical laboratories. The exceptions were groundwater field parameters, all of which were measured in the field using analyte-specific equipment. These were completed by project personnel from Scissortail Environmental Solutions or GSI Environmental using site-specific protocols.

5.5.4 Decontamination Procedures

Standard decontamination measures were employed during sampling activities and all other investigations associated with the project. These were implemented by site contractors or project

personnel using site specific protocols. Procedures employed included the one-time use of sample and method-appropriate containers, as well as decontamination of all sampling equipment (pump tubing) prior to sampling and between collecting each sample. Gloves were worn by all sampling personnel and changed out between each sample to minimize cross-contamination. All IDW was collected and disposed of per site regulations.

5.5.5 Sample Documentation

Sample containers provided by the laboratories for this project were shipped by common carrier or other suitable method in sealed coolers to locations designated by the project team. The laboratory included a shipping form/laboratory chain-of-custody listing containers shipped and the purpose of each container. Containers were not considered in the custody of the laboratory until received by a designated representative. Upon receipt, the shipment was checked to verify that all containers were intact and that the labels were legible and match the custody form. The containers were maintained in the custody of the receiver in a clean, secure area until used for sample processing.

Procedures described below address custody during field sample collection, laboratory analysis, and file storage for the data collected as part of the project.

1) Field sampling personnel were personally responsible for the care and custody of the samples until transferred or properly dispatched.
2) Sample bottles and vessels were labeled with sample numbers and locations at the time of sample collection.
3) Sample labels were completed with permanent ink.
4) The sample label affixed to the container were inspected to confirm that all of the required information was provided.
5) Each sample container was sealed in a zip-lock plastic bag, wrapped in bubble pack, and packed in a wet-ice or dry-ice cooler in a manner to minimize shifting or movement.
6) For each set of samples sent to the laboratory, a triplicate chain-of-custody form was completed. Information on the chain-of-custody form and the sample container labels was checked against the field logbook entries and the samples were recounted. The information contained on the chain-of-custody form included the following:
    - Site name and address or location;
    - Project number;
    - Date of sample collection;
    - Name of sampler responsible for sample submittal;
    - Identification of samples that accompany the form including
    - Field ID number,
    - Number of samples,
    - Date/time collected,
    - Sample container type, volume, preservative,
    - Parameters/methods of interest,
    - Comments about sample conditions;

- Signature of person relinquishing custody and signature of person accepting custody, plus date and time; and
- Identification of common carrier.

7) When a commercial courier service (e.g., Federal Express) transported samples to the laboratory, the chain-of-custody form was signed by a member of the field team, and a copy retained by the field team. The remaining two copies of the form were sealed in a zip-type plastic bag and placed in the cooler with the samples. The cooler was sealed with packaging tape and two custody seals signed and dated by a member of the field team. Custody seals were placed on the exterior of the cooler over the lid and sides. Package routing documentation maintained by the courier service served as chain-of-custody documentation during shipment,

8) If samples were picked up by another third-party representative, a member of the field team signed the chain-of-custody record indicating that the samples were transferred to the courier. The courier will also sign the form, indicating that the samples were transferred to his or her custody. One copy of the chain-of-custody form were retained by the field team and the remaining two copies were sealed in a zip-type plastic bag and placed in the cooler chest with the samples.

9) All documentation is stored at the Scissortail and/or GSI offices. Access is limited to concerned project personnel.

5.6 LAB STUDY RESULTS

Lab studies were performed to determine 1,4-dioxane rate constants using groundwater samples from 10 locations. Rate constants were measured using an assay based on measuring rates of degradation product formation using ^{14}C-1,4-dioxane. This section describes the development of the assay and the results for the groundwater samples. Development including testing the assay with a culture that biodegrades 1,4-dioxane as a sole substrate (CB1190) and a propanotrophic enrichment culture (ENV487) that cometabolizes 1,4-dioxane. The complete dataset from this portion of the project is included in **Table A.1** (Appendix A).

5.6.1 Materials and Methods

5.6.1.1 Microcosms

Groundwater samples from four or five monitoring wells were retrieved from the ten sites described in section 4. For each well, groundwater (100 mL) with low or no sediment was placed in triplicate 160 mL borosilicate glass serum bottles (Wheaton®, Millville, New Jersey), and sealed with polytetrafluoroethylene (PTFE)-faced gray butyl rubber septa (Sun-Sri™, Rockwood, Tennessee; 20 mm) and aluminum crimp caps. Groundwater from each well was also added to two 1-L glass bottles. Samples were shipped overnight to Clemson University. Upon arrival, bottles were immediately removed from the coolers and allowed to warm to room temperature (22 ± 2°C) overnight. The initial concentration of 1,4-dioxane and volatile organic compounds (VOCs) was measured.

Groundwater from the 2-L bottles was filtered (0.2 µm, 47 mm; Whatman™; Millipore Sigma, St. Louis, Missouri) and 100 mL was added to triplicate sterile serum bottles to create filter sterilized groundwater (FSGW) controls. These were used to assess the rate of ^{14}C product formation in the absence of microbial activity. Initial concentrations of 1,4-dioxane and VOCs were measured in the FSGW controls. Purified ^{14}C-1,4-dioxane (described below) was then added to the six serum bottles prepared for each well (i.e., triplicates with groundwater, triplicates with FSGW).

5.6.1.2 Chemicals

^{14}C-1,4-dioxane (0.65 mCi) was custom-synthesized by Moravek Biochemicals, Inc. (Brea, California) and dissolved in butanol. The specific activity was 6.7 mCi per mmol and the reported radiochemical purity was 97.6%. A stock solution was prepared in a 60 mL glass vial by adding the ^{14}C-1,4-dioxane/butanol solution into 49 mL of 1 mM sodium bicarbonate (ACS grade), in order to reduce the specific activity of the ^{14}C-1,4-dioxane and minimize autoradiolysis. The vial was sealed with a Mininert valve cap and stored at 4°C. The total amount of ^{14}C activity recovered in the stock solution was 650 µCi. An ULTIMA GOLD XR (PerkinElmer LAS Inc.) was used for liquid scintillation counting. NaOH (ACS grade) pellets were obtained from VWR (Randor, Pennsylvania); dichloromethane (99.95%) from Omnisolve; acetone (99.5%), sodium acetate (99.7%), and ethylene glycol from Mallinckrodt; methanol from Fisher Scientific; glyoxylate (40% in water) and glyoxal (40% in water) from TCI America; glycolate (70% in water) from MP; and glycoaldehyde and oxalate (99.5%) from EM.

The following VOCs were used: 1,1-DCE (99.5%) from Chem Services; 1,1-DCA (95%) and *cis*-1,2-DCE (99%) from TCI America; 1,1,1-TCA (>99%) from Fisher Scientific; TCE (99.5%) from Aldrich; methane (99.99%) from Matheson; CA (99.7%) from Aldrich; ethane (99.99%) and VC (<1 ppm of nitrogen) from Matheson; ethene (99.5%) from National Welders.

5.6.1.3 Analytical Techniques

1,4-Dioxane was monitored by gas chromatography (GC). Samples from the microcosms (0.3 mL) were filtered (13 mm, 0.2 µm PTFE, VWR) and placed in 500 µL glass inserts in 2.0 mL GC vials. Injections by an autosampler (1.0 µL) were made onto a Zebron ZB-624, 60 m x 0.32 mm ID column with a 1.80 µm film thickness (Phenomenex) in a Hewlett Packard 5890 Series II Plus gas chromatograph, equipped with a flame ionization detector (FID). Hydrogen was used as the carrier gas (1.0 mL/min). The temperature program was 40 ºC for 5 min, then increased to 90 ºC at 6.0 ºC per minute, and held for 3 min, for a total run time of 16.33 min. The injector and detector temperatures were 180 ºC and 260 ºC, respectively. The lowest quantifiable concentration was 1 mg/L. To achieve a lower quantifiable concentration, an alternate method of sample preparation method was used. Micro-frozen extractions of aqueous samples using dichloromethane (DCM) were prepared by adding 3.0 mL of filtered aqueous samples to a 4 mL glass vial in which 0.6 mL of DCM was added. This gives a volumetric sample to DCM ratio of 5.0. The mixture was then capped with a screw-on lid equipped with a Teflon-faced septum. The vials were vortexed for 15 s to ensure adequate mixing of both liquid phases. The vials were then placed upside down in a glass beaker, to allow the DCM phase to be in contact with the septum. The beaker with the samples was placed in a freezer (-20°C) at a 45° angle for one hour. After the aqueous phase in the vials was frozen, an aliquot of approximately 300 µL from the DCM phase was taken rapidly and carefully to prevent any melting of the water phase and then placed into a GC vial. The GC method used to quantify 1,4-dioxane in DCM was the same as that described above, except that the injection volume was set at 3.0 µL. The lowest detectable concentration was 25 µg/L.

For propane, headspace samples (0.5 mL) from microcosms were injected onto a GC equipped with an FID and a RESTEK, Stainless Packed Column (Part No. 80473-800, C42898-01, Molesieve 5A 60/80, 6 ft, 2 mm ID). The carrier gas was N_2 (30 mL/min). An isothermal program was set at 80 ºC for 5 min. The injector and detector temperatures were set at 200 ºC. Response factors were determined based on the GC response and the total amount of propane in the bottle. Assuming the headspace and the aqueous phases were in equilibrium, the total propane present was converted to an aqueous phase concentration:

$$C_l = \frac{M}{V_l + H_C V_g} \qquad 5\text{-}1$$

where C_l is the concentration in the liquid phase (µM); M is the total mass present (µmol/bottle); V_l is the volume of the liquid in the bottle (L); V_g is the volume of the headspace in the bottle (L); and H_C is the Henry's constant ((mol/m^3·gas concentration)/(mol/m^3 liquid concentration)) at 23°C (LaGrega et al., 2010).

Oxygen was monitored by injecting a headspace sample (0.5 mL) onto a GC equipped with a thermal conductivity detector (TCD) and a 3.175-mm x 3.25-m 100/120 Carbosieve SII column (Supelco). N_2 was used as the carrier and reference gas (50 mL/min). The oven and TCD were isothermal (both 105°C).

Protein was quantified using a modified version of the MicroBCA assay (Thermo Scientific®). Bovine serum albumin (BSA) was used as a standard. Samples (0.9 mL) were mixed with sodium hydroxide (0.1 mL, 10 M) by vortexing and placed in a water bath (90ºC, 10 min). Aliquots of the lysate were neutralized (pH 6.5-7.5) using HCl (80-95 µL, 11.64 M) and bicarbonate (0.5 mL, 0.5 mM $NaHCO_3$). Samples (1 mL) were then mixed with MicroBCA Working Reagent (1 mL), incubated in a water bath (60ºC, 1 h), cooled (25 min), and checked for absorbance (Genesys 20 UV-visible spectrophotometer, Thermo Scientific®) at 562 nm. Protein was assumed to constitute 50% of the mass of biomass (Grady et al., 2011).

5.6.1.4 Cultures

Pseudonocardia dioxanivorans CB1190 was provided courtesy of Dr. Shaley Mahendra at the University of California at Los Angeles. Colonies from agar plates were transferred to bottles containing ammonium mineral salts medium (AMSM) amended with 100 mg/L of 1,4-dioxane. The bottles were sealed with gray butyl rubber septa and screw caps and incubated on a shaker table (100 rpm) at room temperature. The culture was maintained by transferring it periodically to fresh AMSM with concentrations of 1,4-dioxane up to 500 mg/L, as well as to agar plates with AMSM and 1,4-dioxane. After growing the culture in medium, cells were harvested close to the stationary phase to obtain the inoculum used in the experiments.

ENV487, a mixed culture of propanotrophs, was provided courtesy of Dr. Robert Stefan at Chicago Bridge & Iron, Inc. The culture was grown in basal salts mineral medium (BSM) (Hareland et al., 1975), modified in order to reduce the amount of organic chelator (nitrilotriacetic acid, NTA) (Barajas-Rodriguez, 2016). ENV487 was grown at room temperature (22-24°C) in 2.6 L glass bottles containing 1.5 L of BSM and 20% propane/80% air (v/v) in the headspace. The bottles were incubated at room temperature on a shaker table (100 rpm). Oxygen in the headspace was maintained between 5-21% during biomass growth by periodic addition of pure oxygen. After growing the culture in medium, harvesting was done when microbial activity was close to the stationary phase to obtain the inoculum used in the experiments. It was assumed that cells harvested during this phase still produced the necessary enzymes to carry out cometabolism of 1,4-dioxane. Washing the cells removed any residual dissolved propane.

5.6.1.5 ^{14}C-1,4-Dioxane Purification

To facilitate detection of low levels of ^{14}C-1,4-dioxane degradation products, it was necessary to remove ^{14}C-labeled impurities before adding it to the serum bottles. The impurities may interfere with detection of low levels of 1,4-dioxane degradation products. ^{14}C-1,4-dioxane purification was achieved by injecting an aliquot (100 µL) of the stock solution onto a Dionex UltiMate 3000 high-performance liquid chromatograph (HPLC) equipped with an Aminex HPX-87H column (300 mm x 7.6 mm; BioRad) and a Refractive Index (RI) or UV-Vis detector. H_2SO_4 was used as the eluant

(0.01 N, 0.6 mL per min). 1,4-Dioxane eluted between 22.4 and 26.0 min; 2.16 mL was collected. Butanol eluted from 33 to 39 min.

Recovery of ^{14}C-1,4-dioxane was calculated based on the ^{14}C activity in the trapped interval (22.4-26.0 min) that corresponded to 1,4-dioxane, divided by the ^{14}C activity in the stock solution injected onto the HPLC (Equation 5-2):

$$\%\ Recovery\ = \left(\frac{dpm\ trapped \left(\frac{2.16\ mL}{0.1\ mL}\right)}{dpm\ injected\ onto\ HPLC \left(\frac{100\ \mu L}{20\ \mu L}\right)} \right) (100) \qquad 5\text{-}2$$

where 2.16 mL is the volume collected from the HPLC; 0.1 mL is the volume of stock solution injected onto the HPLC; 100 µL is the volume of stock solution injected onto the HPLC; 20 µL is the volume of stock solution added directly to liquid scintillation cocktail (LSC) to determine ^{14}C activity in the stock solution; and dpm is disintegration per minute.

The total initial amount of ^{14}C present per bottle was determined based on:

$$C_{tot} = \frac{S_l}{V_{l,s}} \cdot V_{l,b} \qquad 5\text{-}3$$

where C_{tot} is the total ^{14}C present (dpm/bottle); S_l is the ^{14}C in a liquid sample (dpm/sample); $V_{l,s}$ is the volume of the sample; and $V_{l,b}$ is the initial volume of liquid in the bottle (~100 mL).
The efficiency of purification was determined based on the presence of impurities at time zero. Impurities were quantified in terms of $^{14}CO_2$ and ^{14}C-labeled non-strippable residue (NSR) (see below) measured at time zero and a direct count in the liquid phase (0.1 mL of liquid sample), as follows:

$$\%\ Impurities = \left(\frac{A + B}{C}\right) (100) \qquad 5\text{-}4$$

where A = $^{14}CO_2$/bottle (including in the aqueous phase and headspace); B = ^{14}C-NSR; and C = the total ^{14}C added to the bottles. The procedures used to determine A and B are described below. C was determined based on the ^{14}C activity in a sample (0.1 mL) of the purified stock solution added directly to LSC.

Controls were prepared with bottles containing media (AMSM or BSM) and no inoculum. Percentage impurities from experimental bottles were compared against the controls to evaluate the statistical significance of the impurities.

5.6.1.6 Monitoring and ^{14}C-product Distribution

Experiments were started by adding the purified ^{14}C-1,4-dioxane stock solution (0.5 mL) into serum bottles containing 100 mL of groundwater or medium, resulting in ~0.27 µCi per bottle (~600,000 dpm). Addition of purified ^{14}C-1,4-dioxane increased the total concentration of 1,4-

dixane by ~163 µg/L. The actual total amount of ^{14}C present per bottle at time zero was determined based on samples of the aqueous phase and using equation 5-3.

The protocol used to quantify the rate of ^{14}C product formation was adapted from the method developed by Mills et al. (2018). **Figure 5.6.1** shows a schematic of the assay. Aqueous and gaseous samples were removed from the microcosms at approximately weekly intervals over 42 days of incubation to measure the formation of degradation products. Liquid samples were added to 20 mL borosilicate glass scintillation vials with screw caps (VWR). A headspace sample (0.5 mL) was removed with a gas-tight syringe and injected into a 20 mL borosilicate glass scintillation vial. The cap on the vial was lined with a PTFE-faced gray butyl rubber septum and a hole (2.38 mm hole) was drilled in the cap to permit passage of the needle. The vial contained 15 mL of LSC and 1 mL of 1 M NaOH (to ensure the $^{14}CO_2$ in the gas sample dissolved in the scintillation cocktail).

A liquid sample (5.1 mL) from the serum bottles was removed and 0.1 mL was added to a scintillation vial with 15 mL of LSC to determine the total ^{14}C removed in the sample. The remaining 5.0 mL was added to a scintillation vial with a drilled screw cap and a Teflon-faced septum. HCl (24 µL of 6 M) was injected to lower the pH (< 2, confirmed using a pH strip; BDH® VWR Analytical). CO_2 was expunged from the liquid by inserting a needle into the liquid with a flow of N_2 (500 mL/min). A second needle was inserted into the headspace of the vial and transferred the gas through latex tubing a second vial containing 5 mL of 1 M NaOH, to trap the $^{14}CO_2$. A fritted gas dispersion tube was used in the second vial to promote contact between the gas and liquid phases. Both vials were tilted on a 30° angle to facilitate contact between the N_2 and liquid. The second vial was left open to the atmosphere. The sparging was performed in a hood. A schematic of the sparging apparatus is shown in **Figure 5.6.2.** After 30 min of sparging, the contents of the acidified vial were processed as described below; the contents of the second vial were added to 15 mL of LSC and presumptively represented $^{14}CO_2$.

Although 1,4-dioxane is not readily sparged from water, enough volatilizes such that it was necessary to insert a sorbent (described below) in the tubing between the acid and basic vials to prevent any ^{14}C-1,4-dioxane from reaching the second vial. This was confirmed by measuring ^{14}C levels in the second vial when sparging a sample in the first vial that contained ^{14}C-1,4-dioxane (but no $^{14}CO_2$), with and without the cartridge installed. Without the cartridge, 2% of the ^{14}C-1,4-dioxane was detected in the second vial; with the cartridge installed between the vials, no ^{14}C was detected in the second vial.

Because of the low volatility of 1,4-dioxane, it cannot be separated from degradation products by sparging with N_2, as Mills et al. (2018) were able to do when the test compound was ^{14}C-labeled trichloroethene. Separation of the unreacted 1,4-dioxane from its degradation products was accomplished by a combination of acidic stripping with N_2 to remove $^{14}CO_2$, followed by passage through a selective sorbent (ENVI-Carb Plus, Supelco Inc., Sigma-Aldrich) to remove 1,4-dioxane. Cartridges were placed in a Visiprep Solid Phase Extraction Vacuum Manifold (Supelco Inc.). Preliminary tests were run to confirm that the sorbent removed 1,4-dioxane by treating samples containing the same level of ^{14}C-1,4-dioxane as was added to the groundwater and other samples. Samples (5.0 mL) were passed through the cartridge and ^{14}C was measured in the eluant. From 246 samples evaluated, the eluant contained less than 0.05% of the ^{14}C-1,4-dioxane that was

applied. Cartridges were re-used for all bottles from a single well throughout the incubation period (42 days). The effectiveness of the cartridges for repeated use was tested; they did not show any leakage of ^{14}C-1,4-dioxane when reused up to 20 times (**Figure 5.6.3**).

Soluble products that passed through the cartridges are referred to as non-strippable residue (NSR). For 1,4-dioxane, this includes several compounds that have been shown to be formed during metabolic and cometabolic biodegradation, including glyoxylate, glycolate, glyoxal, oxalate, and ethylene glycol (Grostern et al., 2012). Preliminary tests were performed to evaluate the passage of these compounds through the cartridges, based on measurement of their concentration in the eluant. Overall, 63-100% of these potential soluble products passed through the cartridges. Other sorbents were evaluated (i.e., SDB-L and ENVI-Carb) that effectively removed 1,4-dioxane but also retained higher percentages of the potential degradation products. The fact that some of the compounds tested were retained indicates that the NSR portion of the total ^{14}C products formed may have been underestimated, at least to the extent that NSR was a product.

Two sets of duplicate bottles with 100 mL of 1 mM HCO_3^- amended with and without ^{14}C-labeled material (0.5 mL of the purified stock solution) were constructed to verify the effectiveness of the cartridges for retaining ^{14}C-1,4-dioxane and separating it from potential degradation products. Counts from before (direct count from liquid phase) and after (CO_2 and NSR) the ^{14}C assay were compared to evaluate the cartridge effectiveness. The assay extracted 99.95% of the 1,4-dioxane present in the samples, indicating successful retention of the 1,4-dioxane.

The sorbent needed to be conditioned prior to processing the samples. This entailed 1) adding 1 mL of dichloromethane (DCM) and pulling it through the cartridge under a vacuum (15-20 in Hg, Visiprep solid phase extraction vacuum manifold); 2) adding 4 mL of methanol; 3) adding 3 mL of distilled deionized (DDI) water (pH 7). Enough of the DDI water was left in the cartridge to keep the sorbent wetted. At this point, the cartridges were ready for processing samples.

Prior to passage of the acidified sample through the cartridges, the pH was neutralized using 10 M NaOH. pH adjustment decreased the potential for retention of degradation products on the sorbent. Samples were filtered (13 mm, 0.2 µm PTFE, VWR) to remove any particulates that may contain sorbed 1,4-dioxane. An aliquot (0.1 mL) of the liquid sample was evaluated for ^{14}C in LSC (15 mL). The rest of the sample (4.9 mL) was drawn through the cartridge and into a collection vial at a rate of 1 drop per second. Eluant collected from the cartridges was transferred to a scintillation vial with 15 mL of LSC. Subsequently, 2 mL of DCM was added to the cartridges to extract the adsorbed ^{14}C-1,4-dioxane. The cartridges were then allowed to dry and were stored for the next sampling event.

On the final day that microcosms were incubated, routine measurements were made (i.e., ^{14}C present in the headspace and liquid, oxygen in the headspace, and ^{14}C products in a 5.0 mL liquid sample). A mass balance for ^{14}C was calculated at the end of the incubation period based on the percent recovery of ^{14}C remaining in the bottle plus the ^{14}C removed, divided by the initial amount of ^{14}C added:

$$\%\, Recovery = \frac{dpm_{present} + dpm_{removed}}{dpm_{initial}} \qquad 5\text{-}5$$

where $dpm_{present}$ represents the amount of ^{14}C remaining in the liquid plus headspace, $dpm_{removed}$ represents the cumulative ^{14}C removed in the liquid and headspace, and $dpm_{initial}$ represents the initial ^{14}C added. Equation 5-5 was used to determine the recovery of labeled products and unreacted 1,4-dioxane divided by the total ^{14}C added to the bottles ($dpm_{initial}$). The overall recovery for groundwater samples was 102.3% ± 8.3% and 101.2% ± 6.6% for the FSGW controls. The contribution of degradation products (i.e., CO_2 and NSR) to the numerator in equation 5-5 was 0.1 ± 0.1% for experimental bottles and 0.1 ± 0.0% for controls. This indicated that most of the ^{14}C remaining in the microcosms was unreacted 1,4-dioxane. Of the products measured, $^{14}CO_2$ contributed 50.7 ± 34.3% for the microcosms and 38.2 ± 29.2% for the FSGW controls.

5.6.1.7 ^{14}C Assay Validation

The functionality of the ^{14}C assay was confirmed using a propanotrophic enrichment culture (ENV487) that cometabolically oxidizes 1,4-dioxane and a culture that metabolizes 1,4-dioxane, *Pseudonocardia dioxanivorans* CB1190. Following growth of ENV487 on propane in basal salts medium (BSM) to the stationary phase (~10^{10} cells·mL^{-1}, based on measurement of protein and a conversion factor of $9.5x10^{-13}$ g of biomass per cell), the cells were harvested by centrifugation (10,750x*g*, 15 min) and re-suspended in BSM. The cells were serially diluted in 160 mL serum bottles (100 mL of culture/bottle) to 10^{-8} (i.e., 1 mL of the original culture diluted in 10^8 mL of BSM) to provide a wide range of cell concentrations. The same was done with CB1190, following growth on 1,4-dioxane as its sole source of carbon and energy in ammonium mineral salt medium (AMSM). The intent of serial dilutions was to determine the lowest rate of biodegradation of 1,4-dioxane that can be quantified using the ^{14}C assay. Purified ^{14}C-1,4-dioxane was added to the dilutions and rates of accumulation of ^{14}C-labeled degradation products were measured. Total 1,4-dioxane was also monitored using micro-frozen extraction of a water sample followed by analysis on a gas chromatograph (GC) with a flame ionization detector, as described above. A treatment without dilution of the cells served as a positive control, to ensure that biodegradation of 1,4-dioxane and accumulation of ^{14}C-labeled products proceeded to completion within several days of incubation. Bottles were placed on a shaker table (100 rpm) at room temperature and were monitored weekly for 42 days for accumulation of ^{14}C-labeled products.

To validate the role of monooxygenases in biodegradation of 1,4-dioxane during the ^{14}C assay, inhibition tests with acetylene and the absence of oxygen were performed. Monooxygenase enzymes are critical to biodegradation of 1,4-dioxane via metabolic and cometabolic pathways (Gedalanga et al., 2014; Li et al., 2013; Mahendra and Alvarez-Cohen, 2006; Sales et al., 2013). As the simplest alkyne, acetylene inactivates a variety of bacterial monooxygenases (Li et al., 2013). Oxygenase inhibition with acetylene was tested with CB1190 and ENV487 (~10^{10} cells·per mL) in media (AMSM and BSM, respectively). Serum bottles (160 mL) with 100 mL of media amended with 10 mg/L of unlabeled 1,4-dioxane (triplicates) were incubated using four treatments: 1) in the absence of oxygen; 2) aerobic, 8% acetylene added to the headspace (Li et al., 2013); 3) aerobic, acetylene added and then removed, to evaluate if inhibition is reversible; and 4) aerobic, no acetylene added (positive control). Anaerobic conditions for treatment #1 were established by sparging the headspace with high purity N_2, scrubbed of traces of oxygen by passage through a titanium citrate solution. For treatments #2 and #3, 4.8 mL of acetylene was injected into the headspace. For treatment #3, acetylene was kept in the bottle for 30 min, followed by stripping

with high purity N_2 for 5 min; the bottles were then left open to room air as a supply of oxygen. Treatments #1, #2, and #4 received purified ^{14}C-1,4-dioxane; another set did not. The initial concentration of 1,4-dioxane was approximately 10 mg/L. Bottles were placed on a shaker table (100 rpm) at room temperature and were monitored weekly for 42 days for ^{14}C-labeled products and for total 1,4-dioxane.

5.6.1.8 Sample Handling Effect

The ^{14}C assay begins with obtaining groundwater samples in the field and shipping those overnight on ice to the laboratory, where the bottles are then warmed to room temperature overnight. Experiments were performed to evaluate if this method of sample handling had an impact on the rate of 1,4-dioxane biodegradation. ENV487 and CB1190 were used to assess the impact of sample handling. The cultures were grown in medium and then CB1190 was added to groundwater samples from Site 6, Well 2 and ENV487 was added to samples from Site 6, Well 3. These groundwater samples contained no detectable levels for VOCs, which could potentially inhibit 1,4-dioxane biodegradation. Three treatments were used to test the effects of temperature, each in triplicate: 1) the assay was started immediately after adding the cultures, with no cooling and reheating; 2) the cultures were added and the bottles were stored on ice overnight, then warmed to room temperature in 25 min by immersing the bottles in room temperature water; 3) the cultures were added and the bottles were stored on ice overnight (to replicate what occurred during overnight shipping of the groundwater samples), then warmed to room temperature over the next night (to replicate how groundwater samples were handled when they were received at the laboratory). Purified ^{14}C-1,4-dioxane was added once the bottles were at room temperature. Following growth in medium to a density of ~10^{10} cells·mL^{-1}, the experiments were inoculated CB1190 (10^{-2}) and ENV487 (10^{-1}). These concentrations were selected based on results from the assay validation experiments described above. Positive controls were set up with medium (AMSM or BSM) inoculated with the culture; negative controls consisted of bottles with groundwater only (no inoculation). For the set inoculated with CB1190, two batches were prepared at different initial concentration of 1,4-dioxane; one set received 10 mg/L, the other received 0.163 mg/L. Bottles were placed on a shaker table (100 rpm) at room temperature and were monitored weekly for ^{14}C-labeled products over 42 days of incubation.

5.6.1.9 Nutrient Effect

The effect of nutrient amendments on the rate of 1,4-dioxane biodegradation was evaluated using groundwater samples from three sites that contained low levels of VOCs. As will be shown, groundwater from these wells did not have a detectable level of 1,4-dioxane biodegradation. To increase the likelihood of biodegradation, treatments were prepared that were inoculated with CB1190 and ENV487, with varying levels of nutrients added. As with the sample handling experiments, the cultures were grown in medium and then CB1190 was added to groundwater samples from Site 6, Well 2 and ENV487 was added to samples from Well 3. Groundwater from Site 8 Well 4 was also evaluated, but only with CB1190. Microcosms were prepared following the standard assay protocol (i.e., 100 mL of groundwater added to 160 mL serum bottles). The bottles were inoculated with cultures grown to ~10^{10} cells·mL^{-1}; 10^{-2} for CB1190 and 10^{-1} for ENV487. These inoculum levels were selected based on the results from the validation experiments described above. Four treatments were evaluated: 1) groundwater only; 2) groundwater inoculated

with CB1190 or ENV487; 3) groundwater inoculated with CB1190 or ENV487 and amended with 5% (v/v) AMSM in bottles with CB1190 and BSM for bottles with ENV487; and 4) 100% medium inoculated with CB1190 or ENV487 (positive controls). For the bottles with samples from an industrial site (i.e., not one of the 10 described in this report), an additional treatment of 5% nutrients added to groundwater without inoculation was used to monitor the effect of nutrients on indigenous 1,4-dioxane-degraders. Purified ^{14}C-1,4-dioxane was added to all bottles, which were then placed on a shaker table (100 rpm) at room temperature and were monitored weekly for ^{14}C-labeled products over 42 days of incubation. Total 1,4-dioxane was measured by GC analysis in a parallel set of bottles with groundwater amended with an initial 1,4-dioxane level comparable to bottles amended with the ^{14}C-1,4-dioxane. Duplicates were prepared for all treatments except for triplicates with the 100% medium treatment.

5.6.1.10 Modeling to Determine First-Order Rate Coefficients

Pseudo-first order rate coefficients for biodegradation of 1,4-dioxane were determined following the mass balance approach used by Mills et al. (2018) for trichloroethene. Since 1,4-dioxane is essentially nonvolatile, one of the modifications was to eliminate the part of the mass balance that keeps track of ^{14}C-1,4-dioxane in the headspace of the bottles. For a majority of the groundwater samples tested, the concentration of 1,4-dioxane already present plus the amount added with the ^{14}C-1,4-dioxane, resulted in initial concentrations below 1 mg/L. The initial concentrations were therefore below the half saturation constant (~6-15 mg/L) for known metabolic and cometabolic 1,4-dioxane-degraders (Barajas-Rodriguez and Freedman, 2018). Consequently, first-order kinetics were selected to represent the rate of 1,4-dioxane degradation. However, since the amount of 1,4-dioxane degrading cells in the groundwater microcosms was not determined, and the rate coefficients developed through the model could not be normalized to the amount of biomass. Therefore, the assumption was made that the amount of biomass present remained unchanged during the assay, which is consistent with the assumption of pseudo first-order reaction kinetics (Mills IV et al., 2018).

The accumulation of ^{14}C-products over time was used to determine the pseudo first-order rate coefficients (k) by fitting experimental data to a mass balance model for ^{14}C in the microcosms. Rate coefficients were determined using the optimization solver MATLAB by minimizing the sum of squared errors between the prediction and the ^{14}C product accumulation data over time. Triplicate bottles were fit simultaneously to obtain a single value for k (Mills et al., 2018). MATLAB rate coefficients were compared to ones obtained through a spreadsheet in Excel to validate calculations performed in the script. Overall, the difference between the two methods was not significant (0.04 ± 0.06%).

k values and 95% confidence intervals were determined with MATLAB for the groundwater microcosms ($k_{experimental}$) and the FSGW microcosms (k_{FSGW}). When the rate coefficients for both were statistically significant, a net rate coefficient for the groundwater (k_{net}) was calculated by subtraction:

$$k_{net} = k_{experimenal} - k_{FSGW} \quad \text{5-6}$$

The 95% confidence interval (CI_{net}) for k_{net} was calculated by propagation of error:

$$Std_{net} = \sqrt{\frac{(n_1 - 1)std_1{}^2 + (n_2 - 1)std_2{}^2}{n_1 + n_2 - 2}} \quad 5\text{-}7$$

$$CI_{net} = \left(t_{\frac{\alpha}{2},df}\right)(std_{net})\sqrt{\frac{1}{n_1} + \frac{1}{n_2}} \quad 5\text{-}8$$

where Std_{net} is the standard deviation for k_{net}; n_1 and n_2 are the number of measurements taken for the experimental and FSGW bottles, respectively; std_1 and std_2 are the standard deviations for the experimental and FSGW bottles, respectively; and $t_{\alpha/2,df}$ is the critical Student's t value for the pooled sample.

When the net value for k was not statistically significant, a minimum half-life was calculated using the value of k for the groundwater samples and adding to it the 95% confidence interval, as follows:

$$t_{½} = \frac{\ln(0.5)}{k + CI_{95}} \quad 5\text{-}9$$

where $t_{½}$ is the minimum half-life (year), k is the first order rate constant (per year), and CI_{95} is the one-tailed 95% confidence interval for k. For a typical groundwater sample, the number of samples used to determine k was 21, so the degrees of freedom were 20 for this calculation.

5.6.1.11 Liquid Scintillation Counting

Beta radiation measurements were made using a Liquid Scintillation Analyzer (Tri-Carb® 2910 TR, PerkinElmer). QuantaSmart™ was used as the interface. The assay parameters were set up for DPM (single) measurements. The instrument protocol was as follows:

- Count conditions: ^{14}C radionuclide
- Count Mode: Normal
- Quench Set: ^{14}C; Quench Indicator: tSIE/AEC
- External Std Terminator: 0.5 2s%; Pre-count Delay (min): 0.00; Count Time (min): 15
- Assay Count Cycles: 1; Repeat Sample Count: 1
- #Vials/Sample: 1
- Count Corrections: Special Conditions: Static Controller
- Coincidence Time (nsec): 18; Delay Before Burst (nsec): 75

5.6.2 Results

5.6.2.1 ^{14}C-1,4-Dioxane Purification and Addition

During the purification process, >90% of the ^{14}C activity injected onto the HPLC was recovered in the 1,4-dioxane fraction (22.4 to 26.0 min). None of the other fractions that were collected contained ^{14}C activity above background. The activity level in the purified ^{14}C-1,4-dioxane from each trapping event was relatively consistent, with an average of 976,473 ± 62,366 dpm/mL (Ramos-Garcia, 2020).

Addition of the purified ^{14}C-1,4-dioxane to the microcosms (0.5 mL per bottle) increased the initial concentration of 1,4-dioxane by ~165 µg/L. The average initial dpm added per bottle was 548,333 ± 25,807, with no significant change during the period of use (Ramos-Garcia, 2020).

The initial purity of the ^{14}C-1,4-dioxane was assessed by adding 0.5 mL to triplicate bottles containing 1 mM HCO_3^- in DDI water. Another set received no ^{14}C-1,4-dioxane. Aqueous and headspace samples were then subjected to analysis of ^{14}C degradation products. The sum of the $^{14}CO_2$ and ^{14}C-NSR was 0.064% of the total ^{14}C added to the bottles (**Figure 5.6.4**). This is above an initial goal of having less than 0.01% impurities. Nevertheless, the impurities (409 dpm/bottle, after subtracting activity in the bottles with no ^{14}C added) were minor in relation to the total ^{14}C added to the bottles (634,653 dpm/bottle).

Each time an experiment was started, the purity of the stock added was assessed in the same manner. Based on 16 samples analyzed, the average level of impurities at time zero for samples with media (AMSM and BSM) and non-inoculated was 0.023 ± 0.015%.

Based on 246 of the bottles analyzed, the average level of impurities at time zero was 0.038 ± 0.031%. This was considered to be sufficiently low to allow for detection of biodegradation activity over six weeks of incubation.

5.6.2.2 ^{14}C Assay Validation

The assay was evaluated with metabolic and cometabolic cultures (i.e., CB1190 and ENV487). Following six weeks of incubation, statistically significant increases in ^{14}C products occurred in all but the 10^{-8} dilution for CB1190. **Figure 5.6.5** shows the ^{14}C-product accumulation trends. The lowest detectable pseudo first order rate coefficient obtained (using the 10^{-7} dilution) for CB1190 was 0.016 per year, corresponding to a half-life of 43 years. For ENV487, the lowest rate coefficient measured (using the 10^{-5} dilution) was 0.021 per year, corresponding to a half-life of 33 years (**Table 5.6.1**). The trends for ^{14}C product accumulation are shown in **Figure 5.6.6**. These results confirmed that the assay is adequately sensitive to detect rate coefficients that are meaningful in the context of assessing *in situ* remediation. As will be shown below in the results for groundwater samples, even lower statistically significant rate coefficients were detectable, corresponding to half lives in excess of 100 years.

The involvement of monooxygenases in biodegradation of 1,4-dioxane was assessed using acetylene as an inhibitor, as well as incubation in the absence of oxygen. Both CB1190 and ENV487 were evaluated. As shown in **Figure 5.6.7** for CB1190 with only unlabeled 1,4-dioxane present, 8% acetylene added to the headspace inhibited biodegradation of 1,4-dioxane by CB1190 over 40 days of incubation, compared to complete removal of the contaminant in 12 days with no acetylene added. The effect of acetylene was reversible when the acetylene was flushed out after

30 min of exposure. Incubation in the absence of oxygen also prevented biodegradation. When the experiment was repeated with ^{14}C added at a lower initial starting concentration of 1,4-dioxane (0.163 mg/L), acetylene slowed the rate of product formation relative to the positive control with no acetylene added, although the rate of accumulation was statistically significant. Likewise, incubation in the absence of oxygen significantly slowed but did not completely stop accumulation of ^{14}C degradation products (**Figure 5.6.8**).

Similar results were obtained with ENV487. Acetylene and anaerobic incubation inhibited biodegradation of unlabeled 1,4-dioxane (**Figure 5.6.9**). In this case, however, acetylene inhibition was not reversible. When ^{14}C was added, acetylene and anaerobic conditions completely stopped accumulation of products in comparison to the positive control with no inhibition (**Figure 5.6.10**). The results for CB1190 and ENV487 confirmed the involvement of monooxygenases and the potential to use acetylene inhibition and/or anaerobic incubation in the ^{14}C assay if there is any doubt about the role of oxygenases in producing a positive rate coefficient.

The effect of sample handling on measurement of rate coefficients by comparing the standard protocol (shipping overnight on ice, warming to room temperature overnight) to ones in which the length of time on ice and the length of time to warm the sample were decreased. There was no statistically significant difference among rate coefficients for the standard protocol and the variants. Results for ^{14}C product accumulation are presented in **Figures 5.6.11, 5.6.12A,** and **5.6.13**. An average pseudo first order rate coefficient of 0.25 ± 0.0093 per year was obtained for the treatments inoculated with CB1190, and 2.1 ± 0.17 per year for ENV487. Similar results were obtained when a higher initial concentration of 1,4-dioxane (10.2 mg/L, **Figure 5.6.12B**) was used.

5.6.2.3 Pseudo First Order Rates Constants for Groundwater Samples

Representative results for ^{14}C product accumulation in three of the groundwater samples are shown in **Figure 5.6.14**, along with the corresponding FSGW controls; results for all of the samples are available in Ramos-Garcia (2020). **Figure 5.6.14** spans a range of results, i.e., a well with a comparatively high net rate coefficient (k_{net} = 0.0957 per year; Site 3, Well 4); a sample with a relatively low rate coefficient (k_{net} = 0.0209 per year; Site 3, Well 5); and a sample for which there was no statistically significant difference between the groundwater and the FSGW control (Site 5, Well 3).

Of the 54 groundwater samples analyzed, statistically significant rate coefficients were determined for 24 (Table 5.6.2); no significant rate of degradation was observed in 30 of the samples. The median rate coefficient observed using the ^{14}C assay was 0.0137 per year (half-life = 51 year); the maximum rate coefficient was 0.367 per year (half-life = 1.9 year) and the lowest observed was 0.0021 per year (half-life = 328 year). These results indicate that for many of the wells examined, biodegradation of 1,4-dioxane is occurring at a relatively slow rate or not at all.

One notable outlier in the results occurred with the groundwater from Site 4 Well 1. After 42 days of incubation, there was no statistically significant rate of ^{14}C product accumulation. This was unexpected because prior samples from this site yielded the isolate *P. dioxivorans* BERK-1 (Ramos-Garcia et al., 2018). The microcosms were allowed to incubate after the typical 42 day period used for the ^{14}C assay. On day 243, the concentration of 1,4-dioxane was checked using the

gas chromatography method and was below detection (25 µg/L). There was no detectable ^{14}C-1,4-dioxane remaining and the main ^{14}C-labeled compound remaining was $^{14}CO_2$. At some point between days 42 and 243, microbes in the groundwater consumed the 1,4-dioxane, from an initial concentration of 252 µg/L. A half-life of 0.28 year was estimated, assuming a linear rate of consumption between days 42 and 243; from that a rate coefficient of 2.5 per year was calculated. This was approximately seven times higher than the highest rate coefficient determined with the ^{14}C assay.

Based on the result described above, 1,4-dioxane levels in 24 other microcosms were examined by gas chromatography after 160-317 days of incubation. A statistically significant decrease in 1,4-dioxane was observed in microcosms from four of these wells (Site 3, Wells 3, 4, and 5; Site 4 Well 2), along with five other wells that were checked after 42 days of incubation (Table 5.6.3). For each of these wells, the ^{14}C assay yielded a statistically significant rate coefficient, indicating good agreement between the observations. For the other well samples that yielded a statistically significant rate coefficient based on the ^{14}C assay, the magnitude of decrease in total 1,4-dioxane was not significant. However, the rate coefficients for these wells were sufficiently low that a measurable decrease in 1,4-dioxane based on the GC method was not expected. For example, the rate coefficient for Site 5 Well 2 determined by the ^{14}C assay was 0.0042 per year. With that coefficient, the 1,4-dioxane concentration would be expected to decrease by only 0.6 µg/L after 208 days of incubation; that is too small a decrease to detect with the GC method used.

The percentage of oxygen in the headspace of the microcosms was evaluated at the end of the incubation period for the ^{14}C assay, and after extended incubation in a subset of the microcosms. Oxygen consumption was expected to serve as a surrogate for biodegradation of any organic compound, not just 1,4-dioxane. However, a statistically significant decrease in the percent oxygen occurred in microcosms for only three groundwater samples (Site 3, Wells 3 and 4 and Site 4, Well 4 for the second sampling event). The samples from Site 3 also had statistically significant rate coefficients according to the ^{14}C assay (**Table 5.6.2**) and both experienced a statistically significant decrease in 1,4-dioxane based on the end of incubation GC analysis (**Table 5.6.3**). The low level of positives for oxygen consumption suggested that the majority of groundwater samples were low in biodegradable organic matter.

Duplicate samples were obtained from six wells (Site 1, Wells 2 and 3; Site 4, Wells, 1, 3, and 4; Site 5, Well 4) and triplicates from one well (Site 4, Well 2). The time between sampling events ranged from 12 to 16 months. One of the motivations for doing so was to determine if the first set of ^{14}C results could be replicated at a later date. For three of the wells, the net rate coefficient was not statistically significant in samples taken the first or second time (Site 1, Wells 2 and 4; Site 5, Well 4). For Site 4, there was considerable variability in the rate coefficients from replicate samples. The outcome for Well 1 for the first sampling event is described above and resulted in an estimated rate coefficient of 2.5 per year, while the result for the second sampling event one year later was 0.0159 ± 0.0110 per year. For Well 2, the rate coefficients were 0.0061 ± 0.0051 per year for the first sampling event, 0.0143 ± 0.0117 per year for the second event, and 0.297± 0.0580 per year for the third event. For Well 3, the rate coefficients were not statistically significant for the first or second sample. For Well 4, the first sampling event yielded a rate coefficient of 0.0073 ± 0.0028 per year and not significant for the second event. These results indicate good reproducibility in the ^{14}C assay when there is an absence of 1,4-dioxane biodegradation. Results

were more variable when degradation was detectable, suggesting that repeat samples may be advisable to gain greater confidence in positive results.

Chlorinated VOCs in general, and 1,1-DCE in particular, are known to inhibit aerobic biodegradation of 1,4-dioxane (Mahendra et al., 2013; Zhang et al., 2016; Zhao et al., 2018). The groundwater samples had variable levels of CVOCs, ranging from non-detect to ~70 µM (**Figure 5.6.15**). This represents the sum of the chlorinated ethenes and ethanes. The higher rate coefficients for 1,4-dioxane biodegradation were detected at low or no CVOCs. However, one of the rate coefficients was determined in the presence of ~7 µM CVOCs. A similar pattern emerged for 1,1-DCE, with the highest rate coefficients occurring when 1,1-DCE was non-detect. However, rate coefficients were detected with as much as 160 µg/L of 1,1-DCE present. These results indicate that, in groundwater samples, CVOCs partially inhibited 1,4-dioxane biodegradation, i.e., the presence of CVOCs does not preclude the occurrence of degradation, but they are likely to reduce the rate.

5.6.2.3 Nutrients Effect

One of the potential limitations of using only groundwater in the ^{14}C assay is a lack of nutrients needed for biodegradation of 1,4-dioxane, especially for microbes that use the compound as a growth substrate. Experiments were therefore performed to evaluate the effect of nutrient supplements in microcosms with only groundwater, or groundwater inoculated with CB1190 or ENV487. Nutrients were provided in the form of the medium used to grow each culture (i.e., AMSM for CB1190 and BSM for ENV487).

Three groundwater samples were evaluated: An auxiliary industrial site with groundwater containg 1,4-dioxane at less than 100 µg/L (**Figure 5.6.16**), Site 6 Well 2 (**Figure 5.6.17**), and Site 6 Well 3 (**Figure 5.6.18**). For the auxiliary industrial site, the microcosms with only groundwater and groundwater plus 5% nutrient medium did not exhibit statistically significant rates of 1,4-dioxane biodegradation. Both treatments with groundwater inoculated with CB1190 exhibited statistically significant rates of ^{14}C product formation. The treatment that received 5% addition of nutrient medium plus CB1190 exhibited a rate of accumulation approximately four times higher than groundwater plus CB1190 alone, indicating the groundwater was lacking in nutrients needed by CB1190. The treatment with 100% nutrient medium exhibited a rate that was an order of magnitude higher than the treatment with 5% added. Thus, a 5% addition of nutrient medium had an effect but was still below the amount needed for the highest possible rate of biodegradation. The percent degradation of 1,4-dioxane at the end of the incubation period (42 days) was as follows: 12% for bottles with CB1190 and nutrient added (5% by volume); 27% for CB1190 in AMSM; and no removal in the other treatments relative to the groundwater only treatment (**Figure 5.6.16B**).

Results for Site 6 Well 2 (**Figure 5.6.17**) were similar. There was no statistically significant accumulation of ^{14}C products in the treatment with only groundwater. Both of the treatments with groundwater that were inoculated with CB1190 exhibited statistically significant rates of ^{14}C product formation. The treatment that received a 5% addition of nutrient medium exhibited a rate of accumulation approximately two times higher than groundwater alone, indicating that the groundwater was lacking in nutrients needed by CB1190. The treatment with 100% nutrient

medium exhibited a rate that was an order of magnitude higher than the treatment with 5% added. Thus, a 5% addition of nutrient medium had an effect but was still below the amount needed for the highest possible rate of biodegradation. Biodegradation of 1,4-dioxane for each treatment at the end of the incubation period (42 days) was 58% for the treatment inoculated with CB1190 only, and 100% for bottles with CB1190 in AMSM (**Figure 5.6.17B**).

The effect of nutrients on ENV487 was explored with groundwater from Site 6 Well 3 (**Figure 5.6.18**). Treatments with groundwater inoculated with ENV487 exhibited statistically significant rates of ^{14}C product formation. The treatment that received 5% addition of nutrient medium and inoculated with ENV487 exhibited a rate of accumulation approximately two times higher than groundwater and ENV487 alone, indicating that the groundwater was lacking in nutrients needed by ENV487. The treatment with 100% nutrient medium exhibited a rate that was an order of magnitude higher than the treatment with 5% added. Thus, a 5% addition of nutrient medium had an effect but was still below the amount needed for the highest possible rate of biodegradation. Biodegradation of 1,4-dioxane for each treatment at the end of the incubation period (42 days) reached 32% for bottles inoculated with ENV487 only; 54% for bottles with ENV487 and 5% nutrient; and 100% for ENV487 in BSM (**Figure 5.6.18B**). Taken together, these results suggest that a deficiency in nutrients may be limiting biodegradation of 1,4-dioxane in the ^{14}C assay when only groundwater is used.

5.6.3 Discussion

Implementation of a ^{14}C assay to quantify biodegradation rate coefficients requires that the labeled material be of sufficiently high purity so that detection of degradation products is not obscured by the presence of impurities. Manufacturers of ^{14}C labeled compounds typically provide material that is in the range of 96-98% radiochemical pure. That means that 2-4% impurities may be present. Also, the purity is determined shortly after synthesis. Some degree of auto-degradation is likely before the material is ready for use in the assay. The results of this research have validated that passage of a stock solution through an HPLC column yields a product that is adequately pure for use in a ^{14}C assay, with only ~0.038 ± 0.031% impurities detected when microcosms were initiated. Although this exceeds the goal of 0.01%, the difference between the measured level and the target is not statistically significant ($p > 0.05$).

One of the uncertainties with a positive result from the ^{14}C assay is whether or not the degradation activity can be attributed to bacteria that express monooxygenases. This is especially relevant because measuring monooxygenases is faster and less costly, and the presence of monooxygenases could be used as a surrogate to estimate the rate of 1,4-dioxane biodegradation. A similar approach has been proposed for TCE (Wilson et al., 2019). Two of the ways to assess the involvement of monooxygensases is inhibition with acetylene and incubation in the absence of oxygen. In experiments with CB1190 and ENV487 added to groundwater samples, acetylene and lack of oxygen did inhibit accumulation of ^{14}C degradation products. This indicates that a positive test with groundwater could be followed up with inhibition tests to validate the involvement of monooxygenases. The reversible inhibition for CB1190 following removal of acetylene has been reported previously (Gedalanga et al., 2014; Mahendra and Alvarez-Cohen, 2006). Non- reversible inhibition was observed with ENV487. The extent of acetylene inhibition varies with the type of monooxygenases present (Li et al., 2013).

The ^{14}C assay appears to be robust with respect to how the groundwater samples are handled, which includes shipping on ice overnight, followed by gradually warming the samples to room temperature overnight, prior to adding ^{14}C-1,4-dioxane. To assess this, the assay was performed with groundwater to which CB1190 or ENV487 was added. Regardless of how the samples were handled, including no cooling and immediate addition of ^{14}C-1,4-dioxane, there was no difference in the rates of ^{14}C product accumulation. In contrast, Mills et al. (2018) observed a modest decrease in rate coefficients between standard sampling processing and starting the ^{14}C-TCE assay with no cooling or rewarming. The results of this study indicate it would be possible to start the assay on the same day that samples are received, rather than waiting to warm the samples overnight.

Having validated the assay, it was deployed to test groundwater samples from 10 sites across the U.S. Statistically significant rate coefficients were determined for approximately one-half of the samples. For Site 3, which had some of the higher rate coefficients, propane biosparging had been used in the recent past, so the significant activity in those samples is likely related. The highest rate constant from the ^{14}C assay was from Site 4, in a microcosm that had sediment present along with groundwater. For other locations, the role of sediment is less definitive; in general, having sediment present likely improves the chances of seeing biodegradation activity, although this was not always the case. Notably, the third highest first order rate coefficient was observed in groundwater from Site 10. The sample from Well 4 developed an orange color during incubation and the presence of Fe(II) was confirmed, indicating that a portion of the degradation activity was likely attributable to an abiotic Fenton's reaction. This is important because there has been limited evidence presented to date that links in situ degradation of 1,4-dioxane to abiotic activity. Further investigation is warranted on the importance of this abiotic processes.

For the other samples with statistically significant rate coefficients, most of the corresponding half-lives were in excess of 50 years. This suggests that biodegradation is occurring, but at a rate that may not be meaningful in terms of natural attenuation. Alternatively, the ^{14}C assay may be underestimating the *in situ* degradation rates, as suggested by results with nutrient amendments. A key advantage for a groundwater-only assay is the lower cost and complexity associated with collecting groundwater alone versus soil cores. However, the soil likely harbors many of the nutrients needed for metabolic degradation of 1,4-dioxane. On this basis, the groundwater ^{14}C assay may be viewed as a screening tool, i.e., in the event that a statistically significant rate is detected, it may be worthwhile to construct microcosms with soil and groundwater. The presence of the soil will allay any concerns about a lack of nutrients, as well concerns regarding a low level of microbes. The ^{14}C assay is very sensitive, so the likelihood of missing the fact that 1,4-dioxane biodegradation is occurring seems remote.

5.6.4 Conclusions

It was confirmed that custom synthesized ^{14}C-1,4-dioxane can be adequately purified from a stock solution of ^{14}C-1,4-dioxane/butanol via HPLC.

The ^{14}C assay was validated with metabolic and cometabolic cultures (i.e., CB1190 and ENV487, respectively). Detection limits for both cultures were on the same order of magnitude, with half-lives of 43 and 33 years for CB1190 and ENV487, respectively.

Oxygenase activity required for biodegradation of 1,4-dioxane was confirmed by inhibition with acetylene and anaerobic incubation. A reversible inhibition effect was seen with CB1190 once acetylene was removed, whereas the cometabolic culture ENV487 presented an irreversible inhibition effect. Both cultures remained inhibited when acetylene was retained in the bottle and under anaerobic conditions.

The process used to handle the groundwater samples did not affect determination of the rate coefficients. Cooling of the sample (as required for shipment overnight), and rapid warming (versus gradual warming overnight) did not have a statically significant impact on the rate coefficients. In contrast, adding nutrients did have a significant impact on metabolic and cometabolic cultures. Nutrient limitation must therefore be considered when interpreting rate coefficients from the ^{14}C assay based only on groundwater, i.e., in the absence of soil.

Of the 54 groundwater samples analyzed, statistically significant rate coefficients were determined for 24 (**Table 5.6.2**); no significant rate of degradation was observed in 30 of the samples. The median rate coefficient observed using the ^{14}C assay was 0.0137 per year (half-life = 51 year); the maximum rate coefficient was 0.367 per year (half-life = 1.9 year) and the lowest observed was 0.0021 per year (half-life = 328 year). These results indicate that for many of the wells examined, biodegradation of 1,4-dioxane is occurring at a relatively slow rate or not at all.

The reproducibility of the ^{14}C assay with groundwater samples taken one year or more apart was variable. For groundwater samples that were below detection the first time, that result was usually confirmed in the second sample. When activity was quantifiable, there was considerable variability in the repeat measurements made on samples one year or more later. This suggests that repeat samples may be needed to build confidence in the rate coefficient used for modeling purposes.

Table 5.6.1. Rate coefficients determined with CB1190 and ENV487.

Culture	Dilution	Protein (mg/L)	k (yr^{-1})	Half-Life (yr)
CB1190	10^{-2}	1.20E+00	1.80E+00	0.40
	10^{-3}	1.20E-01	5.50E-01	1.3
	10^{-4}	1.20E-02	1.10E-01	6.1
	10^{-5}	1.20E-03	4.00E-02	17
	10^{-6}	1.20E-04	3.50E-02	20
	10^{-7}	1.20E-05	1.60E-02	44
	10^{-8}	1.20E-06	NS[a]	-
ENV487	10^{-2}	1.40E-01	2.20E+00	0.3
	10^{-3}	1.40E-02	2.30E-01	3.1
	10^{-4}	1.40E-03	6.20E-02	11
	10^{-5}	1.40E-04	2.10E-02	33

[a] NS = not statistically significant.

Table 5.6.2. Summary of statistically significant net rate coefficients. [a]

Site No.	Well No. [b]	Groundwater *k* (yr^{-1})	FSGW *k* (yr^{-1})	Net *k* (yr^{-1})	Net $t_{1/2}$ (yr)
2	1	0.0041 ± 0.0018	0.0020 ± 0.0012	0.0021 ± 0.0021	328 ± (165, 27514)
3	3	0.0156 ± 0.0033	0.0042 ± 0.0019	0.0114 ± 0.0033	61 ± (46, 90)
	4	0.109 ± 0.0153	0.0137 ± 0.0019	0.0957 ± 0.0149	7.2 ± (6.3, 8.6)
	5	0.0286 ± 0.0048	0.0077 ± 0.0024	0.0209 ± 0.0052	33 ± (27, 44)
4	1[ii]	0.0250 ± 0.0098	0.0091 ± 0.0056	0.0159 ± 0.0110	44 ± (26, 141)
	2	0.0142 ± 0.0043	0.0081 ± 0.0030	0.0061 ± 0.0051	113 ± (62, 651)
	2[ii]	0.0234 ± 0.0107	0.0091 ± 0.0056	0.0143 ± 0.0117	49 ± (27, 271)
	2[iii]	0.362 ± 0.0574	0.0643± 0.0172	0.297± 0.0580	2.3 ± (2.0, 2.9)
	4	0.0127 ± 0.0025	0.0054 ± 0.0015	0.0073 ± 0.0028	95 ± (69, 153)
	5[c]	0.181 ± 0.0515	0.0643 ± 0.0172	0.117 ± 0.0526	5.9 ± (4.1, 11)
	6[c]	0.431 ± 0.0512	0.0643± 0.0172	0.367 ± 0.0484	1.9 ± (1.7, 2.2)
5	1	0.0070 ± 0.0023	0.0033 ± 0.0023	0.0037 ± 0.0023	189 ± (102, 1254)
	2	0.0086 ± 0.0029	0.0044 ± 0.0014	0.0042 ± 0.0032	164 ± (94, 650)
6	3	0.0081 ± 0.0018	0.0054 ± 0.0016	0.0026 ± 0.0023	262 ± (140, 2001)
7	4	0.0076 ± 0.0029	0.0026 ± 0.0014	0.0050 ± 0.0031	140 ± (86, 376)
8	2[c]	0.0169 ± 0.0069	0.0078 ± 0.0039	0.0091 ± 0.0077	76 ± (41, 510)
	3[c]	0.0206 ± 0.0030	0.0096 ± 0.0029	0.0110 ± 0.0040	63 ± (46, 99)
9	1[c]	0.0446 ± 0.0072	0.0191 ± 0.0044	0.0255 ± 0.0082	27 ± (21, 40)
	2[c]	0.0210 ± 0.0046	0.0078 ± 0.0039	0.0132 ± 0.0058	53 ± (37, 94)
	3 [c]	0.0103 ± 0.0020	0.0045 ± 0.0019	0.0058 ± 0.0027	120 ± (82, 222)
10	2	0.0768 ± 0.0170	0.0081 ± 0.0034	0.0688 ± 0.0141	10 ± (8.4, 13)
	2[c]	0.0333 ± 0.0097	0.0081 ± 0.0034	0.0252 ± 0.0099	27 ± (20, 45)
	4	0.0948 ± 0.0136	0.0081 ± 0.0034	0.0868 ± 0.0136	8.0 ± (6.9, 9.5)
	4[c]	0.203 ± 0.0378	0.0081 ± 0.0034	0.195 ± 0.0368	3.6 ± (3.0, 4.4)

[a] ± represents the 95% confidence limit.
[b] First sampling event unless followed by [ii] = 2nd sampling event; [iii] = 3rd sampling event.
[c] Soil or sediment present along with the groundwater.

Table 5.6.3. 1,4-Dioxane GC measurements for groundwater samples that exhibited a statistically significant decrease during the incubation period.[a]

Site No.	Well No.	Days Incubated	1,4-Dioxane (µg/L)	
			Initial	Final
3	3	265	417 ± 10	325 ± 36
3	4	264	163 ± 4.7	98 ± 17
3	5	264	257 ± 6.3	196 ± 31
4	1	243	252 ± 554	0 ± 0.0
4	2	228	512 ± 15	480 ± 14
4	4	42	294 ± 18	180 ± 132
4	6	42	168 ± 6.2	137 ± 11
10	2	42	453 ± 26	300 ± 10
10	4	42	321 ± 22	259 ± 20
10	4[b]	42	321 ± 22	198 ± 6.4

[a] ± represents standard deviations for triplicate microcosms.
[b] Soil or sediment present along with the groundwater.

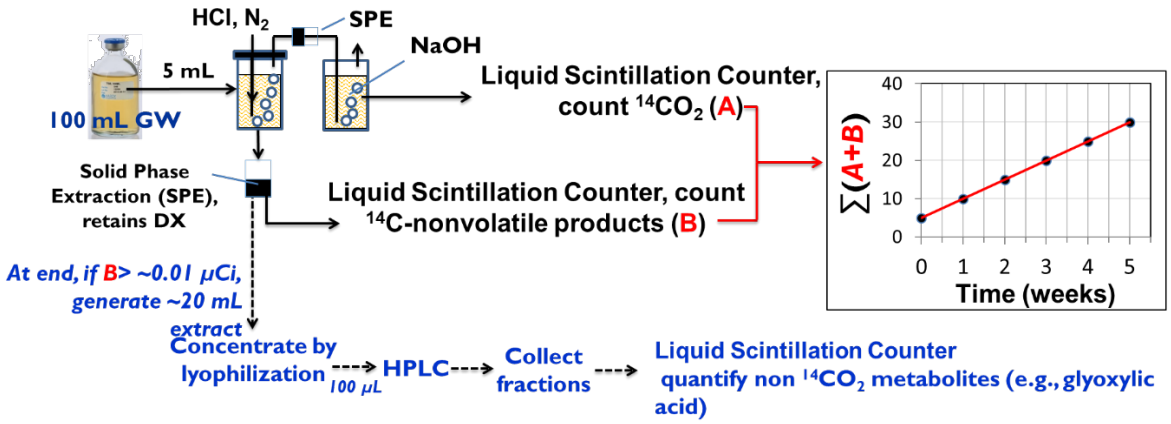


Figure 5.6.1. Schematic of the ^{14}C-assay.

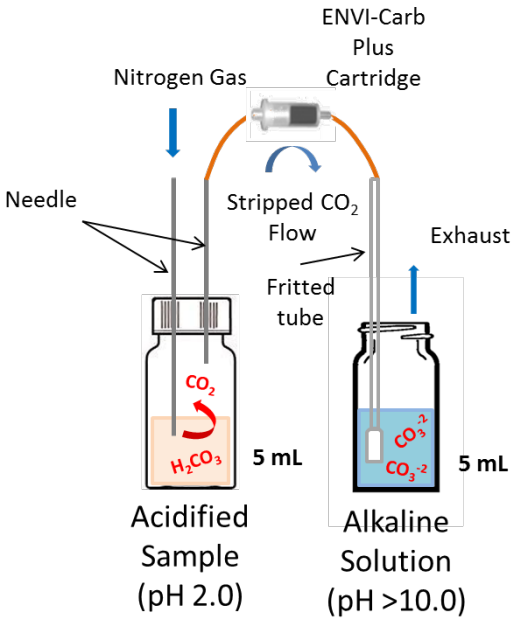


Figure 5.6.2. Schematic of the apparatus used to separate $^{14}CO_2$.
CO_2 was stripped from the acidified solution (containing residual ^{14}C-1,4-dioxane and nonstrippable ^{14}C products) with nitrogen gas and was trapped in the alkaline solution.

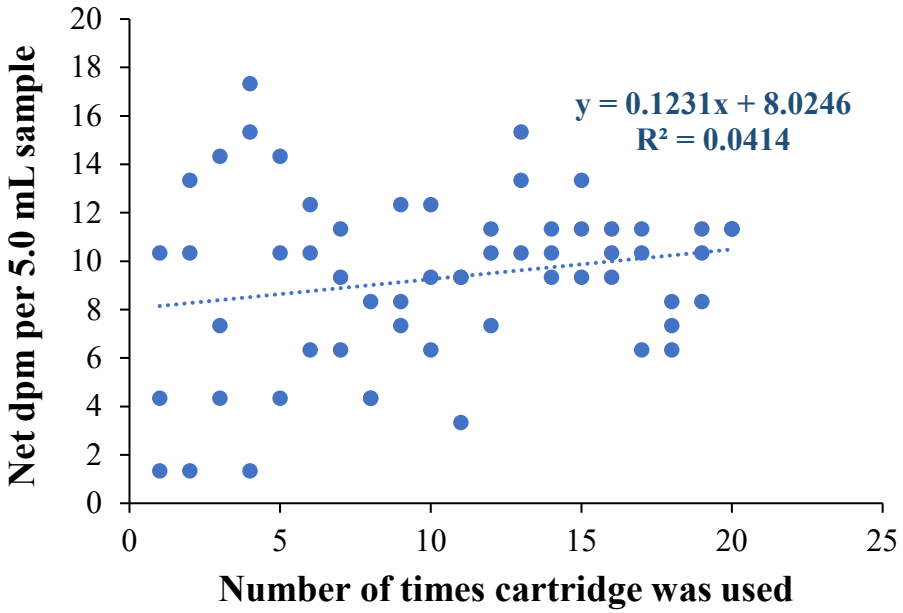


Figure 5.6.3. ^{14}C activity from 5 mL samples that were passed through the SPE cartridges repeated times.

The results demonstrated that the cartridges continued to function even after 20 uses. There was no statistically significant increase in the ^{14}C activity passing through the cartridges.

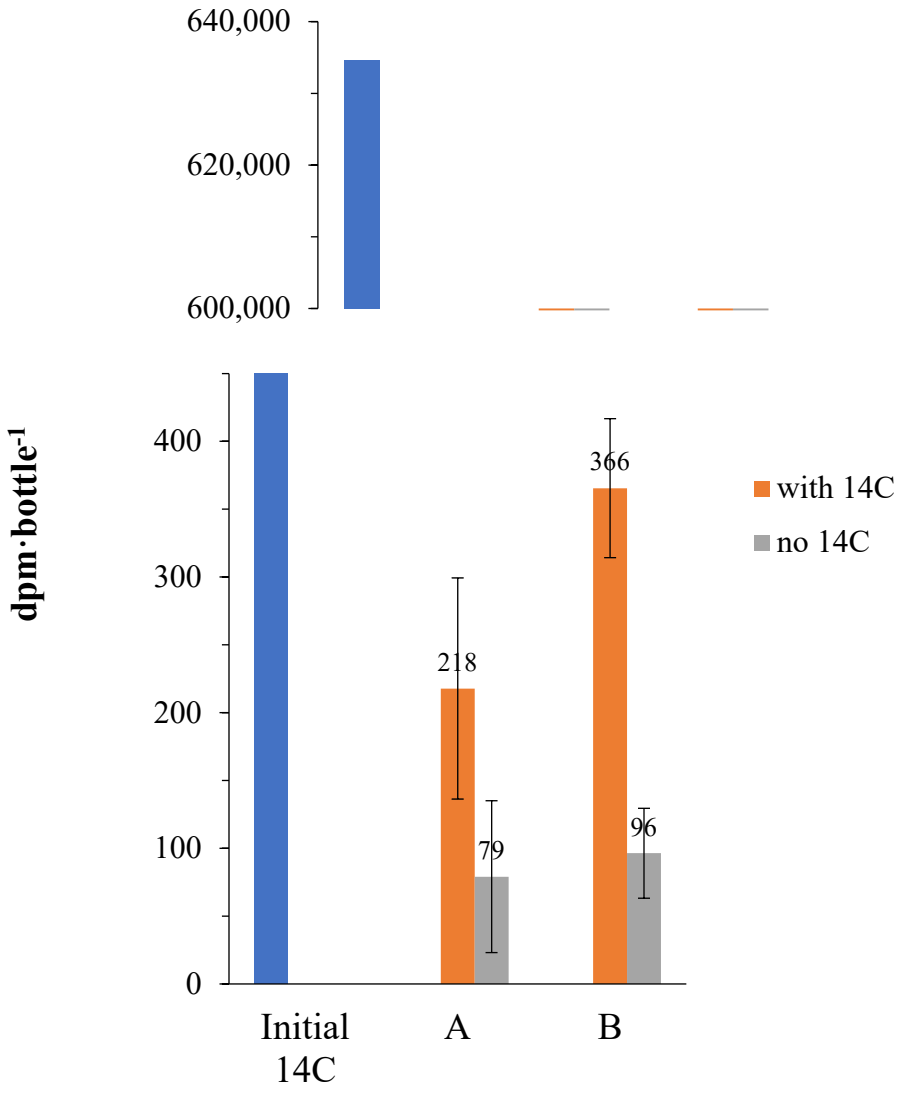


Figure 5.6.4. Analysis of the purity of the ^{14}C-1,4-dioxane stock solution.
Blue bar is for the total ^{14}C added to replicate serum bottles. The orange bars are for products recovered (A = $^{14}CO_2$, B = ^{14}C-NSR) in bottles to which the ^{14}C-1,4-dioxane was added. The grey bars represent the ^{14}C activity in bottles to which no ^{14}C-1,4-dioxane was added, i.e., corresponding to background activity. The net amount of contaminants equals the orange minus the grey bars. Error bars represent the standard deviation.

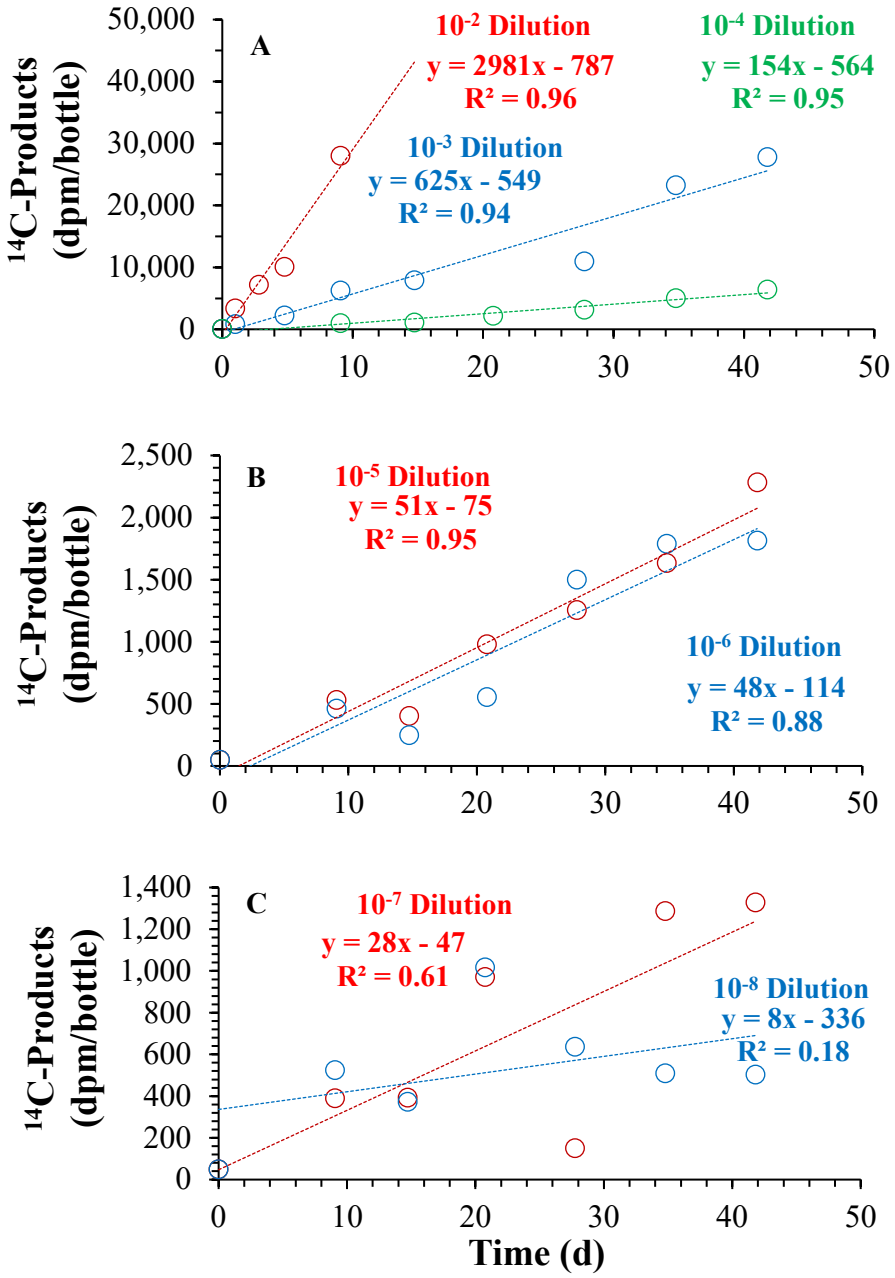


Figure 5.6.5. Evaluation of the ^{14}C assay using the CB1190 culture grown on 1,4-dioxane at varying dilution levels.
Cells were suspended in AMSM.

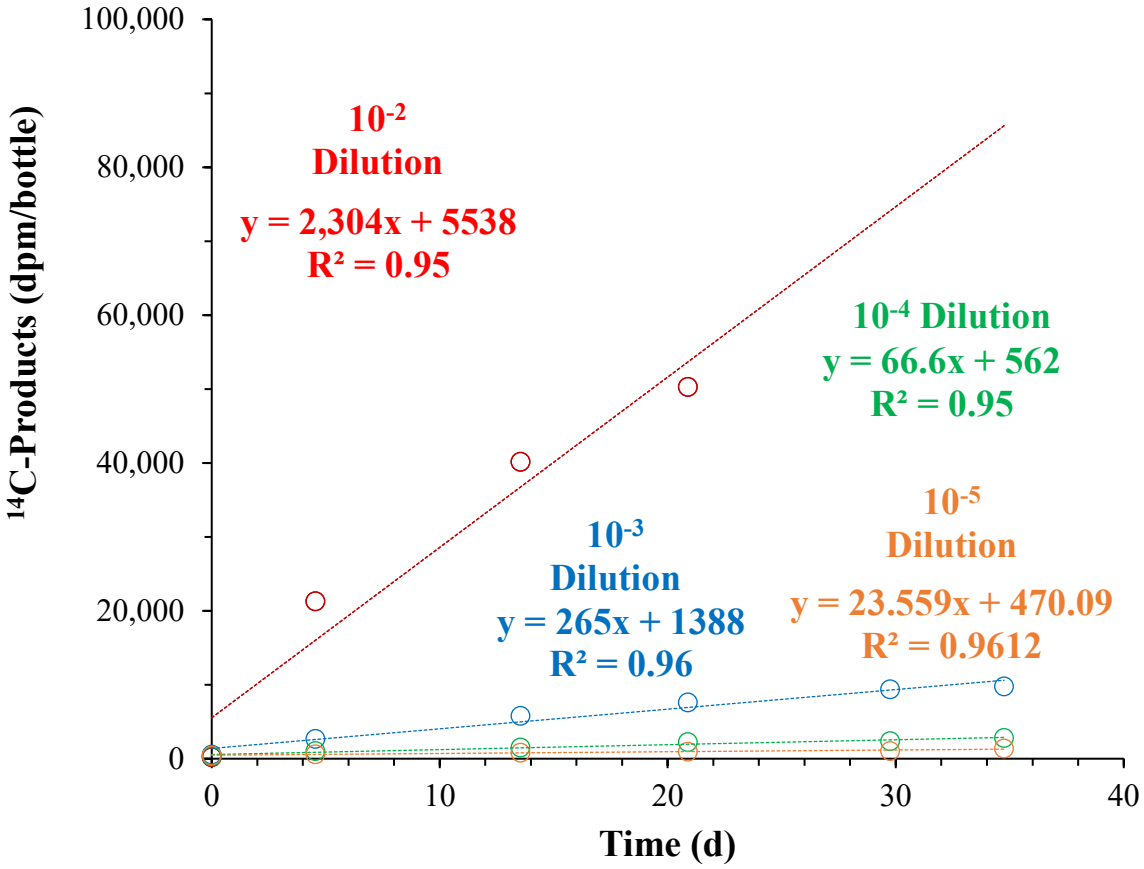


Figure 5.6.6. Evaluation of the ^{14}C assay using the ENV487culture grown on propane at varying dilution levels.
Cells were suspended in BSM.

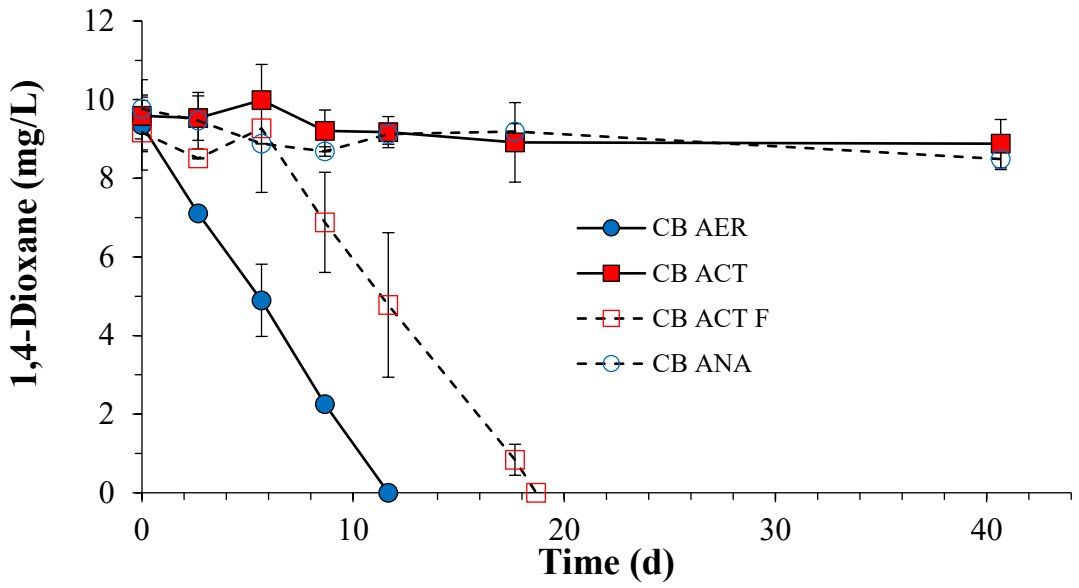


Figure 5.6.7. Inhibition test using acetylene for the CB1190 culture in AMSM.
"CB" stands for the CB1190 culture, "AER" for incubation under aerobic conditions, "ACT" for aerobic incubation in the presence of acetylene, "ACT F" aerobic incubation in the presence of acetylene followed by flushing of the headspace to remove the acetylene, and "ANA" for incubation under anaerobic conditions.

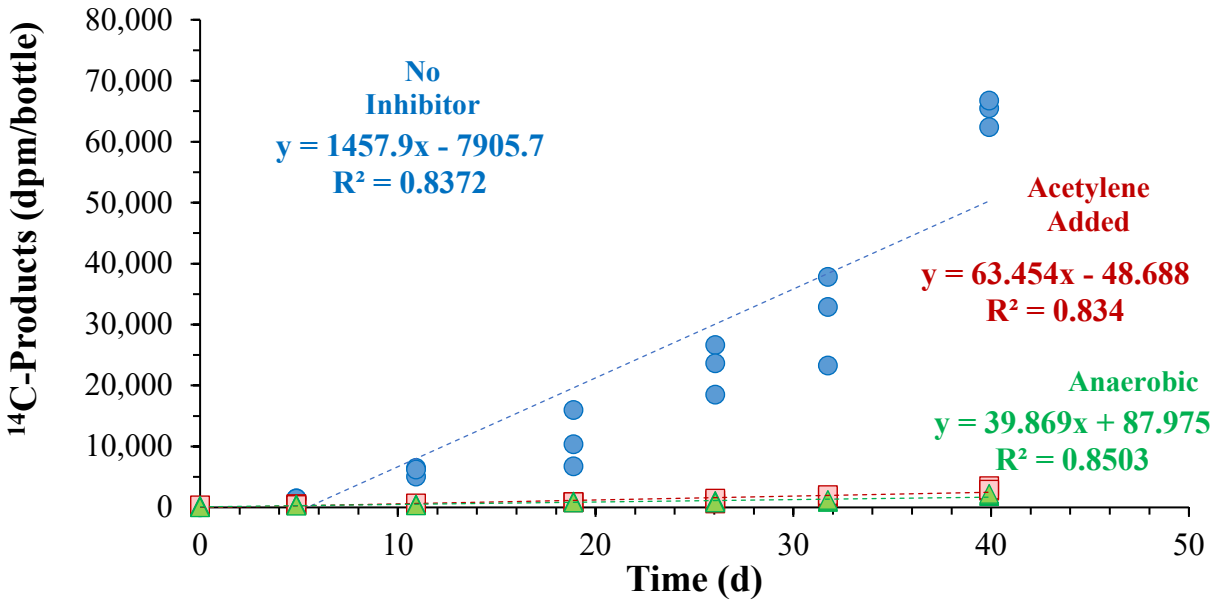


Figure 5.6.8. ^{14}C product accumulation from ^{14}C-1,4-dioxane by the CB1190 culture in AMSM under aerobic conditions (blue), under aerobic conditions with acetylne present (red), and under anaerobic conditions (green).

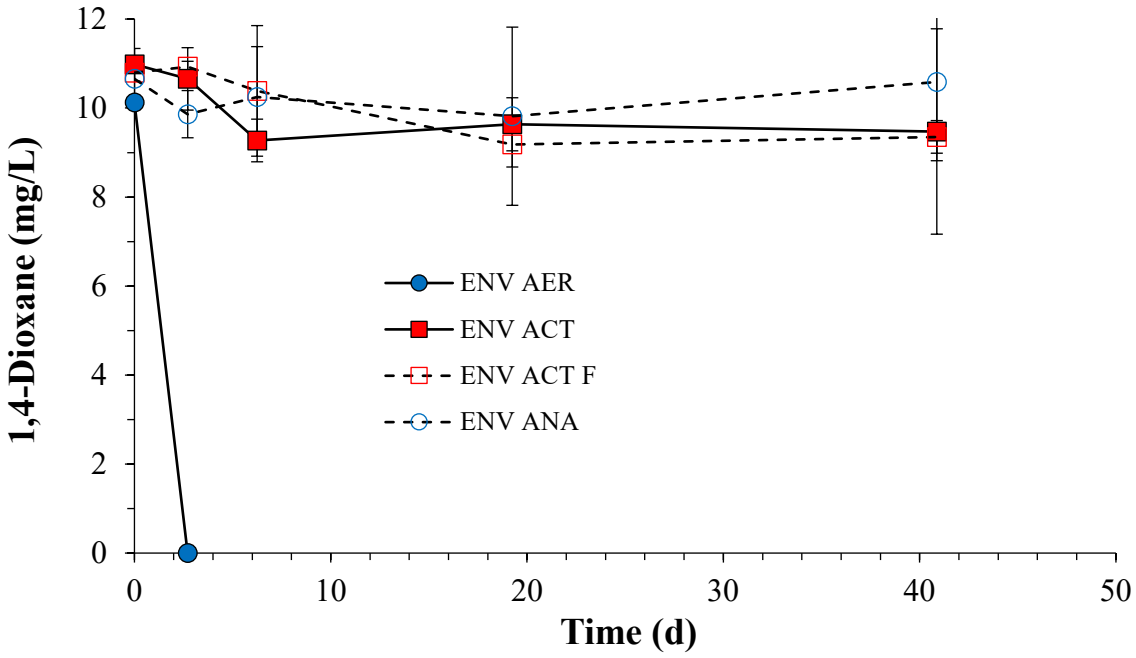


Figure 5.6.9. Inhibition test using acetylene for ENV487 culture in BSM.
"ENV" stands for the ENV487 culture, "AER" for incubation under aerobic conditions, "ACT" for aerobic incubation in the presence of acetylene, "ACT F" aerobic incubation in the presence of acetylene followed by flushing of the headspace to remove the acetylene, and "ANA" for incubation under anaerobic conditions.

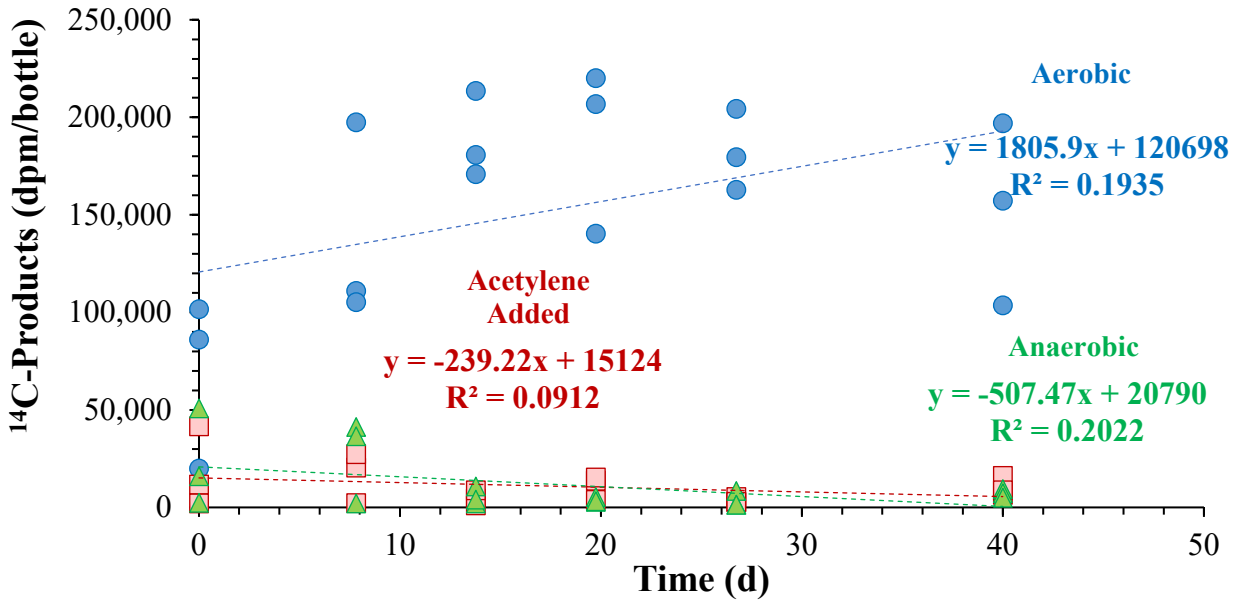


Figure 5.6.10. ^{14}C product accumulation from ^{14}C-1,4-dioxane by the ENV487 culture in BSM under aerobic conditions (blue), under aerobic conditions with acetylene present (red), and under anaerobic conditions (green).

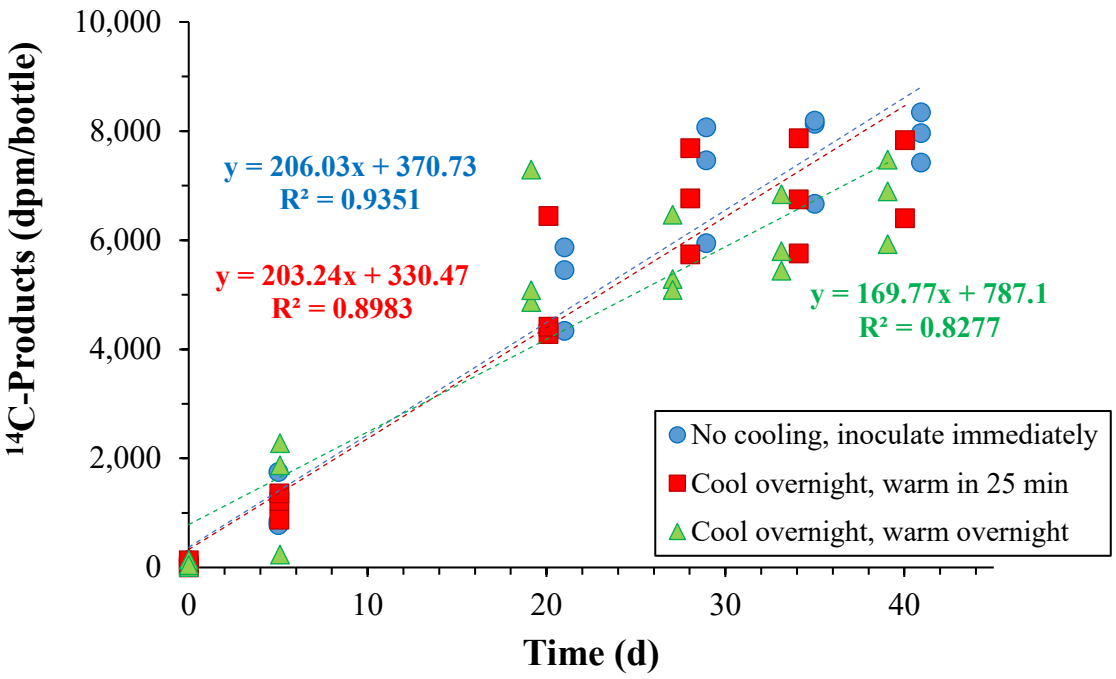


Figure 5.6.11. Effect of sample handling on biodegradation of 1,4-dioxane by the CB1190 culture at an initial concentration of 163 µg/L in groundwater (Site 6, Well 2).
The rate of biodegradation was assessed based on the rate of ^{14}C product accumulation.

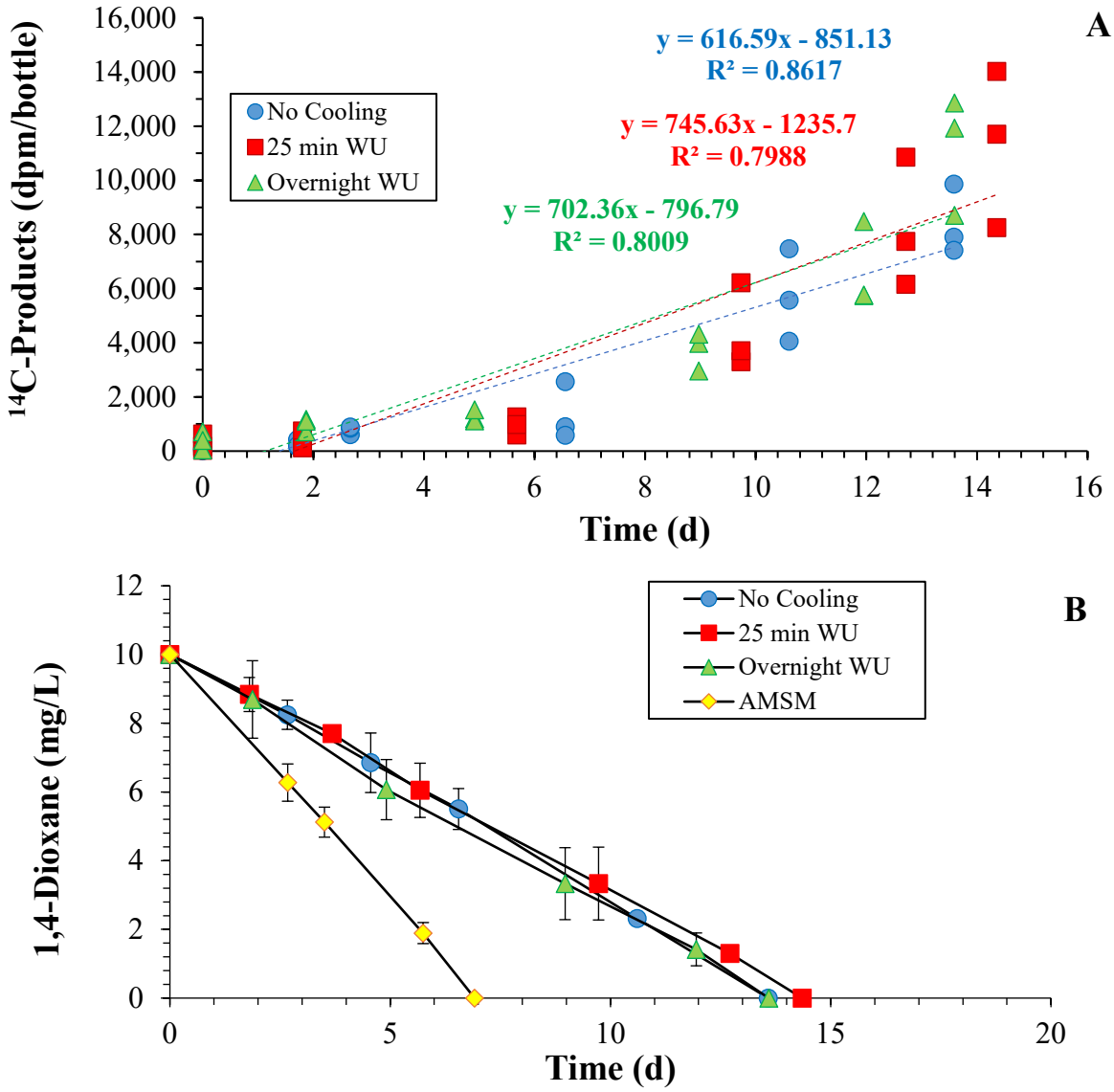


Figure 5.6.12. Effect of sample handling on biodegradation of 1,4-dioxane by the CB1190 culture at an initial concentration of 10.2 mg/L in groundwater (Site 6, Well 2).
(A) The rate of biodegradation assessed based on the rate of ^{14}C product accumulation; (B) the rate of biodegradation based on GC analysis. "WU" stands for warmed up.

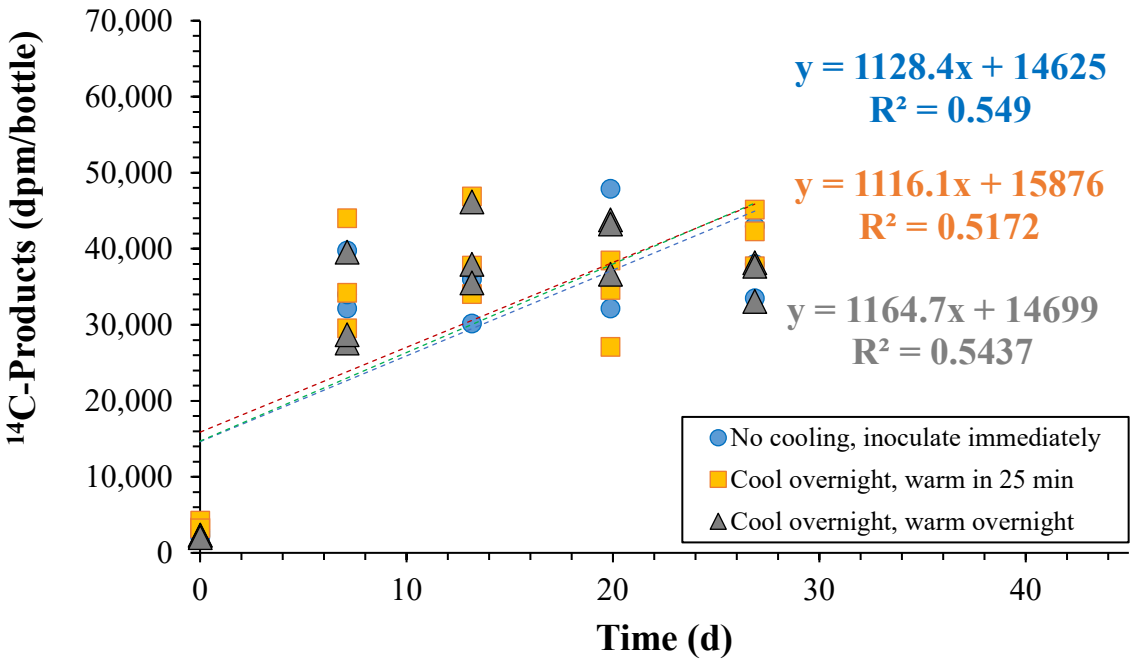


Figure 5.6.13. Effect of sample handling on biodegradation of 1,4-dioxane by the ENV487 culture at an initial concentration of 163 µg/L in groundwater (Site 6, Well 3).
The rate of biodegradation was assessed based on the rate of ^{14}C product accumulation.

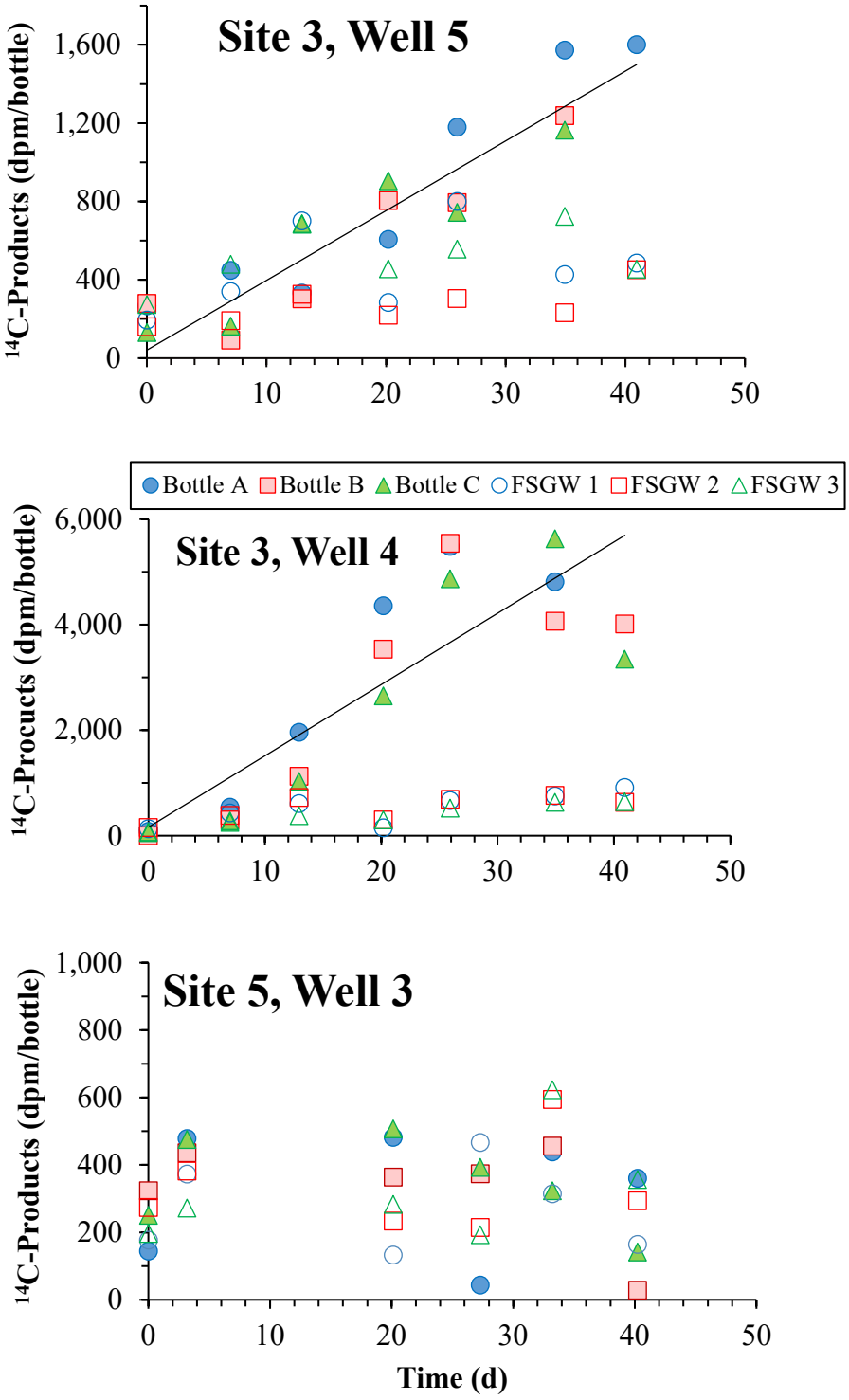


Figure 5.6.14. ^{14}C-product accumulation for Site 3, Well 4; a lower rate constant for Site 3, Well 5; and a non-significant result for Site 5, Well 3.
Lines represents the modeled rate fits.

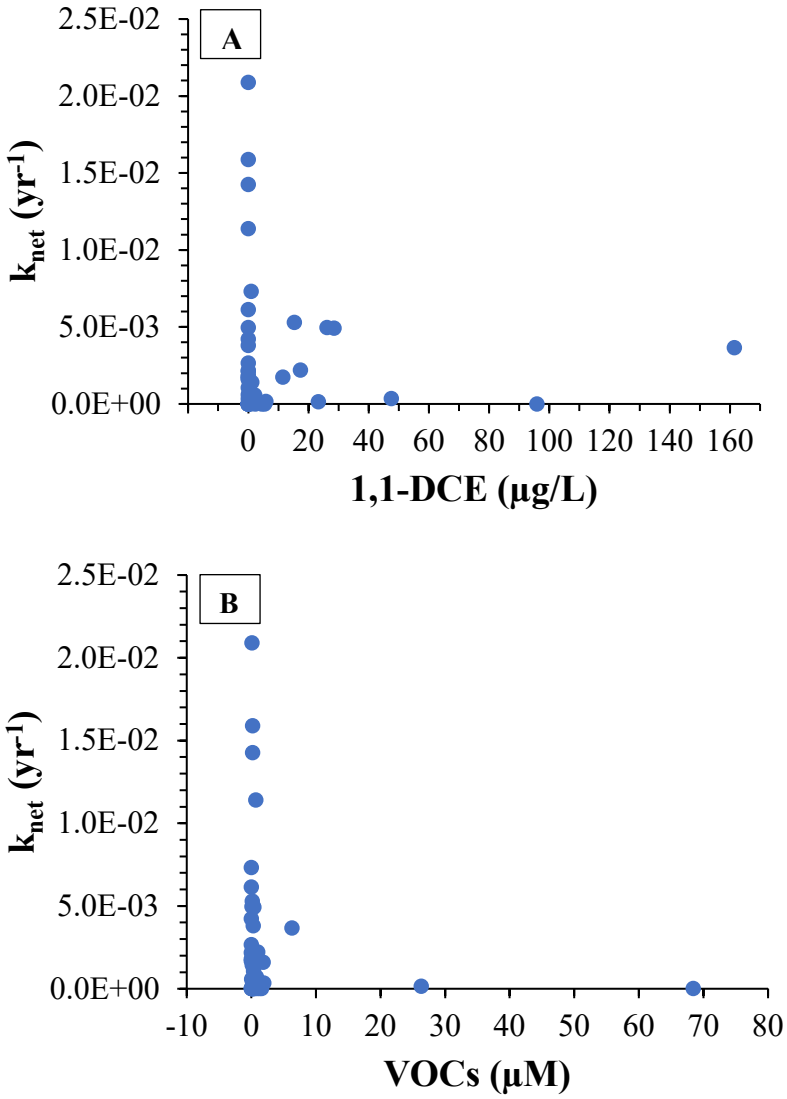


Figure 5.6.15. Correlation between first order degradation rate coefficients and the concentration of A) 1,1-DCE; and B) total VOCs.

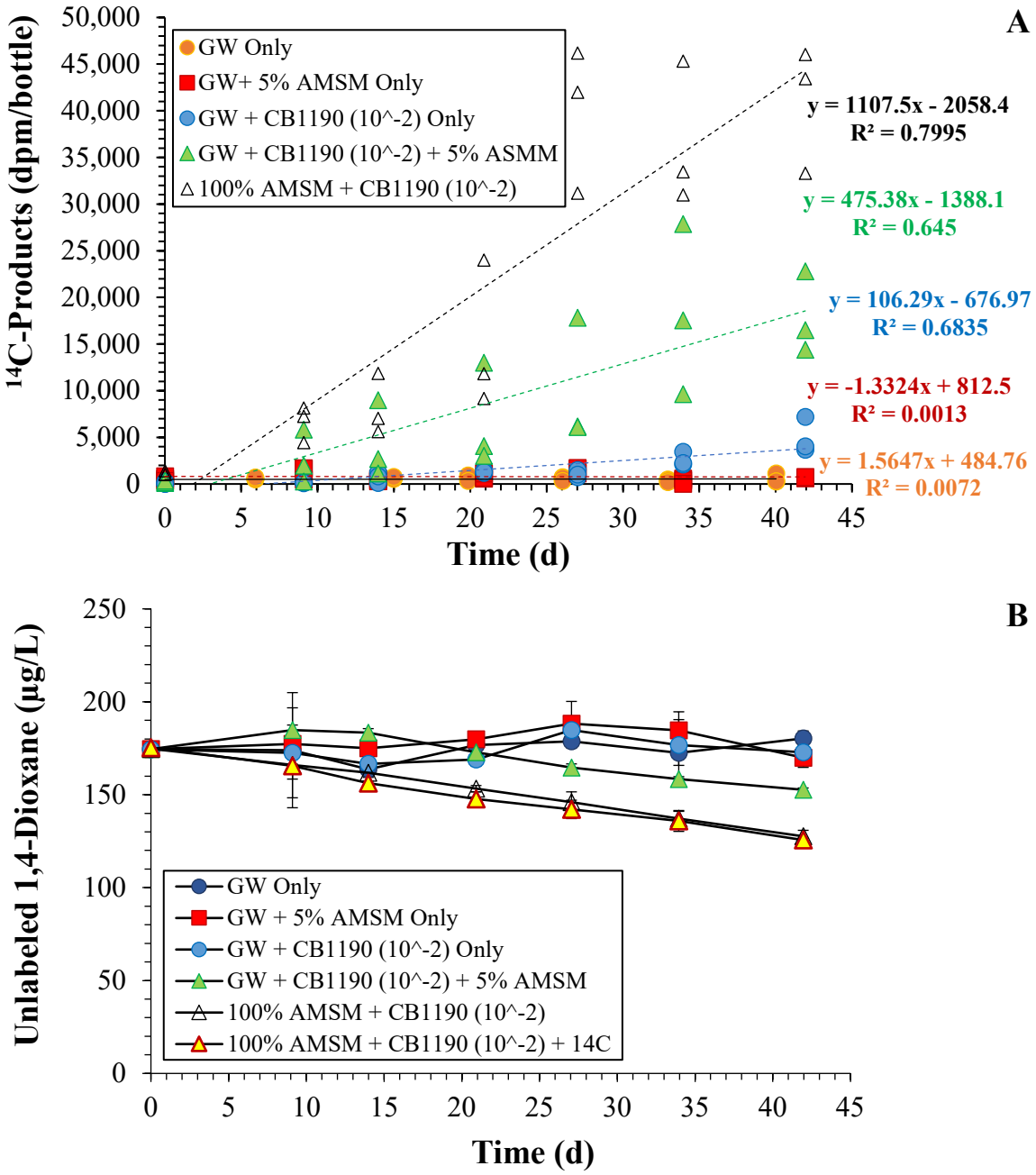


Figure 5.6.16. Effect of nutrient addition in microcosms (auxiliary industrial site) bioaugmented with CB1190.

A) accumulation of ^{14}C products; B) total unlabeled-1,4-dioxane based on GC measurements. "GW" stands for groundwater.

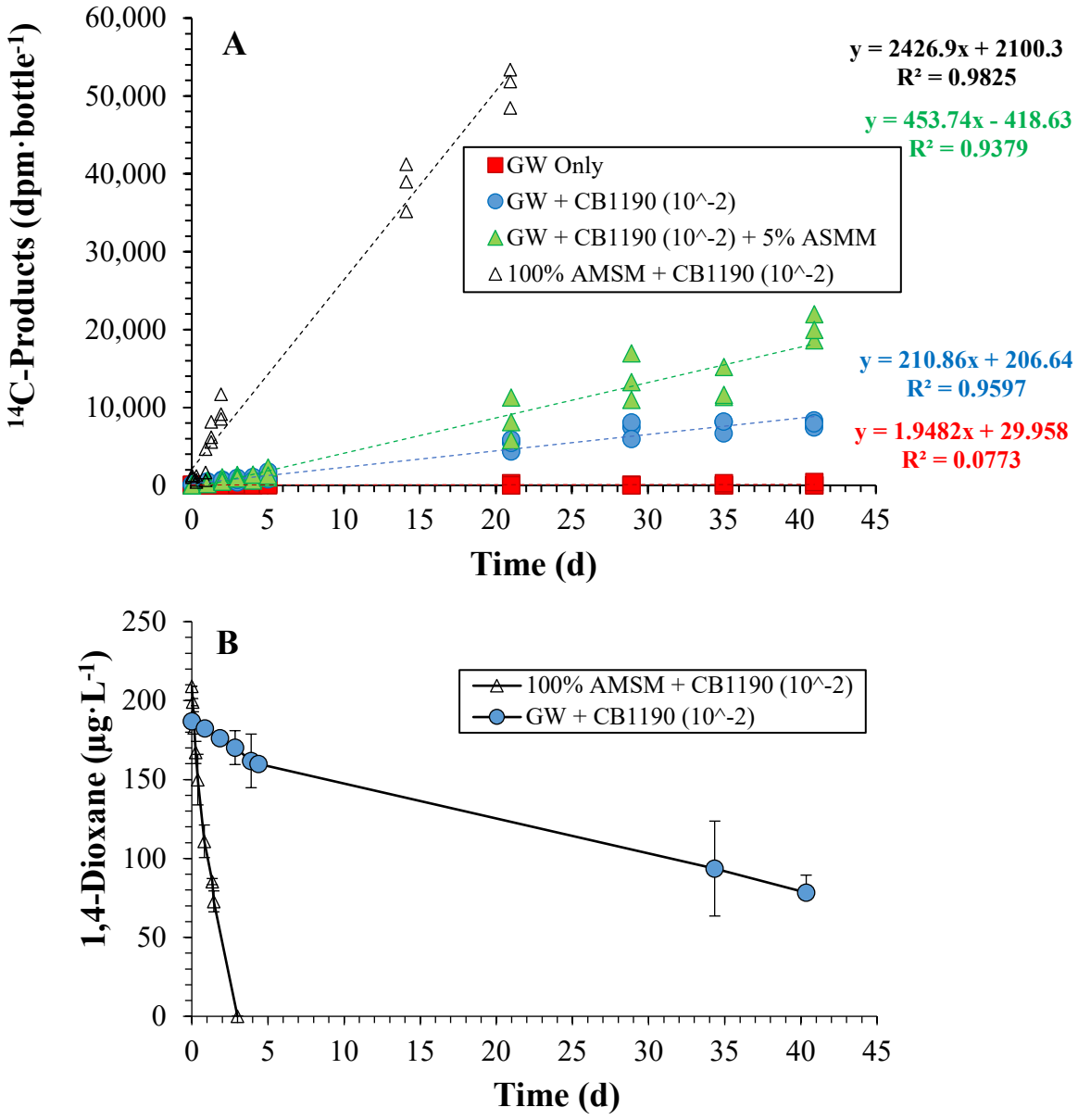


Figure 5.6.17. Effect of nutrient addition in microcosms (Site 6, Well 2) bioaugmented with CB1190.

A) accumulation of ^{14}C products; B) total unlabeled-1,4-dioxane based on GC measurements. "GW" stands for groundwater.

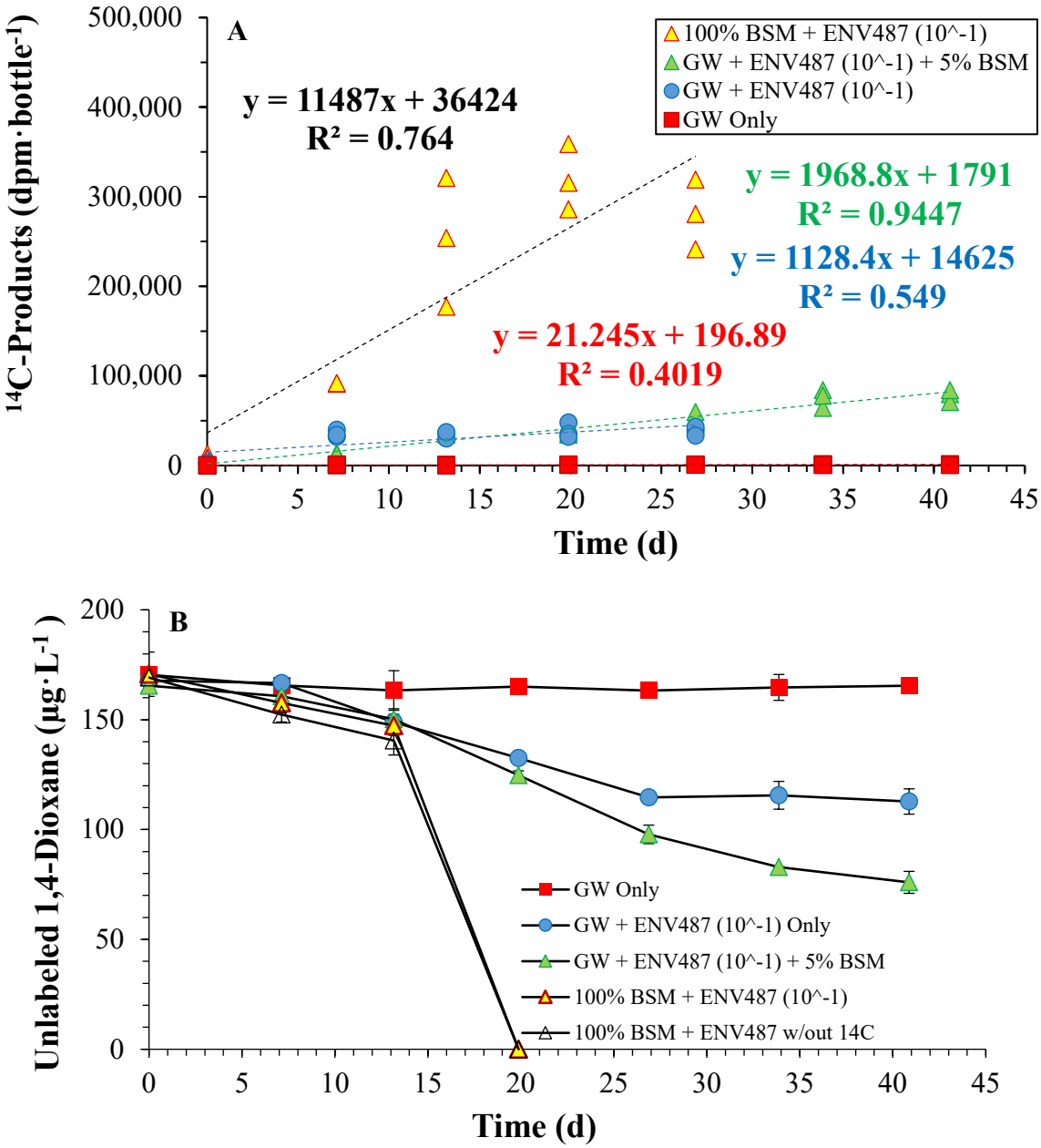


Figure 5.6.18. Effect of nutrient addition in microcosms (Site 6, Well 3) bioaugmented with ENV487.

A) accumulation of ^{14}C products; B) total unlabeled-1,4-dioxane based on GC measurements. "GW" stands for groundwater.

5.7 SAMPLING RESULTS

The full results from all sites included in the field program are included in **Table A.2** in **Appendix A**. Results for each site are briefly summarized in the following subsections. A compilation of key results from across the sites is included in Section 5.8.

5.7.1 Site 1 (DoD)

5.7.1.1 - 1,4-Dioxane

The evaluation of the sampling results from each site follows the Decision Framework developed as part of this project. See **Section 5.9** for full description of the Decision Framework.

An evaluation of 1,4-dioxane was performed for all ten sites, and an evaluation of chlorinated solvents was performed for a subset of these sites.

Overview: This site is located within an industrial area with historic aircraft testing/maintenance shops, tanks, and pipelines. It is suspected that multiple releases in different areas (including one reportedly in early 1990s) contributed to LNAPL/DNAPL impacted area and a chlorinated ethene plume. Project data confirmed that 1,4-dioxane concentration were relatively high but that the plume was likely stable. As described below, reasonable evidence for 1,4-dioxane biodegradation was observed (primarily in the model-predicted rate constant and CSIA data) despite the presence of high concentration of chlorinated solvents and a lack of significant activity in ^{14}C assays.

Is 1,4-dioxane below regulatory standards? **No**
The concentration of 1,4-dioxane in the most contaminated well (the source well) at Site 1 was 970 µg/L in 2018. This is above the regulatory standard for the site.

Is the plume stable (i.e., is the 1st line of evidence for MNA met)? **Yes**
The site was sampled for 1,4-dioxane in 2014, 2015, 2018 and 2019. The distribution of 1,4-dioxane is presented in **Figure 5.7.1.1**. In the Source Well, the most contaminated well, the concentration of 1,4-dioxane was 480 µg/L in March of 2014, then 1,800 µg/L in November of 2015 and 970 µg/L in August1 of 2018. In the perimeter wells (Wells A, B, C and D) 1,4-dioxane was not detected in 2014 and 2015. In Well 2, the most downgradient well where 1,4-dioxane was detected, the concentration of 1,4-dioxane was 29 µg/L in March of 2014, then 15 µg/L in November of 2015 and 8.7 µg/L in December of 2019.

The release of chlorinated solvents that produced the plume is thought to have occurred in the 1990s. The expected time of travel of contamination from the source well to the most distant well in the monitoring network is approximately ten years. The most distant well has not been impacted. The trend in concentrations of 1,4-dioxane in the most distant wells that have been impacted is down. Based on these data, the 1,4-dioxane plume is apparently stable.

Is 1,4-dioxane being degraded based on model predictions? **Yes**
See Panel A of **Figure 5.7.1.2** for the distribution of 1,4-dioxane and chlorinated hydrocarbons at the site. Data on concentrations of 1,4-dioxane from all four wells were used in the MNA Rate Constant Estimator to estimate a rate constant for degradation (Panel B). For each simulation, the MNA Rate Constant Estimator calculated the root mean square error (RMSE) between the

simulated concentrations and the field data. Values of rate constants between 0.24 per year and 0.37 per year produced the lowest value. The center of the range in values is 0.31 per year.

Site 1 has an identified and defined receptor at 2,700 feet from the source. Allowing time for 1,4-dioxane in groundwater to move from the source to the receptor, the simulation using the MNA Rate Constant Estimator indicates that biodegradation and dispersion will bring concentrations of 1,4-dioxane below the acceptance criteria before the groundwater reaches the receptor.

Does the biomarker abundance explain the model-estimated 1,4-dioxane biodegradation rate constant? **No**

The relevant biomarkers for direct metabolism of 1,4-dioxane are DXMO, ALDH, and possibly *prmA*. Biomarkers that are potentially relevant to cometabolism are *prmA*, RMO, RDEG, PHE, SCAM and SMMO. Their abundance is presented in Panel A of **Figure 5.7.1.3**. If the abundance was below the reporting limit, the abundance was plotted at the reporting limit. Neither DXMO nor ALDH was detected in any of the four wells at Site 1. Biomarker RMO was detected in every well but Well 3. Biomarkers RDEG and PHE were detected in every well. The abundance of these biomarkers was higher in Well 4 and Well 1, which are closer to the source, and orders of magnitude lower in Well 3 and Well 2, which are further from the source. The biomarker SCAM as detected in three of the four wells, but at low abundance. Well 1 also contained low but measurable levels of *prmA*. The biomarker SMMO was only detected in Well 4, but at high abundance.

The MNA Rate Constant Estimator can estimate a rate constant for biodegradation from the abundance of the DXMO, *prmA*, RMO and RDEG biomarkers. The abundance of gene copies for these biomarkers was entered into Box 6b of the MNA Rate Constant Estimator. If a marker was not detected, the reporting limit was entered. All markers were determined at an abundance of 20 gene copies per mL or less. The sum of the predicted rate constants for biodegradation was 0.0019 per year. This is one hundred-fold lower than the apparent rate constant for degradation of 0.31 per year that was extracted as the best fit to the field data.

If the MNA Rate Constant Estimator is calibrated to concentrations in the Source Well and Well 1 and biomarker abundances in Well 1, the apparent rate constant for degradation along the flow path is 1.0 per year, and the rate constant estimated from the abundance of biomarkers is 0.001 per year.

The abundance of the biomarkers cannot explain the apparent rate constant for degradation of 1,4-dioxane at Site 1.

Is the model-estimated 1,4-dioxane biodegradation rate constant consistent with rate constants estimated using the ^{14}C assay? **No**

The production of ^{14}C-label in potential degradation products in the living microcosms was not greater than the production in filter-sterilized controls at 95% confidence (**Table 5.7.1.1**). A production that would have been greater at 95% confidence would have resulted in a rate constant for degradation of 0.005 per year or less, equivalent to a half-life greater than 140 years. The rate constants estimated from the biodegradation assay are not consistent with the rate constants

extracted from the model. The assay cannot explain the apparent rate constant for degradation of 1,4-dioxane at Site 1.

Are ^{2}H and/or ^{13}C enriched along the flow path? **Yes**

The site data confirmed that ^{2}H and/or ^{13}C are enriched in 1,4-dioxane along the flow path (see Panel B of **Figure 5.7.1.3**). The values of $\delta^{13}C$ and $\delta^{2}H$ cannot be distinguished in 1,4-dioxane in the Source Well and Well 4. However, values in Well 3, Well 2 and Well 1 are enriched compared to the Source Well, indicating that 1,4-dioxane has been degraded along the flow path to these wells. Panel C of **Figure 5.7.1.3** is a plot that was produced using the CSIA_14D.xlsx calculator in BioPIC software that was updated as part of this project. The pathway compares the relative changes in values for $\delta^{13}C$ and $\delta^{2}H$ in 1,4-doxane in the wells to changes that were seen during biodegradation of 1,4-dioxane by cultures of several different bacteria. The pattern in the wells at Site 1 most closely matched the pattern for degradation by *Pseudonocardia tetrahydrofuranoxidans* K1 (Bennett et al., 2018).

Are geochemical conditions supportive of 1,4-dioxane biodegradation? **Yes**

The geochemistry of the water samples from the wells at Site 1 is provided in **Table 5.7.1.2**. In water samples acquired in 2018 for the ^{14}C biodegradation assay, the temperature, pH, conductivity as a proxy for dissolved electrolytes, dissolved oxygen and ORP were all adequate to support biodegradation of 1,4-dioxane. The absence of detectable rates of biodegradation cannot be attributed to the geochemical conditions. The wells were resampled in 2019 for concentrations of chlorinated organic compounds. In 2019 the concentration of dissolved oxygen was low in Well 2 and Well 3.

Are inhibitory CVOCs present below inhibitory levels and/or decreasing with time/distance? **No**

Mahendra et al. (2013) determined half saturation constants for inhibition of aerobic biodegradation of 1,4-dioxane (K_i) by 1,1-DCE and 1,1,1-TCA. *Pseudonocardia dioxanivorans* CB1190 carries out direct metabolism of 1,4-dioxane using dioxane monooxygenase which corresponds to the DXMO biomarker. For a culture of *Pseudonocardia dioxanivorans* CB1190, the K_i for 1,1-DCE was 320 µg/L and the K_i for 1,1,1-TCA was 160 µg/L. The CB1190 culture did not degrade 1,1-DCE or 1,1,1-TCA, and the inhibition was reversable when the 1,1-DCE and 1,1,1-TCA were removed. *Pseudomonas mendocina* KR1 carries out cometbolism of 1,4-dioxane using a toluene-4-monooxygenase which corresponds to the RMO biomarker. For a culture of *Pseudomonas mendocina* KR1, the K_i for 1,1-DCE was 50 µg/L and the K_i for 1,1,1-TCA was 507 µg/L. The 1,1-DCE and 1,1,1-TCA were degraded by the *P. mendocina*, and the inhibition was not reversible, indicating that the degradation intermediates had combined at the active site of the enzyme and destroyed it.

Although the DXMO marker was not detected in samples, the RMO marker was present (**Figure 5.7.1.2**). The concentration of 1,1-DCE was above 100 µg/L in the Source Well, Well 4, and Well 3 (**Figure 5.7.1.2**). This concentration may have inactivated toluene monooxygenase and prevented cometabolism of 1,4-dioxane.

5.7.1.2 - 1,1,1-TCA, 1,1-DCA, and 1,1-DCE

Overview: Degradation of 1,1,1-TCA via abiotic and (presumably biotic) pathways has occurred at this site based on the low concentrations of 1,1,1-TCA and high concentrations of 1,1-DCE. This was supported by model simulations that established meaningful biodegradation rate constants for these reactions, although the geochemical and other data suggest that a significant portion of the observed attenuation was attributable to abiotic and aerobic processes.

Are 1,1,1-TCA, 1,1-DCA and 1,1-DCE below regulatory standards? **No (1,1-DCE and 1,1-DCA)**

The concentration of 1,1,1-TCA in the most contaminated well (the source well) at Site 1 was 20 µg/L in 2019. This is below the regulatory standard for the site (200 µg/L). However, the concentration of 1,1-DCA was 100 µg/L, and the concentration of 1,1-DCE was 690 µg/L. These concentrations are above the regulatory standards.

Are 1,1,1-TCA and 1,1-DCA and 1,1-DCE being degraded based on model predictions? **Yes**

See Panel A of **Figure 5.7.1.2** for the distribution of 1,1,1-TCA, 1,1-DCA and 1,1-DCE at the site. **Figure 5.7.1.4** compares the distribution of biomarkers DHBt. DHC, and DCA (Panel A) to attenuation in concentrations with distance from the source (Panels B, C and D). The concentration of 1,1,1-TCA was below detection in all the wells downgradient of the source well. The MNA Rate Constant Estimator uses the temperature of the groundwater to set and apply a rate constant for abiotic degradation of 1,1,1-TCA. The rate constant for biodegradation of 1,1,1-TCA was set to 0. The abiotic rate constant would have brought the concentration of 1,1,1-TCA below the detection limit in the downgradient wells (Panel B of **Figure 5.7.1.4**).

Data from all four wells were used in the MNA Rate Constant Estimator to estimate a rate constant for degradation of 1,1-DCA. For each simulation, the MNA Rate Constant Estimator calculated the root mean square error (RMSE) between the simulated concentrations and the field data. The rate constants with the lowest RMSE fell in the range of 0.003 per year or lower.

At a rate constant for degradation of 0.003 per year, the simulation using the MNA Rate Constant Estimator projects that a concentration of 1,1-DCA will reach the receptor at a concentration near the acceptance criteria (Panel C of **Figure 5.7.1.4**). The projection likely overestimates the concentrations at the receptor because the simulation assumes that there is no attenuation of concentrations of 1,1-DCA in the source over time.

Rate constants for degradation of 1,1-DCE with the lowest RMSE fell into the range of 0.010 to 0.019 per year. The center of the range is 0.015 per year. The simulation for 1,1-DCE using the calibrated rate constant of 0.019 per year indicates that 1,1-DCE will reach the receptor at concentrations near the acceptance level (Panel D). Again, the simulation assumes no attenuation of 1,1-DCE in the source.

Does the biomarker abundance explain the model-estimated biodegradation rate constants? **No***

The DHBt biomarker targets *Dehalobacter* species that are capable of reductive dechlorination of 1,1,1-TCA to 1,1-DCA and 1,1-DCA to chloroethane. The DCA biomarker targets the 1,1-DCA reductive dehalogenase gene found in some strains of *Dehalobacter*.

The abundance of DHBt, DCA, and DHC biomarkers in well water from Site 1 is presented in Panel A of **Figure 5.7.1.4**. The MNA Rate Constant Estimator can estimate a rate constant for biodegradation 1,1,1-TCA and 1,1-DCA from the abundance of the DHBt biomarker. The abundance of gene copies for these biomarkers was entered into Box 6b of the MNA Rate Constant Estimator. If a marker was not detected, the reporting limit was entered. All markers were determined at an abundance of 20 or fewer gene copies per mL.

The abundance of DBHt in Well 3 was over 100 gene copies per mL, in Well 2 was over 1000 per mL, and in Well 1 was over 10,000 per mL. However, the predicted rate constants for biodegradation of 1,1,1-TCA was relatively low, a value of 0.001 per year. The rate constant for abiotic degradation determined from the temperature of the water was 0.7 per year. The rate constant for biodegradation that was predicted from the abundance of the DHBt biomarker does not contribute in any substantial way to the overall rate constant.

The rate constant for 1,1-DCA degradation estimated from the abundance of the DHBt biomarker was 0.00012 per year. The range of rate constants that best fit the field data are 0.003 per year or lower. This is roughly an order of magnitude lower than the rate constant for 1,1-DCA biodegradation that was extracted from the filed data (0.003 per year, see Panel C of **Figure 5.7.1.4**). The rate constant predicted from the biomarker data is not inconsistent with the apparent field scale rate constant.

The DHC biomarker targets a structural gene in *Dehalococcoides* bacteria. In plumes with chlorinated alkenes, *Dehalococcoides* bacteria often have an enzyme, vinyl chloride reductase, that can reductively dechlorinate 1,1-DCE. The *vcrA* biomarker targets vinyl chloride reductase. The MNA Rate Constant Estimator can estimate a rate constant for biodegradation of 1,1-DCE from the abundance of the *vcrA* biomarker. We assumed that the abundance of *vcrA* in water from Site 1 was the same as the abundance of the DHC biomarker. The abundance of gene copies for DHC was entered into Box 6b of the MNA Rate Constant Estimator. If DHC was not detected, the reporting limit was entered. The rate constant estimated from the abundance of the *vcrA* marker was 4.5E-05 per year. This is much less than the rate constant of 0.015 per year that was estimated from the field data.

The abundance of the DHBt biomarker is irrelevant to predicting the apparent rate constant for abiotic degradation of 1,1,1-TCA. The abundance of the DHBt is not inconsistent with the apparent rate constant for degradation of 1,1-DCA. The abundance of DHC cannot predict the apparent rate constant for biodegradation of 1,1-DCE at Site 1.

Are geochemical conditions supportive of biodegradation of 1,1,1-TCA or 1,1-DCA or 1,1-DCE? **Yes***

The geochemistry of the water samples from the wells at Site 1 is provided in **Table 5.7.1.2**. The conditions are appropriate for *Dehalobacter* bacteria, and there were high abundances of *Dehalobacter* bacteria in the water. The concentrations of dissolved oxygen were high enough to prevent the growth of *Dehalococcoides* bacteria, and *Dehalococcoides* bacteria were not detected or present at low abundance in groundwater from Site 1 (Panel A of **Figure 5.7.1.4**). The abundance was never above 1 gene copy per mL.

5.7.1.3 PCE, TCE, cis-DCE, and Vinyl Chloride

Overview: As described below, the project data suggest that PCE is biodegrading but that little or no biodegradation of the daughter products are occurring within the plume.

Are Chlorinated Alkenes below regulatory standards? **No**
In the most contaminated well (the source well) at Site 1 in 2018, the concentration of PCE was 17 µg/L, the concentration of TCE was 5,100 µg/L, the concentration of cis-DCE was 7,000 µg/L (Panel a of **Figure 5.7.1.2**). The concentration of vinyl chloride in Well 3 was 370 µg/L. These concentrations are above regulatory standards.

Are PCE, TCE, cis-DCE and Vinyl Chloride being degraded based on model predictions? **Yes***
See Panel A of Figure 5.2b for the distribution of PCE, TCE, *cis*-DCE and vinyl chloride at the site.

Data from all four wells were used in the MNA Rate Constant Estimator to estimate a rate constant for degradation of PCE, then TCE, then *cis*-DCE and then vinyl chloride. For each simulation, the MNA Rate Constant Estimator calculated the root mean square error (RMSE) between the simulated concentrations and the field data. For PCE, the rate constants with the lowest RMSE fell in the range of 0.050 per year or lower. The rate constants with the lowest RMSE for TCE fell in a range of 0.005 per year or less, for *cis*-DCE in the range of 0.0004 or less and for vinyl chloride in the range of 0.02 or less. Panel A of **Figure 5.7.1.5** provides the simulations in the MNA Rate Constant Estimator for the upper limit of values with the lowest RMSE. The rate constants that best minimize the RMSE did a poor job of simulating the high concentration of vinyl chloride in Well 3.

The rate constants were adjusted in the MNA Rate Constant Estimator to project the measured concentration of vinyl chloride in Well 3. The rate constants and projections are provided in Panel B of **Figure 5.7.1.5**. Even with the adjustments, the projection for concentrations of vinyl chloride is a poor match to the field data.

The simulations using the MNA Rate Constant Estimator do not provide compelling evidence that TCE, *cis*-DCE or vinyl chloride are being degraded in groundwater at Site 1. If they are not being degraded, there is a possibility that their concentrations will exceed the acceptance level at the point of compliance at some time in the future.

Does the biomarker abundance explain the model-estimated biodegradation rate constants? **No**
The DHC biomarker targets a structural gene in *Dehalococcoides* bacteria. Many *Dehalococcoides* bacteria can degrade *cis*-DCE to vinyl chloride, and then vinyl chloride to ethene.

The MNA Rate Constant Estimator can estimate rate constants for biodegradation of *cis*-DCE from the abundance of the vcrA biomarker. We assumed that the abundance of *vcrA* in water from Site 1 was the same as the abundance of the DHC biomarker. The abundance of gene copies for DHC was entered into Box 6b of the MNA Rate Constant Estimator. If DHC was not detected, the reporting limit was entered. The rate constant for *cis*-DCE estimated from the abundance of the

DHC marker was 0.0011 per year. This is much less than the rate constant of 0.050 per year (Panel B of **Figure 5.7.1.5**) that would be necessary to produce 370 µg/L of vinyl chloride in Well 3.

The rate constant for vinyl chloride estimated from the abundance of the DHC marker was 0.0018 per year. This slow rate constant cannot explain the low concentration of vinyl chloride in Well 2, down gradient of Well 3.

The biomarker abundance does not explain the model-estimated biodegradation rate constants for cis-DCE and vinyl chloride.

Are geochemical conditions supportive of biodegradation of PCE, TCE, cis-DCE and Vinyl Chloride? **No**

The geochemistry of the water samples from the wells at Site 1 is provided in **Table 5.7.1.2**. The concentrations of dissolved oxygen were high enough to prevent the growth of *Dehalococcoides* bacteria, and *Dehalococcoides* bacteria were not detected or present at low abundance in groundwater from Site 1 (Panel A of **Figure 5.7.1.4**). The abundance was never above 1 gene copy per mL.

Table 5.7.1.1. Rate constants for 1,4-dioxane degradation provided by ^{14}C assay for Site 1.

Well #	Sample Date	First order rate constant (per year)	95% Confidence Interval (per year)
1	8/6/2018	<0.0007	0.0005*
2	8/7/2018	<0.0020	0.0007*
2	12/15/2019	<0.0054	0.0024*
3	8/8/2018	<0.0017	0.0008*
3	12/15/2019	<0.0080	0.0030*
4	8/9/2018	<0.0004	0.0005*

* 95% confidence interval on rate for microcosm; rate for microcosm was not significantly different than filter-sterilized groundwater control.

Table 5.7.1.2. Geochemistry of water samples from Site 1.

Well #	Sample Date	Temp.	pH	Conductivity	Dissolved Oxygen	ORP	Ferrous Iron
		˚C		µS/cm	mg/L	mV	mg/L
1	8/6/2018	25.8	6.84	980	3.38	198	-
1	3/23/2019	19.7	7.08	1410	0.002	18	0.42
2	8/7/2018	23.8	7.44	1624	1.39	314	-
2	3/23/2019	22.6	6.88	1450	0.05	165	0.73
2	12/15/2019	23.1	7.73	1390	0.18	156	0.30
3	8/8/2018	39.4	7.39	1885	0.44	360	-
3	3/23/2019	42.3	8.01	2060	0.23	88	0.09
3	12/15/2019	36.7	8.41	2070	0.65	109	0.23
4	8/9/2018	29.3	6.70	1185	1.65	361	-
source	8/8/2018	26.4	7.47	1062	0.76	318	--

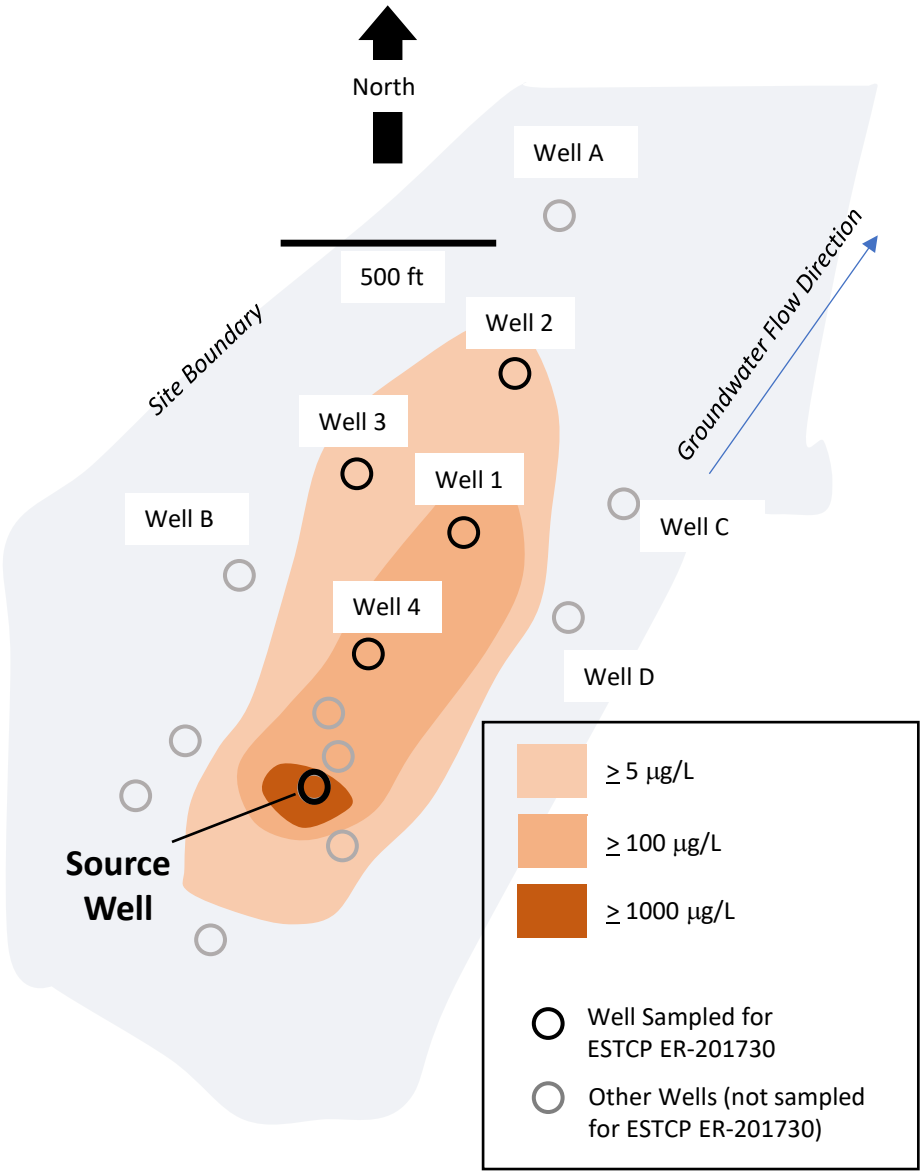
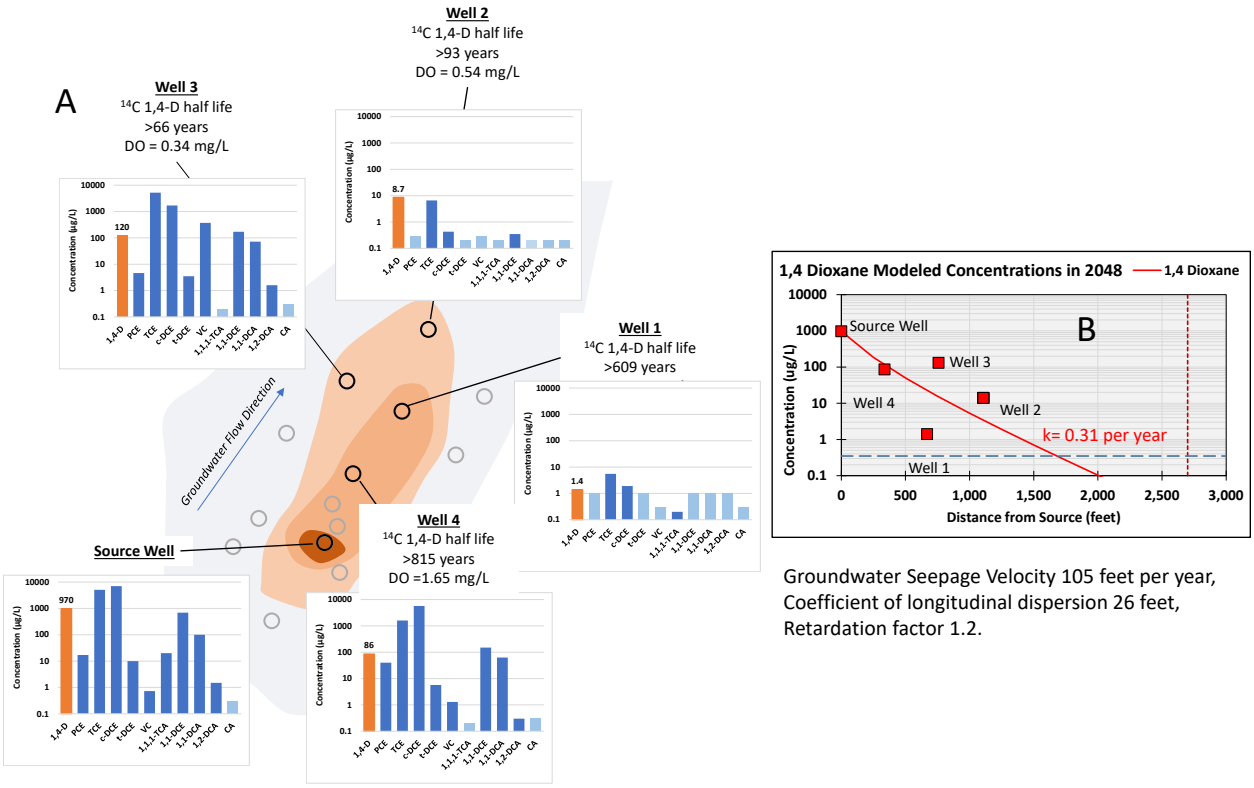


Figure 5.7.1.1. Site 1 (DoD) Distribution of Concentrations of 1,4-Dioxane.



Groundwater Seepage Velocity 105 feet per year, Coefficient of longitudinal dispersion 26 feet, Retardation factor 1.2.

Figure 5.7.1.2. Site 1 (DoD) Summary of 1,4-Dioxane/CVOC Concentrations and Attenuation of 1,4-Dioxane with Distance from the Source.
Pale blue values in Panel A are plotted at the reporting limit.

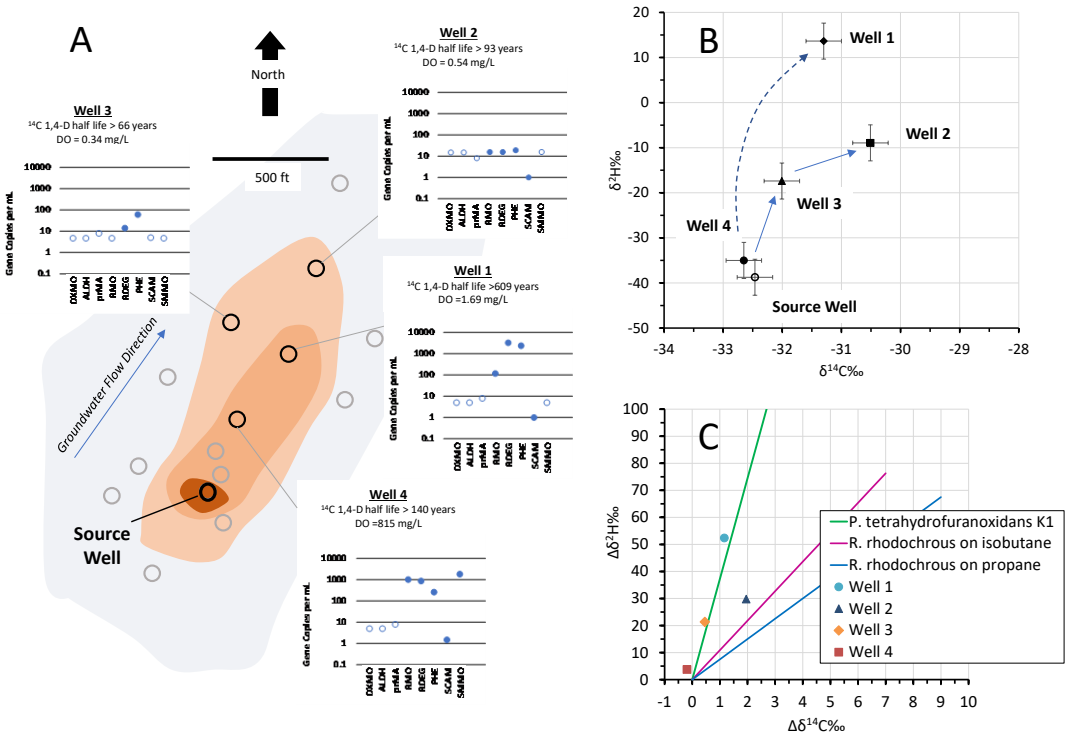
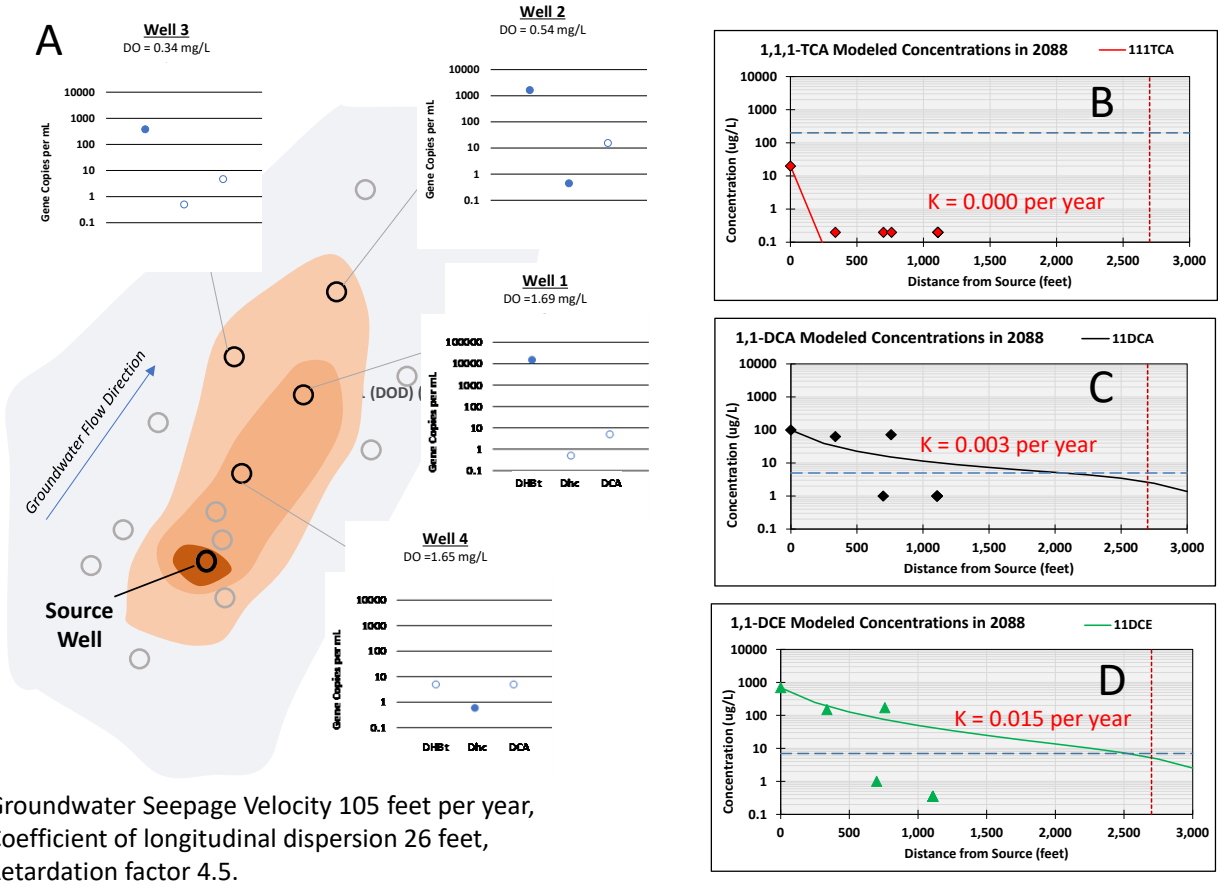


Figure 5.7.1.3. Site 1 (DoD) Summary of Biomarker and Isotope Data for 1,4-Dioxane.
Open circles in abundance of qPCR biomarkers in Panel A indicate the limit of quantitation. Error bars in Panel C represent one standard deviation.



Groundwater Seepage Velocity 105 feet per year,
Coefficient of longitudinal dispersion 26 feet,
Retardation factor 4.5.

Figure 5.7.1.4. Site 1 (DoD) Summary of Biomarkers for CVOCs and Concentration Trends and Rate Constants for 1,1,1-TCA and 1,1-DCA and 1,1-DCE.

Open circles in abundance of qPCR biomarkers in Panel A indicate the limit of quantitation.

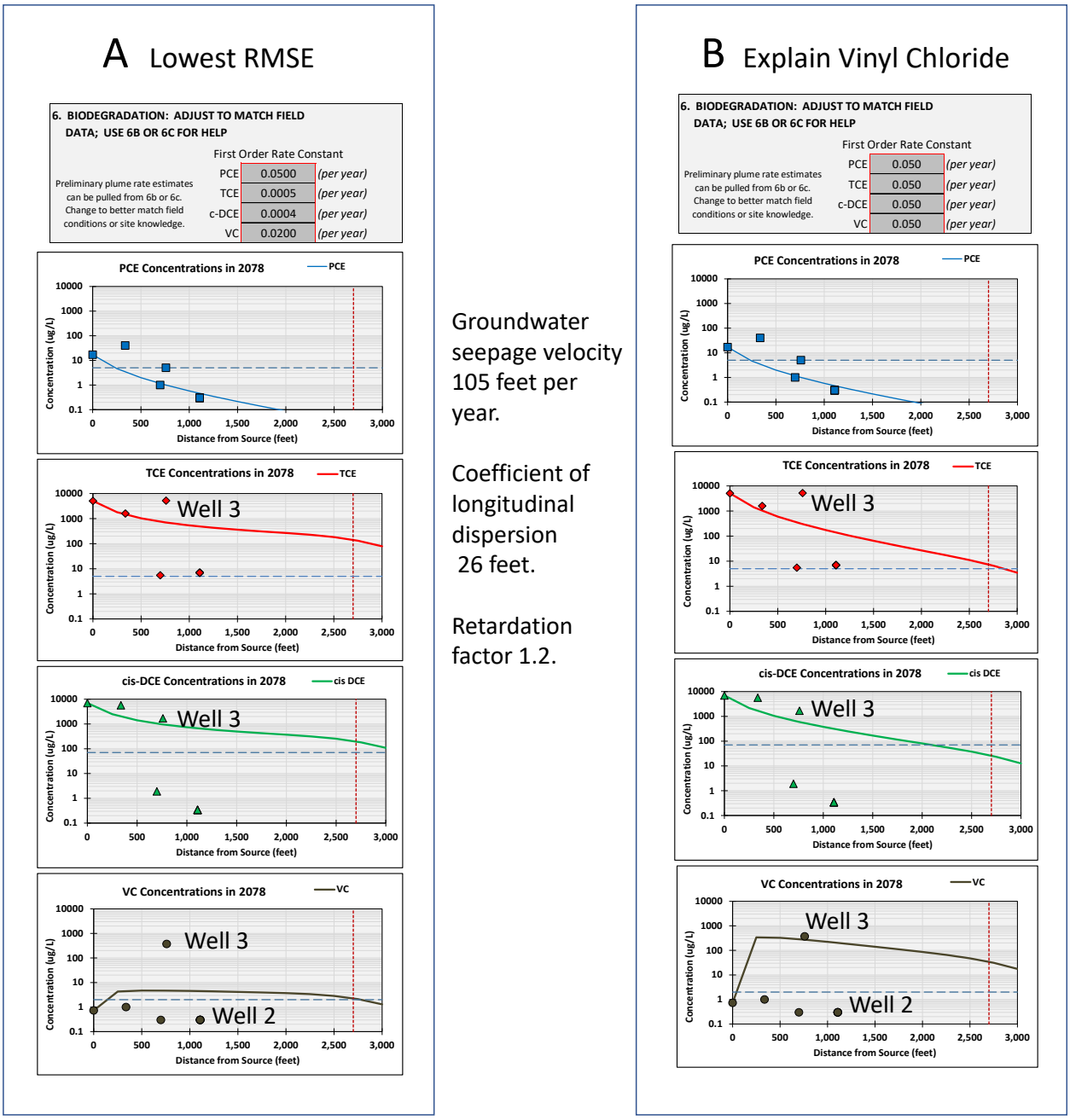


Figure 5.7.1.5. Site 1 (DoD) Summary of Concentration Trends and Rate Constants for PCE, TCE, *cis*-DCE and Vinyl Chloride.

5.7.2. Site 2 (DoD)

Overview: At this site 1,4-dioxane was used to stabilize a chlorinated solvent such as 1,1,1-TCA or TCE. The distribution of concentrations of 1,4-dioxane at Site 4 is presented in **Figure 5.7.2.1**. Well 4 is not the source of the contamination. The source is an additional 7,000 feet up gradient.

When the groundwater was sampled for this project in 2018, the concentrations of 1,4-dioxane in all four wells was below the method reporting limit of 0.25 µg/L. The brown shape in Panel A covers the area where 1,4-dioxane was detected by the site owner in the past in monitoring wells. Panel B presents data on concentrations of 1,4-dioxane in 2014, 2015 and 2016 that exceeded the regulatory standard. The concentrations of 1,4-dioxane were low compared to many other sites (compare Figure 1 of Adamson et al., 2015).

Is 1,4-dioxane below regulatory standards? **Yes**
All locations sampled in 2018 were below the reporting limit for 1,4-dioxane (0.15 µg/L), which means that they also fell below the groundwater regulatory standard for the state where this site is located (0.3 µg/L).

Is the plume stable (i.e., is the 1st line of evidence for MNA met)? **Not assessed**
In the earlier samples, the plume of 1,4-dioxane extended all the way to the point of discharge to surface water. The estimated seepage velocity of groundwater at the site approaches 900 feet per year. This means that groundwater is expected to move from the upgradient well (Well 4) to the most downgradient well (Well 2) within 7 or 8 years. The wells in the study were only sampled twice for 1,4-dioxane. As a result, there is not sufficient data to establish trends in concentrations of 1,4-dioxane in the monitoring wells.

The plume also contained PCE, TCE, and *cis*-DCE, but no detectable vinyl chloride. Panel C of **Figure 5.7.2.1** presents the concentrations of PCE, TCE and *cis*-DCE in samples collected between 1997 to 1999 from wells along the centerline of the plume of chlorinated ethenes (labelled 1999 in the chart). The unit for concentration is the sum of molar concentrations of PCE, TCE, and *cis*-DCE multiplied by the molecular weight of PCE.

Starting in 1999, the groundwater in the plume has been treated for chlorinated ethenes by pumping the water, treating it with activated carbon, and then infiltrating the water back into the aquifer. The plume of chlorinated ethenes has been extensively diminished by the effective pump and treat system. Panel C also presents the distribution of PCE, TCE, and *cis*-DCE in 2016 or the date of last sampling (labelled 2016 in the chart). The active remedy has reduced the concentrations of chlorinated ethenes by roughly an order of magnitude.

Is 1,4-dioxane being degraded based on model predictions? **No**
Panel A of **Figure 5.7.2.2** depicts the distribution of 1,4-dioxane and chlorinated hydrocarbons in the four wells that were sampled for this project in 2018. Because the concentrations of 1,4-dioxane were below the reporting limits in 2018, the MNA Rate Constant Estimator was calibrated to the "centerline" data in Panel A of **Figure 5.7.2.1**, which were collected during earlier sampling events. Results of the simulation are presented in Panel B of **Figure 5.7.2.2**. The concentrations of 1,4-dioxane are much higher than would be expected from reasonable values of the coefficient

of longitudinal dispersion and retardation due to sorption. The model assumes that concentrations are controlled by flow of groundwater, dispersion along the flow path, degradation along the flow path, and sorption to aquifer solids. After 19 years of a pump-and-treat remedy, the concentrations seem to be controlled by back diffusion of sequestered material in the aquifer matrix. As a result, the model cannot be used to extract a prediction of degradation from the monitoring data at this site.

Does the biomarker abundance explain the model-estimated 1,4-dioxane biodegradation rate constant? **No**

Data on the abundance of the biomarkers were collected in 2018. The relevant biomarkers for direct metabolism of 1,4-dioxane are DXMO, ALDH, and possibly *prmA*. Biomarkers that are potentially relevant to cometabolism are *prmA*, RMO, RDEG, PHE, SCAM and SMMO. Their abundance is presented in **Figure 5.7.2.3.** If the abundance was below the reporting limit, the abundance was plotted at the reporting limit. The biomarkers for direct biodegradation of 1,4-dioxane (DXMO and ALDH) were not detected in groundwater at Site 2. The abundance of one or several of the biomarkers that have been associated with cometabolism of 1,4-dioxane were above 1000 gene copies per mL in Well 4, above 100 gene copies per mL in Well 2, but all the biomarkers were below 100 genes copies per mL in Well 1 and Well 3.

The MNA Rate Constant Estimator was used to extract an overall rate constant for biodegradation of 1,4-dioxane from the abundance of the DXMO, *prmA*, RMO and RDEG biomarkers in 2018. The abundance of gene copies for these biomarkers was entered into Box 6b of the MNA Rate Constant Estimator. If a marker was not detected, the reporting limit was entered. The sum of the predicted rate constants for biodegradation was 0.001 per year. The contribution of a rate constant of 0.001 per year to attenuation with distance along the flow path would be minimal compared to the effects of dispersion along the flow path and the effects of the treatment system starting in 1999.

The abundance of biomarkers for degradation of chlorinated hydrocarbons was low, never more than 100 gene copies per mL. The rate constant for degradation of *cis*-DCE predicted from the abundance of the DHC biomarker using the MNA Rate Constant Estimator was 0.005 per year. The effect of biodegradation on attenuation in concentrations of *cis*-DCE would be minimal compared the effects of dispersion along the flow path and the effects of the treatment system starting in 1999.

Is the model-estimated 1,4-dioxane biodegradation rate constant consistent with rate constants estimated using the ^{14}C assay? **No**

The ^{14}C assay was performed on samples acquired in 2018. The rate constants and 95% confidence intervals are provided in **Table 5.7.2.1.** Biodegradation was detected in Well 1, but the rate constant was low (0.002 per year). Biodegradation was not detected in samples from Well 2, Well 3 or Well 4. It was not possible to extract a rate constant using the MNA Rate Constant Estimator. However, detectable concentrations of 1,4-dioxane were found 19,000 feet downgradient of the source of contamination in water that left the source 20 years ago. The small rate constant of biodegradation in water from Well 1 is consistent with the persistence of 1,4-dioxane in the plume for an extended period of time.

Are ^{2}H and/or ^{13}C enriched along the flow path? **No***
The values of $\delta^{13}C$ in 1,4-dioxane along the flow path from Well 4 to Well 3 to Well 1 and then Well 2 was -32.1‰, then -31.8‰, then -32.7‰, and then -32.2‰. With an uncertainty in the measurement of 0.5‰, and a difference of 1.0‰ necessary to be statistically different at 95% confidence, there was no evidence of enrichment of $\delta^{13}C$ along the flow path.

It was only possible to determine ^{2}H in 1,4-dioxane in Well 4, the first well in the flow path. The data are not available to evaluate enrichment of ^{2}H along the flow path.

Panel B of **Figure 5.7.2.3** compares $\delta^{13}C$ and $\delta^{2}H$ in 1,4-dioxane in Well 4 at Site 2 to values for the most contaminated wells at the other nine sites in the study. The most contaminated wells were the best information available on values of $\delta^{13}C$ and $\delta^{2}H$ at the source of contamination at these sites. If the fill of the symbol is black, the concentration of 1,4-dioxane was greater than 1,000 µg/L. If the fill is blue, the concentration was between 1,000 and 100 µg/L. If the fill is while, the concentration was less than 100 µg/L. The value of $\delta^{2}H$ in 1,4-dioxane in Well 4 at Site 2 was heavier than the source of contamination at eight of the other nine sites. This comparison suggests, but does not prove, that 1,4-dioxane was degraded in water from Well 4 at Site 2.

Bennett and Aravena (2020) compiled values for $\delta^{2}H$ in 1,4-dioxane that had not been degraded. The maximum value of $\delta^{2}H$ in 22 samples was -17‰. The value for $\delta^{2}H$ in 1,4-dioxane from Well 4 was +0.5‰, which is higher than the maximum value in the undegraded samples, which would suggest that the 1,4-dioxane in Well 4 was degraded. However, Bennett and Aravena (2020) made the following statement: *Based on the large range in $\delta^{2}H$ values for source 1,4-dioxane and given the larger uncertainty in analytical results, the use of $\delta^{2}H$ values alone as an indicator for biodegradation is not recommended*.

Are geochemical conditions supportive of 1,4-dioxane biodegradation? **Yes**
The geochemistry of the water samples from the wells at Site 2 is provided in **Table 5.7.2.2**. In water samples acquired in 2018 for the ^{14}C biodegradation assay, the temperature, pH, conductivity as a proxy for dissolved electrolytes, dissolved oxygen and ORP were all adequate to support biodegradation of 1,4-dioxane. The absence of detectable rates of biodegradation in three of the ^{14}C assays cannot be attributed to the geochemical conditions. The concentration of methane in all the wells was low, indicating that all the flow paths to the wells provided oxygenated groundwater.

Are inhibitory CVOCs present below inhibitory levels and/or decreasing with time/distance? **Yes**
Mahendra et al. (2013) determined half saturation constants for inhibition of aerobic biodegradation of 1,4-dioxane (K_i) by 1,1-DCE and 1,1,1-TCA. Values of K_i for 1,1-DCE were 50 µg/L for cometabolism and 320 µg/L for direct metabolism while values for 1,1,1-TCA were 507 µg/L for cometabolism and 160 µg/L for direct metabolism. The maximum concentration of 1,1-DCE at Site 2 was 1.2 µg/L and the concentration of 1,1,1-TCA was less than 4 µg/L. Inhibitory CVOCs were not present at concentrations that would be expected to impair biodegradation or cometabolism of 1,4-dioxane.

Table 5.7.2.1. Rate constants for 1,4-dioxane degradation provided by ^{14}C assay for Site 2.

Sampled	Well	First order rate constant (per year)	95% Confidence Interval (per year)
2018	Well 1 (GW only)	0.00211	0.00209
2018	Well 2 (GW only)	<0.00147	0.00096*
2018	Well 3 (GW only)	<0.00249	0.00099*
2018	Well 4 (GW only)	<0.00289	0.00091*

*95% confidence interval on rate constant for microcosm; rate constant for microcosm was not significantly different than filter-sterilized groundwater control; yellow highlighted cells represent rate constants for microcosms that were significantly different than filter-sterilized groundwater control.

Table 5.7.2.2. Geochemistry of water samples from Site 2.

Well #	Sample Date	Temp.	pH	Conductivity	Dissolved Oxygen	ORP	Methane
		˚C		µS/cm	mg/L	mV	mg/L
1	8/20/2018	18.5	7.01	213	0.359	195	<0.01
2	8/21/2018	17.9	6.73	159	0.462	72	<0.01
3	8/22/2018	20.1	6.05	184	0.773	313	<0.01
4	8/23/2018	18.5	6.21	234	0.657	253	<0.01

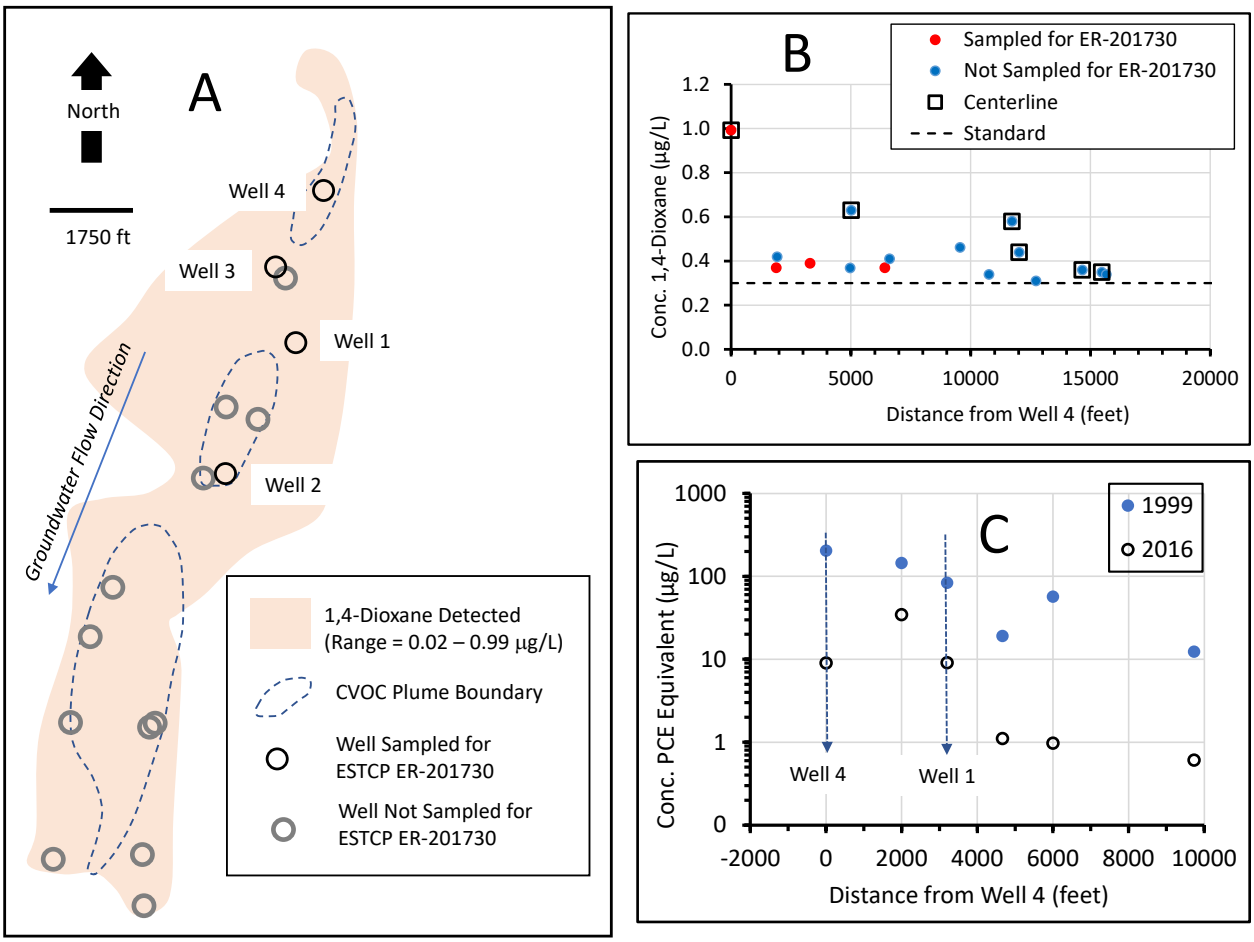


Figure 5.7.2.1. Site 2 (Industrial) Distribution of Concentrations of 1,4-Dioxane.

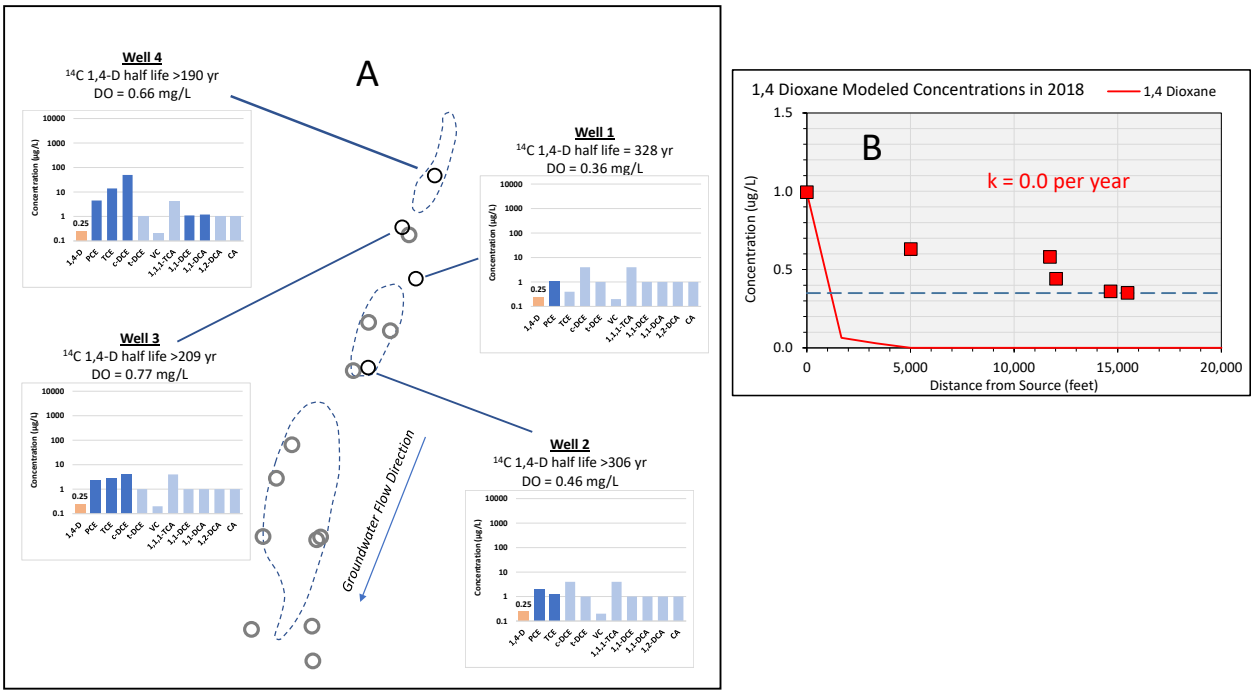


Figure 5.7.2.2. Site 4 (Industrial) Summary of 1,4-Dioxane/CVOC Concentrations and Attenuation of 1,4-Dioxane with Distance from the Source.

Pale values in Panel A are plotted at the reporting limit. Values for 1,4-dioxane in Panel B are historical values, not the values in Panel A.

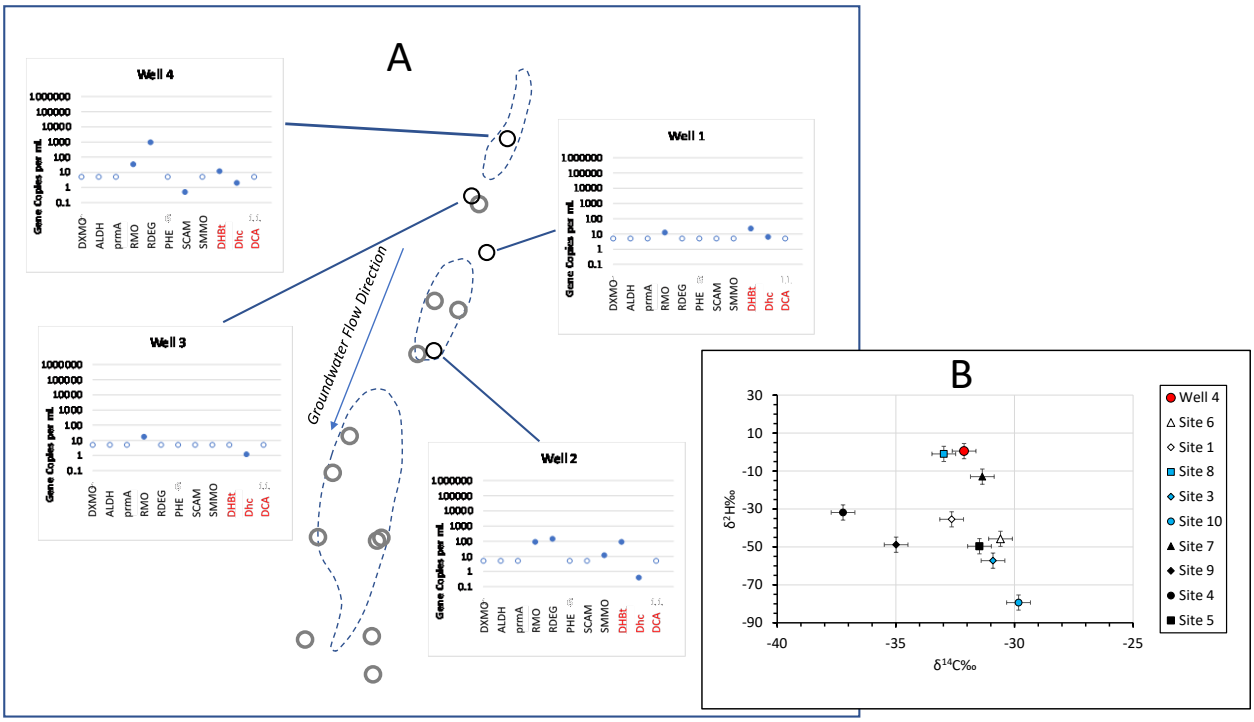


Figure 5.7.2.3. Site 1 (DoD) Summary of Biomarker Data For 1,4-Dioxane and CVOCs.
Open circles in abundance of qPCR biomarkers in Panel A indicate the limit of quantitation. Error bars in Panel B are one sample standard deviation.

5.7.3. Site 3 (Municipal Landfill)

Overview: At this site, manufacturing waste containing 1,4-dioxane was disposed to an unlined municipal landfill. The distribution of concentrations of 1,4-dioxane at Site 3 is presented in Panel A of **Figure 5.7.3.1**. The plume of 1,4-dioxane is co-distributed with a plume of tetrahydrofuran, benzene (near source), and *t*-butyl alcohol (Panel B). The concentrations of 1,4-dioxane are moderate compared to many other sites (compare Figure 1 of Adamson et al., 2015). In comparison to sites where 1,4-dioxane was used to stabilize a chlorinated solvent, the concentrations of the chlorinated solvents were low or not detected (Panel A of **Figure 5.7.3.2**).

Is 1,4-dioxane below regulatory standards? **No**
When the wells were sample in 2018 for this study, the concentration of 1,4-dioxane varied from 310 to 10 µg/L (Panel A of **Figure 5.7.3.1**). These concentrations were above the applicable regulatory standard.

Is the plume stable (i.e., is the 1st line of evidence for MNA met)? **Yes**
As a corrective action, the landfill was capped to prevent recharge of groundwater by water that infiltrates the fill material. As a result, concentrations of contaminants in the groundwater at the margin of the landfill have declined dramatically. The concentrations in Well 2 are stable over time (Panel C of **Figure 5.7.3.1**). The concentrations in Well 5 and Well 1 were stable or were approaching stability at the time samples were taken in 2018.

Is 1,4-dioxane being degraded based on model predictions? **Yes***
The concentration of 1,4-dioxane in Well 3 is the highest concentration that remains in the plume. The MNA Rate Constant Estimator was calibrated to the flow path from Well 3 to Well 1. For each simulation, the MNA Rate Constant Estimator calculated the root mean square error (RMSE) between the simulated concentrations and the field data in 2018. The best fit was obtained when biodegradation was not included in the simulation. The value of RMSE was lowest whenever the values of the biodegradation rate constant was 0.005 per year of less. The simulations could not distinguish a contribution of biodegradation over the contributions of dispersion unless the rate constant was greater than 0.005 per year. Based on the screening-level simulations with the MNA Rate Constant Estimator, there was no evidence that 1,4-dioxane was being degraded at rates faster than 0.005 per year.

Gedalanga et al. (2016) used MODFLOW (a numerical, three-dimensional, finite-difference groundwater flow model) to simulate the groundwater flow in the study area. They used MT3DMS (a modular three-dimensional transport model) to simulate advection, dispersion, and chemical reactions and predict the distribution of 1,4-dioxane at field scale. To best match the field data, it was necessary to use a degradation rate constant of 0.07 per year in the simulation. The simulation done by Gedalanga et al. (2016) using MODFLOW and MT3DMS indicates that 1,4-dioxane is being degraded at the site.

The MNA Rate Constant Estimator is intended as a simple screening model. The calibration of MODFLOW to Site 3 as reported in Gedalanga et al. (2016) is more comprehensive and more robust. The biodegradation rate constant for 1,4-dioxane that is necessary to calibrate MODFLOW to concentrations at the site will be taken as the best estimate for the rate constant at field scale.

Does the biomarker abundance explain the model-estimated 1,4-dioxane biodegradation rate constant? **No**

The relevant putative biomarkers for direct metabolism of 1,4-dioxane are DXMO, ALDH, and possibly *prmA*. Putative biomarkers that are potentially relevant to cometabolism are *prmA*, RMO, RDEG, PHE, SCAM and SMMO. Their abundance is presented in **Figure 5.7.3.3**. If the abundance was below the reporting limit, the abundance was plotted at the reporting limit.

Four conventional monitoring wells were sampled at the site. At the time the conventional wells were sampled, contractors to the site owner had just finished a pilot study of cometabolic bio-stimulation with oxygen and propane infusion into the subsurface. Well 4 was the injection well for the bio-stimulation project. The DXMO biomarker was not detected in groundwater from the four conventional monitoring wells at Site 3. However, it was detected at 775 gene copies per mL in Well 4m, which also had measurable levels of *prmA*. Similarly, the ALDH biomarker was also detected at Well 4, but it was also detected at the conventional monitoring wells MW-2 and MW-3.

Gedalanga et al. (2016) reported the abundance of the DXMO biomarker in water samples collected in 2015 at Site 3. The abundance of the DXMO biomarker in Well-1 was 1.1E+03 gene copies per mL, in Well-2 was 6.4E+03 gene copies per mL, and in Well-3 was <1.5E+01 gene copies per mL. Well-4 did not exist in 2015. There is no obvious explanation for the difference in the abundance of the DXMO biomarker in samples from 2015 and 2018.

In the conventional monitoring wells, the abundance of several of the biomarkers that have been associated with cometabolism of 1,4-dioxane were above 100 gene copies per mL in Well 2, Well 3 and Well 5, but all the biomarkers except SMMO were below 10 genes copies per mL in Well 1, the most distal well in the plume. The abundances in the bio-stimulation well (Well 4) were above 10,000 gene copies per mL for most of the biomarkers and above 100,000 gene copies per mL for RMO and RDEG.

The MNA Rate Constant Estimator was used to extract an overall rate constant for biodegradation of 1,4-dioxane from the abundance of the DXMO, *prmA*, RMO and RDEG biomarkers in the four conventional monitoring wells. The abundance of gene copies for these biomarkers was entered into Box 6b of the MNA Rate Constant Estimator. If a marker was not detected, the reporting limit was entered. A rate constant of 0.00069 per year was predicted from the abundance of the DXMO marker. The *prmA* marker predicted 0.00012 per year, the RMO marker predicted 0.00067 per year and the RDEG marker predicted 0.00014 per year. The sum of the predicted rate constants for biodegradation was 0.0016 per year. This is almost fifty-fold less than the rate constant predicted from the MT3DS simulation (0.07 per year). The biomarkers did not explain the rate constant that was extracted from the field data with the MT3DMS model.

If the DXMO abundance reported in Gedalanga et al. (2016) is used in the MNA Rate Constant Estimator, the predicted rate constant for degradation of 1,4-dioxane is 0.37 per year, which is greater than the rate constant predicted from the MT3DS simulation (0.07 per year).

The abundance of biomarkers in 2018 did not explain the rate constant that was extracted from the field data with the MT3DMS model. However, the abundance of the DXMO biomarker reported

by Gedalanga et al. (2016) for samples collected in 2015 does explain the rate constant extracted from the field data.

The MNA Rate Constant Estimator was also used to extract an overall rate constant for biodegradation of 1,4-dioxane from the abundance of the DXMO, *prmA*, RMO and RDEG biomarkers in Well 4, the injection well for the bio-stimulation project. The DXMO marker predicted 0.10 per year, the *prmA* marker predicted 0.14 per year, the RMO marker 0.25 per year and the RDEG marker 0.15 per year. The sum of the predicted rate constants for biodegradation was 0.52 per year.

Is the model-estimated 1,4-dioxane biodegradation rate constant consistent with rate constants estimated using the ^{14}C assay? **Yes**

The ^{14}C assay was performed on samples acquired in 2018. The rate constants and 95% confidence intervals are provided in **Table 5.7.3.1**. The values are calculated from the net rate of production of ^{14}C-labelled product in excess of label produced from a filter-sterilized control. If the rate constant is greater than zero at 95% confidence, the cell in **Table 5.7.3.1** is highlighted in yellow. Biodegradation was detected in Well 3 and Well 5 of the conventional monitoring wells. The rate constants for Well 3 (0.0114 per year) and Well 5 (0.021 per year) were reasonably consistent with the rate constant estimated by the MT3DMS model (0.07 per year). The rate constant in groundwater from Well 4, which had undergone bio-stimulation, was 0.096 per year, which is roughly an order of magnitude higher than the rate constants in the conventional wells.

For the bio-stimulation well (Well 4), there was reasonable agreement between the rate constant from the ^{14}C assay (0.09 per year) and the rate constant predicted from the biomarkers (0.52 per year).

Biodegradation was not detected in samples from Well 1 and Well 2.

Are ^{2}H and/or ^{13}C enriched along the flow path? **Yes**

Both ^{13}C and ^{2}H are enriched in 1,4-dioxane along the flow path (see Panel B of **Figure 5.7.3.3**). If the error bars do not overlap, the values are different at 95% confidence. Values in Well 1 are enriched compared to the values in Well 3 (the source well), indicating that 1,4-dioxane has been degraded along the flow path to this well. Also notice that values in the bio-stimulation well (Well 4) are enriched compared to the source well.

Panel C of **Figure 5.7.3.3** is a plot that was produced using the CSIA_14D.xlsx calculator in the BioPIC software that was updated as part of this project. The pathway compares the relative changes in values for $\delta^{13}C$ and $\delta^{2}H$ in 1,4-doxane in the wells to changes that were seen during biodegradation of 1,4-dioxane by cultures of several different bacteria. The pattern in the wells at Site 3 most closely matched the pattern for degradation by *Pseudonocardia tetrahydrofuranoxidans* K1 (Bennett et al., 2018). This is the pattern that is expected from direct metabolism.

Are geochemical conditions supportive of 1,4-dioxane biodegradation? **Yes**

The geochemistry of the water samples from the wells at Site 3 is provided in **Table 5.7.3.2**. In water samples acquired from Well 3, Well 4 and Well 5 for the ^{14}C biodegradation assay, the

temperature, pH, conductivity as a proxy for dissolved electrolytes, dissolved oxygen and ORP were all adequate to support biodegradation of 1,4-dioxane. The high concentration of ferrous iron and methane in Well 3 indicated that the well sampled a mixture of oxygenated groundwater and anaerobic groundwater. The anaerobic groundwater would not be supportive of biodegradation of 1,4-dioxane, and the ^{14}C rate constant for Well 3 was lower than Well 5 where the concentrations of methane and ferrous iron were lower (**Table 5.7.3.2**). A lower abundance of organisms that can degrade 1,4-dioxane in the anaerobic water could explain the slower rate constant for biodegradation. However, the abundances of the DNA markers were very similar in Well 3 compared to Well 5 (compare **Table 5.7.3.2** and Panel A of **Figure 5.7.3.3**).

In Well 2, the concentration of oxygen is low, the concentration of methane and ferrous iron is high, and the abundance of biomarkers is low. The geochemistry of the water was not supportive of 1,4-dioxane biodegradation, and this was reflected in the slow rate constant from the ^{14}C assay. The absence of detectable rates of biodegradation in Well 1 in the ^{14}C assay cannot be attributed to the geochemical conditions.

Are inhibitory CVOCs present below inhibitory levels and/or decreasing with time/distance? **Yes**
Mahendra et al. (2013) determined half saturation constants for inhibition of aerobic biodegradation of 1,4-dioxane (K_i) by 1,1-DCE and 1,1,1-TCA. Values of K_i for 1,1-DCE were 50 µg/L for cometabolism and 320 µg/L for direct metabolism while values for 1,1,1-TCA were 507 µg/L for cometabolism and 160 µg/L for direct metabolism. The concentration of 1,1-DCE or 1,1,1-TCA at Site 3 was less than 1 µg/L. Inhibitory CVOCs were not present at concentrations that would be expected to impair biodegradation or cometabolism of 1,4-dioxane.

Table 5.7.3.1. Rate constants for 1,4-dioxane degradation provided by ^{14}C assay at Site 3.

Sampled	Well	First order rate constant (per year)	95% Confidence Interval (per year)
2018	Well 1 (Conventional Well)	<0.0075*	0.0024***
2018	Well 2 (Conventional Well)	0.00182**	0.0023
2018	Well 3 (Conventional Well)	0.0114	0.0033
2018	Well 4 (Bio-stimulation Well)	0.096	0.0149
2018	Well 5 (Conventional Well)	0.021	0.0052

Yellow highlighted cells represent rate constants for microcosms that were significantly different than filter-sterilized groundwater control.
* Rate constant for microcosms was less than the rate constant for the filter-sterilized water control.
** Net rate constant not greater than zero at 95% confidence.
*** 95% confidence interval on rate for microcosm.

Table 5.7.3.2. Geochemistry of water samples from Site 3.

Well #	Net Rate Constant from the ^{14}C assay	Temp.	pH	Conduc-tivity	Dissolved Oxygen	ORP	Ferrous Iron	Methane
	per year	˚C		µS/cm	mg/L	mV	mg/L	mg/L
1	<0.0075*	13.2	7.97	483	0.295	260	-	0.017
2	0.00182**	14.5	8.02	581	0.166	60	2.34	11.1
3	0.0114	14.3	7.99	1664	1.44	131	2.19	5.4
4	0.096	14.1	7.66	590	15.8	296	0.109	0.024
5	0.021	14.8	7.84	479	0.45	187	0.446	0.016

Yellow highlighted cells represent rate constants for microcosms that were significantly different than filter-sterilized groundwater control.
* Rate constant for microcosms was less than the rate constant for the water control.
** Net rate constant not greater than zero at 95% confidence.

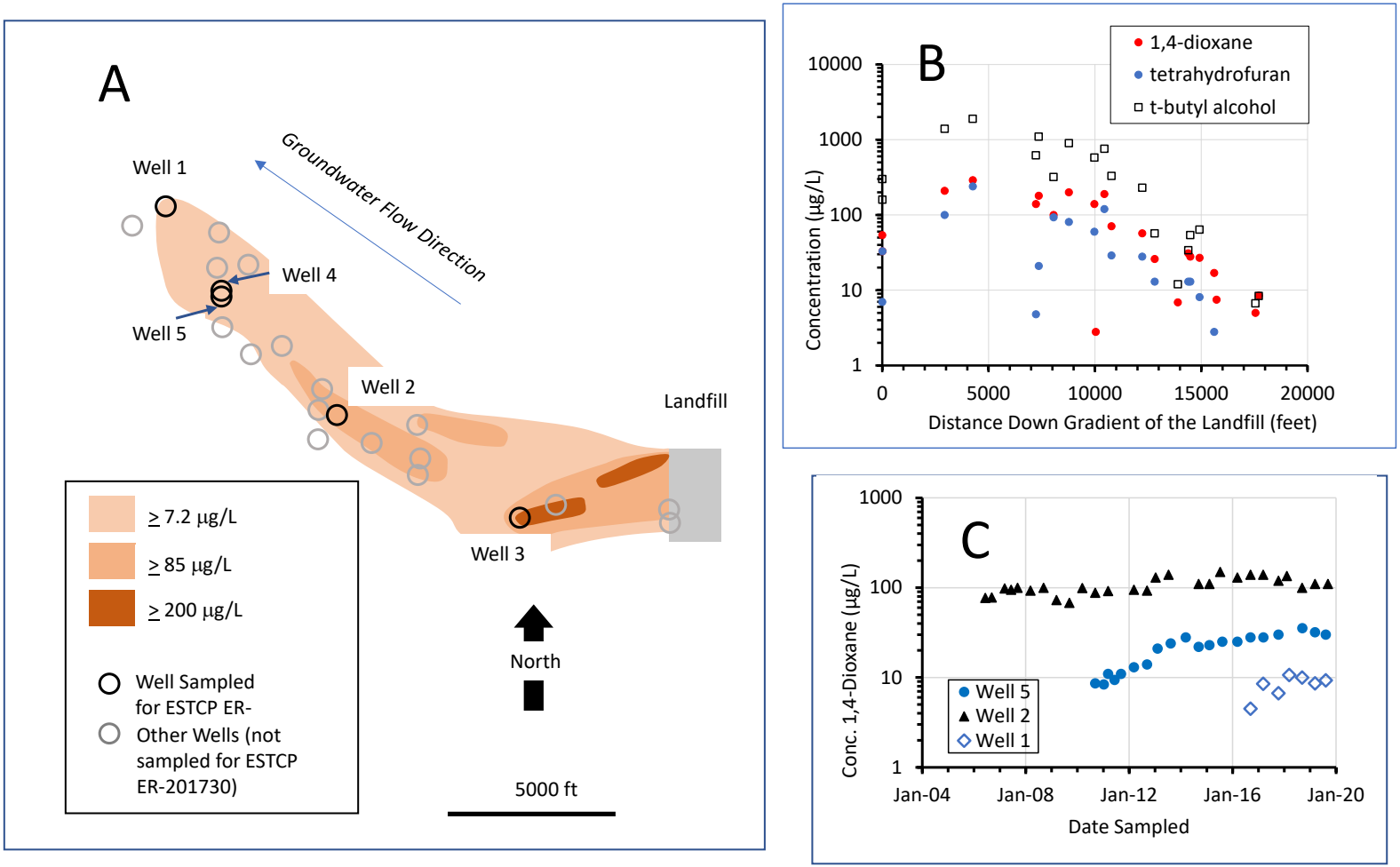


Figure 5.7.3.1. Site 3 (Industrial) Distribution of Concentrations of 1,4-Dioxane.

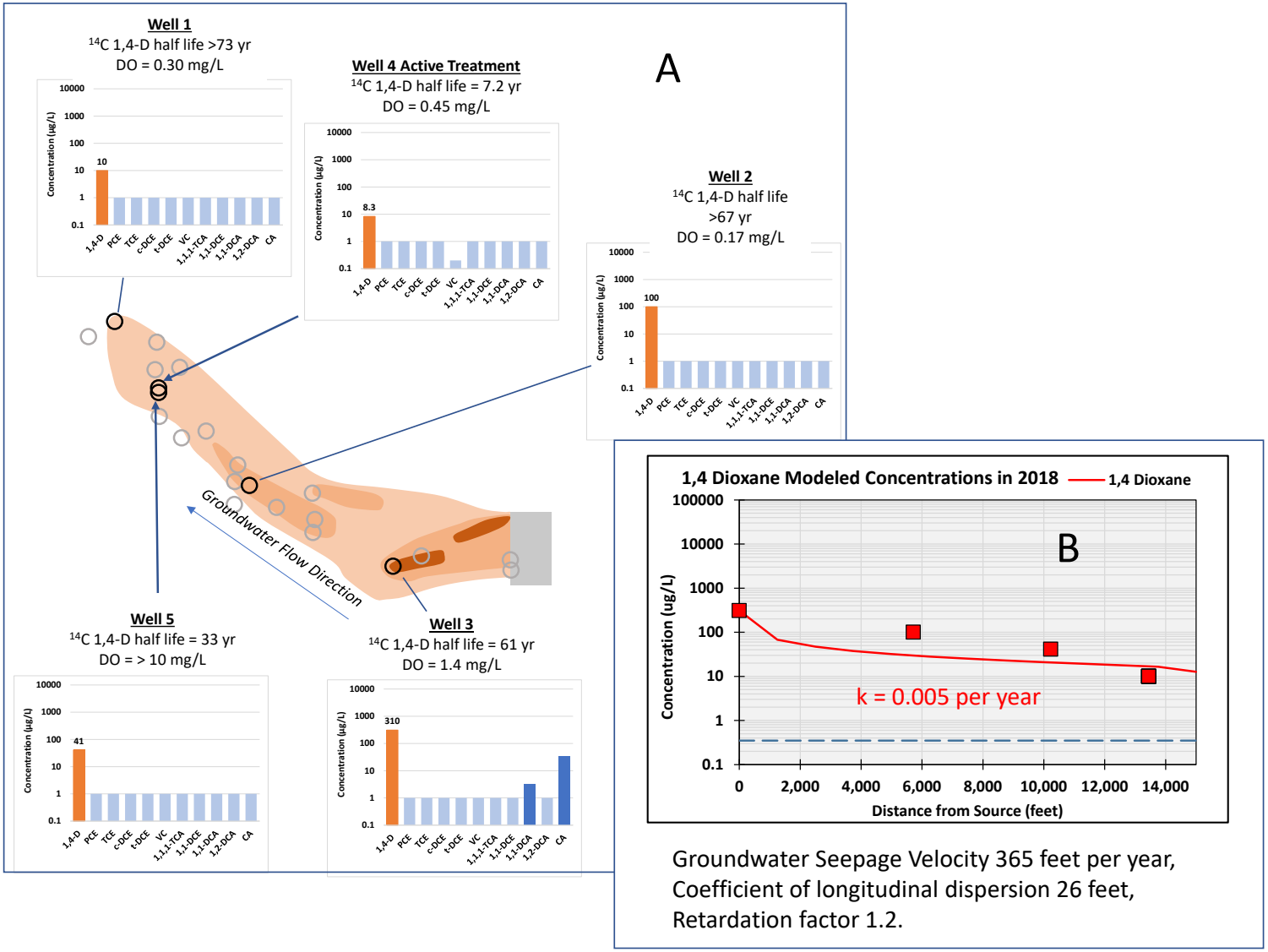


Figure 5.7.3.2. Site 3 (Industrial) Summary of 1,4-Dioxane/CVOC Concentrations and Attenuation of 1,4-Dioxane with Distance from the Source.

Pale blue values in Panel A are plotted at the reporting limit.

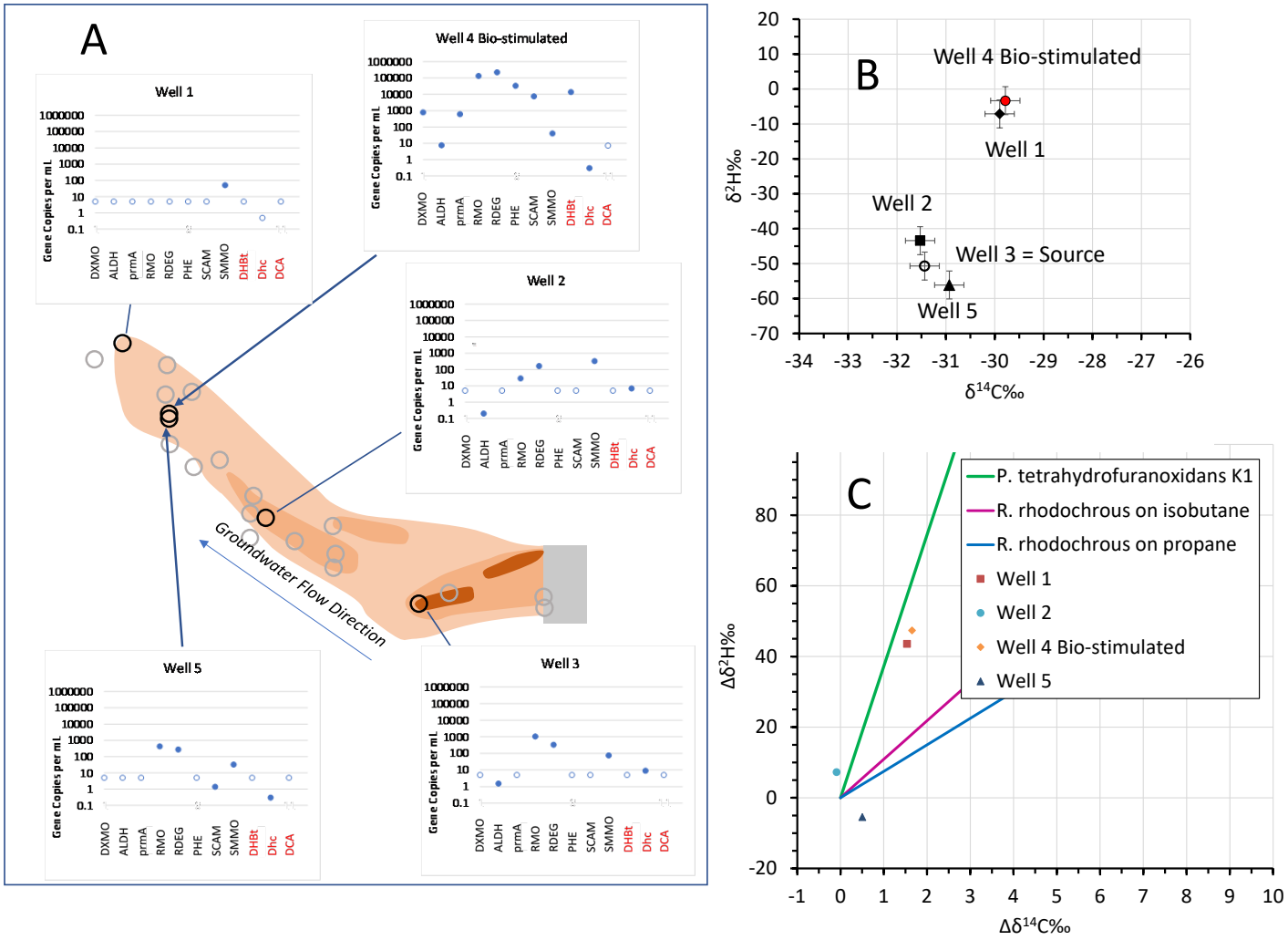


Figure 5.7.3.3. Site 3 (DoD) Summary of Biomarker Data for 1,4-Dioxane and CVOCs.
Open circles in abundance of qPCR biomarkers in Panel A indicate the limit of quantitation. Error bars in Panel B represent one sample standard deviation.

5.7.4. Site 4 (Industrial)

Overview: At this site, the primary use of 1,4-dioxane was as a solvent in a manufacturing process, instead of being used to stabilize a chlorinated solvent such as 1,1,1-TCA or TCE. The distribution of concentrations of 1,4-dioxane at Site 4 is presented in Panel A of **Figure 5.7.4.1**. The concentrations of 1,4-dioxane are high compared to many other sites (compare Figure 1 of Adamson et al., 2015). In comparison to sites where 1,4-dioxane was used to stabilize a chlorinated solvent, the concentrations of the chlorinated solvents were low, never more than 4.7 µg/L.

Is 1,4-dioxane below regulatory standards? **No**
When the wells were sampled in 2018, the maximum concentration of 1,4-dioxane was 10,000 µg/L. The concentration of 1,4-dioxane in all the wells was above the applicable regulatory standard (7.2 µg/L).

Is the plume stable (i.e., is the 1st line of evidence for MNA met)? **Yes**
The estimated seepage velocity of groundwater at the site in near 40 feet per year. Groundwater would move from the source well (Well 3) to the most down gradient well (Well 1) in approximately 8 years. Concentrations have been monitored in the wells for 16 years. Over that time the concentrations of 1,4-dioxane in the wells have been stable or declining (Panel B of **Figure 5.7.4.1**). There are no sentry wells downgradient of plume where 1,4-dioxane has never been present at a concentration above the regulatory standard. As a result, there is no documentation that the plume is stable in three-dimensional space. However, the concentrations are declining over time in the wells that are being monitored, and if that behavior is representative of the rest of the plume, the plume is stable or declining.

Is 1,4-dioxane being degraded based on model predictions? **Yes**
The monitoring wells at the site were arranged in transects down gradient of the source of the release. This configuration means that wells are not arranged along the most common flow direction from the source. As a result, the MNA Rate Constant Estimator was calibrated to data representing a flow path between two separate pairs of wells. The path from Well 3 to Well 1 represents the portion of the plume with the highest concentrations of 1,4-dioxane (Panel A of **Figure 5.7.4.2**). At Well 3, the concentration of 1,4-dioxane was 10 mg/L, which would have a theoretical oxygen demand of 18 mg/L. Cometabolism would require 3.6 mg/L of oxygen. Only 0.6 mg/L of oxygen was available in Well 3 and 3.5 mg/L was available in Well 1. Aerobic biodegradation or cometabolism would have required resupply of oxygen to the groundwater. Assuming that a resupply of oxygen was available, the calibration using the MNA Rate Constant Estimator indicated that a rate constant of 0.35 per year would be necessary to explain the distribution of 1,4-dioxane along the flow path (Panel B in **Figure 5.7.4.2**).

The path from Well 4 to Well 2 represented conditions in the more dilute portions of plume. The theoretical oxygen demand of the concentration of 1,4-dioxane consumed between Well 4 and Well 2 was 0.27 mg/L. The oxygen required for cometabolism was 0.05 mg/L. The concentration of oxygen in Well 4 and Well 2 was 0.59 mg/L and 1.22 mg/L respectively. There was enough oxygen in the path from Well 4 to Well 2 to support aerobic biodegradation or cometabolism of 1,4-dioxane without any need for resupply of oxygen to the groundwater. The calibration using

the MNA Rate Constant Estimator indicated that a rate constant of 0.35 per year would be necessary to explain the distribution of 1,4-dioxane along the flow path (Panal C in **Figure 5.7.4.2**).

The rate constants from the two calibrations were in reasonable agreement. However, both calibrations were based on two wells, and the flow paths between the wells was askew of the general direction of groundwater flow. The rate constants extracted from the MNA Rate Constant Estimator should not be used to inform a site conceptual model until and unless they can be validated using other lines of evidence.

Does the biomarker abundance explain the model-estimated 1,4-dioxane biodegradation rate constant? **No**

Data on the abundance of the biomarkers were collected in 2019 and 2020. The relevant biomarkers for direct metabolism of 1,4-dioxane are DXMO, ALDH, and possibly *prmA*. Biomarkers potentially relevant to cometabolism are *prmA*, RMO, RDEG, PHE, SCAM and SMMO. Their abundance is presented in **Figure 5.7.4.3.** If the abundance was below the reporting limit, the abundance was plotted at the reporting limit. The biomarkers for direct biodegradation of 1,4-dioxane (DXMO and ALDH) were not detected in groundwater at Site 4. The abundance of several of the biomarkers that have been associated with cometabolism of 1,4-dioxane were above 1000 gene copies per mL in Well 4, above 100 gene copies per mL in Well 2 and Well 3, but all the biomarkers were below 10 genes copies per mL in Well 1. The *prmA* gene was not detected in any wells from this site.

The MNA Rate Constant Estimator was used to extract an overall rate constant for biodegradation of 1,4-dioxane from the abundance of the DXMO, *prmA*, RMO and RDEG biomarkers in 2018. The abundance of gene copies for these biomarkers was entered into Box 6b of the MNA Rate Constant Estimator. If a marker was not detected, the reporting limit was entered. The sum of the predicted rate constants for biodegradation was 0.00074 per year. Based on the abundance of biomarkers in 2019, the predicted rate constant was 0.00067 per year. These rate constants are more than one hundred-fold lower than the apparent rate constants for degradation that were extracted from the field data (0.35 and 0.25 per year).

The biomarker abundance does not explain the rate constants estimated from the calibrations of the MNA Rate Constant Estimator.

Is the model-estimated 1,4-dioxane biodegradation rate constant consistent with rate constants estimated using the ^{14}C assay? **Yes***

The ^{14}C assay was performed on samples acquired in 2018, 2019 and 2020. The rate constants and 95% confidence intervals are provided in **Table 5.7.4.1**. If the rate constant is greater than zero at 95% confidence, the cell in **Table 5.7.4.1** is highlighted in yellow. In samples acquired in 2018, biodegradation was detected in Well 2 and Well 4, but the rate constants were low (0.006 and 0.007 per year). Biodegradation was not detected in samples from Well 1 and Well 3. The rate constants produced by the ^{14}C assays were much lower than the apparent rate constants for degradation that were extracted from the field data (0.35 and 0.25 per year).

When the assays were repeated in 2019, statistically significant rate constants were obtained in groundwater from Well 1 and Well 2. The rate constants from the ^{14}C assay were higher, but still an order of magnitude lower than the rates extracted from the field data.

The assays were repeated a third time in 2020. In this case the rate constant for the sample from Well 2 was statistically significant, and of the same order of magnitude as the rate constants extracted from the field data. The rate constants for groundwater from two new wells that were screened across the water table were also of the same order at the field scale rate constants. For the samples acquired in 2020, the rate constant provided by the ^{14}C assays were consistent with the rate constants provided from calibration of the field data to the MNA Rate Constant Estimator.

To determine whether microorganisms in the samples could acclimate to degrade the 1,4-dioxane, the microcosms that were constructed in 2018 were stored from 228 to 243 days in the presence of oxygen, and then analyzed for the concentration of 1,4-dioxane remaining. In all three microcosms constructed with groundwater from Well 1, the concentration of 1,4-dioxane was below the detection limit. This would have required a first order rate constant greater than 2.5 per year. There was no evidence of acclimation in water from the other three wells.

Are ^{2}H and/or ^{13}C enriched along the flow path? **Yes**
^{2}H is enriched in 1,4-dioxane along the flow path (see Panel B of **Figure 5.7.4.3**). Values in Well 2, Well 4 and Well 1 are enriched compared to the values in Well 3 (the source well), indicating that 1,4-dioxane has been degraded along the flow path to these wells.

Panel C of **Figure 5.7.4.3** is a plot that was produced using the CSIA_14D.xlsx calculator in the BioPIC software that was updated as part of this project. The pathway compares the relative changes in values for $\delta^{13}C$ and $\delta^{2}H$ in 1,4-doxane in the wells to changes that were seen during biodegradation of 1,4-dioxane by cultures of several different bacteria. The pattern in the wells at Site 1 most closely matched the pattern for degradation by *Pseudonocardia tetrahydrofuranoxidans* K1 (Bennett et al., 2018). Prior to this ESTCP project, microcosms prepared with soil and groundwater from this site led to isolation of *Pseudonocardia dioxivorans* BERK-1 (Ramos-Garcia et al., 2018). Strain BERK-1 is able to use 1,4-dioxane as a sole source of carbon and energy under aerobic conditions.

Are geochemical conditions supportive of 1,4-dioxane biodegradation? **Yes**
Geochemical data from samples collected from the Site 4 are provided in **Table 5.7.4.2**. In water samples acquired in 2018 for the ^{14}C biodegradation assay, the temperature, pH, conductivity as a proxy for dissolved electrolytes, dissolved oxygen and ORP were all adequate to support biodegradation of 1,4-dioxane. The absence of detectable rates of biodegradation in certain of the ^{14}C assays cannot be attributed to the geochemical conditions. The high concentration of ferrous iron and methane in Well 3, as well as measurable DO and high ORP levels, indicated that the well sampled both oxygenated groundwater and anaerobic groundwater, presumably because it was screened across several sub-layers with differing redox conditions. The anaerobic groundwater would not be supportive of biodegradation of 1,4-dioxane, although it may be possible for abiotic degradation to occur via a Fenton reaction (Sekar and DiChristina, 2014).

Are inhibitory CVOCs present below inhibitory levels and/or decreasing with time/distance? **Yes**

Mahendra et al. (2013) determined half saturation constants for inhibition of aerobic biodegradation of 1,4-dioxane (K_i) by 1,1-DCE and 1,1,1-TCA. Values of K_i for 1,1-DCE were 50 µg/L for cometabolism and 320 µg/L for direct metabolism while values for 1,1,1-TCA were 507 µg/L for cometabolim and 160 µg/L for direct metabolism. The maximum concentration of 1,1-DCE at Site 4 was 4.2 µg/L and the concentration of 1,1,1-TCA was less than 0.1 µg/L. Inhibitory CVOCs were not present at concentrations that would be expected to impair biodegradation or cometabolism of 1,4-dioxane.

Table 5.7.4.1. Rate constants for 1,4-dioxane degradation provided by ^{14}C assay at Site 4.

Sampled	Well	First order rate constant (per year)	95% Confidence Interval (per year)
2018	Well 1 (GW only)	0.0045**	0.0052***
2018	Well 1 (GW only) after acclimation	2.5	--
2018	Well 2 (GW only)	0.0061	0.0051
2018	Well 3 (GW only)	0.0014**	0.003***
2018	Well 4 (GW only)	0.0073	0.0028
2019	Well 1 (GW only)	0.016	0.011
2019	Well 2 (GW only)	0.014	0.012
2019	Well 3 (GW only)	0.002**	0.009***
2019	Well 4 (GW only)	-0.005*	0.009***
+2020	Well 2 (GW only)	0.30	0.058
+2020	North of Well 2 (GW + soil)	0.12	0.053
+2020	South of Well 4 (GW + soil)	0.37	0.048

Yellow highlighted cells represent rate constants for microcosms that were significantly different than filter-sterilized groundwater control.
* Rate constant for microcosms was less than the rate constant for the filter-sterilized water control.
** Net rate constant not greater than zero at 95% confidence.
*** 95% confidence interval on rate for microcosm.

Table 5.7.4.2. Geochemistry of water samples from Site 4.

Well #	Sample Date	Temp.	pH	Conduc-tivity	Dissolved Oxygen	ORP	Ferrous Iron	Methane
		˚C		µS/cm	mg/L	mV	mg/L	mg/L
1	11/12/2018	18.5	8.25	391	3.48	215	2.6	0.02
2	11/13/2018	18.5	7.32	658	1.22	248	<0.05	<0.01
3	11/14/2018	16.9	6.88	875	0.56	67	6.1	2.1
4	11/15/2018	15.0	7.22	491	0.59	186	0.79	0.18

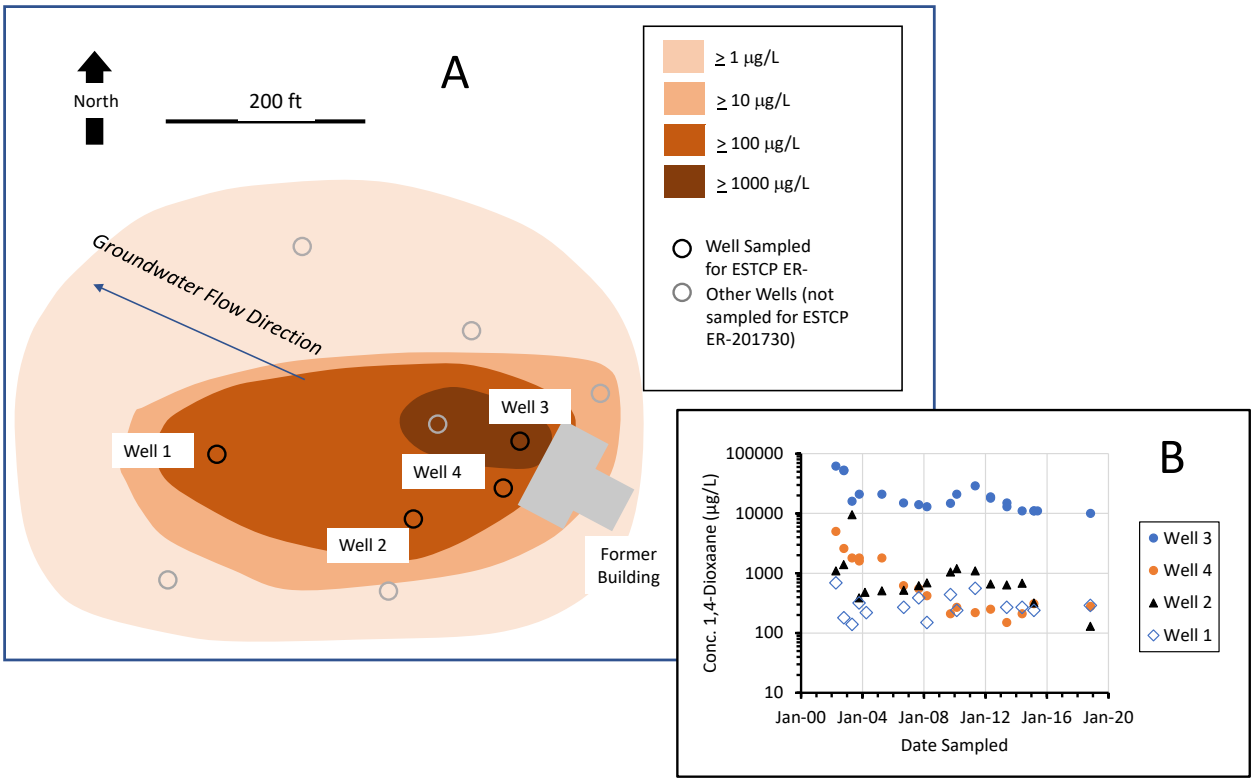
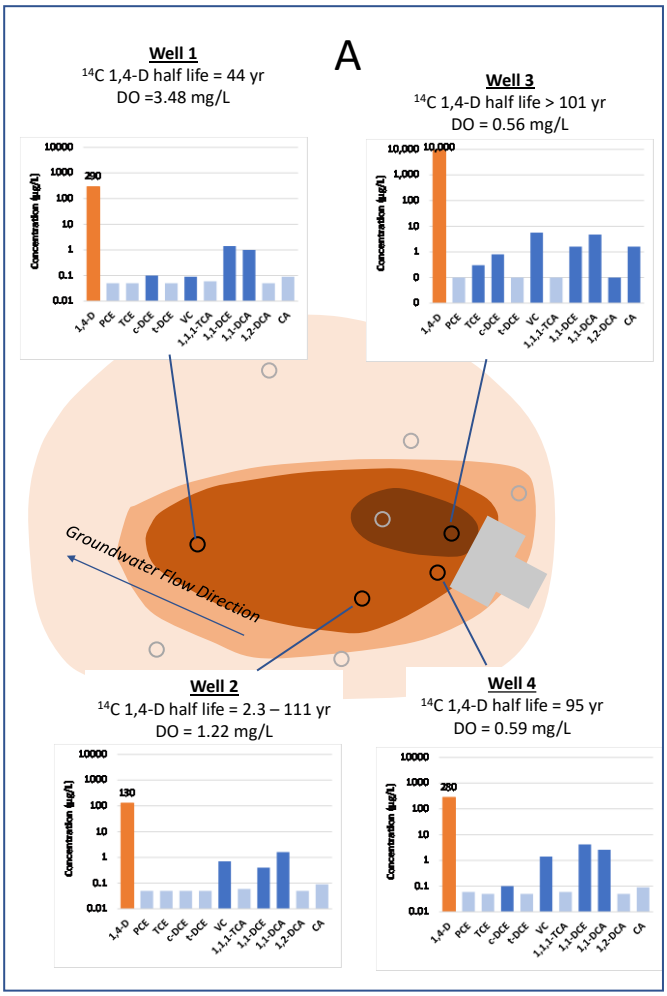
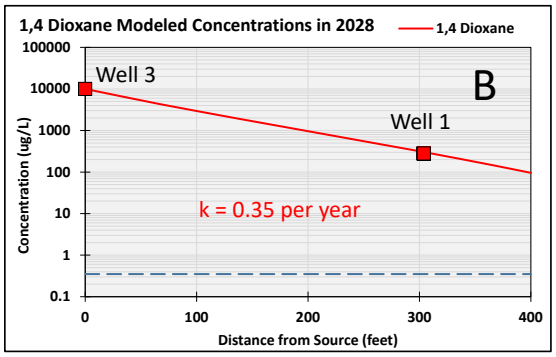
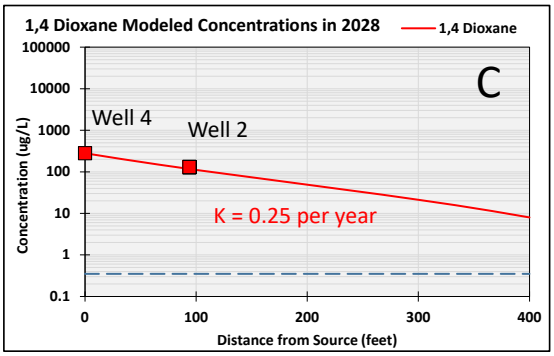


Figure 5.7.4.1. Site 4 (Industrial) Distribution of Concentrations of 1,4-Dioxane







Groundwater Seepage Velocity 39 feet per year, Coefficient of longitudinal dispersion 26 feet, Retardation factor 1.2.

Figure 5.7.4.2. Site 4 (Industrial) Summary of 1,4-Dioxane/CVOC Concentrations and Attenuation of 1,4-Dioxane with Distance from the Source.
Pale blue values in Panel A are plotted at the reporting limit.

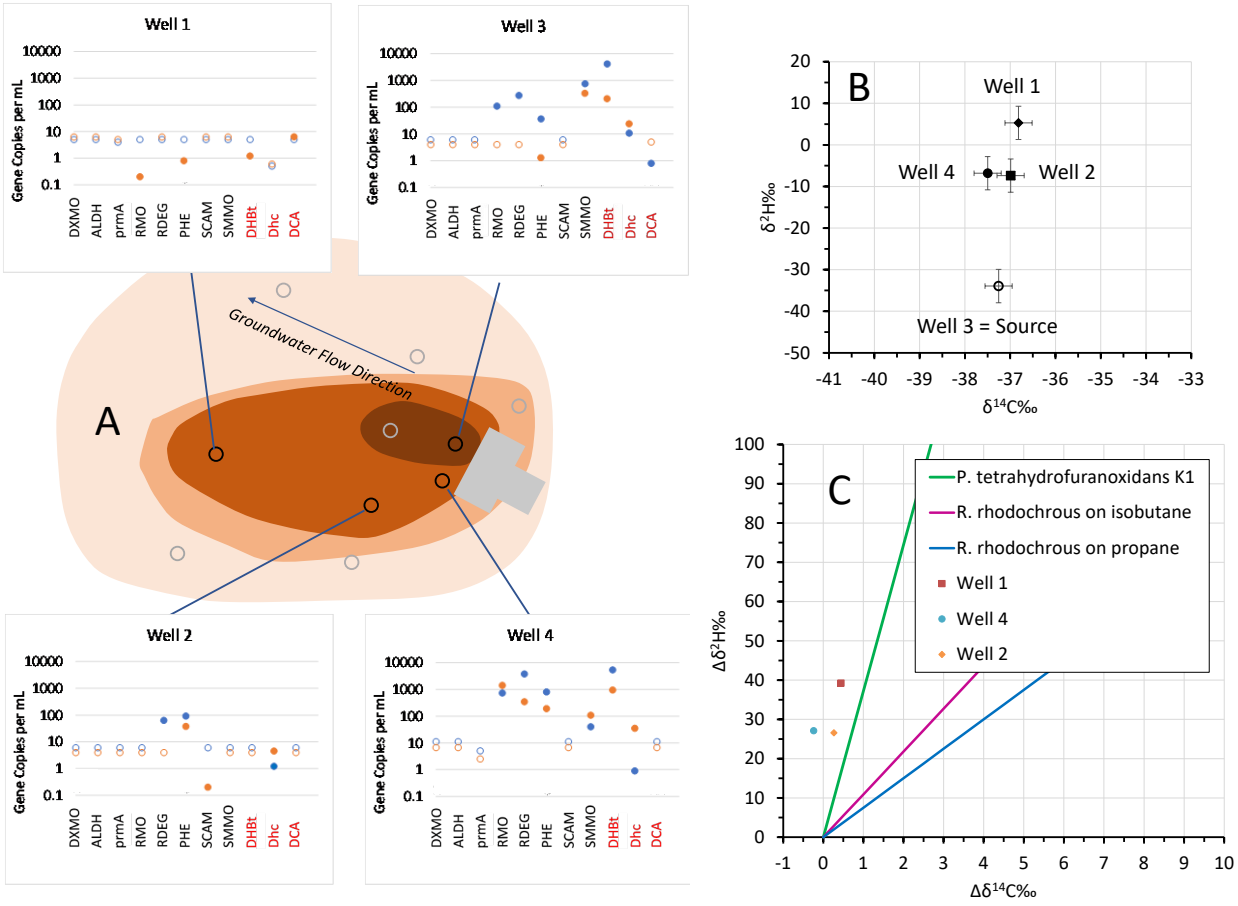


Figure 5.7.4.3. Site 4 (DoD) Summary of Biomarker Data for 1,4-Dioxane and CVOCs.
Open circles in abundance of qPCR biomarkers indicate the limit of quantitation. Blue symbols are data collected in 2018, orange symbols are data collected in 2019.

5.7.5. Site 5 (DoD)

5.7.5.1 – 1,4-Dioxane

Overview: Groundwater contamination originates from sludge beds and waste sludge basins associated with a former Industrial Waste Treatment Plant (IWTP) constructed in 1972 and a former Oily Waste Treatment Plant constructed in 1977. The former IWTP processed rinse waters from plating, stripping, and other industrial processes. The rinse waters were contaminated with organic and inorganic materials.

Is 1,4-dioxane below regulatory standards? **No***
When sampled in 2019, the concentration of 1,4-dioxane in the most contaminated well at Site 5 was 11,000 µg/L. There are currently no applicable regulatory standard for 1,4-dioxane in groundwater at this site. However, 1,4-dioxane is being monitored as a COC.

Is the plume stable (i.e., is the 1st line of evidence for MNA met)? **Unknown**
The distribution of the five wells that were sampled for 1,4-dioxane as part of this project is presented in **Figure 5.7.5.1**. Well 1, Well 2, Well 3 and Well 4 were sampled in 2018. Well 4 and Well 5 were sampled in 2019. The concentrations of 1,4-dioxane in the five wells is presented in Panel B of **Figure 5.7.5.1**.

Over an eight-to-nine-year interval, the concentrations of 1,4-dioxane were stable in Well 1, Well 3, Well 4 and Well 5. Data prior to 2018 were obtained by the site owner using an analytical protocol with a method detection limit for 1,4-dioxane that varied from 40 µg/L to 80 µg/L; and all analyses for 1,4-dioxane in Well 2 were below the MDL. To avoid clutter in Panel B, these data are not plotted. The MDL for the sample collected as part of this project was 1 µg/L, and 1,4-dioxane was not detected in water from Well 2 at the lower MDL.

The distance from Well 5 to the down gradient wells Well 3 and Well 4 is approximately 1100 feet. The upper end of the estimated seepage velocity of groundwater in the plume is 33 feet per year. Travel from Well 5 to Well 4 or Well 3 would take approximately 33 years. Although the concentrations of 1,4-dioxane are stable over time, the plume has not been monitored for a sufficient time to determine if the plume is stable in its distribution along the flow path.

Is 1,4-dioxane being degraded based on model predictions? **Yes**
The distribution of 1,4-dioxane and chlorinated hydrocarbons at the site are shown in Panel A of **Figure 5.7.5.2**. Well A is the most contaminated well immediately downgradient of the source of contamination. It was not sampled as part of this project. The data for Well A were acquired by the site owner, and the data for Well 5 in 2017 were acquired by the site owner.

Data on concentrations of 1,4-dioxane from a flow path extending from Well A to Well 5, then to Well 3 and Well 4, and then to Well 2 were used in the MNA Rate Constant Estimator to estimate a rate constant for 1,4-dioxane degradation (Panel B). The simulation in Panel B represents the expected distribution along the flow path if there was no appreciable degradation. Based on the simulation, the plume of contaminated groundwater should have reached Well 3 and Well 4, but the plume is still advancing along the flow path and concentrations have not come to a steady state.

Simulations were made using the MNA Rate Constant Estimator using different values for the rate constant for degradation until the simulation matched the concentration in the Well 3 and Well 4. The rate constant necessary to fit the data is 0.06 per year (Panel C).

When a plume comes to steady state along a flow path, adsorption comes to equilibrium and has no effect on the distribution of concentrations. As will be discussed later, to successfully simulate the distribution of chlorinated alkenes and alkanes along the flow path, it was necessary to assume that there was no adsorption of the chlorinated alkenes and alkanes to aquifer sediment. Under these circumstances there would be no adsorption of 1,4-dioxane as well.

It is possible that the plume at Site 5 has not come to a steady state, and it is also possible that adsorption does contribute to the attenuation of concentrations of 1,4-dioxane along the flow path. The rate constant is an upper boundary on the contribution of degradation. If sorption contributes to attenuation along the flow path, the rate of degradation would be lower.

Does the biomarker abundance explain the model-estimated 1,4-dioxane biodegradation rate constant? **No**

The relevant biomarkers for direct metabolism of 1,4-dioxane are DXMO, ALDH, and possibly *prmA*. Biomarkers that are potentially relevant to cometabolism are *prmA*, RMO, RDEG, PHE, SCAM and SMMO. Their abundance is presented in Panel A of **Figure 5.7.5.3**. If the abundance was below the reporting limit, the abundance was plotted at the reporting limit. Neither DXMO nor ALDH was detected in any of the four wells at Site 5. Biomarker RMO was detected in every well. Biomarkers RDEG and PHE were detected in every well but Well 1. The abundance of these biomarkers was higher in Well 3 and Well 4, which are along the plume centerline. The biomarker SCAM as only detected in Well 2, at low abundance. The biomarker SMMO was only detected in Well 4, at low abundance. The *prmA* gene was not detected in any of the well samples.

The MNA Rate Constant Estimator can estimate a rate constant for biodegradation from the abundance of the DXMO, *prmA*, RMO and RDEG biomarkers. The abundance of gene copies for these biomarkers was entered into Box 6b of the MNA Rate Constant Estimator. Data on biomarker abundance was not available for Well 5 and Well A. To provide an upper plausible boundary on the rate of degradation associated with biomarkers, the maximum abundance of biomarkers in Well 3 or Well 4 was used to estimate the abundance in Well A and Well 5. If a marker was not detected, the reporting limit was entered. All markers were determined at an abundance of 20 gene copies per mL or less. The sum of the predicted rate constants for biodegradation of 1,4-dioxane was 0.0013 per year. This is fifty-fold lower than the apparent rate constant for degradation of 0.06 per year that was extracted as the best fit to the field data.

The abundance of the biomarkers cannot explain the apparent rate constant for degradation of 1,4-dioxane at Site 5.

Is the model-estimated 1,4-dioxane biodegradation rate constant consistent with rate constants estimated using the ^{14}C assay? **No**

In microcosms constructed with groundwater from Well 1 and Well 2, the production of ^{14}C-label in potential degradation products in the living microcosms was greater than the production in filter-

sterilized controls at 95% confidence (**Table 5.7.5.1**). The rate constants were 0.0037 and 0.0042 per year. These values are between ten-fold and one-hundred-fold lower than the rate constant derived from the model (0.06 per year). In microcosms from Well 3 and Well 4, degradation was not detected. The rate constants were all <0.0124 per year at 95% confidence. The rate constants estimated from the biodegradation assay are not consistent with the rate constants extracted from the model. The assay cannot explain the apparent rate constant for degradation of 1,4-dioxane at Site 5.

Are ^{2}H and/or ^{13}C enriched along the flow path? **Yes**
^{2}H is enriched in 1,4-dioxane along the flow path (see Panel B of **Figure 5.7.5.3**). Values in Well 1 and Well 3 are enriched compared to the upgradient well (Well 5) indicating that 1,4-dioxane has been degraded along the flow path from Well 5 to Well 1 and Well 3. Panel C of **Figure 5.7.5.3** is a plot that was produced using the CSIA_14D.xlsx calculator in the BioPIC software that was updated as part of this project. The pathway compares the relative changes in values for $\delta^{13}C$ and $\delta^{2}H$ in 1,4-doxane in the wells to changes that were seen during biodegradation of 1,4-dioxane by cultures of several different bacteria. The pattern in the wells at Site 5 are not a close match to any of the cultures, but the pattern more closely matches the pattern for degradation by *Pseudonocardia tetrahydrofuranoxidans* K1 (Bennett et al., 2018).

Are geochemical conditions supportive of 1,4-dioxane biodegradation? **Yes**
The geochemistry of the water samples from the wells at Site 5 is provided in **Table 5.7.5.2**. In water samples acquired for the ^{14}C biodegradation assay, the temperature, pH, conductivity as a proxy for dissolved electrolytes, and ORP were all adequate to support biodegradation of 1,4-dioxane. In water where degradation of 1,4-dioxane was detected by the ^{14}C assay (Well 1 and Well 2), the concentration of dissolved oxygen was 1 mg/L or greater. The concentration of dissolved oxygen was 1 mg/L in Well 3 as well. The absence of detectable rates of biodegradation in water from Well 3 cannot be attributed to the geochemical conditions. The concentration of dissolved oxygen in Well 4 was near 0.1 mg/L and may have been limiting for biodegradation of 1,4-dioxane.

The concentrations of methane and ferrous iron were low, indicating that all the flow paths to the well provided aerobic groundwater.

Are inhibitory CVOCs present below inhibitory levels and/or decreasing with time/distance? **Yes**
Mahendra et al. (2013) determined half saturation constants for inhibition of aerobic biodegradation of 1,4-dioxane (K_i) by 1,1-DCE. Values of K_i for 1,1-DCE were 50 µg/L for cometabolism and 320 µg/L for direct metabolism. Although the DXMO marker was not detected in samples, the RMO marker was present (**Figure 5.7.5.3**). The concentration of 1,1-DCE was above 1000 µg/L in Well A and Well 5, and above 100 µg/L in Well 4 (**Figure 5.7.5.2**). This concentration would have inactivated toluene monooxygenase and prevented cometabolism of 1,4-dioxane in most portions of the plume.

5.7.5.2 – PCE, TCE, cis-DCE, and Vinyl Chloride

Are PCE, TCE, cis-DCE and Vinyl Chloride being degraded based on model predictions? **Yes***

The distribution of PCE, TCE, *cis*-DCE and vinyl chloride at the site is shown in Panel A of **Figure 5.7.5.4**. Data from a flow path from Well A to Well 5, then Well 3 and Well 4, and then Well 2 was used in the MNA Rate Constant Estimator to estimate a rate constant for degradation of PCE, then TCE, then *cis*-DCE and then vinyl chloride. In the MNA Rate Constant Estimator, the default value for fraction organic carbon in the aquifer sediments is 0.0018, resulting in a retardation coefficient of 2.2. With this extent of retardation due to adsorption to soil organic matter, even with a trivial value for degradation, TCE would not reach Well 3 or Well 4 at the highest estimate of groundwater seepage velocity in the time since the Industrial Waste Treatment Plant was installed (Panel B). If there is no adsorption and the retardation coefficient is 1.0, and there is no degradation, then reasonable concentrations of TCE would reach Well 3 and Well 4 (Panel C). To incorporate these observations into the model, simulations using the MNA Rate Constant Estimator were run where sorption was omitted (i.e., with a retardation coefficient of 1.0). The values for the TCE biodegradation constant were selected to minimize the RMSE between the simulation and the field data. The fitted value was 0.001 per year. This value represents an upper value on the rate constant that can distinguished from the contribution of longitudinal dispersion. This value and lower values produced the minimum RMSE. Higher values had increased RMSE.

Although the fitted rate constant for biodegradation of TCE was low and had no appreciable effect on the distribution of TCE along the flow path, this was not true for PCE, *cis*-DCE or vinyl chloride. The fitted rate constants necessary to match the concentration of PCE down gradient in Well 4 was 0.04 per year (Panel E). The rate constant for *cis*-DCE was 0.05 per year (Panel F), and the fitted rate constant for vinyl chloride was 3 per year (Panel G). Based on model predictions, TCE is not being degraded, but PCE, *cis*-DCE and vinyl chloride are being degraded.

Does the biomarker abundance explain the model-estimated biodegradation rate constants? **Yes (cis-DCE) and No (Vinyl Chloride)**
The DHC biomarker targets a structural gene in *Dehalococcoides* bacteria. Many *Dehalococcoides* bacteria can degrade *cis*-DCE to vinyl chloride, and then vinyl chloride to ethene.

The MNA Rate Constant Estimator can estimate rate constants for biodegradation of *cis*-DCE from the abundance of the *vcrA* biomarker. We assumed that the abundance of *vcrA* in water from Site 5 was the same as the abundance of the DHC biomarker. The abundance of gene copies for DHC was entered into Box 6b of the MNA Rate Constant Estimator. Data on biomarker abundance was not available for Well 5 and Well A. To provide an upper plausible boundary on the rate of degradation associated with biomarkers, the maximum abundance of biomarkers in Well 3 or Well 4 was used to estimate the abundance in Well A and Well 5. If DHC was not detected, the reporting limit was entered. Based on the assumptions for the abundance of DHC in Well A and Well 5, the upper boundary on the rate constant for *cis*-DCE estimated from the abundance of the DHC marker was 0.01 per year. This is within an order of magnitude of the rate constant of 0.050 per year estimated using the model (Panel F of **Figure 5.7.5.4**)

The rate constant for vinyl chloride estimated from the abundance of the DHC marker was 0.03 per year. This slow rate constant cannot explain the rate constant of 3 per year estimated from the model (Panel G of **Figure 5.7.5.4**).

The biomarker abundance is a plausible explanation for the model-estimated biodegradation rate constants for *cis*-DCE, but not for vinyl chloride.

Are geochemical conditions supportive of biodegradation of PCE, TCE, cis-DCE and Vinyl Chloride? **Yes (only for aerobic cis-DCE and Vinyl Chloride biodegradation)**
The geochemistry of the water samples from the wells at Site 1 is provided in **Table 5.7.5.2**. The concentrations of dissolved oxygen were high enough to prevent the growth of *Dehalococcoides* bacteria in Well 1, Well 2 and Well 3. The geochemical conditions are not supportive of anaerobic biodegradation of PCE, TCE, *cis*-DCE and vinyl chloride. However, they are supportive of aerobic biodegradation of *cis*-DCE and vinyl chloride, and aerobic biodegradation probably accounted for the rate constants necessary to make the model fit the field data. In contrast, TCE is not expected to degrade aerobically, which accounts for the low upper boundary on the rate constant for TCE that was extracted using the model. The geochemistry is not supportive of biodegradation of PCE, even though the model indicated that PCE was biodegraded.

5.7.5.3 - 1,1,1-TCA, 1,1-DCA and 1,1-DCE:

Are 1,1,1-TCA, 1,1-DCA and 1,1-DCE below regulatory standards? **No (1,1-DCA and 1,1-DCE)**
The concentration of 1,1,1-TCA in the wells at Site 5 that were sampled for this project were all < 0.2 µg/L in 2018. This is below the regulatory standard of 200 µg/L for the site. However, the maximum concentration of 1,1-DCE was 270 µg/L, and the maximum concentration of 1,1-DCA was 7.1 µg/L. The concentration of 1,1-DCE in Well 5 in 2017 was 5700 µg/L and the concentration of 1,1-DCA was 1500 µg/L. Theses concentrations are above the regulatory standards of 7 µg/L and 5 µg/L, respectively.

Are 1,1-DCA and 1,1-DCE being degraded based on model predictions? **Yes**
1,1,1-TCA has completely degraded based on its absence in samples collected during this project. The distribution of the by-products of the degradation reaction, 1,1-DCE and 1,1-DCA are shown in Panel A of **Figure 5.7.5.5**. Data from a flow path from Well A to Well 5, then Well 3 and Well 4, and then Well 2 was used in the MNA Rate Constant Estimator to estimate a rate constant for degradation of 1,1-DCA. Panel B of **Figure 5.7.5.5** presents the simulation with the default concentration of native organic material. The expected retardation due to sorption would have prevented the plume of 1,1-DCA from reaching Well 3 or Well 4. Further simulations were done with the coefficient of retardation set to 1.0, as was done for TCE. In this situation, the rate constant for degradation of 1,1-DCA that produced the best match to the concentration in downgradient Well 4 is 1.0 per year (Panel C). Assuming no retardation due to sorption, the rate constant for degradation of 1,1-DCE was 0.03 per year.

Does the biomarker abundance explain the model-estimated biodegradation rate constants? **No**
The DHBt biomarker targets *Dehalobacter* species that are capable of reductive dechlorination of 1,1-DCA and 1,1-DCE to chloroethane. The DCA biomarker targets the 1,1-DCA reductive dehalogenase gene found in some strains of *Dehalobacter*.

The abundance of DHBt, DCA, and DHC biomarkers in well water from Site 1 is presented in Panel A of **Figure 5.7.5.3**. The MNA Rate Constant Estimator can estimate a rate constant for

biodegradation of 1,1-DCA and 1,1-DCE from the abundance of the DHBt biomarker. The abundance of gene copies for these biomarkers was entered into Box 6b of the MNA Rate Constant Estimator. Data on biomarker abundance was not available for Well 5 and Well A. To provide an upper plausible boundary on the rate of degradation associated with biomarkers, the maximum abundance of biomarkers in Well 3 or Well 4 was used to estimate the abundance in Well A and Well 5. If a marker was not detected, the reporting limit was entered. All markers were determined at an abundance of 20 gene copies per mL or less.

The rate constant for 1,1-DCA degradation estimated from the abundance of the DHBt biomarker was 0.000003 per year. The rate constant that best fit the field data was 0.1 per year. The rate constant predicted from the DHBt biomarker data was five orders of magnitude lower than the rate constant predicted by the model.

The DHC biomarker targets a structural gene in *Dehalococcoides* bacteria. In plumes with chlorinated alkenes, *Dehalococcoides* bacteria often have an enzyme, vinyl chloride reductase, that can reductively dechlorinate 1,1-DCE. The biomarker *vcrA* targets vinyl chloride reductase. The MNA Rate Constant Estimator can estimate a rate constant for biodegradation of 1,1-DCE from the abundance of the *vcrA* biomarker. We assumed that the abundance of *vcrA* in water from Site 5 was the same as the abundance of the DHC biomarker. The abundance of gene copies for DHC was entered into Box 6b of the MNA Rate Constant Estimator. If DHC was not detected, the reporting limit was entered. The rate constant estimated from the abundance of the *vcrA* marker was 0.0004 per year. This is much less than the rate constant of 1.0 per year that was estimated with the model from the field data.

The abundance of the DHBt did not predict the apparent rate constant for degradation of 1,1-DCA at Site 5. The abundance of Dhc did not predict the apparent rate constant for biodegradation of 1,1-DCE.

Are geochemical conditions supportive of biodegradation of 1,1-DCA or 1,1-DCE? **Yes**
The concentrations of oxygen in Well 1, Well 2 and Well 3 would support aerobic biodegradation of 1,1-DCA (**Table 5.7.5.2**). Aerobic biodegradation of 1,1-DCA would explain the rate constants for 1,1-DCA extracted from the field data using the MNA Rate Constant Estimator.

Table 5.7.5.1. Rate constants for 1,4-dioxane degradation provided by ^{14}C assay for Site 5.

Sampled	Well	First order rate constant (per year)	95% Confidence Interval (per year)
2018	Well 1	0.0037	0.0018
2018	Well 2	0.0042	0.0032
2018	Well 3	<0.0044	0.0023*
2018	Well 4	<0.0065	0.0021*
2019	Well 4	<0.0124	0.0026*

* 95% confidence interval on rate for microcosm.
Yellow highlighted cells represent rate constants for microcosms that were significantly different than filter-sterilized groundwater control.

Table 5.7.5.2. Geochemistry of water samples from Site 5.

Well #	Sample Date	Temp.	pH	Conduc-tivity	Dissolved Oxygen	ORP	Ferrous Iron	Methane
		˚C		µS/cm	mg/L	mV	mg/L	mg/L
1	12/11/2018	21.2	7.59	3250	1.08	191	1.783	<0.01
2	12/12/2018	22.6	7.67	807	4.7	219	0.077	<0.01
3	12/13/2018	22.7	7.36	4260	1.09	198	0.341	<0.01
4	12/13/2018	21.7	7.42	5770	0.168	212	<0.05	<0.01
4	12/15/2019	22.5	7.87	5960	0.11	165	0.13	0.023
5	12/14/2019	24.3	7.75	2600	0.13	119	0.176	-
A	3Q 2011	23.4	**6.55**	**4064**	**0.03**	-	-	-
A	3Q 2013	23.5	**6.82**	**3703**	**0.4**	-	-	-

Value in **bold** from sampling conducted by the site owner.

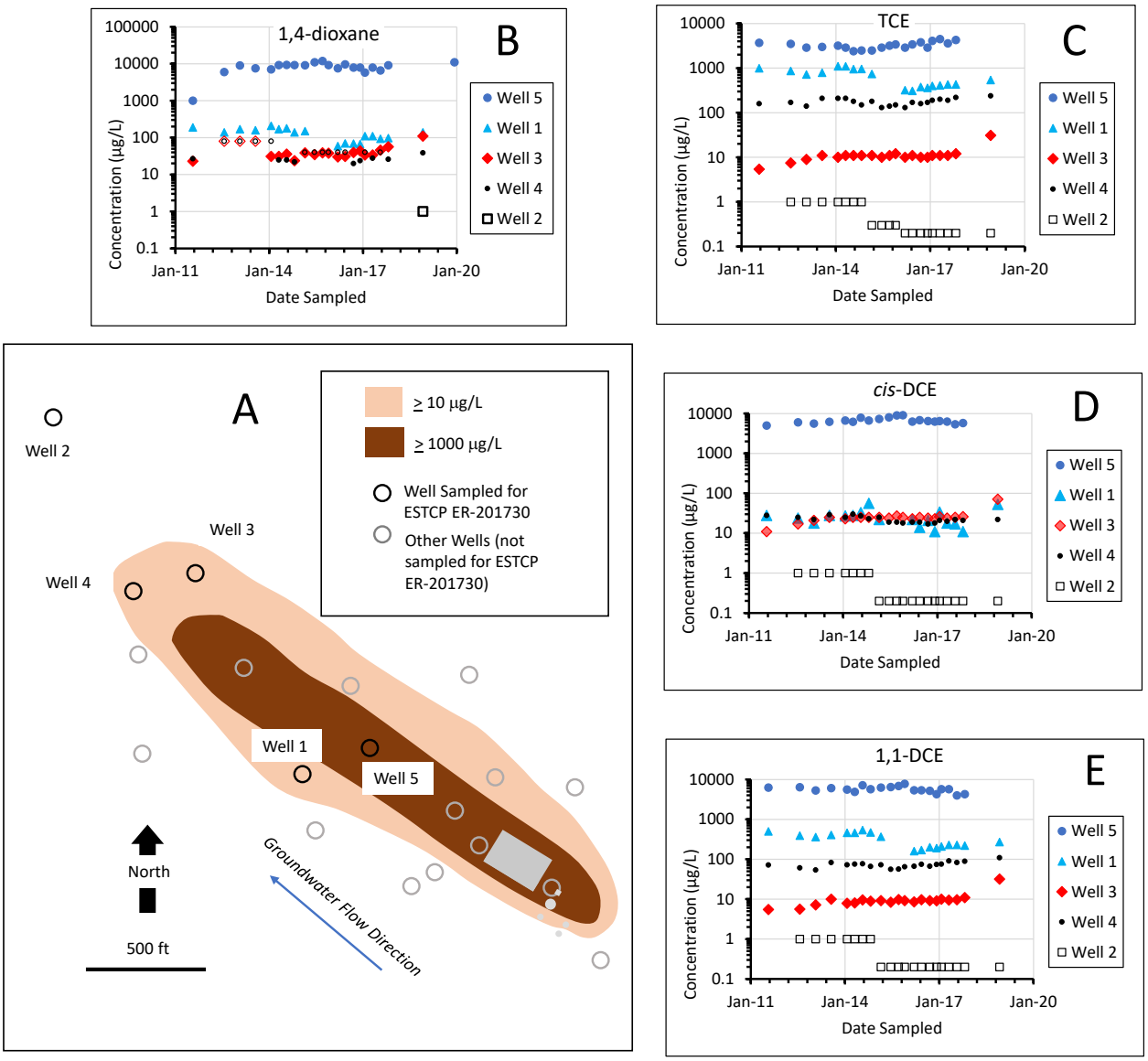
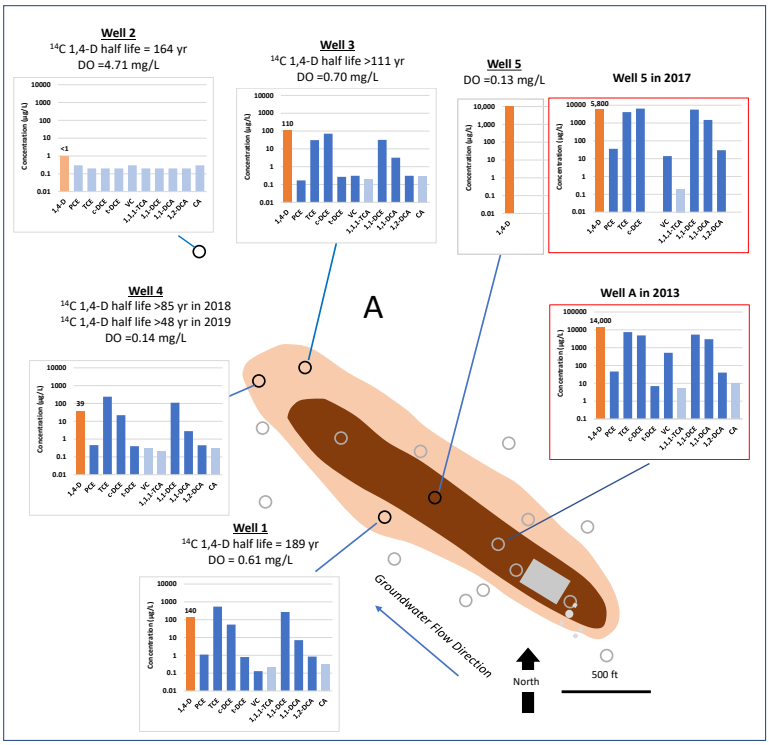
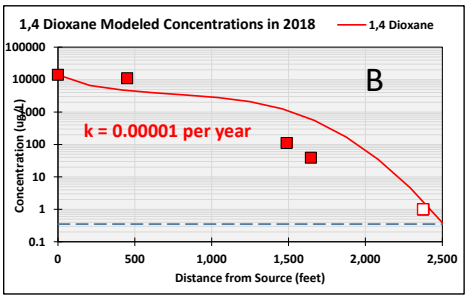
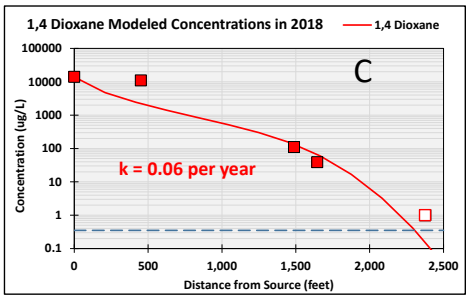


Figure 5.7.5.1. Site 1 (DoD) Changes in Concentrations of 1,4-Dioxane and CVOCs over time.

Open symbols in Panels B through E are plotted at the reporting limit.







Groundwater Seepage Velocity 33 feet per year, Coefficient of longitudinal dispersion 33 feet, Retardation factor 1.0.

Figure 5.7.5.2. Site 5 (DoD) Summary of 1,4-Dioxane/CVOC Concentrations and Attenuation of 1,4-Dioxane with Distance from the Source.

Pale values in Panel A and open symbols in other panels are plotted at the reporting limit.

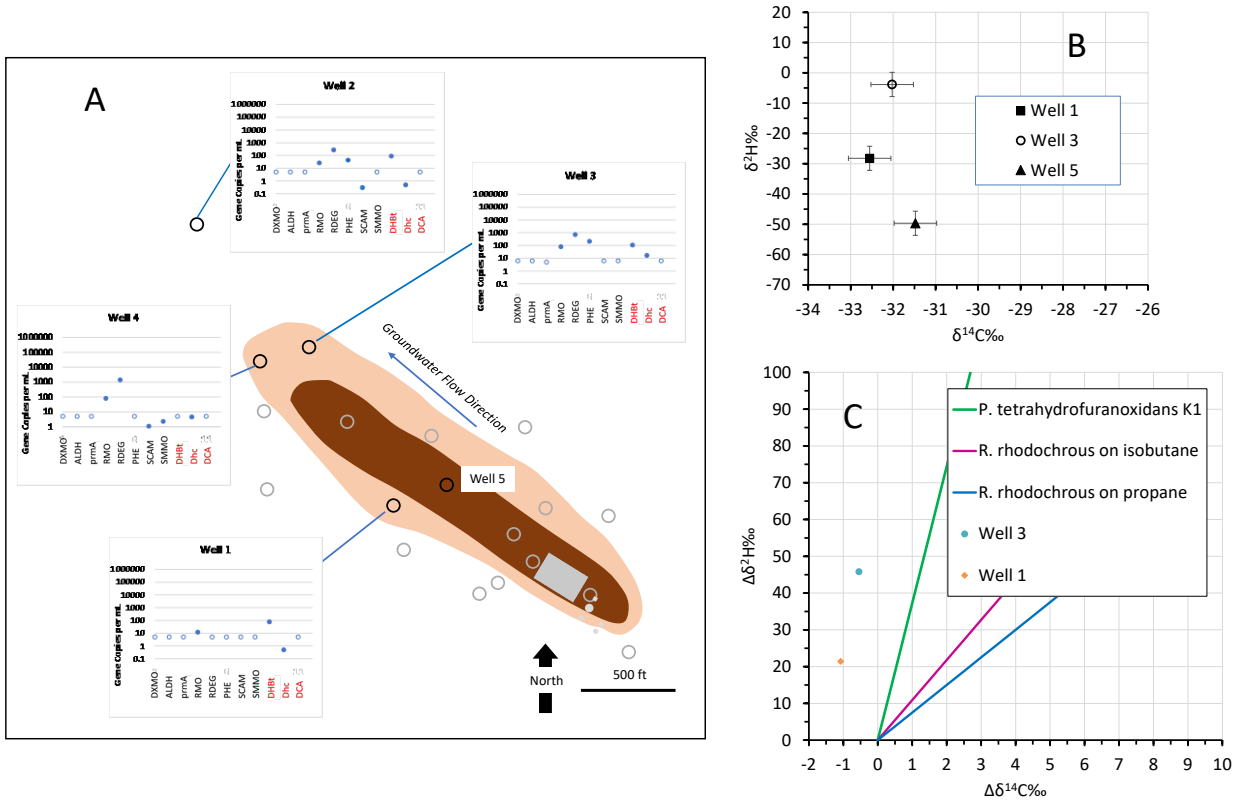


Figure 5.7.5.3. Site 5 (DoD) Summary of Biomarker Data and Isotope Data for 1,4-Dioxane. Open circles in abundance of qPCR biomarkers in Panel A indicate the limit of quantitation. Error bars in Panel B are one standard deviation.

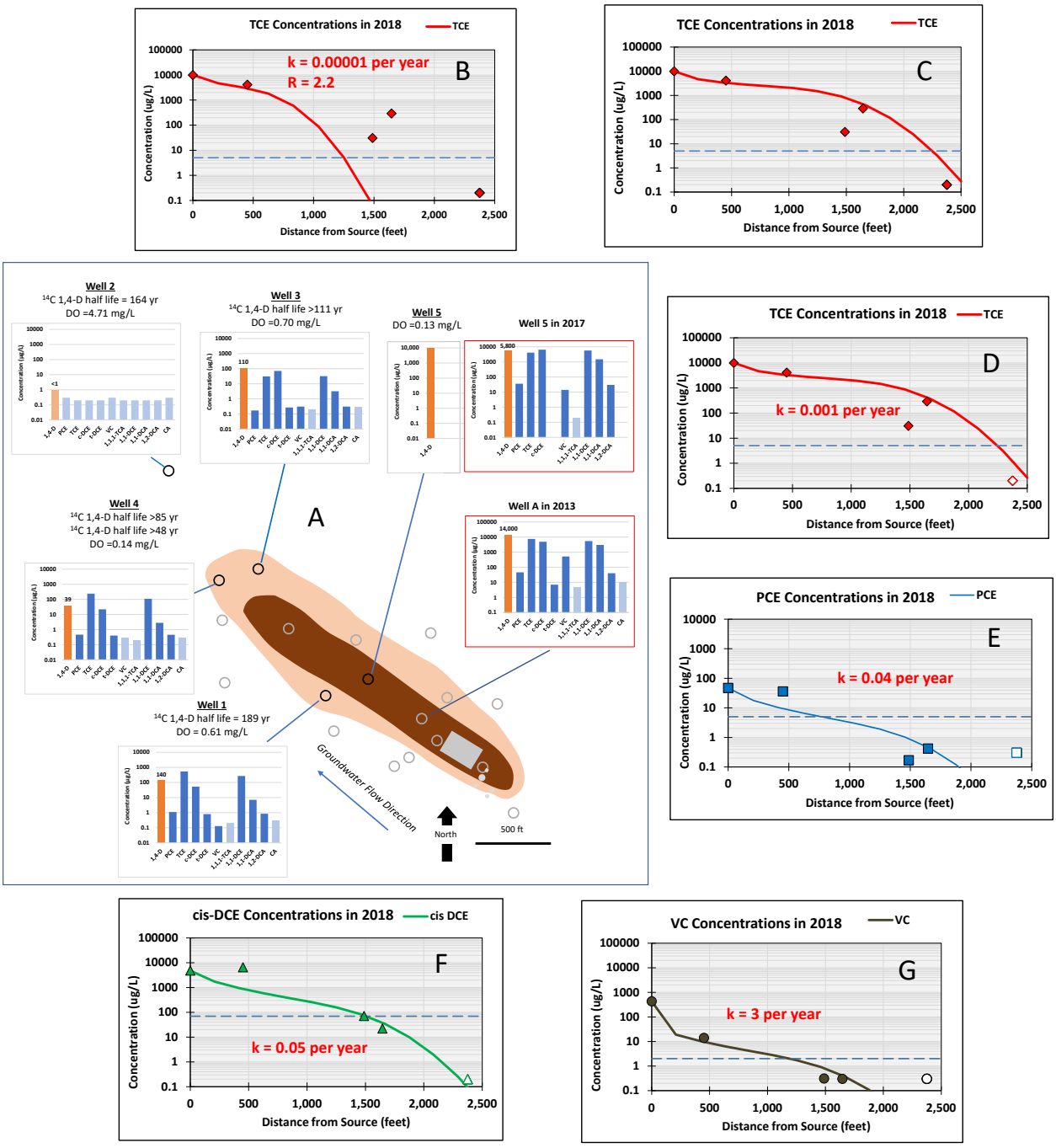


Figure 5.7.5.4. Site 5 (DoD) Summary of 1,4-Dioxane/CVOC Concentrations and Attenuation of PCE, TCE, *cis*-DCE and Vinyl Chloride with Distance from the Source.
Pale values in Panel A and open symbols in other panels are plotted at the reporting limit.

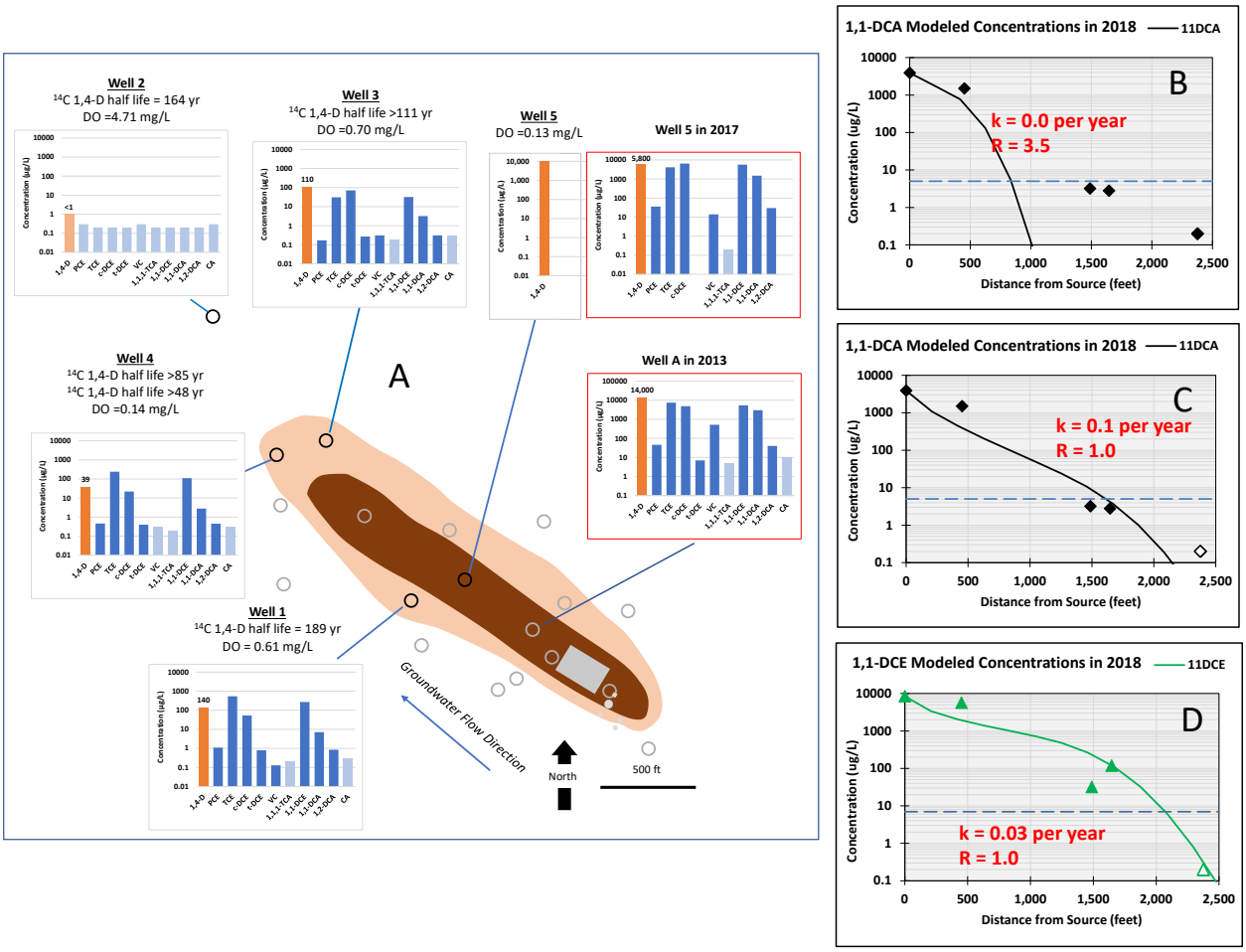


Figure 5.7.5.5. Site 5 (DoD) Summary of 1,4-Dioxane/CVOC Concentrations and Attenuation of PCE, TCE, *cis*-DCE and Vinyl Chloride with Distance from the Source.
Pale values in Panel A and open symbols in other panels are plotted at the reporting limit.

5.7.6. Site 6 (DoD)

Overview: As noted in Section 4, this site is part of an industrial operations area that included multiple inactive and active waste disposal units. Based on the project sampling data, 1,4-dioxane concentrations at this site were relatively low (max concentration of 16 µg/L at Well 4) and decreased modestly with distance (**Figure 5.7.6.1**). The 1,4-dioxane concentration at the farthest downgradient well location (Well 1; 4.5 µg/L) was actually slightly higher than was observed at the two wells located within the middle of the plume. The 1,4-dioxane concentrations measured during this project were substantially lower than those reported in previous monitoring events; for example, the 1,4-dioxane concentration in Well 4 (near the presumed source) was 160 µg/L in 2016. These results suggest that the 1,4-dioxane source is being depleted and/or attenuated. Positive evidence for 1,4-dioxane natural attenuation capacity was established using the ^{14}C assay at only one of the four wells (Well 3; equivalent half life of 262 year). This mid-plume well had relatively low 1,4-dioxane concentration (3.2 µg/L) but had the highest levels of possible biomarkers for 1,4-dioxane degradation. It also had the highest DO concentration (0.87 mg/L), though DO levels were generally low throughout the site, potentially limiting the development of 1,4-dioxane degradation capacity. Biomarkers for possible cometabolism of 1,4-dioxane were detected at all wells (along with DHC as a biomarker of chlorinated ethene degradation), though all were present at relatively modest levels. Possible evidence for 1,4-dioxane attenuation based on hydrogen isotope fractionation was observed in Well 3 (the same well where degradation was confirmed using the ^{14}C assay) due to its high δ^2H value relative to the source well. However, no clear fractionation pattern was observed in downgradient wells, suggesting that 1,4-dioxane biodegradation was likely localized and occurring at relatively slow rates.

Is 1,4-dioxane below regulatory standards? **Not assessed**
The maximum 1,4-dioxane concentration during this event was 16 µg/L and had decreased to 4.5 µg/L at the farther downgradient location; however, compliance points and/or concentration thresholds have not been established at this site to our knowledge.

Is the plume stable (i.e., is the 1st line of evidence for MNA met)? **Not assessed**
Based on a comparison to the available long-term monitoring data, the project data suggest that the 1,4-dioxane plume footprint has not expanded. As noted above, 1,4-dioxane concentrations during this event were significantly lower than those reported previously (including an order of magnitude lower near the source), suggesting 1,4-dioxane mass is being depleted. However, a projection of future plume trends was not attempted due to uncertainty and/or limited information on critical input parameters (e.g., source decay rates, biodegradation rate constants).

Is 1,4-dioxane being degraded based on model predictions? **Yes***
Calibrating the model (the MNA Rate Constant Estimator) to obtain a 1,4-dioxane biodegradation rate constant was challenging due to two factors: 1) uncertainty in critical site-specific parameters, specifically the groundwater seepage velocity; and 2) uncertainty in the source decay rates. To fit the field data, it was necessary to increase the reported groundwater velocity by an order of magnitude to ensure that 1,4-dioxane would reach the downgradient locations. A source decay term of 0.06 per year was also used to reflect the apparent change in 1,4-dioxane concentration at the near source well over time. After these changes to the base case, the best fit was obtained using 1,4-dioxane biodegradation rate constant of 0.002 per year, (half-life ≥ 347 year) (**Figure 5.7.6.1**),

and no significant change in the fit was obtained within the range of 0.000 to 0.005 per year. This suggests that 1,4-dioxane biodegradation is occurring at a site-wide rate of between 0 and 0.005 per year (half-life ≥ 139 year).

Does the biomarker abundance explain the model-estimated 1,4-dioxane biodegradation rate constant? **Yes***

At this site, data from all wells could be used to support the biomarker abundance/rate constant correlation included in the model because RDEG and RMO were detected as possible biomarkers for cometabolism of 1,4-dioxane (**Figure 5.7.6.2**). Based on these data, a site-wide 1,4-dioxane biodegradation rate constant of 0.0002 per year was predicted using the correlation in the MNA Rate Constant Estimator, which is within the range predicted by fitting the concentration vs. distance data. Given that the ^{14}C assay was only able to establish 1,4-dioxane degradation at one location and it was at a similarly low rate (0.003 per year), it is plausible that these enzymes are contributing to a portion of the apparent degradation activity.

Is the model-estimated 1,4-dioxane biodegradation rate constant consistent with rate constants estimated using the ^{14}C assay? **Yes**

The range of 1,4-dioxane rate constants was estimated to be between 0.000 to 0.005 per year based on model simulations using the MNA Rate Constant Estimator, which are similar to the range of rate constants observed during the ^{14}C assays for this set of wells (0 to 0.003 per year) (**Table 5.7.6.1**). The rate constants estimated using both of these approaches are relatively slow, and the ^{14}C assay results suggest that measurable activity may be localized to certain portions of the site.

Are ^{2}H and/or ^{13}C enriched along the flow path? **Yes***

The $\delta^{2}H$ value of -0.2 ‰ in Well 3 (i.e., the well where 1,4-dioxane biodegradation activity was definitively established using the ^{14}C assay) was modestly higher than $\delta^{2}H$ values measured elsewhere at the site (-45.8 to -18.6 ‰) (**Figure 5.7.6.2**). Well 3 is located in the middle of the plume, and the farthest downgradient well (Well 1) did not exhibit similar hydrogen isotope fractionation. These data suggest that some localized 1,4-dioxane biodegradation is occurring (presumably near Well 3) but that this activity is not widespread enough to significantly alter the isotopic signature across the site (i.e., due to mixing of degraded and undegraded 1,4-dioxane as groundwater is transported downgradient). The $\delta^{13}C$ values fell within a narrow range and exhibited no consistent pattern along the apparent flow path. Consequently, the carbon isotope data could not be used as supporting evidence for 1,4-dioxane degradation. Variability in $\delta^{13}C$ patterns can occur due to the lower enrichment factors for carbon, different source signatures, and mixing between degraded and undegraded 1,4-dioxane within the aquifer.

Are geochemical conditions supportive of 1,4-dioxane biodegradation? **No**

Based on low dissolved oxygen levels (< 1 mg/L in all four wells), geochemical conditions were apparently reducing and would generally not be supportive of oxidative 1,4-dioxane biodegradation. For example, ORP readings were all > 0 mV, and little or no methane was observed (max concentration = 5.7 µg/L. 2 mg/L in all three of the four wells. Similarly, the presence of detectable levels of multiple genes encoding both oxygenases and reductases (e.g., DHC) suggests that both aerobic and anaerobic activity may be supported within portions of the aquifer (perhaps within narrower depth intervals) (**Table 5.7.6.2**).

Are inhibitory CVOCs present below inhibitory levels and/or decreasing with time/distance? **Yes**
Both chlorinated ethenes and ethanes are present at this site but at low levels. Total CVOC concentrations were less than 10 µg/L in all wells. 1,1-DCE was detected at a maximum concentration of 2.5 µg/L, and this occurred in the same well with the highest 1,4-dioxane concentration (Well 4). Both Total CVOC and 1,1-DCE concentrations were consistently lower than 1,4-dioxane concentrations at individual wells and generally decreased with distance. The 1,1-DCE levels are also significantly lower than reported half saturation constants for inhibition of aerobic biodegradation of 1,4-dioxane (K_i) by 1,1-DCE for both cometabolism and direct metabolism (Mahendra et al., 2013). Collectively, the data suggest that chlorinated solvents are present at relatively low levels, such that inhibition is unlikely to be a major influence on observed 1,4-dioxane degradation patterns.

Table 5.7.6.1. Rate constants for 1,4-dioxane degradation provided by ^{14}C assay for Site 6.

Sampled	Well	First order rate constant (per year)	95% Confidence Interval (per year)
2019	Well 1	<0.0063	0.0028*
2019	Well 2	<0.0039	0.0016*
2019	Well 3	0.002647	0.0023
2019	Well 4	<0.0137	0.0033*

Yellow highlighted cells represent rate constants for microcosms that were significantly different than filter-sterilized groundwater control.
* 95% confidence interval on rate for microcosm.

Table 5.7.6.1. Geochemistry of water samples from Site 6.

Well #	Sample Date	Temp.	pH	Conduc-tivity	Dissolved Oxygen	ORP	Ferrous Iron	Methane
		˚C		µS/cm	mg/L	mV	mg/L	mg/L
1	1/28/2019	21.9	7.05	2090	0.522	287	0.106	5.68
2	1/29/2019	20.1	7.47	927	0.301	200	0.102	0.036 J
3	1/30/2019	18.3	7.31	1947	0.869	182	0.744	0.377
4	1/31/2019	20.1	7.14	2110	0.479	170	<0.05	2.74

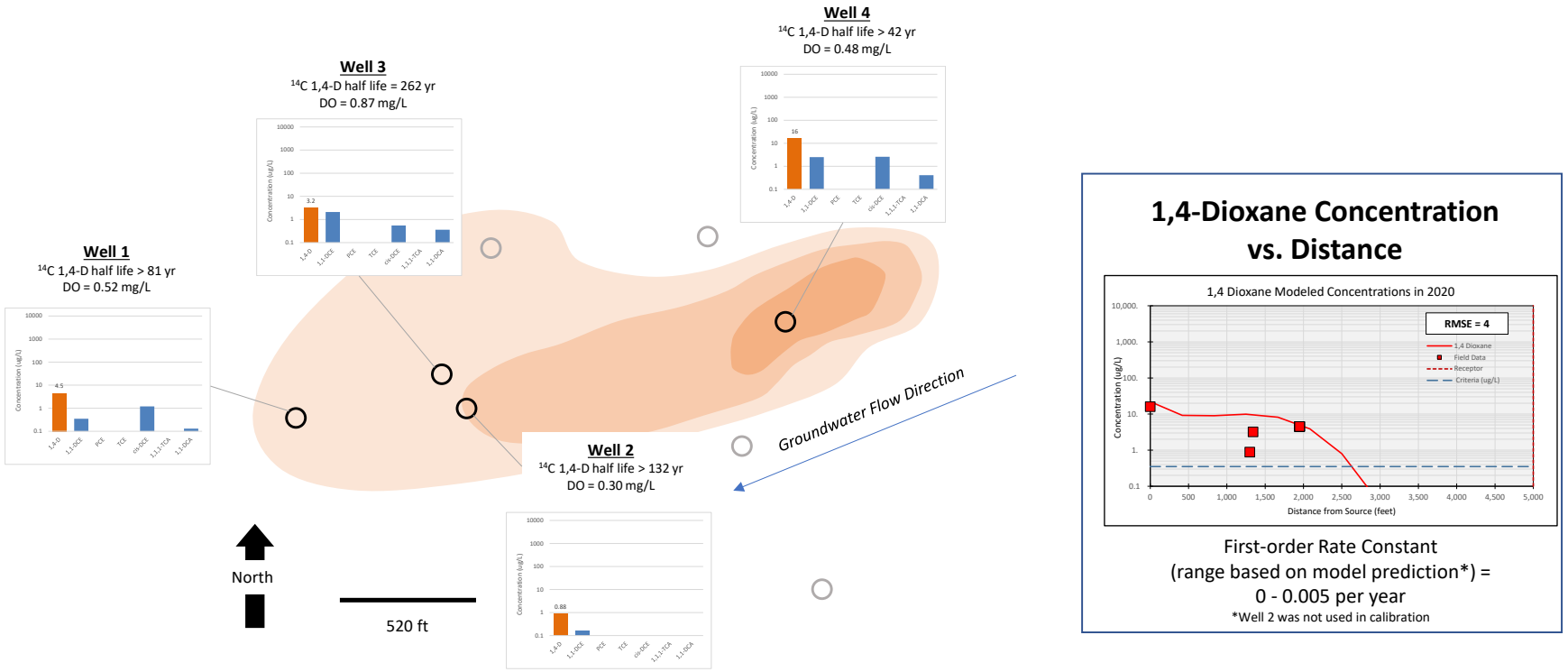


Figure 5.7.6.1. Site 6 (DoD) Summary of 1,4-Dioxane/CVOC Concentration Trends and Rate Constants.

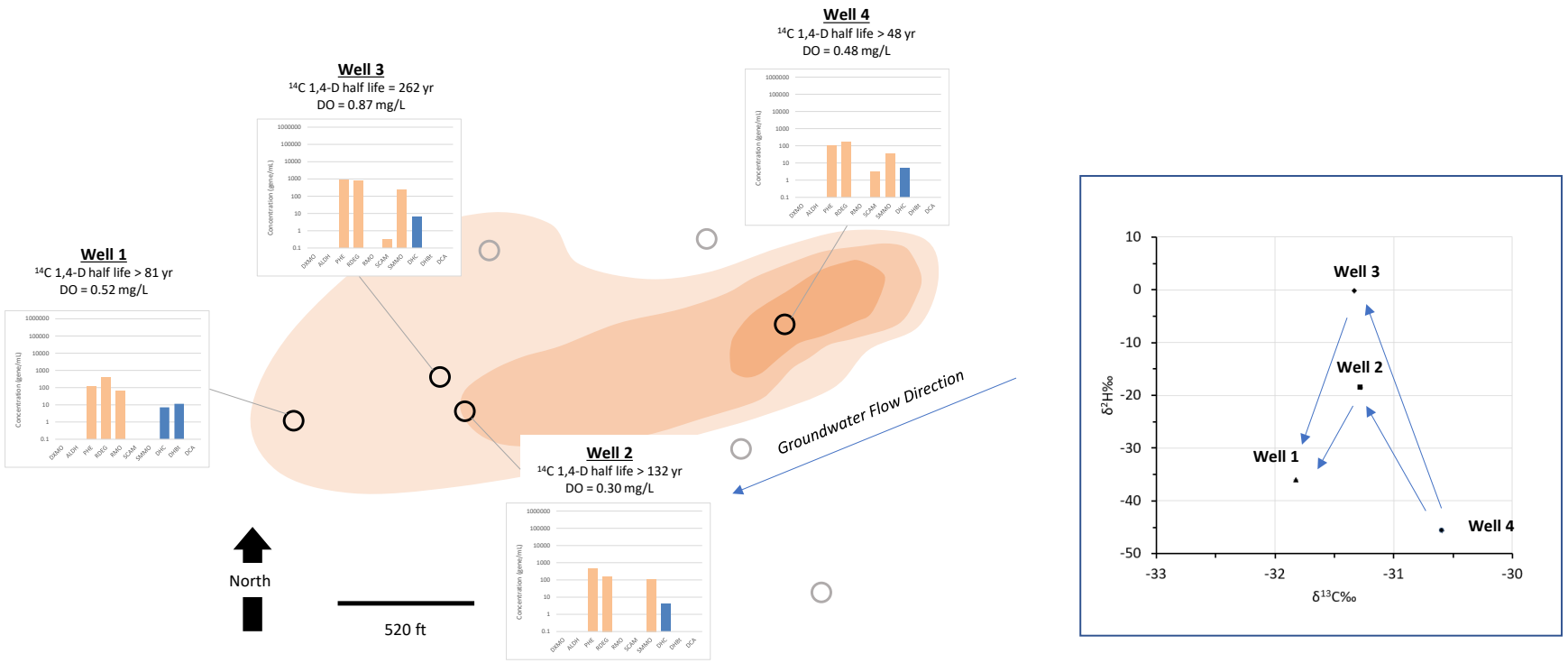


Figure 5.7.6.2. Site 6 (DoD) Summary of Biomarker and Isotope Data.

5.7.7. Site 7 (DoD)

Overview: The site is former chemical waste disposal area. Chemical wastes including paints, solvents and oils were disposed in uncontrolled landfills at several locations on the site.

Is 1,4-dioxane below regulatory standards? **No**

Figure 5.7.7.1 depicts the distribution of 1,4-dioxane and chlorinated hydrocarbons in the four wells that were sampled for this project in 2019, with additional data from the site owner for the most contaminated well in 2017. Well 5 is in an area with residual NAPL. When the wells were sampled in 2019, the concentration of 1,4-dioxane was above the regulatory standard in Well 2 (1,400 µg/L) and Well 5 (320 µg/L).

Is the plume stable (i.e., is the 1st line of evidence for MNA met)? **Yes**

Figure 5.7.7.2 plots historical data on concentrations of 1,4-dioxane and chlorinated hydrocarbons over time at the five monitoring wells. Except for data from 2019, the data were acquired by the site owner. Approximately nine years of data are available. In Well 5 and Well 2 in the source area of the plume, concentrations appear to be stable or increasing. In Well 4, Well 3 and Well 2 in the periphery of the plume, the concentrations appear to be stable or decreasing over time. Down gradient sentry wells have not been impacted.

Contaminants were released starting in the late 1940s and extending to the mid-1970s. The seepage velocity of groundwater is approximately 19 feet per year. Over this time, groundwater could have moved approximately 1300 feet. The distance from Well 5 at the source to Well 1 down gradient is 670 feet.

Is 1,4-dioxane being degraded based on model predictions? **Not assessed**

Based on the hydraulic gradient, the general flow direction of groundwater would be from the source at Well 5 to Well 2 and then to Well 1. Note that wells were selected for this project based on the potential that biodegradation of 1,4-dioxane would be detected in the ^{14}C assay. Well 3 and Well 4 are not in the flow path from Well 5 and Well 2.

When sampled in 2019, the concentration of 1,4-dioxane increased from Well 5 to Well 2, then decreased dramatically in Well 1. The concentrations of chlorinated hydrocarbons were high in Well 5 and Well 2. Both wells were screened in or near NAPL that acts as a source of contamination. In Well 1 the chlorinated hydrocarbons were not detected. It is impossible to determine whether the much lower concentration of 1,4-dioxane and chlorinated hydrocarbons in Well 1 compared to Well 5 and Well 2 were caused by degradation of the 1,4-dioxane and chlorinated hydrocarbons, or by hydrological parameters such as Well 1 lying askew of the flow path, or the groundwater not moving fast enough to reach the well. A simple one-dimensional screening model such as the MNA Rate Constant Estimator is not appropriate to model degradation with the limited data that are available (3 wells) and the uncertainty on the groundwater flow characteristics. Therefore, no determination can be made whether 1,4-dioxane is being degraded based on model predictions.

Does the biomarker abundance explain the model-estimated 1,4-dioxane biodegradation rate constant? **No**

Data on the abundance of the biomarkers in 2019 are presented in **Figure 5.7.7.3**. The relevant biomarkers for direct metabolism of 1,4-dioxane are DXMO and ALDH, and possibly *prmA*. Biomarkers that are potentially relevant to cometabolism are RMO, RDEG, PHE, SCAM, SMMO, and possibly *prmA*. If the abundance was below the reporting limit, the abundance was plotted at the reporting limit. The biomarkers for direct biodegradation of 1,4-dioxane (DXMO and ALDH) were not detected in groundwater at Site 7. The abundance of one or several of the biomarkers that have been associated with cometabolism of 1,4-dioxane were above 1000 gene copies per mL in Well 3, above 100 gene copies per mL in Well 2, but all the biomarkers were below 100 genes copies per mL in Well 1 and Well 4.

The MNA Rate Constant Estimator is designed to calculate an overall rate constant for biodegradation along a flow path. It calculates a rate constant for each well in the flow path based on the abundance of the biomarkers and the concentration of the organic compound of concern, and then calculates a weighted average of the well-specific rate constants based on the distribution of the wells along the flow path. The MNA Rate Constant Estimator was used to predict the rate constant that would be expected for groundwater from each of the four wells. The MNA Rate Constant Estimator was calibrated with the conditions that pertain to a particular well as the source well. A dummy well was included with the same conditions, 100 feet down the flow path. The abundance of gene copies for the biomarkers was entered into section 6b of the MNA Rate Constant Estimator. If a marker was not detected, the reporting limit was entered.

The predicted rate constants for degradation of 1,4-dioxane based on biomarker abundance are provided in **Table 5.7.7.1**. The predicted rate constants are low (≤0.0017 per year) and would not be expected to make a useful contribution to attenuation of concentrations of 1,4-dioxane along the flow path.

Is the model-estimated 1,4-dioxane biodegradation rate constant consistent with rate constants estimated using the ^{14}C assay? **No***

The ^{14}C assay was performed on samples acquired in 2019. Biodegradation was detected in Well 4, but the rate constant was low (0.005 per year). Biodegradation was not detected in samples from Well 1, Well 2 or Well 3. The rate constants for biodegradation were all less than 0.0124 per year in these wells (equivalent half-lives all > 48 year after accounting for 95% confidence levels) and were not significantly different than filter-sterilized controls. As described above, the model could not be fully calibrated because representative transport data were not available to fit a concentration vs. distance profile. However, the small rate constants from the biomarker assays (≤0.0017 per year) are consistent with the small rate constants predicted from the ^{14}C assay.

Are ^{2}H and/or ^{13}C enriched along the flow path? **No***

Values for $\delta^{13}C$ and $\delta^{2}H$ were obtained for 1,4-dioxane in Well 2, Well 3 and Well 5 (Panel B of **Figure 5.7.7.3**). The values of $\delta^{13}C$ in 1,4-dioxane varied from -30.57‰ in Well 3 to -31.35‰ in Well 2. With a sample standard deviation of 0.5‰, and a difference of 1.0‰ necessary to be statistically different at 95% confidence, there was no evidence of enrichment of $\delta^{13}C$ along the flow path.

There was enrichment of ^{2}H, but it did not increase systematically along the flow path. Values varied from -12.94‰ in Well 2 to -4.06‰ in Well 5 to 9.2‰ in Well 3, with a difference of 8‰ being required to be statistically different at 95% confidence.

Panel B of **Figure 5.7.7.3** compares $\delta^{13}C$ and $\delta^{2}H$ in 1,4-dioxane in the three wells at Site 7 to values for the most contaminated wells at the other nine sites in this study. The most contaminated wells were the best information available on values of $\delta^{13}C$ and $\delta^{2}H$ at the source of contamination at these sites. If the fill of the symbol is black, the concentration of 1,4-dioxane was greater than 1,000 µg/L. If the fill is blue, the concentration was between 1,000 and 100 µg/L. If the fill is white, the concentration was less than 100 µg/L. The value of $\delta^{2}H$ in 1,4-dioxane in wells at Site 7 was heavier than the source of contamination at seven of the other nine sites. This comparison suggests, but does not prove, that 1,4-dioxane was degraded in water from all the wells at Site 7.

Bennett and Aravena (2020) compiled values for $\delta^{2}H$ in 1,4-dioxane that had not been degraded. The maximum value of $\delta^{2}H$ in 22 samples was -17‰ (the dotted red line in Panel B). The value for $\delta^{2}H$ in 1,4-dioxane from all the wells at Site 7 is higher than the maximum value in the undegraded samples, which would suggest that the 1,4-dioxane in all the wells at Site 7 was degraded. However, Bennett and Aravena (2020) made the following statement: *Based on the large range in $\delta^{2}H$ values for source 1,4-dioxane and given the larger uncertainty in analytical results, the use of $\delta^{2}H$ values alone as an indicator for biodegradation is not recommended.*

Are geochemical conditions supportive of 1,4-dioxane biodegradation? **No***
The geochemistry of the water samples from the wells at Site 7 is provided in **Table 5.7.7.2**. In water samples from Well 4, the temperature, pH, conductivity as a proxy for dissolved electrolytes, dissolved oxygen and ORP were all adequate to support biodegradation of 1,4-dioxane. This is the only well where biodegradation was detected in the ^{14}C assay. Oxygen was not available in water from Well 2, Well 3 or Well 5. The pH was 9.2 in Well 1. High concentrations of ferrous iron in Well 5 and methane in Well 1 indicated that some of the flow paths to these wells did not have oxygenated water.

The geochemical conditions either prohibited or inhibited biological degradation of 1,4-dioxne in four of the five wells that were sampled.

Are inhibitory CVOCs present below inhibitory levels and/or decreasing with time/distance? **No (plume)**
Mahendra et al. (2013) determined half saturation constants for inhibition of aerobic biodegradation of 1,4-dioxane (K_i) by 1,1-DCE. Values of K_i for 1,1-DCE were 50 µg/L for cometabolism and 320 µg/L for direct metabolism. The concentration of 1,1-DCE was 620 µg/L at Well 2 and 880 µg/L at Well 5. Inhibitory CVOCs were present in the source area of the plume at concentrations that would be expected to impair biodegradation or cometabolism of 1,4-dioxane. In the periphery of the plume the concentrations of 1,1-DCE were <0.2 µg/L. In the periphery of the plume CVOCs would not be expected to inhibit biodegradation of 1,4-dioxane.

Are CVOCs below regulatory standards? **No**
Figure 5.7.7.1 depicts the distribution of chlorinated hydrocarbons in the four wells that were sampled for this project in 2019, and additional data from the site owner for the most contaminated

well in 2017. When the wells were sample in 2019, the concentration of TCE, *cis*-DCE, vinyl chloride, 1,1-DCA and 1,1-DCE were above the applicable regulatory standards in Well 2.

Does the biomarker abundance predict biodegradation of CVOCs? **No**

The biomarkers relevant to biodegradation of the CVOCs are DHBt, DHC, and DCA. Their abundance in wells at Site 7 is provided in Panel A of **Figure 5.7.7.3**. If the abundance was below the reporting limit, the abundance was plotted at the reporting limit. The abundance of DHBt and DHC were high in the source area, near 10,000 gene copies per mL in Well 2. In Well 4 in the periphery, they were low, less than 20 gene copies per mL. In the other two wells in the periphery, they were less than 1000 gene copies per mL.

As described above, the MNA Rate Constant Estimator was calibrated to estimate a rate constant for conditions that applied to the individual wells, instead of a flow path. The abundance of gene copies for the biomarkers was entered into Box 6b of the MNA Rate Constant Estimator. The DHBt biomarker used in the MNA Rate Constant Estimator to predict degradation of 1,1-DCA, and *vcrA* was used to predict degradation of 1,1-DCE, *cis*-DCE and vinyl chloride. The abundance of *vcrA* was assumed to be the same as the abundance of DHC. If a marker was not detected, the reporting limit was entered.

The predicted rate constants for degradation of the CVOCs are provided in **Table 5.7.7.1**. The predicted rate constants for 1,1-DCA are low (≤5E-04 per year) and would not be expected to make a useful contribution to attenuation of concentrations along the flow path. In contrast, the predicted rate constants for degradation of *cis*-DCE and vinyl chloride in water from Well 1, Well 2 and Well 3 and the rate constant for 1,1-DCE in Well 2 approach or exceed the criteria established by Lu et al. (2016) for rate constants that are generally useful for MNA (0.3 per year). The rate constants for *cis*-DCE and vinyl chloride in Well 4 are slower, but they would make a contribution over long residence times.

Table 5.7.7.1. Biodegradation rate constants predicted from the abundance of biomarkers in groundwater from monitoring wells.

Compound	Biomarker	Well 1	Well 2	Well 3	Well 4
		Predicted rate constant (per year)			
1,4-dioxane	DXMO+RMO+RDEG	1.6E-03	1.6E-03	1.7E-03	8.5E-04
1,1-DCA	DHBt	5.2E-07	5.1E-04	1.3E-06	7.1E-07
1,1-DCE	Assume DHC = *vcrA*	5.1E-03	3.7E-01	3.7E-02	2.3E-04
cis-DCE	Assume DHC = *vcrA*	2.3E-01	4.3E+00	1.7E+00	1.0E-02
Vinyl Chloride	Assume DHC = *vcrA*	3.0E-01	1.8E+00	2.2E+00	1.3E-02

Table 5.7.7.2. Geochemistry of water samples from Site 7.

Well #	Sample Date	Temp.	pH	Conduct-ivity	Dissolved Oxygen	ORP	Ferrous Iron	Methane
		˚C		µS/cm	mg/L	mV	mg/L	mg/L
1	2019	20.3	9.2	862	0.36	101	0.31	1.2
2	2019	21.3	8.5	3060	0.03	-54	0.43	0.006
3	2019	23.4	7.6	958	0.00	-63	0.47	0.41
4	2019	22.3	8.0	670	0.67	75	0.11	0.068
5	2019	22.8	7.3	4140	0.00	-124	> 6.00	--

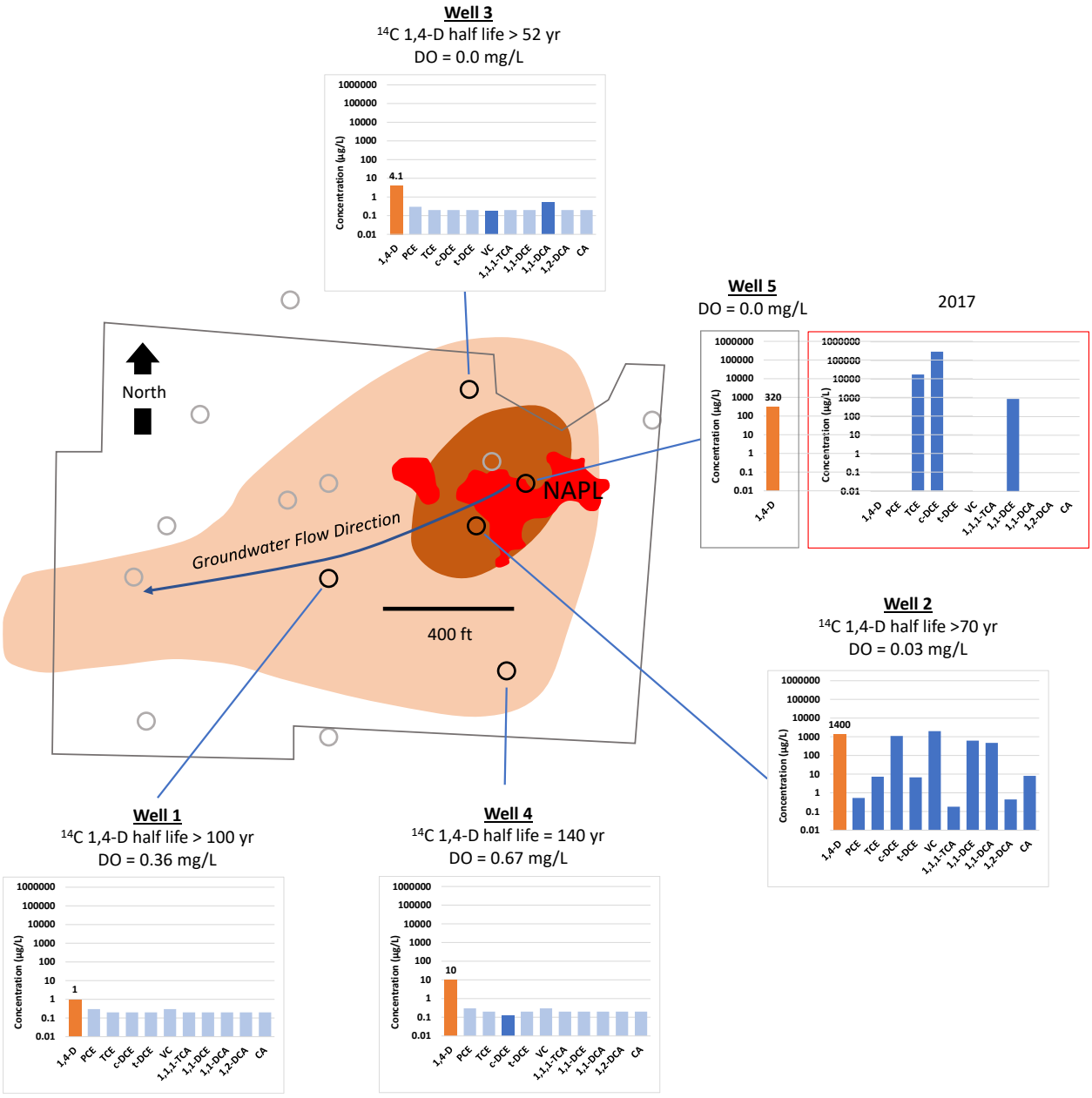


Figure 5.7.7.1. Site 7 (DoD) Distributions of 1,4-Dioxane/CVOCs in 2017 and 2019.
Pale values are plotted at the reporting limit.

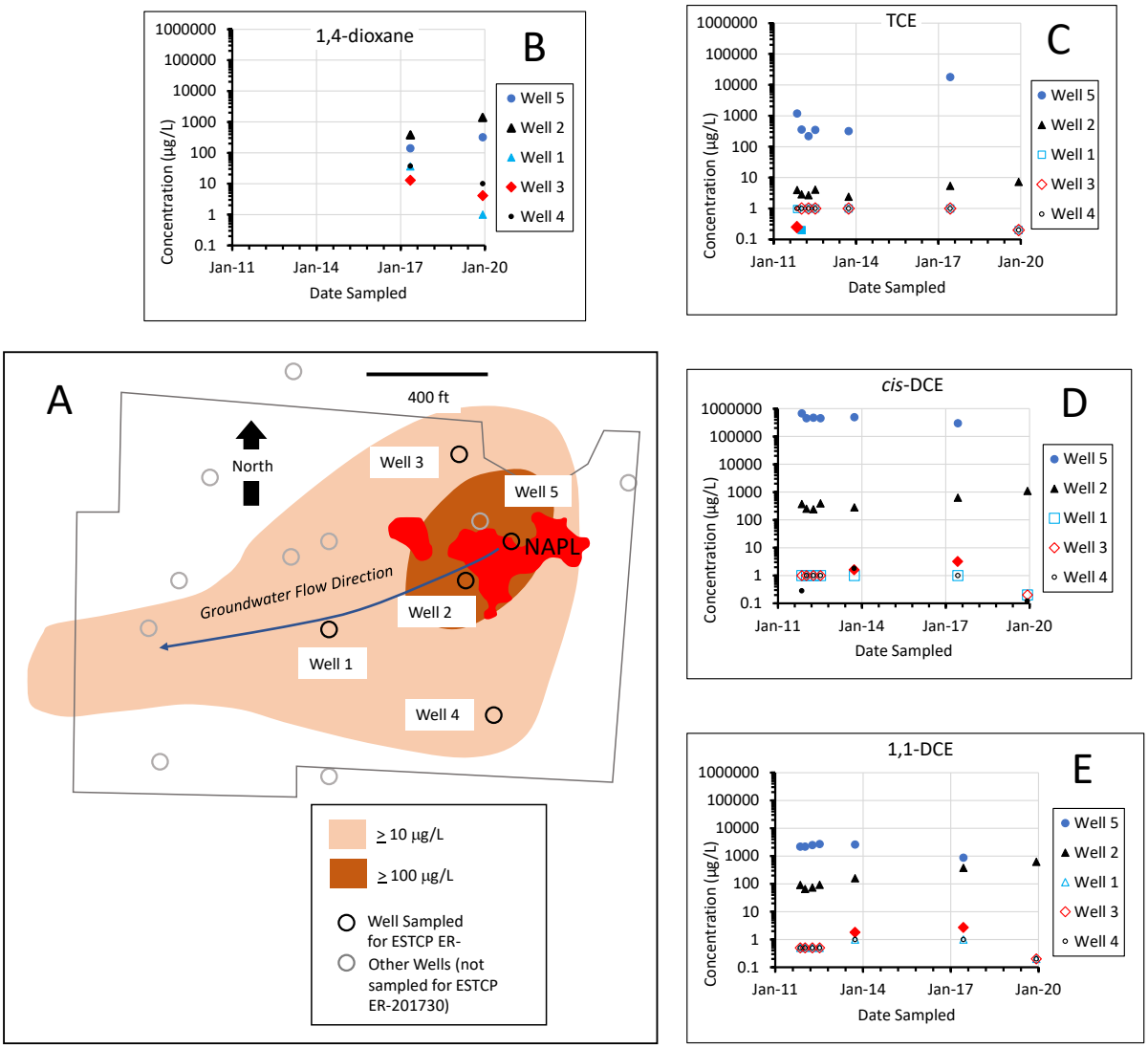


Figure 5.7.7.2. Changes in Concentrations of 1,4-Dioxane and CVOC over time.
Open symbols in Panel C through E are plotted at the reporting limit.

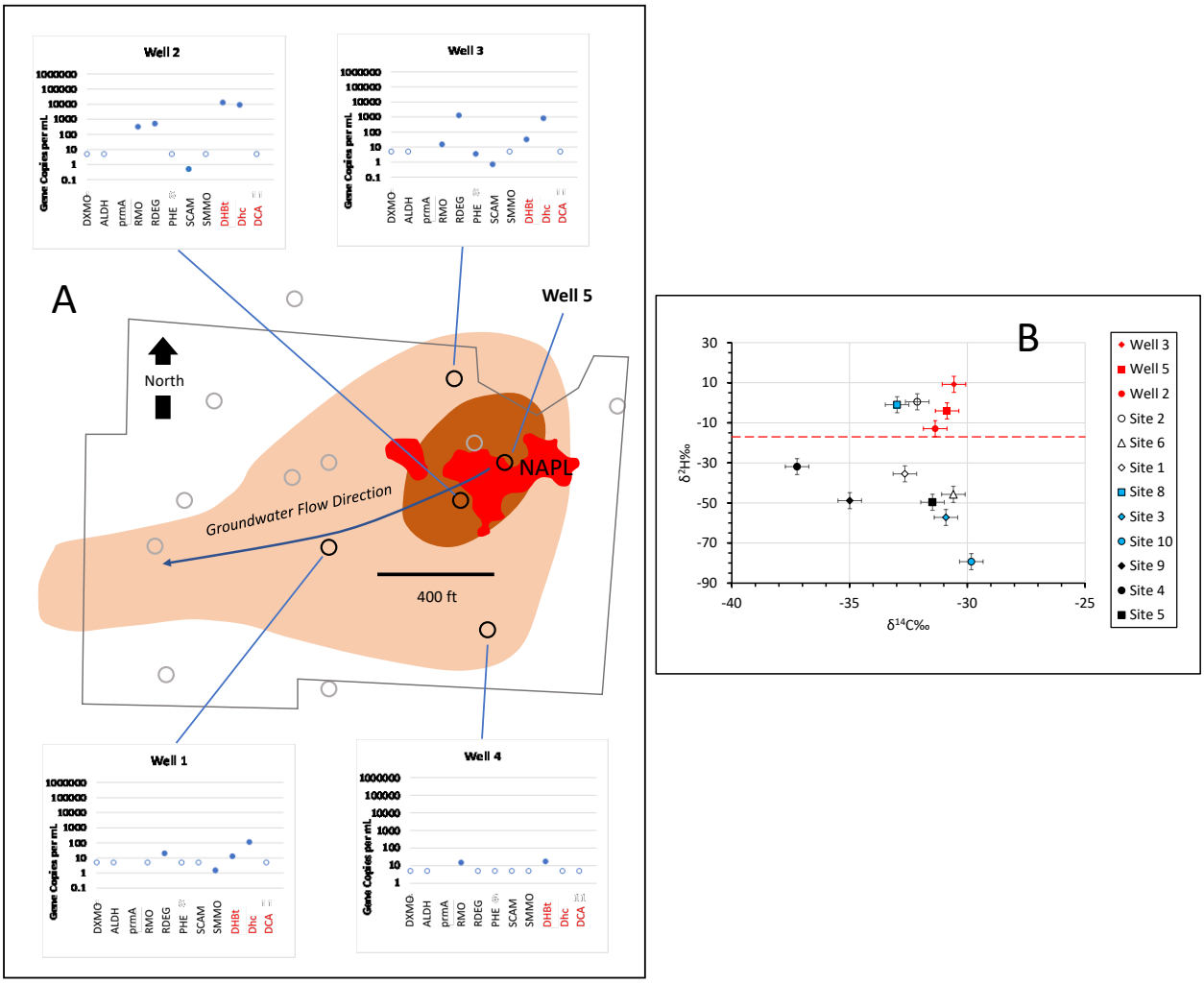


Figure 5.7.7.3. Site 7 (DoD) Summary of Biomarker Data and Isotope Data For 1,4-Dioxane.

Open circles in abundance of qPCR biomarkers in Panel A indicate the limit of quantitation. Error bars in Panel B are one standard deviation.

5.7.8. Site 8 (DoD)

Overview: This site is a former chemical waste disposal area where relatively large volumes of solvents and other chemical wastes were disposed for several decades. 1,4-dioxane concentrations measured at Site 8 during this project exhibited a decreasing pattern with distance and were relatively consistent with those reported previously. Positive evidence for 1,4-dioxane natural attenuation capacity was established using the ^{14}C assay at the two wells with the highest 1,4-dioxane concentrations (near the presumed source), with equivalent half-lives of 63 to 76 years (**Figure 5.7.8.1**). These two wells had significant dissolved oxygen, as did all wells that were part of the sampling program. Possible biomarkers for 1,4-dioxane degradation were not present at levels that could be quantified at any well; biomarkers for chlorinated solvent biodegradation were also absent (although qPCR targets based on RNA were present). However, evidence for 1,4-dioxane attenuation based on hydrogen isotope fractionation was observed, based on the difference in the values observed at the source well (Well 3) vs. the far downgradient well (Well 1).

Is 1,4-dioxane below regulatory standards? **Not assessed**
The maximum 1,4-dioxane concentration was 120 µg/L but compliance points and/or concentration thresholds have not been established at this site to our knowledge.

Is the plume stable (i.e., is the 1st line of evidence for MNA met)? **Not assessed**
Based on a comparison to the available long-term monitoring data, the project data suggest that the 1,4-dioxane plume footprint has not expanded. A projection of future plume trends was not attempted due to uncertainty and/or limited information on critical input parameters (e.g., source decay rates, biodegradation rate constants).

Is 1,4-dioxane being degraded based on model predictions? **Yes**
Based on calibrating the model (the MNA Rate Constant Estimator) with site-specific parameters, no significant change in the fit was obtained until the rate constant was increased above 0.017 per year. This suggests that 1,4-dioxane biodegradation is occurring at a rate of between 0 and 0.017 per year (half-life ≥ 41 year) (**Figure 5.7.8.1**).

Does the biomarker abundance explain the model-estimated 1,4-dioxane biodegradation rate constant? **No**
Biomarkers for 1,4-dioxane biodegradation were below quantification limits in all samples (**Figure 5.7.8.2**). This includes both DNA-based and RNA-based qPCR targets for direct metabolism, as well as those that have been implicated for cometabolic degradation.

Is the model-estimated 1,4-dioxane biodegradation rate constant consistent with rate constants estimated using the ^{14}C assay? **Yes**
As described above, the range of 1,4-dioxane rate constants was estimated to be between 0 to 0.017 per year based on the model, which was slightly greater than the range of rates established using the ^{14}C assay (0 to 0.011 per year).

Are ^{2}H and/or ^{13}C enriched along the flow path? **Yes**
The $\delta^{2}H$ values increased from -0.97 ‰ at the source well to 53.8 ‰ at the far downgradient well (**Figure 5.7.8.2**). Given that an intermediate value was also observed at a mid-plume well, this

fractionation pattern suggest that 1,4-dioxane was being degraded during groundwater transport. Fractionation patterns for the carbon isotope could not be used as supporting evidence since $\delta^{13}C$ values actually decreased slightly along the flow path. Variability in $\delta^{13}C$ patterns can occur due to the lower enrichment factors for carbon, different source signatures, and mixing between degraded and undegraded 1,4-dioxane within the aquifer.

Are geochemical conditions supportive of 1,4-dioxane biodegradation? **Yes**
Geochemical conditions were generally supportive, particularly given that dissolved oxygen was above 2 mg/L in all four wells (and above 5 mg/L in all but one well), with consistently positive ORP readings, and low methane and iron levels (**Table 5.7.8.1**). The wells where biodegradation capacity was established using the ^{14}C assay actually had lower DO readings than the wells where activity could not be established, suggesting that oxygen availability was not a rate limiting step in 1,4-dioxane degradation at this site.

Are inhibitory CVOCs present below inhibitory levels and/or decreasing with time/distance? **Yes**
The primary CVOCs are chlorinated ethanes; the highest concentration measured during this project was 1,1-DCE at 32 µg/L at the near-source well (Well 3) (**Figure 5.7.8.1**). This was still 4X lower than the 1,4-dioxane concentration at this well, and 1,1-DCE concentrations decreased along the groundwater flow path similarly to 1,4-dioxane. The 1,1-DCE levels are also significantly lower than reported half saturation constants for inhibition of aerobic biodegradation of 1,4-dioxane (K_i) by 1,1-DCE for both cometabolism and direct metabolism (Mahendra et al., 2013). 1,1,1-TCA was detected only at trace levels, such that additional 1,1-DCE formation is likely to be very limited. As a result, the presence of chlorinated solvents may be slowing observed 1,4-dioxane degradation rates but the relatively low levels are not completely inhibiting degradation.

Table 5.7.8.1. Geochemistry of water samples from Site 8.

Well #	Net Rate Constant from the ^{14}C assay	Temp.	pH	Conduc-tivity	Dissolved Oxygen	ORP	Ferrous Iron	Methane
	per year	˚C		µS/cm	mg/L	mV	mg/L	µg/L
1	<0.00012	25.11	7.08	422	5.48	164	<0.05	<0.34
2	0.0091	22.06	6.44	458	5.13	147	0.3	<0.34
3	0.0110	23.16	5.91	465	2.77	84	0.2	<0.34
4	<0.00117	19.53	6.66	429	9.12	209	<0.05	0.82 J

Yellow highlighted cells represent rate constants for microcosms that were significantly different than filter-sterilized groundwater control.

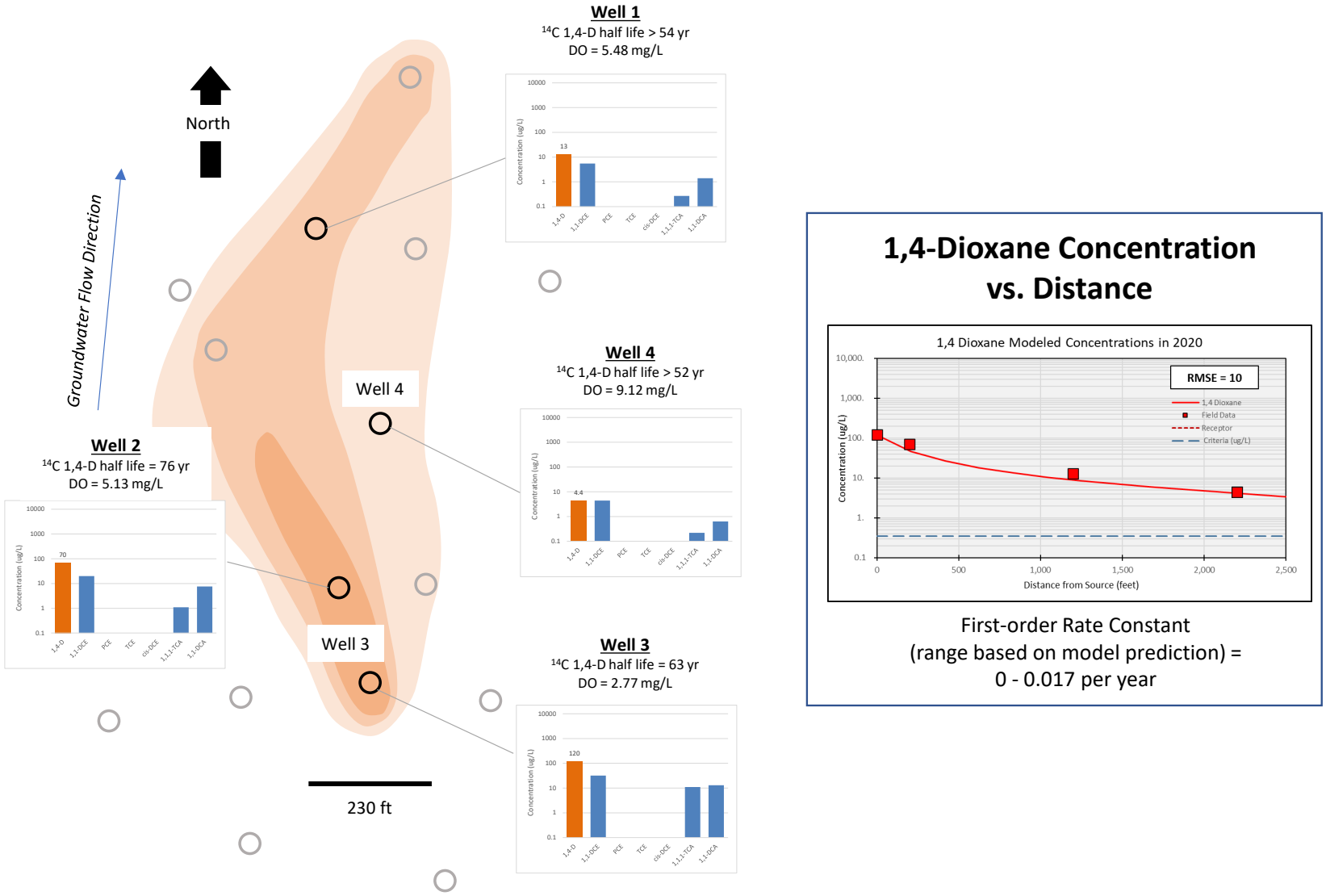


Figure 5.7.8.1. Site 8 (DoD) Summary of 1,4-Dioxane/CVOC Concentration Trends and Rate Constants.

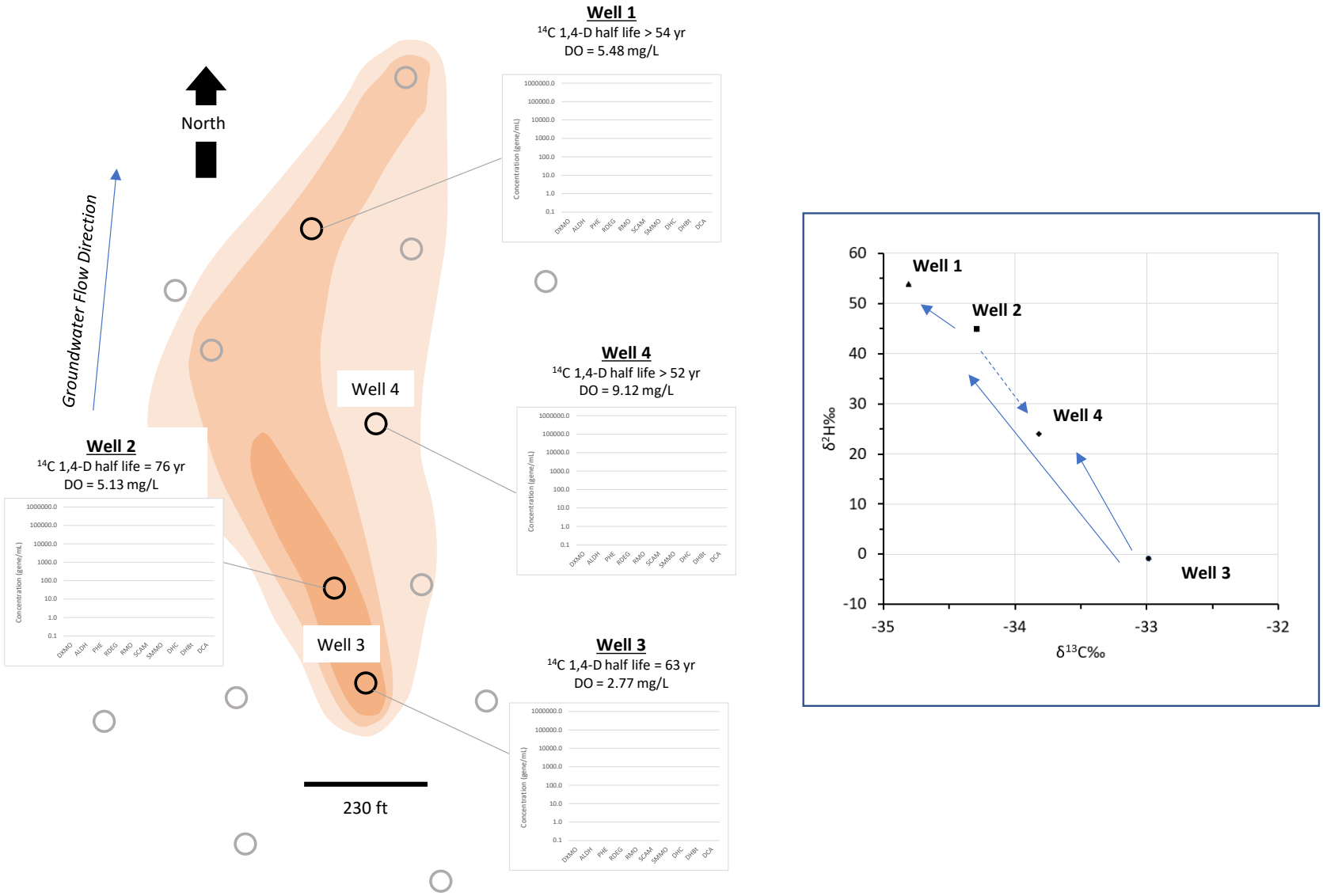


Figure 5.7.8.2. Site 8 (DoD) Summary of Biomarker and Isotope Data.
All qPCR biomarkers were below limits of quantitation.

5.7.9. Site 9 (DoD)

Overview: Solvents and other chemicals were suspected to have been released from a former underground storage tank at this site. A decreasing 1,4-dioxane concentration trend with distance was observed based on the project field data; the results were consistent with those reported previously (**Figure 5.7.9.1**). Positive evidence for 1,4-dioxane natural attenuation capacity was established using the ^{14}C assay at the three of the four wells (equivalent half-lives ranging from 53 to 272 years). The exception was the well with the highest 1,4-dioxane concentrations (Well 4, near the presumed source), which also had the lowest dissolved oxygen level (0.86 mg/L) and the highest CVOC levels (including > 1000 µg/L of both TCE and cis-DCE). The remaining wells with positive ^{14}C assay results had > 2 mg/L of DO, and this positive biodegradation capacity was established at wells both upgradient and downgradient of the series of infiltration trenches that are present at the site. Possible biomarkers for 1,4-dioxane degradation, as well as those for chlorinated solvents degradation, were largely absent from the sampled locations with the exception of Well 3. Well 3 is located slightly downgradient of the area with the highest 1,4-dioxane concentration and notably had much lower CVOC concentrations as well (including no 1,1-DCE). Evidence for 1,4-dioxane attenuation based on hydrogen isotope fractionation was observed, specifically the difference between the values at the far downgradient well (Well 1) vs. the wells located nearer the source.

Is 1,4-dioxane below regulatory standards? **Not assessed**
The maximum 1,4-dioxane concentration was 4,000 µg/L and had decreased to 5 µg/L at the farther downgradient location; however, compliance points and/or concentration thresholds have not been established at this site to our knowledge.

Is the plume stable (i.e., is the 1st line of evidence for MNA met)? **Not assessed**
Based on a comparison to the available long-term monitoring data, the project data suggest that the 1,4-dioxane plume footprint has not expanded. A projection of future plume trends was not attempted due to uncertainty and/or limited information on critical input parameters (e.g., source decay rates, biodegradation rate constants).

Is 1,4-dioxane being degraded based on model predictions? **Yes**
Based on calibrating the model (the MNA Rate Constant Estimator) with site-specific parameters, the best fit was obtained using 1,4-dioxane biodegradation rate constant of 0.08 per year, (half-life ≥ 8.8 year) (**Figure 5.7.9.1**), and no significant change in the fit was obtained within the range of 0.05 to 0.11 per year.

Does the biomarker abundance explain the model-estimated 1,4-dioxane biodegradation rate constant? **No**
At this site, only data from Well 3 (where RDEG and RMO were detected as possible biomarkers for cometabolism of 1,4-dioxane; see **Figure 5.7.9.2**) could be used to support the biomarker abundance/rate constant correlation included in the model. Based on these data, a site-wide 1,4-dioxane biodegradation rate constant of 0.001 per year was predicted using the correlation, which is substantially lower than the rate constant predicted by fitting the concentration vs. distance data, or the rate constants obtained using the ^{14}C assay. These results suggest that: 1) other (unidentified)

enzymes are responsible for the observed degradation; or 2) that the observed in situ activity is greater than what would be expected based on lab-derived relationships.

Is the model-estimated 1,4-dioxane biodegradation rate constant consistent with rate constants estimated using the ^{14}C assay? **No***
The range of 1,4-dioxane rate constants was estimated to be between 0.05 to 0.11 per year based on model simulations using the MNA Rate Constant Estimator, which are considerably higher than the range of rate constants observed during the ^{14}C assays for this set of wells (0 to 0.013 per year) (**Table 5.7.9.1**). As noted in Section 5.6, the ^{14}C rate constants may be conservative due to several factors (e.g., absence of solid-phase, nutrient limitations, etc.).

Are ^{2}H and/or ^{13}C enriched along the flow path? **Yes**
The $\delta^{2}H$ values were all within the range of -48.8 to -37.0 ‰ at the three wells located within and immediately downgradient of the source area but increased substantially to 45.1 ‰ at the far downgradient well (Well 1) (**Figure 5.7.9.2**). Given the distance between Well 1 and the three wells located upgradient of Well 1, the difference in $\delta^{2}H$ values likely suggests that 1,4-dioxane was being degraded during groundwater transport. There was no consistent pattern in the $\delta^{13}C$ values and thus these data could not be used as supporting evidence for 1,4-dioxane degradation. Variability in $\delta^{13}C$ patterns can occur due to the lower enrichment factors for carbon, different source signatures, and mixing between degraded and undegraded 1,4-dioxane within the aquifer.

Are geochemical conditions supportive of 1,4-dioxane biodegradation? **Yes**
Geochemical conditions were generally supportive, with dissolved oxygen levels above 2 mg/L in all three of the four wells (**Table 5.7.9.1**). Significantly, the wells where biodegradation capacity was established using the ^{14}C assay were the same wells with DO > 2 mg/L. The well with lowest DO (Well 4) also had the highest methane concentration (0.74 mg/L) as well as detectable levels of ethene and ethane. These apparent reducing conditions may have contributed to the lack of activity observed in this well using the ^{14}C assay.

Are inhibitory CVOCs present below inhibitory levels and/or decreasing with time/distance? **Yes**
Both chlorinated ethenes and ethanes are present at this site. Based on the project-generated data, both TCE and cis-DCE were above 1000 µg/L at the near source well (Well 4) but decreased below 100 µg/L by the next downgradient well (Well 3) (**Figure 5.7.9.1**). 1,1-DCE concentrations never exceeded 10 µg/L and were ND in the two wells located farthest downgradient. Infiltration trenches are located downgradient of Well 2 and may have contributed to the concentration trends between Well 2 and Well 1. These patterns are consistent with the other lines of evidence (particularly the ^{14}C data) that show that biodegradation activity is likely occurring within the plume. Similarly, these patterns suggest that any inhibition of 1,4-dioxane capacity or rates was most likely to have occurred in the near source well (Well 4) but may not have been a factor as groundwater moved farther downgradient, especially beyond Well 2.

Table 5.7.9.1. Geochemistry of water samples from Site 9.

Well #	Net Rate Constant from the ^{14}C assay	Temp.	pH	Conduc-tivity	Dissolved Oxygen	ORP	Ferrous Iron	Methane
	per year	˚C		µS/cm	mg/L	mV	mg/L	µg/L
1	0.00255	15.0	7.30	8570	2.27	158	0.4	<0.34
2	0.0132	15.3	6.87	11700	9.22	240	0.3	190
3	0.00579	13.7	7.12	10500	10.5	-19	2.0	84
4	<0.00203	17.2	7.00	16500	0.86	209	0.4	740

Yellow highlighted cells represent rate constants for microcosms that were significantly different than filter-sterilized groundwater control.

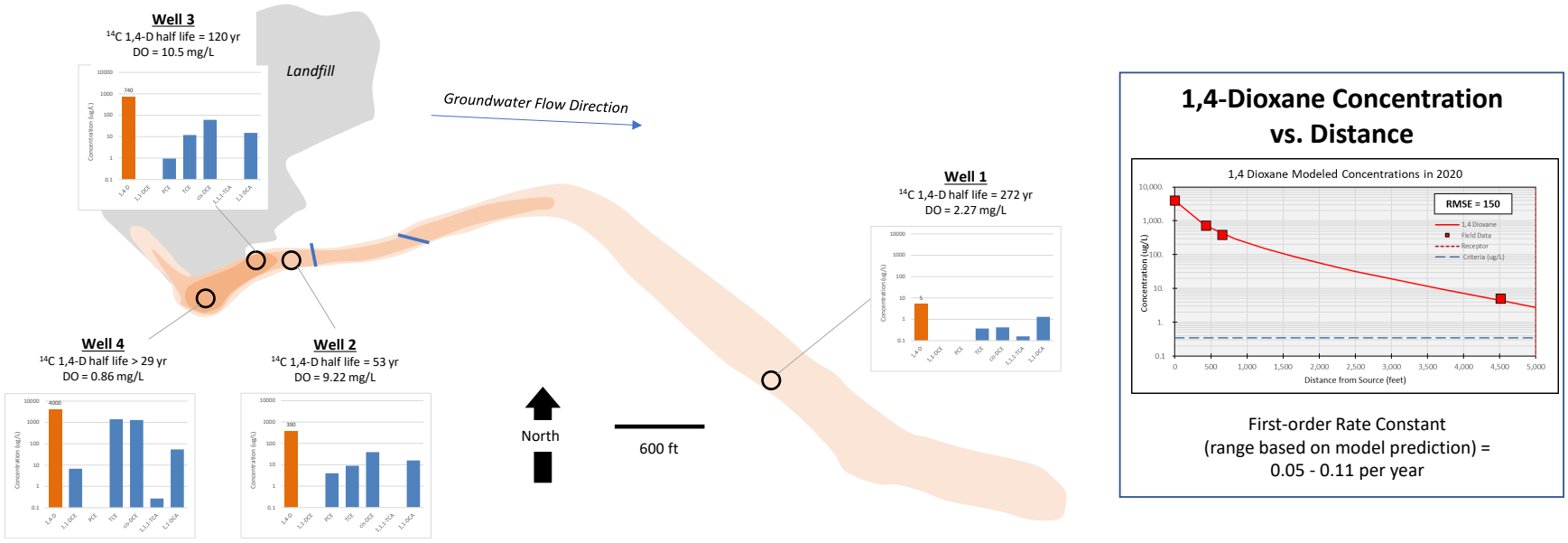


Figure 5.7.9.1. Site 9 (DoD) Summary of 1,4-Dioxane/CVOC Concentration Trends and Rate Constants.

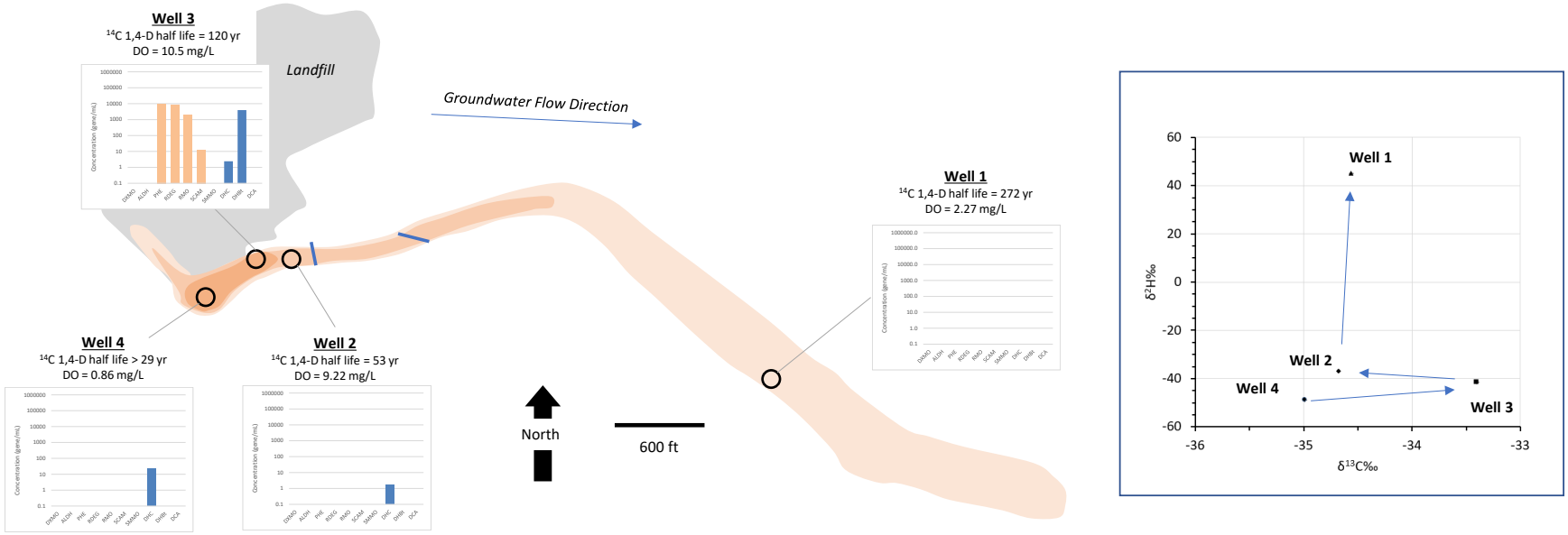


Figure 5.7.9.2. Site 9 (DoD) Summary of Biomarker and Isotope Data.

5.7.10. Site 10 (DoD)

Overview: This site includes a solid waste management unit that is part of a larger landfill. Based on project data, the 1,4-dioxane concentrations decreased with distance in a similar manner to available historical monitoring data (**Figure 5.7.10.1**). Positive evidence for 1,4-dioxane natural attenuation capacity was established using the ^{14}C assay at the two of the four wells at rates that were relatively rapid (equivalent half-lives ranging from 3.5 to 27.5 years). These were wells that had low to moderate levels of dissolved oxygen but elevated levels of dissolved iron that suggested that iron cycling was contributing to a combined biotic/abiotic degradation pathway. One of these (Well 2) was the far downgradient extraction well. Degradation could not be established in the near source using the ^{14}C assay. Data associated with several other lines of evidence would not have been supportive of attenuation at this site without the ^{14}C rate data. For example, all wells contained elevated levels of CVOCs (> 100 µg/L of multiple compounds, including 1,1-DCE), and these concentrations were consistently higher than the 1,4-dioxane measured in the same well. Similarly, there was no evidence of isotope fractionation along the groundwater flow path, although mixing of multiple and/or undegraded sources may have occurred to mask these effects.

Is 1,4-dioxane below regulatory standards? **Not assessed**
The maximum 1,4-dioxane concentration was 980 µg/L and had decreased to 59 µg/L at the extraction well that served as the farther downgradient location that was sampled. However, no compliance points and/or concentration thresholds have been established at this site to our knowledge.

Is the plume stable (i.e., is the 1st line of evidence for MNA met)? **Not assessed**
The project data could not be used to determine if the 1,4-dioxane plume footprint has expanded. Both source and plume concentrations were slightly higher than those observed during previous monitoring events, but further plume expansion is prevented by the downgradient extraction well. A projection of future plume trends was not attempted due to uncertainty and/or limited information on critical input parameters (e.g., source decay rates, biodegradation rate constants).

Is 1,4-dioxane being degraded based on model predictions? **Yes**
Based on calibrating the model (the MNA Rate Constant Estimator) with site-specific parameters, the best fit was obtained using 1,4-dioxane biodegradation rate constant of 0.32 per year, (half-life ≥ 2.2 year) (**Figure 5.7.10.1**), and no significant change in the fit was obtained within the range of 0.30 to 0.34 per year. Because the concentration at the far downgradient well may have been influenced by its use as an extraction well, the process was repeated with this well omitted. For this case, the best fit was obtained with a 1,4-dioxane biodegradation rate constant of 0.31 per year. Since similar values were obtained with both approaches, the rate constant based on four well locations (0.32 per year) was retained during further evaluation of the data.

Does the biomarker abundance explain the model-estimated 1,4-dioxane biodegradation rate constant? **No**
Biomarkers that are possible indicators of direct metabolism of 1,4-dioxane (DXMO, ALDH) were not detected at any well (**Figure 5.7.10.2**). As a result, only biomarker data for potential cometabolic processes (RDEG and RMO) could be used to support the biomarker abundance/rate constant correlation included in the model. Because detections of these biomarkers were sporadic

and low, the correlation predicted a site-wide 1,4-dioxane biodegradation rate constant that was < 0.001 per year. This is substantially lower than the rate constant predicted by fitting the concentration vs. distance data, or the positive rate constants obtained using the ^{14}C assay. These results suggest that: 1) other enzymes (including possibly unidentified enzymes) are responsible for the observed degradation; or 2) that the observed in situ activity is greater than what would be expected based on lab-derived relationships.

Is the model-estimated 1,4-dioxane biodegradation rate constant consistent with rate constants estimated using the ^{14}C assay? **Yes**

The range of 1,4-dioxane rate constants was estimated to be between 0.30 to 0.34 per year based on model simulations, which are substantially higher than any of the rate constants observed during the ^{14}C assays for this set of wells (0 to 0.195 per year; see **Table 5.7.10.1**). The upper-end of the ^{14}C-based rate constants is approximately half of the model-predicted rate. However, this rate was applicable to a single well, and a rate-constant weighted based on site-wide values would be somewhat lower.

Are ^{2}H and/or ^{13}C enriched along the flow path? **No**

The $\delta^{2}H$ values for this set of wells all fell within a relatively narrow range (-79.3 to -60.3‰) and did not exhibit any pattern with distance along the groundwater flow path (**Figure 5.7.10.2**). Carbon isotope data also provided no evidence of enrichment. Collectively, the isotope results contradict the other lines of evidence that showed strong attenuation of 1,4-dioxane (^{14}C assays, model predictions). This suggests that mixing of 1,4-dioxane with different isotopic source signatures and different degrees of degradation across the site may be masking any evidence of isotopic fractionation. This is plausible given that the sampling location located farthest downgradient (Well 2) is an extraction well that likely captures groundwater from a wide area.

Are geochemical conditions supportive of 1,4-dioxane biodegradation? **No***

Geochemical conditions differed widely across the site, though three of the four wells were clearly installed in relatively anoxic groundwater due to low dissolved oxygen, elevated methane, and/or high levels of iron (**Table 5.7.10.1**). The exception was the downgradient extraction well that contained high DO (4.5 mg/L) as expected. However, the wells with elevated dissolved iron (Well 4, Well 2) exhibited significant 1,4-dioxane biodegradation rates during the ^{14}C assay. This suggests that the iron cycling that was observed within these samples contributed to positive attenuation activity, and the ^{14}C assay data suggested that at least a portion of this activity was abiotic. As a result, the geochemical conditions appeared to be highly influential on promoting 1,4-dioxane attenuation, albeit through a different pathway than was expected.

Are inhibitory CVOCs present below inhibitory levels and/or decreasing with time/distance? **No**

Both chlorinated ethenes and (to a lesser extent) chlorinated ethanes are present at this site. Based on the project-generated data, the highest levels of PCE, TCE, and cis-DCE (>1000 µg/L) were observed at Well 1 near the middle of the 1,4-dioxane plume (**Figure 5.7.10.1**). Because the maximum concentrations of 1,1-DCA (290 µg/L) and 1,1-DCE (880 µg/L) were found in the same well with the maximum 1,4-dioxane concentration (Well 3), it is presumed that there were multiple points of release and/or multiple solvents used in this area. Total CVOC concentrations across the site were relatively high, with maximums that exceeded those associated with any of the other sites that were part of this project. Further, the 1,1-DCE levels are also significantly higher than reported

half saturation constants for inhibition of aerobic biodegradation of 1,4-dioxane (K_i) by 1,1-DCE for both cometabolism and direct metabolism (Mahendra et al., 2013). Regardless, positive evidence of 1,4-dioxane biodegradation was observed in multiple wells with hundreds to thousand ppb of CVOCs. These results suggest that while some type of inhibition of 1,4-dioxane biodegradation rates might be expected, the presence of CVOCs is not preventing measurable 1,4-dioxane attenuation from occurring.

Table 5.7.10.1. Geochemistry of water samples from Site 10.

Well #	Net Rate Constant from the ^{14}C assay	Temp.	pH	Conduc-tivity	Dissolved Oxygen	ORP	Ferrous Iron	Methane
	per year	˚C		µS/cm	mg/L	mV	mg/L	µg/L
1	<0.0101	28.1	6.27	292	0.34	-28	> 10	1300
2	0.0688	22.9	6.35	136	4.32	161	0.1	8
2 (repeat)	0.0252							
3	<0.00438	20.9	4.56	104	0.76	270	0.6	420
3 (repeat)	<0.010							
4	0.0948	24.2	6.72	461	0.57	-77	> 10	5400
4 (repeat)	0.1948							

Yellow highlighted cells represent rate constants for microcosms that were significantly different than filter-sterilized groundwater control.

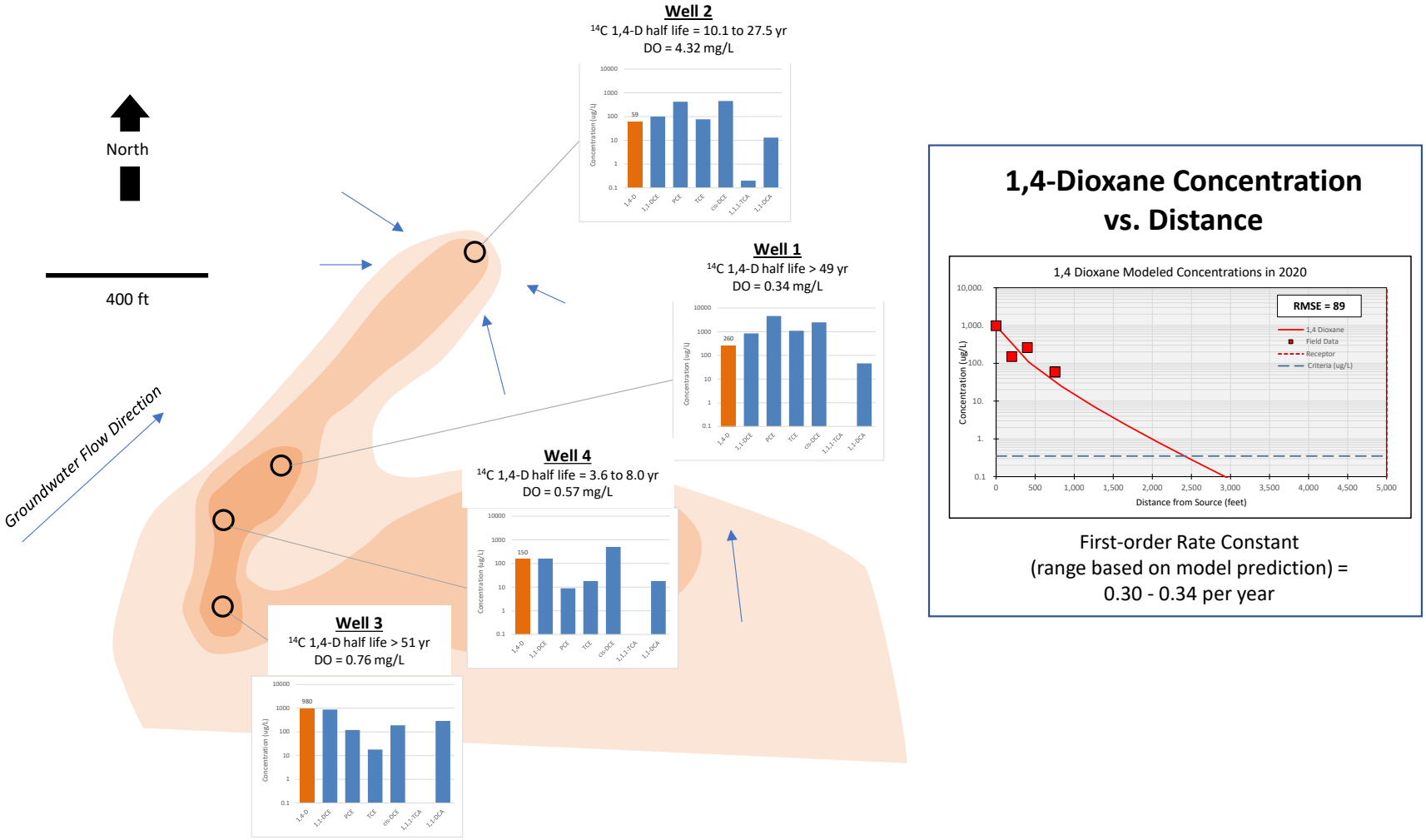


Figure 5.7.10.1. Site 10 (DoD) Summary of 1,4-Dioxane/CVOC Concentration Trends and Rate Constants.

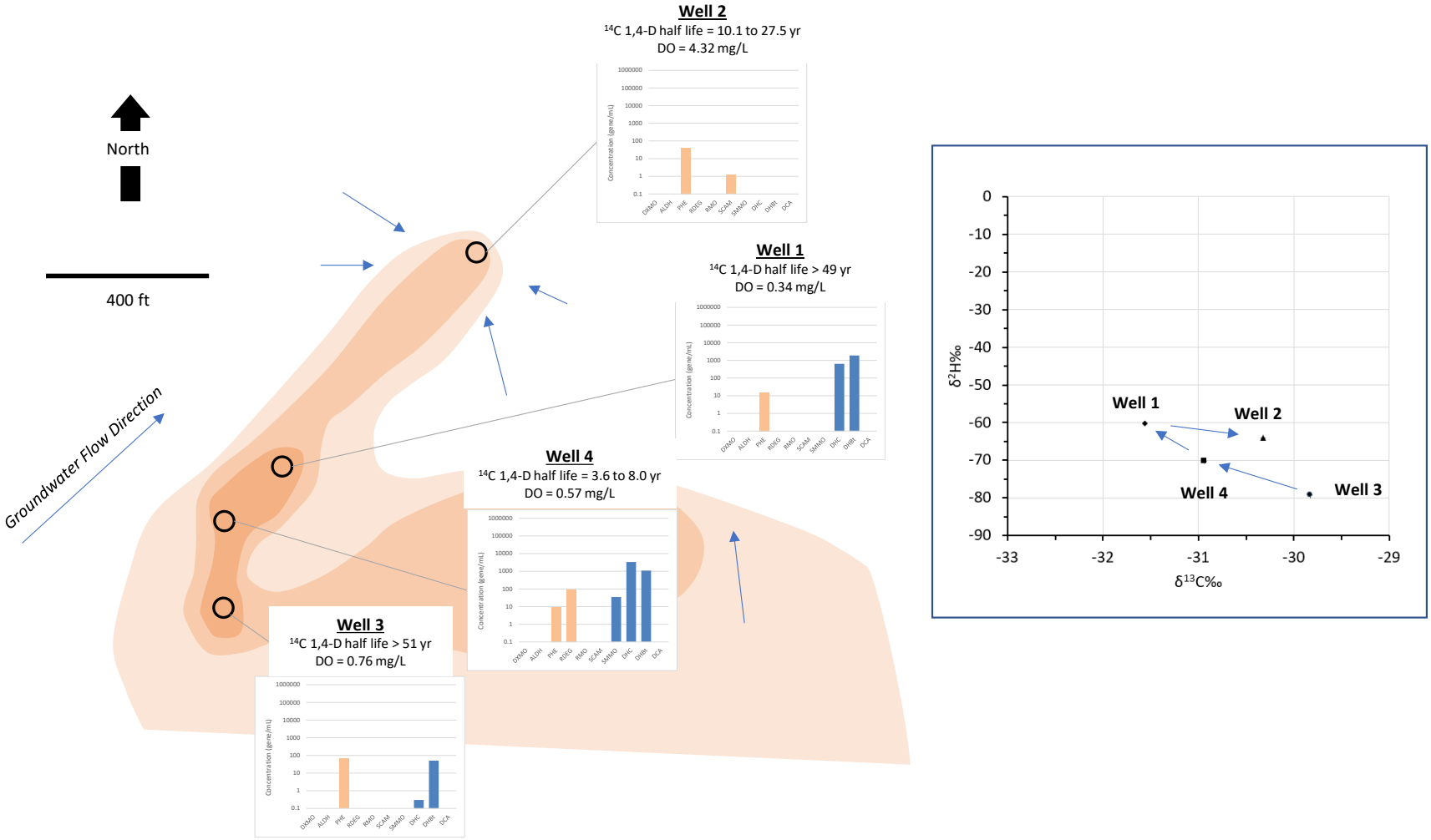


Figure 5.7.10.2. Site 10 (DoD) Summary of Biomarker and Isotope Data.

5.8 COMPILATION AND COMPARISON OF ALL LAB AND FIELD DATA

One of the primary objectives for this project was to: 1) collect data on multiple lines of evidence for 1,4-dioxane attenuation to better understand the prevalence of this activity; and 2) evaluate the extent to which these lines of evidence converged and/or complemented each other. Note that within an MNA remedy evaluation framework, these serve as the second/third lines of evidence for attenuation.

As a first step, the results from each field site (including the results of the ^{14}C lab assay that were performed on field samples) were compiled into a single table (**Table 5.8**). For screening purposes, a color-coding scheme was used, where YES/green cells signify a positive line of evidence for 1,4-dioxane biodegradation. In contrast, NO/gray cells represent cases where the data were not supportive of biodegradation.

For the purposes of site evaluation and this project, these data can be placed three broad categories:

1. ***Site-specific confirmation that the capacity for 1,4-dioxane biodegradation is present and an estimate of the biodegradation rate***
    a. Statistically significant 1,4-dioxane biodegradation rate constant using the ^{14}C assay – *successfully obtained at 9 of the 10 sites in this study.*

2. ***Site-specific evidence that 1,4-dioxane attenuation is occurring***
    a. Biodegradation rate constant based on model calibration of concentration vs. distance data – *successfully obtained at 8 of the 10 sites in this study*
    b. Isotope fractionation along the groundwater flow path – *successfully obtained at 7 of 10 sites in this study*

3. ***Site-specific data that are supportive of the proposed attenuation mechanism (i.e., 1,4-dioxane degradation)***
    a. Consistency between ^{14}C-based rate constant and the model-predicted rate constants – *successfully obtained at 5 of 9 sites where positive ^{14}C activity was observed*
    b. Consistency between biomarker abundance and model-predicted rate constants – *successfully obtained at 1 of 8 sites with model-predicted rate constants*
    c. Presence of favorable geochemical conditions – *included 7 of 10 sites in this study (and 6 of 9 sites where ^{14}C-based degradation was observed*
    d. Low levels of potentially inhibitory CVOCs – *included 7 of 10 sites in this study (and 7 of 9 sites where ^{14}C-based degradation was observed)*

As shown in **Table 5.8** summary, the data for these lines of evidence (particularly in the third category) were not always able to corroborate the ^{14}C assay results at some sites. However, aggregating the data did help identify which of these analyses provided more useful information. This is discussed further in the following subsections.

Table 5.8. Summary of Site-Specific Lines of Evidence for 1,4-Dioxane Attenuation at Project Field Sites

Site	1,4-D biodegradation in ^{14}C Assay?	Is 1,4-D biodegrading based on model predictions?	Does biomarker abundance explain model-estimated 1,4-D biodegradation rate?	Is model-estimated 1,4-dioxane biodegradation rate constant consistent with rate constants from ^{14}C assay?	Are ^{2}H and/or ^{13}C enriched along the flow path?	Are geochemical conditions supportive of 1,4-dioxane biodegradation?	Are inhibitory CVOCs present at low levels and/or decreasing with time/distance?
1	NO	YES	NO	NO	YES	YES	NO
2	YES (1 of 4 wells)	NO	NO	NO	NO*	YES	YES
3	YES (3 of 4 wells)	YES*	NO	YES	YES	YES	YES
4	YES (3 of 4 wells)	YES	NO	YES	YES	YES	YES
5	YES (2 of 4 wells)	YES	NO	NO	YES	YES	YES
6	YES (1 of 4 wells)	YES*	YES*	YES	YES*	NO	YES
7	YES (1 of 4 wells)	N/A	NO	NO*	NO*	NO*	NO*
8	YES (2 of 4 wells)	YES	NO	YES	YES	YES	YES
9	YES (3 of 4 wells)	YES	NO	NO*	YES	YES	YES
10	YES (2 of 4 wells)	YES	NO	YES	NO	NO*	NO

Note: Cells marked with * indicate lines of evidence where a more tentative categorization was made. This was typically due to variability within the site (different parameter values amongst the set of wells that were evaluated) and/or because uncertainty in the parameter values influence the interpretation. See individual site descriptions in Section 5.7 for more details.

5.8.1. Comparison of 1,4-Dioxane Biodegradation Rates

The results of the ^{14}C assay confirmed that the capacity for 1,4-dioxane biodegradation existed at 90% of the sites included in this study. This is considered strong evidence for 1,4-dioxane attenuation because the activity can be attributed to a distinct mechanism (biodegradation) due to the parallel testing of filter-sterilized groundwater to control for abiotic and other non-biological decay processes.

These data also suggest that 1,4-dioxane biodegradation capacity is relatively widespread, corroborating empirical data that suggests that 1,4-dioxane attenuation is occurring at a significant number of sites (e.g., Adamson et al., 2015). The site selection process may have influenced the prevalence observed during the current study (e.g., by selecting sites with favorable characteristics), but the 90% rate is encouraging evidence for 1,4-dioxane biodegradation capacity in the environment.

Within sites, ^{14}C-based 1,4-dioxane biodegradation capacity was observed at some but not all the wells that were sampled (i.e., 25% to 75% of the sampled wells). This finding suggests that activity may be localized to certain portions of a site where conditions are favorable for 1,4-dioxane degraders to establish an ecological niche.

The 1,4-dioxane biodegradation rate constants obtained from the ^{14}C assay spanned a wide range but were typically slow. This may explain why wells at some sites did not exhibit a statistically significant rate constant; rates for slower reactions are generally more challenging to quantify.

As noted previously, the ^{14}C assays are typically performed with groundwater that contains little or no solids or supplemental nutrients, such that the 1,4-dioxane rate constants likely underestimate in situ biodegradation rates. This hypothesis was tested by comparing the ^{14}C-derived rate constants with those generated during model calibration (**Figure 5.8.1**). The rate constants obtained from these two different approaches were reasonably consistent at 5 of the 9 sites that had sufficient data to evaluate. At sites where they were not consistent, the model-calibrated rate was typically much higher. This includes Site 1, where the model predicted relatively rapid 1,4-dioxane biodegradation (~0.3 per year) while no rate constant could be obtained using the ^{14}C assay. These data support the assumption that the rates obtained from the ^{14}C are generally consistent with predictions made using site concentration data but are likely more conservative.

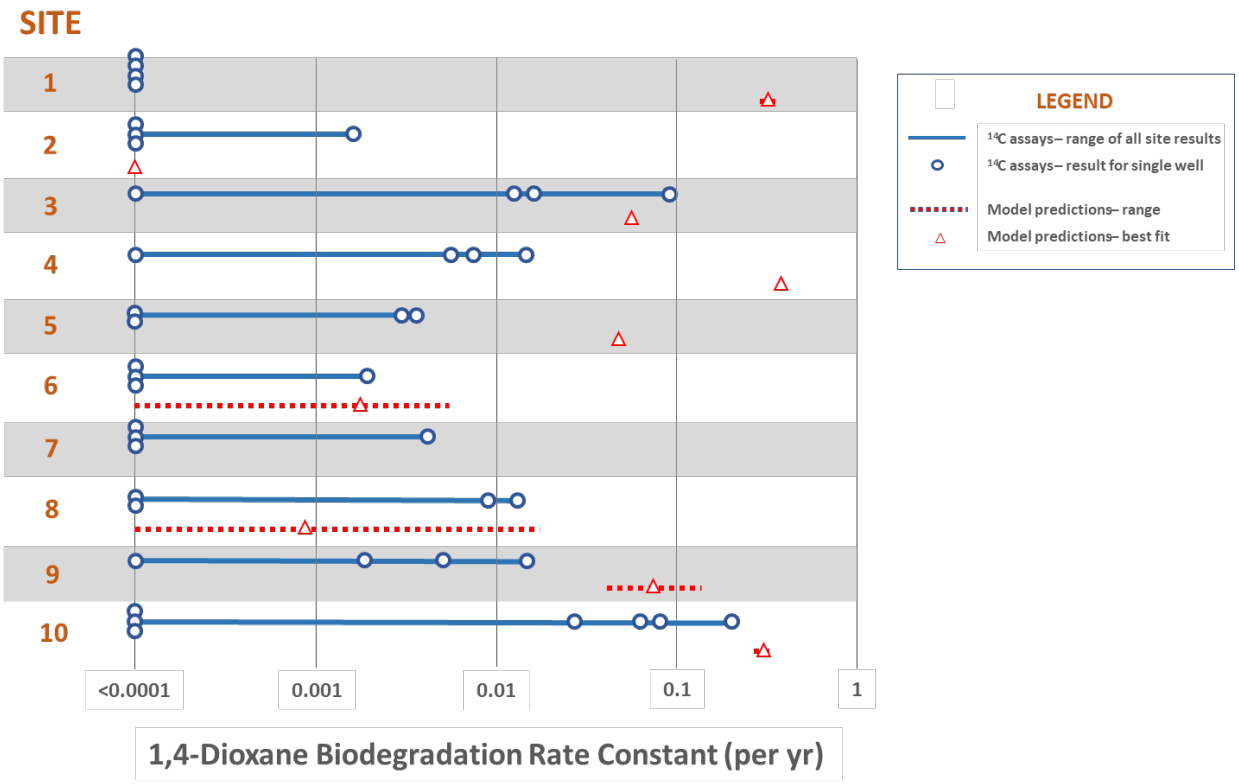


Figure 5.8.1. Comparison of 1,4-Dioxane Biodegradation Rate Constants Obtained From ^{14}C Assays (Blue) and Model Predictions (Red).
Note that no model-predicted rate constant could be obtained for Site 7, and ranges for model-predicted rate constants were not obtained for some other sites.

5.8.2. Influence of Geochemical Conditions of Observed 1,4-Dioxane Biodegradation Rates

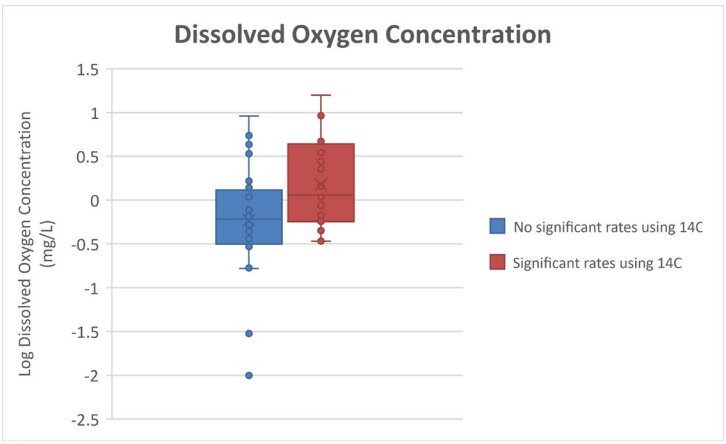
As noted above, at individual sites, 1,4-dioxane biodegradation using the ^{14}C assay and/or model predictions did not always align with expectations based on other site conditions. This includes the geochemical data that was obtained through a combination of lab and field analyses. Evaluating these data on a well-by-well basis is likely to encounter discrepancies, particularly for geochemical parameters that are evaluated using groundwater from long-screened monitoring wells. The samples may represent mixed groundwater from zones with different redox conditions, which can influence both the geochemical parameter values and abundance/activity of any 1,4-dioxane degraders that are present at that location.

To compensate for these potential discrepancies, geochemical data from all wells where positive ^{14}C-based degradation was obtained were aggregated and log transformed, and then compared to data from all wells where ^{14}C activity was absent. For several key geochemical parameters, the resulting data are displayed as box plots (**Figure 5.8.2**) and discussed below

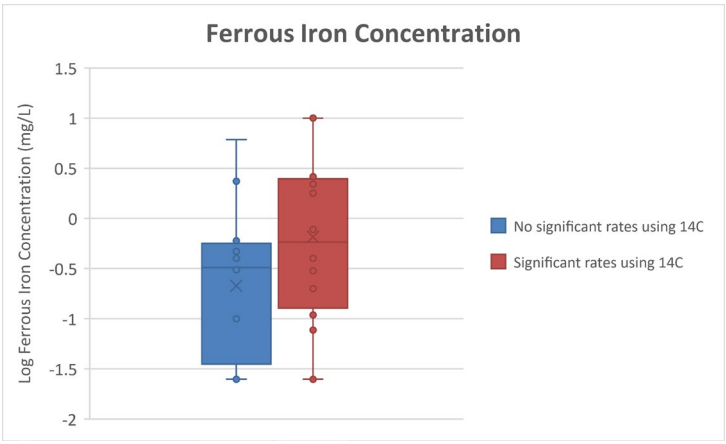
Dissolved Oxygen: The median DO concentration at wells with a significant 1,4-dixoane rate constant (1.15 mg/L) was higher than the median DO concentration at wells where no rate constant could be obtained (0.61 mg/L). This was consistent with expectations that sites with higher dissolved oxygen would be beneficial for promoting activity of the presumed aerobic biodegradation pathway for 1,4-dioxane. This difference was significant at the 90% level but did not meet the 95% significance level (Wilcoxon Rank Sum test; $p = 0.052$).

Ferrous Iron: The median Fe(II) concentration at wells with a significant 1,4-dioxane rate constant based on the ^{14}C assay (0.58 mg/L) was higher than the median value at those wells where 1,4-dioxane degradation could not be established (0.32 mg/L). This difference was significant at the 90% level but did not meet the 95% significance level (Wilcoxon Rank Sum test; $p = 0.097$). While Fe(II) is typically associated with an anaerobic process (iron reduction), the ^{14}C data did suggest that active iron cycling was beneficial for 1,4-dioxane degradation.

Methane: Similar to the influence of Fe(II), the median methane concentration at wells with a significant 1,4-dioxane degradation rate constant (21.8 µg/L) was higher than the median values at wells where no rate constant could be obtained (5.9 µg/L). This difference was not statistically significant (Wilcoxon Rank Sum test; $p=0.63$), reflecting the wide variability in methane levels observed in both categories. The presence of methane suggests that geochemical conditions are generally favorable for biological activity (e.g., pH, organic carbon) although oxygen itself may be limiting.



A



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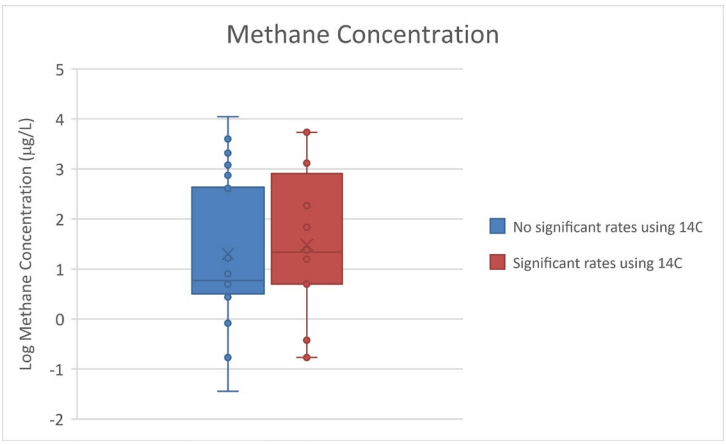


C

Figure 5.8.2. Influence of Geochemical Indicator Parameters on Observed 1,4-Dioxane Biodegradation Rates Constant from ^{14}C Assays.

Y-axis shows log concentration of individual parameters (Panel A = dissolved oxygen (mg//L); Panel B = ferrous iron (µg/L); Panel C = methane (µg/L)). Blue data points represent concentration distribution at wells where no significant 1,4-dioxane rate could be established using ^{14}C assay (box shows 25th and 75th percentile; top and bottom whiskers show min and max). Red data points represent concentration distribution at wells where significant 1,4-dioxane rate could be established using ^{14}C assay.

5.8.3. Influence of Initial 1,4-Dioxane Concentrations on Observed 1,4-Dioxane Biodegradation Rates

The 1,4-dioxane concentrations measured during commercial lab analysis of field samples were used to determine if the initial 1,4-dioxane concentration influenced the 1,4-dioxane biodegradation capacity measured in ^{14}C assays. **Figure 5.8.3** shows the 1,4-dioxane concentration data aggregated into two groups. Locations where a significant 1,4-dioxane rate constant could be established using the ^{14}C assay were associated with higher initial 1,4-dioxane concentrations (median = 135 µg/L) than locations where no activity could be established (median = 14.4 µg/L). This difference was not statistically significant (Wilcoxon Rank Sum test; p=0.17) which reflects the variability in the data and the possibility that different mechanisms for 1,4-dioane biodegradation may have occurred at this set of sites. For example, the presence of higher initial 1,4-dioxane concentrations would be beneficial for organisms that can use 1,4-dioxane as a source of carbon/energy. These data generally support a hypothesis that this pathway was active at some locations, although the lack of statistical significance is consistent with other site data (e.g., biomarkers) that suggest that the abundance of identifiable 1,4-dioxane metabolizers is limited at this set of sites. Organisms that co-metabolize 1,4-dioxane would be expected to be much less influenced by initial 1,4-dioxane concentrations.

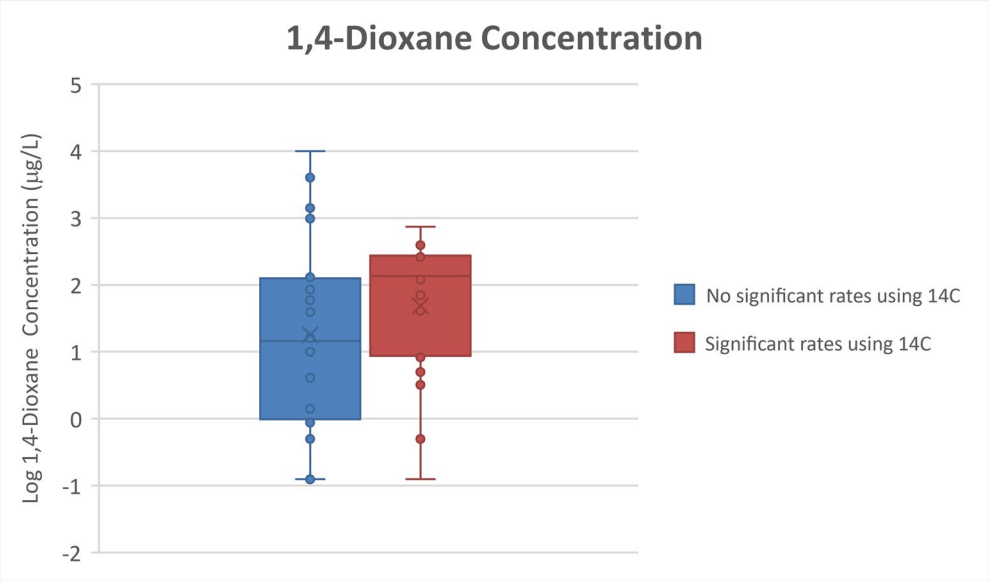


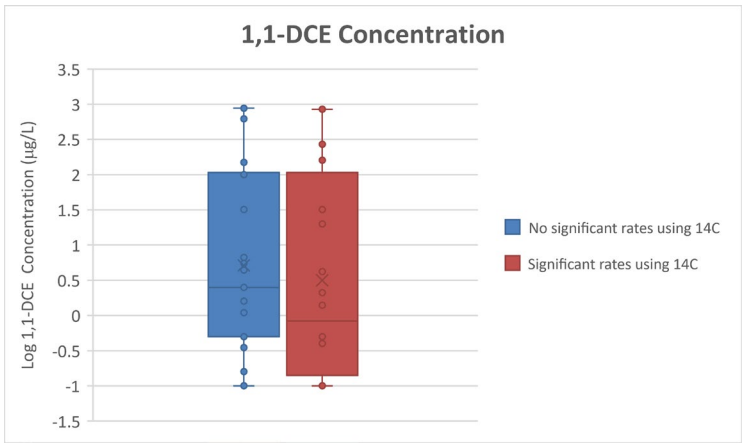
Figure 5.8.3. Influence of Initial 1,4-Dioxane Concentration on Observed 1,4-Dioxane Biodegradation Rates Constant from ^{14}C Assays.

Y-axis shows log concentration of 1,4-dioxane concentration (µg/L) measured in field samples. Blue data points represent concentration distribution at wells where no significant 1,4-dioxane rate could be established using ^{14}C assay (box shows 25th and 75th percentile; top and bottom whiskers show min and max). Red data points represent concentration distribution at wells where significant 1,4-dioxane rate could be established using ^{14}C assay.

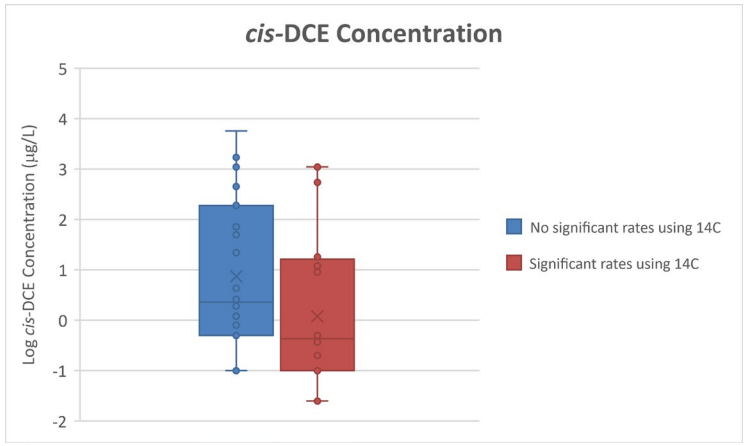
5.8.4. Influence of Site CVOC Concentrations on Observed 1,4-Dioxane Biodegradation Rates

The influence of potentially inhibitory CVOCs was also evaluated by aggregating and log-transforming the initial concentration of several different individual CVOCs, and then comparing the concentration distributions from all wells where ^{14}C degradation was obtained to data from all wells where ^{14}C activity was absent.

The results for 1,1-DCE, cis-DCE, and TCE are shown in **Figure 5.8.4**. In general, the comparisons supported the hypothesis that higher concentrations of these compounds negatively impacted 1,4-dioxane degradation capacity. For example, the median 1,1-DCE concentration at wells with a significant 1,4-dioxane rate constant (0.84 μg/L) was lower than the median 1,1-DCE concentrations at wells where no rate constant could be established (2.5 μg/L). A similar pattern was observed for both cis-DCE and TCE. However, these differences were not statistically significant (Wilcoxon Rank Sum test; $p>0.05$ in all cases). This may be due to the relatively low concentrations of CVOCs that were present at many of these wells. Similarly, 1,4-dioxane degradation capacity was established even at sites where CVOC concentrations were well above 100 μg/L (e.g., Site 10), suggesting that 1,4-dioxane degradation may still proceed at sites with elevated CVOC levels.



A



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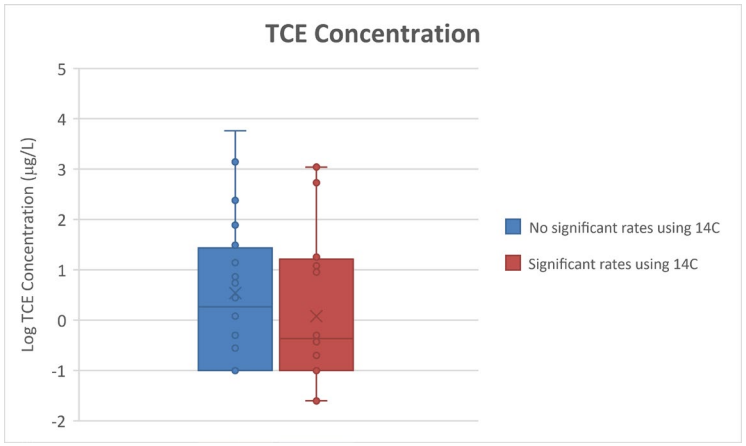


C

Figure 5.8.4. Influence of Initial CVOC Concentrations on Observed 1,4-Dioxane Biodegradation Rates Constant from ^{14}C Assays.

Y-axis shows log concentration (µg/L) of individual parameters (Panel A = 1,1-DCE; Panel B = cis-DCE; Panel C = TCE). Blue data points represent concentration distribution at wells where no significant 1,4-dioxane rate could be established using ^{14}C assay (box shows 25th and 75th percentile; top and bottom whiskers show min and max). Red data points represent concentration distribution at wells where significant 1,4-dioxane rate could be established using ^{14}C assay.

To further evaluate the potential influence of CVOC concentrations, the same comparison was performed using the ratios of 1,4-dioxane/1,1-DCE concentrations **(Figure 5.8.5)**. In this case, the assumption is that higher ratios would be favorable for 1,4-dioxane degradation and minimize the effects of various inhibition mechanisms, particularly for 1,1-DCE which has been identified as a major inhibitor (e.g., Zhang et al., 2016). The results of this evaluation show that this 1,4-dioxane/1,1-DCE concentration ratio was higher at wells with ^{14}C based 1,4-dioxane degradation capacity (median ratio = 9.1) than at wells where ^{14}C based rate constant could not be established (median = 2.3). While the difference was not statistically significant (Wilcoxon Rank Sum test; p=0.13), the results do emphasize that 1,4-dioxane concentrations are generally higher than 1,1-DCE concentrations at these sites, which helps minimize potential inhibitory effects.

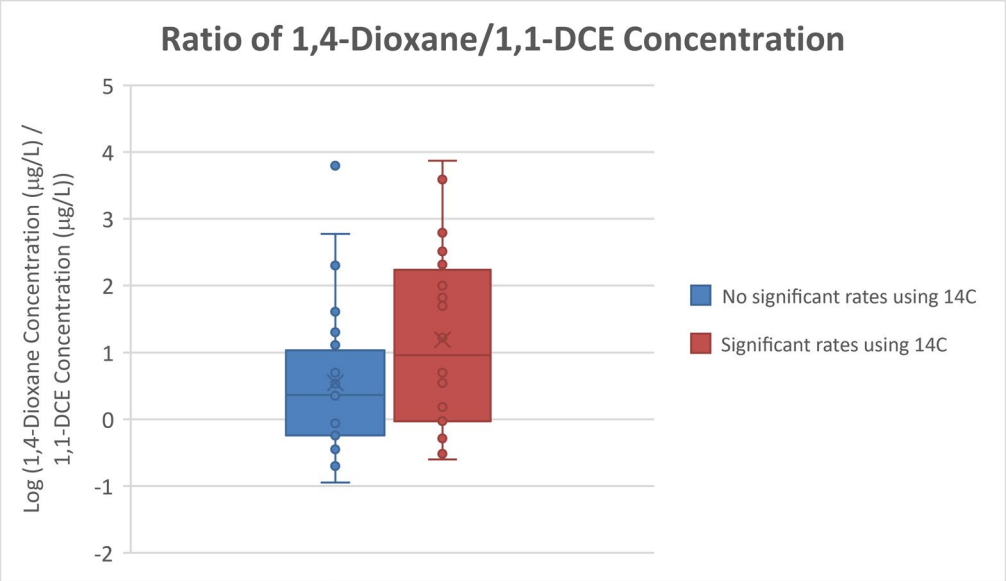


Figure 5.8.5. Influence of Ratio of 1,4-Dioxane/1,1-DCE Concentrations on Observed 1,4-Dioxane Biodegradation Rates Constant from ^{14}C Assays.

Y-axis shows ratio of log-transformed 1,4-dioxane concentration (µg/L) divided by 1,1-DCE concentration (µg/L) in same well. Blue data points represent concentration distribution at wells where no significant 1,4-dioxane rate could be established using ^{14}C assay (box shows 25th and 75th percentile; top and bottom whiskers show min and max). Red data points represent concentration distribution at wells where significant 1,4-dioxane rate could be established using ^{14}C assay.

5.8.5. Influence of Biomarker Abundance on Observed 1,4-Dioxane Biodegradation Rates

Like the parameter data associated with the other lines of evidence, the biomarker abundances were aggregated to see if they influenced observations of 1,4-dioxane degradation capacity from the ^{14}C assays. In this case, the focus was on potential biomarkers for cometabolism of 1,4-dioxane (SCAM, PHE, RDEG, RMO, SMMO, *prmA*) because the potential biomarkers for direct metabolism (DXMO/THFMO, ALDH) were only detected at one site. Note that *prmA* is potentially a biomarker for direct metabolism and cometabolism of 1,4-dioxane. All samples from

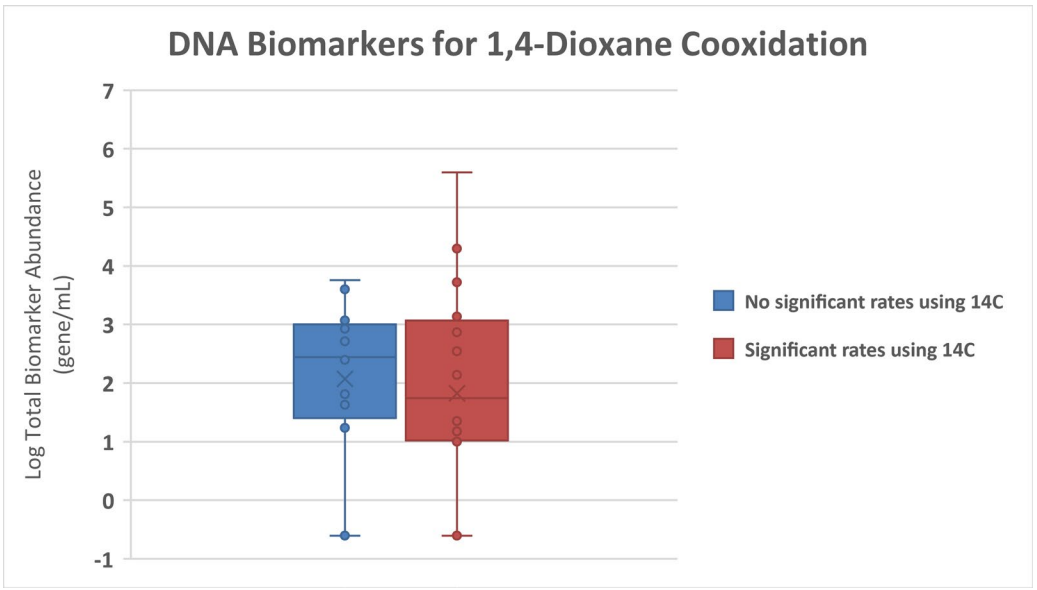
Sites 1 – 6 were analyzed for prmA, but it was only detected in 2 samples (Well 1 at Site 1, Well 4 at Site 3).

The resulting distributions are shown in **Figure 5.8.6** for DNA-based qPCR testing (Panel A) and RNA-based qPCR. In both cases, the presence of higher levels of these biomarkers did not have a positive influence on 1,4-dioxane degradation capacity. As noted previously, rate predictions based on lab-derived kinetic correlations with biomarker abundance generally underestimated the rates predicted by the fate and transport model (MNA Rate Constant Estimator) and/or the rates predicted by the ^{14}C assay. Bi-variate plots of the rate constants and the sum of these potential biomarkers for cometabolism of 1,4-dioxane also did not indicate a clear relationship (**Figure 5.8.7**). However, the genes themselves were relatively prevalent, with detection rates ranging between 25% (RMO) and 75% (PHE) of all field groundwater samples. SCAM was detected in 33% of the entire set of wells that were sampled, including at least one well at 90% of the sites.

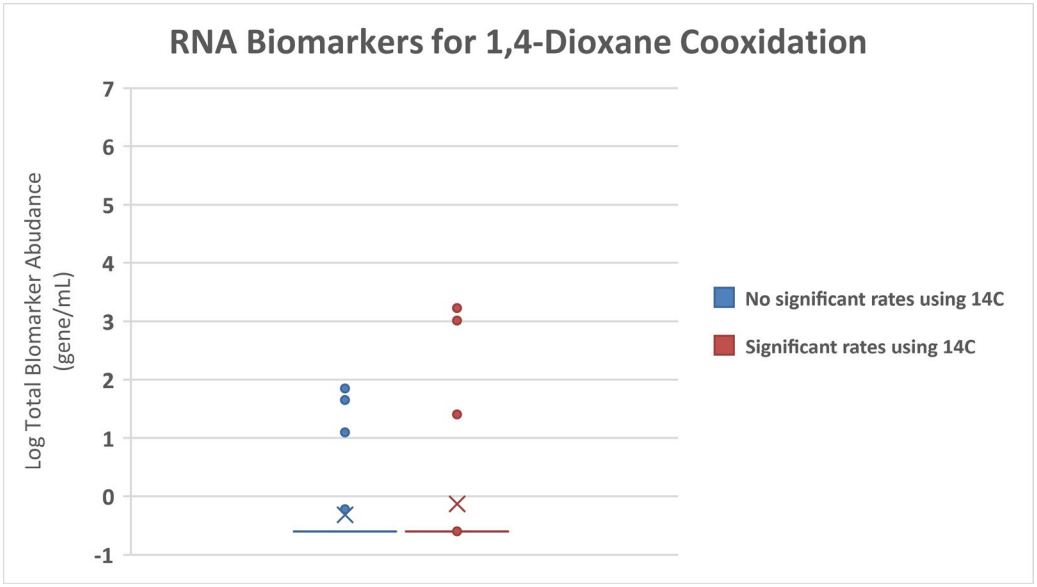
It is also notable that RNA-based biomarkers were less prevalent than DNA-based biomarkers for 1,4-dioxane cometabolism. While both target functional genes, the latter attempt to quantify gene transcripts and thus should be more reflective of activity of the targeted enzymes. The use of RNA to assay 1,4-dioxane activity has been documented in a previous study (Gedalanga et al., 2014). However, there is some uncertainty about the quality and longevity of RNA obtained from field samples (ITRC, 2013), which may be reflected in the lack of detections in the current dataset.

In addition, DXMO/THFMO and ALDH were detected infrequently; DNA-based qPCR targets were present in 3 of 54 samples that were analyzed, all of which were from the same site (Site 3). These biomarkers have been used in the past to demonstrate activity in controlled lab testing of field samples (e.g., Li et al., 2014) but it is still not clear if in situ measurements also reflect activity and/or capacity. Similarly, the prevalence of organisms with these genes at environmental field sites has yet to be fully documented. This study found these genes in groundwater samples from 1 of 10 sites, while the Li et al. (2014) study found them in soils from 5 different sites. However, the latter only reported data from the end of 3- to 5-month microcosm tests, meaning that extended incubation and/or the presence of soil likely influenced the observed abundance. As noted above, *prmA* was also infrequently detected (2 of 24 samples that were analyzed). This gene encodes for propane monooxygenases, some of which have been shown to degrade 1,4-dioxane through a metabolic pathway.

Collectively, the data suggest that the biomarkers were—at best—a secondary line of evidence for 1,4-dioxane degradation capacity and should not be viewed as sufficient, standalone evidence without corroborating evidence from other analyses.



A



B

Figure 5.8.6. Influence of Potential Biomarkers for 1,4-Dioxane Cometabolism on Observed 1,4-Dioxane Biodegradation Rates Constant from ^{14}C Assays.

Y-axis shows log concentration of sum of all potential biomarkers for 1,4-dioxane cometabolism (SCAM, RDEG, RMO, PHE, SMMO) (Panel A = DNA based; Panel B = RNA based). Blue data points represent concentration distribution at wells where no significant 1,4-dioxane rate could be established using ^{14}C assay (box shows 25th and 75th percentile; top and bottom whiskers show min and max). Red data points represent concentration distribution at wells where significant 1,4-dioxane rate could be established using ^{14}C assay.

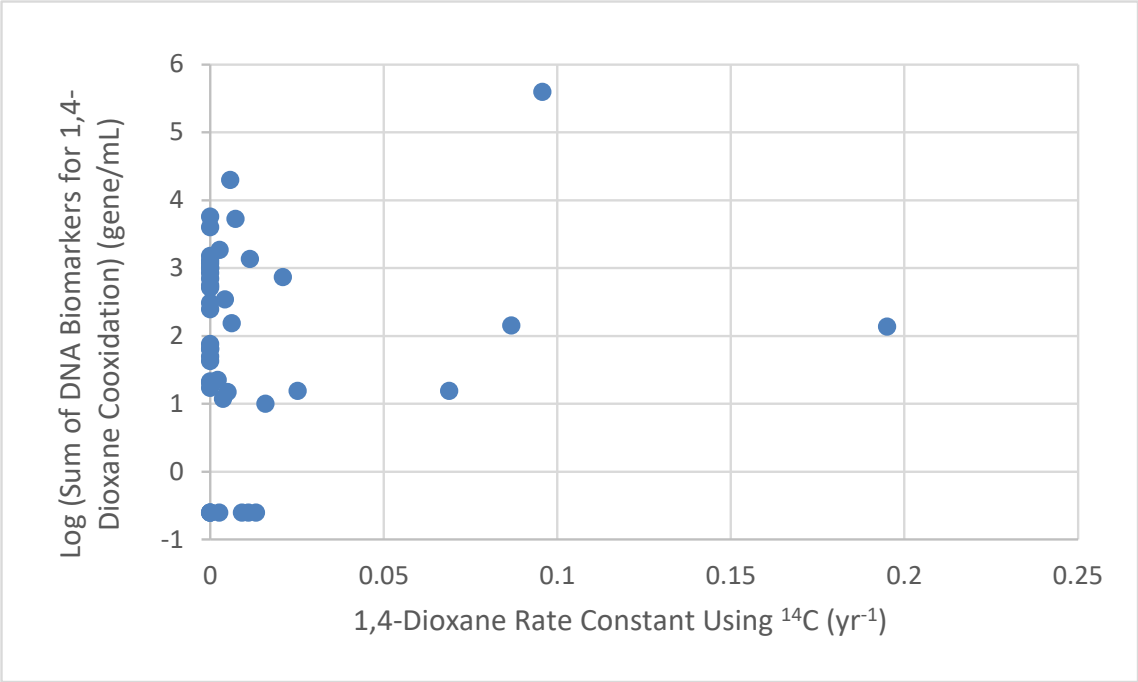
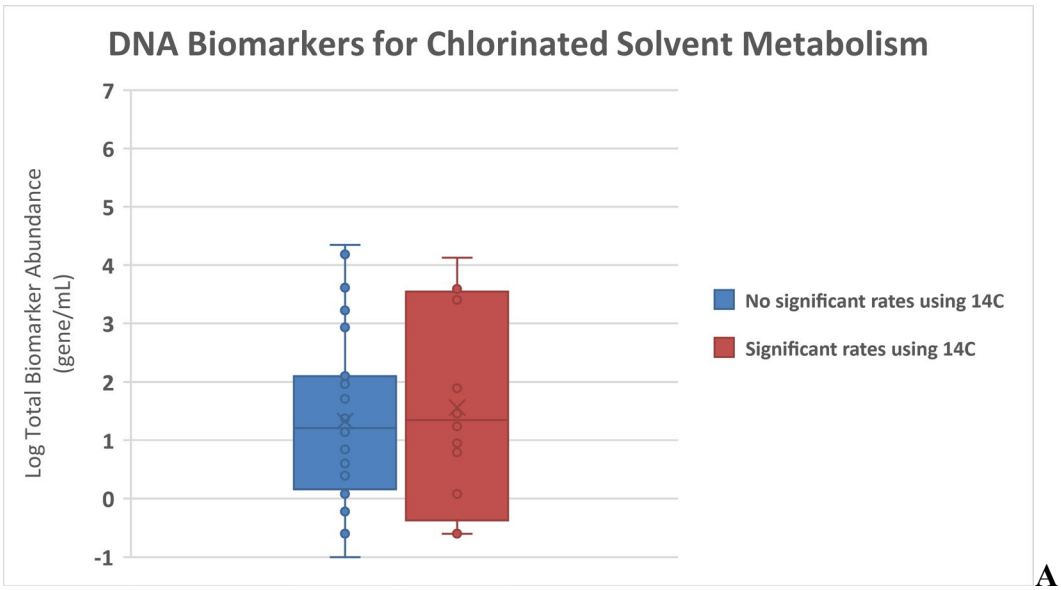


Figure 5.8.7. Comparison of DNA Biomarkers for 1,4-Dioxane Cometabolism and 1,4-Dioxane Rate Constants Obtained Using ^{14}C Assay.
No clear relationship could be established.

Data associated with biomarkers for chlorinated solvent degradation were also aggregated and evaluated similarly. In this case, the distributions shown in **Figure 5.8.8** for both DNA-based qPCR testing (Panel A) and RNA-based testing (Panel B) both suggest no apparent relationship with 1,4-dioxane degradation. In other words, the median values at the wells where a 1,4-dioxane rate constant was obtained using the ^{14}C assay were similar to the values at wells where no rate constant could be established. In part, this reflects the orders of magnitude differences in biomarker abundance that were observed across this set of wells (**Figure 5.8.8**). DHC and DHBt were widely occurring (79% and 50% detection rates for the genes; 85% and 81% detection rates for the gene transcripts), while the DCA gene (2%) and gene transcript (0%) were infrequently detected. These data suggest widespread dechlorinating capacity, including organisms capable of dechlorinating multiple different types of chlorinated parent compounds and by-products. This is despite the co-occurrence of many monooxygenases in these same samples, suggesting that mixed redox conditions may be present in the areas where these monitoring wells are screened. While the presence of biomarkers for dechlorination could not be correlated to 1,4-dioxane degradation capacity, they may help alleviate inhibition by co-occurring chlorinated solvents. In fact, the presence of significantly higher levels of RNA-based biomarkers (relative to genes themselves) suggests that these dechlorinating organisms may be active at the field sites. In the case of chlorinated solvent biomarkers, there was a more reasonable association between the abundance of genes and gene transcripts in groundwater samples (**Figure 5.8.9**) than was observed for 1,4-dioxane.



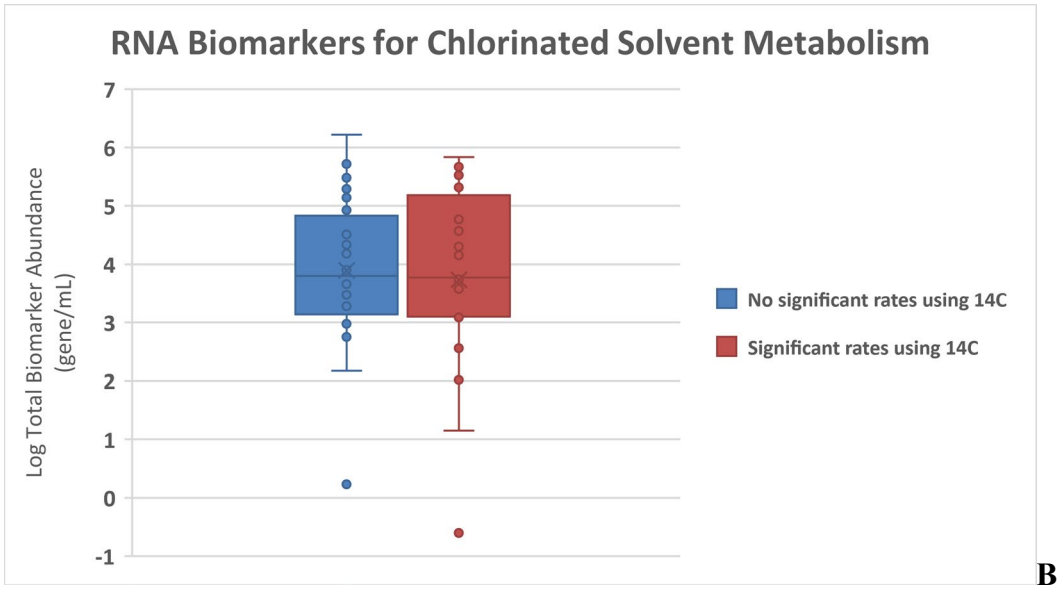


Figure 5.8.8. Influence of Potential Biomarkers for Chlorinated Solvent Degradation on Observed 1,4-Dioxane Biodegradation Rates Constant from ^{14}C Assays.

Y-axis shows log concentration (gene/mL) of sum of all potential biomarkers for chlorinated solvent degradation (DHC, DHBt, DCA) (Panel A = DNA based; Panel B = RNA based). Blue data points represent concentration distribution at wells where no significant 1,4-dioxane rate could be established using ^{14}C assay (box shows 25th and 75th percentile; top and bottom whiskers show min and max). Red data points represent concentration distribution at wells where significant 1,4-dioxane rate could be established using ^{14}C assay.

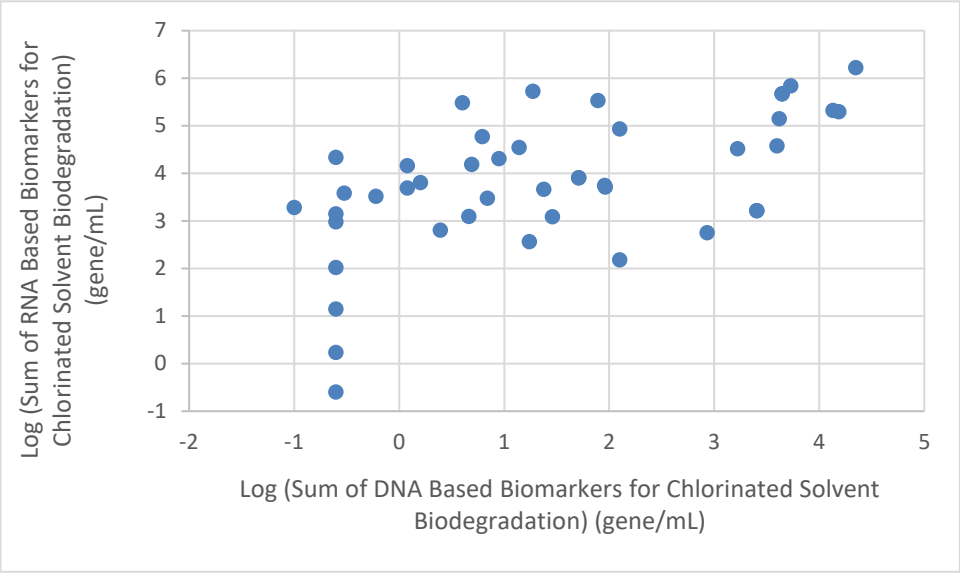


Figure 5.8.9. Comparison of DNA Based and RNA Based Biomarkers for Chlorinated Solvent Degradation.

5.9 DECISION FRAMEWORK

A series of Decision Frameworks were developed to aid in the evaluation of MNA as a remedy for 1,4-dioxane, chlorinated ethanes, and chlorinated ethenes. The approach was based on the successful development of a decision framework for MNA of chlorinated ethenes from ESTCP ER-201129 ("Development and Validation of a Quantitative Framework and Management Expectation Tool for the Selection of Bioremediation Approaches at Chlorinated Solvent Sites"). Note that framework for chlorinated ethenes was included in the original BioPIC tool that was also developed for ESTCP ER-201129, and it was built on a lines of evidence approach that was consistent with USEPA guidance on MNA for chlorinated solvents (e.g., USEPA, 1998; USEPA, 1999). Specifically, the goal is to establish whether the first line of evidence for MNA has been achieved (i.e., historical data that demonstrate a clear and meaningful trend of decreasing contaminant mass and/or concentration over time at appropriate monitoring or sampling points" (USEPA, 1999)), and then guide the users on how to collect data that will serve as secondary and tertiary lines of evidence for MNA.

For the current project, a similar lines of evidence approach was developed, using information from literature and project-specific data to identify the relevant lines of evidence. While these lines of evidence differ slightly depending on the class of compounds, they generally included the following:

- Is the compound being degraded based on model predictions?
- Does biomarker abundance explain the model-predicted degradation rate?
- Do isotope data suggest that the compound is being degraded?
- Is there confirmatory evidence for degradation based on lab-based studies (e.g., ^{14}C assays)?
- Are geochemical conditions supportive of the targeted degradation pathways?
- Are other biomarkers for biodegradation present?
- Are other compounds present that would be expected to inhibit the targeted degradation pathway?

For each class of compounds, these elements were then incorporated into a Decision Framework that can be visualized as a flowchart. A copy of the flowcharts is included in this section for 1,4-dioxane (**Figure 5.9.1**), chlorinated ethanes (**Figure 5.9.2)**, and chlorinated ethenes (**Figure 5.9.3**). These flowcharts were then converted into a "guided tour" within the Microsoft Excel-based BioPIC tool. This updated version of BioPIC is one of the deliverables for this project, and it contains detailed explanations of the decision criteria as well as other help text (see **Appendix B** for BioPIC User's Guide).

As described earlier, the Decision Framework for 1,4-dioxane was applied to each of the 10 sites where field data were collected as part of this project. It is important to note that even when several lines of evidence for 1,4-dioxane attenuation are positive at a particular site, it does not necessarily imply that all lines of evidence will necessarily be met. In part, this reflects the current limitations in some of our diagnostic tools, including the use of groundwater from long-screened monitoring wells to evaluate degradation capacity and geochemical conditions that may be localized to specific intervals within an aquifer.

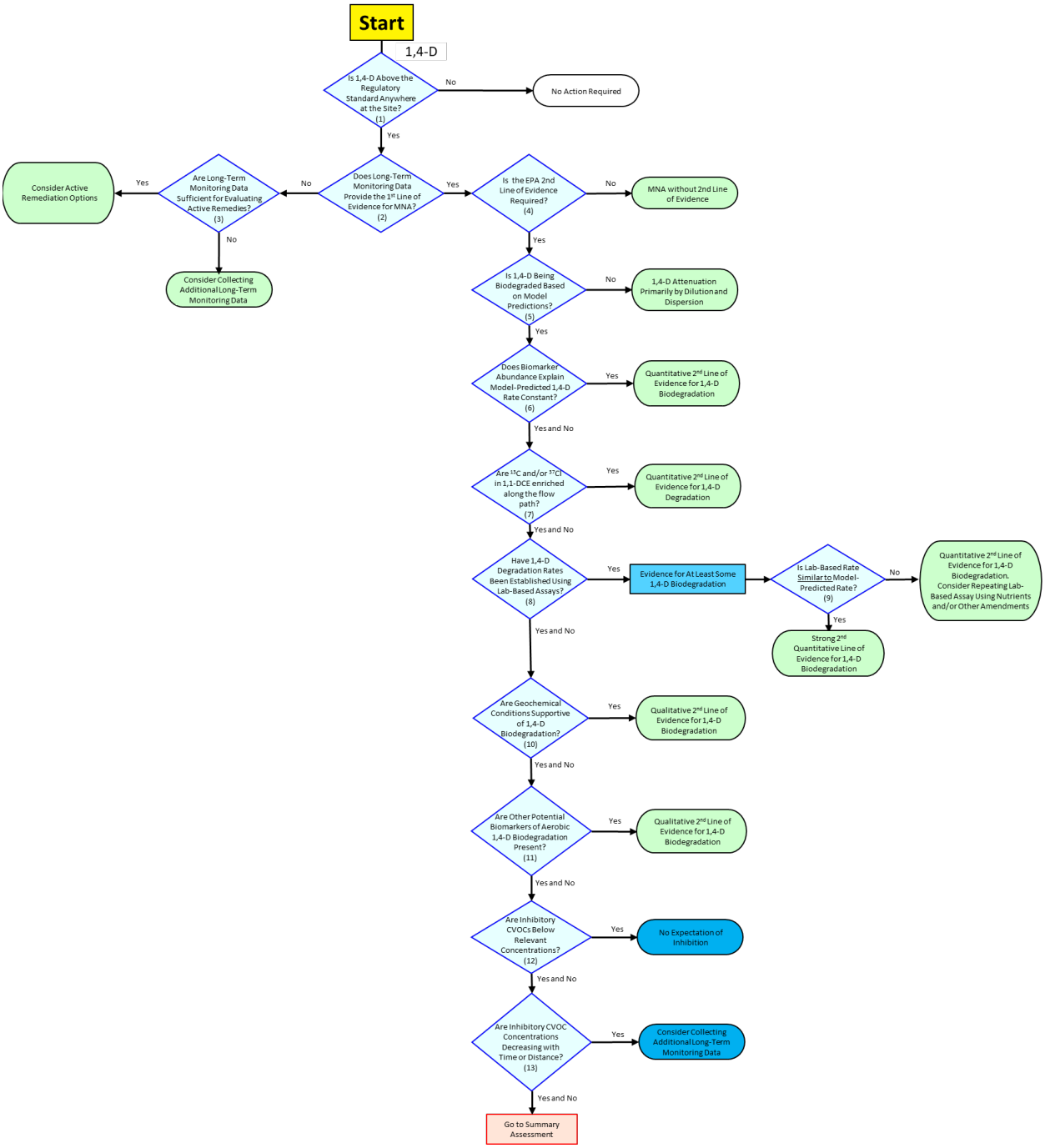


Figure 5.9.1. Decision Framework for 1,4-Dioxane.
This flowchart was coded into the updated BIOPIC tool that was one of the project deliverables.

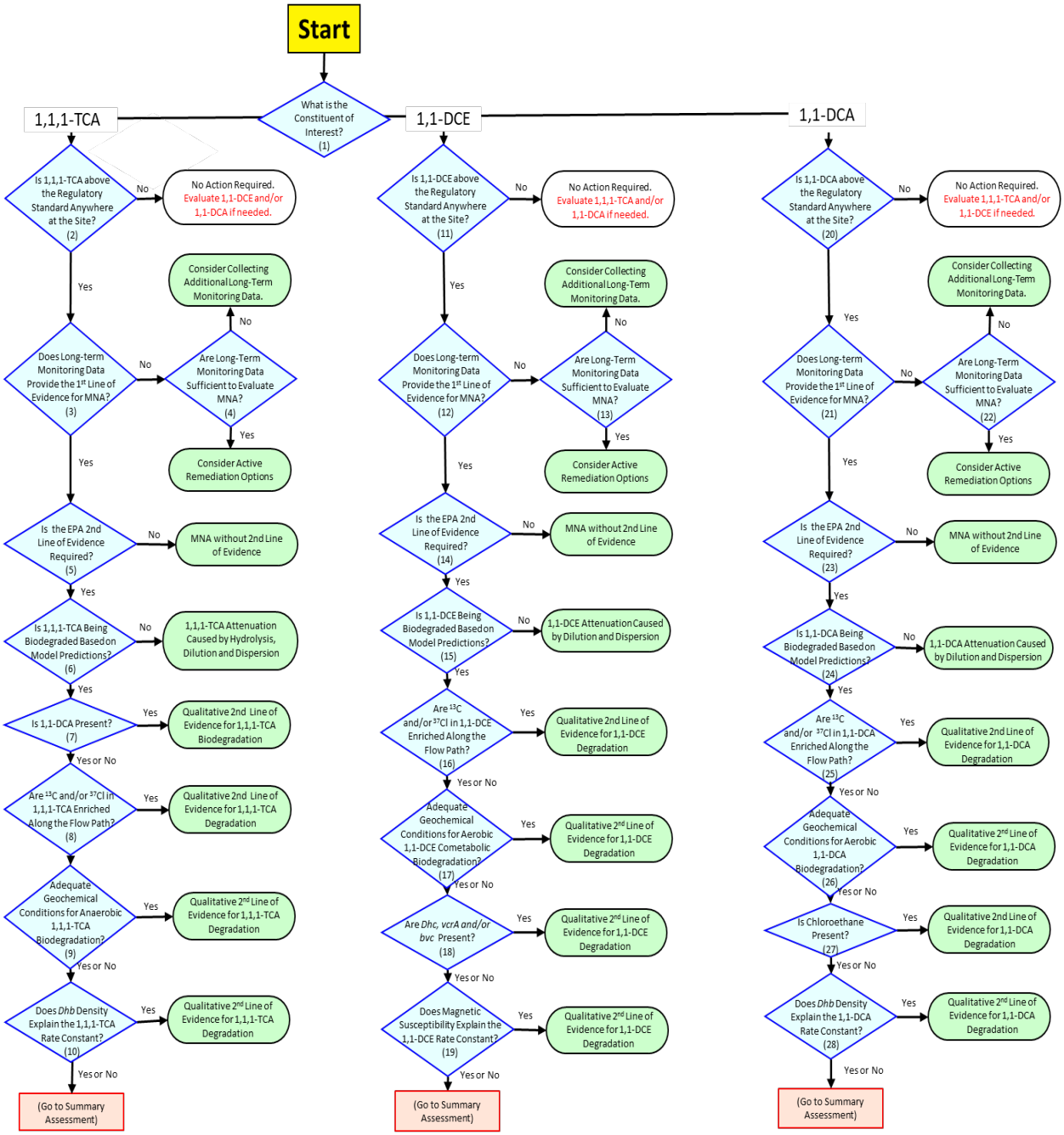


Figure 5.9.2. Decision Framework for Chlorinated Ethanes

1,1-DCE is a chlorinated ethene but is included in the decision framework for chlorinated ethanes because it is a key degradation product of the parent compound 1,1,1-TCA. This flowchart was coded into the updated BIOPIC tool that was one of the project deliverables.

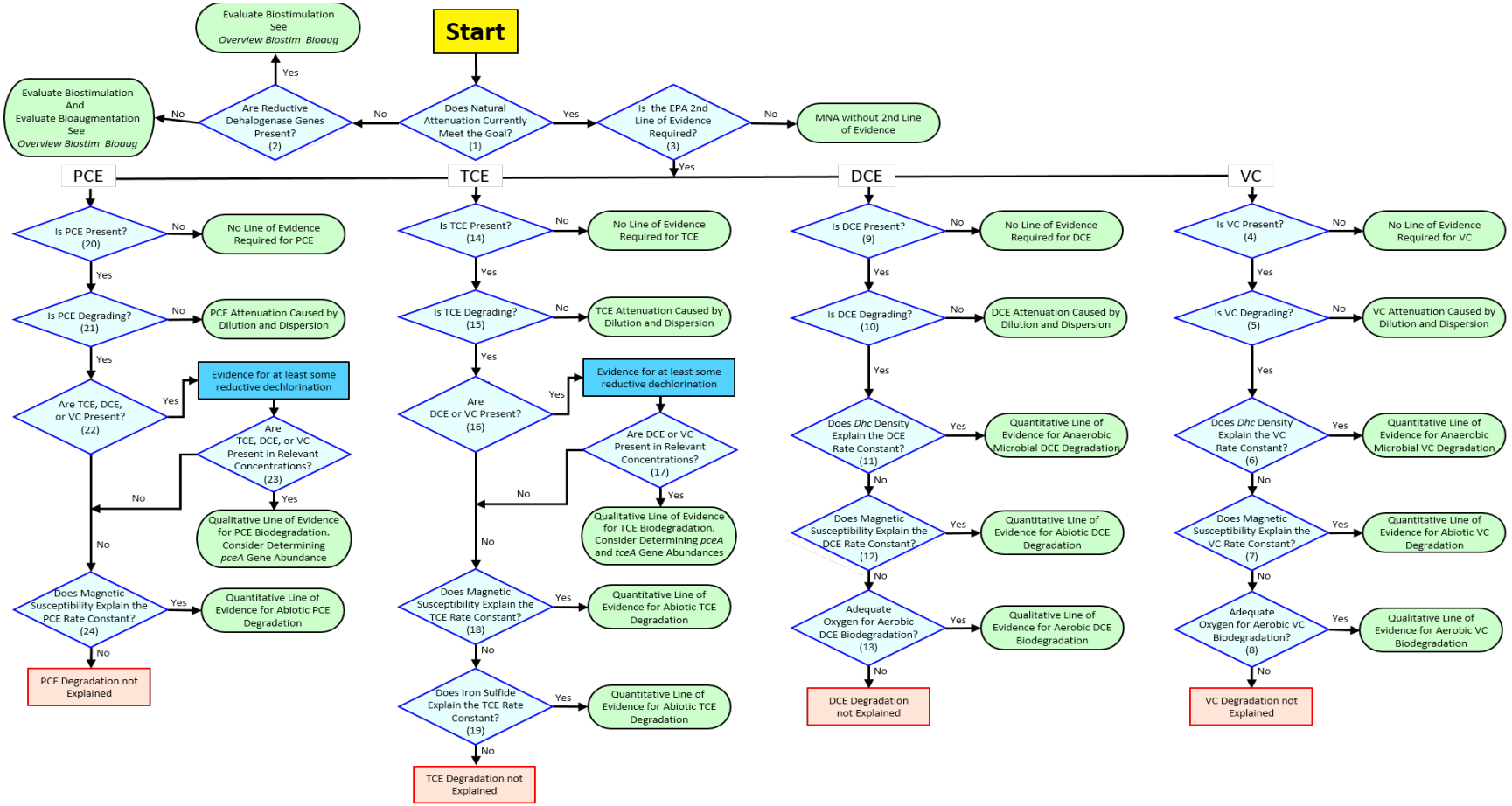


Figure 5.9.3. Decision Framework for Chlorinated Ethenes.

This flowchart was created as part of a previous ESTCP project (ER-201129) and was transferred into the updated BIOPIC tool that was one of the project deliverables.

6.0 PERFORMANCE ASSESSMENT

A summary of the performance objectives for this demonstration was presented in Section 3. This section includes a further assessment of technology performance based on the quantitative data presented in Section 5 and qualitative information collected during project development, as needed. The evaluation of each individual performance objective is discussed below.

6.1 Modify BIOCHLOR (or develop new but similar fate and transport model) to Account for Biological Degradation of 1,4-Dioxane

Success Criteria Achieved? **YES**

The project team develop a new software tool (titled "MNA Rate Constant Estimator") to serve as a simple fate and transport model for determining 1,4-dioxane rate constants and predicting source and plume behavior over time. It represents an improvement over the original platform that was going to be used (BIOCHLOR) because it is more compatible with newer versions of Microsoft Excel.

As described in Section 3, success criteria were based on incorporating the relevant processes for 1,4-dioxane natural attenuation into the model. This was accomplished using an analytical solution for solute transport equations based on advection, dispersion, linear equilibrium adsorption, and biological degradation of 1,4-dioxane (see User's Guide in **Appendix C** for more information). A screenshot of the model interface is shown in **Figure 6.1** to highlight how these processes were incorporated.

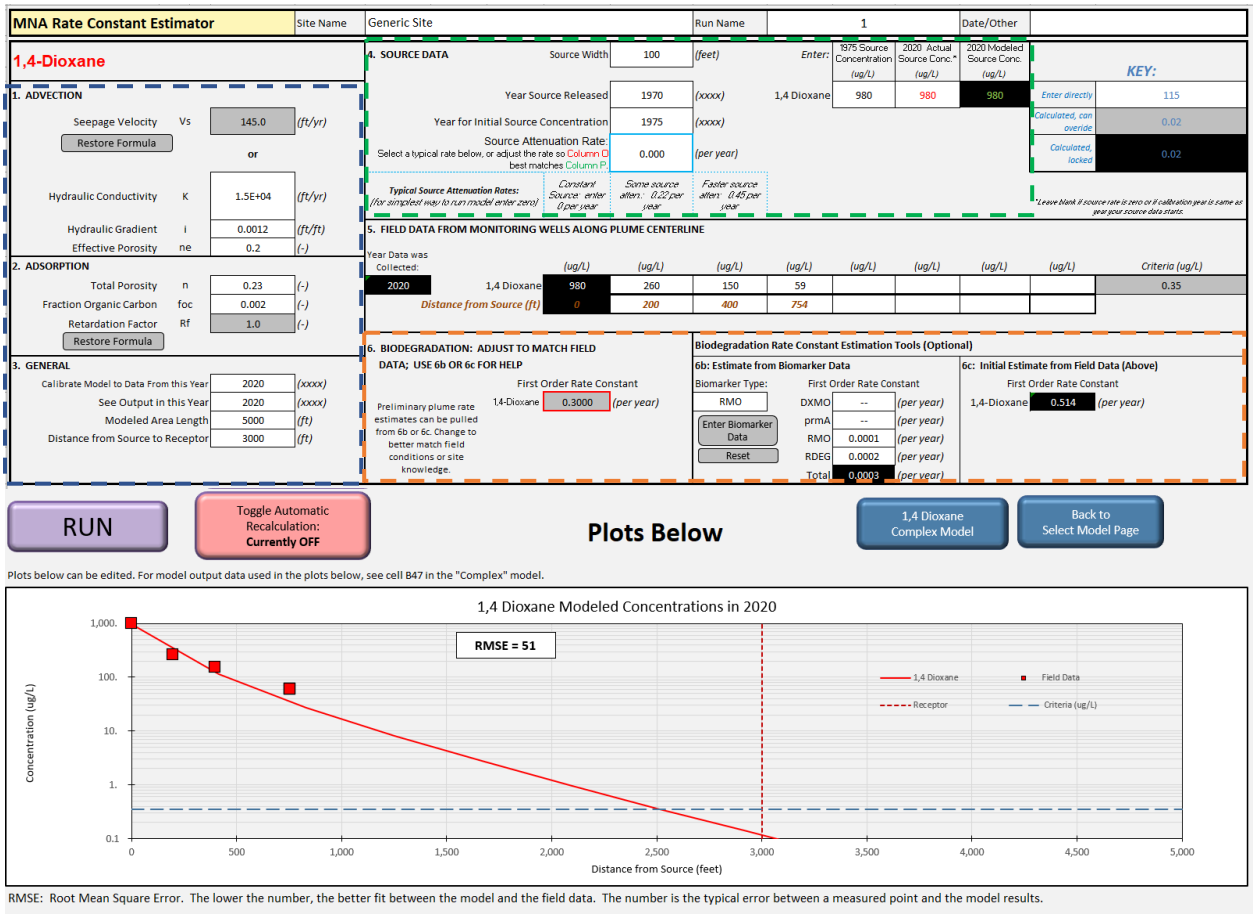


Figure 6.1. Interface for the Simple 1,4-Dioxane Model in the "MNA Rate Constant Estimator".

Blue box shows input parameters associated with modeling advection/dispersion/sorption. Green box shows input parameters associated with source decay. Orange box shows input/calibration parameter associated with 1,4-dioxane biodegradation based on aerobic pathway.

6.2 Modify BIOCHLOR (or develop new but similar fate and transport model) to Account for Biological and Abiotic Degradation of 1,1,1-TCA

Success Criteria Achieved? **YES**

As with 1,4-dioxane, the new software tool (MNA Rate Constant Estimator) is able to model processes that were not previously captured by the software that served as inspiration for this project (BIOCHLOR). In additional to mathematically modeling physical migration (advection, dispersion, and sorption), it can model the biological degradation of 1,1,1-TCA to 1,1-DCA and then the subsequent biodegradation of 1,1-DCA to chloroethane and ethane, as well as the abiotic degradation of 1,1,1-TCA to acetic acid and 1,1-DCE, and then the subsequent biological degradation of 1,1-DCE to vinyl chloride, and the subsequent biological degradation of vinyl chloride to ethene.

As with 1,4-dioxane, this involved coding analytical solutions of 3-D solute transport equations containing the relevant processes. A screenshot of the model interface is shown in **Figure 6.2** to highlight how these processes were incorporated.

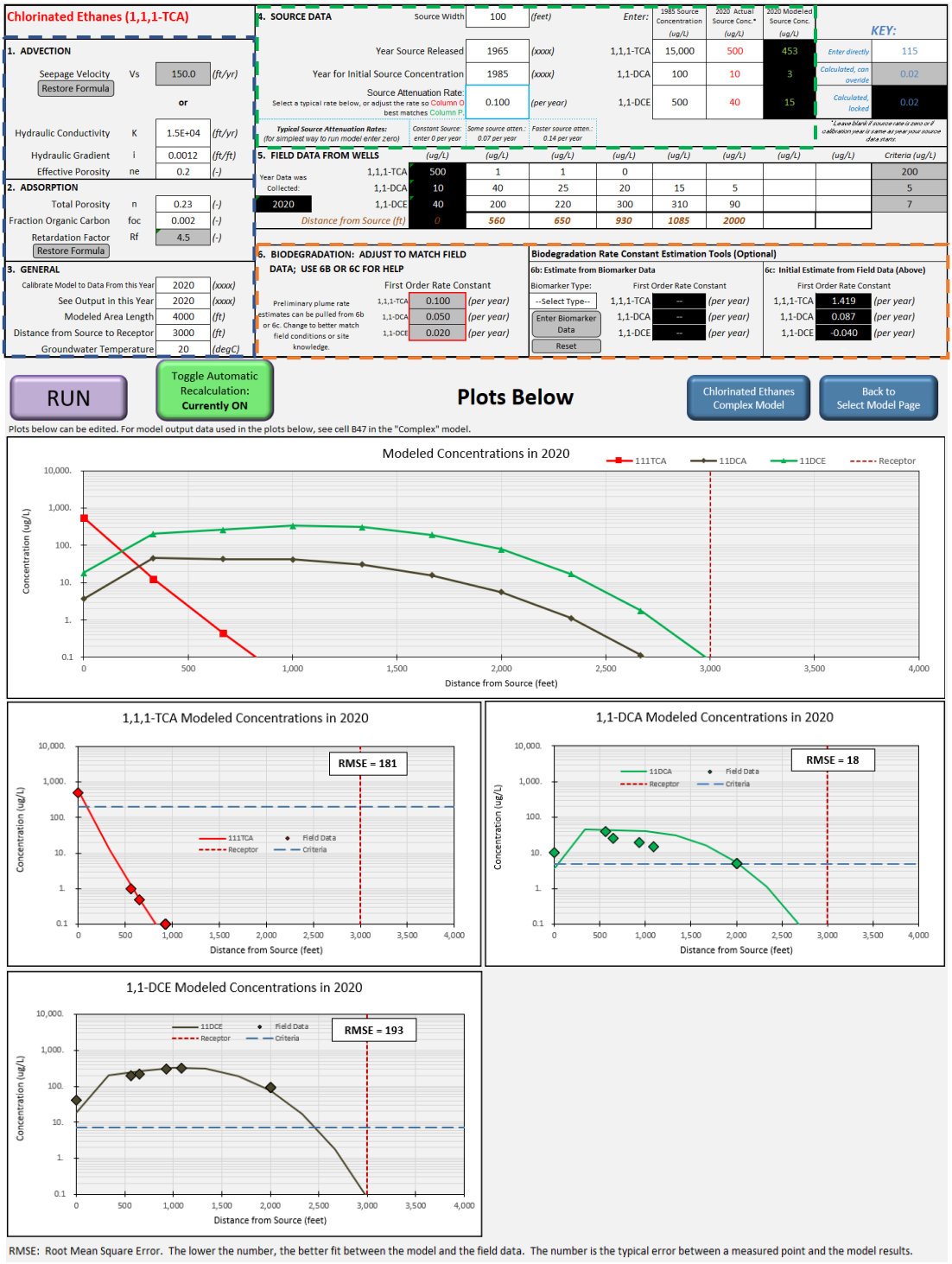


Figure 6.2 Interface for the Simple Chlorinated Ethane Model in the "MNA Rate Constant Estimator".

Blue box shows input parameters associated with modeling advection/dispersion/sorption and abiotic pathway for 1,1,1-TCA (temperature dependent). Green box shows input parameters associated with source decay. Orange box shows input/calibration parameter associated with 1,1,1-TCA, 1,1-DCA, and 1,1-DCE biodegradation based on anaerobic pathways.

6.3 Development of a Quantitative Decision Matrix to Elucidate Degradation Pathways and Select the Most Efficacious Remediation Approach for 1,4-Dioxane

Success Criteria Achieved? **PARTIALLY**

Because site conditions (and thus the viability of MNA at a particular site) were beyond the control of the project team, an alternative approach for evaluating model performance was developed. Specifically, it was determined that the model must support the following key elements of MNA remedy selection: (1) it can make projections using the MNA Rate Constant Estimator that will indicate whether the site-specific goals (e.g., a concentration threshold at a point of compliance) can be attained; and (1) for cases where goals are not currently met, it can determine how much source decay or source treatment is needed to achieve downgradient concentration goals.

As described in Section 3, these success criteria were achieved. **Figure 6.3** shows a projection for a theoretical 1,4-dioxane plume over time. In this case, the model was first calibrated to current data (from 2020), and then the calibrated 1,4-dioxane biodegradation rate was used to show that the plume extent stabilized sometime between 1990 and 2000. The goal concentration (0.35 µg/L) was being achieved at the downgradient point of compliance, such that the primary and secondary lines of evidence to support MNA could be established with the help of the model.

If the point of compliance for the example shown in **Figure 6.3** was moved from 3000 ft downgradient to 2000 ft downgradient, then the target concentration would no longer be achieved through natural attenuation. To determine how much source reduction would be required, the source concentration for the model (typically in cell O6 for 1,4-dioxane module) could be reduced successively until the goal concentration would be achieved at 2000 ft. In this case, reducing the concentration by approximately 88% would be sufficient. It should be understood plume concentrations would remain elevated until the effects of source reduction propagated downgradient (due to transport of cleaner groundwater from the source). This transient behavior is not captured by the model, but a rough estimate of the time required can be made using the compound-specific retardation factors.

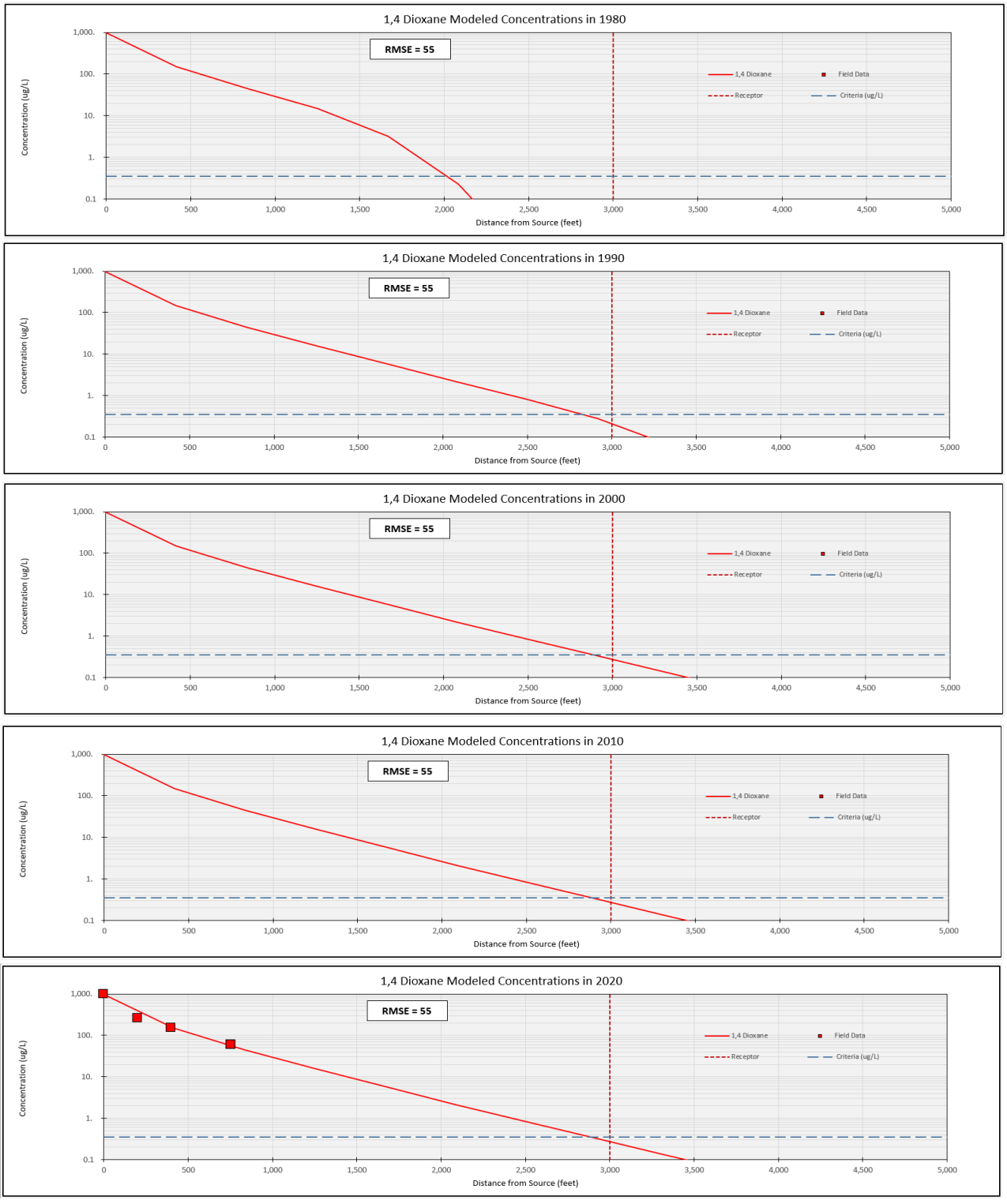


Figure 6.3. Example Simulation Showing 1,4-Dioxane Plume Extent Over Time (Rate Constant = 0.32 per year).

Visual inspection of concentration vs. distance plots confirms that 1,4-dioxane plume would be predicted to stabilize between 1990 and 2000. Field data from 2020 are included on only the 2020 plot.

A parallel success criterion for this performance objective involved establishing correlations between the first-order rate constants from the ^{14}C assays and biomarker abundance. This criterion was not achieved, and these correlations were not included in the decision matrix. In part, this was due to the lack of detections of biomarkers for direct metabolism (3 detections of THFXO/DXMO and ALDH in the 54 well samples that were analyzed). In addition, the correlation between potential biomarkers of 1,4-dioxane cometabolism and ^{14}C rate constants was weak (see **Figure 5.8.7**).

At the outset of the project, these correlations were envisioned as a way to generate an "initial guess" for the 1,4-dioxane rate constant (that could later be refined with the model). As an alternative, the project team developed an analytical expression that predicts rate constants based on lab-derived kinetic parameters (Appendix D). For the sites included in this study, the predicted rate constants for 1,4-dioxane were typically much lower than the rate constants that were based on fitting the concentration vs. distance data and/or the rate constants from ^{14}C assays. This highlight that: (1) in situ activity may be related to organisms/processes that are not well captured by the current suite of qPCR targets; and (2) differences in the analytical protocols (e.g., ^{14}C assay involves extended monitoring; biomarker measurements represent a snapshot from a single monitoring event) may be contributing to the lack of correlations.

6.4 Development of a Quantitative Decision Matrix to Elucidate Degradation Pathways and Select the Most Efficacious Remediation Approach for 1,1,1-TCA. 1,1-DCA, and 1,1-DCE

Success Criteria Achieved? **YES**

As described in Section 6.3 for 1,4-dioxane, the model was constructed to do the following for multiple different compounds, including 1,1,1-TCA, 1,1-DCA, and 1,1-DCE: (1) it can make projections using the MNA Rate Constant Estimator that will indicate whether the site-specific goals (e.g., a concentration threshold at a point of compliance) can be attained; and (1) for cases where goals are not currently met, it can determine how much source decay or source treatment is needed to achieve downgradient concentration goals. As a result, the success criteria for this objective were achieved.

6.5 Ease of Use of New Version of BioPIC

Success Criteria Achieved? **INCOMPLETE**

Beta testing of the project deliverables (updated BioPIC including new MNA Rate Constant Estimator) is scheduled for late April 2021. Feedback from beta testers will be incorporated into the software and documented in an Appendix to the Final Report. Note that a detailed User's Guide for both BioPIC and the MNA Rate Constant Estimator have already been developed (see **Appendix B** and **Appendix C**).

6.6 Validate Rate Constants for 1,4-Dioxane Degradation Using a ^{14}C Assay Conducted with Groundwater from the Study Sites

Success Criteria Achieved? **PARTIALLY**

The development and validation of the ^{14}C assay for 1,4-dioxane biodegradation was a major focus of this project. To reflect its importance, a series of 5 sub-objectives were created to evaluate the results from this task. A number of these were added after the Demonstration Plan was submitted.

The success criteria for four of the five objectives were successfully met, as described in detail in Section 3.6. Collectively, the results showed that the ^{14}C assay has the requisite sensitivity to quantify degradation rate constants that may be relatively slow (including those with equivalent half-lives in excess of 100 years). These are important for MNA remedy evaluations, where slow *in situ* degradation may still be meaningful if initial concentrations are low, particularly if the data can be used to show that concentration goals will continue to be met at a downgradient point of compliance.

The assay was also able to unequivocally document when biodegradation was responsible for 1,4-dioxane losses over time. This was based on statistically documenting when rate constants in live microcosms were different than those obtained in parallel filter-sterilized groundwater controls. In addition, the uncertainty associated with the estimated rate constants were within acceptable ranges at 75% of the wells, and different methods for calculating the net rate constant resulted in statistically similar answers. Finally, while the radiolabel impurity levels did not meet the original success criteria, a newly developed criterion was met to show that these levels did not significantly impact the rate determinations.

6.7 Determine Whether Aerobic Biological Cometabolism or Biological Degradation of 1,4-Dioxane Explains the Degradation Rate Constant for Attenuation of 1,4-Dioxane at Field Scale

Success Criteria Achieved? **PARTIALLY**

One goal of the project was to provide site managers with multiple ways to quantify biodegradation rate constants as secondary lines of evidence for natural attenuation. This included both a fate and transport model that could be calibrated based on field data (i.e., concentration vs. distance data, relevant site-specific hydrogeologic parameters) and a ^{14}C assay that provides strong evidence for location-specific degradation capacity along with some indication of the degradation rate.

Table 6.1 summarizes the statistically significant 1,4-dioxane rate constants obtained from the ^{14}C assay on a well-by-well basis, along with the site-wide biodegradation rates predicted through model calibration. The two success criteria for this performance objective were fairly stringent, and only 3 of 9 sites had one well with a rate constant that met one or more of these criteria. As discussed in Section 5.8, considerable variability in the ^{14}C-based rate constants were observed within sites, but most sites (90%) had at least one location with a statistically significant rate constant. Similarly, the model was able to generate a 1,4-dioxane biodegradation rate constant at 8 of 9 sites that had sufficient data for calibration. But in most cases, the rates based on model calibration were much larger than those from the ^{14}C assay, which is presumably influenced by the experimental protocol (i.e., relying on groundwater with low initial biomass and nutrients). At two other sites, the ^{14}C rate constants were all within an order of magnitude of the model-predicted site-wide rate constant (though generally still lower). This suggests that both methods do generate reasonable data for evaluating MNA. As part of the decision framework developed for this project, a method is described for using the ^{14}C assay as part of a step-wise approach. The first step would be a screening-level test using groundwater to determine initial estimates of capacity/rate. If the initial testing yields positive net rates, but they are much lower than model-predicted rate, then the test can be re-run using higher sediment content and/or nutrients. This second step would provide a more refined 1,4-dioxane biodegradation rate estimate that may also be useful in calibrating the fate and transport model.

Table 6.1. Summary of Model-Predicted 1,4-Dioxane Rate Constants vs. ^{14}C-Based 1,4-Dioxane Rate Constants

Site No.	Well No. [b]	Net 1,4-D Rate Constant for Based on ^{14}C Assay (± 95% CI) (yr^{-1})	Model-Predicted 1,4-D Rate Constant (yr^{-1})	Criterion #1: Is Model-Predicted Rate within 95% CI for ^{14}C-Based Rate?	Criterion #2: Is ^{14}C-Based Rate > Model-Predicted Rate?
2	1	0.0021 ± 0.0021	Model predicted no degradation	No	No
3	3	0.0114 ± 0.0033	0.07	No	No
	4	0.0957 ± 0.0149		No	Yes
	5	0.0209 ± 0.0052		No	No
4	1[ii]	0.0159 ± 0.0110	0.35	No	No
	2	0.0061 ± 0.0051		No	No
	2[ii]	0.0143 ± 0.0117		No	No
	2[iii]	0.297± 0.0580		No	No
	4	0.0073 ± 0.0028		No	No
	5[c]	0.117 ± 0.0526		No	No
	6[c]	0.367 ± 0.0484		Yes	Yes
5	1	0.0037 ± 0.0023	0.06	No	No
	2	0.0042 ± 0.0032		No	No
6	3	0.0026 ± 0.0023	0.002	Yes	Yes
7	4	0.0050 ± 0.0031	Insufficient data to calibrate model	--	--
8	2[c]	0.0091 ± 0.0077	0.017	No	No
	3[c]	0.0110 ± 0.0040		No	No
9	1[c]	0.0255 ± 0.0082	0.08	No	No
	2[c]	0.0132 ± 0.0058		No	No
	3[c]	0.0058 ± 0.0027		No	No
10	2	0.0688 ± 0.0141	0.32	No	No
	2[c]	0.0252 ± 0.0099		No	No
	4	0.0868 ± 0.0136		No	No
	4[c]	0.195 ± 0.0368		No	No

[a] ± represents the 95% confidence limit.
[b] First sampling event unless followed by [ii] = 2nd sampling event; [iii] = 3rd sampling event.
[c] Soil or sediment present along with the groundwater.

7.0 COST ASSESSMENT

An objective of this project was to track costs that would be associated with a full-scale implementation of the decision framework described in this report. This includes developing a cost model that identifies and incorporates the key cost drivers.

Note that the goal of this decision framework is to improve the technical basis for selecting MNA as a site management strategy for sites with 1,4-dioxane and chlorinated ethanes. As a result, there is an opportunity for substantial cost-savings if the use of MNA is justified vs. the implementation of more aggressive remedial options (e.g., biostimulation, bioaugmentation). The cost model does not attempt to project the cost savings associated with this type of outcome. However, it should be understood that MNA is generally a cost-effective technology in terms of capital and O&M costs, and it also can reduce the environmental impact at these sites. By incorporating the decision framework into an easy-to-use tool (BioPIC), this project should facilitate its application and increase the number of sites where a remedy decision can be made.

7.1 COST MODEL

The framework developed as part of this project requires an assessment of site-specific data. **Table 7.1** summarizes the cost elements that were considered in developing the model, and then identifies whether or not they were retained. Note that many of the analytical data required for use of BioPIC and the decision framework are routinely measured groundwater parameters and would already be necessary parts of any MNA assessment that followed existing protocols (e.g., USEPA, 1998; USEPA, 1999). As a result, only those costs that are unique to this technology were retained as part of this cost assessment.

Table 7.1. Summary of Elements Considered for the Project Cost Model

Cost Element	Data Tracked	Unit Costs (for Retained Cost Elements only)
Site Access and Coordination	No unique requirements; no cost tracking necessary	--
Groundwater Sampling	No unique requirements; assumed that the data can be collected from spilt samples from routine monitoring event	--
Soil, Surface Water, and Sediment Sampling	Not applicable; data are solely associated with groundwater sampling and analysis	--
Groundwater Analyses	Unit costs per location for extra analyses: Biomarkers Isotopes ^{14}C Treatability Testing	Biomarkers: $950/sample for series of DNA-based biomarkers; $1150/sample for series RNA-based biomarkers Isotopes: $160/sample for low-level 13C on 1,4-dioxane; $175/sample for low-level 2H on 1,4-dioxane ^{14}C Treatability Testing: $1500/sample for 1,4-dioxane (assuming costs for controls amortized across multiple samples)
Material Cost and Expenses	Unit costs by location for extra analyses, including method-specific sampling kits and shipping charges	$1000/site (assumes overnight shipping to 3 different labs with costs amortized across multiple samples)
Operation and Maintenance Costs	Not Applicable	--
Waste Disposal and Decommissioning	Not Applicable	--
Long-Term Monitoring	Not Applicable; data can be collected during single monitoring event	--
Application of Decision Framework	Labor for experienced personnel to use software tool (and associated model)	Including all phases: 30 hr for Staff Environmental Engineer/Geologist; 25 hr for Sr. Environmental Engineer/Geologist; 5 hr for Principal (review/oversight)

7.2 COST DRIVERS

The main cost drivers for implementation of the decision framework are summarized in **Table 7.1** and include:

- ***The cost for collecting data on specific parameters that are not normally collected as part of a standard MNA assessment.*** This includes biomarkers for 1,4-dioxane and chlorinated solvent degradation, stable isotopes associated with 1,4-dioxane, and a ^{14}C assay that focuses on 1,4-dioxane biodegradation. Note that all these data may not be collected as part of every evaluation. As discussed in Section 8 of this report, some lines of evidence may provide inconclusive and/or contradictory evidence at some sites. Other data may be considered supportive but not necessary (e.g., certain non-specific biomarkers). As a result, an incremental approach may be used, with the initial focus on methods that are likely to be more conclusive with respect to demonstrating attenuation capacity. In these cases, the costs associated with individual sampling events would decrease.
- ***The labor cost associated with learning how to run the BioPIC software tool.*** This would include time for both a staff and senior environmental engineer/geologist/scientist to familiarize themselves with the software, including the interface, input parameters, rate constant estimation process, and output. It is assumed that the user would already have experience with Microsoft Excel and possibly some familiarity with fate and transport models such as BIOCHLOR. It is also assumed that the user would have a general understanding of MNA processes and how lines of evidence may be used to support remedy decision making. However, it is assumed that the user would not already be familiar with the original or the updated versions of BioPIC, so the labor estimate includes time to review the user's guide, as well as install and test out the software package.
- ***The labor cost associated with using the BioPIC software tool to assess site-specific data.*** This includes labor costs for the same personnel described above to perform a site-specific application of the tool. Site data would be compiled and then used as input data for the model to estimate a biodegradation rate constant for 1,4-dioxane and other applicable compounds. The decision framework would be followed for these same sets of compounds, resulting in a compilation of positive and negative lines of evidence for attenuation to support the MNA decision. Also included within these costs is a brief report that summarizes the findings of the MNA assessment. It is assumed that this report could serve as a standalone deliverable for internal uses, or as an appendix to a more detailed deliverable that may be needed for regulatory purposes.

7.3 COST ANALYSIS

The cost model was applied for a single site using the following assumptions:

- The MNA assessment includes both 1,4-dioxane and chlorinated solvents.
- The site being evaluated contains a reasonably well delineated 1,4-dioxane plume and chlorinated solvent plume. Ideally, this means that a source area has been identified, the plume extent has been determined, and a downgradient point of compliance has been established (or can be reasonably assumed).

- The groundwater flow direction is known and the velocity can be reasonably estimated (using site-specific data such as hydraulic gradient and hydraulic conductivity).
- The existing plume data can be used to select a set of existing monitoring wells along the groundwater flow path that exhibit a decreasing trend of concentration vs. distance.
- Four samples will be collected from the pre-selected wells as part of a routine groundwater monitoring events where other standard parameters are already being analyzed. In other words, split samples can be collected and sent to separate labs to support the decision framework.
- Data are collected to evaluate all of the lines of evidence for 1,4-dioxane attenuation that were investigated as part of this project.
- The assessment can be completed using data from a single sampling event. This means that life-cycle costs and/or other time frame considerations are not applicable.

Based on these assumptions, the total cost for the assessment of MNA of a single site using this tool was estimated to be $31,039. **Table 7.2** provides a summary of the cost modeling results, including the unit costs.

Table 7.2. Results of Cost Modeling

COST ELEMENT	DATA TRACKED OR ESTIMATED	UNIT COST	QUANTITY	COST
Groundwater Analyses		**Unit Cost**		
Biomarkers	Unit costs/number of samples analyzed; labor not tracked because samples collected as part of routine event			
DNA-based biomarkers	--Includes DHC, SCAM, PHE, RMO, ALDH, EDGE, SMMO, DXMO, DHBt'	$950	4 samples per site	$3,800
RNA-based biomarker	--Includes DHC, SCAM, PHE, RMO, ALDH, EDGE, SMMO, DXMO, DHBt'	$1,150	4 samples per site	$4,600
Isotopes	Unit costs/number of samples analyzed; labor not tracked because samples collected as part of routine event			
13C for low level 1,4-dioxane		$160	4 samples per site	$640
2H for low level 1,4-dioxane		$175	4 samples per site	$700
14C Assay	Unit costs/number of samples analyzed; labor not tracked because samples collected as part of routine event			
1,4-dioxane	--Includes controls amortized across 4 samples; costs for radiolabeled 1,4-dioxane included in unit cost'	$1,500	4 samples per site	$6,000
Miscellaneous costs	Sampling kits			
Sampling kits	--if not otherwise covered by existing sampling program	$25	4 samples per site	$100
Shipping	--Overnight shipping of 1 cooler to 3 labs (amortized)	$300	3 coolers per site	$900
Task 1 Total				**$16,740**
Application of Decision Framework				
Project management	Labor hours	$250.00	5 hours per site	$1,250
Software training	Labor hours for running software on 1 site; including reporting and senior review			
ESG III	--10 hr for reviewing manual and gaining proficiency	$150.00	10 hours per site	$1,500
Sr. ESG	--10 hr for reviewing manual and gaining proficiency	$180.00	10 hours per site	$1,800
Software application	Labor hours for running software on 1 site; including reporting and senior review			
ESG III	--10 hr for gathering data and preliminary application	$150.00	20 hours per site	$3,000
Sr. ESG	--10 hr for final application and review reporting	$180.00	15 hours per site	$2,700
TASK 2 Total				**$10,250**
CONTINGENCY (15%)				$4,049
TOTAL COST				**$31,039**

8.0 IMPLEMENTATION ISSUES/LESSONS LEARNED

8.1 IMPLEMENTATION ISSUES

The project developed a decision framework for evaluating MNA at sites with 1,4-dioxane, 1,1,1-TCA, 1,1-DCA, and 1,1-DCE. Implementation issues for the MNA decision framework are expected to be minimal, as described below.

- ***Regulatory:*** No permits are required. Obtaining approval from applicable regulatory bodies to collect the required data is likely to be straightforward; it involves collecting groundwater samples using conventional protocols that would follow standard work plans. The primary regulatory issue will be educating RPMs and regulators on the protocol, which helps addresses a gap in the existing MNA guidance (i.e., lack of protocols for evaluating 1,4-dioxane and chlorinated ethanes). The goal was to develop a decision framework that builds on previous frameworks for chlorinated ethenes, relies on a similar "lines of evidence" approach, and has a strong quantitative basis for establishing secondary and tertiary lines of evidence (e.g., fate and transport model to help estimate biodegradation rates). These elements were all successfully incorporated into the resulting decision framework and should facilitate regulatory acceptance.
- ***Procurement:*** This approach can be implemented using standard equipment (groundwater sampling). Data required to support this approach can be generated using analyses that are either currently offered by commercial labs (1,4-dioxane and CVOC analysis, geochemical parameters, stable isotopes, hydrogeologic parameters, and possible biomarkers for biodegradation) or expected to be offered through commercial/academic lab partnering agreements (the ^{14}C assay for 1,4-dioxane degradation).
- ***End-User Concerns:*** Data to support this approach can be collected during relatively short mobilizations and do not require any short-term or long-term changes or disruptions to site operations. The goal is to provide a site with the technical basis for evaluating MNA as a viable remedy. As a result, the decision to collect these data should be made in context with the timeline for remedial investigations/feasibility studies (or similar site assessments). For a compound of emerging concern like 1,4-dioxane, there may be some reluctance from site managers to generate such a robust dataset if the site-specific regulatory drivers have yet to be established. At some sites—especially those where insufficient data is available to fully evaluate the primary line of evidence for natural attenuation—a screening-level approach may be warranted. This would involve initially collecting limited data on the secondary lines of evidence (focusing on one or two types of data), and then expanding efforts as more concentration vs. time/distance data become available.

8.2 LESSONS LEARNED

The following lessons learned are focused on the lab and field data interpretations, as well as bigger-picture issues associated with our understanding of natural attenuation at field sites

- ***Widespread prevalence of 1,4-dioxane degradation capacity.*** 1,4-dioxane degradation capacity was established at 9 of 10 sites that were sampled as part of this study. The primary

line of evidence for this finding was the ^{14}C assays. While the rates were generally slow, the observed prevalence and associated rates may actually be conservative. In part, this is because the protocol for the ^{14}C assays during this study relied on groundwater samples, and thus may have omitted biofilm-associated organisms that appear to be important for 1,4-dioxane degradation (Zhao et al., 2018; Johnson et al., 2020).

- ***Evidence for 1,4-dioxane degradation capacity at monitoring locations/sites that are anoxic.*** Evidence for 1,4-dioxane degradation and/or degradation capacity was observed at several sites where the apparent dissolved oxygen levels were relatively limited. While the reaction requires oxygen to proceed, field-measured dissolved oxygen levels may not reflect the actual availability of oxygen. This may be due to performance issues for dissolved oxygen probes (particularly at low levels). But perhaps more importantly, it also suggests that typical monitoring wells (with 10-ft or longer screens) yield mixed groundwater samples that come from several sub-intervals with different redox conditions. This mixing may lower the bulk dissolved oxygen measured in a field sample. It should be noted that this type of mixing may also mask the isotope fractionation signal. In addition, the oxygen demand of dilute concentrations of 1,4-dioxane is relatively low; 1 mg/L of DO could theoretically oxidize more than 500 µg/L of 1,4-dioxane to CO_2. For the purposes of the decision framework, a dissolved oxygen level of 0.1 mg/L is considered the screening-level threshold for establishing whether geochemical conditions are favorable for 1,4-dioxane degradation. Installing shorter-screened monitoring wells, particularly in shallow portions of the aquifer where infiltration may enhance oxygen availability, is recommended because it should provide more refined data for evaluating 1,4-dioxane degradation.

- ***Prevalence of in situ 1,4-dioxane degradation based on model predictions/isotope fractionation.*** 1,4-Dioxane biodegradation rate constants could be established using model predictions for 8 of the 10 study sites. These site-wide, first-order rate constants ranged from 0 to 0.32 per year, with the upper end equivalent to a half-life of 2.2 year. A total of 6 sites had model-predicted rate constants that were greater than 0.06 per (equivalent half-life of < 12 year). Evidence for isotope fractionation was obtained at 7 of 10 sites. These data provide solid evidence that in situ biodegradation of 1,4-dioxane is actually occurring at a significant percentage of sites.

- ***Slow 1,4-dioxane rates are common but may be an artifact of experimental protocol.*** The rate constants based on concentration vs. distance data described above are derived from a model that accounts for the effects of non-destructive processes. However, they are still calibrated parameters that may be influenced by uncertainty in other input parameters. In contrast, the rates obtained using the ^{14}C assay (which is designed to control for all other processes) were much slower, with a median value of 0.0137 per year (equivalent half-life of 51 year) at the locations where a significant rate constant could be established. Furthermore, no rate constant could be established at 56% of the well locations where groundwater was tested using this assay. However, the use of groundwater likely limits the initial biomass (including attached-growth organisms that would be associated with soil) and nutrient availability within the microcosms. These factors would not only lower the observed degradation rates but make it more challenging to establish a statistically significant rate constant (i.e., increases the chance for a false negative). As noted in Section 5.6, it may be worthwhile to use groundwater-based assays as a screening step and/or construct microcosms with soil and groundwater to provide more refined rate data.

- ***Lack of prevalence of direct biomarkers for 1,4-dioxane.*** Possible biomarkers for direct metabolism of 1,4-dioxane (THFMO/DXMO, ALDH) were only detected at one of the ten study sites (Site 3). These data suggest that either: 1) these biomarkers are not sufficiently prevalent to serve as a valuable line of evidence for 1,4-dioxane biodegradation; and/or 2) organisms capable of direct metabolism are relatively rare when compared to organisms capable of cometabolizing 1,4-dioxane. It should be noted that the one site where the biomarkers for direct oxidation were detected also had several other positive lines of evidence for natural attenuation. This indicates that the THFMO/DXMO and ALDH biomarkers may have some value as corroborating evidence.
- ***Lines of evidence for 1,4-dioxane natural attenuation did not always converge.*** While some sites had many converging lines of evidence for natural attenuation, others had lines of evidence that diverged. This included the data associated with potential biomarkers for 1,4-dioxane cometabolism, which were not particularly illustrative of 1,4-dioxane attenuation trends suggested by the other datasets. At 7 of the 10 study sites, a biodegradation rate constant was obtained with both the ^{14}C rate assay and the model calibration. This is an important convergence because the ^{14}C rate assay suggests that the capacity exists for biodegradation (with some indication of the relative rate by location), while the model calibration suggests that in situ biodegradation is occurring (with an estimate of the site-wide rate). These two analyses, along with the isotope fractionation patterns, are likely to be the strongest lines of evidence for natural attenuation (as described in Section 5.8). Given that each of the individual analyses has some limitations, the data from this study highlight the importance of not relying on a single line of evidence for documenting natural attenuation. Within the decision framework, this is accomplished by making the user go through all possible lines of evidence (and documenting which ones are supportive) as opposed to prescriptive outcomes based on each individual line of evidence.
- ***Variability within sites was observed:*** At the sites where the ^{14}C assay generated positive results, a statistically significant rate constant was obtained at 25% to 75% of sampled locations. This highlights that: 1) 1,4-dioxane degradation capacity at a particular site may be localized; and 2) collecting data from multiple locations is critical. The finding that 1,4-dioxane degradation capacity was variable within a site is perhaps not surprising, given that this is an aerobic process that is reliant on oxygen availability, which is expected to vary due to infiltration and other redox-driven processes. Similarly, sampling multiple locations (including within the source and the plume) is important not only for increasing representativeness of the data, but for making sure that evaluations based on spatial patterns (specifically isotope fractionation patterns and calibration of the fate and transport model) can be performed.
- ***Lack of predictive power for biomarkers:*** As noted above, the biomarkers for direct metabolism of 1,4-dioxane were infrequently detected and therefore did not provide much help in developing predictive relationships for rate constants. Similarly, no clear relationship could be established between rate constants obtained using the ^{14}C assay and the abundance of possible biomarkers for 1,4-dioxane cometabolism. A parallel approach for developing a correlation generally resulted in underpredictions of the model-calibrated rate constant. At this stage, it appears that sampling groundwater for the current set of

functional gene targets has limited ability to predict rate constants, either because other (unidentified) enzymes are responsible for the observed activity and/or because of the limitations of assaying groundwater samples (e.g., less biomass to establish abundance or rates). Consequently, other methods (e.g., models) are likely to serve as better secondary lines of evidence for evaluating MNA.

- ***Limited availability of long-term 1,4-dioxane data at many sites may influence ability to show plume stability.*** Several of the sites included in this study had very limited monitoring data on 1,4-dioxane. This included sites where the plume had yet to be fully delineated, as well as sites where only a single comprehensive monitoring event had been completed. The situation at these sites is obviously dynamic, and it is expected that additional data has been collected at many of them. In some cases, data are unlikely to be sufficient to establish the 1st line of evidence for natural attenuation (*"Historical groundwater and/or soil chemistry data that demonstrate a clear and meaningful trend of decreasing contaminant mass and/or concentration over time at appropriate monitoring or sampling points"*, USEPA 1999). However, collecting data that would serve as supporting lines of evidence for natural attenuation may prove valuable at these types of sites, particularly since some data like stable isotopes can be obtained relatively quickly and support further characterization/monitoring efforts.

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APPENDICES

Appendix A:
Results Summary Tables

TABLE A.1
SUMMARY OF ALL ANALYTICAL RESULTS FROM ^{14}C LAB ASSAY

Development of a Quantitative Framework for Evaluating Natural Attenuation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-Dioxane in Groundwater
ESTCP ER-201730

** Significant Rate or Regression Slope* | *Not significant* | *Significant* | *Significant, GC data*

			MATLAB k (yr^{-1})							MATLAB Half Life (yr)			Regression			Predicted 1,4-Dioxane Change		
Samples Received	Exp. Set Up	Sample	GW	GW 95% CI	FSGW	FSGW 95% CI	Lower 95% Half-Life on GW	Net	Net 95% CI	Net $t_{1/2}$ (yr)	Net $t_{1/2}$ (yr) Lower	Net $t_{1/2}$ (yr) Upper	Net Significance	GW Slope	FSGW Slope	According to Net k	Lower Level	Upper Level
8/7/2018	8/8/2018	Site 1 Well 1	6.97E-04	5.33E-04	1.42E-03	6.26E-04	609	0	-	-	-	-	No	NS	-	-	-	-
8/8/2018	8/9/2018	Site 1 Well 2	1.97E-03	6.50E-04	3.11E-03	9.73E-04	276	0	-	-	-	-	No	3.8	4.7	-	-	-
12/18/2019	12/18/2019	Site 1 Well 2, resampled	5.41E-03	2.43E-03	8.59E-03	4.05E-03	93	0	-	-	-	-	No	NS	NS	-	-	-
8/9/2018	8/10/2018	Site 1 Well 3	1.67E-03	8.12E-04	1.52E-03	6.13E-04	296	1.51E-04	9.86E-04	-	-	-	No	NS	-	-	-	-
12/18/2019	12/18/2019	Site 1 Well 3, resampled	8.01E-03	3.02E-03	8.59E-03	4.05E-03	66	0	-	-	-	-	No	9.0	NS	-	-	-
8/10/2018	8/11/2018	Site 1 Well 4	4.35E-04	5.02E-04	2.70E-03	6.37E-04	815	0	-	-	-	-	No	NS	-	-	-	-
8/21/2018	8/22/2018	Site 2 Well 1	0.0041	0.0018	0.0020	0.0012		**0.0021**	**0.0021**	328	165	27,514	No	NS	-	162.5	162.8	162.2
8/22/2018	8/23/2018	Site 2 Well 2	1.47E-03	9.61E-04	2.68E-03	8.89E-04	306	0	-	-	-	-	No	NS	-	-	-	-
8/23/2018	8/24/2018	Site 2 Well 3	2.49E-03	9.93E-04	3.22E-03	1.36E-03	209	0	-	-	-	-	No	NS	-	-	-	-
8/24/2018	8/25/2018	Site 2 Well 4	2.89E-03	9.14E-04	2.18E-03	1.15E-03	190	7.09E-04	1.42E-03	-	-	-	No	NS	-	-	-	-
10/2/2018	10/3/2018	Site 3 Well 1	7.50E-03	2.38E-03	7.63E-03	1.91E-03	73	0	-	-	-	-	No	NS	-	-	-	-
10/3/2018	10/4/2018	Site 3 Well 2	9.05E-03	1.56E-03	7.24E-03	1.85E-03	67	1.82E-03	2.34E-03	-	-	-	No	12.2	NS	-	-	-
10/4/2018	10/5/2018	Site 3 Well 3	0.0156	0.0033	0.0042	0.0019		**0.0114**	**0.0033**	61	46	90	Yes	16.8	NS	413.3	414.3	412.3
10/5/2018	10/6/2018	Site 3 Well 4	0.1094	0.0153	0.0137	0.0019		**0.0957**	**0.0149**	7.2	6.3	8.6	Yes	136.3	13.4	151.9	153.6	150.3
10/5/2018	10/6/2018	Site 3 Well 5	0.0286	0.0048	0.0077	0.0024		**0.0209**	**0.0052**	33	27	44	Yes	41.8	NS	253.5	254.5	252.6
11/13/2018	11/14/2018	Site 4 Well 1 (Set I, GW only)	2.52E+00	-	-	-		-	-	0.28	-	-	-	-	-	-	-	-
11/14/2018	11/15/2018	Site 4 Well 2 (Set I, GW only)	0.0142	0.0043	0.0081	0.0030		**0.0061**	**0.0051**	113	62	651	No	12.2	NS	277.9	278.8	277.1
11/15/2018	11/16/2018	Site 4 Well 3 (Set I, GW only)	4.58E-03	2.79E-03	3.17E-03	2.22E-03	101	1.41E-03	2.79E-03	-	-	-	No	NS	-	-	-	-
11/16/2018	11/17/2018	Site 4 Well 4 (Set I, GW only)	1.27E-02	2.46E-03	5.37E-03	1.49E-03		**7.32E-03**	**2.79E-03**	95	69	153	Yes	13.8	4.8	509.5	510.4	508.6
11/21/2019	11/22/2019	Site 4 Well 1, resampled (Set II, GW only)	0.0250	0.0098	0.0091	0.0056		**0.0159**	**0.0110**	44	26	141	No	NS	NS	165.5	165.7	165.3
11/21/2019	11/22/2019	Site 4 Well 2, resampled (Set II, GW only)	0.0234	0.0107	0.0091	0.0056		**0.0143**	**0.0117**	49	27	271	No	NS	NS	293.1	293.5	292.7
11/21/2019	11/22/2019	Site 4 Well 3, resampled (Set II, GW only)	1.07E-02	4.30E-03	9.13E-03	5.59E-03	49	1.59E-03	6.83E-03	-	-	-	No	NS	NS	-	-	-
11/21/2019	11/23/2019	Site 4 Well 4, resampled (Set II, GW only)	8.65E-03	3.34E-03	9.13E-03	5.59E-03	61	0	-	-	-	-	No	NS	NS	546.5	-	-
11/20/2020	11/20/2020	Site 4 Well 2 (Set III, GW only)	0.3616	0.0574	0.0643	0.0172		**0.2973**	**0.0580**	2.3	2.0	2.9	Yes	468.9	63.7	284.5	286.4	282.6
11/20/2020	11/20/2020	Site 4 Well 5, North MW-26 (Set III, GW + soil)	0.1813	0.0515	0.0643	0.0172		**0.1170**	**0.0526**	5.9	4.1	11	Yes	209.8	63.7	166.2	167.2	165.2
11/20/2020	11/20/2020	Site 4 Well 6, South MW-6 (Set III, GW + soil)	0.4309	0.0512	0.0643	0.0172		**0.3666**	**0.0484**	1.9	1.7	2.2	Yes	283.7	63.7	161.5	162.4	160.6
12/12/2018	12/13/2018	Site 5 Well 1	0.0070	0.0023	0.0033	0.0023		**0.0037**	**0.0023**	189	102	1,254	No	NS	-	259.0	259.3	258.6
12/13/2018	12/14/2018	Site 5 Well 2	0.0086	0.0029	0.0044	0.0014		**0.0042**	**0.0032**	164	94	650	No	NS	-	168.4	168.7	168.1
12/14/2018	12/15/2018	Site 5 Well 3	4.37E-03	2.27E-03	4.20E-03	1.92E-03	111	1.65E-04	2.87E-03	-	-	-	No	NS	-	-	-	-
12/14/2018	12/15/2018	Site 5 Well 4	6.46E-03	2.08E-03	6.12E-03	2.35E-03	85	3.42E-04	3.02E-03	-	-	-	No	NS	-	-	-	-
12/18/2019	12/18/2019	Site 1 Well 4, resampled	1.24E-02	2.64E-03	8.59E-03	4.05E-03	48	3.80E-03	#REF!	-	-	-	No	11.9	NS	-	-	-
1/29/2019	1/30/2019	Site 6 Well 1	6.30E-03	2.79E-03	4.51E-03	1.56E-03	81	1.79E-03	3.10E-03	-	-	-	No	NS	-	-	-	-
1/30/2019	1/31/2019	Site 6 Well 2	3.93E-03	1.62E-03	2.27E-03	1.26E-03	132	1.66E-03	1.99E-03	-	-	-	No	NS	-	-	-	-
1/31/2019	2/1/2019	Site 6 Well 3	0.0081	0.0018	0.0054	0.0016		**0.0026**	**0.0023**	262	140	2,001	No	6.7	5.0	168.6	168.8	168.4
2/1/2019	2/2/2019	Site 6 Well 4	1.37E-02	3.28E-03	1.15E-02	3.05E-03	42	2.17E-03	4.34E-03	-	-	-	No	9.8	6.1	-	-	-
12/14/2019	12/15/2019	Site 7 Well 1	5.00E-03	2.30E-03	7.94E-03	1.93E-03	100	0	-	-	-	-	No	NS	4.1	-	-	-
12/14/2019	12/15/2019	Site 7 Well 2	7.44E-03	2.91E-03	6.83E-03	2.35E-03	70	6.09E-04	3.63E-03	-	-	-	No	10.1	11.0	-	-	-
12/14/2019	12/15/2019	Site 7 Well 3	1.09E-02	2.98E-03	1.06E-02	4.12E-03	52	3.30E-04	4.93E-03	-	-	-	No	8.9	NS	-	-	-
12/14/2019	12/15/2019	Site 7 Well 4	0.0076	0.0029	0.0026	0.0014		**0.0050**	**0.0031**	140	86	376	No	NS	NS	168.7	168.8	168.6
8/21/2020	8/21/2020	Site 8 Well 1	9.90E-03	3.55E-03	8.70E-03	3.71E-03	54	1.20E-03	4.97E-03	-	-	-	No	NS	-	-	-	-
8/21/2020	8/21/2020	Site 8 Well 2	0.0169	0.0069	0.0078	0.0039		**0.0091**	**0.0077**	76	41	510	No	NS	-	207.4	207.6	207.2
8/21/2020	8/21/2020	Site 8 Well 3	0.0206	0.0030	0.0096	0.0029		**0.0110**	**0.0040**	63	46	99	Yes	21.7	NS	162.6	162.7	162.5
8/21/2020	8/21/2020	Site 8 Well 4	9.34E-03	4.80E-03	8.18E-03	2.42E-03	52	1.17E-03	5.21E-03	-	-	-	No	NS	-	-	-	-
8/28/2020	8/28/2020	Site 9 Well 1	0.0446	0.0072	0.0191	0.0044		**0.0255**	**0.0082**	27	21	40	Yes	40.0	18.9	167.9	168.1	167.8
8/28/2020	8/28/2020	Site 9 Well 2	0.0210	0.0046	0.0078	0.0039		**0.0132**	**0.0058**	53	37	94	No	9.4	NS	8,333.0	8,338.6	8,327.5
8/28/2020	8/28/2020	Site 9 Well 3	0.0103	0.0020	0.0045	0.0019		**0.0058**	**0.0027**	120	82	222	Yes	10.8	NS	1,032.0	1,032.3	1,031.7
8/28/2020	8/28/2020	Site 9 Well 4	2.03E-02	4.10E-03	2.16E-02	1.02E-02	29	0	-	-	-	-	No	19.3	NS	1,294.3	-	-
11/11/2020	11/11/2020	Site 10 Well 1	1.01E-02	4.77E-03	0.0081	0.0034	49	2.05E-03	5.66E-03	-	-	-	No	NS	-	267.0	267.2	266.8
11/11/2020	11/11/2020	Site 10 Well 2	0.0768	0.0170	0.0442	0.0142		**0.0688**	**0.0141**	10	8.4	13	No	72.1	61.8	448.9	449.7	448.2
11/11/2020	11/11/2020	Site 10 Well 3	4.38E-03	2.07E-03	1.87E-02	6.08E-03	114	0	-	-	-	-	No	NS	-	1171.8	-	-
11/11/2020	11/11/2020	Site 10 Well 4	0.0948	0.0136	0.2152	0.0273		**0.0868**	**0.0136**	8.0	6.9	9.5	No	216.7	389.4	318.3	318.8	317.8
11/11/2020	11/11/2020	Site 10 Well 2	0.0333	0.0097	0.0431	0.0081		**0.0252**	**0.0099**	27	20	45	No	15.0	58.7	451.2	451.7	450.7
11/11/2020	11/11/2020	Site 10 Well 3	1.00E-02	4.19E-03	1.87E-02	6.08E-03	51	1.99E-03	5.22E-03	-	-	-	No	NS	-	1171.6	-	-
11/11/2020	11/11/2020	Site 10 Well 4	0.2029	0.0378	0.2152	0.0273		**0.1948**	**0.0368**	3.6	3.0	4.4	No	293.8	389.4	314.4	315.7	313.0

TABLE A.2
SUMMARY OF ALL ANALYTICAL RESULTS FROM FIELD SAMPLES

Development of a Quantitative Framework for Evaluating Natural Attenuation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-Dioxane in Groundwater
ESTCP ER-201730

Site #	Well #	Sample Date	**14C Assay Data**	**CSIA Data**		**1,4-D/CVOC Concentration Data**										**Dissolved Gas Concentration Data**		
			Net 1,4-Dioxane biodegradation rate per year	1,4-Dioxane Average δ^{13C}	1,4-Dioxane Average δ^{2H}	1,4-Dioxane µg/L	PCE µg/L	1,1-DCE µg/L	TCE µg/L	*cis* -DCE µg/L	*trans* -DCE µg/L	1,1,1-TCA µg/L	1,1-DCA µg/L	1,2-DCA µg/L	CA µg/L	Ethane µg/L	Ethene µg/L	Methane µg/L
1	1	8/6/2018	0	-31.30	13.63	1.4 J	--	--	--	--	--	--	--	--	--	--	--	--
1	1	3/23/2019	--	--	--	--	<1	<1	5.5	1.9	<1	<0.2	<1	<1	<0.3	<0.64	<0.60	<0.34
1	2	8/7/2018	0	-30.37	-8.93	14	--	--	--	--	--	--	--	--	--	--	--	--
1	2	3/23/2019	--	--	--	6.8	<1	0.27 J	2.5	0.28 J	<1	<0.2	<1	<1	<0.3	<0.64	<0.60	<0.34
1	2	12/15/2019	0	--	--	8.7	<0.3	0.35 J	6.6	0.43 J	<0.2	<0.2	<0.2	<0.2	<0.2	<0.64	<0.60	<0.34
1	3	8/8/2018	0	-32.01	-16.86	130	--	--	--	--	--	--	--	--	--	--	--	--
1	3	3/23/2019	--	--	--	120	<1	150	5800	1700	4.1	<0.2	67	1.3	<0.3	0.49 J	4.4	430
1	3	12/15/2019	0	--	--	120	4.6	170	5200	1700	3.5	<0.2	72	1.6	<0.3	0.66 J	3.9	210
1	4	8/9/2018	0	-32.65	-35.46	86	40	150	1600	5700	5.7	<0.2	63	0.3 J	<0.3	23	0.60 J	4,000
2	1	8/20/2018	0.002114	-32.56	BAL	<0.25	1.1	<1	<0.4	<4	<1	<4	<1	<1	<1	<10	<10	<10
2	2	8/21/2018	0	-32.24	BAL	<0.25	2	<1	1.2	<4	<1	<4	<1	<1	<1	<10	<10	<10
2	3	8/22/2018	0	-31.84	BAL	<0.25	2.3	<1	2.8	4.3	<1	<4	<1	<1	<1	<10	<10	<10
2	4	8/23/2018	0	-32.13	0.50	<0.25	4.5	1.1	13.9	49.5	<1	<4	1.2	<1	<1	<10	<10	<10
3	1	10/1/2018	0	-29.87	-10.52	10	<1	<1	<1	<1	<1	<1	<1	<1	<1	<10	<10	16.5
3	2	10/2/2018	0	-31.56	-44.11	100	<1	<1	<1	<1	<1	<1	<1	<1	<1	10.5	<10	11,100
3	3	10/3/2018	0.01139	-31.54	-50.69	310	<1	<1	<1	<1	<1	<1	3.2	<1	33	<10	<10	5420
3	4	10/4/2018	0.09574	-29.78	-3.33	8.3	<1	<1	<1	<1	<1	<1	<1	<1	<1	<10	<10	23.9
3	5	10/5/2018	0.020891	-30.91	-57.24	41	<1	<1	<1	<1	<1	<1	<1	<1	<1	<10	<10	15.8
4	1	11/12/2018	0.015882	-36.77	5.69	290	<0.05	1.4	<0.05	0.1 J	<0.05	<0.06	1	<0.05	<0.09	<10	<10	19.9
4	1	11/21/2019	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
4	2	11/13/2018	0.00613	-36.99	-7.38	130	<0.05	0.4 J	<0.05	<0.05	<0.05	<0.06	1.6	<0.05	<0.09	<10	<10	<10
4	2	11/20/2019	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
4	3	11/14/2018	0	-37.23	-31.85	10,000	<0.1	1.6	0.3 J	0.8 J	<0.1	<0.1	4.7	0.1	1.6	<10	<10	2080
4	3	11/20/2019	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
4	4	11/15/2018	0.007316	-37.50	-9.21	280	<0.06	4.2	<0.05	0.1 J	<0.05	<0.06	2.6	<0.05	<0.09	<10	<10	184
4	4	11/20/2019	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
5	1	12/11/2018	0.003658	--*	--*	140	1.1	270	540	53	0.80 J	<0.2	7.1	0.85 J	<0.3	<10	<10	<10
5	1	12/14/2019	--	-32.55	-28.21	120	--	--	--	--	--	--	--	--	--	--	--	--
5	2	12/12/2018	0.004224	--*	--*	<1	<0.3	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.3	<10	<10	<10
5	3	12/13/2018	0	--*	--*	110	0.17J	32	31	71	0.27 J	<0.2	3.2	0.31 J	<0.3	<10	<10	<10
5	3	12/14/2019	--	-32.02	-3.84	56	--	--	--	--	--	--	--	--	--	--	--	--
5	4	12/13/2018	0	--*	--*	39	0.46 J	110	240	22	0.40 J	<0.2	2.8	0.45 J	<0.3	<10	<10	<10
5	4	12/15/2019	--	BAL	BAL	42	0.42 J	110	290	22	0.33	<0.2	3	0.51 J	<0.3	<0.64	<0.60	0.23 J
5	source	12/14/2019	--	-31.48	-49.65	11,000	--	--	--	--	--	--	--	--	--	--	--	--
6	1	1/28/2019	0	-31.82	-36.00	4.5	<0.5	0.35 J	<0.2	1.2	<0.1	<0.1	0.13 J	<0.1	<0.2	<0.064	<0.081	5.68
6	2	1/29/2019	0	-31.28	-18.56	0.88 J	<0.5	0.16 J	<0.2	<0.2	<0.1	<0.1	<0.1	<0.1	<0.2	<0.064	<0.081	0.036 J
6	3	1/30/2019	0.002647	-31.33	-0.22	3.2	<0.5	2.1	<0.2	0.55	<0.1	<0.1	0.36 J	<0.1	<0.2	<0.064	<0.081	0.377
6	4	1/31/2019	0	-30.59	-45.73	16	<0.5	2.5	<0.2	2.6	0.16 J	<0.1	0.41 J	<0.1	<0.2	<0.064	<0.081	2.74
7	1	12/13/2019	0	--	--	<1	<0.3	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.64	<0.60	1200
7	2	12/13/2019	0	-31.36	-12.94	1400	0.53 J	620	7.3	1100	6.7	0.18 J	470	0.44 J	8.1	1.0 J	42	6.1
7	3	12/13/2019	0	-30.57	9.20	4.1	<0.3	<0.2	<0.2	<0.2	<0.2	<0.2	0.5 J	<0.2	<0.2	2.3	<0.60	410
7	4	12/13/2019	0.004958	BAL	BAL	10	<0.3	<0.2	<0.2	0.12	<0.2	<0.2	<0.2	<0.2	<0.2	<0.64	<0.60	68
7	source	12/13/2019	--	-30.98	-4.06	320	--	--	--	--	--	--	--	--	--	--	--	--
8	1	8/19/2020	0	-34.81	53.86	13	<0.3	5.5	<0.2	<0.2	<0.2	0.27 J	1.4	<0.2	<1	<0.64	<0.60	<0.34
8	2	8/20/2020	0.00909	-34.29	44.81	70	<0.3	20	<0.2	<0.2	<0.2	1.1	7.6	0.28 J	<1	<0.64	<0.60	<0.34
8	3	8/20/2020	0.011	-32.98	-0.97	120	<0.3	32	<0.2	<0.2	<0.2	11	13	0.47 J	<1	<0.64	<0.60	<0.34
8	4	8/19/2020	0	-33.82	24.00	4.4	<0.3	4.4	<0.2	<0.2	<0.2	0.22 J	0.63 J	<0.2	<1	<0.64	<0.60	0.82 J
8	DUP-1	8/20/2020	-	-	-	-	<0.3	26	<0.2	<0.2	<0.2	10	12	0.45 J	<1	-	-	-
9	1	8/26/2020	0.00255	-34.56	45.06	5	<0.3	<0.2	0.37 J	0.42 J	<0.2	0.16 J	1.3	<0.2	<1	<0.64	<0.60	<0.34
9	2	8/26/2020	0.0132	-34.68	-37.00	390	4	<0.2	9	39	2.7	<0.2	16	0.69 J	1.7 J	<0.64	<0.60	190
9	3	8/27/2020	0.00579	-33.40	-41.62	740	0.95 J	<0.2	12	61	6.1	<0.2	15	0.43 J	1.2 J	<0.64	<0.60	84
9	4	8/27/2020	0	-34.99	-48.85	4000	<0.3	6.7	1400	1300	120	0.27 J	54	2.3	0.54 J	0.81 J	7.9 J	740
9	DUP-1	8/27/2020	--	--	--	-	<0.3	8 J	1700	1700	140	0.41 J	68	3.4	0.79 J	-	-	-
10	1	11/9/2020	0	-31.56	-60.29	260	4600	850	1100	2500	12	<0.2	46	6.8	<1	14	160	1300
10	2	11/9/2020	0.0688	-30.32	-64.01	59	420	100	77	450	2.1	0.2 J	13	0.81 J	<2	<0.64	<0.60	8
10	2	11/9/2020	0.0252 (repeat)	-30.32	-64.01	59	420	100	77	450	2.1	0.2 J	13	0.81 J	<2	<0.64	<0.60	8
10	3	11/10/2020	0	-29.83	-79.31	980	120	880	18	190	1.9	<1	290	14	<2	2.2	13 J	420
10	3	11/10/2020	0 (repeat)	-29.83	-79.31	980	120	880	18	190	1.9	<1	290	14	<2	2.2	13 J	420
10	DUP-1	11/10/2020	--	--	--	1000	-	-	-	-	-	-	-	-	-	-	-	-
10	4	11/10/2020	0.0868	-30.94	-70.29	150	8.9	160	18	500	1.6	<1	18	2.5	<2	97	300	5400
10	4	11/10/2020	0.195 (repeat)	-30.94	-70.29	150	8.9	160	18	500	1.6	<1	18	2.5	<2	97	300	5400

Notes:
1. "< or U" = not detected at the SQL; J = analyte is positively identified and the result is less than LOQ/RL but greater than LOD/MDL/DL.
2. Cells marked with "--" represent analytes that were -- from a particular well during that sampling event OR replicate analysis.
3. BAL = Below analytical limit.
4. Wells with 0 values as the Net 1,4-Dioxane biodegradation rate represent wells where the 14C live microcosm rates are not significantly different than the rates from the 14C filter-sterilized groundwater control.
5. Wells with * in the CSIA data represent wells where initial analyses were did not generate quantifiable data.

TABLE A.2
SUMMARY OF ALL ANALYTICAL RESULTS FROM FIELD SAMPLES

Development of a Quantitative Framework for Evaluating Natural Attenuation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-Dioxane in Groundwater
ESTCP ER-201730

Site #	Well #	Sample Date	**14C Assay Data**	**1,4-dioxane direct metabolism**		**1,4-dioxane cooxidation**						**Chlorinated hydrocarbon metabolism**			**1,4-dioxane direct metabolism**		**1,4-dioxane cooxidation**					**Chlorinated hydrocarbon metabolism**		
			Net 1,4-Dioxane biodegradation rate	DXMO	ALDH	PHE	RDEG	RMO	SCAM	prmA**	SMMO	DHC	DHBt	DCA	DXMO	ALDH	PHE	RDEG	RMO	SCAM	SMMO	DHC	DHBt	DCA
			per year	DNA gene/mL	DNA gene/mL	DNA gene/mL	DNA gene/mL	DNA gene/mL	DNA gene/mL	DNA gene/mL	DNA gene/mL	DNA gene/mL	DNA gene/mL	DNA gene/mL	RNA tran/mL	RNA tran/mL	RNA tran/mL	RNA tran/mL	RNA tran/mL	RNA tran/mL	RNA tran/mL	RNA tran/mL	RNA tran/mL	RNA tran/mL
1	1	8/6/2018	0	<5	<5	2370	3260	117	0.6 J	9.9	<5	<0.5	15300	<5	<5	<5	<5	<5	<5	0.6 J	<5	<0.5	196000	<5
1	1	3/23/2019	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
1	2	8/7/2018	0	<15.6	<15.6	19	<15.6	<15.6	1.5 J	< 10	<15.6	0.45 J	1660	<15.6	<17.2	<17.2	<17.2	<17.2	<17.2	<17.2	<17.2	22.6	32400	<17.2
1	2	3/23/2019	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
1	2	12/15/2019	0	<11.1	<11.1	<11.1	<11.1	<11.1	<11.1	--	<11.1	2.1	<11.1	<11.1	<9.8	<9.8	<9.8	<9.8	<9.8	<9.8	<9.8	66.5	<9.8	<9.8
1	3	8/8/2018	0	<4.7	<4.7	61.6	14.2	<4.7	<4.7	< 10	<4.7	<0.5	2.46 J	<4.7	<7.9	<7.9	<7.9	<7.9	<7.9	<7.9	<7.9	<0.8	639	<7.9
1	3	3/23/2019	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
1	3	12/15/2019	0	<5	<5	57.4	140	<5	3.7 J	--	<5	0.5 J	384	<5	<11.6	<11.6	<11.6	<11.6	<11.6	<11.6	<11.6	51	<11.6	<11.6
1	4	8/9/2018	0	<5	<5	262	871	1020	1.5 J	< 10	1840	0.6	<5	<5	<5	<5	<5	<5	<5	<5	70.4	36.7	3250	<5
2	1	8/20/2018	0.002114	<5	<5	12.4	<5	<5	<5	< 10	<5	6.4	22.4	<5	<5	<5	<5	<5	<5	<5	<5	466	749	<5
2	2	8/21/2018	0	<5	<5	90.7	145	<5	<5	< 10	12	0.4 J	90.9	<5	<5	<5	<5	<5	<5	<5	<5	52.4	5020	<5
2	3	8/22/2018	0	<5	<5	17.2	<5	<5	<5	< 10	<5	1.2	<5	<5	<5	<5	<5	<5	<5	<5	<5	412	4490	<5
2	4	8/23/2018	0	<5	<5	33.8	964	<5	0.5 J	< 10	<5	2	11.9	<5	<5	<5	<5	<5	<5	<5	<5	375	34200	<5
3	1	10/1/2018	0	<5	<5	<5	<5	<5	<5	< 10	49.9	<0.5	<5	<5	<5	<5	<5	<5	<5	<5	<5	48.1	21600	<5
3	2	10/2/2018	0	<5	0.2 J	28.6	163	<5	<5	< 10	325	6.9	<5	<5	<5	<5	<5	<5	<5	<5	<5	580	2410	<5
3	3	10/3/2018	0.01139	<5	1.5 J	1030	328	<5	<5	< 10	75	8.9	<5	<5	<5	<5	<5	<5	<5	<5	<5	2110	18000	<5
3	4	10/4/2018	0.09574	775	7.5	133000	222000	33200	7360	6420	40	0.3 J	13500	<7.1	<7.1	<7.1	271	1090	<7.1	94	244	173	208000	<7.1
3	5	10/5/2018	0.020891	<5	<5	427	276	<5	1.4 J	< 10	32.6	0.3 J	<5	<5	<5	<5	<5	<5	<5	<5	<5	33.4	3770	<5
4	1	11/12/2018	0.015882	<5	<5	<5	<5	<5	<5	< 10	<5	<0.5	<5	<5	<5.6	<5.6	<5.6	<5.6	<5.6	<5.6	<5.6	<0.6	1400	<5.6
4	1	11/21/2019	--	<6.3	<6.3	0.8 J	<6.3	0.2 J	<6.3	--	<6.3	<0.6	1.2 J	<6.3	<5	<5	<5	<5	<5	<5	<5	3.7	1650	<5
4	2	11/13/2018	0.00613	<5	<5	91.8	62.7	<5	<5	< 10	<5	1.2	<5	<5	<5	<5	<5	<5	<5	<5	<5	125	14200	<5
4	2	11/20/2019	--	<5	<5	37.7	<5	<5	0.2 J	--	<5	4.5	<5	<5	<5	<5	<5	<5	<5	<5	<5	149	21400	<5
4	3	11/14/2018	0	<5	<5	36.7	275	110	<5	< 10	754	10.7	4130	0.8 J	<5	<5	<5	<5	<5	<5	12.6	11600	127000	<5
4	3	11/20/2019	--	<5	<5	1.3 J	<5	<5	<5	--	331	23.9	208	<5	<5	<5	<5	<5	<5	<5	<5	3050	12100	<5
4	4	11/15/2018	0.007316	<11.1	<11.1	799	3760	734	<11.1	< 10	40.1	0.9 J	5350	<11.1	<12	<12	<12	<12	<12	<12	25.5	1610	686000	<12
4	4	11/20/2019	--	<6.7	<6.7	192	348	1410	<6.7	--	108	35.3	944	<6.7	<7.1	<7.1	<7.1	<7.1	<7.1	<7.1	<7.1	682	44600	<7.1
5	1	12/11/2018	0.003658	<5	<5	11.9	<5	<5	<5	< 10	<5	0.5	77.4	<5	<5	<5	<5	<5	<5	<5	<5	<0.5	336000	<5
5	1	12/14/2019	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
5	2	12/12/2018	0.004224	<5	<5	26.2	278	42.8	0.3 J	< 10	<5	0.5 J	90	<5	<5	<5	78.6	833	128	<5	<5	558	4980	<5
5	3	12/13/2018	0	<6.3	<6.3	81.2	712	211	<6.3	< 10	<6.3	16.7	109	<6.3	<6.3	<6.3	<6.3	<6.3	<6.3	<6.3	<6.3	2080	82900	<6.3
5	3	12/14/2019	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
5	4	12/13/2018	0	<5	<5	81.2	1420	<5	1.1 J	< 10	2.3 J	4.6	<5	<5	<5	<5	44.5	<5	<5	0.4 J	<5	238	991	<5
5	4	12/15/2019	--	<5	<5	<5	<5	<5	<5	< 10	<5	2.8	<5	<5	<5	<5	<5	<5	<5	<5	<5	137	<5	<5
5	source	12/14/2019	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
6	1	1/28/2019	0	<5	<5	113	380	65.8	<5	< 10	<5	7.2	11.5	<5	<5	<5	<5	<5	<5	<5	<5	219	525000	<5
6	2	1/29/2019	0	<5	<5	426	159	<5	<5	< 10	112	4	<5	<5	<5	<5	<5	<5	<5	<5	<5	34.4	302000	<5
6	3	1/30/2019	0.002647	<5	<5	840	761	<5	0.3 J	< 10	245	6.2	<5	<5	<5	<5	<5	<5	<5	<5	<5	126	58500	<5
6	4	1/31/2019	0	<5	<5	107	166	<5	3.2 J	< 10	33.4	4.9	<5	<5	<5	<5	<5	<5	<5	<5	<5	126	15100	<5
7	1	12/13/2019	0	<5	<5	<5	19.9	<5	<5	--	1.5 J	12.8	113	<5	<5	<5	<5	<5	<5	<5	<5	150	<5	<5
7	2	12/13/2019	0	<5	<5	322	524	<5	0.5 J	--	<5	13000	9200	<5	<5	<5	<5	<5	<5	<5	<5	1660000	<5	<5
7	3	12/13/2019	0	<5	<5	15	1280	3.5 J	0.7 J	--	<5	31.9	825	<5	<5	<5	<5	<5	<5	<5	<5	564	<5	<5
7	4	12/13/2019	0.004958	<5	<5	15	<5	<5	<5	--	<5	17.3	<5	<5	<5.6	<5.6	<5.6	<5.6	<5.6	<5.6	<5.6	362	<5.6	<5.6
7	source	12/13/2019	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
8	1	8/19/2020	0	<3.7	<3.7	<3.7	<3.7	<3.7	<3.7	--	<3.7	0.1 J	<3.7	<3.7	<4.6	<4.6	<4.6	<4.6	<4.6	<4.6	<4.6	<5	1910	<4.6
8	2	8/20/2020	0.00909	<5	<5	<5	<5	<5	<5	--	<5	<0.5	<5	<5	<5	<5	<5	<5	<5	<5	<5	1.00	103	<5
8	3	8/20/2020	0.011	<5	<5	<5	<5	<5	<5	--	<5	<0.5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	14	<5
8	4	8/19/2020	0	<4.3	<4.3	<4.3	<4.3	<4.3	<4.3	--	<4.3	<0.4	<4.3	<4.3	<4.1	<4.1	<4.1	<4.1	<4.1	<4.1	<4.1	3.15	948	<4.1
8	DUP-1	8/20/2020	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	1	8/26/2020	0.00255	<6.3	<6.3	<6.3	<6.3	<6.3	<6.3	-	<6.3	<0.6	<6.3	<6.3	<5	<5	<5	<5	<5	<5	<5	<0.5	<5	<5
9	2	8/26/2020	0.0132	<16.1	<16.1	<16.1	<16.1	<16.1	<16.1	-	<16.1	1.6 J	<16.1	<16.1	<13.3	<13.3	<13.3	<13.3	<13.3	<13.3	<13.3	35.7	6300	<13.3
9	3	8/27/2020	0.00579	<8.2	<8.2	10000	8000	1890	12.7	-	<8.2	2.4	3950	<8.2	<7.1	<7.1	<7.1	<7.1	<7.1	<7.1	<7.1	21	37500	<7.1
9	4	8/27/2020	0	<11.1	<11.1	<11.1	<11.1	<11.1	<11.1	-	<11.1	23.8	<11.1	<11.1	<17.2	<17.2	<17.2	<17.2	<17.2	<17.2	<17.2	3420	1170	<17.2
9	DUP-1	8/27/2020	--	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	1	11/9/2020	0	<5.00	<5.00	15.5	<5.00	<5.00	<5.00	-	<5.00	637	1900	<5.00	<5.00	<5.00	<5.00	<5.00	<5.00	<5.00	<5.00	27.5	1600	<5.00
10	2	11/9/2020	0.0688	<4.80	<4.80	41.4	<4.80	<4.80	1.20 J	-	<4.80	<0.5	<4.80	<4.80	<5.00	<5.00	<5.00	<5.00	<5.00	<5.00	<5.00	1.70	<5.00	<5.00
10	2	11/9/2020	0.0252 (repeat)	<4.80	<4.80	41.4	<4.80	<4.80	1.20 J	-	<4.80	<0.5	<4.80	<4.80	<5.00	<5.00	<5.00	<5.00	<5.00	<5.00	<5.00	1.70	<5.00	<5.00
10	3	11/10/2020	0	<4.20	<4.20	64.7	<4.20	<4.20	<4.20	-	<4.20	0.3 J	50.9	<4.20	<5.00	<5.00	<5.00	<5.00	<5.00	<5.00	<5.00	1860	6140	<5.00
10	3	11/10/2020	0	<4.20	<4.20	64.7	<4.20	<4.20	<4.20	-	<4.20	0.3 J	50.9	<4.20	<5.00	<5.00	<5.00	<5.00	<5.00	<5.00	<5.00	1860	6140	<5.00
10	DUP-1	11/10/2020	--	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	4	11/10/2020	0.0868	<5.40	<5.40	9.00	93.00	<5.40	<5.40	-	34.9	3330	1080	<5.40	<6.30	<6.30	<6.30	<6.30	<6.30	<6.30	<6.30	446000	20700	<6.30
10	4	11/10/2020	0.195 (repeat)	<5.40	<5.40	9.00	93.00	<5.40	<5.40	-	34.9	3330	1080	<5.40	<6.30	<6.30	<6.30	<6.30	<6.30	<6.30	<6.30	446000	20700	<6.30

Notes:
1. "< or U" = not detected at the SQL; J = analyte is positively identified and the result is less than LOQ/RL but greater than LOD/MDL/DL.
2. Cells marked with "--" represent analytes that were -- from a particular well during that sampling event OR replicate analysis.
3. BAL = Below analytical limit.
4. Wells with 0 values as the Net 1,4-Dioxane biodegradation rate represent wells where the 14C live microcosm rates are not significantly different than the rates from the 14C filter-sterilized groundwater control.
5. * = initial CSIA did not generate quantifiable data.
6. ** = prmA gene encodes propane monoxygenases for cometabolism and/or direct metabolism.

TABLE A.2
SUMMARY OF ALL ANALYTICAL RESULTS FROM FIELD SAMPLES

Development of a Quantitative Framework for Evaluating Natural Attenuation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-Dioxane in Groundwater
ESTCP ER-201730

Site #	Well #	Sample Date	**14C Assay Data**	**Field Parameters**						
			Net 1,4-Dioxane biodegradation rate	Temperature	Temperature	pH	Conductivity	Dissolved Oxygen	ORP	Ferrous Iron
			per year	˚C	˚F		µS/cm	mg/L	mV	mg/L
1	1	8/6/2018	0	25.8	78.4	6.84	980	3.382	198	-
1	1	3/23/2019	--	19.7	67.4	7.08	1410	0.002	18	0.422
1	2	8/7/2018	0	23.8	74.8	7.44	1624	1.385	314	-
1	2	3/23/2019	--	22.6	72.7	6.88	1450	0.050	165	0.727
1	2	12/15/2019	0	23.1	73.5	7.73	1390	0.180	156	0.293
1	3	8/8/2018	0	39.4	102.9	7.39	1885	0.442	360	-
1	3	3/23/2019	--	42.3	108.1	8.01	2060	0.230	88	0.091
1	3	12/15/2019	0	--	--	--	--	--	--	--
1	4	8/9/2018	0	29.3	84.7	6.70	1185	1.651	361	-
2	1	8/20/2018	0.002114	18.5	65.3	7.01	213	0.359	195	-
2	2	8/21/2018	0	17.9	64.2	6.73	159	0.462	72	-
2	3	8/22/2018	0	20.1	68.2	6.05	184	0.773	313	-
2	4	8/23/2018	0	18.5	65.3	6.21	234	0.657	253	-
3	1	10/1/2018	0	13.2	55.8	7.97	483	0.295	260	-
3	2	10/2/2018	0	14.5	58.1	8.02	581	0.166	60	2.343
3	3	10/3/2018	0.01139	14.3	57.7	7.99	1664	1.443	131	2.193
3	4	10/4/2018	0.09574	14.8	58.6	7.84	479	0.450	187	0.436
3	5	10/5/2018	0.020891	14.1	57.4	7.66	590	15.800	296	0.109
4	1	11/12/2018	0.015882	18.5	65.3	8.25	391	3.483	215	2.601
4	1	11/21/2019	--	-	-	-	-	-	-	-
4	2	11/13/2018	0.00613	18.5	65.3	7.32	658	1.217	248	<0.05
4	2	11/20/2019	--	-	-	-	-	-	-	-
4	3	11/14/2018	0	16.9	62.4	6.88	875	0.561	67	6.102
4	3	11/20/2019	--	-	-	-	-	-	-	-
4	4	11/15/2018	0.007316	15.0	59.0	7.22	491	0.590	186	0.788
4	4	11/20/2019	--	-	-	-	-	-	-	-
5	1	12/11/2018	0.003658	21.2	70.2	7.59	3250	1.081	191	1.783
5	1	12/14/2019	--	23.3	73.9	8.23	2770	0.140	109	0.169
5	2	12/12/2018	0.004224	22.6	72.7	7.67	807	4.711	219	0.077
5	3	12/13/2018	0	22.7	72.9	7.36	4260	1.092	198	0.341
5	3	12/14/2019	--	22.9	73.3	7.84	3230	0.300	117	0.341
5	4	12/13/2018	0	21.7	71.1	7.42	5770	0.168	212	<0.05
5	4	12/15/2019	--	22.5	72.5	7.87	5960	0.110	165	0.130
5	source	12/14/2019	--	24.3	75.7	7.75	2600	0.130	119	0.176
6	1	1/28/2019	0	21.9	71.4	7.05	2090	0.522	287	0.106
6	2	1/29/2019	0	20.1	68.2	7.47	927	0.301	200	0.102
6	3	1/30/2019	0.002647	18.3	64.9	7.31	1947	0.869	182	0.774
6	4	1/31/2019	0	20.1	68.2	7.14	2110	0.479	170	<0.05
7	1	12/13/2019	0	20.3	68.5	9.20	862	0.360	101	0.307
7	2	12/13/2019	0	21.7	71.0	8.52	3060	0.030	-54	0.429
7	3	12/13/2019	0	23.4	74.1	7.63	958	0.000	-63	0.470
7	4	12/13/2019	0.004958	22.3	72.1	8.00	670	0.670	75	0.110
7	source	12/13/2019	--	22.8	73.1	7.25	4140	0.000	-124	> 6.00
8	1	8/19/2020	0	25.11	77.198	7.08	422	5.48	164	0
8	2	8/20/2020	0.00909	22.06	71.708	6.44	458	5.13	147	0.3
8	3	8/20/2020	0.011	23.16	73.688	5.91	465	2.77	84	0.2
8	4	8/19/2020	0	19.53	67.154	6.66	429	9.12	209	0
8	DUP-1	8/20/2020	-	-	-	-	-	-	-	-
9	1	8/26/2020	0.00255	15.0	58.9	7.30	8570	2.270	158	0.400
9	2	8/26/2020	0.0132	15.3	59.5	6.87	11700	9.220	240	0.300
9	3	8/27/2020	0.00579	13.7	56.7	7.12	10500	10.500	-19	2.000
9	4	8/27/2020	0	17.2	63.0	7.00	16500	0.860	209	0.400
9	DUP-1	8/27/2020	--	-	-	-	-	-	-	-
10	1	11/9/2020	0	28.1	82.6	6.27	292	0.340	-28	>10
10	2	11/9/2020	0.0688	22.9	73.3	6.35	136	4.320	161	0.100
10	2	11/9/2020	0.0252 (repeat)	22.9	73.3	6.35	136	4.320	161	0.100
10	3	11/10/2020	0	20.9	69.7	4.56	104	0.760	270	0.600
10	3	11/10/2020	0	20.9	69.7	4.56	104	0.760	270	0.600
10	DUP-1	11/10/2020	--	-	-	-	-	-	-	-
10	4	11/10/2020	0.0868	24.2	75.6	6.72	461	0.570	-77	>10
10	4	11/10/2020	0.195 (repeat)	24.2	75.6	6.72	461	0.570	-77	>10

Notes:
1. "< or U" = not detected at the SQL; J = analyte is positively identified and the result is less than LOQ/RL but greater than LOD/MDL/DL.
2. Cells marked with "--" represent analytes that were -- from a particular well during that sampling event OR replicate analysis.
3. BAL = Below analytical limit.
4. Wells with 0 values as the Net 1,4-Dioxane biodegradation rate represent wells where the 14C live microcosm rates are not significantly different than the rates from the 14C filter-sterilized groundwater control.
5. Wells with * in the CSIA data represent wells where initial analyses were did not generate quantifiable data.

TABLE A.1

SUMMARY OF ALL ANALYTICAL RESULTS FROM ^{14}C LAB ASSAY

Development of a Quantitative Framework for Evaluating Natural Attenuation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-Dioxane in Groundwater
ESTCP ER-201730

			GC Analysis		Initial 1,4-Dioxane* (µg/L) Before 14C		Initial 1,4-Dioxane* (µg/L)		Final 1,4-Dioxane* (µg/L)		Oxygen Analysis		VOCs (µg/L)										
Samples Received	Exp. Set Up	Sample	1,4-Dioxane Change	Days of Incubation	Ave	Stdev	Ave	Stdev	Ave	Stdev	Oxygen Change	Days of Incubation	PCE	TCE	1,1-DCE	1,2-cDCE	1,2-DCA	VC	1,1,1-TCA	1,1-DCA	CA	Ethane	Ethene	Methane
8/7/2018	8/8/2018	Site 1 Well 1	No	317	0.0	0.0	163	4.7	171	13	No	383	0.0	0.0	0.0	1.9	0.47	0.0	4.9	0.0	0.0	0.0	0.0	0.0
8/8/2018	8/9/2018	Site 1 Well 2	No	316	0.0	0.0	163	4.7	167	22	No	383	0.0	4.7	0.84	0.0	2.1	0.0	7.3	0.0	0.0	0.0	0.0	0.0
12/18/2019	12/18/2019	Site 1 Well 2, resampled	No	45	0.0	0.0	163	4.7	176	10	No	46	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8/9/2018	8/10/2018	Site 1 Well 3	No	315	146	8.7	308	7.7	307	39	No	383	10.7	33	23	894	854	82	865	11	0.0	0.0	0.0	0.0
12/18/2019	12/18/2019	Site 1 Well 3, resampled	No	45	146	8.7	273	6.0	280	15	No	46	0.0	0.0	4.5	0.0	88	0.0	0.0	0.0	0.0	0.19	1.1	0.39
8/10/2018	8/11/2018	Site 1 Well 4	No	314	140	14	302	11	298	7.9	No	383	24.4	0.56	96	5991	520	0.44	0.0	23	0.0	2.1	0.0	0.0
8/21/2018	8/22/2018	Site 2 Well 1	No	307	0.0	0.0	163	4.7	164	10	No	369	0.0	0.0	0.0	3.4	0.0	0.0	10	0.0	0.65	0.0	0.0	0.0
8/22/2018	8/23/2018	Site 2 Well 2	No	306	0.0	0.0	163	4.7	164	17.9	No	369	0.0	0.0	0.0	0.0	0.0	0.0	12	0.0	0.0	0.0	0.0	0.0
8/23/2018	8/24/2018	Site 2 Well 3	No	305	0.0	0.0	163	4.7	163	18	No	369	0.0	0.0	0.0	2.9	0.11	0.0	11	0.0	0.0	0.0	0.0	0.0
8/24/2018	8/25/2018	Site 2 Well 4	No	304	0.0	0.0	163	4.7	173	20	No	369	2.5	0.0	0.38	41	2.4	0.0	45	0.06	0.0	0.0	0.0	0.0
10/2/2018	10/3/2018	Site 3 Well 1	No	267	0.0	0.0	163	4.7	171	21	No	327	13	0.0	0.0	0.0	0.0	0.0	15	0.0	0.5	0.0	0.0	0.0
10/3/2018	10/4/2018	Site 3 Well 2	No	266	50	3.1	213	5.2	199	13	No	327	0.0	0.0	0.0	3.1	0.0	0.0	13	0.0	0.50	2.5	0.0	17
10/4/2018	10/5/2018	Site 3 Well 3	Yes	265	254	12	417	10	325	36	Yes	327	0.0	0.0	0.0	17	0.0	0.0	3.7	5.4	29	3.0	0.0	14
10/5/2018	10/6/2018	Site 3 Well 4	Yes	264	0.0	0.0	163	4.7	98	17	Yes	327	0.0	0.0	0.0	0.0	0.0	0.0	4.6	0.0	0.0	0.0	0.0	0.0
10/5/2018	10/6/2018	Site 3 Well 5	Yes	264	95	6.0	257	6.3	196	31	No	327	0.0	0.0	0.0	0.0	0.0	0.0	12	0.0	0.0	0.0	0.0	0.0
11/13/2018	11/14/2018	Site 4 Well 1 (Set I, GW only)	Yes	243	89	77	252	55	0	0.0	No	285	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
11/14/2018	11/15/2018	Site 4 Well 2 (Set I, GW only)	No	229	116	10	279	8.5	279	15	No	285	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
11/15/2018	11/16/2018	Site 4 Well 3 (Set I, GW only)	No	228	11005	93	11,168	66	11,296	301	No	285	0.0	0.0	1.2	0.0	0.0	2.6	0.0	3.9	2.7	0.0	0.26	1.4
11/16/2018	11/17/2018	Site 4 Well 4 (Set I, GW only)	Yes	228	349	21	512	15	480	14	No	285	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
11/21/2019	11/22/2019	Site 4 Well 1, resampled (Set II, GW only)	No	41	0.0	0.0	166	5	175	19	No	41	0.0	0.0	0.0	0.0	19	0.0	0.0	0.0	0.0	0.0	0.0	0.0
11/21/2019	11/22/2019	Site 4 Well 2, resampled (Set II, GW only)	No	41	128	8.7	294	8	312	40	No	41	0.0	0.0	0.0	0.0	19	0.0	0.0	0.0	0.0	0.0	0.0	0.0
11/21/2019	11/22/2019	Site 4 Well 3, resampled (Set II, GW only)	No	41	8678	553	8,844	391	8,582	268	No	41	0.0	135	0.0	0.0	80	0.0	0.0	0.0	0.0	0.0	0.0	0.5
11/21/2019	11/23/2019	Site 4 Well 4, resampled (Set II, GW only)	No	42	381	24	547	18	437	110	Yes	41	0.0	0.0	0.0	0.0	38	0.0	0.0	0.0	0.0	0.0	1.3	0.0
11/20/2020	11/20/2020	Site 4 Well 2 (Set III, GW only)	Yes	42	126	23	294	18	180	32	No	42	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
11/20/2020	11/20/2020	Site 4 Well 5, North MW-26 (Set III, GW + soil)	No	42	0.0	0.0	168	6	170	16	No	42	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
11/20/2020	11/20/2020	Site 4 Well 6, South MW-6 (Set III, GW + soil)	Yes	42	0.0	0.0	168	6	137	11	No	42	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
12/12/2018	12/13/2018	Site 5 Well 1	No	209	91	11	260	10	276	10	No	256	0.0	0.0	161	60	171	0.0	296	3.7	0.0	0.0	0.0	0.0
12/13/2018	12/14/2018	Site 5 Well 2	No	208	0.0	0.0	169	6.2	160	12	No	256	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
12/14/2018	12/15/2018	Site 5 Well 3	No	208	0.0	0.0	169	6.2	174	5.0	No	256	0.0	0.0	5.9	24	2.7	0.0	23	0.0	0.0	0.0	0.0	0.0
12/14/2018	12/15/2018	Site 5 Well 4	No	207	0.0	0.0	169	6.2	175	8.1	No	256	0.0	0.0	48	18	65	0.0	77	0.39	0.0	0.0	0.0	0.0
12/18/2019	12/18/2019	Site 1 Well 4, resampled	No	45	0.0	0.0	163	4.7	170	24	No	46	11.3	0.0	0.0	0.0	23	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1/29/2019	1/30/2019	Site 6 Well 1	No	163	0.0	0.0	169	6.2	173	5.6	No	208	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1/30/2019	1/31/2019	Site 6 Well 2	No	162	0.0	0.0	169	6.2	169	8.8	No	208	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1/31/2019	2/1/2019	Site 6 Well 3	No	161	0.0	0.0	169	6.2	188	20	No	208	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2/1/2019	2/2/2019	Site 6 Well 4	No	160	0.0	0.0	169	6.2	170	20.1	No	208	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
12/14/2019	12/15/2019	Site 7 Well 1	No	48	0.0	0.0	169	6.2	177	8	No	49	0.0	77	0.0	0.0	99	0.0	0.0	0.0	0.0	0.0	0.0	1.4
12/14/2019	12/15/2019	Site 7 Well 2	No	48	1475	99	1644	70.5	1,680	44	No	49	0.0	0.0	0.0	0.0	30	0.0	0.0	0.0	0.0	0.0	0.0	0.0
12/14/2019	12/15/2019	Site 7 Well 3	No	48	0.0	0.0	169	6.2	169	12	No	49	0.0	0.0	0.0	0.0	18	0.0	0.0	0.0	0.0	0.0	0.0	0.0
12/14/2019	12/15/2019	Site 7 Well 4	No	48	0.0	0.0	169	6.2	170	19	No	49	0.0	0.0	0.0	0.0	11	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8/21/2020	8/21/2020	Site 11 Well 1 (Site 8)	No	42	0.0	0.0	163	6.6	170	2	No	41.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8/21/2020	8/21/2020	Site 11 Well 2 (Site 8)	No	42	45	1.3	208	4.8	206	6	No	41.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8/21/2020	8/21/2020	Site 11 Well 3 (Site 8)	No	42	0.0	0.0	163	6.6	164	6	No	41.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8/21/2020	8/21/2020	Site 11 Well 4 (Site 8)	No	42	0.0	0.0	163	6.6	159	4	No	41.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8/28/2020	8/28/2020	Site 12 Well 1 (Site 9)	No	42	0.0	0.0	168	6.2	162	4.0	No	41.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8/28/2020	8/28/2020	Site 12 Well 2 (Site 9)	No	42	8177	234	8346	165.4	8036	536.4	No	41.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8/28/2020	8/28/2020	Site 12 Well 3 (Site 9)	No	42	864	78	1033	55.8	951	56.1	No	41.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8/28/2020	8/28/2020	Site 12 Well 4 (Site 9)	No	42	1126	36	1294	26.0	1155	98.0	No	41.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
11/11/2020	11/11/2020	Site 13 Well 1 (Site 10)	No	42	99	6.7	267	8	259	18	No	42.0	0.0	0.0	0.0	0.0	119.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
11/11/2020	11/11/2020	Site 13 Well 2 (Site 10)	Yes	42	284	36	453	26	300	10	No	42.0	0.0	0.0	0.0	0.0	31.1	0.0	0.0	0.0	0.0	2.7	33.6	2.5
11/11/2020	11/11/2020	Site 13 Well 3 (Site 10)	No	42	1003	49	1172	35	1142	69	No	42.0	0.0	0.0	0.0	0.0	11.1	0.0	0.0	0.0	0.0	0.0	2.7	0.0
11/11/2020	11/11/2020	Site 13 Well 4 (Site 10)	Yes	42	153	30	321	22	259	20	No	42.0	0.0	0.0	5.6	0.0	11.8	0.0	0.0	0.0	0.0	13.8	58.0	8.4
11/11/2020	11/11/2020	Site 13 Well 2 (Site 10)	No	42	284	36	453	26	409	19	No	42.0	0.0	0.0	0.0	0.0	31.1	0.0	0.0	0.0	0.0	2.7	33.6	2.5
11/11/2020	11/11/2020	Site 13 Well 3 (Site 10)	No	42	1003	49	1172	35	1144	25	No	42.0	0.0	0.0	0.0	0.0	11.1	0.0	0.0	0.0	0.0	0.0	2.7	0.0
11/11/2020	11/11/2020	Site 13 Well 4 (Site 10)	Yes	42	153	30	321	22	198	6	No	42.0	0.0	0.0	5.6	0.0	11.8	0.0	0.0	0.0	0.0	13.8	58.0	8.4

**** Final 1.4-Dioxane concentration on last monitoring day.***
***** Bottle with GW and CB1190 (10^-2).***
****** Bottle with GW and CB1190 (10^-2) AND %5 AMSM.***

Appendix B:
User's Guide for BioPIC (Decision Framework)

Quick BioPIC User Guide

Updated October 2021

This Quick Guide is intended for users of the application BioPIC (Bio Pathway Identification Criteria), which uses the Microsoft Excel 2020 platform. This is an updated version of the original BioPIC, which was first developed under ESTCP Project ER-201129 for evaluating Monitored Natural Attenuation (MNA) for chlorinated ethenes. Separate modules for 1,4-dioxane (1,4-D) and chlorinated ethanes have recently been added to BioPIC under ESTCP Project ER-201730 (note that no change to the decision framework for chlorinated ethenes were made as part of this update).

OBJECTIVE

The tool is intended to help users follow OSWER directive 9200.4-17P on MNA of chlorinated ethenes. While the USEPA has yet to develop a similar directive for chlorinated ethanes and 1,4-dioxane, the tool follows a very similar technical approach in evaluating MNA for these compounds.

OVERVIEW OF FRAMEWORK

BioPIC is organized around the USEPA lines of evidence for MNA Framework (USEPA, 1998 and 1998) where the first line of evidence is *Historical groundwater … data that demonstrates a clear and meaningful trend of decreasing contaminant* … Therefore, use of BioPIC requires that the user first applies a groundwater fate and transport model to determine whether the rate of attenuation of the contaminants will bring the highest concentrations of the contaminants in groundwater to acceptable concentrations before the groundwater reaches a receptor or a sentry well. If the predicted concentrations are acceptable, MNA is appropriate. As part of the 2021 update, a model for predicting contaminant trends over time and distance, including a method to estimate site-specific biodegradation rate constants for chlorinated ethenes, chlorinated ethanes, and 1,4-dioxane, has been included within BioPIC.

If MNA is appropriate, BioPIC offers guidance on developing information that can meet the USEPA requirement for a second lines of evidence *that can be used to demonstrate indirectly the type(s) of natural attenuation processes active at the site, and the rate at which such processes will reduce contaminant concentrations to required levels*. For chlorinated ethenes, BioPIC offers guidance on alternative remedies in cases where MNA is not appropriate, specifically the use of in situ bioremediation, and whether it is useful to bioaugment the site with active microorganisms as well as biostimulate with nutrients.

BIOPIC START-UP AND HOME PAGE

Please begin by opening the file titled BioPIC_2021.xlsm. When the screen first opens, click on “enable macros” or enable these within the application settings. These macros are required for using the software; consult with IT or system administrators as needed.

Upon opening the file, you’ll see 3 different red “Start” buttons and 3 different blue “Overview” buttons.

- By clicking on one of the red “Start” buttons for a set of target compounds, you will be led to a stepwise diagnostic process with several YES/NO questions following the framework logic available in the blue “Overview” buttons.

- By clicking on the one of the blue "Overview" buttons for a set of target compounds, you will see a flowchart representation of the entire Decision Framework for that set of compounds. This serves as a reference for users so that they get a sense of the decision logic, and it may be valuable to print out and include in deliverables.

You'll also see 5 tabs (worksheets) at the bottom of the screen. The first tab—the Home tab—is the starting point. A user can always click the Home tab to return to the home page and chose another option (or to start over). The three "Guided Tour" tabs lead to the same screens as the red "Start" tabs for each of the targeted compounds; these are redundant but are included for users who were accustomated to the previous version of BioPIC. The final tab is a "FILES" tab that contains several useful calculators (described in more detail below) that can be launched or downloaded separately as needed.

NAVIGATION TIPS:

After clicking on one of the red "Start" buttons, you are taken to a separate page that provides a guided tour through the relevant decision framework. A few simple rules for navigating these pages:

- Most questions can be answered by clicking on one of 5 (five) potential options: YES, NO, Decision Criteria, Help and Back.
- When a YES or NO button is chosen, the next question will appear.
- If users are uncertain how to answer the question, a click on the Decision Criteria or Help button displays more detailed background information that should help the user to select the appropriate answer.
- By clicking the Back button, the user will be directed back to the previous question.

"FILES" TAB (last tab on the Home Page):

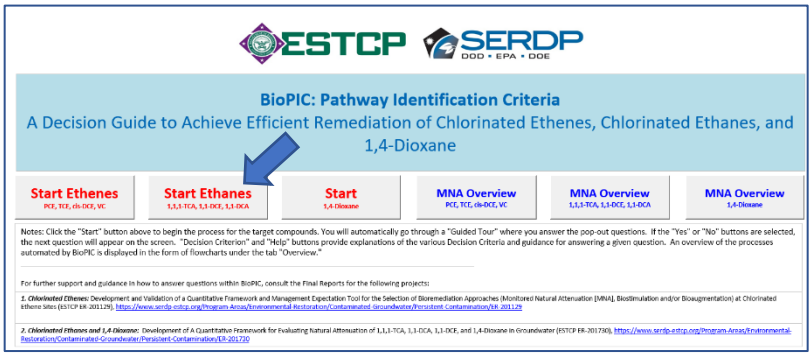
The last (5th) tab (worksheet) on the BioPIC Home Page is titled "Files" and contains several Excel files as separate objects to aid users to enter data for further analysis. Users, for example, can click the "CSIA.XLSX", "Dhc.XLSX", "FeS.XLSX", "Magnetic Susceptibility.XLSX" or "Mole Percent Calculator.XLSX" buttons in the "Decision Criteria" box, and will be automatically directed to these tab "Files." By double-clicking the Excel button, the corresponding Excel file will be displayed. Note that this includes all files that were part of the original release of BioPIC, as well as several new files developed as part of the 2021 update. The latter include a new "MNA Rate Constant Estimator" that serves as a standalone contaminant fate and transport model. It was patterned after BIOCHLOR (though using a slightly different code) but incorporates more compounds and some other features (described in more detail in the User's Guide for this model, which is also Appendix C of the project report for ESTCP ER-201730).

Users are encouraged to provide feedback and report incidents for continuous improvement of the BioPIC tool to Carmen A. Lebron (lebron.carmen.a@gmail.com), John Wilson (john@scissortailenv.com) or David Adamson (dtadamson@gsienv.com).

Detailed BioPIC User Guide:

Chlorinated Ethanes

The following describes the Decision Framework for the compounds that are part of the Ethanes module, including 1,1,1-TCA and 1,1-DCA, as well as 1,1-DCE. The latter compound is part of this module because it is primarily of interest as a by-product of a chlorinated ethane degradation pathway (i.e., the abiotic degradation of 1,1,1-TCA).



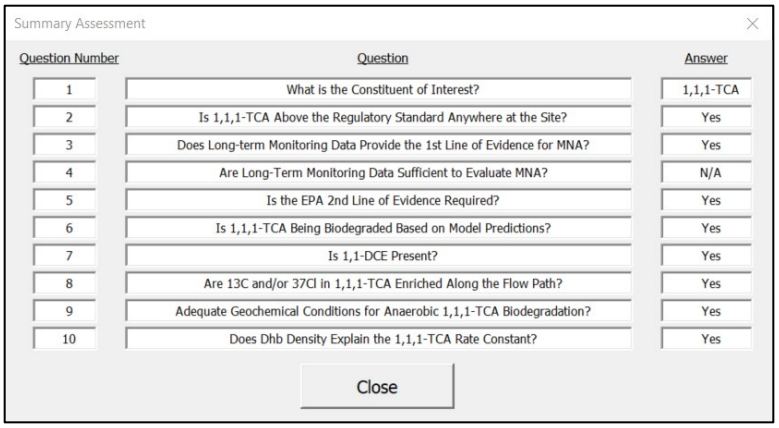
BioPIC Home Page showing start button for entering the chlorinated ethane decision framework

Each of the numbered questions below corresponds to a number in the flowchart/guided tour. After each number, the decision criteria are explained. For most, further information is provided in the Help text. Note that these text descriptions are shown as pop-up boxes within the tool. A graphic showing the entire decision flowchart for these compounds is reproduced at the end of this section.

1. What is the constituent of interest?

Decision Criteria:

Choose the appropriate constituent of interest. Options are 1,1,1-TCA, 1,1-DCA, and 1,1-DCE. This will take the user through the decision logic for that particular compound (i.e., to Question #2 if 1,1,1-TCA is selected). Once finished with the logic for the selected compound, a summary assessment will be displayed that shows the results for that particular compound (see example graphic at right). The process can be then repeated for the remaining compound(s). Note that once the user has selected a constituent of interest and starts answering the subsequent questions, the summary assessment can also be pulled up by clicking the View Summary box that appears to the right of each question. In these cases, it will display answers to only those questions that have been completed.



Example of Summary Assessment pop-up box after completing the stepwise decision framework for 1,1,1-TCA

2. Is 1,1,1-TCA above the regulatory standard anywhere at the site?

Decision Criteria:

Answer YES if: The 1,1,1-TCA concentration at any groundwater monitoring location at the site is above the applicable concentration-based regulatory standard. Note that this standard is site-specific. If no standard has been established for your site, then select a value based on guidance from EPA or other states (see **HELP**) for planning purposes.

Answer NO if: The 1,1,1-TCA concentration at each groundwater monitoring location at the site is already below the concentration-based regulatory standard. The decision tool is intended for sites where the concentration of 1,1,1-TCA must fall below a site-specific regulatory standard before contaminated groundwater reaches the point of compliance (POC). If the 1,1,1-TCA concentration is already below the regulatory standard across the site (including the POC), then it is highly likely that it will remain below this standard in the future. This assumes that the site has been reasonably well characterized (especially the source area) and that no significant changes in conditions at the site are anticipated.

HELP

In the absence of site-specific regulatory standards for 1,1,1-TCA the user may wish to select the federal MCL (200 µg/L) for 1,1,1-TCA to proceed with the BioPIC evaluation.

If 1,1,1-TCA is already below the established or assumed standard, then a future exceedance would likely be associated with one or more of the following: 1) the site is poorly characterized; 2) a new release of 1,1,1-TCA occurs; 3) active remediation is on-going or recently completed, such that steady state conditions have not yet been reached; or 4) any other change in site conditions has occurred that enhance 1,1,1-TCA mass transfer to the aquifer and inhibit 1,1,1-TCA attenuation.

3. Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?

Decision Criteria

Answer YES if: The 1,1,1-TCA concentration is currently below the regulatory standard at the point of compliance (POC) and is predicted to be below the concentration-based regulatory standard at the POC at any time in the future. Use the model provided as part of this tool (see **FILES** for "MNA Rate Constant Estimator") to predict if the concentration will be below the standard at any time in the future (see **HELP** for additional explanation).

Answer NO if: At any time in the future, the 1,1,1-TCA concentration will exceed the regulatory standard at the POC. Use the model provided as part of this tool to predict the concentration in the future at the POC. Note there usually is also a temporal component in the regulatory goals, which involves establishing how long it will take the concentration at a particular location to achieve a regulatory goal. The implementation of more aggressive remedies may reduce time to achieve remediation goals, thereby reducing the overall cost. However, this tool primary deals with the spatial aspects of remediation goals (i.e., will the goal be achieved at a POC) rather than the temporal components.

HELP

If sufficient historical contaminant concentration data are not available to determine if the 1,1,1-TCA plume will reach a POC, then a groundwater flow and solute transport model, such as the "MNA Rate Constant Estimator" provided as part of this tool (see **FILES**) should be used to predict solute plume behavior. In this case, the simulation should account for the effects of advective groundwater flow, dispersion of the relevant solutes, sorption, and degradation of 1,1,1-TCA in groundwater at the site.

For more information on using this type of model for 1,1,1-TCA, consult the project report (Development of a Quantitative Framework for Evaluating Natural Attenuation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-Dioxane in Groundwater (ESTCP ER-201730)), which can be found on the project page (copy link into web browser): https://serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/Persistent-Contamination/ER-201730/ER-201730.

A similar approach for chlorinated ethenes can be found in another project report (Development of a Quantitative Framework and Management Expectation Tool for the Selection of Bioremediation Approaches at Chlorinated Ethene Sites (ESTCP Project ER-201129)), which can be downloaded from the project page (copy link into web browser): https://www.serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/Persistent-Contamination/ER-201129/ER-201129. In the ER-201129 report, Section 5.2.3 illustrates the process of calibrating a groundwater flow and transport model (in this case, the "BIOCHLOR" model), and Section 5.2.4 Step 1 illustrates the use of a model to apply the decision criteria.

If available, a robust historical database of contaminant concentrations can be used as an alternative to a computer model. Spatial and temporal trends in solute concentrations can be utilized to determine if the plume is stable or receding and therefore will not reach the POC. When sufficient data are available, using empirical data to ascertain trends is much better than using a model. In many cases, sufficient 1,1,1-TCA concentration data are available to evaluate plume behavior and to determine if solute concentrations will exceed cleanup goals at a regulatory POC.

4. Is Long-Term Monitoring Data Sufficient to Evaluate MNA?

Decision Criteria

Answer YES if: The current long-term monitoring data confirm that one or more of the following conditions are met:

1. The plume is currently beyond the point of compliance at a concentration that is above the applicable standard.
2. The plume is still expanding and is predicted to extend beyond the point of compliance in the future based on modeling.
3. Concentration-based goals have been established within the plume (i.e., upgradient of the point of compliance), and model predictions suggest that these will not be achieved with a reasonable timeframe.

Answer NO if: The current long-term monitoring data confirm that ALL of the following conditions are met:

1. The plume is currently not beyond the point of compliance.
2. The current dataset is too limited to evaluate if plume is expanding vs. receding.
3. The current dataset is too limited to predict using a model whether the plume will expand or whether concentration-based goals will be achieved.
4. There is no current regulatory requirement for active remediation.

HELP

Long-term monitoring data are an important component of site management, and they are particularly important for demonstrating the site-specific viability of MNA.

It is possible that a site's existing dataset for this compound may be limited if it is a recent addition to the monitoring program. It may not be possible to establish a clear trend in the attenuation in concentration of 1,1,1-TCA or its degradation products along a flow path in groundwater at appropriate monitoring points. This means that the data are inadequate to permit a thorough evaluation for the primary line of evidence for MNA. This is typically because one or more of the following apply: 1) data are highly variable across the site; 2) data are highly variable from event-to-event; or 3) data are available from a relatively limited number of monitoring points or events. In each case, these data limitations make it difficult to establish trends with any degree of statistical certainty.

In that case, collecting additional data may be beneficial to provide more certainty that current trends are statistically significant and sustainable. These additional monitoring events may also include data that would be used to support the second line of evidence for MNA (e.g., geochemical data, biomarkers, stable isotopes) or even hydrogeologic parameters to improve fate and transport model predictions.

At sites where the number of monitoring locations is relatively small (e.g., 4 or less wells), this type of analysis will likely benefit by including additional monitoring locations along the plume transect. In part, this is because it can be more challenging to establish that a trend is significant using standard approaches (e.g., that the slope of a best-fit regression line is different than zero). As a result, adding more monitoring locations along the plume transect may provide value.

It is necessary to use modeling software to evaluate if current trends in natural attenuation will meet the standard at the point of compliance (POC). An example is the "MNA Rate Constant Estimator" that has been developed as part of this decision tool (see **FILES**). This type of model allows the user to predict concentrations along a plume transect. However, it relies on the user to calibrate to the model predictions based on field data, and it also assumes that those field data are representative. At sites where data vary considerably from event to event, this type of calibration can be challenging. In any case, the goal is to demonstrate that the plume footprint is stable and/or shrinking and will not result in concentrations at a downgradient POC that exceed an acceptable level. This may require additional monitoring locations (particularly if the plume extent is not yet delineated) or additional monitoring events to demonstrate longer-term stability. Data from additional events are also important to establish that trends are sustainable even if minor (or major) changes in site conditions (e.g., groundwater flow directions, redox conditions) occur that might impact attenuation and/or plume stability.

In some cases where MNA is proposed as a remedy, an estimate of the remediation timeframe is also required by the regulatory agency. The remediation timeframe is typically the date when some or all wells are projected to achieve a concentration goal. Estimating the remediation timeframe is often done using

linear regression of concentration vs. time data at a specific well (or wells), but this requires data from enough events to ensure that the result is statistically significant. Additional monitoring events may be required to achieve this objective at sites with limited concentration vs. time data.

For the case where the dataset is already robust and confirms that MNA is unlikely to be effective, active remediation is likely to be the next course of action. Active remediation should be selected and implemented based on appropriate, well-defined objectives, but one primary goal should be to reduce concentration in the source area enough so that concentrations are below the site-specific standard at the point of compliance. The required reduction in the source concentration can be estimated using models, including the one provided as part of this tool (see **FILES**). Another good option is REMChlor-MD, which allows the user to input the extent of source reduction and then compare the plume behavior for cases with and without remediation. REMChlor-MD also incorporates the effects of matrix diffusion (i.e., contaminants diffusing into and out of lower-permeability intervals within the saturated zone), which has implications for contaminant transport and persistence at many sites.

Additional resources for understanding and developing monitoring objectives for MNA include the following (copy link into web browser):

https://clu-in.org/download/contaminantfocus/dnapl/Treatment_Technologies/performance_monitoring_mns600R04027.pdf

https://www.serdp-estcp.org/content/download/25789/262545/file/FAQ%20ER-201211.V2%20February%202014.pdf

https://clu-in.org/download/techfocus/na/NA-approach-for-eval-2011.pdf

https://www.epa.gov/sites/production/files/2014-02/documents/d9200.4-17.pdf

https://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=10004674.TXT

https://pubs.usgs.gov/wri/wri034057/

5. Is the EPA Second Line of Evidence Required?

Decision Criteria

Answer YES if: The appropriate regulatory authority has requested that multiple lines of evidence for 1,1,1-TCA attenuation should be collected before approval of MNA as a site remedy will be granted. The first direct line of evidence requires data that demonstrate a clear and meaningful trend of decreasing contaminant mass and/or concentration over time at appropriate monitoring or sampling points. The second line of evidence originally included "hydrogeologic and geochemical data that can be used to indirectly demonstrate the type(s) of natural attenuation processes active at the site, and the rates at which such processes will reduce contaminant concentrations to required levels".

Answer NO if: The appropriate regulatory authority has specifically requested that only the first/primary line of evidence for natural attenuation is required for approval of MNA as a site remedy. The first direct line of evidence requires data that demonstrate a clear and meaningful trend of decreasing contaminant

mass and/or concentration over time at appropriate monitoring or sampling points. Note that the final decision to require, or to not require, the second line of evidence is made by the appropriate regulatory authority. If the regulatory authority has not signaled what lines of evidence will be required, then a more conservative approach would be to answer "YES" to this question and proceed with collecting additional lines of evidence.

HELP

As part of EPA's MNA guidance, the agency has listed three lines of evidence that may be part of an MNA evaluation. Collecting data to support the first line of evidence is always required, and data to support the second line of evidence is typically required. The rest of this decision tool is focused on the second and third lines of evidence. It is recommended that the user go through these decision points even if the applicable data are not available.

For sites where the second line of evidence is required, it is expected that one focus will be on establishing that geochemical conditions are favorable for targeted reactions and on estimating degradation rates. However, it should be noted that 1,1,1-TCA will naturally attenuate in aquifers via hydrolysis/dehydrohalogenation, and this reaction occurs at a predictable rate based on the groundwater temperature. This information should be used to support other secondary lines of evidence in supporting natural attenuation.

The following resources provide more information on the lines of evidence approach, including definitions and how they are used (copy link into web browser):

https://www.epa.gov/sites/production/files/2014-02/documents/d9200.4-17.pdf

https://www.serdp-estcp.org/content/download/25789/262545/file/FAQ%20ER-201211.V2%20February%202014.pdf

6. Is 1,1,1-TCA biodegrading based on model predictions?

Decision Criteria

Answer YES if: Using 1,1,1-TCA degradation rate constants greater than zero in the model provides a better fit than the fit when the rate constant is set to zero. This can be evaluated using the same simulation in the "MNA Rate Constant Estimator" model that was used in "Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?" Prepare a new simulation where the rate constant for degradation of 1,1,1-TCA is set to zero (note that the model automatically incorporates degradation due to hydrolysis). Compare the actual in situ concentrations of 1,1,1-TCA against the new simulation. Then enter trial values for the rate constant for 1,1,1-TCA degradation into the simulation to determine if the model projections provide a better fit to the actual field-measured concentrations. A better fit is defined as having a lower value of RMSE (root mean square error) between the field data and the concentrations predicted by the model). If rate constants greater than zero provide a better fit, then 1,1,1-TCA is degrading. Note that this may have already been established as part of the earlier evaluation of the criterion "Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?"

Answer NO if: Setting the 1,1,1-TCA degradation rate constant to zero in the model provides a better fit than the fit when the rate constant is greater than zero. This is evaluated using the same model and simulations described above for the "YES" answer.

7. Is 1,1-DCE present?

Decision Criteria

Answer YES if: 1,1-DCE has been present above the reporting limit at any groundwater monitoring location at the site. This compound is a by-product of 1,1,1-TCA hydrolysis and therefore serves as a confirmatory line of evidence that this reaction is actively contributing to 1,1,1-TCA attenuation.

Answer NO if: 1,1-DCE has not been present at any groundwater monitoring location at the site, either currently or historically.

8. Are ^{13}C and/or ^{37}Cl in 1,1,1-TCA enriched along the flow path?

Decision Criteria

Answer YES if: A clear pattern of carbon and/or chlorine isotope fractionation can be observed in samples collected along a groundwater flow path. For 1,1,1-TCA, values of $\delta^{13}C$ and $\delta^{37}Cl$ can be obtained for individual samples via commercial lab analysis (see **HELP** for further explanation of CSIA principles and analytical considerations). If samples are taken from the source area and then at several locations downgradient, a 2-dimensional plot of these values can then be generated (see CSIA_111TCA in **FILES**). If the values of both $\delta^{13}C$ and $\delta^{37}Cl$ generally increase along the groundwater flow path (i.e., become "less negative" due to depletion of the lighter isotope), then this is taken as evidence for degradation of 1,1,1-TCA.

Answer NO if: No isotope data are available or if there is no clear trend in samples collected along a groundwater flow path. Again, this is best visualized by creating a 2-D plot of the $\delta^{13}C$ and $\delta^{37}Cl$ values (see HELP and FILES for more guidance).

HELP

Additional lines of evidence for 1,1,1-TCA attenuation can be provided by site-specific analysis of samples for stable isotopes of carbon and chloride. Data from a single sample may not provide sufficient evidence for 1,1,1-TCA degradation. This is because little is known about the natural variation in in the isotopic composition of the 1,1,1-TCA that was originally released to groundwater. Collecting multiple samples along the groundwater flow path is a more appropriate approach for establishing degradation because it relies on site-specific isotopic data to document 1,1,1-TCA degradation.

If values for $\delta^{13}C$ and $\delta^{37}Cl$ are available for 1,1,1-TCA, open the tab Files and select the spreadsheet CSIA_111TCA.xlsx. Enter your data in the tab Input. The user can enter data for up to 8 wells. Data from a well located within the source area or in an upgradient area should be entered in the first row (Well 0); this provides a site-specific estimate of the isotopic composition of undegraded 1,1,1-TCA and serves as a baseline for further comparisons. This well should have the lowest (most negative) values for $\delta^{13}C$ and

$\delta^{37}Cl$ (prioritize the well with the lowest $\delta^{37}Cl$). Data from other wells are entered in the remaining rows, following the order that they fall along the groundwater flow path.

Once the available data have been entered, the user can first consult the tab 2-D Chart_simple. Your data should plot on the chart. If not, you may need to extend the scales of the x and/or y axes. Degradation of 1,1,1-TCA is indicated if the data points generally proceed up and/or to the right within the plot in the direction of groundwater flow. This occurs due to the preferential degradation of bonds that contain lighter isotopes, such that the lighter isotopes become depleted and the heavier isotopes become enriched within the remaining portion of the compound as it is transported downgradient. The degree of enrichment can vary depending on the compound, the isotope, and the transformation pathway.

The error bars represent uncertainty in the determination of $\delta^{13}C$ and $\delta^{37}Cl$. The values can be said to increase between two samples if either one or both of the vertical or horizontal error bars do not overlap.

For 1,1,1-TCA, the user can also roughly estimate the amount of 1,1,1-TCA that has been degraded based on different possible degradation pathways (see Step 2 in the Input tab). This relies on published isotopic enrichment factors (Ɛ) for carbon and/or chlorine for three different abiotic transformation pathways: (1) hydrolysis/dehydrohalogenation; (2) reductive dechlorination by zero-valent iron; (3) oxidation via persulfate; and 4) biological reductive dechlorination of 1,1,1-TCA is also known to cause fractionation of carbon isotopes, but the effect is relatively small. Note that for Pathway 4, the chlorine isotope enrichment factor for biological reduction has yet to be established, so it is not included in the 2-D plots described below.

For each of the four possible pathways listed above, the percent of 1,1,1-TCA degraded is presented as a range based on the uncertainty in the isotopic enrichment factors, as well as a user-input uncertainty factor. The latter can be used to perform a limited sensitivity analysis on the degradation estimates.

To better understand how the data compare to the expected isotopic fractionation patterns for each pathway, the user can consult the tab 2-D delta from upgradient. In this chart, the origin is the isotopic composition of the upgradient/source well (Well 0), and the rest of the site-specific data are plotted as symbols. The three solid lines represent the fractionation pattern associated with each of the first three pathways described above as degradation proceeds. The slopes of these lines reflect changes to both elements (carbon and chloride) and are minimally influenced by retardation and other non-destructive processes that may occur during groundwater transport. If the data adhere to a specific pathway line, then this is plausible evidence that this specific pathway may be contributing to the observed fractionation. It should be understood that alternate or multiple transformation pathways may be occurring and cause data to not adhere to any of the plotted lines.

9. Are geochemical conditions adequate for anaerobic 1,1,1-TCA biodegradation?

Decision Criteria

Answer YES if: Dissolved oxygen is routinely absent in groundwater samples from one or more wells in the 1,1,1-TCA plume or in the area downgradient of the plume. This is a qualitative line of evidence that conditions are favorable to support anaerobic reductive dechlorination but does not imply that 1,1,1-TCA is actually being biodegraded. A threshold (maximum) DO value that would preclude anaerobic

biodegradation has not been established, and because of field sampling limitations, dissolved oxygen concentration data on well water are often unreliable. For the purposes of this decision tool, conditions are considered generally favorable for anaerobic biodegradation of 1,1,1-TCA when one of the following criteria are met: Dissolved oxygen concentrations measured in the field are less than 0.1 mg/L, ferrous iron (Fe^{2+}) concentrations are greater than 0.5 mg/L, and methane concentrations are greater than 0.005 mg/L.

Answer NO if: Dissolved oxygen is typically present at elevated levels (> 1 mg/L) across the entire site. This might include sites where the impacted intervals are shallow, unconfined, and/or organic-rich. This type of determination would also rely on other corroborating geochemical data, such as highly positive ORP readings (≥ +100 mV against the AgCl reference electrode), ferrous iron (Fe^{2+}) < 0.5 mg/L or methane < 0.005 mg/L.

HELP

1,1,1-TCA can be naturally attenuated by reactions that occur in primarily anaerobic conditions (e.g., biological reductive dechlorination to 1,1-DCA and abiotic degradation by reactive minerals via several different pathways). 1,1,1-TCA can also be naturally attenuated by a hydrolysis/dehydrohalogenation reaction that will proceed regardless of the redox conditions.

In assessing whether geochemical conditions are favorable for anaerobic vs. aerobic processes, it should be noted that field methods for measuring dissolved oxygen may generate inconsistent and/or erroneous results. One contributing factor is the common use of long-screened (≥ 10 ft) monitoring wells that may be collecting water from multiple zones with different redox conditions. This mixing of groundwater can make it difficult to quantify zones with higher dissolved oxygen that may promote 1,1,1-TCA biodegradation. Consequently, field dissolved oxygen measurements should be used with caution and supported by other lines of evidence. Other data that would corroborate that geochemical conditions are favorable for reductive dechlorination of 1,1,1-TCA include negative ORP readings and elevated dissolved iron and methane concentrations. Total organic carbon (> 20 mg/L) is also a positive indicator because it provides a carbon source and electron donor to promote microbial reductive dechlorination. In addition, portions of the site where groundwater transitions between anaerobic and aerobic should be delineated to identify areas that might be best managed by different natural attenuation pathways.

10. Does *Dhb* Density Explain the 1,1,1-TCA Rate Constant?

Decision Criteria

Answer YES if: The 1,1,1-TCA biodegradation rate constant used in the model is consistent with correlations based on the abundance of the Dhb biomarker (also referred to as DHBt) for strains of *Dehalobacter* bacteria that degrade 1,1,1-TCA. The correlations were derived from other studies and kinetic data. To do this, first refer to the simulation in the "MNA Rate Constant Estimator" model that was used to evaluate the criterion "Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?". Note that this model has the option to use biomarker data to estimate the biodegradation rate constant (i.e., it uses a correlation to predict the representative rate constant based on the biomarker levels measured at the site). If this option was employed and it resulted in a reasonable fit to the actual

data, then this confirms that "YES" is the appropriate answer. See **HELP** for additional guidance on determining if the fit was reasonable.

Answer NO if: No data on the abundance of the Dhb biomarker are available, or if the 1,1,1-TCA biodegradation rate constant used to optimize the model is inconsistent with rate constants predicted using the biomarker correlations. To evaluate the latter condition, first refer to the model simulation that was used to evaluate the criterion "Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?". If the option to use the correlation was not employed OR did not result in an optimal fit, then "NO" is the appropriate answer.

HELP

For 1,1,1-TCA, the "MNA Rate Constant Estimator" model (see **FILES**) has an option to estimate rate constants based on the abundance of Dhb (also referred to as DHBt), which is a qPCR-based biomarker for degradation of this compound. The correlations are designed to help calibrate the model, and they are intended as a starting point for improving the fit between the actual field data and the model simulations. Consequently, they should not be considered a true prediction of the actual degradation rate that is occurring at the site. This is because they are based on empirical data from other studies where conditions may be quite different than those observed at the site being evaluated.

To use the biomarker correlations as part of this decision tool, the following process is recommended:

1. In Box 6b, select the specific biomarker Dhb from the dropdown menu. On the chlorinated ethanes module of this model, this is one of only two biomarker options; only Dhb is applicable for 1,1,1-TCA. Selecting a biomarker from the menu will launch a pop-up box where biomarker abundance data can be entered.
2. In the pop-up box, enter the abundance of the selected biomarker for those wells where data are available. The model will perform a spatial interpolation to estimate a representative biomarker abundance for the site (i.e., a single value that is weighted based on the distance between wells with biomarker data).
3. The rate constant associated with this biomarker abundance will then be automatically entered in the appropriate location in Box 6b.
4. Enter the rate constant from Box 6b into Box 6. This is the rate constant that is used for the model simulation (i.e., to generate a simulated concentration vs. distance curve).
5. The user should then evaluate the fit between the actual field data and the model simulation that is based on this estimated rate constant. Manually adjust the rate constant in Box 6 until an optimal fit between the actual field data and the model simulation is obtained. Use the Root Mean Square Error (RMSE) that is overlaid on the plot as a guide; lower RMSE values generally indicate a better fit. Record the rate constant that provided the optimal fit.
6. Compare the recorded rate constant from the biomarker correlation with the "optimal" rate constant from Box 6. If the optimal rate constant from Box 6 is within a factor of 3 to 5 of the rate constant that was generated from the biomarker correlation, then this is considered reasonable evidence that these biodegradation processes are contributing to the actual field trend in 1,1,1-TCA concentrations.

The derivation of these correlations is described in Appendix D of the project report. They are based on an assumption that anaerobic reductive dechlorination of 1,1,1-TCA follows Michaelis-Menten (Haldane)

kinetics. The rate equation for Michaelis-Menten kinetics can be rearranged to solve for a first-order rate constant that is a function of other kinetic parameters (specifically Km and Vmax expressed in terms of gene copies), the biomarker abundance (expressed in gene copies per mL) and the concentration of the organic chemical being degraded (in this case, 1,1,1-TCA). Derived values for the kinetic parameters for each biomarker are also detailed in Appendix D of the ESTCP ER-201730 project report.

11. Is 1,1-DCE above the regulatory standard anywhere at the site?

Decision Criteria:

Answer YES if: The 1,1-DCE concentration at any groundwater monitoring location at the site is above the applicable concentration-based regulatory standard. Note that this standard is site-specific. If no standard has been established for your site, then select a value based on guidance from EPA or other states (see **HELP**) for planning purposes.

Answer NO if: The 1,1-DCE concentration at each groundwater monitoring location at the site is already below the concentration-based regulatory standard and 1,1,1-TCA is not detected at the site. The decision tool is intended for sites where the concentration of 1,1-DCE must fall below a site-specific regulatory standard before contaminated groundwater reaches the point of compliance (POC). If the 1,1-DCE concentration is already below the regulatory standard across the site (including the POC), then it is likely that it will remain below this standard in the future (see **HELP** for possible exceptions). This assumes that the site has been reasonably well characterized (especially the source area) and that no significant changes in conditions at the site are anticipated.

HELP

In the absence of site-specific regulatory standards for 1,1-DCE, the user may wish to select the federal MCL (7 µg/L) for 1,1-DCE to proceed with the BioPIC evaluation.

If 1,1-DCE is already below the established or assumed standard, then a future exceedance would likely be associated with one or more of the following: 1) the site is poorly characterized; 2) a new release of a highly chlorinated ethene or ethane occurs and results in the formation of 1,1-DCE; 3) active remediation is on-going or recently completed, such that steady state conditions have not yet been reached; or 4) any other change in site conditions has occurred that would contribute to 1,1-DCE formation or inhibit 1,1-DCE attenuation.

12. Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?

Decision Criteria

Answer YES if: The 1,1-DCE concentration is currently below the regulatory standard at the point of compliance (POC) and is predicted to be below the concentration-based regulatory standard at the POC at any time in the future. Use the model provided as part of this tool (see **FILES** for "MNA Rate Constant Estimator") to predict if the concentration will be below the standard at any time in the future (see **HELP** for additional explanation).

Answer NO if: At any time in the future, the 1,1-DCE concentration will exceed the regulatory standard at the POC. Use the model provided as part of this tool to predict the concentration in the future at the POC. Note there usually is also a temporal component in the regulatory goals, which involves establishing how long it will take the concentration at a particular location to achieve a regulatory goal. The implementation of more aggressive remedies may reduce time to achieve remediation goals, thereby reducing the overall cost. However, this tool primary deals with the spatial aspects of remediation goals (i.e., will the goal be achieved at a POC) rather than the temporal components.

HELP

If sufficient historical contaminant concentration data are not available to determine if the 1,1-DCE plume will reach a POC, then a groundwater flow and solute transport model, such as the "MNA Rate Constant Estimator" provided as part of this tool (see **FILES**), should be used to predict solute plume behavior. In this case, the simulation should account for the effects of advective groundwater flow, dispersion of the relevant solutes, sorption, and degradation of 1,1-DCE in groundwater at the site.

For more information on using this type of model for 1,1-DCE, consult the project report (Development of a Quantitative Framework for Evaluating Natural Attenuation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-Dioxane in Groundwater (ESTCP ER-201730)), which can be found on the project page (copy link into web browser): https://serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/Persistent-Contamination/ER-201730/ER-201730.

A similar approach for chlorinated ethenes can be found in another project report (Development of a Quantitative Framework and Management Expectation Tool for the Selection of Bioremediation Approaches at Chlorinated Ethene Sites (ESTCP Project ER-201129)), which can be downloaded from the project page (copy link into web browser): https://www.serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/Persistent-Contamination/ER-201129/ER-201129. In the ER-201129 report, Section 5.2.3 illustrates the process of calibrating a groundwater flow and transport model (in this case, the "BIOCHLOR" model), and Section 5.2.4 Step 1 illustrates the use of a model to apply the decision criteria.

If available, a robust historical database of contaminant concentrations can be used as an alternative to a computer model. Spatial and temporal trends in solute concentrations can be utilized to determine if the plume is stable or receding and therefore will not reach the POC. When sufficient data are available, using empirical data to ascertain trends is much better than using a model. In many cases, sufficient 1,1-DCE concentration data are available to evaluate plume behavior and to determine if solute concentrations will exceed cleanup goals at a regulatory POC.

13. Is Long-Term Monitoring Data Sufficient to Evaluate MNA?

Decision Criteria

Answer YES if: The current long-term monitoring data confirm that one or more of the following conditions are met:

1. The plume is currently beyond the point of compliance at a concentration that is above the applicable standard.

2. The plume is still expanding and is predicted to extend beyond the point of compliance in the future based on modeling.
3. Concentration-based goals have been established within the plume (i.e., upgradient of the point of compliance), and model predictions suggest that these will not be achieved with a reasonable timeframe.

Answer NO if: The current long-term monitoring data confirm that ALL of the following conditions are met:

1. The plume is currently not beyond the point of compliance.
2. The current dataset is too limited to evaluate if plume is expanding vs. receding.
3. The current dataset is too limited to predict using a model whether the plume will expand or whether concentration-based goals will be achieved.
4. There is no current regulatory requirement for active remediation.

HELP

Long-term monitoring data are an important component of site management, and they are particularly important for demonstrating the site-specific viability of MNA.

It is possible that a site's existing dataset for this compound may be limited if it is a recent additional to the monitoring program. It may not be possible to establish a clear trend in the attenuation in concentration of 1,1-DCE or its degradation products along a flow path in groundwater at appropriate monitoring points. This means that the data are inadequate to permit a thorough evaluation for the primary line of evidence for MNA. This is typically because one or more of the following apply: 1) data are highly variable across the site; 2) data are highly variable from event-to-event; or 3) data are available from a relatively limited number of monitoring points or events. In each case, these data limitations make it difficult to establish trends with any degree of statistical certainty.

In that case, collecting additional data may be beneficial to provide more certainty that current trends are statistically significant and sustainable. These additional monitoring events may also include data that would be used to support the second line of evidence for MNA (e.g., geochemical data, biomarkers, stable isotopes) or even hydrogeologic parameters to improve fate and transport model predictions.

At sites where the number of monitoring locations is relatively small (e.g., 4 or less wells), this type of analysis will likely benefit by including additional monitoring locations along the plume transect. In part, this is because it can be more challenging to establish that a trend is significant using standard approaches (e.g., that the slope of a best-fit regression line is different than zero). As a result, adding more monitoring locations along the plume transect may provide value.

It is necessary to use modeling software to evaluate if current trends in natural attenuation will meet the standard at the point-of-compliance. An example is the "MNA Rate Constant Estimator" that has been developed as part of this decision tool (see **FILES**). This type of model allows the user to predict concentrations along a plume transect. However, it relies on the user to calibrate to the model predictions based on field data, and it also assumes that those field data are representative. At sites, where data vary considerably from event to event, this type of calibration can be challenging. In any case, the goal is to demonstrate that the plume footprint is stable and/or shrinking and will not result in concentrations at a downgradient POC that exceed an acceptable level. This may require additional monitoring locations

(particularly if the plume extent is not yet delineated) or additional monitoring events to demonstrate longer-term stability. Data from additional events are also important to establish that trends are sustainable even if minor (or major) changes in site conditions (e.g., groundwater flow directions, redox conditions) occur that might impact attenuation and/or plume stability.

In some cases where MNA is proposed as a remedy, an estimate of the remediation timeframe is also required by the regulatory agency. The remediation timeframe is typically the date when some or all wells are projected to achieve a concentration goal. Estimating the remediation timeframe is often done using linear regression of concentration vs. time data at a specific well (or wells), but this requires data from enough events to ensure that the result is statistically significant. Additional monitoring events may be required to achieve this objective at sites with limited concentration vs. time data.

For the case where the dataset is already robust and confirms that MNA is unlikely to be effective, active remediation is likely to be the next course of action. Active remediation should be selected and implemented based on appropriate, well-defined objectives, but one primary goal should be to reduce concentration in the source area enough so that concentrations are below the site-specific standard at the point of compliance. The required reduction in the source concentration can be estimated using models, including the one provided as part of this tool (see "MNA Rate Constant Estimator" in **FILES**). Another good option is REMChlor-MD, which allows the user to input the extent of source reduction and then compare the plume behavior for cases with and without remediation. REMChlor-MD also incorporates the effects of matrix diffusion (i.e., contaminants diffusing into and out of lower-permeability intervals within the saturated zone) which has implications for contaminant transport and persistence at many sites.

Additional resources for understanding and developing monitoring objectives for MNA include the following (copy link into web browser):

https://clu-in.org/download/contaminantfocus/dnapl/Treatment_Technologies/performance_monitoring_mns600R04027.pdf

https://www.serdp-estcp.org/content/download/25789/262545/file/FAQ%20ER-201211.V2%20February%202014.pdf

https://clu-in.org/download/techfocus/na/NA-approach-for-eval-2011.pdf

https://www.epa.gov/sites/production/files/2014-02/documents/d9200.4-17.pdf

https://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=10004674.TXT

https://pubs.usgs.gov/wri/wri034057/

14. Is the EPA Second Line of Evidence Required?

Decision Criteria

Answer YES if: The appropriate regulatory authority has requested that multiple lines of evidence for 1,1-DCE attenuation should be collected before approval of MNA as a site remedy will be granted. The first

direct line of evidence requires data that demonstrate a clear and meaningful trend of decreasing contaminant mass and/or concentration over time at appropriate monitoring or sampling points. The second line of evidence originally included "hydrogeologic and geochemical data that can be used to indirectly demonstrate the type(s) of natural attenuation processes active at the site, and the rates at which such processes will reduce contaminant concentrations to required levels".

Answer NO if: The appropriate regulatory authority has specifically requested that only the first/primary line of evidence for natural attenuation is required for approval of MNA as a site remedy. The first direct line of evidence requires data that demonstrate a clear and meaningful trend of decreasing contaminant mass and/or concentration over time at appropriate monitoring or sampling points. Note that the final decision to require, or to not require, the second line of evidence is made by the appropriate regulatory authority. If the regulatory authority has not signaled what lines of evidence will be required, then a more conservative approach would be to answer "YES" to this question and proceed with collecting additional lines of evidence.

HELP

As part of EPA's MNA guidance, the agency has listed three lines of evidence that may be part of an MNA evaluation. Collecting data to support the first line of evidence is always required, and data to support the second line of evidence is typically required. The rest of this decision tool is focused on the second and third lines of evidence. It is recommended that the user go through these decision points even if the applicable data are not available.

The following resources provide more information on the lines of evidence approach, including definitions and how they are used (copy link into web browser):

https://www.epa.gov/sites/production/files/2014-02/documents/d9200.4-17.pdf

https://www.serdp-estcp.org/content/download/25789/262545/file/FAQ%20ER-201211.V2%20February%202014.pdf

15. Is 1,1-DCE biodegrading based on model predictions?

Decision Criteria

Answer YES if: Using 1,1-DCE degradation rate constants greater than zero in the model provide a better fit than the fit when the rate constant is set to zero. This can be evaluated using the same simulation in the "MNA Rate Constant Estimator" model that was used to "Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?" Prepare a new simulation where the rate constant for degradation of 1,1-DCE is set to zero. Compare the actual field-measured concentrations of 1,1-DCE against the new simulation. Then enter trial values for the rate constant for 1,1-DCE degradation into the simulation to determine if the model projections provide a better fit to the actual field-measured concentrations. A better fit is defined as having a lower value of RMSE (root mean square error) between the field data and the concentrations predicted by the model). If rate constants greater than zero provide a better fit, then 1,1-DCE is degrading. Note that this may have already been established as part of the earlier evaluation of the criterion "Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?" In addition,

the model will also have to be calibrated to fit the field-measured concentrations of 1,1,1-TCA and 1,1-DCA.

Answer NO if: Setting the 1,1-DCE degradation rate constant to zero in the model provides a better fit than the fit when the rate constant is greater than zero. This is evaluated using the same model and simulations described above for the "YES" answer.

16. Are ^{13}C and/or ^{37}Cl in 1,1-DCE enriched along the flow path?

Decision Criteria

Answer YES if: A clear pattern of carbon and/or chlorine isotope fractionation can be observed in samples collected along a groundwater flow path. For 1,1-DCE, values of $\delta^{13}C$ and $\delta^{37}Cl$ can be obtained for individual samples via commercial lab analysis (see HELP for further explanation of CSIA principles and analytical considerations). If samples are taken from the source area and then at several locations downgradient, a 2-dimensional plot of these values can then be generated (see CSIA_11DCA_11DCE in **FILES**). If the values of both $\delta^{13}C$ and $\delta^{37}Cl$ generally increase along the groundwater flow path (i.e., become "less negative" due to depletion of the lighter isotope), then this is taken as evidence for degradation of 1,1-DCE.

Answer NO if: No isotope data are available or if there is no clear trend in samples collected along a groundwater flow path. Again, this is best visualized by creating a 2-D plot of the $\delta^{13}C$ and $\delta^{37}Cl$ values (see **HELP** and **FILES** for more guidance).

HELP

Additional lines of evidence for 1,1-DCE attenuation can be provided by site-specific analysis of samples for stable isotopes of carbon and chloride. Data from a single sample may not provide sufficient evidence for 1,1-DCE degradation. This is because it is often a by-product of releases of other contaminants, and isotopic patterns may be difficult to distinguish if data are limited. Collecting multiple samples along the groundwater flow path is a more appropriate approach for establishing degradation because it relies on site-specific isotopic data to document 1,1-DCE degradation. Collecting isotopic data for parent compounds (e.g., 1,1,1-TCA) is also recommended to better establish trends across the site.

If values for $\delta^{13}C$ and $\delta^{37}Cl$ are available for 1,1-DCE, open the tab Files and select the spreadsheet CSIA_11DCA_11DCE.xlsx. Enter your data in the tab Input. The user can enter data for up to 8 wells. Data from a well located within the source area or in an upgradient area should be entered in the first row (Well 0); this provides a site-specific estimate of the isotopic composition of 1,1-DCE at the source and serves as a baseline for further comparisons. This well should have the lowest (most negative) values for $\delta^{13}C$ and $\delta^{37}Cl$ (prioritize the well with the lowest $\delta^{37}Cl$). Data from other wells are entered in the remaining rows, following the order that they fall along the groundwater flow path.

Once the available data have been entered, the user can consult the tab 2-D Chart_simple. Your data should plot on the chart. If not, you may need to extend the scales of the x and/or y axes. Degradation of 1,1-DCE is indicated if the data points generally proceed up and/or to the right within the plot in the direction of groundwater flow. This occurs due to the preferential degradation of bonds that contain lighter isotopes, such that the lighter isotopes become depleted and the heavier isotopes become

enriched within the remaining portion of the compound as it is transported downgradient. The degree of enrichment can vary depending on the compound, the isotope, and the transformation pathway.

The error bars represent uncertainty in the determination of $\delta^{13}C$ and $\delta^{37}Cl$. The values can be said to increase between two samples if either one or both of the vertical or horizontal error bars do not overlap.

Note that the user may also enter isotopic data for 1,1,1-TCA in the Input tab and plot it on the same chart as the 1,1-DCE data. If the $\delta^{13}C$ and $\delta^{37}Cl$ values for 1,1-DCE are less than the corresponding $\delta^{13}C$ and $\delta^{37}Cl$ values for 1,1,1-TCA at the source and near-source wells, then this is confirmatory evidence that the 1,1-DCE originated from 1,1,1-TCA degradation. This pattern occurs because the 1,1-DCE is formed from the preferential degradation of the lighter isotopes in 1,1,1-TCA, which is then reflected in the isotopic signature of the 1,1-DCE in these wells. If the $\delta^{13}C$ and $\delta^{37}Cl$ values for 1,1-DCE in the far downgradient wells exceed those of 1,1,1-TCA in the source wells, then this suggests that 1,1-DCE is degrading during groundwater transport.

17. Are geochemical conditions adequate for aerobic 1,1-DCE cometabolic biodegradation?

Decision Criteria

Answer YES if: Dissolved oxygen is routinely present in groundwater samples from one or more wells in the plume or in the area downgradient of the plume. This is a qualitative line of evidence that conditions are favorable to support aerobic cometabolic oxidation but does not imply that 1,1-DCE is actually being biodegraded. A threshold (minimum) DO value that would preclude aerobic biodegradation has not been established, and because of field sampling limitations, dissolved oxygen concentration data on well water are often unreliable. For the purposes of this decision tool, conditions are considered generally favorable for aerobic biodegradation of 1,1-DCE when one of the following criteria are met: Dissolved oxygen concentrations measured in the field are greater than 1 mg/L, ORP readings are > + 100 mV (against the AgCl reference electrode), ferrous iron (Fe^{2+}) concentrations are less than 0.5 mg/L, and methane concentrations are less than 0.005 mg/L. However, field parameter data should be evaluated with appropriate caution (see HELP).

Answer NO if: Dissolved oxygen is typically present at low levels (< 0.1 mg/L) across the entire site. This might include sites where the impacted intervals are deep, confined, and/or organic-rich. This type of determination would also rely on other corroborating geochemical data, such as highly negative ORP readings (< -100 mV against the AgCl reference electrode), elevated ferrous iron (Fe^{2+}) > 1 mg/L, or methane > 0.5 mg/L.

HELP

1,1-DCE can be naturally attenuated by reactions that occur in both anaerobic conditions (e.g., biological reductive dechlorination) and aerobic conditions. The former reaction can be evaluated as part of the evaluation of other chlorinated ethenes or 1,1,1-TCA and yields transformation products (vinyl chloride, ethene) that are similar to other chlorinated ethenes. Aerobic oxidation of 1,1-DCE results in products that are not easily measurable, so documenting favorable geochemical conditions is an important secondary line of evidence.

In assessing whether geochemical conditions are favorable for anaerobic vs. aerobic processes, it should be noted that field methods for measuring dissolved oxygen may generate inconsistent and/or erroneous results. One contributing factor is the common use of long-screened (≥ 10 ft) monitoring wells that may be collecting water from multiple zones with different redox conditions. This mixing of groundwater can make it difficult to quantify zones with higher dissolved oxygen that may promote 1,1-DCE biodegradation. Consequently, field dissolved oxygen measurements should be used with caution and supported by other lines of evidence. Other data that would corroborate that geochemical conditions are favorable for aerobic biodegradation of 1,1-DCE include positive ORP readings and little or no dissolved iron and methane. Elevated levels of total organic carbon (TOC) (e.g., > 20 mg/L) is also a positive secondary indicator because it provides a carbon source and electron donor to promote biological cometabolic oxidation of 1,1-DCE. To date, there is little evidence that 1,1-DCE can be used as a sole carbon and energy source for microbial activity, so the presence of organic co-substrates is important.

18. Are *Dhc*, *vcrA*, and *bvc* present?

Decision Criteria

Answer YES if: Any of these qPCR-based biomarkers for chlorinated ethene degradation are present in one or more wells at the site. These are gene targets that are associated with organisms and/or enzymes that can reductively dechlorinate several chlorinated ethenes, including 1,1-DCE, and their abundance in site samples can be quantified by several analytical laboratories. If these biomarkers are present, a supplemental evaluation can rely on correlations between biomarker abundance and rate constants developed for chlorinated ethenes (see **HELP**).

Answer NO if: No data on the abundance of qPCR biomarkers are available, or analytical results confirm that none are present above detection limits.

HELP

The presence or absence of biomarkers for chlorinated ethene biodegradation is a starting point for evaluating MNA. When analytical labs quantify the abundance of specific biomarkers, they can typically provide information on how the measured levels compare to those from other sites. At sites where this abundance is comparably high, this helps support the second line of evidence for MNA. It should be understood that the degradation rate needed to achieve a goal concentration at one site may be much different than that at another site. As a result, a relatively high biomarker abundance does not guarantee that MNA will be successful; these data need to be combined with the primary line of evidence for MNA (meaningful concentration/mass trends).

Another approach is to use the biomarker data to help refine model predictions of the biodegradation rate constant. The "MNA Rate Constant Estimator" model (see **FILES**) has an option to estimate rate constants based on the abundance of several different qPCR-based biomarkers for degradation. These correlations are designed to help calibrate the model, and they are intended as a starting point for improving the fit between the actual field data and the model simulations. Consequently, they should not be considered a true prediction of the actual degradation rate that is occurring at the site. This is because they are based on empirical data from other studies where conditions may be quite different than those observed at the site being evaluated.

To use the biomarker correlations as part of this decision tool, the following process is recommended:

1. In Box 6b, select the specific biomarker vcrA from the dropdown menu. On the chlorinated ethanes module of this model, this is one of only two biomarker options; only vcrA is applicable for 1,1-DCE. Selecting a biomarker from the menu will launch a pop-up box where biomarker abundance data can be entered.
2. In the pop-up box, enter the abundance of the selected biomarker for those wells where data are available. The model will perform a spatial interpolation to estimate a representative biomarker abundance for the site (i.e., a single value that is weighted based on the distance between wells with biomarker data).
3. The rate constant associated with this biomarker abundance will then be automatically entered in the appropriate location in Box 6b.
4. Enter the rate constant from Box 6b into Box 6. This is the rate constant that is used for the model simulation (i.e., to generate a simulated concentration vs. distance curve).
5. The user should then evaluate the fit between the actual field data and the model simulation that is based on this estimated rate constant. Manually adjust the rate constant in Box 6 until an optimal fit between the actual field data and the model simulation is obtained. Use the Root Mean Square Error (RMSE) that is overlaid on the plot as a guide; lower RMSE values generally indicate a better fit. Record the rate constant that provided the optimal fit.
6. Compare the recorded rate constant from the biomarker correlation with the "optimal" rate constant from Box 6. If the optimal rate constant from Box 6 is within a factor of 3 to 5 of the rate constant that was generated from the biomarker correlations, then this is considered reasonable evidence that these biodegradation processes are contributing to the actual field trend in 1,1-DCE concentrations.

The derivation of these correlations is described in Appendix XX of the project report. They are based on an assumption that anaerobic biodegradation of 1,1-DCE follows Michaelis-Menten (Haldane) kinetics. The rate equation for Michaelis-Menten kinetics can be rearranged to solve for a first-order rate constant that is a function of other kinetic parameters (specifically Km and Vmax expressed in terms of gene copies), the biomarker abundance (expressed in gene copies per mL) and the concentration of the organic chemical being degraded (in this case, 1,1-DCE). Derived values for the kinetic parameters for each biomarker are also detailed in Appendix D of the project report.

19. Does Magnetic Susceptibility Explain the 1,1-DCE Rate Constant?

Decision Criteria

Answer YES if: The 1,1-DCE degradation rate constant and value of magnetic susceptibility from the field site of concern are in the same range as known values from microcosm studies or from other field sites. This evaluation can be performed using the worksheet provided as part of this tool (see Magnetic Susceptibility_11DCE in the **FILES** tab), and the process is described in the **HELP** screen. If this correlation is observed, then abiotic degradation by magnetite is a plausible mechanism to explain the bulk attenuation rate at the field site of concern.

Answer NO if: No site-specific magnetic susceptibility data are available OR if the 1,1-DCE degradation rate constant and value of magnetic susceptibility from the field site of concern are not in the same range as known values from microcosm studies or from other field sites. The latter can be evaluated using the same worksheet described above for the "YES" answer.

HELP

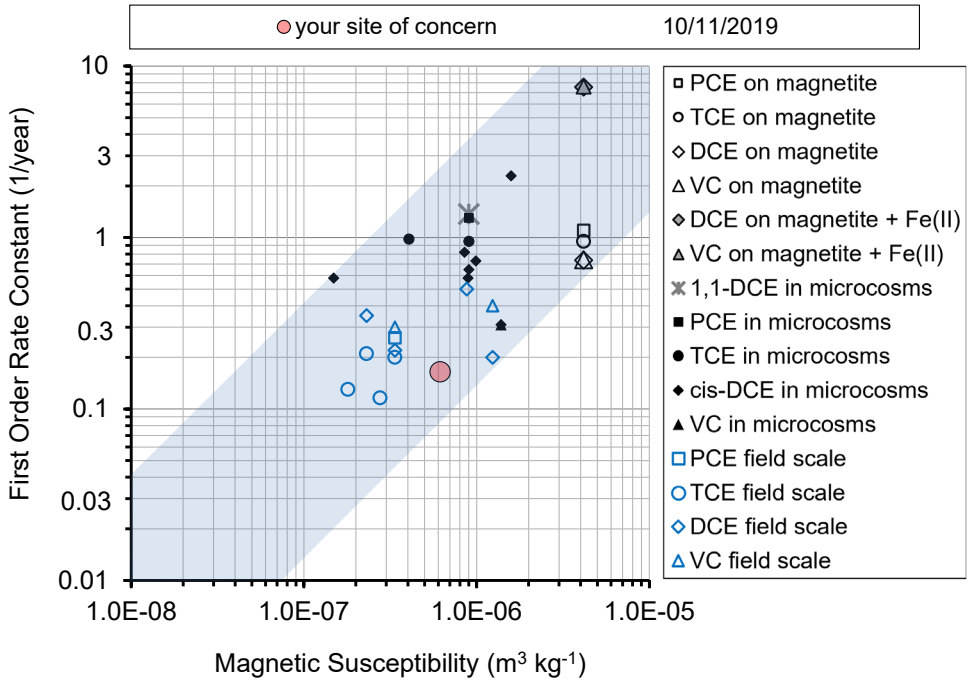
Chlorinated alkenes can be degraded by abiotic reactions with magnetite (He et al., 2009; Lee and Batchelor, 2002; Ferrey et al., 2004). The quantity of magnetite in aquifer sediments can be determined from a measurement of the mass magnetic susceptibility of the sediment. He et al. (2009) summarized rate constants for abiotic degradation of PCE, TCE, *cis*-DCE, and Vinyl Chloride in laboratory microcosm studies that were constructed with sediment with known values of magnetic susceptibility.

Lebrón et al. (2015) developed a worksheet to determine if bulk rate constants for attenuation of PCE, TCE, cis-DCE, and Vinyl Chloride in plumes of contaminated ground water could plausibly be attributed to abiotic degradation by magnetite.

The worksheet compared the field scale rate constant for attenuation of PCE, TCE, cis-DCE or Vinyl Chloride and the magnetic susceptibility of the aquifer sediment to the rate constants and magnetic susceptibilities in the sediments described in He et al. (2009), and to rate constants that had been fitted several field-scale plumes where data were available on magnetic susceptibility. If the rate constant and value of magnetic susceptibility from the field site of concern was in the same range as the values from the microcosm studies or from the other field sites, then abiotic degradation by magnetite was a plausible mechanism to explain the bulk attenuation rate at the field site of concern.

The rate constants for degradation of PCE, TCE, cis-DCE, and Vinyl Chloride by magnetite were very similar (Lee and Batchelor, 2002). There is only one report in the literature that provides a rate constant for abiotic degradation of 1,1-DCE in aquifer material with known magnetic susceptibility (Ferrey et al., 2004). The rate constants for degradation of 1,1-DCE and *cis*-DCE were very similar. The decision logic will assume that the rate constants for abiotic degradation of 1,1-DCE by magnetite are the same as the rate constants for the other chlorinated ethenes.

The **Magnetic Susceptibility_11DCE** worksheet compares the field scale rate constant for degradation of 1,1-DCE at a site of concern and the magnetic susceptibility of the aquifer sediment to the available literature. Data from the field site of concern are entered in the tab ***Data Input***. The evaluation is provided in the tab ***Mag Susceptibility Explain Rate (s***ee figure below for an example)



*Example of the chart in the Tab **Mag Susceptibility Explains Rate** from the **Magnetic Susceptibility Worksheet.xlsx.***

The blue shape encompasses a linear extrapolation of data available in the peer-reviewed literature on the relationship between rate constants and magnetic susceptibility. If the data from the site of concern falls within the blue shape, then abiotic degradation of 1,1-DCE by magnetite is a plausible explanation for the bulk rate constant for attenuation at field scale. Note that the one data point for degradation of 1,1-DCE microcosms constructed with aquifer sediment is consistent with rate constants for degradation of the other chlorinated ethenes in aquifer sediment.

The data in Figure 1 on field scale rate constants includes additional data published in Wiedemeier et al. (2015). The laboratory studies of Lee and Batchelor (2002) on synthetic magnetite are also included in Figure 1**.** Surface area specific first order rate constants reported in Lee and Batchelor (2002) were converted to first order rate constants by multiplying the surface area specific rate constant by the mass of magnetite per unit volume of water in their experimental reactor, and then by the specific surface area of the magnetite suspended in the water.

The following assumptions were used to estimate the magnetic susceptibility of aquifer sediment that would be equivalent to the experimental reactor. The milligram of magnetite per liter of water in the experimental reactor was assumed to be the milligram of magnetite exposed to each liter of pore water in the sediment. Porewater was assumed to occupy 25% of the total volume of the sediment, the dry bulk density of the sediment was assumed to be 2.0 kg/Liter, and magnetite was assumed to represent all the magnetic material in the aquifer sediment. Based on these assumptions, the milligrams of magnetite per kilogram of sediment was calculated, and the equations on page 77 of He et al. (2009) were used to

estimate the magnetic susceptibility of the equivalent aquifer sediment. The calculations are performed in Tab ***Synthetic Magnetite Calculation***.

20. Is 1,1-DCA above the regulatory standard anywhere at the site?

Decision Criteria:

Answer YES if: The 1,1-DCA concentration at any groundwater monitoring location at the site is above the applicable concentration-based regulatory standard. Note that this standard is site-specific. If no standard has been established for your site, then select a value based on guidance from EPA or other states (see **HELP**) for planning purposes.

Answer NO if: The 1,1-DCA concentration at each groundwater monitoring location at the site is already below the concentration-based regulatory standard and 1,1,1-TCA is not detected at the site. The decision tool is intended for sites where the concentration of 1,1-DCA must fall below a site-specific regulatory standard before contaminated groundwater reaches the point of compliance (POC). If the 1,1-DCA concentration is already below the regulatory standard across the site (including the POC), then it is likely that it will remain below this standard in the future (see **HELP** for possible exceptions). This assumes that the site has been reasonably well characterized (especially the source area) and that no significant changes in conditions at the site are anticipated.

HELP

In the absence of site-specific regulatory standards for 1,1-DCA, the user may wish to select one of the following values to proceed with the BioPIC evaluation. Note that there is considerable variability in state-level groundwater and drinking water standards for 1,1-DCA. The information below was compiled on 1 January 2021, so it should also be understood that states are likely to promulgate and/or revised standards over time.

- USEPA Reference Concentration for Drinking Water (10^{-6} risk) = 6.14 µg/L
- California MCL in Drinking Water = 5 µg/L
- North Carolina Groundwater Standard = 6 µg/L
- New Jersey Groundwater Standard = 50 µg/L
- Wisconsin Groundwater Preventive Action Limit = 85 µg/L

If 1,1-DCA is already below the established or assumed standard, then a future exceedance would likely be associated with one or more of the following: 1) the site is poorly characterized; 2) a new release of a highly chlorinated ethane occurs and results in the formation of 1,1-DCA; 3) active remediation is on-going or recently completed, such that steady state conditions have not yet been reached; or 4) any other change in site conditions has occurred that would contribute to 1,1-DCA formation or inhibit 1,1-DCA attenuation.

21. Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?

Decision Criteria

Answer YES if: The 1,1-DCA concentration is currently below the regulatory standard at the point of compliance (POC) and is predicted to be below the concentration-based regulatory standard at the POC at any time in the future. Use the model provided as part of this tool (see **FILES** for "MNA Rate Constant Estimator") to predict if the concentration will be below the standard at any time in the future (see **HELP** for additional explanation).

Answer NO if: At any time, the 1,1-DCA concentration will exceed the regulatory standard at the POC. Use the model provided as part of this tool to predict the concentration in the future at the POC. Note there usually is also a temporal component in the regulatory goals, which involves establishing how long it will take the concentration at a particular location to achieve a regulatory goal. The implementation of more aggressive remedies may reduce time to achieve remediation goals, thereby reducing the overall cost. However, this tool primary deals with the spatial aspects of remediation goals (i.e., will the goal be achieved at a POC) rather than the temporal components.

HELP

If sufficient historical contaminant concentration data are not available to determine if the 1,1-DCA plume will reach a POC, then a groundwater flow and solute transport model, such as the "MNA Rate Constant Estimator" provided as part of this tool (see **FILES**) should be used to predict solute plume behavior. In this case, the simulation should account for the effects of advective groundwater flow, dispersion of the relevant solutes, sorption, and degradation of 1,1-DCA in groundwater at the site.

For more information on using this type of model for 1,1-DCA, consult the project report (Development of a Quantitative Framework for Evaluating Natural Attenuation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-Dioxane in Groundwater (ESTCP ER-201730)), which can be found on the project page (copy link into web browser): https://serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/Persistent-Contamination/ER-201730/ER-201730.

A similar approach for chlorinated ethenes can be found in another project report (Development of a Quantitative Framework and Management Expectation Tool for the Selection of Bioremediation Approaches at Chlorinated Ethene Sites (ESTCP Project ER-201129)), which can be downloaded from the project page (copy link into web browser): https://www.serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/Persistent-Contamination/ER-201129/ER-201129. In the ER-201129 report, Section 5.2.3 illustrates the process of calibrating a groundwater flow and transport model (in this case, the "BIOCHLOR" model), and Section 5.2.4 Step 1 illustrates the use of a model to apply the decision criteria.

If available, a robust historical database of contaminant concentrations can be used as an alternative to a computer model. Spatial and temporal trends in solute concentrations can be utilized to determine if the plume is stable or receding and therefore will not reach the POC. When sufficient data are available, using empirical data to ascertain trends is much better than using a model. In many cases, sufficient 1,1-DCA concentration data are available to evaluate plume behavior and to determine if solute concentrations will exceed cleanup goals at a regulatory POC.

22. Is Long-Term Monitoring Data Sufficient to Evaluate MNA?

Decision Criteria

Answer YES if: The current long-term monitoring data confirm that one or more of the following conditions are met:

1. The plume is currently beyond the point of compliance at a concentration that is above the applicable standard.
2. The plume is still expanding and is predicted to extend beyond the point of compliance in the future based on modeling.
3. Concentration-based goals have been established within the plume (i.e., upgradient of the point of compliance), and model predictions suggest that these will not be achieved with a reasonable timeframe.

Answer NO if: The current long-term monitoring data confirm that ALL of the following conditions are met:

1. The plume is currently not beyond the point of compliance.
2. The current dataset is too limited to evaluate if plume is expanding vs. receding.
3. The current dataset is too limited to predict using a model whether the plume will expand or whether concentration-based goals will be achieved.
4. There is no current regulatory requirement for active remediation.

HELP

Long-term monitoring data are an important component of site management, and they are particularly important for demonstrating the site-specific viability of MNA.

It is possible that a site's existing dataset for this compound may be limited if it is a recent additional to the monitoring program. It may not be possible to establish a clear trend in the attenuation in concentration of 1,1-DCA or its degradation products along a flow path in groundwater. This means that the data are inadequate to permit a thorough evaluation for the primary line of evidence for MNA. This is typically because one or more of the following apply: 1) data are highly variable across the site; 2) data are highly variable from event-to-event; or 3) data are available from a relatively limits number of monitoring points or events. In each case, these data limitations make it difficult to establish trends with any degree of statistical certainty.

In that case, collecting additional data may be beneficial to provide more certainty that current trends are statistically significant and sustainable. These additional monitoring events may also include data that would be used to support the second line of evidence for MNA (e.g., geochemical data, biomarkers, stable isotopes) or even hydrogeologic parameters to improve fate and transport model predictions.

At sites where the number of monitoring locations is relatively small (e.g., 4 or less wells), this type of analysis will likely benefit by including additional monitoring locations along the plume transect. In part, this is because it can be more challenging to establish that a trend is significant using standard approaches (e.g., that the slope of a best-fit regression line is different than zero). As a result, adding more monitoring locations along the plume transect may provide value.

It is necessary to use modeling software to evaluate if current trends in natural attenuation will meet the standard at the point of compliance. An example is the "MNA Rate Constant Estimator" that has been developed as part of this decision tool (see **FILES**). This type of model allows the user to predict concentrations along a plume transect. However, it relies on the user to calibrate to the model predictions based on field data, and it also assumes that those field data are representative. At sites where data vary considerably from event to event, this type of calibration can be challenging. In any case, the goal is to demonstrate that the plume footprint is stable and/or shrinking and will not result in concentrations at a downgradient POC that an unacceptable level. This may require additional monitoring locations (particularly if the plume extent is not yet delineated) or additional monitoring events to demonstrate longer-term stability. Data from additional events are also important to establish that trends are sustainable even if minor (or major) changes in site conditions (e.g., groundwater flow directions, redox conditions) occur that might impact attenuation and/or plume stability.

In some cases where MNA is proposed as a remedy, an estimate of the remediation timeframe is also required by the regulatory agency. The remediation timeframe is typically the date when some or all wells are projected to achieve a concentration goal. Estimating the remediation timeframe is often done using linear regression of concentration vs. time data at a specific well (or wells), but this requires data from enough events to ensure that the result is statistically significant. Additional monitoring events may be required to achieve this objective at sites with limited concentration vs. time data.

For the case where the dataset is already robust and confirms that MNA is unlikely to be effective, active remediation is likely to be the next course of action. Active remediation should be selected and implemented based on appropriate, well-defined objectives, but one primary goal should be to reduce concentration in the source area enough so that concentrations are below the site-specific standard at the point of compliance. The required reduction in the source concentration can be estimated using models, including the one provided as part of this tool (see **FILES**). Another good option is REMChlor-MD, which allows the user to input the extent of source reduction and then compare the plume behavior for cases with and without remediation. REMChlor-MD also incorporates the effects of matrix diffusion (i.e., contaminants diffusing into and out of lower-permeability intervals within the saturated zone), which has implications for contaminant transport and persistence at many sites.

Additional resources for understanding and developing monitoring objectives for MNA include the following (copy link into web browser):

https://clu-in.org/download/contaminantfocus/dnapl/Treatment_Technologies/performance_monitoring_mns600R04027.pdf

https://www.serdp-estcp.org/content/download/25789/262545/file/FAQ%20ER-201211.V2%20February%202014.pdf

https://clu-in.org/download/techfocus/na/NA-approach-for-eval-2011.pdf

https://www.epa.gov/sites/production/files/2014-02/documents/d9200.4-17.pdf

https://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=10004674.TXT

https://pubs.usgs.gov/wri/wri034057/

23. Is the EPA Second Line of Evidence Required?

Decision Criteria

Answer YES if: The appropriate regulatory authority has requested that multiple lines of evidence for 1,1-DCA attenuation should be collected before approval of MNA as a site remedy will be granted. The first direct line of evidence requires data that demonstrate a clear and meaningful trend of decreasing contaminant mass and/or concentration over time at appropriate monitoring or sampling points. The second line of evidence originally included "hydrogeologic and geochemical data that can be used to indirectly demonstrate the type(s) of natural attenuation processes active at the site, and the rates at which such processes will reduce contaminant concentrations to required levels".

Answer NO if: The appropriate regulatory authority has specifically requested that only the first/primary line of evidence for natural attenuation is required for approval of MNA as a site remedy. The first direct line of evidence requires data that demonstrate a clear and meaningful trend of decreasing contaminant mass and/or concentration over time at appropriate monitoring or sampling points. Note that the final decision to require, or to not require, the second line of evidence is made by the appropriate regulatory authority. If the regulatory authority has not signaled what lines of evidence will be required, then a more conservative approach would be to answer "YES" to this question and proceed with collecting additional lines of evidence.

HELP

As part of EPA's MNA guidance, the agency has listed three lines of evidence that may be part of an MNA evaluation. Collecting data to support the first line of evidence is always required, and data to support the second line of evidence is typically required. The rest of this decision tool is focused on the second and third lines of evidence. It is recommended that the user go through these decision points even if the applicable data are not available.

The following resources provide more information on the lines of evidence approach, including definitions and how they are used (copy link into web browser):

https://www.epa.gov/sites/production/files/2014-02/documents/d9200.4-17.pdf

https://www.serdp-estcp.org/content/download/25789/262545/file/FAQ%20ER-201211.V2%20February%202014.pdf

24. Is 1,1-DCA biodegrading based on model predictions?

Decision Criteria

Answer YES if: Using 1,1-DCA degradation rate constants greater than zero in the model provide a better fit than the fit when the rate constant is set to zero. This can be evaluated using the same simulation in the "MNA Rate Constant Estimator" model that was used to "Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?" Prepare a new simulation where the rate constant for degradation of 1,1-DCA is set to zero. Compare the actual in situ concentrations of 1,1-DCA against the new simulation.

Then enter trial values for the rate constant for 1,1-DCA degradation into the simulation to determine if the model projections provide a better fit to the actual field-measured concentrations. A better fit is defined as having a lower value of RMSE (root mean square error) between the field data and the concentrations predicted by the model). If rate constants greater than zero provide a better fit, then 1,1-DCE is degrading. Note that this may have already been established as part of the earlier evaluation of the criterion "Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?" In addition, the model will also have to be calibrated to fit the field-measured concentrations of 1,1,1-TCA and 1,1-DCE.

Answer NO if: Setting the 1,1-DCA degradation rate constant to zero in the model provides a better fit than the fit when the rate constant is greater than zero. This is evaluated using the same model and simulations described above for the "YES" answer.

25. Are ^{13}C and/or ^{37}Cl in 1,1-DCA enriched along the flow path?

Decision Criteria

Answer YES if: A clear pattern of carbon and/or chlorine isotope fractionation can be observed in samples collected along a groundwater flow path. For 1,1-DCA, values of $\delta^{13}C$ and $\delta^{37}Cl$ can be obtained for individual samples via commercial lab analysis (see HELP for further explanation of CSIA principles and analytical considerations). If samples are taken from the source area and then at several locations downgradient, a 2-dimensional plot of these values can then be generated (see CSIA_11DCA_11DCE in **FILES**). If the values of both $\delta^{13}C$ and $\delta^{37}Cl$ generally increase along the groundwater flow path (i.e., become "less negative" due to depletion of the lighter isotope), then this is taken as evidence for degradation of 1,1-DCA.

Answer NO if: No isotope data are available or if there is no clear trend in samples collected along a groundwater flow path. Again, this is best visualized by creating a 2-D plot of the $\delta^{13}C$ and $\delta^{37}Cl$ values (see **HELP** and **FILES** for more guidance).

HELP

Additional lines of evidence for 1,1-DCA attenuation can be provided by site-specific analysis of samples for stable isotopes of carbon and chloride. Data from a single sample may not provide sufficient evidence for 1,1-DCA degradation. This is because 1,1-DCA is often a by-product of releases of other contaminants, and isotopic patterns may be difficult to distinguish if data are limited. Collecting multiple samples along the groundwater flow path is a more appropriate approach for establishing degradation because it relies on site-specific isotopic data to document 1,1-DCA degradation. Collecting isotopic data for parent compounds (e.g., 1,1,1-TCA) is also recommended to better establish trends across the site.

If values for $\delta^{13}C$ and $\delta^{37}Cl$ are available for 1,1-DCA, open the tab Files and select the spreadsheet CSIA_11DCA_11DCE.xlsx. Enter your data in the tab Input. The user can enter data for up to 8 wells. Data from a well located within the source area or in an upgradient area should be entered in the first row (Well 0); this provides a site-specific estimate of the isotopic composition of 1,1-DCA at the source and serves as a baseline for further comparisons. This well should have the lowest (most negative) values for

$\delta^{13}C$ and $\delta^{37}Cl$ (prioritize the well with the lowest $\delta^{37}Cl$). Data from other wells are entered in the remaining rows, following the order that they fall along the groundwater flow path.

Once the available data have been entered, the user can consult the tab 2-D Chart_simple. Your data should plot on the chart. If not, you may need to extend the scales of the x and/or y axes. Degradation of 1,1-DCA is indicated if the data points generally proceed up and/or to the right within the plot in the direction of groundwater flow. This occurs due to the preferential degradation of bonds that contain lighter isotopes, such that the lighter isotopes become depleted and the heavier isotopes become enriched within the remaining portion of the compound as it is transported downgradient. The degree of enrichment can vary depending on the compound, the isotope, and the transformation pathway.

The error bars represent uncertainty in the determination of $\delta^{13}C$ and $\delta^{37}Cl$. The values can be said to increase between two samples if one or both of the error bars do not overlap. Note that the user may also enter isotopic data for 1,1,1-TCA in the Input tab and plot it on the same chart as the 1,1-DCA data. If the $\delta^{13}C$ and $\delta^{37}Cl$ values for 1,1-DCA are less than the corresponding $\delta^{13}C$ and $\delta^{37}Cl$ values for 1,1,1-TCA at the source and near-source wells, then this is confirmatory evidence that the 1,1-DCA originated from 1,1,1-TCA degradation. This pattern occurs because the 1,1-DCA is formed from the preferential degradation of the lighter isotopes in 1,1,1-TCA, which is then reflected in the isotopic signature of the 1,1-DCA in these wells. If the $\delta^{13}C$ and $\delta^{37}Cl$ values for 1,1-DCA in the far downgradient wells exceed those of 1,1,1-TCA in the source wells, then this suggests that 1,1-DCA is degrading during groundwater transport.

26. Are geochemical conditions adequate for aerobic 1,1-DCA biodegradation?

Decision Criteria

Answer YES if: Dissolved oxygen is routinely present in groundwater samples from one or more wells in the plume or in the area downgradient of the plume. This is a qualitative line of evidence that conditions are favorable to support aerobic oxidation but does not imply that 1,1-DCA is actually being degraded. A threshold (minimum) DO value that would preclude aerobic biodegradation has not been established, and because of field sampling limitations, dissolved oxygen concentration data on well water are often unreliable. For the purposes of this decision tool, conditions are considered generally favorable for aerobic biodegradation of 1,1-DCE when one of the following criteria are met: Dissolved oxygen concentrations measured in the field are greater than 1 mg/L, ORP readings are > + 100 mV (against the AgCl reference electrode), ferrous iron (Fe^{2+}) concentrations are less than 0.5 mg/L, and methane concentrations are less than 0.005 mg/L. However, field parameter data should be evaluated with appropriate caution (see **HELP**).

Answer NO if: Dissolved oxygen is typically present at low levels (< 0.1 mg/L) across the entire site. This might include sites where the impacted intervals are deep, confined, and/or organic-rich. This type of determination would also rely on other corroborating geochemical data, such as highly negative ORP readings (< -100 mV against the AgCl reference electrode), elevated ferrous iron (Fe^{2+}) > 1 mg/L, or methane > 0.5 mg/L.

HELP

1,1-DCA can be naturally attenuated by reactions that occur in both anaerobic conditions (e.g., biological reductive dechlorination) and aerobic conditions. The former reaction can be evaluated as part of the evaluation of 1,1,1-TCA. Aerobic oxidation of 1,1-DCA can occur via direct metabolism (i.e., 1,1-DCA is used as a carbon and energy source by the microbes that perform the reaction) or via co-metabolism (i.e., 1,1-DCA is transformed fortuitously and does not support growth). In either case, the products of these reactions are not easily measurable, so documenting favorable geochemical conditions is an important secondary line of evidence.

In assessing whether geochemical conditions are favorable for anaerobic vs. aerobic processes, it should be noted that field methods for measuring dissolved oxygen may generate inconsistent and/or erroneous results. One contributing factor is the common use of long-screened (≥ 10 ft) monitoring wells that may be collecting water from multiple zones with different redox conditions. This mixing of groundwater can make it difficult to quantify zones with higher dissolved oxygen that may promote 1,1-DCA biodegradation. Consequently, field dissolved oxygen measurements should be used with caution and supported by other lines of evidence. Other data that would corroborate that geochemical conditions are favorable for aerobic biodegradation of 1,1-DCA include positive ORP readings and little or no dissolved iron and methane. Total organic carbon (TOC) may also be a positive indicator because it provides a carbon source and electron donor to promote biological cometabolic oxidation of 1,1-DCA; TOC > 20 mg/L may also serve as a positive line of evidence for the anerobic natural attenuation pathway. In addition, portions of the site where groundwater transitions between anaerobic and aerobic should be delineated to identify areas that might be best managed by different natural attenuation pathways.

27. Is chloroethane present?

Decision Criteria

Answer YES if: Chloroethane has been present above the reporting limit at any groundwater monitoring location at the site. This compound is a by-product of 1,1-DCA reductive dechlorination and therefore serves as a confirmatory line of evidence that this reaction is actively contributing to 1,1-DCA attenuation.

Answer NO if: Chloroethane has not been present at any groundwater monitoring location at the site, either currently or historically.

28. Does *Dhb* Density Explain the 1,1-DCA Rate Constant?

Decision Criteria

Answer YES if: The 1,1-DCA biodegradation rate constant used in the model is consistent with correlations based on the abundance of the Dhb biomarker (also referred to as DHBt) for strains of *Dehalobacter* bacteria that degrade 1,1-TCA. The correlations were derived from other studies and kinetic data. To do this, first refer to the simulation in the "MNA Rate Constant Estimator" model that was used to evaluate the criterion "Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?". Note that this model has the option to use biomarker data to estimate the biodegradation rate constant (i.e., it uses a

correlation to predict the representative rate constant based on the biomarker levels measured at the site). If this option was employed and it resulted in a reasonable fit to the actual data, then this confirms that "YES" is the appropriate answer. See **HELP** for additional guidance on determining if the fit was reasonable.

Answer NO if: No data on the abundance of the Dhb biomarker are available, or if 1,1-DCA biodegradation rate constant used to optimize the model is inconsistent with rate constants predicted using the biomarker correlations. To evaluate the latter condition, first refer to the model simulation that was used to evaluate the criterion "Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?". If the option to use the correlation was not employed OR did not result in an optimal fit, then "NO' is the appropriate answer.

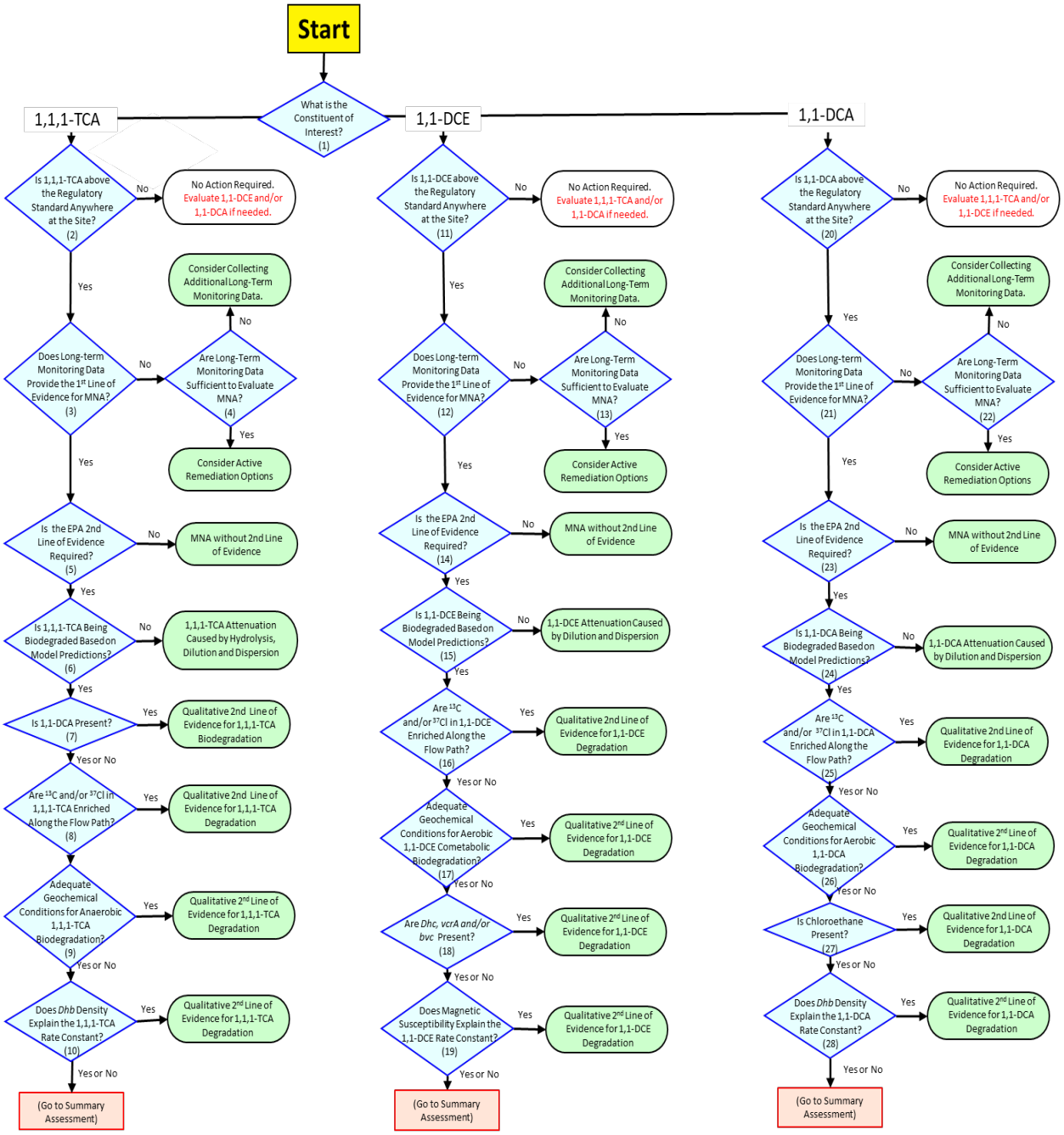
HELP

For 1,1-DCA, the "MNA Rate Constant Estimator" model (see **FILES**) has an option to estimate rate constants based on the abundance of Dhb (also referred to as DHBt), which is a qPCR-based biomarker for degradation of this compound (as well as 1,1,1-TCA). The correlations are designed to help calibrate the model, and they are intended as a starting point for improving the fit between the actual field data and the model simulations. Consequently, they should not be considered a true prediction of the actual degradation rate that is occurring at the site. This is because they are based on empirical data from other studies where conditions may be quite different than those observed at the site being evaluated.

To use the biomarker correlations as part of this decision tool, the following process is recommended:

1. In Box 6b, select the specific biomarker Dhb from the dropdown menu. On the chlorinated ethanes module of this model, this is one of only two biomarker options; only Dhb is applicable for 1,1-DCA. Selecting a biomarker from the menu will launch a pop-up box where biomarker abundance data can be entered.
2. In the pop-up box, enter the abundance of the selected biomarker for those wells where data are available. The model will perform a spatial interpolation to estimate a representative biomarker abundance for the site (i.e., a single value that is weighted based on the distance between wells with biomarker data).
3. The rate constant associated with this biomarker abundance will then be automatically entered in the appropriate location in Box 6b.
4. Enter the rate constant from Box 6b into Box 6. This is the rate constant that is used for the model simulation (i.e., to generate a simulated concentration vs. distance curve).
5. The user should then evaluate the fit between the actual field data and the model simulation that is based on this estimated rate constant. Manually adjust the rate constant in Box 6 until an optimal fit between the actual field data and the model simulation is obtained. Use the Root Mean Square Error (RMSE) that is overlaid on the plot as a guide; lower RMSE values generally indicate a better fit. Record the rate constant that provided the optimal fit.
6. Compare the recorded rate constant from the biomarker correlation with the "optimal" rate constant from Box 6. If the optimal rate constant from Box 6 is within a factor of 3 to 5 of the rate constant that was generated from the biomarker correlation, then this is considered reasonable evidence that these biodegradation processes are contributing to the actual field trend in 1,1-DCA concentrations.

The derivation of these correlations is described in Appendix D of the project report. They are based on an assumption that anaerobic reductive dechlorination of 1,1-DCA follows Michaelis-Menten (Haldane) kinetics. The rate equation for Michaelis-Menten kinetics can be rearranged to solve for a first-order rate constant that is a function of other kinetic parameters (specifically Km and Vmax expressed in terms of gene copies), the biomarker abundance (expressed in gene copies per mL) and the concentration of the organic chemical being degraded (in this case, 1,1-DCA). Derived values for the kinetic parameters for each biomarker are also detailed in Appendix D of the project report.



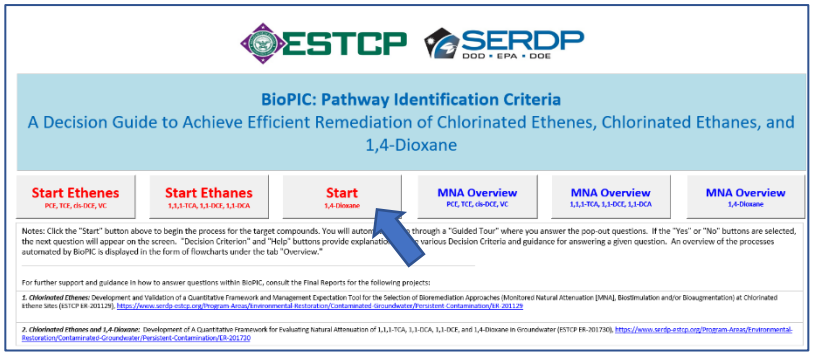
Decision Framework for Chlorinated Ethanes

1,1-DCE is a chlorinated ethene but is included in the decision framework for chlorinated ethanes because it is a key degradation product of the parent compound 1,1,1-TCA. This flowchart was coded into the updated BIOPIC tool.

Detailed BioPIC User Guide:

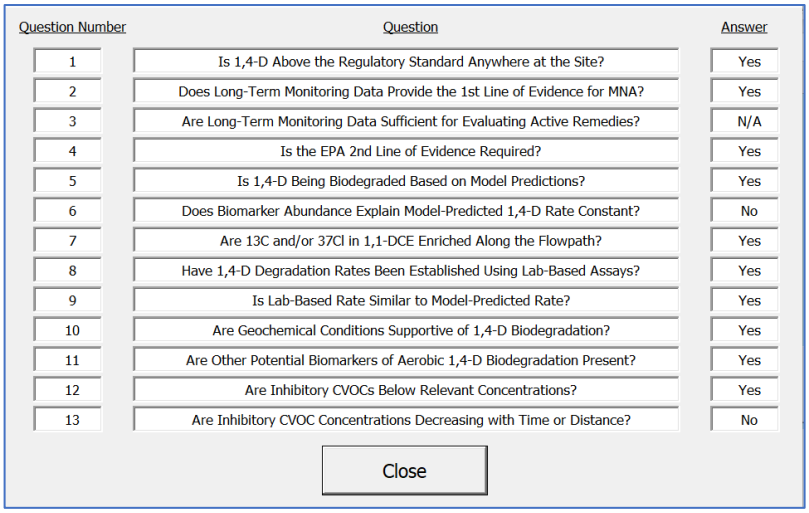
1,4-Dioxane

The following describes the Decision Framework for the 1,4-Dioxane (1,4-D) module.



BioPIC Home Page showing start button for entering the chlorinated ethane decision framework

Each of the numbered questions below corresponds to a number in the flowchart/guided tour. After each number, the decision criteria are explained. For most, further information is provided in the Help text. Note that these text descriptions are shown as pop-up boxes within the tool.



Question Number	Question	Answer
1	Is 1,4-D Above the Regulatory Standard Anywhere at the Site?	Yes
2	Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?	Yes
3	Are Long-Term Monitoring Data Sufficient for Evaluating Active Remedies?	N/A
4	Is the EPA 2nd Line of Evidence Required?	Yes
5	Is 1,4-D Being Biodegraded Based on Model Predictions?	Yes
6	Does Biomarker Abundance Explain Model-Predicted 1,4-D Rate Constant?	No
7	Are 13C and/or 37Cl in 1,1-DCE Enriched Along the Flowpath?	Yes
8	Have 1,4-D Degradation Rates Been Established Using Lab-Based Assays?	Yes
9	Is Lab-Based Rate Similar to Model-Predicted Rate?	Yes
10	Are Geochemical Conditions Supportive of 1,4-D Biodegradation?	Yes
11	Are Other Potential Biomarkers of Aerobic 1,4-D Biodegradation Present?	Yes
12	Are Inhibitory CVOCs Below Relevant Concentrations?	Yes
13	Are Inhibitory CVOC Concentrations Decreasing with Time or Distance?	No

Close

Example of Summary Assessment pop-up box after completing the stepwise decision framework for 1,4-dioxane

As with the other compounds, a summary assessment that shows the all of the results for 1,4-dioxane will be displayed once the user has gone through the entire decision logic (i.e., evaluated all of the possible lines of evidence). A graphic showing the entire decision flowchart for 1,4-dioxane is reproduced at the end of this section. Note that once the user starts answering the questions, the summary assessment can also be pulled up by clicking the View Summary box that appears to the right of each question. In these cases, it will display answers to only those questions that have been completed.

1. Is 1,4-D above the regulatory standard anywhere at the site?

Decision Criteria:

Answer YES if: The 1,4-D concentration at any groundwater monitoring location at the site is above the applicable concentration-based regulatory standard. Note that this standard is site-specific. If no standard has been established for your site, then select a value based on guidance from EPA or other states (see **HELP**) for planning purposes.

Answer NO if: The 1,4-D concentration at each groundwater monitoring location at the site is already below the concentration-based regulatory standard. The decision tool is intended for sites where the concentration of 1,4-D must fall below a site-specific regulatory standard before contaminated groundwater reaches the point of compliance (POC). If the 1,4-D concentration is already below the regulatory standard across the site (including the POC), then it is highly likely that it will remain below this standard in the future. This assumes that the site has been reasonably well characterized (especially the source area) and that no significant changes in conditions at the site are anticipated.

HELP

In the absence of site-specific regulatory standards for 1,4-dioxane, the user may wish to select one of the following values to proceed with the BioPIC evaluation. Note that there is considerable variability in state-level groundwater and drinking water standards for 1,4-dioxane. The information below was compiled on 1 January 2021, so it should also be understood that states are likely to promulgate and/or revised standards over time.

- USEPA Reference Concentration for Drinking Water (10-6 risk) = 0.35 µg/L
- USEPA Reference Concentration for Drinking Water (10-4 risk) = 35 µg/L
- California Notification Level in Drinking Water = 1 µg/L
- Massachusetts Groundwater Standard = 0.3 µg/L
- Colorado Groundwater Standard = 0.35 µg/L
- Florida Groundwater Standard = 3.2 µg/L
- Illinois Groundwater Standard = 7.7 µg/L
- Missouri Groundwater Standard = 61 µg/L
- New Hampshire Groundwater Standard = 0.32 µg/L
- New Jersey Groundwater Standard = 0.4 µg/L
- North Carolina Groundwater Standard = 3 µg/L
- Texas Groundwater Standard = 9.1 µg/L
- Wisconsin Groundwater Preventive Action Limit = 3 µg/L

If 1,4-dioxane is already below the established or assumed standard, then a future exceedance would likely be associated with one or more of the following: 1) the site is poorly characterized; 2) a new release of 1,4-dioxane occurs; 3) active remediation is on-going or recently completed, such that steady state conditions have not yet been reached; or 4) any other change in site conditions has occurred that enhance 1,4-dioxane mass transfer to the aquifer and inhibit 1,4-dioxane attenuation.

2. Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?

Decision Criteria

Answer YES if: The 1,4-D concentration is currently below the regulatory standard at the point of compliance (POC) and is predicted to be below the concentration-based regulatory standard at the POC at any time in the future. Use the model provided as part of this tool (see MNA Rate Constant Estimator in **FILES**) to predict if the concentration will be below the standard at any time in the future (see **HELP** for additional explanation).

Answer NO if: At any time in the future, the 1,4-D concentration will exceed the regulatory standard at the POC. Use the model provided as part of this tool to predict the concentration in the future at the POC. Note there usually is also a temporal component in the regulatory goals, which involves establishing how long it will take the concentration at a particular location to achieve a regulatory goal. The implementation of more aggressive remedies may reduce time to achieve remediation goals, thereby reducing the overall cost. However, this tool primary deals with the spatial aspects of remediation goals (i.e., will the goal be achieved at a POC) rather than the temporal components.

HELP

If sufficient historical contaminant concentration data are not available to determine if the 1,4-D plume will reach a POC, then a groundwater flow and solute transport model, such as the "MNA Rate Constant Estimator" provided as part of this tool (see MNA Rate Constant Estimator in **FILES**), should be used to predict solute plume behavior. In this case, the simulation should account for the effects of advective groundwater flow, dispersion of the relevant solutes, sorption, and degradation of 1,4-D in groundwater at the site.

For more information on using this type of model for 1,4-D, consult the project report (Development of a Quantitative Framework for Evaluating Natural Attenuation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, and 1,4-Dioxane in Groundwater (ESTCP ER-201730)), which can be found on the project page (copy link into web browser): https://serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/Persistent-Contamination/ER-201730/ER-201730. The model is also explained in the User's Guide for BioPIC.

A similar approach for chlorinated ethenes can be found in another project report (Development of a Quantitative Framework and Management Expectation Tool for the Selection of Bioremediation Approaches at Chlorinated Ethene Sites (ESTCP Project ER-201129)), which can be downloaded from the project page (copy link into web browser): https://www.serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/Persistent-Contamination/ER-201129/ER-201129. In the ER-201129 report, Section 5.2.3 illustrates the process of calibrating a groundwater flow and transport model (in this case, the "BIOCHLOR" model), and Section 5.2.4 Step 1 illustrates the use of a model to apply the decision criteria.

If available, a robust historical database of contaminant concentrations can be used as an alternative to a computer model. Spatial and temporal trends in solute concentrations can be utilized to determine if the plume is stable or receding and therefore will not reach the POC. When sufficient data are available, using empirical data to ascertain trends is much better than using a model. In many cases, sufficient 1,4-D concentration data are available to evaluate plume behavior and to determine if solute concentrations will exceed cleanup goals at a regulatory POC.

3. Is Long-Term Monitoring Data Sufficient to Evaluate MNA?

Decision Criteria

Answer YES if: The current long-term monitoring data confirm that one or more of the following conditions are met:

1. The plume is currently beyond the point of compliance at a concentration that is above the applicable standard.
2. The plume is still expanding and is predicted to extend beyond the point of compliance in the future based on modeling.
3. Concentration-based goals have been established within the plume (i.e., upgradient of the point of compliance), and model predictions suggest that these will not be achieved with a reasonable timeframe.

Answer NO if: The current long-term monitoring data confirm that ALL of the following conditions are met:

1. The plume is currently not beyond the point of compliance.
2. The current dataset is too limited to evaluate if plume is expanding vs. receding.
3. The current dataset is too limited to predict using a model whether the plume will expand or whether concentration-based goals will be achieved.
4. There is no current regulatory requirement for active remediation.

HELP

Long-term monitoring data are an important component of site management, and they are particularly important for demonstrating the site-specific viability of MNA.

In the case of 1,4-D, it is possible that a site's existing dataset for this compound may be limited if it is a recent additional to the monitoring program. It may not be possible to establish a clear trend in the attenuation in concentration of 1,4-D along the flow path in groundwater. This means that the data are inadequate to permit a thorough evaluation for the primary line of evidence for MNA. This is typically because one or more of the following apply: 1) data are highly variable across the site; 2) data are highly variable from event-to-event; or 3) data are available from a relatively limited number of monitoring points or events. In each case, these data limitations make it difficult to establish trends with any degree of statistical certainty.

In that case, collecting additional data may be beneficial to provide more certainty that current trends are statistically significant and sustainable. These additional monitoring events may also include data that would be used to support the second line of evidence for MNA (e.g., geochemical data, biomarkers, stable isotopes) or even hydrogeologic parameters to improve fate and transport model predictions.

At sites where the number of monitoring locations is relatively small (e.g., 4 or less wells), this type of analysis will likely benefit by including additional monitoring locations along the plume transect. In part, this is because it can be more challenging to establish that a trend is significant using standard approaches (e.g., that the slope of a best-fit regression line is different than zero). As a result, adding more monitoring locations along the plume transect may provide value.

It is necessary to use modeling software to evaluate if current trends in natural attenuation will meet the standard at the point of compliance (POC). An example is the "MNA Rate Constant Estimator" that has been developed as part of this decision tool (see MNA Rate Constant Estimator in **FILES**). This type of model allows the user to predict concentrations along a plume transect. However, it relies on the user to calibrate to the model predictions based on field data, and it also assumes that those field data are representative. At sites, where data vary considerably from event to event, this type of calibration can be

challenging. In any case, the goal is to demonstrate that the plume footprint is stable and/or shrinking and will not result in concentrations at a downgradient POC that exceed an acceptable level. This may require additional monitoring locations (particularly if the plume extent is not yet delineated) or additional monitoring events to demonstrate longer-term stability. Data from additional events are also important to establish that trends are sustainable even if minor (or major) changes in site conditions (e.g., groundwater flow directions, redox conditions) occur that might impact attenuation and/or plume stability.

In some cases where MNA is proposed as a remedy, an estimate of the remediation timeframe is also required by the regulatory agency. The remediation timeframe is typically the date when some or all wells are projected to achieve a concentration goal. Estimating the remediation timeframe is often done using linear regression of concentration vs. time data at a specific well (or wells), but this requires data from enough events to ensure that the result is statistically significant. Additional monitoring events may be required to achieve this objective at sites with limited concentration vs. time data.

For the case where the dataset is already robust and confirms that MNA is unlikely to be effective, active remediation is likely to be the next course of action. Active remediation should be selected and implemented based on appropriate, well-defined objectives, but one primary goal should be to reduce concentration in the source area enough so that concentrations are below the site-specific standard at the point of compliance. The required reduction in the source concentration can be estimated using models, including the one provided as part of this tool (see MNA Rate Constant Estimator in **FILES**). Another good option is REMChlor-MD, which allows the user to input the extent of source reduction and then compare the plume behavior for cases with and without remediation. REMChlor-MD also incorporates the effects of matrix diffusion (i.e., contaminants diffusing into and out of lower-permeability intervals within the saturated zone) which has implications for contaminant transport and persistence at many sites.

Additional resources for understanding and developing monitoring objectives for MNA include the following (copy link into web browser):

https://clu-in.org/download/contaminantfocus/dnapl/Treatment_Technologies/performance_monitoring_mns600R04027.pdf

https://www.serdp-estcp.org/content/download/25789/262545/file/FAQ%20ER-201211.V2%20February%202014.pdf

https://clu-in.org/download/techfocus/na/NA-approach-for-eval-2011.pdf

https://www.epa.gov/sites/production/files/2014-02/documents/d9200.4-17.pdf

https://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=10004674.TXT

https://pubs.usgs.gov/wri/wri034057/

4. Is the EPA Second Line of Evidence Required?

Decision Criteria

Answer YES if: The appropriate regulatory authority has requested that multiple lines of evidence for 1,4-D attenuation should be collected before approval of MNA as a site remedy will be granted. The first direct line of evidence requires data that demonstrate a clear and meaningful trend of decreasing contaminant mass and/or concentration over time at appropriate monitoring or sampling points. The second line of evidence originally included "hydrogeologic and geochemical data that can be used to indirectly demonstrate the type(s) of natural attenuation processes active at the site, and the rates at which such processes will reduce contaminant concentrations to required levels".

Answer NO if: The appropriate regulatory authority has specifically requested that only the first/primary line of evidence for natural attenuation is required for approval of MNA as a site remedy. The first direct line of evidence requires data that demonstrate a clear and meaningful trend of decreasing contaminant mass and/or concentration over time at appropriate monitoring or sampling points. Note that the final decision to require, or to not require, the second line of evidence is made by the appropriate regulatory authority. If the regulatory authority has not signaled what lines of evidence will be required, then a more conservative approach would be to answer "YES" to this question and proceed with collecting additional lines of evidence.

HELP

As part of EPA's MNA guidance, the agency has listed three lines of evidence that may part of an MNA evaluation. Collecting data to support the first line of evidence is always required, and data to support the second line of evidence is typically required. The rest of this decision tool is focused on the second and third lines of evidence. It is recommended that the user go through these decision points even if the applicable data are not available.

The following resources provide more information on the lines of evidence approach, including definitions and how they are used (copy link into web browser):

https://www.epa.gov/sites/production/files/2014-02/documents/d9200.4-17.pdf

https://www.serdp-estcp.org/content/download/25789/262545/file/FAQ%20ER-201211.V2%20February%202014.pdf

5. Is 1,4-D biodegrading based on model predictions?

Decision Criteria

Answer YES if: Using 1,4-D biodegradation rate constants greater than zero in the model provide a better fit than the fit when the rate constant is set to zero. This can be evaluated using the same simulation in the "MNA Rate Constant Estimator" model that was used to "Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?" Prepare a new simulation where the rate constant for degradation of 1,4-D is set to zero. Compare the actual field-measured concentrations of 1,4-D against the new simulation. Then enter trial values for the rate constant for 1,4-D degradation into the simulation to determine if the model projections provide a better fit to the actual field-measured concentrations. A better fit is defined as having a lower value of RMSE (root mean square error between the field data and the concentrations predicted by the model). If rate constants greater than zero provide a better fit, then

1,4-D is degrading. Note that this may have already been established as part of the earlier evaluation of the criterion "Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?"

Answer NO if: Setting the 1,4-D biodegradation rate constant to zero in the model provides a better fit than the fit when the rate constant is greater than zero. This is evaluated using the same model and simulations described above for the "YES" answer.

6. Does Biomarker Abundance explain model-predicted 1,4-D rate constant?

Decision Criteria

Answer YES if: The 1,4-D biodegradation rate constant used in the model is consistent with biomarker correlations that were derived from other studies and kinetic data. To do this, first refer to the simulation in the "MNA Rate Constant Estimator" model that was used to evaluate the criterion "Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?". Note that this model has the option to use biomarker data to estimate the biodegradation rate constant (i.e., it uses a correlation to predict the representative rate constant based on the biomarker levels measured at the site). If this option was employed and it resulted in a reasonable fit to the actual data, then this confirms that "YES" is the appropriate answer. See **HELP** for additional guidance on determining if the fit was reasonable.

Answer NO if: No data on the abundance of DXMO (also known as THFMO) or other qPCR biomarkers are available, or if 1,4-D biodegradation rate constant used to optimize the model is inconsistent with rate constants predicted using the biomarker correlations. To evaluate the latter condition, first refer to the model simulation that was used to evaluate the criterion "Does Long-Term Monitoring Data Provide the 1st Line of Evidence for MNA?". If the option to use the correlation was not employed OR did not result in an optimal fit, then "NO' is the appropriate answer.

HELP

The "MNA Rate Constant Estimator" model (see MNA Rate Constant Estimator in **FILES**) has an option to estimate rate constants based on the abundance of several different qPCR-based biomarkers for degradation. These correlations are designed to help calibrate the model, and they are intended as a starting point for improving the fit between the actual field data and the model simulations. Consequently, they should not be considered a true prediction of the actual degradation rate that is occurring at the site. This is because they are based on empirical data from other studies where conditions may be quite different than those observed at the site being evaluated.

To use the biomarker correlations as part of this decision tool, the following process is recommended:

1. In Box 6b, select a specific biomarker from the dropdown menu. For 1,4-dioxane, there is an option to enter the following biomarkers: DXMO, prmA, RDEG, and RMO. The DXMO biomarker (also known as THFMO) is associated with organisms that can grow by degrading 1,4-dioxane. The prmA biomarker (also known by the enzyme name PrMO or PPO) is associated with organisms that cometabolize 1,4-dioxane while growing on propane. The RDEG and RMO biomarkers are associated with organism that cometabolize 1,4-dioxane while growing on toluene, or native organic matter. Only 1 biomarker can be entered at a time; start with DXMO if available. Selecting

a biomarker from the menu will launch a pop-up box where biomarker abundance data can be entered.

2. In the pop-up box, enter the abundance of the selected biomarker for those wells where data are available. The model will perform a spatial interpolation to estimate a representative biomarker abundance for the site (i.e., a single value that is weighted based on the distance between wells with biomarker data).
3. The rate constant associated with this biomarker abundance will then be automatically entered in the appropriate location in Box 6b.
4. Enter the rate constant from Box 6b into Box 6. This is the rate constant that is used for the model simulation (i.e., to generate a simulated concentration vs. distance curve).
5. The user should then evaluate the fit between the actual field data and the model simulation that is based on this estimated rate constant.
6. Manually adjust the rate constant in Box 6 until an optimal fit between the actual field data and the model simulation is obtained. Use the Root Mean Square Error (RMSE) that is overlaid on the plot as a guide; lower RMSE values generally indicate a better fit. Record the rate constant that provided the optimal fit.
7. Return to Box 6b and repeat Steps 1 - 3 for all remaining biomarkers. In each case, record the rate constant that is generated in Box 6b (i.e., after the biomarker data are entered in the pop-up box).
8. Compare the recorded rate constants from the biomarker correlations with the "optimal" rate constant from Box 6. If the optimal rate constant from Box 6 is within a factor of 3 to 5 of one or more of the rate constants that were generated from the biomarker correlations, then this is considered reasonable evidence that this particular biodegradation process is contributing to the actual field trend in 1,4-dioxane concentrations.
9. The biomarkers target different genes in different organisms. Ideally, all the organisms could be present in the groundwater at the same time, and act on 1,4-dioxane concomitantly. Add all the rate constants associated with DXMO, prmA, RDEG, and RMO together, and if the optimal rate constant from Box 6 is within a factor of 3 to 5 of the sum of the rate constants that were generated from the biomarker correlations, then this is considered reasonable evidence that biodegradation processes are contributing to the actual field trend in 1,4-dioxane concentrations.

The derivation of these correlations is described in Appendix D of the project report. They are based on an assumption that aerobic biodegradation of 1,4-dioxane follows Michaelis-Menten (Haldane) kinetics (Mahendra and Alvarez-Cohen, 2006; Mahendra et al., 2013; Ye et al. 2017; Grostern et al., 2009; Parthasarathy et al., 2015). The rate equation for Michaelis-Menten kinetics can be rearranged to solve for a first-order rate constant that is a function of other kinetic parameters (specifically Km and Vmax expressed in terms of gene copies), the biomarker abundance (expressed in gene copies per mL) and the concentration of the organic chemical being degraded (in this case, 1,4-dioxane). Derived values for the kinetic parameters for each biomarker are also detailed in Appendix D of the project report.

Additional information on 1,4-dioxane biomarkers is also provided in Question #11.

7. Are ^{13}C and/or ^{2}H in 1,4-dioxane enriched along the flow path?

Decision Criteria

Answer YES if: A clear pattern of carbon and hydrogen isotope fractionation can be observed in samples collected along a groundwater flow path. For 1,4-D, values of $\delta^{13}C$ and $\delta^{2}H$ can be obtained for individual samples via commercial lab analysis (see **HELP** for further explanation of CSIA principles and analytical considerations). If samples are taken from the source area and then at several locations downgradient, a 2-dimensional plot of these values can then be generated (see CSIA_14D in **FILES**). If the values of both $\delta^{13}C$ and $\delta^{2}H$ (particularly the latter) generally increase along the groundwater flow path (i.e., become "less negative" due to depletion of the lighter isotope), then this is taken as evidence for degradation of 1,4-D.

Answer NO if: No isotope data are available or if there is no clear trend in samples collected along a groundwater flow path. Again, this is best visualized by creating a 2-D plot of the $\delta^{13}C$ and $\delta^{2}H$ values (see **HELP** and CSIA_14D in **FILES** for more guidance).

HELP

Additional lines of evidence for 1,4-dioxane attenuation can be provided by site-specific analysis of samples for stable isotopes of carbon and hydrogen. Data from a single sample is unlikely to provide evidence for 1,4-D biodegradation. This is because there is significantly variability in the known isotopic composition of undegraded 1,4-dioxane sources, as well as data that suggests that these known source compositions do not represent the full range that might be encountered at contaminated sites. As a result, any attempt to establish biodegradation by comparing the isotopic composition of a groundwater sample to known source compositions (similar to the CSIA approach described for chlorinated ethenes) is subject to considerable uncertainty for 1,4-D and unlikely to serve as a convincing line of evidence at this time. Collecting multiple samples along the groundwater flow path is a more appropriate approach because it relies on site-specific isotopic data to document 1,4-D degradation.

If values for $\delta^{13}C$ and $\delta^{2}H$ are available for 1,4-D, open the tab **FILES** and select the spreadsheet CSIA_14D.xlsx. Enter your data in the tab Input. The user can enter data for up to 8 wells. Data from a well located within the source area or in an upgradient area should be entered in the first row (Well 0); this provides a site-specific estimate of the isotopic composition of 1,4-dioxane at the source and serves as a baseline for further comparisons. This well should have the lowest (most negative) values for $\delta^{13}C$ and δH (prioritize the well with the lowest $\delta^{2}H$). Data from other wells are entered in the remaining rows, following the order that they fall along the groundwater flow path.

Once the available data have been entered, the user can first consult the tab 2-D Chart_simple. Your data should plot on the chart. If not, you may need to extend the scales of the x and/or y axes. Degradation of 1,4-dioxane is indicated if the data points generally proceed up and/or to the right within the plot in the direction of groundwater flow. This occurs due to the preferential degradation of bonds that contain lighter isotopes, such that the lighter isotopes become depleted and the heavier isotopes become enriched within the remaining portion of the compound as it is transported downgradient. The degree of enrichment can vary depending on the compound, the isotope, and the transformation pathway.

The error bars represent uncertainty in the determination of $\delta^{13}C$ and $\delta^{2}H$. The values can be said to increase between two samples if either one or both of the vertical or horizontal error bars do not overlap.

For 1,4-dioxane, the user can also roughly estimate the amount of 1,4-dioxane that has been degraded based on different possible degradation pathways (see Step 2 in the Input tab). This relies on published isotopic enrichment factors (Ɛ) for carbon and hydrogen for three different biological transformation pathways: (1) co-metabolic oxidation by Rhodococcus rhodochrous strain 21198 grown on propane; (2) co-metabolic oxidation by Rhodococcus rhodochrous strain 21198 grown on isobutane; and (3) co-metabolic oxidation by Pseudonocardia tetrahydrofurans strain K1 grown on tetrahydrofuran (THF).

For each of the three possible pathways listed above, the percent of 1,4-dioxane degraded is presented as a range based on the uncertainty in the isotopic enrichment factors, as well as a user-input uncertainty factor. The latter can be used to perform a limited sensitivity analysis on the degradation estimates.

To better understand how the data compare to the expected isotopic fractionation patterns for each pathway, the user can consult the tab 2-D delta from upgradient. In this chart, the origin is the isotopic composition of the upgradient/source well (Well 0), and the rest of the site-specific data are plotted as symbols. The three solid lines represent the fractionation pattern associated each of the three pathways described above as degradation proceeds. The slopes of these lines reflect changes to both elements (carbon and hydrogen) and are minimally influenced by retardation and other non-destructive processes that may occur during groundwater transport. If the data adhere to a specific pathway line, then this is plausible evidence that this specific pathway may be contributing to the observed fractionation. It should be understood that alternate or multiple transformation pathways may be occurring and cause data to not adhere to any of the plotted lines.

8. Have 1,4-D degradation rates been established using lab-based assays?

Decision Criteria

Answer YES if: A statistically significant 1,4-D degradation rate constant has been established using concentration vs. time data generated from a lab-based test of site material. This can include standard microcosms constructed with site groundwater (and possibly soil) or more advanced techniques such as an assay based on adding radiolabeled ^{14}C-1,4-D (see **HELP**) to site groundwater. In each case, samples are collected from bottles at periodic intervals to monitor 1,4-D disappearance (and in the case of the ^{14}C assay, product accumulation) over time. An abiotic control must also be included to accurately quantify the rate associated with biological activity. The 1,4-D rate constant is then calculated from the concentration vs. time dataset under the assumption that degradation follows a first-order relationship. For MNA studies, this type of testing is traditionally considered a third (or tertiary) line of evidence. However, it also provides rate information that is consistent with the second line of evidence.

Answer NO if: No lab-based tests have been performed, or if the results of lab-based tests are negative. The latter is true if rate constants are not statistically significant (i.e., not greater than zero or if they are not different than controls). If lab-based tests are used to obtain lines of evidence for 1,4-D biodegradation, it is recommended that samples be collected from multiple locations at the site and tested individual. This reduces the possibility of “false negative” results.

HELP

The predominant product formed from biodegradation of 1,4-D is carbon dioxide (CO_2). Because many other processes result in formation of CO_2, it is not possible to document in situ biodegradation of 1,4-D based on product accumulation. It is possible, however, to document this process in the laboratory using ^{14}C-1,4-D in microcosms. Using ^{14}C material makes it possible to identify $^{14}CO_2$ as a product from ^{14}C-1,4-D. It is also possible to identify other biodegradation products that may be released. Furthermore, by measuring the rate at which ^{14}C-labeled products accumulate, it is possible to determine a pseudo-first order biodegradation rate constant. The rate at which 1,4-D biodegrades can also be determined in microcosms without using ^{14}C-1,4-D. However, this often requires at least several months of incubation, in order to detect an adequate level of decrease in 1,4-D. The ^{14}C assay is typically complete within six weeks, and it is sensitive enough to detect rate constants as low as 0.0069 yr^{-1}, equivalent to a half-life of 100 years.

The assay beings by collecting groundwater samples and shipping them overnight on ice to a laboratory that is equipped to use ^{14}C-labeled compounds. In the lab, a purified stock solution of ^{14}C-1,4-D is added to the microcosms and measurements are made at time zero for the amount of ^{14}C initially present, along with GC analysis of total 1,4-D and headspace analysis of VOCs. At weekly intervals, samples of groundwater are removed from the microcosms to determine the amount of ^{14}C-labeled products formed. When the incubation period is complete, the product data is evaluated using a mass balance model to estimate the pseudo first order rate constant. Data from a filter-sterilized control is also evaluated and if the rate of accumulation from the control is statistically significant, a net rate constant is calculated and evaluated for statistical significance. The procedures are very similar to those outlined in Mills IV et al. (Quantification of TCE co-oxidation in groundwater using a ^{14}C–Assay. Groundwater Monitoring & Rem. 2018, 38, 57-67).

In a study performed for ESTCP, groundwater samples were collected from 10 sites and a total of 49 wells. Of these, statistically significant first order rate constants were measured for 1,4-D in groundwater from 15 of the wells based on the ^{14}C assay. It should be noted that most of the half-lives determined were in excess of 50 years. That may be a consequence of the assay being performed with groundwater alone (i.e., no soil present), which may present limitations in terms of the amount of biomass and nutrients available. For this reason, a statistically significant result in the ^{14}C assay may be viewed as justification for performing additional laboratory studies with soil present, to further refine the estimate of a biodegradation rate constant.

9. Is lab-based degradation rate for 1,4-D similar to the model-predicted rate?

Decision Criteria

Answer YES if: If the 1,4-D biodegradation rate constant from one or more locations in the lab-based tests is greater than the biodegradation rate predicted from modeling. To do this, first refer to the model simulation that was used to evaluate the criterion “Does Natural Attenuation Current Meet the Goal?”. The model estimates the biodegradation rate that would result in the actual 1,4-D concentration vs. distance pattern observed at the site being evaluated. The lab-based tests establish a biodegradation rate under controlled conditions and are unequivocal evidence that 1,4-biodegredation can occur.

Consequently, lab-based rates greater than the model-predicted rates are seen as strong quantitative evidence of biodegradation potential, and in some cases, may be used to refine the rate constants estimated by the model. Additional lines of evidence beyond these lab-based assays should be evaluated as needed.

Answer NO if: If the 1,4-D biodegradation rate constant from all locations in the lab-based tests are less than the biodegradation rate predicted from modeling. This is evaluated using the same model and simulation described above for the "YES" answer. For cases where the lab-based tests yield a very slow 1,4-D degradation rate (e.g., half-lives greater than 100 years, degradation rates that are more than an order of magnitude smaller than the model-predicted rate), the user should consider performing supplemental lab-based tests to confirm if nutrient limitations and other factors may have suppressed the 1,4-D biodegradation rate. Additional lines of evidence beyond these lab-based assays should be evaluated as needed.

10. Are geochemical conditions supportive of 1,4-D biodegradation?

Decision Criteria

Answer YES if: Dissolved oxygen is routinely present in groundwater samples from one or more wells in the 1,4-D plume or in the area downgradient of the 1,4-D plume. This is a qualitative line of evidence and does not imply that 1,4-D is actually degrading. Dissolved oxygen is needed to support aerobic 1,4-D biodegradation, although a threshold (minimum) value to support in situ biodegradation has not been established. Lab studies have shown that degradation rates decrease below 2 mg/L, but 1,4-D biodegradation has been observed in wells with lower field measurements of dissolved oxygen. For the purposes of this decision tool, conditions are considered generally favorable for aerobic biodegradation of 1,4-dioxane when one of the following criteria are met: Dissolved oxygen concentrations measured in the field are greater than 0.1 mg/L, ferrous iron (Fe^{2+}) concentrations are less than 0.5 mg/L, and methane concentrations are less than 0.005 mg/L. However, field parameter data should be evaluated with caution (see **HELP**).

Answer NO if: Dissolved oxygen is typically present at low levels (<< 1 mg/L) across the entire site. This might include sites where the impacted intervals are deep, confined, and/or organic-rich. This type of determination would also rely on other corroborating geochemical data, such as highly negative ORP readings, elevated dissolved iron, and methane.

HELP

1,4-dioxane can be naturally attenuated by reactions that occur in primarily aerobic conditions. No naturally occurring abiotic or anaerobic degradation reactions have been established. In assessing whether geochemical conditions are favorable for anaerobic vs. aerobic processes, it should be noted that field methods for measuring dissolved oxygen may generate inconsistent and/or erroneous results. One contributing factor is the common use of long-screened (≥ 10 ft) monitoring wells that may be collecting water from multiple zones with different redox conditions. This mixing of groundwater can make it difficult to quantify zones with higher dissolved oxygen that may promote 1,4-dioxane biodegradation. Consequently, field dissolved oxygen measurements should be used with caution and supported by other lines of evidence. Other data that would corroborate that geochemical conditions are favorable include

positive ORP readings and low dissolved iron and methane concentrations. Total organic carbon is also a positive indicator, although carbon may create reducing conditions if oxygen availability is limited. This highlights the importance of delineating those portions of the site where groundwater transitions between anaerobic and aerobic to identify areas that might be best managed by natural attenuation.

11. Are potential biomarkers of aerobic 1,4-D biodegradation present?

Decision Criteria

Answer YES if: The presence of genes encoding DXMO/THFMO and/or ALDH has been established using quantitative polymerase chain reaction (qPCR) testing OR the presence of several other less-specific biomarkers has been established. DXMO/THFMO and ALDH have been identified as enzymes that are involved in the initial steps of 1,4-dioxane metabolism and/or co-metabolism. Other monooxygenases such as SCAM (short chain alkane monooxygenase), RMO and RDEG (both of which are ring-hydroxylating toluene monooxygenases) have also been identified as enzymes that can be involved in 1,4-dioxane co-metabolism. In addition, various propane monooxygenases have been evaluated for 1,4-dioxane capacity, and at least one (encoded by prmA) may be capable of both metabolic and co-metabolic degradation of 1,4-dioxane. However, it is not well-established if other propane monooxygenases (e.g., PPO) or various methane monooxygenases are capable of degrading 1,4-dioxane. In some cases, these enzymes may be expressed at the same time as other monooxygenases that are more directly involved in 1,4-dioxane degradation, such that they would serve as a secondary indicator that conditions are favorable for biodegradation. See **HELP** for additional information.

Note that genes that encode oxygenases are frequently found in a variety of environmental samples, including groundwater that would be considered anaerobic based on field measurements. These oxygenase enzymes also have broad metabolic capabilities, and the presence of an oxygenase-encoding gene does not ensure that 1,4-dioxane is actually degrading (see **HELP** for additional information). Consequently, qPCR results showing the presence of non-specific oxygenase genes should be used with caution and supported by other lines of evidence.

Answer NO if: No qPCR data are available OR if these biomarkers were not detected in any of the samples analyzed

HELP

The metabolic pathway for aerobic biodegradation of 1,4-D includes several enzymes that appear to be relevant to this process. For that reason, detection of the DNA responsible for coding the formation of these enzymes can provide a useful line of evidence for 1,4-D biodegradation. For example, an aldehyde dehydrogenase (ALDH) has been identified as a secondary biomarker 14D biodegradation by THFMO-expressing strains such as *P. dioxanivorans* CB1190.

Biodegradation of 1,4-D may also occur by a cometabolic process, whereby microbes grow on a substrate other than 1,4-D, but they express non-specific oxygenase enzymes that are capable of initiating oxidation of 1,4-D. There are numerous primary substrates that result in expression of enzymes capable of oxidizing 1,4-D, including tetrahydrofuran, propane, and butane. Monooxygenases investigated as possible or likely to be able to cometabolize 1,4-dioxane include soluble methane monooxygenase (sMMO), ring

hydroxylating toluene monooxygenase (RMO and RDEG), phenol hydroxlyase (PHE), and short-chain alkane monooxygenases (SCAM). Recently, a toluene-oxidizing monooxygenase has been described that can oxidize low concentrations of 1,4-dioxane and can also oxidize propane at sufficiently high rates that its activity can support the growth of the host bacterium using propane as a sole source of carbon and energy (Deng et al. 2020). The majority of these have been classified as soluble di-iron monooxygenases (SDIMO) (He *et al*., 2017; Deng et al., 2018). For example, the SCAM enzyme is frequently found in bacteria that can grow on a broad range of gaseous and short chain alkanes (C_2-C_6). SCAM is thought to catalyze the terminal oxidation of alkanes to primary alcohol products. Bacteria that express SCAM can cometabolically degrade 1,4-dioxane at low, environmentally relevant concentrations (≤100 ppb) and have also been shown to oxidize a wide variety of chlorinated 1,4-dioxane-associated co-contaminants. It has been shown that model strains expressing other monooxygenases such as sMMO or one of several toluene-oxidizing monooxygenases can degrade high (≥50 ppm) concentrations of 1,4-dioxane. However, the activity of sMMO towards 1,4-dioxane has not been reproduced, even at the level of the purified enzyme (Hatzinger et al., 2017). The activity of the model toluene-oxidizing monooxygenases towards lower concentrations of 1,4-dioxane (≤100 ppb) also has not been confirmed.

A *q*PCR assay has been developed for many of the genes that encode the enzymes described above, and in most cases these assays are now commercially available (e.g., Microbial Insights) or can be completed by academic labs (e.g., SCAM at North Carolina State University in Dr. Michael Hyman's research lab).

In the case of 1,4-dioxane, collecting data on multiple gene targets may be useful. For example, the term propane monooxygenase has been widely used in the literature and was historically used to generically describe any undefined propane-oxidizing monooxygenase. More recently, two distinctly different enzymes have been referred to as propane monooxygenase. One of these enzymes is SCAM. The second enzyme is found in a wide diversity of hydrocarbon-oxidizing bacteria including organisms that can grow on substrates including methane, non-methane alkanes, alkenes (e.g., propene or isoprene), MTBE, and even 1,4-dioxane. Unlike SCAM, this enzyme (PrMO) has a restricted substrate range and is thought to sub-terminally oxidize propane to 2-propanol. Although expression of PrMO can enable some bacteria to grow on propane (and potentially ethane and *n*-butane), the only contaminants unequivocally known to be degraded by this enzyme are NDMA and phenol. This enzyme is encoded by the *prm*ABCD gene cluster and can be quantified qPCR using the PPO assay. The dramatically different catalytic capabilities of PrMO and SCAM justifies a nomenclature that distinguishes these two enzymes, especially as genome analyses now indicate that many gaseous alkane-oxidizing bacteria possess genes that encode both PrMO and SCAM. Consequently, qPCR-based analyses demonstrating changes in the abundance of one of these genes can potentially also exhibit quantitatively equivalent changes in the other. This issue of multiple monooxygenases within a single organism also extends to bacteria such as *P. dioxanivorans* CB1190 that also possess genes encoding PrMO in addition to genes encoding THFMO/DXMO.

Detection of these oxygenase genes provides an indirect line of evidence for the capacity for oxidation of 1,4-D. However, just because a gene is detected does not mean that it is being expressed, i.e., the active enzyme needed for oxidation of 1,4-D may not be undergoing synthesis. It is possible to test for mRNA (messenger ribonucleic acid), which is present only if the gene is being expressed (i.e., DNA makes RNA makes proteins). However, it is considerably more challenging to obtain good quantification of mRNA. 1,4-D is often present at levels below 1 mg/L, at which point there is not much substrate available to support growth that will allow for detection of DNA, let alone mRNA.

The appeal of using qPCR to quantify specific genes is the relatively low cost of this measurement. The results may be viewed as supportive but, taken alone, not sufficient to document the occurrence of 1,4-D biodegradation. In other words, if biodegradation is occurring, it is likely that the necessary DNA will be present in a groundwater sample. However, the presence of the DNA does not ensure that biodegradation is occurring, and the absence of the DNA does not exclude the possibility that biodegradation is occurring.

12. Are inhibitory CVOCs present at relevant concentrations?

Decision Criteria

Answer YES if: The concentration of 1,1-DCE currently exceeds 10 µg/L in one or more wells. 1,4-D biodegradation may proceed at this level, but the rate is likely to slow given the various mechanisms by which 1,1-DCE can inhibit 1,4-D biodegradation.

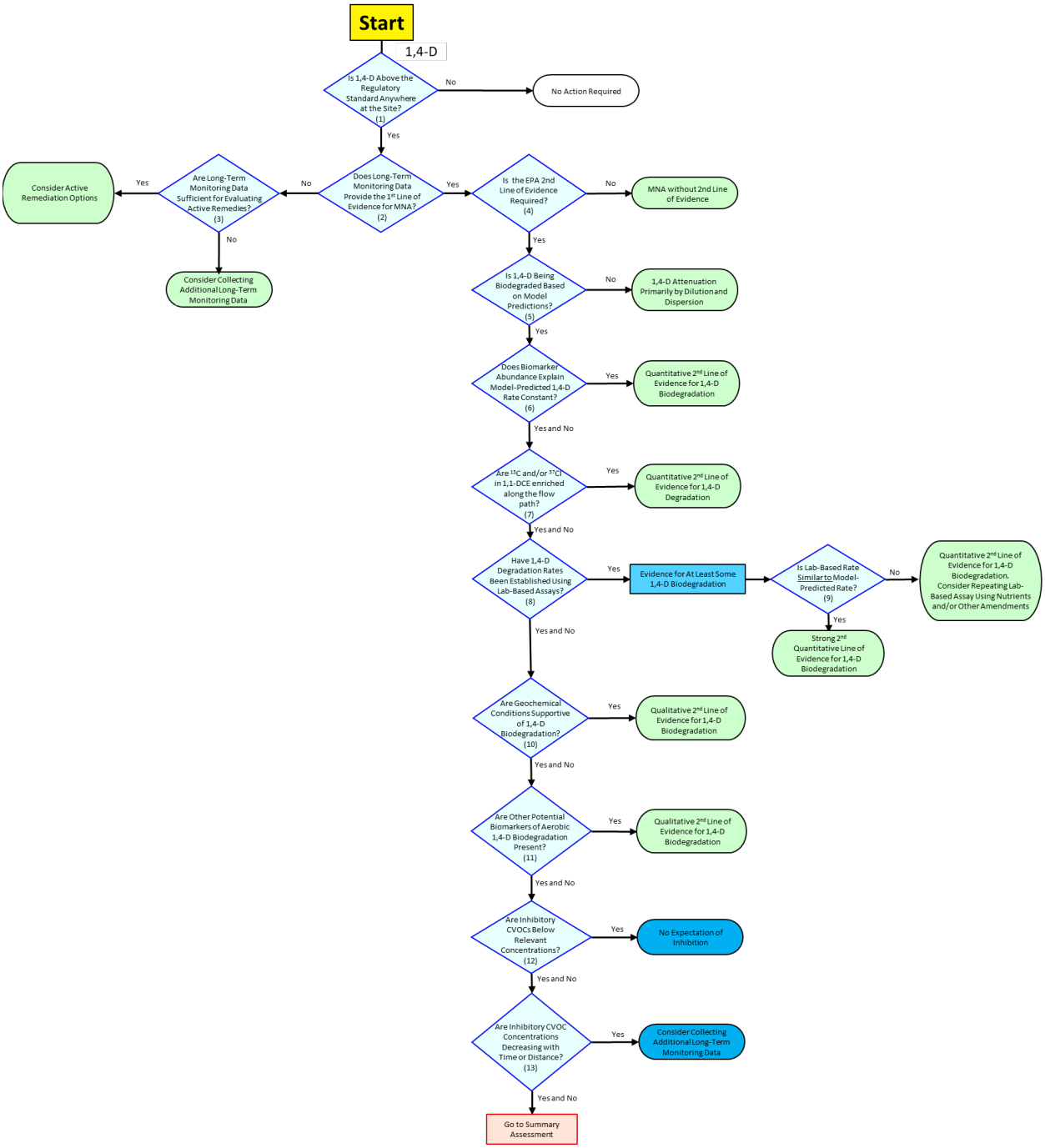
Answer NO if: No CVOCs are currently present in any wells at the site OR if the concentration of individual CVOCs is generally lower than 10 µg/L. Given the uncertainty, low CVOC concentrations should be combined with other lines of evidence that conditions are favorable for 1,4-D biodegradation.

13. Are inhibitory CVOC concentrations declining with time or distance?

Decision Criteria

Answer YES if: Declining trends in CVOC concentrations (total and/or individually) over time or along the groundwater flow path can be established. This can be accomplished using the model provided as part of this tool (see MNA Rate Constant Estimator in **FILES**, or go to the **GUIDED TOUR** for Chlorinated Ethenes and/or Chlorinated Ethanes). It provides evidence that these compounds are degrading, which can lessen their inhibitory effects based on lab and field studies. It also would help delineate portions of the 1,4-D plume where CVOCs are not present (e.g., in the toe of the 1,4-D plume) that might be better candidates for 1,4-D natural attenuation activity. Regardless, this is a qualitative indicator that conditions may be favorable for 1,4-D biodegradation and is more valuable if supported by other lines of evidence. It also suggests that collecting additional long-term monitoring data may be the most appropriate next step to determine if these favorable trends continue and eventually contribute to more rapid 1,4-D attenuation.

Answer NO if: CVOC concentrations exhibit stable trends over time OR along the groundwater flow path.

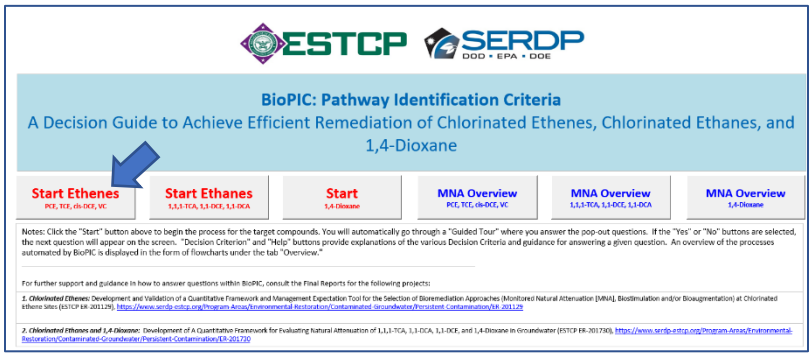


Decision Framework for 1,4-Dioxane.
This flowchart was coded into the updated BIOPIC tool.

Detailed BioPIC User Guide:

Chlorinated Ethenes

The following describes the Decision Framework for the compounds that are part of the Ethenes module, including PCE, TCE, cis-1,2-DCE, and VC.



BioPIC Home Page showing start button for entering the chlorinated ethene decision framework

Each of the numbered questions below corresponds to a number in the flowchart/guided tour. After each number, the decision criteria are explained. For most, further information is provided in the Help text. Note that these text descriptions are shown as pop-up boxes within the tool. A graphic showing the entire decision flowchart for these compounds is reproduced at the end of this section. If the user has selected a constituent of interest for evaluating the 2nd line of evidence for MNA (Question #3), a summary assessment can be displayed that shows the results for that particular compound. The summary assessment can be pulled up by clicking the View Summary box that appears to the right of each question, and it will display answers to only those questions that have been completed.

Note that these descriptions are retained from the 2015 version of BioPIC; no changes to the Chlorinated Ethene decision framework were made as part of the 2021 update to BioPIC. This means that the questions and associated decision criteria/help formats in this module differ somewhat from those found in the Chlorinated Ethane module and in the 1,4-Dioxane module.

1. Does natural attenuation currently meet the goal?

Decision Criteria

If at any time, the concentrations of PCE, TCE, DCE and VC will exceed the regulatory standard at the POC, then natural attenuation will not meet the cleanup goal.

There usually is also a temporal component in the regulatory goals, and the implementation of more aggressive remedies may reduce time to achieve remediation goals, thereby reducing the overall cost. This tool only deals with the spatial, not temporal, aspects of remediation goals.

HELP

If sufficient historical contaminant concentration data are not available to determine if a solute plume will reach a POC, then a groundwater flow and solute transport model such as BIOCHLOR should be used to predict solute plume behavior. In this case, the simulation should account for the effects of advective groundwater flow, dispersion of the relevant solutes, sorption, and degradation of the PCE, TCE, DCE and VC in groundwater at the site.

For more information, consult Development and Validation of a Quantitative Framework and Management Expectation Tool for the Selection of Bioremediation Approaches at Chlorinated Ethene Sites ESTCP Project ER-201129 https://www.serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/Persistent-Contamination/ER-201129/ER-201129. Section 5.2.3 illustrates the process of calibrating a groundwater flow and transport model. Section 5.2.4 Step 1 illustrates the use of a model to apply the decision criteria.

If available, a robust historical database of contaminant concentrations can be used as an alternative to a computer model. Spatial and temporal trends in solute concentrations can be utilized to determine if the plume is stable or receding and therefore will not reach the POC. When sufficient data are available, using empirical data to ascertain trends is much better than using a model. In many cases, sufficient solute concentration data are available to evaluate plume behavior and to determine if solute concentrations will exceed cleanup goals at a regulatory POC.

If historical data are used to determine whether NA currently meets the goal, it is still necessary to build a transport and fate model of the plume. The model is necessary to extract degradation rate constants that will be used in BioPIC to evaluate whether biological reductive dechlorination or abiotic degradation are a second line of evidence for MNA. Any computer application that simulates the fate and migration of PCE, TCE, DCE and VC in groundwater can be used to assess solute plume behavior. The simulation time for the model should be sufficient for concentrations of PCE, TCE, DCE and VC to reach their maximum concentrations at the POC. Most computer applications (i.e., software) cannot distinguish between cDCE, tDCE and 1,1- DCE. If this is true for the software you're using, then the simulations should be run to determine if natural attenuation will meet the remediation goal using the sum of the cDCE, tDCE, and 1,1-DCE isomers. When analyzing the degradation of PCE, TCE, DCE, and VC, different combinations of DCE isomers should be used in the analysis, depending upon the compound for which degradation pathways are being analyzed. This is discussed in the relevant sections that follow. For example, when evaluating degradation of TCE, only the cDCE and tDCE isomers should be included in the analysis because these are the relevant compounds produced from the degradation of TCE. When evaluating DCE degradation and therefore the possible production of VC, the sum of all DCE isomers should be used in the simulations, regardless of DCE origin, because all three DCE isomers can be reduced to VC by specialized bacteria. Again, when DCE is discussed in this document, if one of the isomers is specified, for example, cDCE, then it is that isomer that is relevant and that isomer only that should be considered. If the general term DCE is used, then the reader should assume that all three isomers of DCE should be considered (i.e., cDCE, tDCE, and 1,1-DCE).

2. Are reductive dechlorination genes present?

Decision Criteria

This decision box is reached if natural attenuation does not meet remediation goals. For the purpose of this decision support system, relevant RDase genes (e.g., tceA, bvcA, vcrA) are determined by the quantitative polymerase chain reaction (qPCR). Based on the current qPCR technology, a specific RDase gene is considered to be present if its abundance exceeds 10E+03 gene copies per liter of groundwater.

HELP

Some Dhc strains possess the bvcA or vcrA genes, which encode VC reductive dehalogenases (RDases). Assays to specifically assess bvcA and vcrA gene abundances are commercially available. If bvcA and vcrA can be quantified, Dhc strains with the potential to dechlorinate VC to ethene are present. Dhc can only grow at the expense of reductive dechlorination reactions. Therefore, if Dhc biomarker genes (i.e., specific RDase genes and the Dhc 16S rRNA gene) are detected in samples collected from a chlorinated ethene plume, it is highly probable that these Dhc strains grew with chlorinated ethenes as electron acceptors. Without growth, Dhc biomarkers are unlikely to exceed 10E+03 gene copies per liter of groundwater, and therefore would not be quantified with qPCR.

Note that not all Dhc strains carry VC RDase genes and therefore not all Dhc strains contribute to VC reductive dechlorination to ethene. The vcrA and/or bvcA genes are typically found at sites where ethene is formed; however, not all VC RDases have been identified and it is possible that at some sites ethene formation occurs even in the absence of vcrA and bvcA. Quantitative real-time polymerase reactions (qPCR) targeting Dhc and bacterial 16S rRNA genes should accompany the VC RDase gene analysis. This information is useful to calculate the ratio of Dhc to total bacterial 16S rRNA gene copies and the ratio of VC RDase genes to Dhc cells, which inform about the potential for ethene formation. In general, qPCR assays can detect and enumerate Dhc biomarker genes when at least 100 to 1,000 Dhc cells, respectively, are present per liter of groundwater.

3. Is the EPA 2nd line of evidence required?

Decision Criteria

The final decision to require, or to not require, the second line of evidence is made by the appropriate regulatory authority.

The USEPA may require two lines of evidence before approval of Monitored Natural Attenuation (MNA) as a site remedy will be granted. The first direct line of evidence requires data that demonstrate a clear and meaningful trend of decreasing contaminant mass and/or concentration over time at appropriate monitoring or sampling points. The second line of evidence originally included “hydrogeologic and geochemical data that can be used to indirectly demonstrate the type(s) of natural attenuation processes active at the site, and the rates at which such processes will reduce contaminant concentrations to required levels”.

HELP

Lines of evidence for MNA are described in Use of Monitored Natural Attenuation at Superfund, RCRA Corrective Action, and Underground Storage Tank Sites (EPA. 1999)http://www2.epa.gov/sites/production/files/2014-02/documents/d9200.4-17.pdf. The intent of the second line of evidence was to corroborate that degradation is occurring. Since the 1999 release of the EPA document, several additional methodologies have been developed. These include compound-specific isotope analysis (CSIA) and various molecular biological tools such as qPCR targeting biomarker genes of dechlorinating bacteria. In addition, our understanding of degradation mechanisms affecting chlorinated ethenes has increased, and previously unknown degradation mechanisms, particularly abiotic degradation mechanisms such as degradation using magnetite or FeS, have been identified.

The first line of evidence is always required. A regulator will require the second line of evidence based on the regulator's level of understanding of the processes that control the distribution and fate of the contaminants. If the critical processes for natural attenuation are already well understood and the processes are ubiquitous at sites, and there is extensive experience from other sites that documents that the processes are reliable, then a regulator may not require the second line of evidence.

If the processes are not ubiquitous, or the critical process(es) operate effectively at some sites but not at others, a regulator will often require the second line of evidence. The focus on this decision support system is to evaluate natural attenuation processes and provide a creditable second line of evidence.

There is a third line of evidence, which can be provided by field or microcosm studies, that directly demonstrate the occurrence of a particular natural attenuation process at the site and its ability to degrade the contaminant(s) of concern. Regulators rarely require the third line of evidence, which is usually reserved for compounds that have not been studied and little is known about their fate and transport. This framework or decision support system does not address the third line of evidence.

4. Is VC present?

Decision Criteria

For the purposes of this decision support system, VC is considered present when the concentration of VC exceeds the site-specific VC cleanup goal. If no cleanup goal for VC has been established, VC is considered present when the concentration is equal to or exceeds 2 µg/L. Other criteria may apply depending on the specific site conditions and the regulatory authority.

HELP

The cleanup goal is not always the U.S. EPA MCL established for drinking water. In many cases, the use of risk-based cleanup goals is appropriate. Consult the regulator for the cleanup goals that apply to the site of interest.

5. Is VC degrading?

Decision Criteria

Access the computer simulation that you prepared to evaluate the criterion "Does Natural Attenuation Currently Meet the Goal? Prepare a new simulation where the rate constant for degradation of VC is set to zero. Compare the actual in situ concentrations of VC against the new simulation. Then enter trial values for the rate constant for VC degradation into the simulation to see if the model projections provide a better fit to the in situ concentrations.

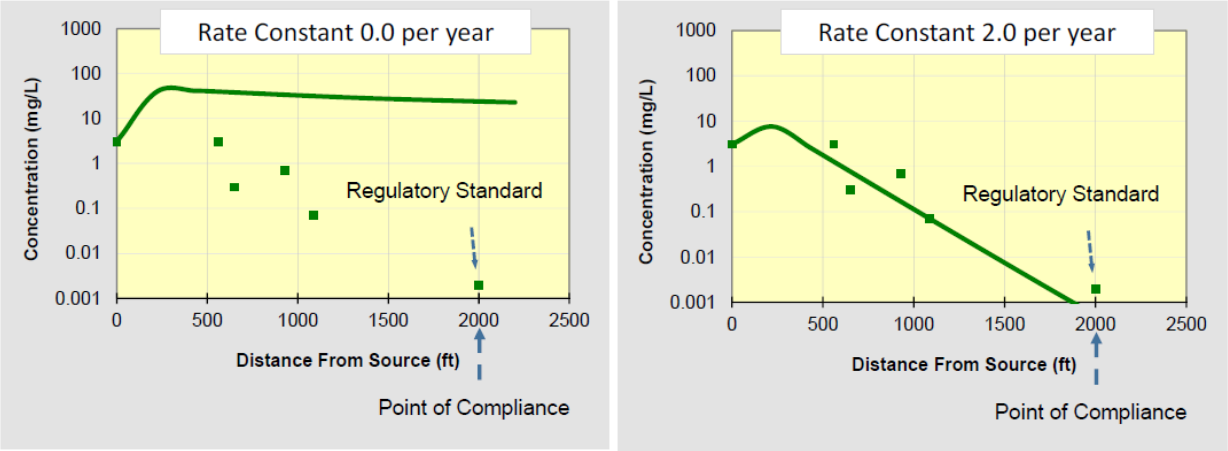
If rate constants greater than zero provide a better fit, then VC degradation is occurring.

Additional information can be provided from an analysis of stable isotopes of carbon in VC. If values of δ13C are available for VC, open the tab Files and select the spreadsheet CSIA.xlsx. Enter your data in the tab Input Data CSIA + Concentration. Then open the tab Kuder Plot VC.

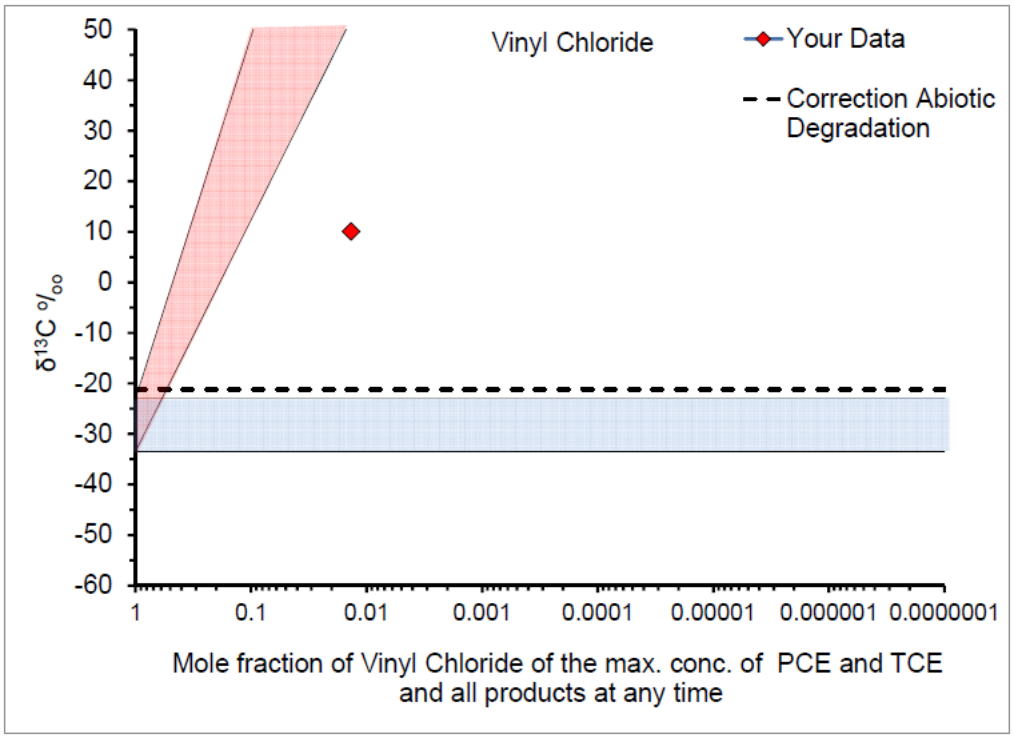
If your data fall above the blue rectangular shape in the chart Kuder Plot for VC, the stable isotopes of carbon in VC have been fractionated, which is evidence that VC degradation has occurred. If your data fall above the blue shape and within the red shape, then microbial reductive dechlorination to ethene can explain the fractionation. If the data falls to the right of red shape, some other process that does not degrade the VC, such as dispersion or dilution, has contributed to the reduction in VC concentrations.

HELP

A computer simulation of the transport and fate of VC can reveal when VC is degrading. The figure below is a hypothetical example where the Point of Compliance (POC) is 2,000 feet from the source of contamination, and the concentrations of VC at the POC are below the MCL for VC. The distance from the source and the acceptable concentration were entered in the input screen of BIOCHLOR so that it would plot in the RUN CENTERLINE output. The value for the rate constant for VC degradation was set at zero to simulate the concentrations that would be expected without VC degradation. The in situ VC concentrations were lower than the simulation with no degradation of VC, indicating that degradation was occurring. Trial values of the rate constant for degradation of VC were selected. The rate constant for degradation of VC that provided the best fit was 2.0 per year.



Data from Compound Specific Isotope Analysis (CSIA) can reveal when DCE is degrading. The figure below is the chart in the spreadsheet CSIA.xlsx under the tab Kuder Plot VC for a hypothetical data set. In this example, the point plotted above the blue box and the dotted line, indicating that degradation had changed the ratio of stable isotopes. The point plotted to the right of the red shape, indicating that processes other than biological reductive dechlorination had contributed to attenuation of the concentrations of VC. These processes can include dispersion in the aquifer or dilution in the monitoring well.



Your data should plot in the chart. If not, you may need to extend the scales of the x and/or y axes.

6. Does Dhc density explain the VC rate constant?

Decision Criteria

Consult the simulation that you prepared to evaluate the criterion "Does Natural Attenuation Currently Meet the Goal?" Identify the rate constant for degradation of VC. Access information about the abundance of Dhc cells in groundwater at the site. Open the tab Files and select the spreadsheet Dhc.xlsx. Input values for the first order rate constant for degradation of VC and the abundance of Dhc biomarker gene copies on the tab Input Dhc data. If you have more than one value for the abundance of Dhc gene copies, input the highest value, not the average. Then open the tab Dhc Explains VC. If your data plot in the blue shape, then the abundance of Dhc in groundwater can explain the in situ rate of VC degradation.

Not every bacterium with the Dhc 16S rRNA gene can degrade VC. A qPCR assay is commercially available for two of the known genes that code for enzymes that reductively dechlorinate VC. The reductase genes have been designated vcrA and bvcA. If there is a concern that the Dehalococcoides strains at your site cannot degrade VC, access information on the abundance of vcrA and bvcA genes in groundwater at the site.

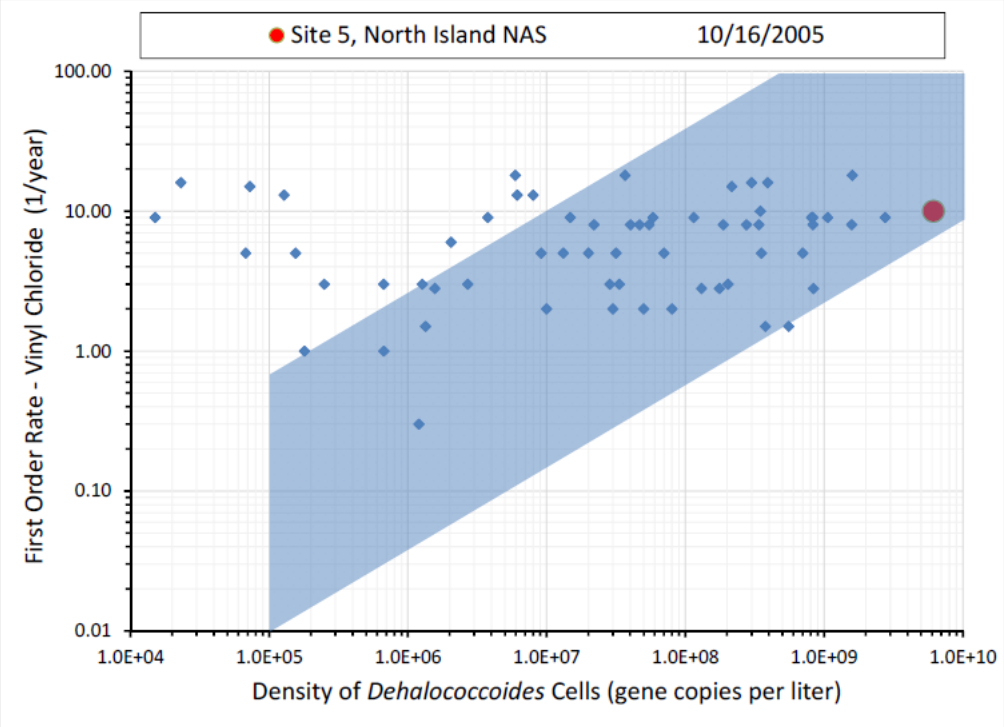
Open the tab Files and select the spreadsheet Reductase Genes.xlsx. Enter your data in the tab Input Data. Then open the tab VC Rase and Dhc. If your data plot in the blue shape, transformation of VC is plausible based on the abundance of the VC reductase genes in the groundwater.

HELP

The figure below is the chart in tab Dhc Explains VC for an example data set. In this example the density of Dehalococcoides gene copies does explain the rate.

Note that the chart has data points that are outside of the blue shape and have first order rate constants that are larger than can be plausibly explained by the Dhc cell abundance in the groundwater. Possible explanations for the observed rates of VC degradation include:

1. The groundwater Dhc analysis underestimates the actual Dhc abundance in the aquifer due to Dhc cell attachment to the aquifer solids.
2. To date, the VC-to-ethene reductive dechlorination step has been exclusively associated with Dhc strains carrying the VC RDase genes vcrA or bvcA; however, it is conceivable that not-yet-recognized bacteria may contribute to VC-to-ethene reductive dechlorination.
3. Microbial VC oxidation can occur at very low dissolved oxygen concentrations, and areas of the aquifer may have sufficient oxygen to sustain aerobic VC (and ethene) degradation.
4. Abiotic VC degradation mediated by reactive iron-bearing mineral phases (e.g., iron sulfides, magnetite) contributes to VC degradation.



7. Does Dhc density explain the VC rate constant?

Decision Criteria

Consult the simulation that you prepared to evaluate the criterion "Does Natural Attenuation Currently Meet the Goal?" Prepare a new simulation. Do not include any portion of the plume where biological reductive dechlorination might contribute to the bulk rate constant that is extracted by the model. Exclude any portion of the flow path where the concentrations of any daughter products are increasing with distance from the source. By trial and error, identify the rate constant for degradation of VC that provides the best match between the new simulation and the field data.

Open the tab Files and select the spreadsheet Magnetic Susceptibilty.xlsx. Enter your values for the rate constant for degradation of VC and for mass magnetic susceptibility in the tab Input Data. Then open the tab Mag. Sus. Explains VC.

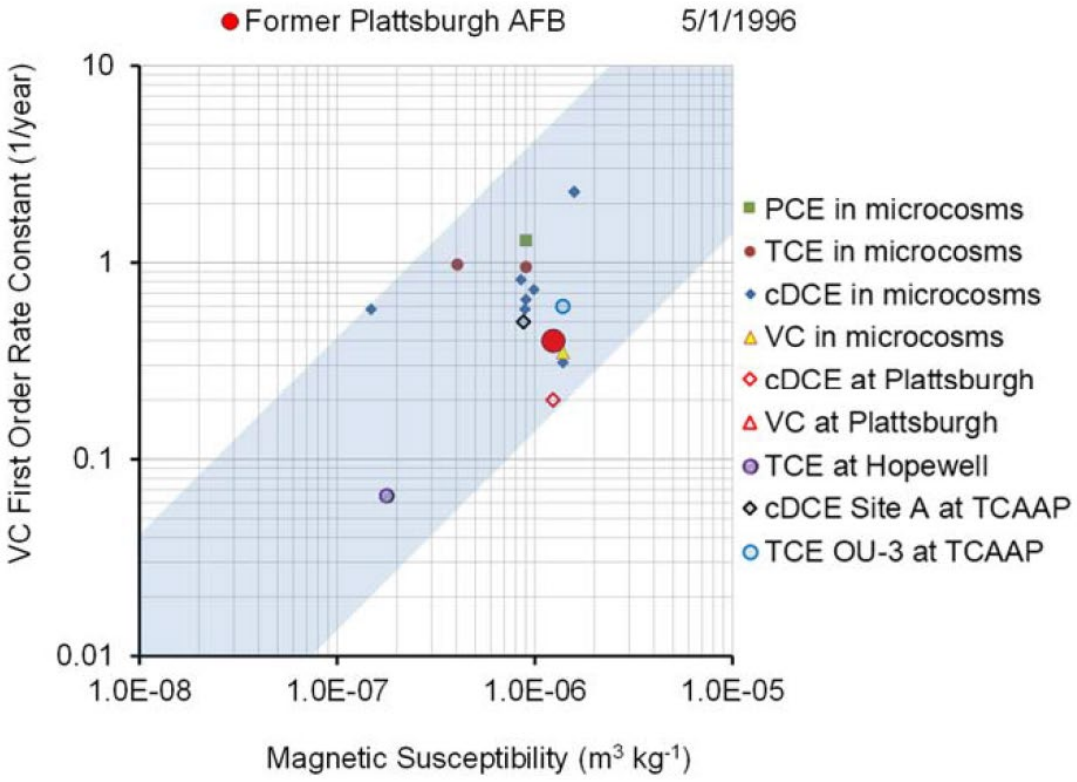
If the site-specific values fall within the blue shape, then mass magnetic susceptibility can explain the apparent in situ rate of VC degradation.

HELP

Magnetite can mediate abiotic degradation of VC. The amount of magnetite in aquifer material can be estimated from the mass magnetic susceptibility of core samples. Empirical data are available that associate degradation rate constants for VC with mass magnetic susceptibility. The available data were used to define the blue shape in the figure. The figure is the chart in tab Mag. Sus. Explains VC for an example data set. If the rate constant plots within the blue shape, then abiotic degradation mediated by magnetite can explain the observed rate constant.

If the rate constant plots above the blue shape, other processes are likely contributing to the rate of VC degradation.

If the rate constant plots below the shape, inappropriate sampling locations may have been selected for mass magnetic susceptibility measurements. Mass magnetic susceptibility should be determined with aquifer material that is most transmissive to water since this is where most solute transport will occur. In addition, the input values used for the rate constant calculation with BIOCHLOR should be verified.



8. Adequate oxygen for aerobic VC biodegradation?

Decision Criteria

Bacteria that degrade VC if oxygen is available are generally present in aquifers. These bacteria require very low dissolved oxygen concentrations to metabolize VC. Because of field sampling limitations, dissolved oxygen concentration data on well water are generally unreliable to determine if sufficient oxygen is available to support oxygen-dependent VC oxidation.

For the purposes of this decision support system, oxygen is considered to be available for aerobic biodegradation of VC when all of the following criteria are met: Dissolved oxygen concentrations measured in the field exceed 0.1 mg/L, ferrous iron (Fe2+) concentrations are below 0.5 mg/L, and methane concentrations are below 0.005 mg/L.

HELP

Bacteria that degrade VC if oxygen is available are generally present in aquifers. These bacteria require very low dissolved oxygen concentrations to metabolize VC. Because of field sampling limitations, dissolved oxygen concentration data on well water are generally unreliable to determine if sufficient oxygen is available to support oxygen-dependent VC oxidation.

It is easy to contaminate a groundwater sample with oxygen because, among other things, the sampling of monitoring wells frequently causes mixing of water from different depth intervals. It is possible the VC in a sample of well water came from one depth interval and the oxygen from another. If this is the case, oxygen may not be available to the VC-degrading bacteria in the aquifer, leading to the erroneous conclusion that VC can be degraded aerobically.

The absence of ferrous iron (Fe2+) and methane are good indicators for the presence of oxygen that supports aerobic biodegradation of organic compounds. The absence of ferrous iron and methane in water collected from a well generally indicates that all of the flowpaths to the well had adequate concentrations of oxygen to support aerobic VC degradation.

Note that aerobic VC oxidizers are able to degrade VC at very low oxygen concentrations. Therefore, aerobic VC oxidation may contribute to VC attenuation in aquifers characterized as "anoxic" (i.e., the answer to the decision criterion is "No"). While aerobic VC degraders will likely contribute to VC degradation in the presence of oxygen, establishing quantitative relationships is difficult. As a result, the presence of oxygen is only a qualitative line of evidence for aerobic biodegradation of VC.

9. Is DCE present?

Decision Criteria

For the purposes of this decision support system, DCE is present when the concentrations of cDCE, tDCE, and/or 1,1-DCE exceed the cleanup goal that has been established for the site. If no cleanup goal for DCE has been established, DCE is considered present when the concentration equals or exceeds 7 µg/L. Other criteria may apply depending on the regulatory authority.

HELP

The cleanup goal is not always the U.S. EPA MCL established for drinking water. Consult the regulator and verify the cleanup goals that apply to the site.

10. Is DCE degrading?

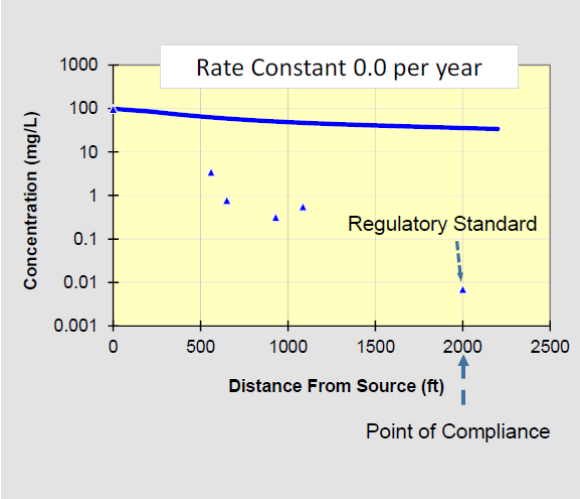
Decision Criteria

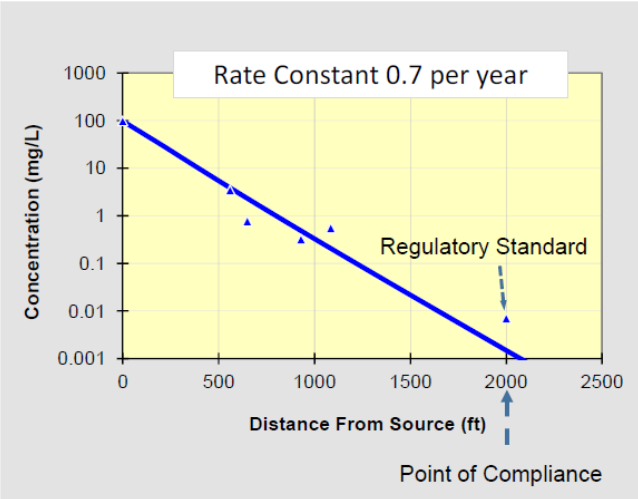
Prepare a new simulation where the rate constant for degradation of DCE is set to zero. Compare the actual in situ concentrations of the sum of cDCE + tDCE + 1,1-DCE against the new simulation. Then enter trial values for the rate constant for DCE degradation into the simulation to see if the model projections provide a better fit to the in situ concentrations.

If rate constants greater than zero provide a better fit, then DCE is degrading.

Additional information can be provided from an analysis of stable isotopes of carbon in DCE. If values for δ13C are available for DCE, open the tab Files and select the spreadsheet CSIA.xlsx. Enter your data in the tab Input Data CSIA + Concentration. Then open the tab Kuder Plot cDCE and examine the chart.

If your data fall above the blue shape, the stable isotopes of carbon in DCE have been fractionated and that is evidence that DCE is degrading. If your data fall above the blue shape and within the red shape, then microbial reductive dechlorination to DCE can explain the fractionation. If the data fall to the right of red shape, some other process that does not degrade the DCE, such as dispersion or dilution, has contributed to the reduction in contaminant concentrations.

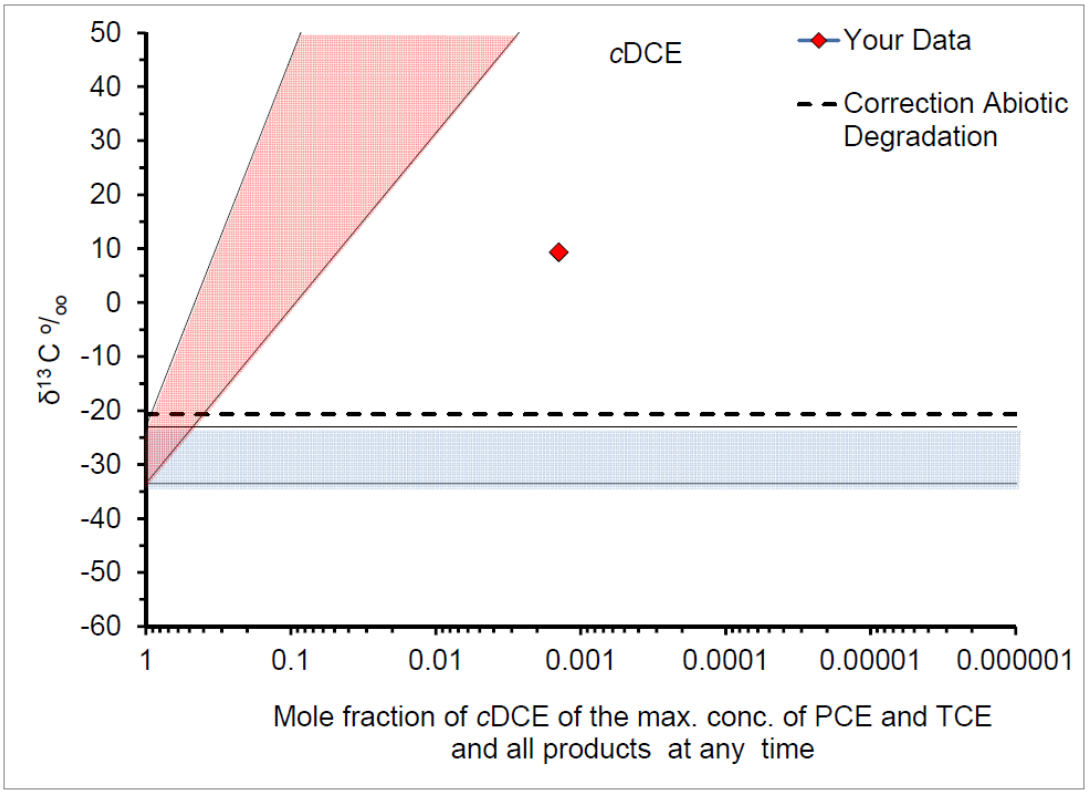




A computer simulation of the transport and fate of DCE can reveal when DCE is degrading. The figure below is a hypothetical example, where the POC is 2,000 feet from the source of contamination, and the acceptable concentration of DCE at the POC was the MCL for 1,1-DCE. The BIOCHLOR model does not discriminate between DCE isomers. The value entered in the model is the sum of the cDCE, tDCE and 1,1-DCE isomers for the total DCE concentration. Regardless of this, in this case the acceptable concentration for DCE was set at the MCL for 1,1-DCE because this isomer has the lowest MCL. The distance from the source and the acceptable concentration was entered in the input screen of BIOCHLOR so that it would plot in the RUN CENTERLINE output. The value for the rate constant for DCE degradation was set at zero to simulate the concentrations that would be expected if there were no degradation of DCE. The concentrations of DCE in the field were lower than the simulation with no degradation of DCE. Trial values of the rate constant for degradation of DCE were selected. The rate constant for degradation of DCE that provided the best fit was 0.7 per year.

Data from Compound Specific Isotope Analysis (CSIA) can reveal when DCE is degrading. The figure below is the chart in the spreadsheet CSIA.xlsx under the tab Kuder Plot DCE for a hypothetical data set. In this example, the point plotted above the blue box and the dotted line, indicating that degradation had changed the ratio of stable isotopes. The point plotted to the right of the red shape, indicating that processes other than biological reductive dechlorination had contributed to attenuation of the concentrations of VC. These processes can include dispersion in the aquifer or dilution in the monitoring well.

Your data should plot in the chart. If not, you may need to extend the scales of the x and/or y axes.



11. Does Dhc density explain the DCE rate constant?

Decision Criteria

Consult the simulation that you prepared to evaluate the criterion "Does Natural Attenuation Currently Meet the Goal?" Identify the rate constant for degradation of DCE. Access information about the abundance of Dhc cells in site groundwater. Open the tab Files and select the spreadsheet Dhc.xlsx. Input values for the first order rate constant for degradation of DCE and the abundance of Dhc biomarker gene copies on the tab Input Dhc data. If you have more than one value for the abundance of Dhc gene copies, input the highest value, not the average. Then open the tab Dhc Explains cDCE. If your data plot in the blue shape, then the abundance of Dhc in groundwater can explain the in situ rate of DCE degradation.

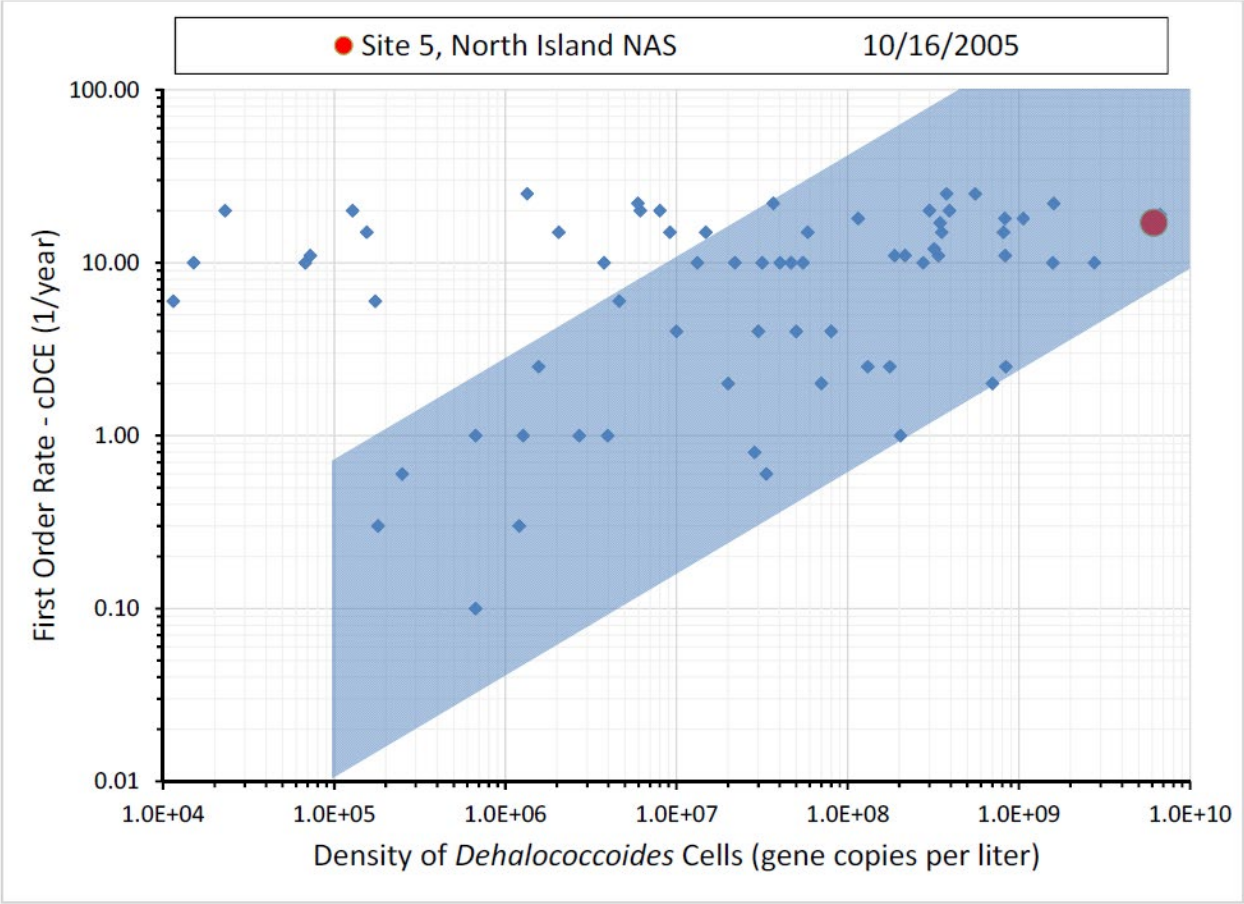
Not every bacterium with the Dhc 16S rRNA gene can degrade DCE. A qPCR assay is commercially available for two of the known genes that code for enzymes that reductively dechlorinate DCE. The reductase genes have been designated vcrA and bvcA. If there is a concern that the Dehalococcoides strains at your site cannot degrade cDCE, access information on the abundance of vcrA and bvcA genes in groundwater at the site.

Open the tab Files and select the spreadsheet Reductase Genes. Input values for the abundance of vcrA, bvcA and Dhc gene copies into the tab Input Data. Then open the tab VC Rase and Dhc. If your data plot in the blue shape, transformation of cDCE to ethene is plausible based on the abundance of the reductase genes in the groundwater.

HELP

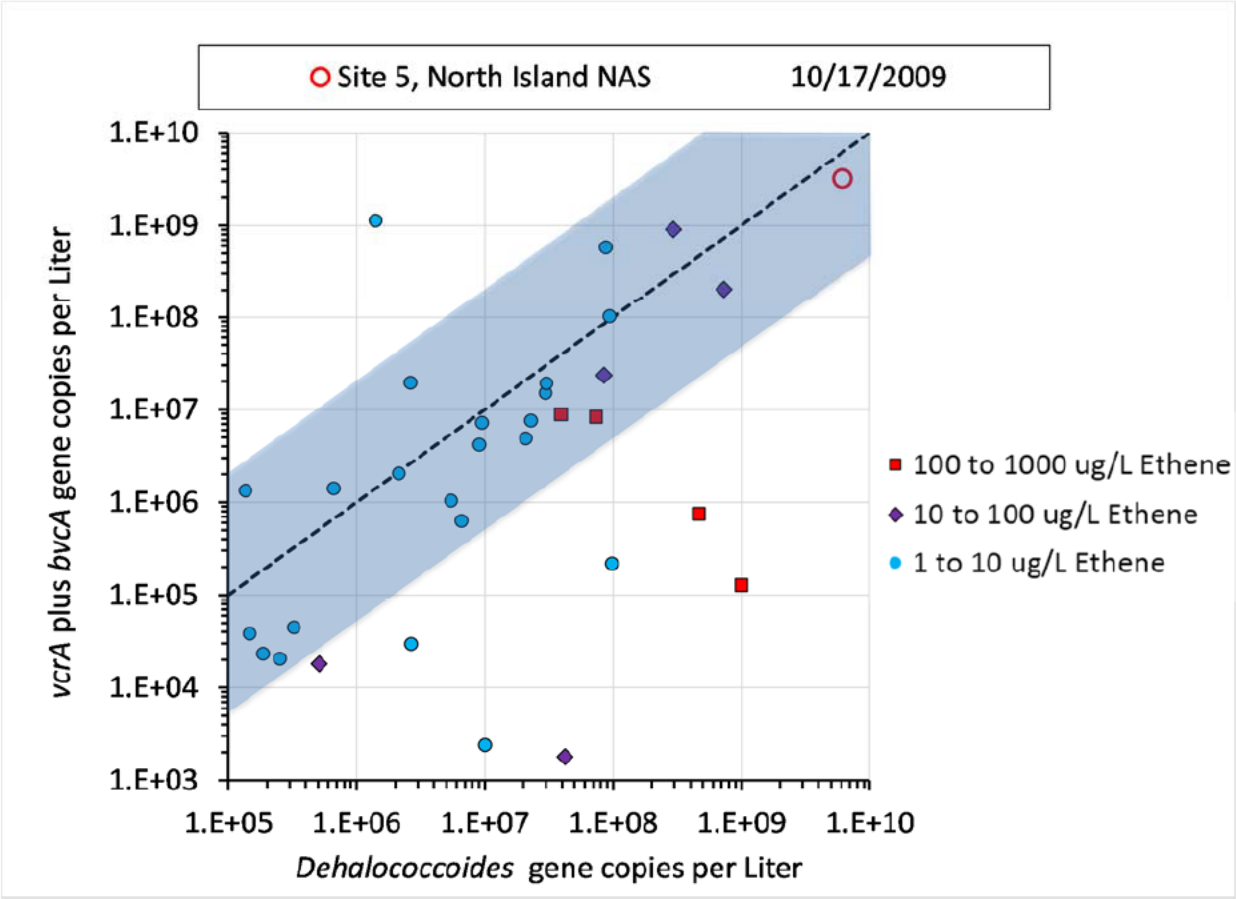
Note that the chart has data points that are outside of the blue shape and have first order rate constants that are larger than can be plausibly explained by the Dhc cell abundance in the groundwater. Possible explanations for the observed rates of cDCE degradation include:

1. The groundwater Dhc analysis underestimates the actual Dhc abundance in the aquifer due to Dhc cell attachment to the aquifer solids.
2. To date, the DCE-to-VC-to-ethene reductive dechlorination step has been exclusively associated with Dhc strains carrying the Reductase genes vcrA or bvcA; however, it is conceivable that not-yet-recognized bacteria may contribute to DCE- to-VC-to-ethene reductive dechlorination.
3. Microbial DCE oxidation can occur at very low dissolved oxygen concentrations, and areas of the aquifer may have sufficient oxygen to sustain aerobic DCE degradation.
4. Abiotic DCE degradation mediated by reactive iron-bearing mineral phases (e.g., iron sulfides, magnetite) contributes to DCE degradation.



Dhc strains have been described that contribute to reductive dechlorination of polychlorinated ethenes but cannot efficiently dechlorinate DCE. If such strains dominate the Dhc population, a high Dhc cell abundance may not correlate with DCE-to-VC-to-ethene reductive dechlorination activity. Two Dhc RDase

genes involved in DCE-to-VC-to-ethene reductive dechlorination have been identified, vcrA and bvcA, and commercial qPCR assays targeting these genes are available. The combined application of Dhc 16S rRNA gene- and RDase gene-targeted qPCR can provide additional valuable information about VC degradation at the site. The figure below is the chart in tab RDase and Dhc for an example data set. If the data plot near the dotted line, the abundance of genes for the reductase enzymes is near the abundance of Dhc cells. In this example, the data plot in the blue shape, and transformation of DCE to ethane is plausible based on the abundance of vcrA and bvcA in the groundwater.



12. Does magnetic susceptibility explain the DCE rate constant?

Decision Criteria

Consult the simulation that you prepared to evaluate the criterion "Does Natural Attenuation Currently Meet the Goal?" Prepare a new simulation. Do not include any portion of the plume where biological reductive dechlorination might contribute to the bulk rate constant that is extracted by the model. Exclude any portion of the flow path where the concentrations of any daughter products are increasing with distance from the source. By trial and error, identify the rate constant for degradation of DCE that provides the best match between the new simulation and the field data.

Open the tab Files and select the spreadsheet Magnetic Susceptibilty.xlsx. Enter your values for the rate constant for degradation of DCE and for mass magnetic susceptibility in the tab Input Data. Then open the tab Mag. Sus. Explains cDCE.

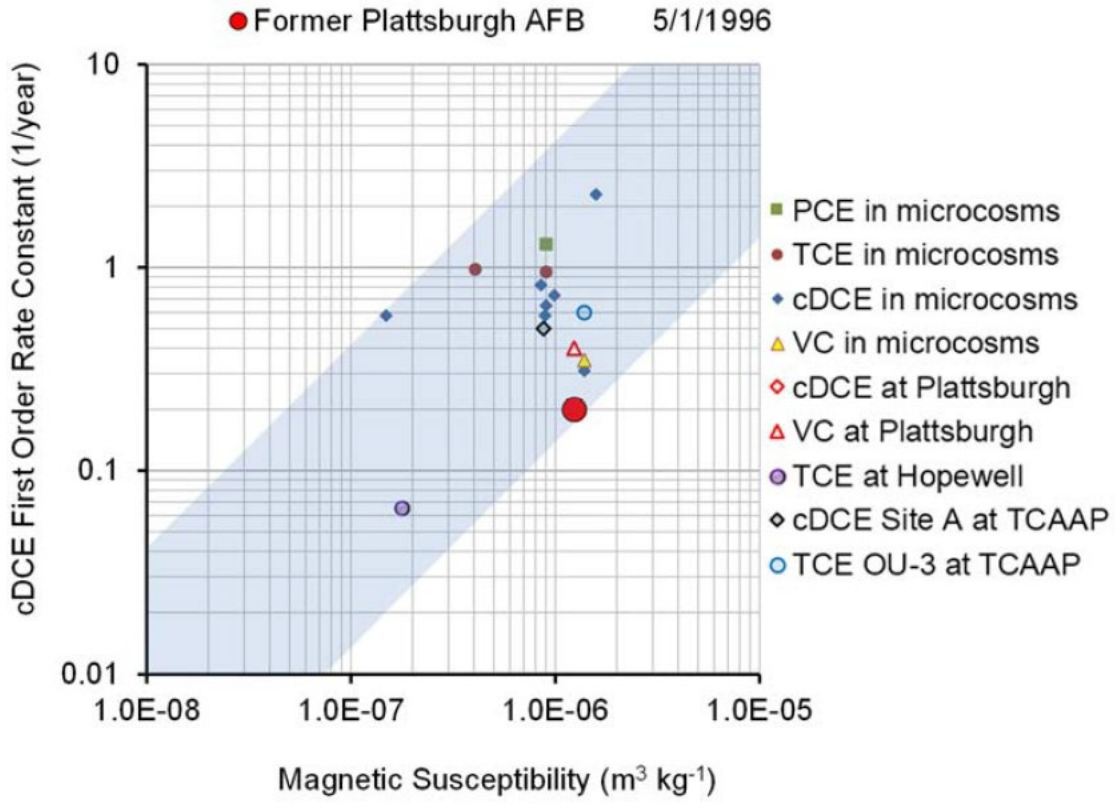
If the site-specific values fall within the blue shape, then mass magnetic susceptibility can explain the apparent in situ rate of DCE degradation.

HELP

Magnetite can mediate abiotic degradation of cDCE. The amount of magnetite in aquifer material can be estimated from the mass magnetic susceptibility of core samples. Empirical data are available that associate degradation rate constants for cDCE with mass magnetic susceptibility. The available data were used to define the blue shape in the figure. The figure is the chart in tab Mag. Sus. Explains DCE for an example data set. If the rate constant plots within the blue shape, then abiotic degradation mediated by magnetite can explain the observed rate constant.

If the rate constant plots above the blue shape, other processes are likely contributing to the rate of VC degradation.

If the rate constant plots below the shape, inappropriate sampling locations may have been selected for mass magnetic susceptibility measurements. Mass magnetic susceptibility should be determined with aquifer material that is most transmissive to water since this is where most solute transport will occur. In addition, the input values used for the rate constant calculation with BIOCHLOR should be verified.



13. Adequate oxygen for aerobic DCE degradation?

Decision Criteria

Bacteria that degrade DCE with oxygen are generally present in aquifers, even when the groundwater has been characterized as anoxic. Because of field sampling limitations, dissolved oxygen concentration data on well water are generally unreliable to determine if sufficient oxygen is available to support oxygen-dependent DCE degradation.

For the purposes of this decision support system, oxygen is considered to be available for aerobic biodegradation of DCE when all of the following criteria are met: Dissolved oxygen concentrations measured in the field exceed 0.1 mg/L, ferrous iron (Fe2+) concentrations are less than 0.5 mg/L, and methane concentrations are less than 0.005 mg/L.

HELP

It is easy to contaminate a groundwater sample with oxygen. Sampling monitoring wells often causes mixing of water from different depth intervals. It is possible the DCE in a sample of well water came from one depth interval and the oxygen from another. If this is the case, oxygen may not be available to the DCE-degrading bacteria in the aquifer, leading to the erroneous conclusion that DCE is degraded aerobically. The absence of ferrous iron (Fe2+) and methane are good indicators for the presence of concentrations of oxygen that support aerobic biodegradation of organic compounds. The absence of ferrous iron or methane in water collected from a well indicates that all of the flow paths to the well had adequate concentrations of oxygen to support aerobic DCE degradation.

14. Is TCE present?

Decision Criteria

For the purposes of this decision support system, TCE is present in groundwater when the concentration of TCE exceeds a cleanup goal for TCE that has been established for the site. If no cleanup goal for TCE has been established, TCE is considered present when the concentration is ≥ 5 µg/L. Other criteria may apply depending on the regulatory authority.

HELP

The cleanup goal is not always the U.S. EPA MCL established for drinking water. Consult the regulator for the cleanup goals that apply to the site of interest.

15. Is TCE degrading?

Decision Criteria

Access the computer simulation that you prepared to evaluate the criterion “Does Natural Attenuation Currently Meet the Goal?” Prepare a new simulation where the rate constant for degradation of TCE is set to zero. Compare the actual in situ concentrations of TCE against the new simulation. Then enter trial

values for the rate constant for TCE degradation into the simulation to see if the model projections provide a better fit to the in situ concentrations.

If rate constants greater than zero provide a better fit, then TCE is degrading.

As an alternative, CSIA can be used to determine if TCE is degrading.

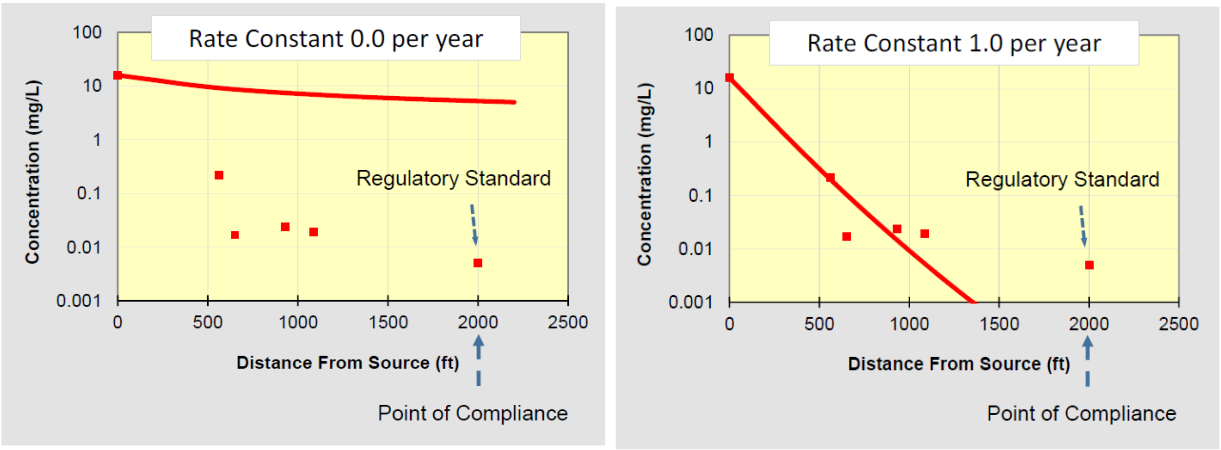
If the value of δ13C of TCE in a down gradient well is larger (less negative) than the value in an up gradient well by more than 0.5‰, that can be taken as evidence for degradation of TCE.

The highest value that has been reported for the δ13C of TCE used in commerce is -23.2‰.

As a general rule, a value of δ13C for TCE that is greater than -22.7‰ can be taken as evidence of degradation of TCE.

HELP

A computer simulation of the transport and fate of TCE can reveal when TCE is degrading. The figure below is a hypothetical example, where the POC is 2,000 feet from the source of contamination, and the acceptable concentration of TCE at the POC was the MCL for TCE. The distance from the source and the acceptable concentration was entered in the input screen of BIOCHLOR so that it would plot in the RUN CENTERLINE output. The value for the rate constant for TCE degradation was set at zero to simulate the concentrations that would be expected if there were no degradation of TCE. The concentrations of TCE in the field were lower than the simulation with no degradation of TCE. Trial values of the rate constant for degradation of DCE were selected. The rate constant for degradation of TCE that provided the best fit was 1.0 per year.



As an alternative, CSIA can be used to determine if TCE is degrading. Microbial degradation of TCE would make the value of δ13C a larger (less negative) number. The precision of the analysis is near 0.5‰. If the value of δ13C of TCE in a down gradient well is larger (less negative) than the value in an up gradient well by more than 0.5‰, that can be taken as evidence for degradation of TCE.

The increase in δ13C in TCE in areas close to a NAPL source area containing TCE may be difficult to discern and may not become apparent until the NAPL source becomes significantly depleted. In addition, the continued formation of TCE from PCE will reduce the value of δ13C for TCE in the pool of TCE until the PCE is consumed, either over time or along the flow path.

16. Are DCE or VC present?

Decision Criteria

Evaluate data on concentrations of TCE, cDCE, tDCE and VC in groundwater. If the sum of cDCE, tDCE and VC is more than 5 mole % of the concentration of TCE, then cDCE, tDCE and VC are present. The presence of cDCE or tDCE or VC indicates that reductive dechlorination of TCE has occurred.

The calculation of mole % can be easily performed using the Excel file Mole Percent Calculator.xlsx, which is included in the BioPIC program, and also found in Appendix D.

HELP

The detection of cDCE or tDCE or VC at TCE-impacted sites suggests that TCE reductive dechlorination has occurred and this process may still be ongoing. Enumeration of pceA genes (present in PCE- and/or TCE-dechlorinating bacteria and implicated in PCE-to-TCE and PCE-to-cDCE reductive dechlorination) and the tceA gene (present in some Dhc strains and implicated in TCE-to-VC reductive dechlorination) with qPCR provides support that bacteria capable of TCE reductive dechlorination to cDCE or VC are present.

17. Are DCE or VC present in relevant concentrations?

Decision Criteria

Evaluate data on concentrations of TCE, cDCE, tDCE and VC in wells downgradient of the source of contamination. If the sum of cDCE, tDCE and VC is more than 25 mole % of the concentration of TCE, then cDCE, tDCE and VC are present in relevant concentrations. The presence of daughter products at these concentrations indicates that microbial reductive dechlorination is an important pathway for TCE fate, and explains in a qualitative manner why TCE is degrading.

The calculation of mole % can easily be performed using the Excel file included with the BioPIC program titled Mole Percent Calculator.xlsx, and also found in Appendix D.

HELP

The detection of cDCE, tDCE or VC at TCE-impacted sites suggests that TCE reductive dechlorination has occurred and this process may still be ongoing. Enumeration of pceA genes with qPCR provides support that bacteria capable of TCE reductive dechlorination to cDCE are present. The pceA gene is present in TCE-dechlorinating bacteria and implicated in TCE-to-cDCE reductive dechlorination. The presence of the Dhc RDase genes tceA, bvcA or vcrA implicated in reductive dechlorination of DCE can explain the formation of VC and ethene.

18. Are DCE or VC present in relevant concentrations?

Decision Criteria

Consult the simulation that you prepared to evaluate the criterion "Does Natural Attenuation Currently Meet the Goal?" Prepare a new simulation. Do not include any portion of the plume where biological reductive dechlorination might contribute to the bulk rate constant that is extracted by the model. Exclude any portion of the flow path where the concentrations of any daughter products are increasing with distance from the source. By trial and error, identify the rate constant for degradation of TCE that provides the best match between the new simulation and the field data.

Open the tab Files and select the spreadsheet Magnetic Susceptibilty.xlsx. Enter your values for the rate constant for degradation of TCE and for mass magnetic susceptibility in the tab Input Data. Then open the tab Mag. Sus. Explains TCE.

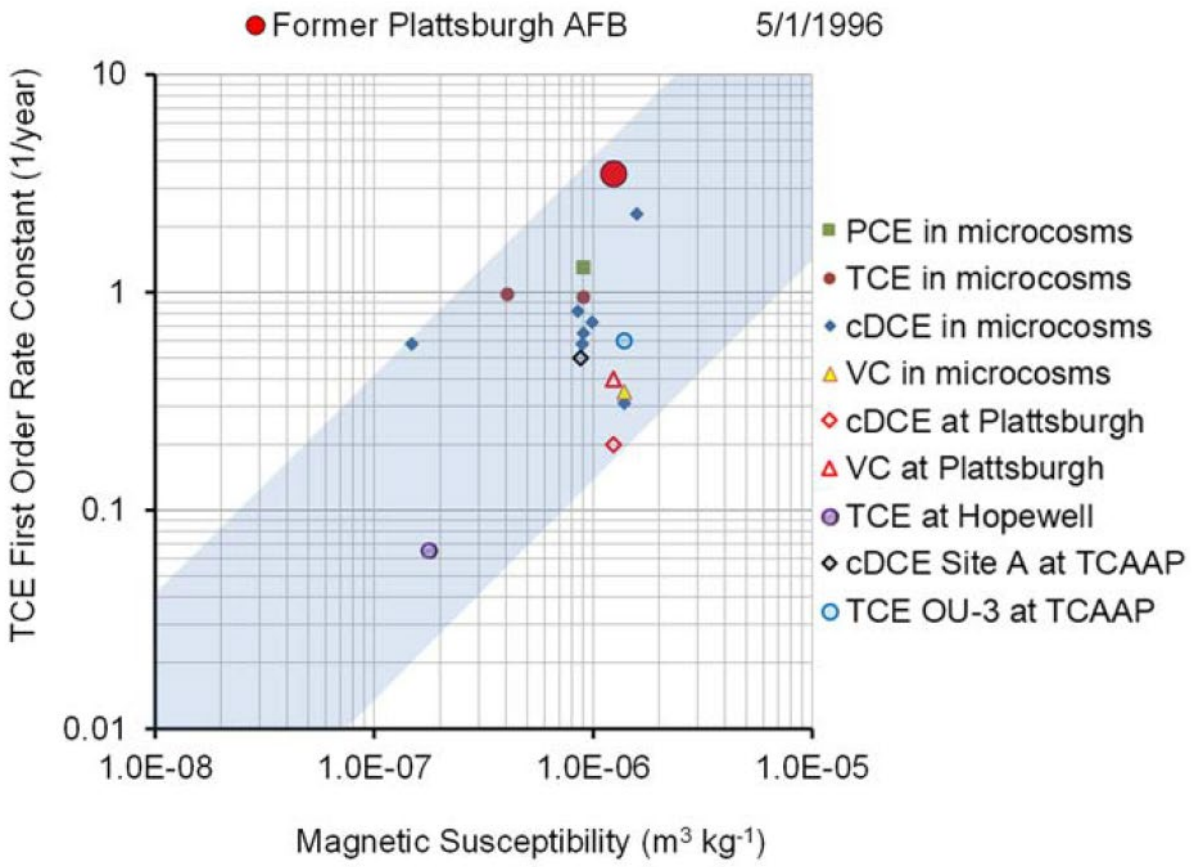
If the site-specific values fall within the blue shape, then mass magnetic susceptibility can explain the apparent in situ rate of TCE degradation.

HELP

Magnetite can mediate abiotic degradation of TCE. The amount of magnetite in aquifer material can be estimated from the mass magnetic susceptibility of core samples. Empirical data are available that associate degradation rate constants for TCE with mass magnetic susceptibility. The available data were used to define the blue shape in the figure. The figure is the chart in tab Mag. Sus. Explains TCE for an example data set. If the rate constant plots within the blue shape, then abiotic degradation mediated by magnetite can explain the observed rate constant.

If the rate constant plots above the blue shape, other processes are likely contributing to the rate of TCE degradation.

If the rate constant plots below the shape, inappropriate sampling locations may have been selected for mass magnetic susceptibility measurements. Mass magnetic susceptibility should be determined with aquifer material that is most transmissive to water since this is where most solute transport will occur. In addition, the input values used for the rate constant calculation with BIOCHLOR should be verified.



19. Does iron sulfide explain the TCE rate constant?

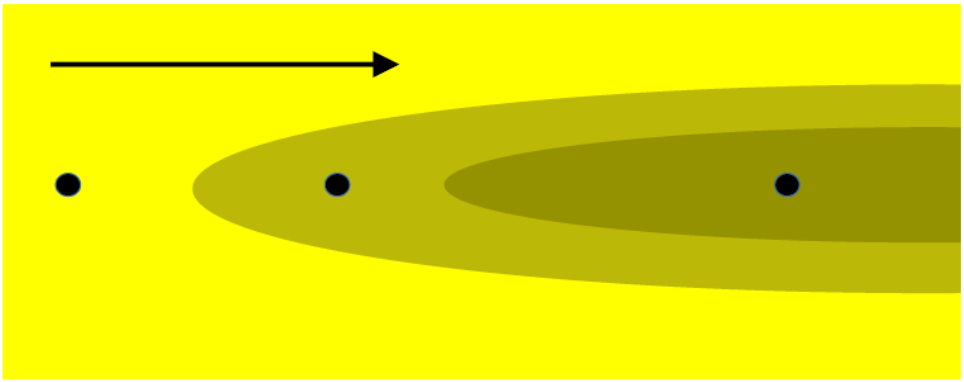
Decision Criteria

Open the tab Files and select the spreadsheet FeS.xlsx. If the distribution of sulfate shows a decrease in sulfate concentration along the flowpath, open the tab Sulfate Sag Along Flow Path and enter values for aquifer properties and for concentrations of sulfate.

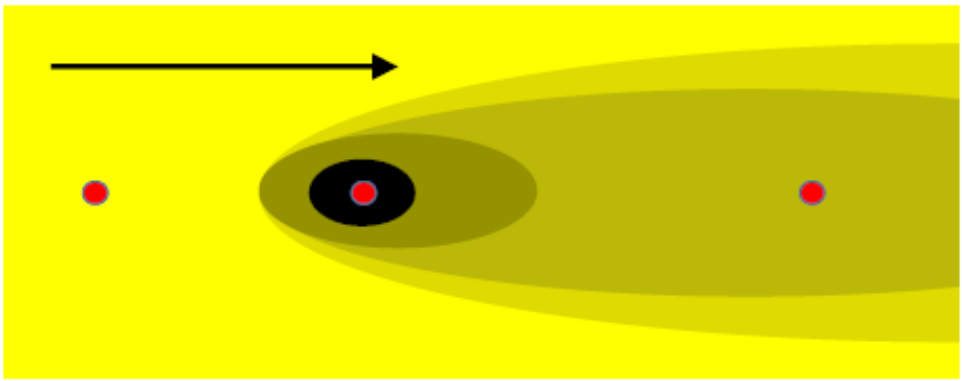
If the value of the rate constant in cell D28 or D29 (whichever is applicable) of the spreadsheet in the tab Sulfate Sag Along Flow Path is equal to or greater than the rate constant estimated using BIOCHLOR, then abiotic degradation by reactive iron sulfide minerals can explain the degradation rate constant for TCE.

If the lowest concentrations of sulfate are at the source of contamination, open the tab Lowest Sulfate at Source and enter values for aquifer properties and for concentrations of sulfate.

If the value of the rate constant in cell D31 or D32 of the tab Lowest Sulfate at Source (whichever is applicable) is equal to or greater than the rate constant from the BIOCHLOR simulation, then abiotic degradation by reactive iron sulfide minerals can explain the degradation rate constant for TCE.



Distribution of sulfate at a site where there is a decrease in sulfate concentration along the flowpath.



Distribution of sulfate at a site where the extent of sulfate depletion is greatest at the source.

HELP

Reactive iron sulfide minerals can mediate TCE degradation. Reactive iron sulfide minerals are formed during sulfate reduction and will form over time as sulfate reduction progresses and ferrous iron is dissolved in the groundwater. However, the reactive iron sulfide minerals are inactivated over time at a rate that is proportional to the amount of reactive minerals that have already accumulated. The pool of reactive iron sulfide will increase until the rate of production from sulfate reduction is balanced by the rate of inactivation. The rate of TCE degradation mediated by reactive iron sulfide minerals is related to the steady-state pool of reactive iron sulfide.

The spreadsheets use data on the effective porosity, hydraulic gradient and hydraulic conductivity to estimate a seepage velocity of groundwater along a flow path. Then the spreadsheet uses the volumetric sulfate loading to estimate the consumption of sulfate and production of sulfide between an up-gradient well and a down-gradient well along the flow path. The spreadsheet assumes that excess Fe (III) is available in minerals in the aquifer matrix, and that the sulfide produced from the reduction of sulfate reacts to form FeS. The spreadsheet calculates the rate of production of FeS over time.

The spreadsheet models the inactivation of FeS as a first order process on the concentration of FeS present at any time. The user provides the elapsed time since sulfate reduction began at the site, and the

spreadsheet uses the volumetric sulfate loading and the rate of FeS inactivation to calculate the pool of accumulated reactive FeS. Then the spreadsheet uses the rate of degradation of TCE on reactive FeS to estimate a rate constant for TCE degradation along the flowpath between the two wells.

20. Is PCE present?

Decision Criteria

For the purposes of this decision support system, PCE is considered present when the concentration of PCE exceeds a cleanup goal for PCE that has been established for the site. If no cleanup goal for PCE has been established, PCE is considered present when the concentration is ≥ 5 µg/L. Other criteria may apply depending on the regulatory authority.

HELP

The cleanup goal is not always the U.S. EPA MCL established for drinking water. Consult the regulator for the cleanup goals that apply to the site of interest.

21. Is PCE degrading?

Decision Criteria

Access the computer simulation that you prepared to evaluate the criterion "Does Natural Attenuation Currently Meet the Goal?" Prepare a new simulation where the rate constant for degradation of PCE is set to zero. Compare the actual in situ concentrations of PCE against the new simulation. Then enter trial values for the rate constant for PCE degradation into the simulation to see if the model projections provide a better fit to the in situ concentrations.

If rate constants greater than zero provide a better fit, then TCE is degrading.

As an alternative, CSIA can be used to determine if PCE is degrading.

If the value of δ13C of TCE in a down gradient well is larger (less negative) than the value in an up gradient well by more than 0.5‰, that can be taken as evidence for degradation of PCE.

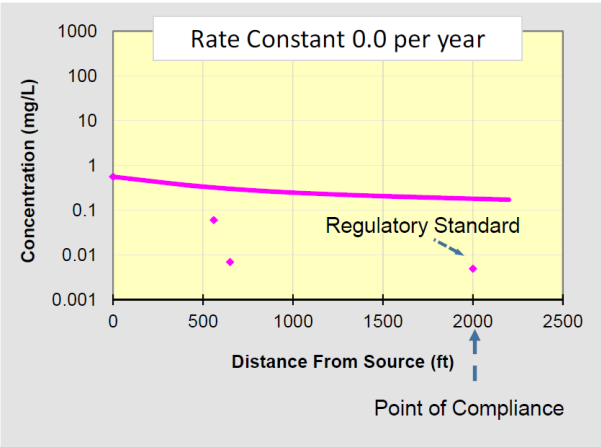
The highest value that has been reported for the δ13C of PCE used in commerce is -23.2‰.

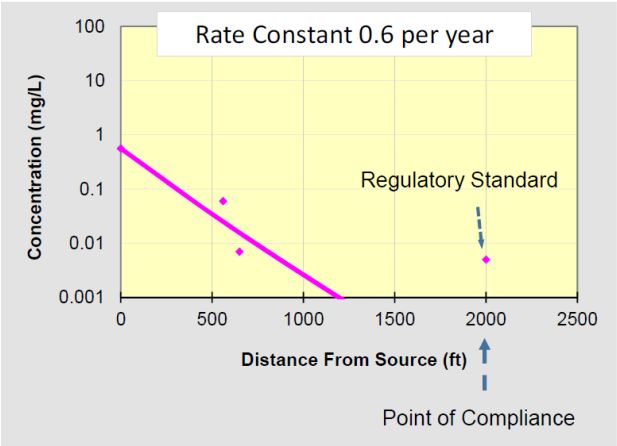
As a general rule, a value of δ13C for PCE that is greater than -22.7‰ can be taken as evidence of degradation of TCE.

HELP

A computer simulation of the transport and fate of PCE can reveal when PCE is degrading. The figure below is a hypothetical example, where the POC is 2,000 feet from the source of contamination, and the acceptable concentration of PCE at the POC was the MCL for PCE. The distance from the source and the acceptable concentration was entered in the input screen of BIOCHLOR so that it would plot in the RUN CENTERLINE output. The value for the rate constant for PCE degradation was set at zero to simulate the concentrations that would be expected if there were no degradation of PCE. The concentrations of PCE

in the field were lower than the simulation with no degradation of PCE. Trial values of the rate constant for degradation of DCE were selected. The rate constant for degradation of PCE that provided the best fit was 0.6 per year.





22. Are TCE, DCE, or VC present?

Decision Criteria

Evaluate data on concentrations of PCE, TCE, cDCE, tDCE and VC in wells down-gradient of the source of contamination. If the sum of TCE, cDCE, tDCE and VC is more than 5 mole % of the concentration of PCE, then TCE, cDCE, tDCE and VC are considered present. The presence of TCE, cDCE, tDCE or VC indicates that reductive dechlorination of PCE has occurred.

The calculation of mole % can easily be performed using the Excel file Mole Percent Calculator.xlsx. This tool is included in the BioPIC program as well as Appendix D.

HELP

The detection of TCE, cDCE, or VC at PCE-impacted sites suggests that PCE reductive dechlorination has occurred and this process may still be ongoing. Enumeration of pceA genes (present in PCE- and/or TCE-dechlorinating bacteria and implicated in PCE-to-TCE and PCE-to-cDCE reductive dechlorination) with qPCR provides support that bacteria capable of PCE reductive dechlorination to TCE or cDCE are present. The presence of the Dhc RDase genes tceA, bvcA or vcrA implicated in reductive dechlorination of DCE can explain the formation of VC and ethene.

23. Are TCE, DCE, or VC present in relevant concentrations?

Decision Criteria

Evaluate data on concentrations of PCE, TCE, cDCE, tDCE and VC in wells downgradient of the source of contamination. If the sum of TCE, cDCE, tDCE and VC is more than 25 mole % of the concentration of PCE,

then TCE, cDCE, tDCE and VC are present at relevant concentrations. The presence of daughter products at these concentrations indicates that microbial reductive dechlorination is an important pathway for TCE fate, and explains in a qualitative manner why TCE is degrading.

The calculation of mole % can easily be performed using the Excel file Mole Percent Calculator.xlsx. This tool is included in the BioPIC program and included in Appendix D.

24. Does magnetic susceptibility explain the PCE rate constant?

Decision Criteria

Consult the simulation that you prepared to evaluate the criterion "Does Natural Attenuation Currently Meet the Goal?" Prepare a new simulation. Do not include any portion of the plume where biological reductive dechlorination might contribute to the bulk rate constant that is extracted by the model. Exclude any portion of the flow path where the concentrations of any daughter products are increasing with distance from the source. By trial and error, identify the rate constant for degradation of TCE that provides the best match between the new simulation and the field data.

Open the tab Files and select the spreadsheet Magnetic Susceptibilty.xlsx. Enter your values for the rate constant for degradation of PCE and for mass magnetic susceptibility in the tab Input Data. Then open the tab Mag. Sus. Explains PCE.

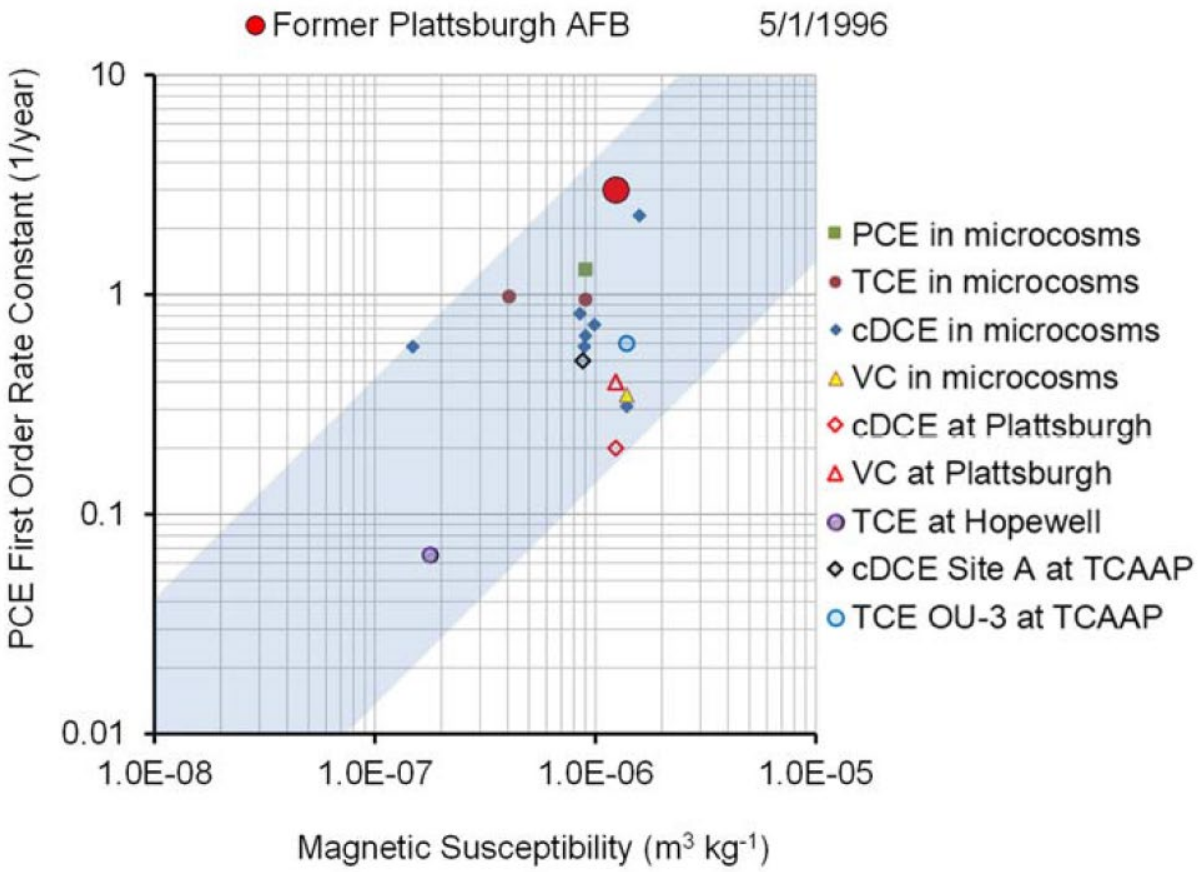
If the site-specific values fall within the blue shape, then mass magnetic susceptibility can explain the apparent in situ rate of PCE degradation.

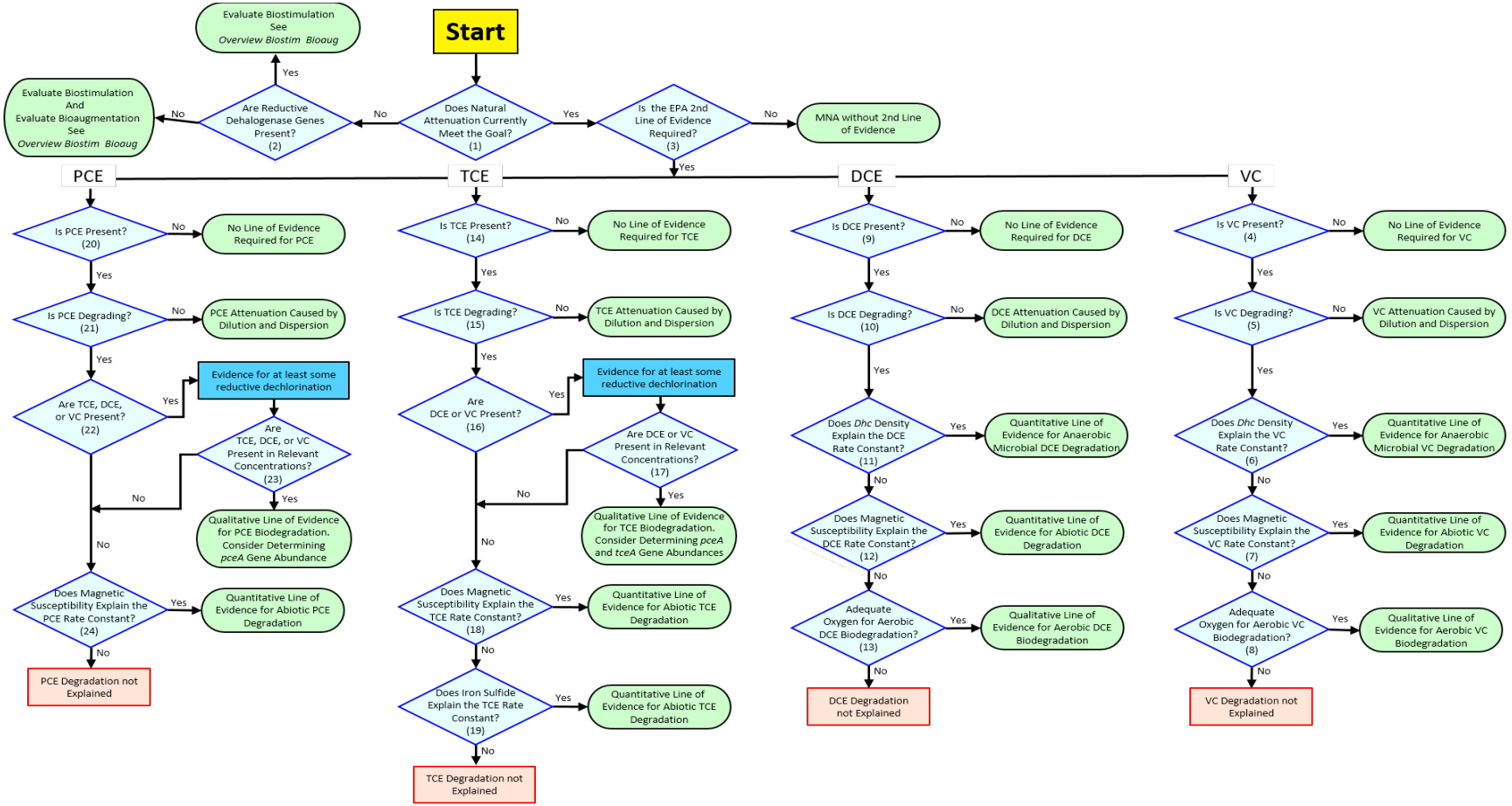
HELP

Magnetite can mediate abiotic degradation of PCE. The amount of magnetite in aquifer material can be estimated from the mass magnetic susceptibility of core samples. Empirical data are available that associate degradation rate constants for PCE with mass magnetic susceptibility. The available data were used to define the blue shape in the figure. The figure is the chart in tab Mag. Sus. Explains PCE for an example data set. If the rate constant plots within the blue shape, then abiotic degradation mediated by magnetite can explain the observed rate constant.

If the rate constant plots above the blue shape, other processes are likely contributing to the rate of PCE degradation.

If the rate constant plots below the shape, inappropriate sampling locations may have been selected for mass magnetic susceptibility measurements. Mass magnetic susceptibility should be determined with aquifer material that is most transmissive to water since this is where most solute transport will occur. In addition, the input values used for the rate constant calculation with BIOCHLOR should be verified.


Former Plattsburgh AFB
5/1/1996
PCE First Order Rate Constant (1/year)
10
1
0.1
0.01
1.0E-08
1.0E-07
1.0E-06
1.0E-05
Magnetic Susceptibility (m3 kg-1)
PCE in microcosms
TCE in microcosms
cDCE in microcosms
VC in microcosms
cDCE at Plattsburgh
VC at Plattsburgh
TCE at Hopewell
cDCE Site A at TCAAP
TCE OU-3 at TCAAP



Decision Framework for Chlorinated Ethenes.

This flowchart was created as part of a previous ESTCP project (ER-201129) and was transferred into the updated BIOPIC tool.

Appendix C:
User's Guide for MNA Rate Constant Estimator

MONITORED NATURAL ATTENUATION (MNA) RATE CONSTANT ESTIMATOR

WHAT THIS SOFTWARE TOOL DOES

- Estimates degradation rate constants for multiple different types of contaminants based on field data (e.g., concentration vs. distance, biomarker abundance correlations) using a simple groundwater fate and transport model
- Generates lines of evidence to support a site-specific evaluation of monitored natural attenuation (MNA)

DISCLAIMER

The MNA Rate Constant Estimator is available "as is". Considerable care has been exercised in preparing this manual and software product; however, no party, including without limitation the United States Government, GSI Environmental Inc., the authors and reviewers, make any representation or warranty regarding the accuracy, correctness, or completeness of the information contained herein, and no such party shall be liable for any direct, indirect, consequential, incidental or other damages resulting from the use of this product or the information contained herein. Information in this publication is subject to change without notice. Implementation of the tool and interpretation of the predictions of the models are the sole responsibility of the user.

This report was prepared under contract to the Department of Defense Environmental Security Technology Certification Program (ESTCP). The publication of this report does not indicate endorsement by the Department of Defense, nor should the contents be construed as reflecting the official policy or position of the Department of Defense. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the Department of Defense.

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OVERVIEW

The MNA Rate Constant Estimator is a screening model that simulates natural attenuation of dissolved compounds in groundwater. The software has been programmed using the Microsoft Excel platform and has the ability to simulate 3-D solute transport that incorporates advection-dispersion, linear adsorption, and various transformation processes using a modification of the analytical solutions developed by Wexler (1992). It includes three different modules:

1. Solute transport of ***1,4-dioxane*** with biotransformation via an oxidative pathway
2. Solute transport of ***chlorinated ethenes*** (PCE, TCE, cis-1,2-DCE, and VC) with biotransformation modeled as a sequential first-order reductive dechlorination process
3. Solute transport of ***chlorinated ethanes*** (1,1,1-TCA, 1,1-DCA) and 1,1-DCE with two degradation pathways: i) biotransformation modeled as a sequential first-order reductive dechlorination; ii) abiotic hydrolysis/dehydrohalogenation of 1,1,1-TCA

GSI Environmental Inc. (GSI) developed the software for the Environmental Security Technology Certification Program (ESTCP) as part of Project Number ER-201730. It is designed to support an evaluation of MNA using BioPIC, but it also functions effectively as a standalone tool.

INTENDED USES

This tool was designed to be similar to BIOCHLOR (USEPA) in terms of its design and application. BIOCHLOR was developed by GSI as a screening model for natural attenuation but focused primarily on chlorinated ethenes. The MNA Rate Constant Estimator has added features and increased functionality for newer versions of Excel, but it can be used to evaluate the same fundamental issues as BIOCHLOR:

Intended Use #1 - Evaluate how far a dissolved plume will extend under a natural attenuation scenario (i.e., if no engineered controls or source area reduction measures are implemented).

The tool uses an analytical solute transport model that includes options for multiple first-order degradation processes depending on the compound. The model will predict the extent of dissolved-phase plume migration over time, which may then be compared to the distance to potential points of compliance or points of exposure (e.g., drinking water wells, groundwater discharge areas, or property boundaries). Analytical ground-water transport models have seen wide application for this purpose (e.g., ASTM, 1995) and experience has shown such models can produce reliable results when site conditions in the plume area are relatively uniform. An evaluation of the plume extent requires an estimate of the rates at which compounds are being degraded, as described below.

Intended Use #2 -Determine appropriate rate constants for relevant degradation processes.

The tool provides a simple method to estimate site-specific degradation rate constants for individual compounds within a groundwater plume. It also allows users to simulate source decay to understand how plumes may expand or contract over time.

The use of an appropriate plume degradation rate constant is important because the model is typically sensitive to the magnitude of the rate constants. Although rate constants can be taken from the literature, the values reported for a given compound can vary by several orders of magnitude. Biotransformation rate constants are site-specific and will be dependent on the size of the microbial population, the availability of electron donors/acceptors/nutrients, and geochemical conditions. Many rate constants in

the general literature are derived from laboratory microcosm studies under idealized conditions. Typically, these laboratory-derived values overestimate the rate of biotransformation seen in the field because of the difficulty in simulating field conditions in a laboratory environment (Aziz et al., 2000b).

The best approach for determining rate constants is to calibrate the model to field data for a given sampling event (see the **Instructions for Calibrating Model** section of this guide). Briefly, to estimate rate constants for a parent compound (e.g., PCE, 1,4-dioxane), change the rate constant for the compound until its predicted concentrations match the field data. The tool displays the Root Mean Square Error (RMSE) between the predicted concentrations and field-measured concentration, and this parameter can be used to optimize the fit. Next, change the rate constant for the next relevant daughter product until the predicted concentrations for that daughter product also match the field data. Continue estimating rate constants for the other constituents in the degradation pathway as needed. Using this approach, site-specific rate constants are estimated, and the model is then considered calibrated. Using the site-specific rate constants, predictive simulations can be conducted by increasing the simulation time to estimate future plume behavior.

To speed the calibration process for rate constants, the tool includes several options:

- Estimate rate constants based on the abundance of one or more qPCR-based biomarkers for degradation of individual compounds. These correlations have been developed using lab-based kinetic parameters and are designed to help calibrate the model. After the user chooses from a list of possible biomarkers and enters the measured abundance, the tool will generate a rate constant that can serve as a starting point for improving the fit between the actual field data and the model simulations.
- Estimate rate constants based on fitting the field concentration vs. distance data to a first order rate constant that lumps all processes. Although this has the potential to overestimate biotransformation rate constants by lumping the effects of multiple processes (including non-destructive processes), it quickly yields a reasonable first approximation of the rate constants. These rate constant estimates can be manually refined subsequently. This approach is most appropriate for parent constituents. Caution should be exercised in using this approach with daughter constituents, as daughter product generation is not accounted for in this method.

The user can also assume that the source is decaying via natural processes, including decreasing rates of dissolution from non-aqueous phase liquids, biotransformation, or any other degradation processes. Decay is modeled as a first order processes where the user estimates the date at which the release occurred, or alternatively the date when data from source area wells are first available. The user can then use the available source area data to estimate the first-order rate constant for source decay, or the user can enter their own estimates of the rate constant to help calibrate the plume data.

Intended Use #3 - Support a lines of evidence approach for evaluating MNA.

Based on existing USEPA and state protocols, establishing plume stability and documenting degradation processes and rates are key primary and secondary lines of evidence for selecting MNA as part of a site management strategy or remedy. The tool generates quantitative data that directly supports this type of approach. It was designed to be used in conjunction with BioPIC, which is another Excel-based tool that serves as a decision framework for MNA. BioPIC essentially provides a step-by-step roadmap for users to determine if MNA is appropriate for a particular site. The MNA Rate Constant Estimator provides key output data that helps answering several of the questions within BioPIC that require estimates of rates

for compounds such as chlorinated ethenes, chlorinated ethanes, and 1,4-dioxane. However, the MNA Rate Constant Estimator also functions well as a standalone tool for evaluating fate and transport of these compounds.

MODEL BASIS

The model uses a modified version of the analytical solutions to 3-D solute transport equations presented in Wexler (1992). The solution assumes uniform flow within an aquifer of infinite width but finite height (to allow for reflection of dispersion at the upper and lower boundaries of the aquifer. It incorporates advection and hydrodynamic dispersion (with uniform constants), linear equilibrium adsorption, and chemical transformation (via first-order processes).

LIMITATIONS

This software tool has the following assumptions and limitations:

- As an analytical model, it assumes simple groundwater flow conditions. The model should not be applied where pumping systems create a complicated flow field. In addition, the model should not be applied where strong vertical flow gradients affect contaminant transport.
- The model assumes uniform hydrogeologic and biogeochemical conditions exist over the entire model area. Because it simplifies actual site conditions, the model should not be applied where extremely detailed, accurate results that closely match site conditions are required. More comprehensive numerical models should be applied in such cases.
- It is primarily designed for simulating the degradation of compounds via a first-order process where the rate does not change over time. This is a simplifying assumption in some cases but is generally reasonable for natural attenuation processes where concentrations are relatively dilute. In some cases, contaminants may be subject to metabolic biodegradation process in more upgradient portions of a plume where the concentration levels are high and subject to cometabolic biodegradation in more downgradient portion of the plume where the concentration levels are lower. Using a single first-order kinetic parameter to simulate the overall plume behavior in may be less accurate. In such cases, one can divide the plume into two portions and analyze them separately.
- It assumes the user is familiar with basic groundwater transport and mass balance concepts. It uses input parameters that should be easily estimated by this audience, although some guidance on parameter values is provided later in this guide.

NAVIGATION

On the home page for the MNA Rate Constant Estimator, the user can pick between several different models that are shown below in **Figure 1**. The user can select models using either the buttons or the tabs (both will lead to the same screen).

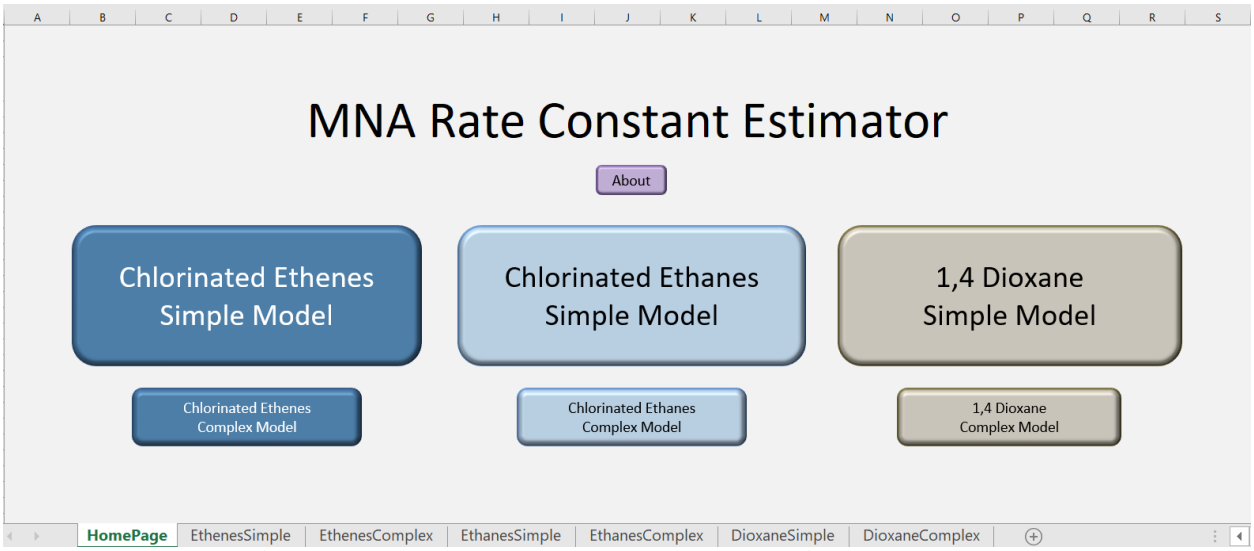


Figure 1. Home Page for MNA Rate Constant Estimator

The user can evaluate chlorinated ethenes, chlorinated ethanes (plus 1,1-DCE), and 1,4-dioxane separately. There is a choice between "Simple" and "Complex" model for each set of compounds. The primary difference between the Simple and Complex models is the interface; the models themselves use the same fate and transport equations.

- Most users should choose the "Simple" models because the interface is more user-friendly and displays only the information needed to run the model. The rest of this User's Guide focuses on how to use the "Simple" models.
- Users can choose the "Complex" model if they want more transparency on the input parameters and tabulations of various results.

DATA ENTRY (SIMPLE MODEL)

For each model, the interface is constructed so that all input and output data are shown on a single tab, although the user will need to scroll down to see the output data. Site-specific information is input on the top portion of the page in Boxes 1 – 6. In all cases, white cells represent parameters for which data can be entered directly, gray cells represent parameters for which values are calculated automatically by the tool but can be overridden, and black cells represent parameters with values that are locked (typically because they were already entered in a previous box).

The following set of screenshots from the 1,4-Dioxane Simple Model provides instructions on how data are entered. For more information on selecting parameter values, consult the Additional Help section of this User's Guide.

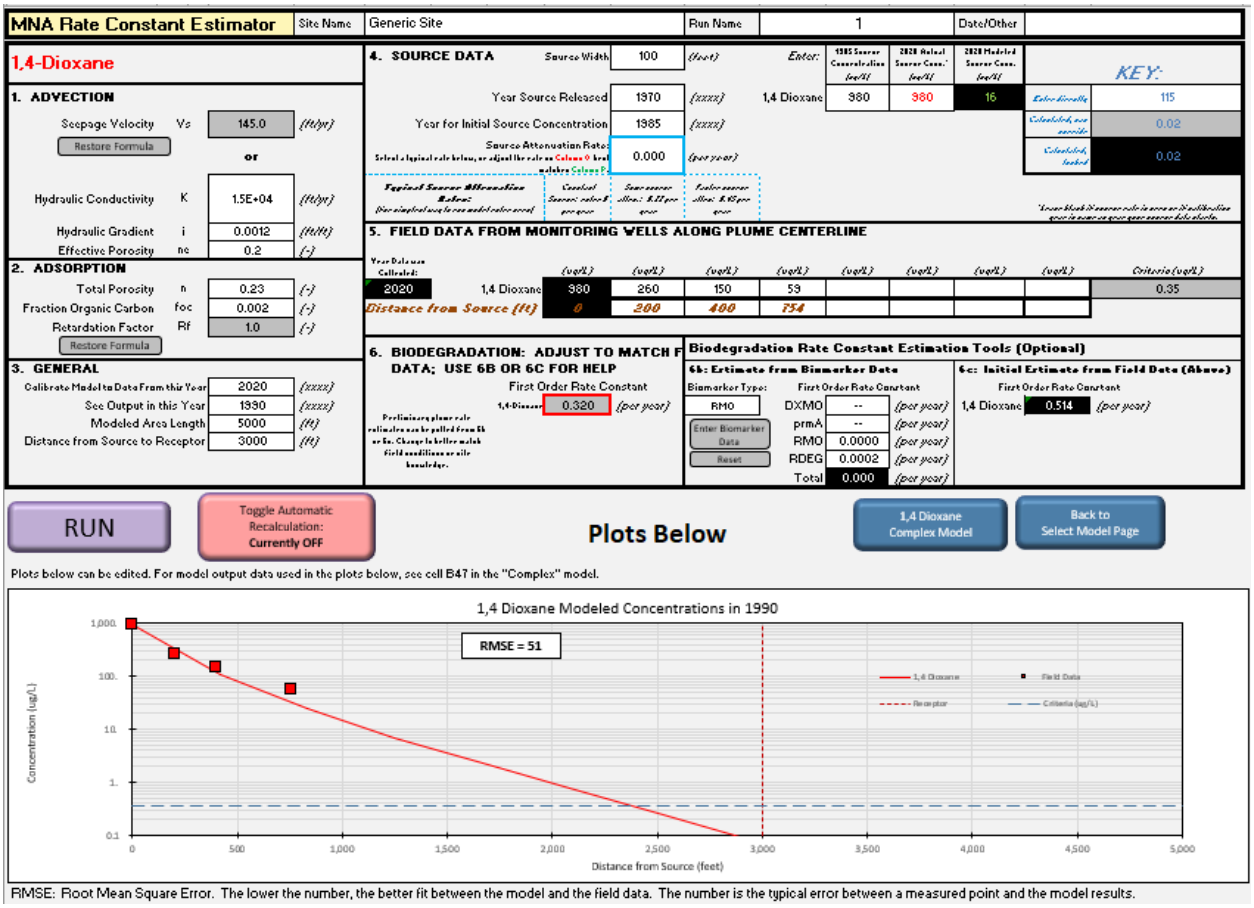


Figure 2. Interface for Simple 1,4-Dioxane Model.

Site specific data are entered in Boxes 1 – 6. Results are shown in plot at bottom of page.

Box 1 – Advection: Enter seepage velocity for groundwater directly into the grey box, if known. Alternatively, the user can enter the hydraulic conductivity, hydraulic gradient, and effective porosity, and the seepage velocity will be automatically calculated from these parameters. If the user has directly entered a seepage velocity into the gray box but would then like the tool to calculate based on the values for the other parameters, click the "Restore Formula" button. See the **Additional Help** section of this User's Guide for additional guidance on selecting appropriate parameter values.

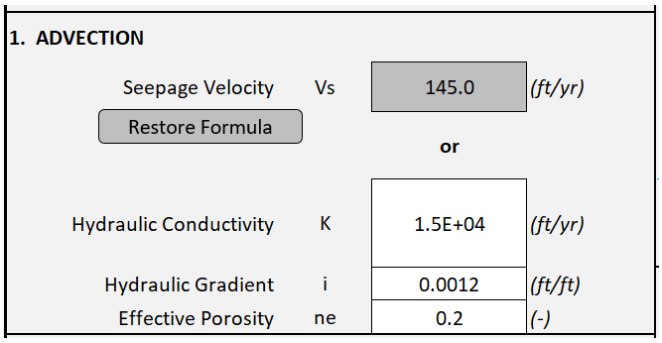


Figure 3. Box 1 Entry (Advection)

Box 2 – Adsorption: Enter the compound-specific retardation factor, if known. This reflects the ratio between the velocity of the contaminant in groundwater (which is influenced by adsorption to aquifer solids) and the groundwater seepage velocity Alternatively, the user can enter the total porosity and the fraction of organic carbon, and the tool will calculate the retardation factor based on stored K_{oc} values for the parent compound. See the **Additional Help** section of this User's Guide for additional guidance on selecting appropriate parameter values.

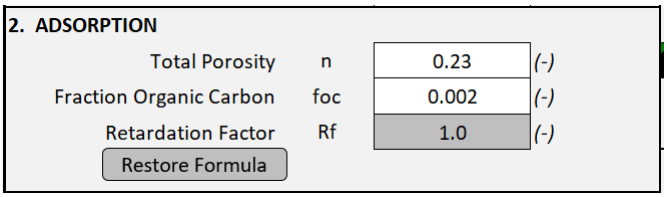


Figure 4. Box 2 Entry (Adsorption)

Box 3 – General: In the top row, enter the year that will be used to calibrate the model—this should correspond to one of the monitoring events with relevant data that will be entered in Box 5. In the second row, the user can select different years to show the historical plume extent or to see if the plume will expand in future years.

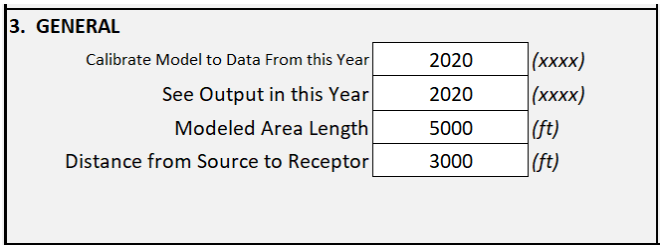
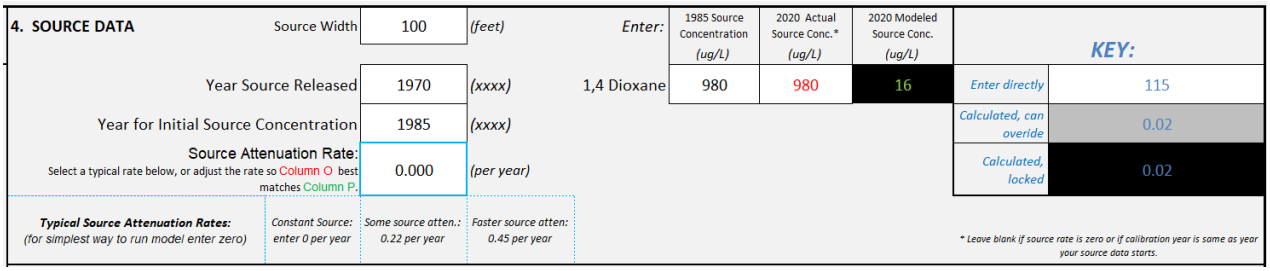


Figure 5. Box 3 Entry (General)

The user can also then specify the length of the modeled area, which should include the maximum downgradient distance that is relevant for evaluating the site (including receptors). The distance from source to receptor (or a Point of Compliance) can be entered in the final row as a visualization aid during plotting of the data.

Box 4 – Source Data: Enter relevant information for the source area. This includes the approximate width of the source and a best estimate of the year that the release occurred. The Year for Initial Source Concentration corresponds to the earliest date when concentration data are available for the monitoring location that is being designated as the source (e.g., 1985 in the screenshot above). The source concentration on that date is also entered, along with the source concentration on the date which will be used for model calibration (i.e., the date entered in the first row of Box 3).



4. SOURCE DATA				Enter:	1985 Source Concentration (ug/L)	2020 Actual Source Conc.* (ug/L)	2020 Modeled Source Conc. (ug/L)	KEY:	
Source Width	100	(feet)							
Year Source Released	1970	(xxxx)		1,4 Dioxane	980	980	16	Enter directly	115
Year for Initial Source Concentration	1985	(xxxx)						Calculated, can overide	0.02
Source Attenuation Rate: Select a typical rate below, or adjust the rate so Column O best matches Column P.	0.000	(per year)						Calculated, locked	0.02
Typical Source Attenuation Rates: (for simplest way to run model enter zero)	Constant Source: enter 0 per year	Some source atten.: 0.22 per year	Faster source atten: 0.45 per year						

* Leave blank if source rate is zero or if calibration year is same as year your source data starts.

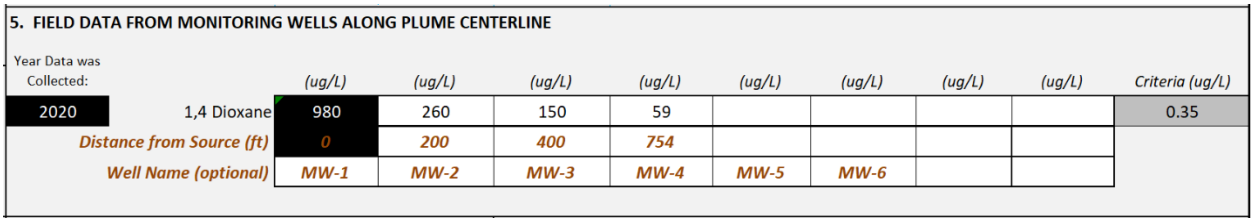
Figure 6. Box 4 Entry (Source Data)

The tool then uses these data to help determine an appropriate Source Attenuation Rate for the parent compound. The Source Attenuation Rate is based on an assumption of first-order decay, meaning that a starting point estimate of the rate can be made using the temporal and concentration data. This is done by manually adjusting the Source Attenuation Rate until the concentration in Column P (modeled source concentration) matches the concentration in Column O (actual source concentration based on field data). Note that this approach is optional; users do not have to enter values in these cells to proceed with the model. This would include cases where the Source Attenuation Rate is expected to be zero or if the calibration year is same as year as when your source data starts. However, if the modeled source concentration (Column P) is substantially different than the actual source concentration (Column O) for the year being simulated, then the calibration is less likely to be accurate.

Alternatively, the user can directly enter a Source Attenuation Rate based on a range of typical compound-specific values provided within Box 4 of the individual modules. For example, the range of Source Attenuation Rates for 1,4-dioxane span a range of 0 to 0.45 yr^{-1} based on the empirical data reported in Adamson et al. (2015).

As noted above, the user should check which of these approaches provide reasonable fit to the field data. In some cases, selecting zero for the Source Attenuation Rate may be appropriate. This would include sites where little change in the source concentration has been observed and/or the user wants to be conservative when estimating the possible extent of the plume.

Box 5 – Field Data from Monitoring Wells Along Plume Centerline: Enter the groundwater concentrations of relevant compounds in monitoring wells near the centerline of the plume. The relative distance of each monitoring location from the source well should also be entered. These data are used to help calibrate the model and are displayed with model results at the same locations.



5. FIELD DATA FROM MONITORING WELLS ALONG PLUME CENTERLINE

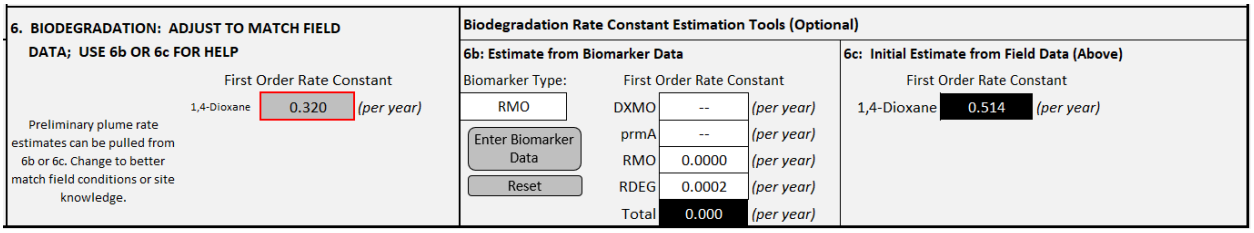
Year Data was Collected:		(ug/L)	(ug/L)	(ug/L)	(ug/L)	(ug/L)	(ug/L)	(ug/L)	(ug/L)	Criteria (ug/L)
2020	1,4 Dioxane	980	260	150	59					0.35
	Distance from Source (ft)	*0*	*200*	*400*	*754*					
	Well Name (optional)	*MW-1*	*MW-2*	*MW-3*	*MW-4*	*MW-5*	*MW-6*			

Figure 7. Box 5 Entry (Field Data)

Concentration and distance data from up to 8 wells can be entered. However, the tool can be used with data from less than 8 wells (generally 4 or more are recommended). To the extent practical, the user should enter data from a source area well (note that the tool will automatically enter the data that was used for the Source Data in Box 5) and data from a well located near the toe of the plume.

It is assumed that users will enter data that were all collected during a single monitoring event in Box 6. If users have access to data from multiple monitoring events, they can calibrate the model to obtain a biodegradation rate constant for each event, if desired (see Option 5 in the **Instructions for Calibrating Model** section).

Box 6 – Biodegradation: One of the primary uses of this tool is to estimate first-order biodegradation constant(s) that best match the observed site concentrations. The user may adopt a trial-and-error procedure to derive a best-fit decay coefficient for each contaminant by varying the decay coefficient until predicted concentrations match measured concentrations.



6. BIODEGRADATION: ADJUST TO MATCH FIELD DATA; USE 6b OR 6c FOR HELP

First Order Rate Constant

1,4-Dioxane 0.320 *(per year)*

Preliminary plume rate estimates can be pulled from 6b or 6c. Change to better match field conditions or site knowledge.

Biodegradation Rate Constant Estimation Tools (Optional)

6b: Estimate from Biomarker Data

Biomarker Type: RMO

Enter Biomarker Data

Reset

First Order Rate Constant		
DXMO	--	*(per year)*
prmA	--	*(per year)*
RMO	0.0000	*(per year)*
RDEG	0.0002	*(per year)*
Total	0.000	*(per year)*

6c: Initial Estimate from Field Data (Above)

First Order Rate Constant

1,4-Dioxane 0.514 *(per year)*

Figure 8. Box 6 Entry (Biodegradation)

The first order rate constant that the model uses to simulate concentration vs. distance data in highlighted in red in the main area on the left-hand side of Box 6. In other words, this rate constant (or rate constants for cases where daughter products are also considered) generates a dataset that can be used for calibration, and changes to these values are made to optimize the fit to the field data.

There are multiple different approaches that can be used to help estimate the rate constant(s), as discussed in the **Instructions for Calibrating Model** section. This includes entering Biomarker data in Box 6b for the compound of interest, as well as an "Initial Estimate" of the rate constant in Box 6c that is automatically generated from the user-provided field data.

OUTPUT DATA (SIMPLE MODEL)

The results of the model simulations are shown in one or more plots that can be found at the bottom of the interface page. These plots show:

1. The simulated concentration vs. distance data as a solid line.
2. The field-measured concentration at each location as a series of points.
3. The calculated RMSE, which is used as an optimization parameter.
4. The location of the receptor (or the point of compliance) as a vertical dashed line.
5. User-specified regulatory criteria (if applicable).

These plots allow the user the visualize the plume trends with respect to receptors and regulatory criteria. Importantly, they allow the user to visualize how well the simulated data (which are based on the user-inputted biodegradation rate constant from Box 6) fit the actual field-measured concentration vs. distance data.

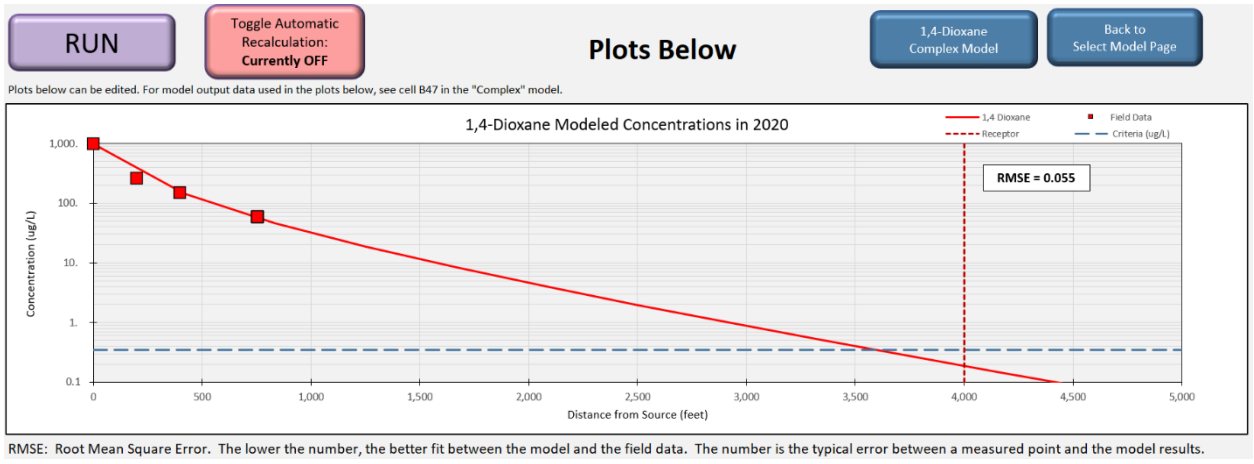


Figure 9. Output Data Plots. Concentration vs. distance are shown for a single year to allow for comparison between simulated data (solid red line) and field-measured data (square points).

The model results are also located in the "Complex Model" tab for each set of compounds (generally in Columns B through F at Rows 44 through 59). These data can be copied and pasted into other Excel spreadsheets or tables for other purposes (e.g., custom plots, reporting) as needed.

INSTRUCTIONS FOR CALIBRATING MODEL

Calibrating the model is based on optimizing the fit between the field data and the simulated concentration by adjusting the biodegradation rate constant. This can be accomplished iteratively using a few different approaches, as described below:

Option 1 – Simple Iteration: Prepare a new simulation where the rate constant for degradation of the parent compound (in Box 6) is set to zero. Compare the actual field-measured concentrations of the parent compound against the simulated concentrations. This can be done by visual inspection of the concentration vs. distance plots and recording the RMSE associated with this baseline case (i.e., the root mean square error between the field data and the concentrations predicted by the model). For example, in the simulation of 1,4-dioxane presented in Figure 10, the rate constant was initially set to zero. From visual inspection of the concentration vs. distance plot, it is clear that the simulated concentrations (the solid red line) are higher at each point along the groundwater flowpath than the field data. This means that the degree of attenuation is being underestimated. The RMSE for this baseline case (0.231) should be recorded and then compared to subsequent iterations, as described below.

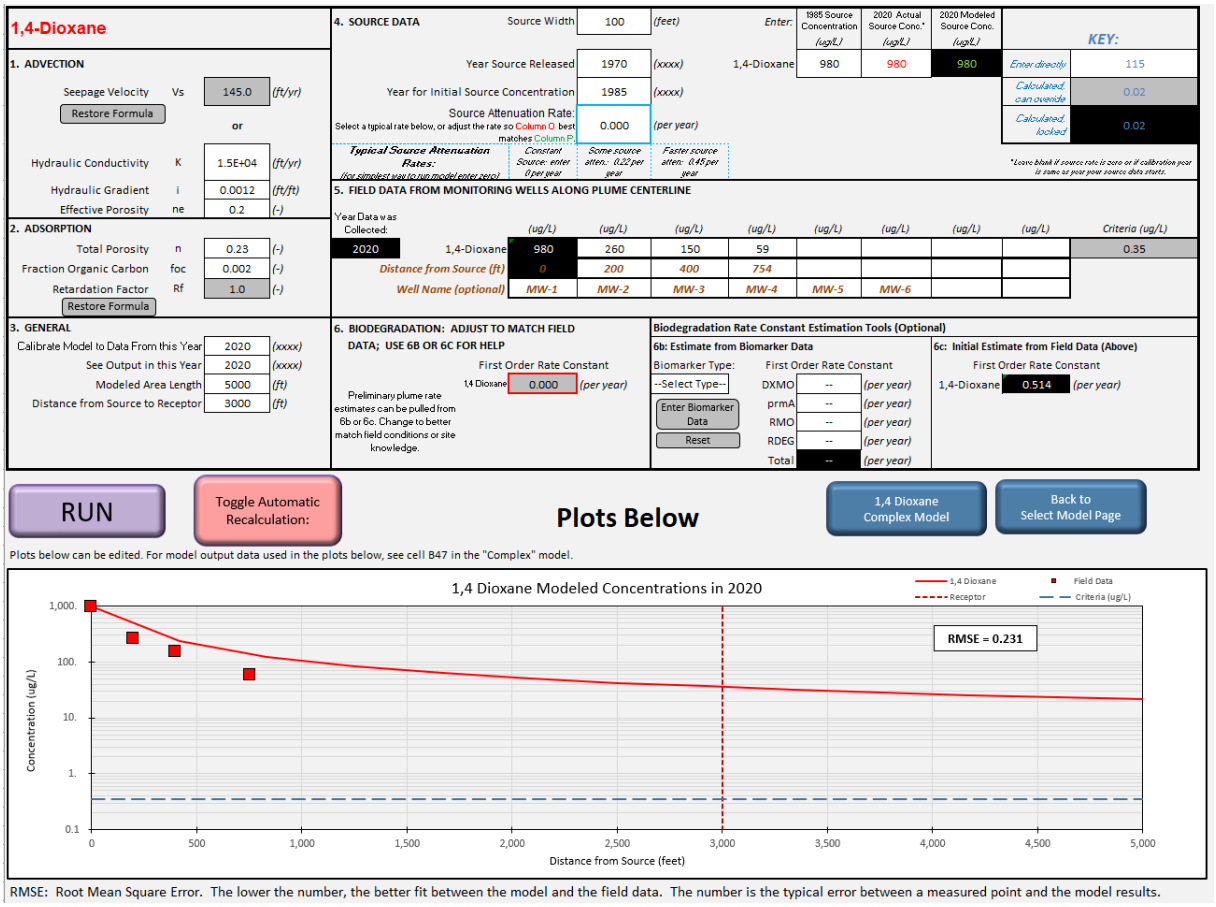


Figure 10. Example Simulation with Assumption of No 1,4-Dioxane Biodegradation (Rate Constant = 0). Visual inspection of concentration vs. distance plot confirms that degradation is underestimated, such that model needs further calibrating.

Next, the user should enter new values for the biodegradation rate constant of the parent compound into Box 6 to determine if the resulting model projections provide a better fit to the actual field-measured concentrations. An initial guess for the rate constant can be entered manually or using one of the "starting point" guesses described in Option 2 or Option 3 below. A better fit is defined as having a lower value of RMSE. If rate constants greater than zero can be shown to provide a better fit, then the parent compound is degrading. Continue to adjust the rate constant until the RMSE is minimized. For example, Figure 11 shows the optimized rate constant (0.19 per year) for the same set of field data that was simulated in Figure 10 where it was assumed that no biodegradation was occurring. In this case, calibration resulted in an improved fit of the data, as confirmed by a significant reduction in the RSME (0.055 vs. 0.231). In this case, the degradation would be expected to reduce concentrations below the specified regulatory criteria (0.35 ug/L) by time the plume reaches the downgradient point of compliance.

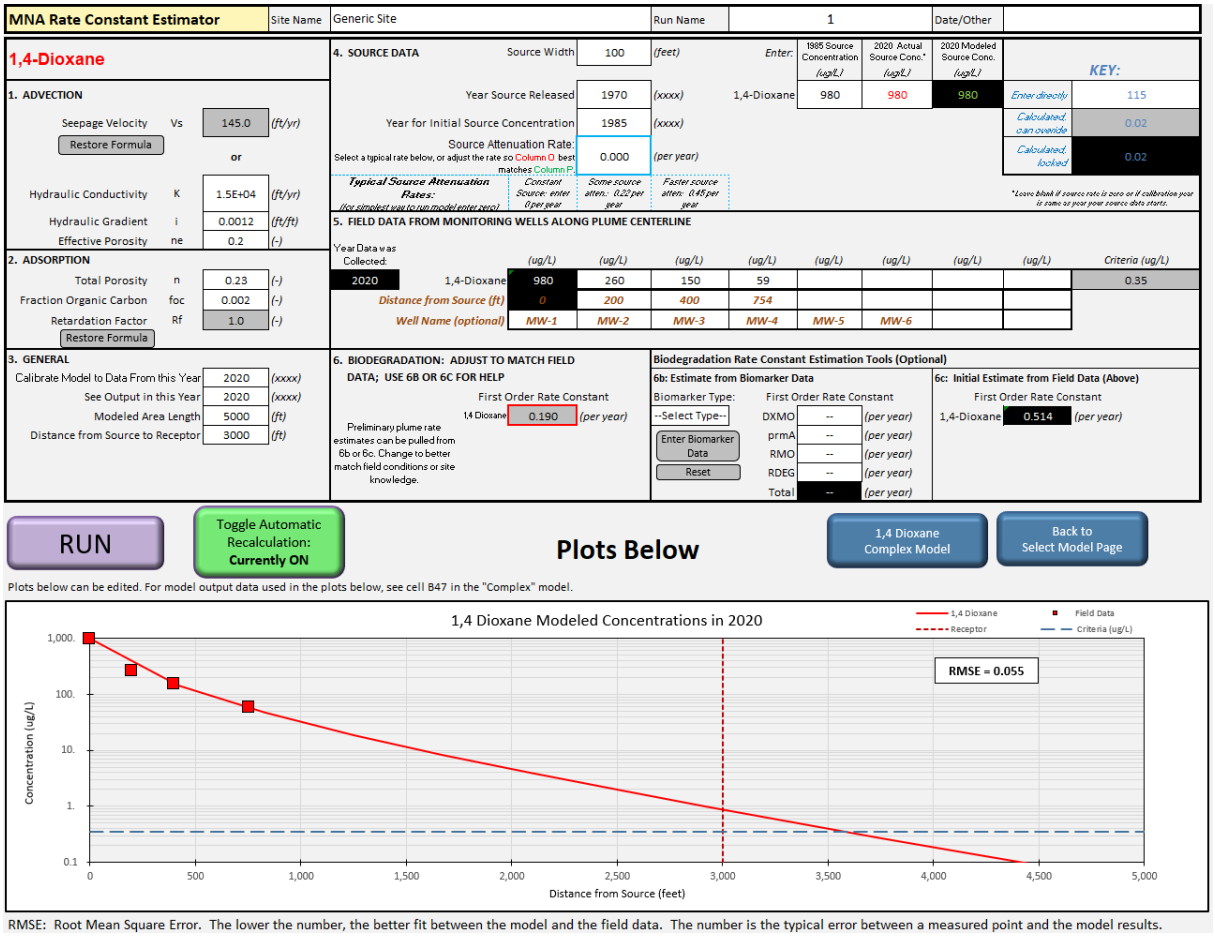


Figure 11. Example Simulation with Evidence of 1,4-Dioxane Biodegradation Based on Model Calibration (Rate Constant = 0.19 per year). Visual inspection of concentration vs. distance plot and low RMSE value confirms that model is calibrated.

For parent compounds that are degraded to daughter products that are also included in the model (e.g., TCE to cis-1,2-DCE), the next step is to change the rate constant for the next relevant daughter product until the predicted concentrations for that daughter product also match the field data. Continue estimating rate constants for the other constituents in the degradation pathway as needed. Using this approach, site-specific rate constants are estimated, and the model is then considered calibrated.

Option 1 is applicable to cases where it is (conservatively) assumed that the source is not decaying. For cases where source decay is assumed, see Option 4 below.

Option 2 – Use the "Initial Estimate" in Box 6c During Iteration: The tool automatically generates an "Initial Estimate" of the biodegradation rate constant in Box 6c from the user-provided field data. It is based on fitting the data to a first-order decay equation where the effects of processes other than biodegradation during transport are ignored. This initial estimate can be entered in Box 6 as a starting point for the iterative process of determining the rate constant that provides the best fit. The initial estimate is likely to be an upper bound estimate, such that the decreasing the rate constant should (at first) produce better RMSE values. As with Option 1, it is necessary to check all fits against the baseline case of no biodegradation (i.e., setting the rate constant of zero).

For example, Figure 12 shows the results of the simulation when the Initial Estimate (0.514 per year) was entered into Box 6. The RMSE value is higher than in the optimized case (Figure 11), and it is clear based on visual inspection that the rate constant is overestimating the extent of degradation, particularly in the far downgradient monitoring location. This suggests that the next iteration should use a lower value for the rate constant to improve the fit.

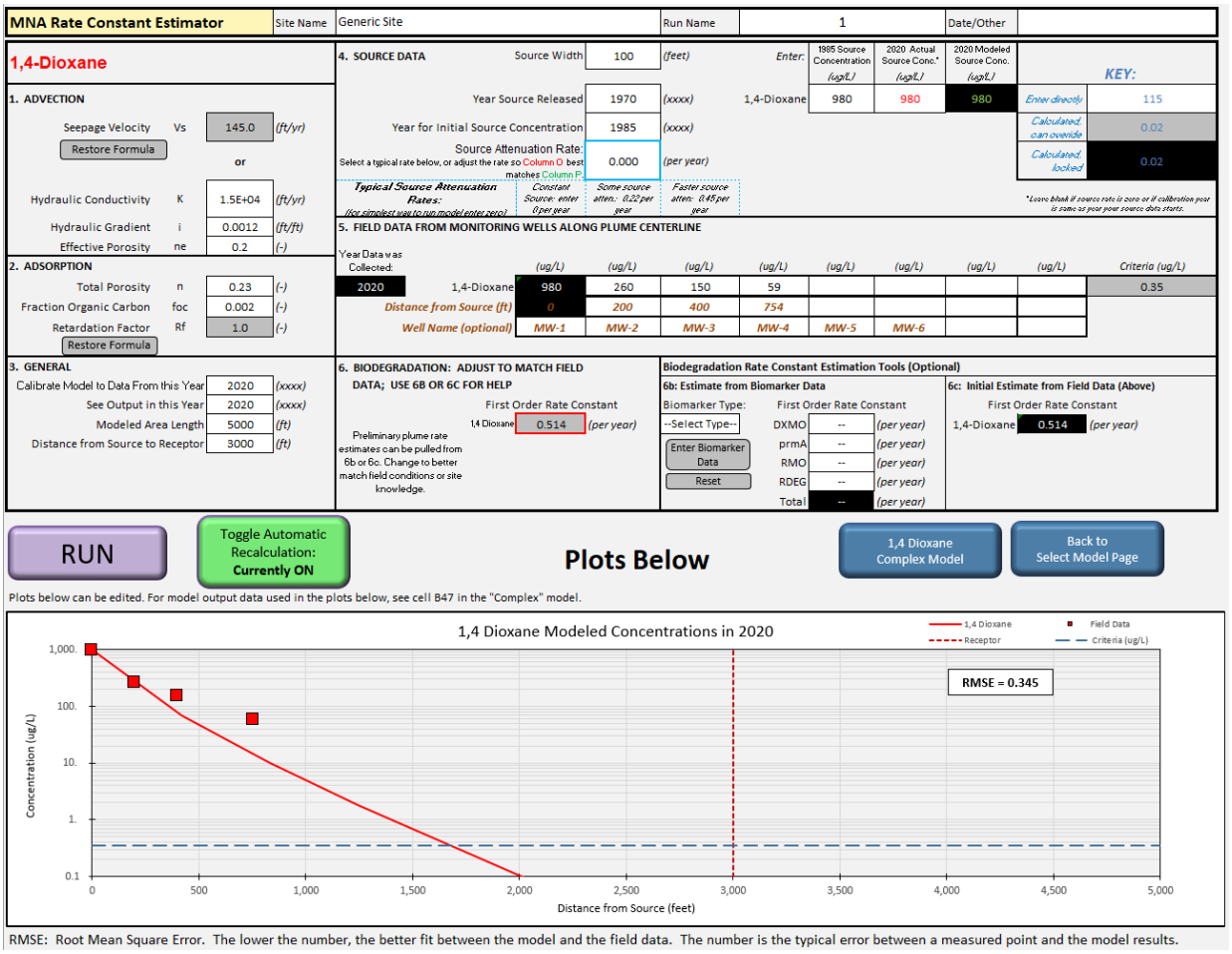


Figure 12. Example Simulation where Initial Estimate from Box 6c Used as Starting Point for 1,4-Dioxane Biodegradation (Rate Constant = 0.514 per year). Visual inspection of concentration vs. distance plot confirms that degradation is overestimated, such that model needs further calibrating.

Option 3 – Use the Biomarker Correlations in Box 6b During Iteration: The model an option to estimate rate constants based on the abundance of several different qPCR-based biomarkers for degradation. The resulting rate constant(s) are generated in Box 6c and are intended as a starting point for improving the fit between the actual field data and the model simulations. Since these correlations are designed to help calibrate the model, they should not be considered a true prediction of the actual degradation rate that is occurring at the site. This is because they are based on empirical data from other studies where conditions may be quite different than those observed at the site being evaluated.

The following describes how to the biomarker correlations developed for 1,4-dioxane as part of this decision tool. A similar process can be followed for chlorinated ethenes and chlorinated ethanes using the biomarkers specific to those compounds (vcrA, Dhb).

1. In Box 6b, select a specific biomarker from the dropdown menu. For 1,4-dioxane, there is an option to enter the following biomarkers: DXMO, prmA, RDEG, and RMO. The DXMO biomarker (also known as THMXO) is associated with organisms that can grow by degrading 1,4-dioxane. The prmA biomarker (also known as PRM or PrMO) is associated with organisms that cometabolize 1,4-dioxane while growing on propane. The RDEG and RMO biomarkers are associated with organism that cometabolize 1,4-dioxane while growing on toluene, or native organic matter. Only 1 biomarker can be entered at a time; start with DXMO if available. Selecting a biomarker from the menu will launch a pop-up box where biomarker abundance data can be entered.

2. In the pop-up box, enter the abundance of the selected biomarker for those wells where data are available. The model will perform a spatial interpolation to estimate a representative biomarker abundance for the site (i.e., a single value that is weighted based on the distance between wells with biomarker data).

3. The rate constant associated with this biomarker abundance will then be automatically entered in the appropriate location in Box 6b.

4. Enter the rate constant from Box 6b into Box 6. This is the rate constant that is used for the model simulation (i.e., to generate a simulated concentration vs. distance curve).

5. The user should then evaluate the fit between the actual field data and the model simulation that is based on this estimated rate constant.

6. Manually adjust the rate constant in Box 6 until an optimal fit between the actual field data and the model simulation is obtained. Use the RMSE that is overlaid on the plot as a guide; lower RMSE values generally indicate a better fit. Record the rate constant that provided the optimal fit.

7. Return to Box 6b and repeat Steps 1 - 3 for all remaining biomarkers. In each case, record the rate constant that is generated in Box 6b (i.e., after the biomarker data are entered in the pop-up box).

8. Compare the recorded rate constants from the biomarker correlations with the “optimal” rate constant from Box 6. If the optimal rate constant from Box 6 is within a factor of 3 to 5 of one or more of the rate constants that were generated from the biomarker correlations, then this is considered reasonable evidence that this particular biodegradation process is contributing to the actual field trend in 1,4-dioxane concentrations.

9. The biomarkers target different genes in different organisms. Ideally, all the organisms could be present in the groundwater at the same time, and act on 1,4-dioxane concomitantly. Add all the rate constants associated with DXMO, prmA, RDEG, and RMO together, and if the optimal rate constant from Box 6 is within a factor of 3 to 5 of the sum of the rate constants that were generated from the biomarker correlations, then this is considered reasonable evidence that biodegradation processes are contributing to the actual field trend in 1,4-dioxane concentrations.

The derivation of these correlations is described in the project report. They are based on an assumption that aerobic biodegradation of 1,4-dioxane follows Michaelis-Menten (Haldane) kinetics (Mahendra and Alvarez-Cohen, 2006; Mahendra et al., 2013; Ye et al. 2017; Grostern et al., 2009; Parthasarathy et al., 2015). The rate equation for Michaelis-Menten kinetics can be rearranged to solve for a first-order rate constant that is a function of other kinetic parameters (specifically Km and Vmax expressed in terms of gene copies), the biomarker abundance (expressed in gene copies per mL) and the concentration of the organic chemical being degraded (in this case, 1,4-dioxane).

The calculations in the MNA rate constant estimator apply for biomarkers with known (published) kinetic parameters. Our work and the work of others shows that there are other enzyme systems that can degrade 1,4-dioxane in groundwater. However, the organisms that carry out this activity are not isolated, the enzyme systems are not characterized, and the kinetic parameters are not available. As a result, the calculations in the MNA Rate Constant Estimator based on the known biomarkers may underestimate the possible rate of biodegradation of 1,4-dioxane.

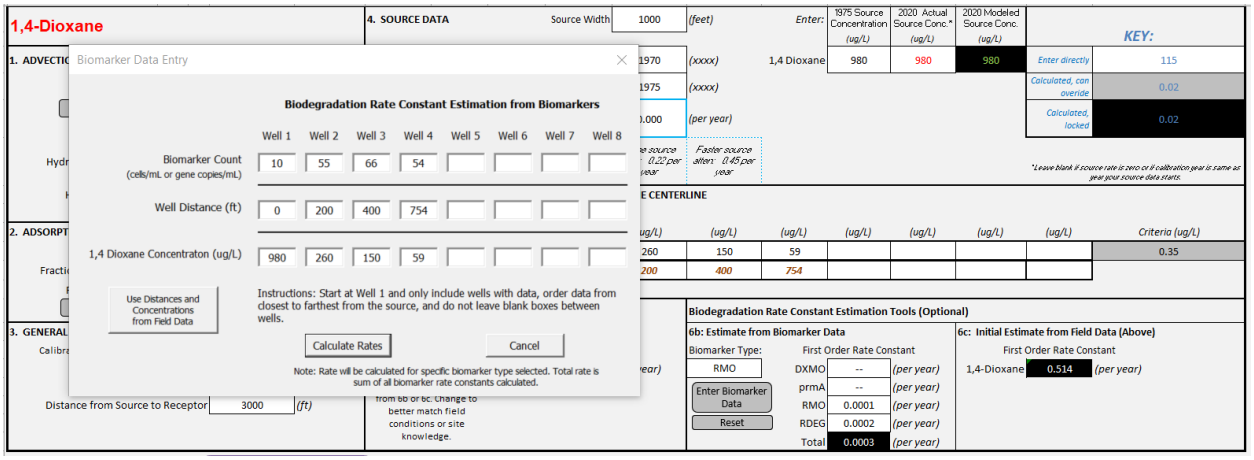


Figure 13. Example Simulation Showing 1,4-Dioxane Biomarker Data Entry. Pop-up box for data entry is visible once the user selects the gray "Enter Biomarker Data" button in Box 6b. After entering the biomarker and 1,4-dioxane concentrations, the "Calculate Rate" button on the pop-up box will automatically enter the corresponding rate constant into Box 6b.

For example, Figure 14 shows the results of the simulation when the rate constant based on the biomarker correlations (0.0003 per year) was entered into Box 6. The RMSE value is very similar to the baseline "no biodegradation" case (Figure 10). Based on visual inspection of the concentration vs. distance data, the rate constant is clearly underestimating the extent of degradation, particularly in the far downgradient monitoring location. This suggests that the next iteration should use a higher value for the rate constant to improve the fit. Note that this finding does not mean that biodegradation is not occurring. Instead, it

suggests that the correlations are not supportive for this particular case and/or that other (unquantified) enzymes are contributing to the observed biodegradation.

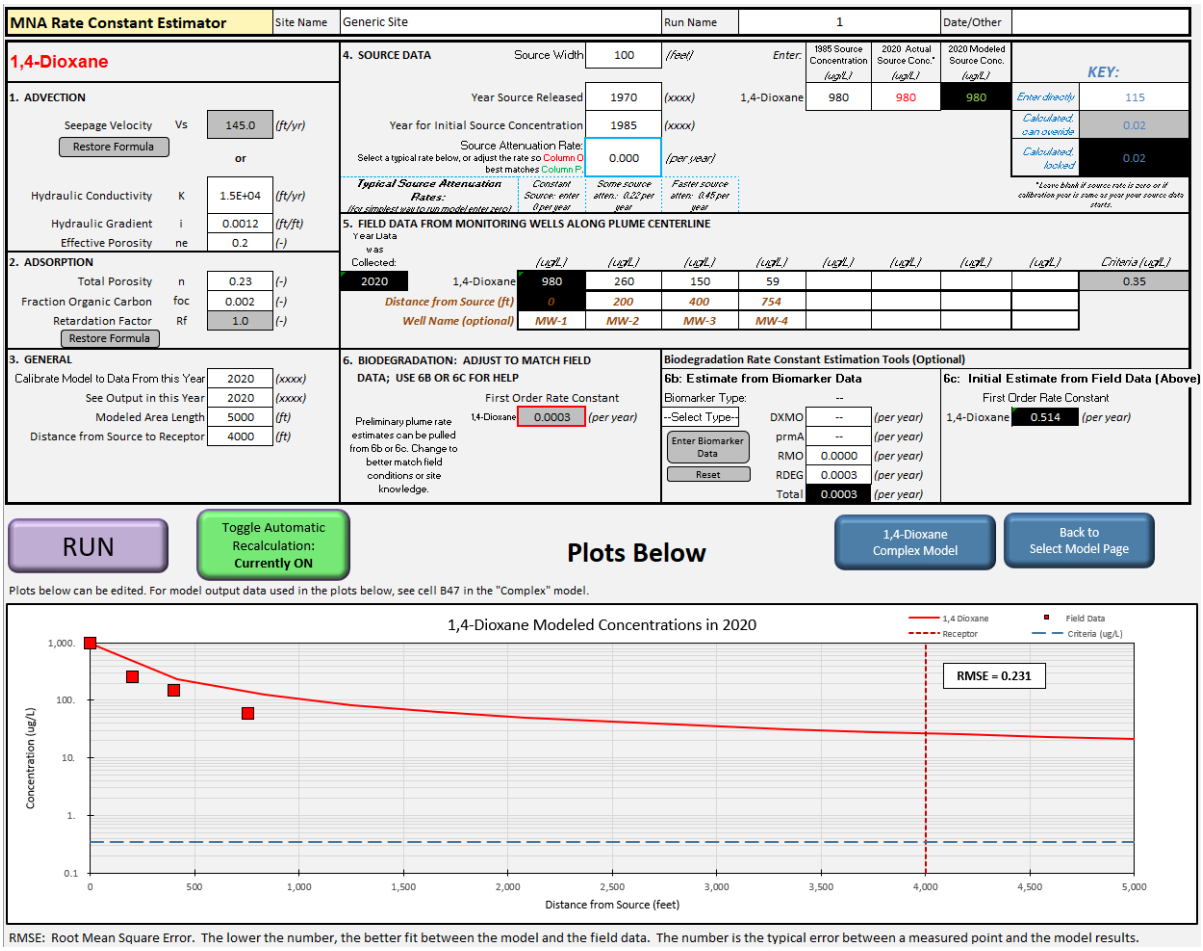


Figure 14. Example Simulation where Biomarker Estimate from Box 6b Used as Starting Point for 1,4-Dioxane Biodegradation (Rate Constant = 0.0003 per year). Visual inspection of concentration vs. distance plot confirms that degradation is underestimated, such that model needs further calibrating.

Option 4 – Include Source Decay During Iteration: A first-order source decay assumption can be incorporated into the simulation using Box 4. This may be appropriate when the field-measured concentration in source area wells has been observed to decline during the available monitoring period. In these cases, a starting point estimate of the rate can be made using the temporal and concentration data by manually adjusting the Source Attenuation Rate until the concentration in Column P (modeled source concentration) matches the concentration in Column O (actual source concentration based on field data). Alternatively, the user can directly enter a Source Attenuation Rate based on a range of typical compound-specific values provided within Box 6 of the individual modules.

In either case, the user should first calibrate the model by entering a field-measured parent concentration for the source area well (i.e., Column J in Box 5) for the specific year that is being used for calibration (e.g.,

2020). This will ensure that the biodegradation rate constant in Box 6 (which represents biodegradation in the plume) is properly calibrated. The user can then do further simulation where source decay is included to see how this parameter influences source and plume trends.

Option 5 – Simple Iterations Using Data from Multiple Events: In many cases, users may have access to data from multiple monitoring events. The user can choose to enter data from a single event and then calibrate the model to obtain a biodegradation rate constant for that event. The calibration process can then be repeated using data from other events, such that different rate constant estimates are generated for each event. The primary advantages of this approach include the following:

- The user can obtain a range of plausible rate constants.
- It helps quantify the variability (or uncertainty) associated with this process and can support statistical testing to confirm that the biodegradation rate constant is greater than zero
- It may help in evaluating if data from certain wells or events are outliers.

Alternatively, the user could convert data from multiple events into set of average values (e.g., arithmetic mean or geomean) for each well. This approach may be useful if the user is trying to identify a single biodegradation rate constant that could be considered representative for a certain time period. However, it may not be as applicable at sites where plumes are rapidly expanding or where other significant changes have been observed over that period. It is likely more applicable for data collected from closely spaced monitoring events (e.g., over the course of a few quarters or years) after the plume footprint has already been established.

EVALUTING PLUME EXTENT OVER TIME

The user can use this tool to simulate how the plume extent changes over time due to transport and degradation processes (including source decay). This is particularly useful for predicting how far a dissolved plume will extend under a natural attenuation scenario assuming no other remedies are implemented. This distance can then be compared to the distance where receptors or compliance points are located. It is also useful for determining if a plume is at steady state and/or expected to contract over time.

For example, the 1,4-dioxane simulation for the example site shown in Figures 10 – 14 was repeated using the calibrated biodegradation rate constant (0.19 per year) and no source decay. In this case, the plume extent was plotted in multiple different years by changing the "See Output in this Year" field in Box 3 of the model interface. Figure 15 shows the progression in the plume footprint in 10-year intervals between 1980 and 2020. These simulations predict that the plume is stable in 2020, and that it reached its maximum extent between 2010 and 2020. This outcome is the result of attenuation in the plume. Since source decay is neglected in the simulation, the plume extent would not be predicted to change over time. If modest source decay was incorporated into the simulation (Box 4), then the plume would be predicted to retreat over time.

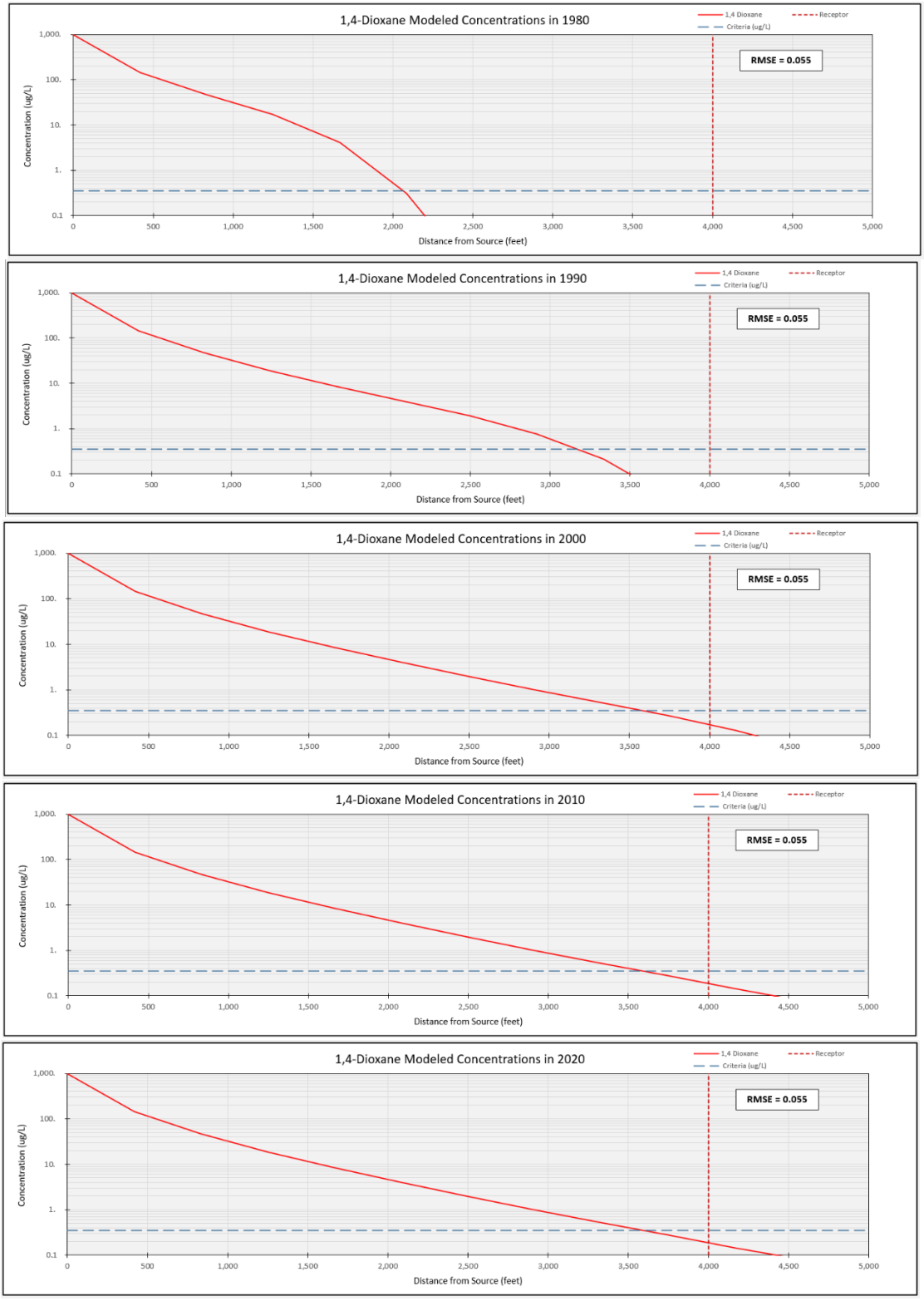


Figure 15. Example Simulation Showing 1,4-Dioxane Plume Extent Over Time (Rate Constant = 0.19 per year). Visual inspection of concentration vs. distance plots confirms that 1,4-dioxane plume would be predicted to stabilize between 2010 and 2020.

TIPS FOR TROUBLESHOOTING

- This software was developed to run on Microsoft Excel 2020. Some loss of functionality may be observed if earlier versions of Excel are used.
- Macros must be enabled for the tool to run properly. If macros are not already enabled by default, then the setting within Excel may need to be adjusted. The procedures for enabling macros will vary depending on the version of Excel that is being used to run the software. Users with security restrictions may need administrator support or approval to enable macros.
- Portions of the interface may not be visible on some screens without scrolling or adjusting the zoom level. The default zoom level was selected to accommodate most sc
- The tool was created in Excel and is not locked for editing. As a result, most features are customizable. This means that the user can adjust plot and cell dimensions based on individual preferences. In particular, the plots can be modified as needed for reporting purposes, and the data can be copied as transferred
- A "Run" button is provided for users who do not want the model to perform automatic calculations. This feature is included because calculation time(s) may take longer than a few seconds for some users, meaning that adjustments to parameter values may slow down the calibration process.
    - For users who do not want automatic calculations, they should first hit the "Toggle Automatic Recalculation" button so that it turns red and reads "Currently OFF". Once this option is selected, no calculations will be performed unless the user hits the purple "Run" button. Both buttons are located in Rows 25 – 27 immediately above the plots.
    - For users who would prefer that simulations are updated automatically every time a parameter value is changed, they should hit the "Toggle Automatic Recalculation" once so that it reads "Currently ON" and turns green.
- In cases where the user has overridden automatic calculations of certain parameters, a "Restore Formula" button is provided so that the model will return to automatic calculations. These parameters are designated by gray cells in the interface, and they include the seepage velocity and the retardation factor. Once the formulas are restored, the parameters will again be calculated using the relevant input parameters (e.g., hydraulic conductivity, gradient, and effective porosity for the seepage velocity).

ADDITIONAL HELP

This section is primarily intended for users who want more information on selecting values for various parameters. Users should seek out additional resources as needed.

PARAMETER	HYDRAULIC CONDUCTIVITY (K)
Units	ft/yr
Description	Measure of the permeability of the aquifer. To characterize concentrations in the target layer utilizing advection, representative measurements are required for both the hydraulic conductivity and the hydraulic flow gradient of the flow system. Representative measurements of the hydraulic conductivity of the target layer should be obtained at one or more locations using appropriate slug test or pumping test methods (Newell et al., 2003).
Typical Values	*Horizontal K* Clay: $<1x10^{-6}$ cm/s Fractured Sandstone: $1x10^{-6}$ - $1x10^{-2}$ cm/s Limestone: $1x10^{-7}$ - $1x10^{-4}$ cm/s Sandstone: $1x10^{-8}$ - $1x10^{-4}$ cm/s Shale: $1x10^{-11}$ - $1x10^{-7}$ cm/s Silt: $1x10^{-6}$ - $1x10^{-3}$ cm/s (Newell *et al*., 1996; Freeze and Cherry, 1979.)
Source of Data	Pump tests or slug tests at the site. It is strongly recommended that actual site data be used for all evaluations.
How to Enter Data	Enter directly after converting to ft/yr units.

PARAMETER	HYDRAULIC GRADIENT (i)
Units	ft/ft
Description	The slope of the potentiometric surface. In unconfined aquifers, this is equivalent to the slope of the water table.
Typical Values	0.0001 – 0.05 ft/ft
Source of Data	Calculated by constructing potentiometric surface maps using static water level data from monitoring wells and estimating the slope of the potentiometric surface.
How to Enter Data	Enter directly.

PARAMETER	EFFECTIVE POROSITY (n_e)
Units	Unitless.
Description	Dimensionless ratio of the volume of interconnected voids to the bulk volume of the aquifer matrix. Note that "total porosity" is the ratio of all voids (included non-connected voids) to the bulk volume of the aquifer matrix. Differences between total and effective porosity reflect lithologic controls on pore structure. In unconsolidated sediments coarser than silt size, effective porosity can be less than total porosity by 2-5% (Smith and Wheatcraft, 1993).
Typical Values	Values for Effective Porosity: Clay 0.01 - 0.20 Sandstone 0.005 - 0.10 Silt 0.01 - 0.30 Unfractured Limestone 0.001 - 0.05 Fine Sand 0.10 - 0.30 Fractured Granite 0.00005 - 0.01 Medium Sand 0.15 - 0.30 Coarse Sand 0.20 - 0.35 Gravel 0.10 - 0.35 (From Wiedemeier et al., 1995; originally from Domenico and Schwartz, 1990 and Walton, 1988).
Source of Data	Typically estimated. One commonly used value for silts and sands is an effective porosity of 0.25. The ASTM RBCA Standard (ASTM, 1995) includes a default value of 0.38 (to be used primarily for unconsolidated deposits)
How to Enter Data	Enter directly.

PARAMETER	TOTAL POROSITY (n)
Units	Unitless.
Description	Dimensionless ratio of the volume of voids to the bulk volume of the surface soil column matrix, but excluding secondary porosity (fractures, solution cavities, etc.). Total porosity is the ratio of all voids (including non-connected voids) to the bulk volume of the aquifer matrix. Effective porosity and any porosity data with secondary porosity information should not be used for this parameter.
Typical Values	Default values: Fine Sand: 0.40 (mid-range of values below) Silt: 0.48 (mid-range of values below) Clay: 0.47 (mid-range of values below) Sandstone/shale: 0.10 (Pankow and Cherry (1996), Table 12.2) Granite: 0.006 (Pankow and Cherry (1996), Table 12.2) Values for total porosity from Domenico and Schwartz (1990), in part from Davis (1969) and Johnson and Morris (1962), and as stated, Payne et al. (2008): **SEDIMENTARY** Porosity (-) Gravel, coarse: 0.24 - 0.36 Gravel, fine: 0.25 - 0.38 Sand, coarse: 0.31 - 0.46 Sand, fine: 0.26 - 0.53 (Payne et al., 2008, Table 2.3) Silt: 0.34 - 0.61 (Payne et al., 2008, Table 2.3) Clay: 0.34 - 0.60 (Payne et al., 2008, Table 2.3) **SEDIMENTARY ROCKS** Sandstone: 0.05 - 0.30 Siltstone: 0.21 - 0.41 Shale: 0 - 0.10 **CRYSTALLINE ROCKS** Dense crystalline rocks: 0 - 0.05 Koerner (1984) reports these values for unit weight for saturated soils (note no dry bulk density values are reported for these materials): Glacial till, very mixed grain: 0.20 Soft glacial clay: 0.57 Stiff glacial clay: 0.37 Soft slightly organic clay: 0.66 Soft very organic clay: 0.75 Soft bentonite: 0.84 One fractured microcrystalline limestone in Virginia had matrix porosities ranging from 0.0004 to 0.0065 (GSI Environmental).
Source of Data	Typically estimated. Occasionally obtained through physical property testing of site soil samples.
How to Enter Data	Enter directly.

PARAMETER	FRACTION ORGANIC CARBON (f_{oc})
Units	Unitless.
Description	Fraction of the aquifer soil matrix comprised of natural organic carbon in uncontaminated areas. More natural organic carbon means more adsorption of organic constituents on the aquifer matrix.
Typical Values	0.0002 – 0.02
Source of Data	The organic carbon value should be measured, if possible, by collecting a sample of aquifer material from an uncontaminated area and performing a laboratory analysis (e.g., ASTM Method 2974-87 or equivalent). If unknown, a default value of 0.001 is often used (LaGrega et al., 1994).
How to Enter Data	Enter directly.

PARAMETER	RETARDATION FACTOR (R_f)
Units	Unitless.
Description	Adsorption to the soil matrix can reduce the concentration of dissolved contaminants moving through the ground water. The retardation factor is the ratio of the groundwater seepage velocity to the rate that organic chemicals migrate in the ground water. A retardation value of 2 indicates that if the ground-water seepage velocity is 100 ft/yr, then the organic chemicals migrate at approximately 50 ft/yr. The degree of retardation depends on both aquifer and constituent properties.
Typical Values	1 to 6
Source of Data	Typically estimated from soil and chemical data (using variables described below) with the following expression: $R = 1 + K_d * \rho_b / n_e$ where $K_d = K_{oc} * f_{oc}$, ρ_b = bulk density, n_e = effective porosity, K_{oc} = organic carbon-water partition coefficient, K_d = distribution coefficient, and f_{oc} = fraction organic carbon in soil When biotransformation rates are insignificant, the retardation factor can be estimated by comparing the plume length of an adsorbed compound to the plume length of a conservative (non-adsorbing) compound.
How to Enter Data	1) Enter directly; or 2) Allow the tool to calculate automatically using constituent parameter values stored in the "Complex Model" tabs

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Appendix D:
Derivation of Correlations Between Biomarker Abundance and Biodegradation Rate Constants

Predicting Rate Constants From qPCR Biomarker Abundance

Aerobic biodegradation of 1,4-dioxane, and anaerobic biodegradation of 1,1,1-TCA; 1,1-DCA; 1,1-DCE and vinyl chloride follows Michaelis-Menten (Haldane) kinetics (Mahendra and Alvarez-Cohen, 2006; Mahendra et al., 2013; Ye et al. 2017; Grostern et al., 2009; Parthasarathy et al., 2015). The equation can be expressed as follows:

$$v_o = \frac{V_{max} * S}{K_m + S} \qquad \text{equation 1}$$

where v_o is the zero-order rate of degradation of the compound (mass per volume of water per time), S is the concentration of the organic chemical being degraded (mass per volume water), V_{max} is the maximum value of v_0 at large values of S, and K_m is the concentration of the chemical being degraded where $v_0 = 0.5\ V_{max}$.

A first-order rate constant describes the fraction of the material present that is degraded in any instant of time. The first order rate constant for biodegradation (k) of the organic compounds in groundwater can be calculated as follows:

$$k = \frac{v_o}{S} = \frac{V_{max}}{K_m + S} \qquad \text{equation 2}$$

Values for K_m and V_{max} are obtained from biodegradation studies using bacterial cultures in laboratories, and S is the concentration of the compound of concern in groundwater at the site.

The value for V_{max} reported in the literature is conventionally normalized to the quantity of cell protein in the experimental system (mass of contaminant degraded per mass of cell protein per time). We shall refer to these normalized parameters as $V_{max\ (protein)}$. The V_{max} in equation 2 is calculated by multiplying the $V_{max\ (protein)}$ by the concentration of cell protein in the system being described (mass per volume of water).

To use qPCR biomarkers to estimate rate constants, it is necessary to convert values of the $V_{max\ (protein)}$ to values of V_{max} normalized to the number of gene copies of the qPCR biomarker. Values of $V_{max\ (gene\ copies)}$ can be calculated by dividing $V_{max\ (protein)}$ by the number of gene copies in a unit mass of cell protein. A value of V_{max} as used in equation 2 is calculated by multiplying $V_{max\ (gene\ copies)}$ by the abundance of DNA targeted by the qPCR biomarker in a sample of groundwater. The equation for k is as follows:

$$k = \frac{v_o}{S} = \frac{V_{\max\ (gene\ copies)} * A}{K_m + S} \qquad \text{equation 3}$$

where A is the abundance of the qPCR biomarker.

When $V_{max\ (gene\ copies)}$ is in units of mg/(year * gene copy), A is in units of gene copies/L, and K_m and S are in units of mg/L, then k is in units of 1/year.

Table 1 summarizes values for $V_{max\ (gene\ copies)}$ and K_m used in equation 3 to calculate a first order rate constant in the decision support tools. The approach to determine the values for $V_{max\ (gene\ copies)}$ for each qPCR biomarker is described in the sections below.

Table 1. Values for $V_{max(gene\ copy)}$ and K_m used in equation 2 to estimate first order rate constants for biodegradation of 1,4-dioxane; 1,1,1-TCA; 1,1-DCA; 1,1-DCE; *cis*-DCE and Vinyl Chloride from their concentration in groundwater and the abundance of the associated qPCR biomarker.

Compound	qPCR biomarker	$V_{max\ (gene\ copy)}$	K_m
		mg/(year * gene copy)	mg/L
1,4-dioxane	DXMO	8.82E-07	6.3
1,4-dioxane	prmA	1.83E-06	78
1,4-dioxane	RMO	5.35E-08	28
1,4-dioxane	RDEG	1.84E-08	28
1,1,1-TCA	Dhb	6.11E-10	1.69
1,1-DCA	Dhb	5.77E-10	14.1
1,1-DCE	vcrA	1.98E-07	4.36
cis-DCE	vcrA	6.63E-07	0.32
Vinyl Chloride	vcrA	4.27E-07	0.16

Rate Constants for Degradation of 1,4-Dioxane from the DXMO, prmA, RDEG and RMO biomarkers

The DXMO biomarker amplifies DNA that codes for a monooxygenase in *Pseudonocardia dioxanivorans* CB1190 (Gedalanga et al., 2014). The prmA biomarker targets a monooxygenase in *Mycobacterium dioxanotrophicus* PH-06 (He et al., 2017). *Pseudonocardia dioxanivorans* CB1190 and *Mycobacterium dioxanotrophicus* PH-06 can grow on 1,4-dioxane as a primary substrate.

The RDEG biomaker targets toluene-2-monooxygenases, and the RMO biomarker target toluene-3- and toluene-4-monooxygenase genes (Wilson et al., 2019). *Berkholderia cepacia* G4 expresses toluene 2 monooxygenase and *Pseudomonas mendocina* KR-1 expresses the toluene 4 monooxygenase. *Berkholderia cepacia* G4 and *Pseudomonas mendocina* KR-1 can degrade 1,4-dioxane, but they cannot grow on it as a primary substrate (Mahendra and Alvarez-Cohen, 2006).

DXMO to Predict Degradation of 1,4-Dioxane

Barajas-Rodriquez and Freedman (2018) determined a normalized value of V_{max} for degradation of 1,4-dioxane by *P. dioxanivorans* CB1190 of 2.11 mg COD/mg COD/day, equivalent to a $V_{max\ (protein)}$ of 1.09E+03 mg 1,4-dioxane /(mg protein * year). Experiments reported in Section ____ determined that cultures of *P. dioxanivorans* CB1190 growing slowly on low concentrations of 1,4-dioxane have 1.24E+09 gene copies targeted by DXMO per mg cell protein. The value of $V_{max(gene\ copies)}$ for degradation of 1,4-dioxane by enzymes targeted by the DXMO biomarker would be 8.8E-07 mg/(gene copy * year).

Barajas-Rodriquez and Freedman (2018) provided a K_m of 11.5 mg COD/L equivalent to 6.3 mg 1,4-dioxane/L.

prmA to Predict Degradation of 1,4-Dioxane

Figure S1 of He et al. (2017) provides a normalized value for V_{max} for degradation of 1,4-dioxane by *M. dioxanotrophicus* PH-06 of 6.2 g/(day * gram protein), equivalent to a $V_{max\ (protein)}$ of 2.3E+03 mg 1,4-dioxane / (mg protein * year). *P. dioxanivorans* CB1190 and *M. dioxanotrophicus* PH-06 are both actinomycetes. If the ratio of gene copies to protein determined for *P. dioxanivorans* CB1190 (Section __) apply to mycobacteria, the value of $V_{max(gene\ copies)}$ for degradation of 1,4-dioxane by enzymes targeted by the prmA biomarker would be 1.8E-06 mg/ (gene copy * year).

Figure S1 of He et al. (2017) provided a K_m of 78 mg/L.

RMO to Predict Degradation of 1,4-Dioxane

Table 1 of Mahendra et al. (2013) provides two normalized value of V_{max} for degradation of 1,4-dioxane by *Pseudomonas mendocina* KR-1. They are 3.2 and 3.4 µmoles dioxane degraded/ (hour * mg protein). They are equivalent to an average $V_{max\ (protein)}$ of 2.6E+03 mg 1,4-dioxane / (mg protein * year).

Wilson et al. (2019) compared experimentally measured first order rate constants for cometabolism of TCE by groundwater bacteria to rates predicted using kinetic parameters. They got a useful match between measured and predicted rate constants if they assumed there was 2.1E-11 mg protein per cell.

The cells expressing the RMO biomarker should presumably be the same native population described in Wilson et al. (2019). Assuming one gene copy of the RMO biomarker per cell, that corresponds to 4.8E+10 gene copies/ mg protein. Dividing $V_{max\ (protein)}$ by the number of gene copies per mg protein produces a value for $V_{max\ (gene\ copies)}$ for degradation of 1,4-dioxane by enzymes targeted by the RMO biomarker of 5.4E-08 mg/ (gene copy * year).

Table 1 of Mahendra et al. (2013) provides values for K_m for 1,4-dioxane degradation by *P. mendocina* KR-1. The listed values are 0.15 and 0.49 µM. These values are only consistent with the literature (and with data in their Fig. 2) if the units for K_m are mM. Converted from units of mM the average value for K_m would be 28 mg/L.

RDEG to Predict Degradation of 1,4-Dioxane

No value of K_m is provided in the literature for biodegradation of 1,4-dioxane by the toluene 2 monooxygenase targeted by the RDEG biomarker. In the absence of a published value, we will assume the K_m for the toluene 2 monooxygenase is 28 mg/L, the same as the K_m for the toluene 2 monooxygenase targeted by the RMO biomarker.

Table 3 of Mehendra and Alvarez-Cohen (2006) provides a normalized rate constant for 1,4-dioxane degradation by *B. cepacia* G4 of 0.10 mg/ hr/mg protein at a 1,4-dioxane concentration of 50 mg/L. This concentration is almost twice the assumed value for K_m. The normalized rate constant is equivalent to $V_{max\ (protein)}$ of 8.8E+02 mg 1,4-dioxane / (mg protein * year). As was done above for the RMO biomarker, assuming one gene copy of the RDEG biomarker per cell, there should be 4.8E+10 gene copies/ mg protein. Dividing $V_{max\ (protein)}$ by the number of gene copies per mg protein produces a value for $V_{max\ (gene}$

$_{copies)}$ for degradation of 1,4-dioxane by enzymes targeted by the RDRG biomarker of 1.8E-08 mg/ (gene copy * year).

The value in Table 3 is v_0, not V_{max}. Using v_0 to calculate $V_{max\ (protein)}$ will underestimate $V_{max\ (protein)}$ and $V_{max\ (gene\ copies)}$. Any error is conservative in that it will underestimate the contribution of cells producing an enzyme targeted by the RDEG biomarker.

Rate Constants for Degradation of 1,1,1-TCA, 1,1-DCA, 1,1-DCE, *cis*-1,2-DCE and Vinyl Chloride from the Dhb and vcrA biomarkers

Sun et al. (2000) showed that a strain of *Dehalobacter* (TCA1) could grow by dechlorinating 1,1,1-TCA to 1,1-DCA, then dechlorinating 1,1-DCA to chloroethane. The Dhb biomarker targets DNA that codes for 16S ribosomal RNA in *Dehalobacter* (Grostern and Edwards, 2006).

The vcrA biomarker targets vinyl chloride reductase. This enzyme degrades 1,1-DCE and *cis*-DCE to vinyl chloride and vinyl chloride to ethene. The enzyme is expressed in some strains of *Dehalococcoides* bacteria, but not all of them (Lee et al., 2008).

Dhb to Predict Degradation of 1,1,1-TCA

Gostern and Edwards (2006) reported on an enrichment culture (MS) that degraded 1,1,1-TCA to 1,1-DCA and then to CA. Microcosms degrading 1,1,1-TCA contained 160 mL of culture media. After 46 µmoles of 1,1,1-TCA was degraded to CA (their FIG. 1), the abundance of the Dhb marker increased 7.4E+08 copies per mL (their FIG. 2) or 1.23E+11 copies per microcosm. Degradation of 46 µmoles of 1,1,1-TCA produced 1.23E+11 copies of the *Dehalobacter* 16S rRNA gene (Dhb). Degradation of 46 µmole of 1,1,1-TCA to CA releases 92 µmole of Cl^-. There were 1.34E+09 copies of Dhb produced per µmole of Cl^- produced.

Sun et al. (2002) reported that growth yield of *Dehalobacter* TCA1 from reductive dechlorination of 1,1,1-TCA to CA was 5.6 ± 1.26 gm (dry weight) cells per mole of chloride released. Assuming the cells are 50% protein on a dry weight basis, the yield is 2.8E-03 mg protein per µmole of chloride released.

Assuming that *Dehalobacter* in the MS culture had the same growth yield as *Dehalobacter* TCA1, dividing the estimate of gene copies of Dhb (per µmoles of Cl^- released) by the estimate of the protein produced (per µmoles of Cl^-released) provides an estimate of 4.8E+11 gene copies of Dhb per mg protein.

Table 2 of Grostern et al. (2009) provides data on the kinetics of biodegradation of 1,1,1-TCA by *Dehalobacter*. They conducted three experiments to determine the effect of TCE, *cis*-DCE and vinyl chloride on degradation of 1,1,1-TCA by a mixed culture dominated by *Dehalobacter* species. Inhibition followed a noncompetative model, which meant that inhibition did not affect the values of V_{max} and K_m for degradation of 1,1,1-TCA. In suspensions of whole cells, the values of a normalized V_{max} were 3.0, 4.5 and 5.0 nmole/ (minute * mg protein), which is equivalent to a value for $V_{max(protein)}$ of 2.9E+02 mg 1,4-dioxane / (mg protein * year). Dividing $V_{max\ (protein)}$ by the number of gene copies per mg protein produces a value for $V_{max\ (gene\ copies)}$ for degradation of 1,1,1-TCA by enzymes targeted by the Dhb biomarker of 6.1E-10 mg/ (gene copy * year).

Table 2 of Grostern et al. (2009) also provides values for Km of 1.5, 1.7, and 1.9 mg/L for an average of 1.7 mg/L.

Dhb to Predict Degradation of 1,1-DCA

Calculation of a rate constant for biodegradation of 1,1-DCA by *Dehalobacter* followed the same logic as described above for 1,1,1-TCA. As described in Grostern and Edwards (2006), microcosms degrading 1,1-DCA contained 120 mL of culture media. After 56 µmoles of 1,1-DCA was degraded to CA (their FIG. 1), the abundance of the Dhb marker increased 5.3E+08 copies per mL (their FIG. 2) or 6.36E+10 copies per microcosm. Degradation of 56 µmoles of 1,1-DCA produced 6.36E+10 copies of the *Dehalobacter* 16S rRNA gene (Dhb). Degradation of 46 µmole of 1,1-DCA to CA releases 46 µmole of Cl-. There were 1.14E+09 copies of Dhb produced per µmole of Cl^- produced.

Sun et al. (2002) reported that growth yield of *Dehalobacter* TCA1 from reductive dechlorination of 1,1,1-TCA to CA was 5.6 ± 1.26 gm (dry weight) cells per mole of chloride released. Assuming the cells are 50% protein on a dry weight basis, the yield is 2.8E-03 mg protein per µmole of chloride released.

Assuming that *Dehalobacter* in the MS culture had the same growth yield as *Dehalobacter* TCA1, dividing the estimate of gene copies of Dhb (per µmoles of Cl^- released) by the estimate of the protein produced (per µmoles of Cl^-released) provides an estimate of 4.1E+11 gene copies of Dhb per mg protein.

Table 2 of Grostern et al. (2009) provides data on the kinetics of biodegradation of 1,1-DCA by *Dehalobacter*. They conducted three experiments to determine the effect of TCE, *cis*-DCE and vinyl chloride on degradation of 1,1-DCA by a mixed culture dominated by *Dehalobacter* species. Inhibition followed a Michaelis-Menten model or a noncompetative model, which meant that inhibition did not affect the values of V_{max} and K_m for degradation of 1,1-DCA. In suspensions of whole cells, the values of a normalized V_{max} were 2.2, 2.2 and 5.6 nmole/ (minute * mg protein), which is equivalent to a value for $V_{max(protein)}$ of 2.3E+02 mg 1,4-dioxane / (mg protein * year). Dividing $V_{max\ (protein)}$ by the number of gene copies per mg protein produces a $V_{max\ (gene\ copies)}$ for degradation of 1,1-TCA by enzymes targeted by the Dhb biomarker of 5.8E-10 mg/ (gene copy * year).

Table 2 of Grostern et al. (2009) also provides values for K_m of 87, 147, and 192 µg/L for an average of 14.1 mg/L.

vcrA to Predict Degradation of *cis*-DCE

Cupples et al. (2004) evaluated the kinetics of dechlorination of *cis*-DCE by a mixed culture containing *Dehalococcoides* strain VS, which expresses vinyl chloride reductase. They reported a value for V_{max} that is normalized to the number of *Dehalococcoides* cells in the culture. That value is 7.8E-10 µmole Cl^- released $(cell*day)^{-1}$. The number of cells was calculated using an indirect method that was ultimately based on the number of gene copies for 16S ribosomal RNA in the culture as determined using the Dhc biomarker. Assuming there is one gene copy targeted by the vcrA biomarker in each cell, the $V_{max\ (gene\ copies)}$ for degradation of *cis*-DCE by enzymes targeted by the vcrA biomarker is 6.6E-07 mg/ (gene copy * year).

Haston and McCarty (1999) provide a value for K_m of 3.3 µM, equal to 0.32 mg/L.

vcrA to Predict Degradation of Vinyl Chloride

Cupples et al. (2004) evaluated the kinetics of dechlorination of vinyl chloride by a mixed culture containing *Dehalococcoides* strain VS, which expresses vinyl chloride reductase. They reported a value for V_{max} that is normalized to the number of *Dehalococcoides* cells in the culture. The value is 7.8E-10 µmole Cl^- released (cell*day)$^{-1}$. Assuming one copy of the gene targeted by vcrA in each cell, the $V_{max\ (gene\ copies)}$ for degradation of vinyl chloride by enzymes targeted by the vcrA biomarker is 4.3E-07 mg/ (gene copy * year).

Haston and McCarty (1999) provide a value for K_m of 2.6 µM, equal to 0.25 mg/L.

vcrA to Predict Degradation of 1,1-DCE

The are no studies in the literature on the kinetics of 1,1-DCE degradation in strains of *Dehalococcoides* bacteria. To facilitate the production of large quantities of vinyl chloride reductase for characterization of the enzyme, Parthasarathy et al. (2015) constructed a plasmid containing DNA for the enzyme, then used the plasmid to create a transformed stain of *Escherichia coli* that produced vinyl chloride reductase. They then isolated and purified the enzyme and studied its properties. In their Table 1, they provide values of a normalized V_{max} for 1,1-DCE and VC. The ratio of the value for V_{max} for 1,1-DCE to the value for vinyl chloride was 0.298. The $V_{max\ (gene\ copies)}$ for vinyl chloride of 2.8E-08 mg/ (gene copy * year) as calculated in the section above was multiplied by 0.298 to estimate a $V_{max\ (gene\ copies)}$ for 1,1-DCE degradation by enzymes targeted by the vcrA biomarker of 2.0E-07 mg/(gene copy * year).

Parthasarathy et al. (2015) reported a K_m for vinyl chloride in their cell-free system of 10 µM, in reasonable agreement with the K_m of 2.6 µM reported by Cupples et al. (2004) for whole cells. Parthasarathy et al. (2015) reported a K_m for 1,1-DCE in their cell-free system of 45 µM or 4.4 mg/L. In the absence of a value of K_m determined on whole cells, the value of 4.4 mg/L will be used to estimate rate constants for degradation.

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Appendix E:
Results of Beta Testing of Decision Framework

Beta Testing Sessions for Project Tools

April 2021

Beta testing sessions for the updated BioPIC and the MNA Rate Constant Estimator were held in April 2021 during two remote, 1-hour video sessions. A total of 10 environmental professionals graciously volunteered their time and were able to attend the training. The list included attendees from USEPA, NAVFAC, Pacific Northwest National Laboratory, state agencies, and private industry. Attendees provided comments during the sessions, and several also provided additional responses to specific questions via email following the sessions. Comments and other feedback were addressed by revising the tools to the extent practical.

Summary of Attendee Responses to Specific Questions

1. Do you work at site(s) where 1,4-dioxane is present with chlorinated solvents?

- All but one attendee answered "yes".

2. Do you work at site(s) where MNA has been used as part of the management strategy?

- All attendees answered "yes".

3. Was the tool easy to use?

- All attendees answered "yes".

4. List primary strength(s) of the tool.

- Simplicity.
- The "help function" with the user guide linking to existing MNA reference documents was very useful in framing the process.
- The tool is well structured and organized.
- It is easy to follow and can help practitioners quickly identify data gaps for MNA evaluation.
- Easy to use.
- Similar to previous tools and therefore provides a degree of built-in familiarity.
- Provides a very useful result with minimal input data requirements.

5. List primary limitation(s) of the tool.

- As the BioPIC software was a type of flowchart, I found myself wanting to be able to reference something in the user guide. (**Project Team Response:** The flowcharts were added to the User's Guide.)
- It would have been nice to be able to add well names to the MNA Rate Constant Estimator. (**Project Team Response:** Since the tool is an unlocked Excel file, it is easy for users to modify if they choose. Since multiple attendees mentioned including well names, this revision was made to Box 5 of the MNA Rate Constant Estimator.)

- I didn't see any governing equations in the user's guide. I noted the citation to Wexlar (1992) on PDF page 4, but the sentence says it's a modified version of Wexler (1992). I think the user guide should either have the equations or cite another document where the all the mathematical details of this particular model can be found (project report maybe?) (**Project Team Response:** The relevant equations were added to the User's Guide.)
- Was any consideration given to including a power-law model for the source term as developed by Falta et al. in REMChlor? I believe that represents the current "state-of-the-art" in screening level approximations of contaminant source zones, although it does come at the price of needing additional input data. (**Project Team Response:** We do agree that the gamma function is likely more applicable behavior for a wider variety of sites. However, for simplicity, we decided to use a simple exponential decay term as opposed to replacing the gamma function. The exponential decay is a "middle of the road" selection within the gamma model, so it is considered relatively representative. We also feel that some users may not be comfortable with the slightly higher level of data input and interpretation that be required with using the gamma term.)
- A minor issue: no page numbers in either user's guide. (**Project Team Response:** Page numbers were added.)
- In future work, there is a real need to include back diffusion into the attenuation rate. (**Project Team Response:** We agree that diffusion is only captured as a bulk attenuation mechanism in the current version of the tool, and it cannot account for longer-term persistence. However, as a screening-level model, including back diffusion in as a separate mechanism was beyond the scope of this project.)
- Need better understanding of how model handles declining source and contaminants are flushed out of source zone. Instead of simple 1st order decay of source, recommend 'Gamma' model from RemChlor. (**Project Team Response:** See response above to similar comment from another attendee.)
- For the BioPIC tool, the paragraph below may also consider the recent knowledge obtained from SERDP ER-2303 about aerobic cometabolic biodegradation of 1,4-dxiaone at the low concentration range (< 100 ug/L). PPO is not likely a primary monooxygenase that degrades 1,4-dxoane, it may be just co-expressed with other monooxygenases. I recommend to solicit Dr. Michael Hyman's opinions on the likely candidate monooxygenase enzymes that may degrade 1,4-dioxane at low concentrations and incorporated into this tool and final report. (**Project Team Response:** Mike Hyman was contacted and graciously provided substantial text that was incorporated both into the BioPIC User's Guide as well as the project report.)

6. *Briefly summarize the results of any site-specific testing of the tool that you did on your own. If you attempted to fit site-specific field data, what were the estimated biodegradation rate constants?*

- Did some site specific testing of a site. This was limited, as the facility is in the process of collecting additional MNA and biomarker info. I hope to be able to go back to the tool following the additional data collection efforts.
- My project sites do not show signs of MNA for 1,4-D
- I had hoped to do this, but time limitations got in the way.

SUMMARY OF OTHER FEEDBACK FROM ATTENDEES

(Paraphrased)

- I think the caveat below (or something equivalent) may also be considered as part of the limitations for the third bullet on p. 4 of the User's Guide for the MNA Rate Constant Estimator "Note that contaminants may be subject to metabolic biodegradation process in more upgradient portion of the plume where the concentration levels are high and subject to cometabolic biodegradation in more downgradient portion of the plume where the concentration levels are lower. Using a single first-order kinetic parameter to simulate the overall plume behavior may be inadequate." In such cases, one can divide the plume into two portions and analyze them separately. You may consider reminding the users about this possibility. (**Project Team Response:** This text was incorporated into the revised User's Guide.).
- For MNA Rate Constant Estimator user friendliness, it would be really nice to have a row in Box 5 where users can enter the name of monitoring wells they are entering data for. I found myself creating a separate spreadsheet just to do this, and it seemed extraneous and possibly more error prone. (**Project Team Response:** Well names can now be entered in Box 5).
- Overall, I found both tools very helpful. I did want to share them with a couple of my colleagues when the beta testing is finished.
- In guidance, provide effective date of any information that might change over time. For example, regulatory values for 14D standards will likely change over time. Need to put date on table so users know how old the data are. (**Project Team Response:** The date when this information was compiled (January 1, 2021) is now included in the User's Guide for BioPIC.)
- My understanding is that BioChlor only allows you to use one concentration at each monitor location. In most cases we have data that extends over many years. It would be very helpful to be able to plot multiple measurements to see the data 'cloud'. If not feasible, can you provide guidance on what type of average should be entered? (arithmetic or geometric mean? Do you leave in outliers). (**Project Team Response:** Including a "data cloud" would be interesting approach but was beyond the scope of this project. Instead, the reviewer's suggestion was addressed by adding text to the User's Guide that explains different approaches when data from multiple events are available.)
- When searching for the coefficient that provides the best fit to the model, I believe it is preferable to use the Normalized root mean square error, not regular RMSE. (**Project Team Response:** This was addressed through a revision in the way the RMSE was calculated. Specifically, the data are now log-normalized before calculating the RMSE. The resulting output is similar to what would be generated if a Normalized root mean square error was used as the optimization parameter.)
- In presentation, qPCR results were presented in copies per liter. Microbial Insights typically reports values in copies per mL. Need to reminder to users to make conversion if needed. (**Project Team Response:** The units in both User's Guide for both BioPIC and the MNA Rate Constant Estimator are copies per mL. It was only the presentation that used copies per L, so no additional changes to the deliverables were required.)