FINAL REPORT

Bioaugmentation for Aerobic Bioremediation of RDX-Contaminated Groundwater

ESTCP Project ER-201207

JUNE 2016

Dr. Mandy Michalsen, P.E.
US Army Engineer and Research Development Center

Dr. Fiona Crocker
Dr. Karl Indest
Dr. Carina Jung
US Army Engineer and Research Development Center

Dr. Mark Fuller
Dr. Paul Hatzinger
CB&I Federal Services

Jack Istok, P.E.
School of Civil and Construction Engineering

Distribution Statement A
This document has been cleared for public release

Page Intentionally Left Blank

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.

Page Intentionally Left Blank

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to the Department of Defense, Executive Services and Communications Directorate (0704-0188). Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.
PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ORGANIZATION.

1. REPORT DATE *(DD-MM-YYYY)*
01-06-2016

2. REPORT TYPE
Technical Report

3. DATES COVERED *(From - To)*

4. TITLE AND SUBTITLE
FINAL REPORT
Bioaugmentation for Aerobic Bioremediation of
RDX-Contaminated Groundwater

5a. CONTRACT NUMBER
N/A

5b. GRANT NUMBER
ER-201207

5c. PROGRAM ELEMENT NUMBER
N/A

6. AUTHOR(S)
Mandy Michalsen; Fiona Crocker; Karl Indest; Carina Jung - ERDC
Mark Fuller; Paul Hatzinger - CB&I
Jonathan Istok - Oregon State University

5d. PROJECT NUMBER
372856

5e. TASK NUMBER
N/A

5f. WORK UNIT NUMBER
N/A

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
US Army Engineer and Research Development Center (ERDC), 3909 Halls Ferry Rd, Vicksburg, MS 39180; CB&I Federal Services, 17 Princess Road, Lawrenceville, NJ 08648; Oregon State University, School of Civil & Const. Engineering, Corvallis, OR 3

8. PERFORMING ORGANIZATION REPORT NUMBER
N/A

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Environmental Security Technology Certification Program (ESTCP)
4800 Mark Center Drive, Suite 17D08, Alexandria, VA 22350-3605

10. SPONSOR/MONITOR'S ACRONYM(S)
ESTCP

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This project demonstrated an innovative application of bioaugmentation to enhance the biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in contaminated groundwater under aerobic conditions. RDX is mobile and persistent in aerobic groundwater and typically forms large, dilute plumes that are difficult and costly to remediate using conventional technologies such as pump and treat or anaerobic biostimulation. The Umatilla Chemical Depot (UMCD) in Umatilla, OR was selected as the field site for this demonstration. The principal demonstration objectives were: (1) to select and optimize RDX-degrading microbial cultures for use in aerobic bioaugmentation at the UCMD, (2) to compare in situ RDX biodegradation rates for aerobic bioaugmentation to those for biostimulation, and (3) to quantify and compare costs of RDX. This final report presents a comprehensive summary of demonstration activities and results.

15. SUBJECT TERMS
Bioaugmentation, groundwater, remediation, explosives, RDX

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE		259	Mandy Michalsen
					19b. TELEPHONE NUMBER *(Include area code)* 206-764-3324

Standard Form 298 (Rev. 8/98)
Prescribed by ANSI Std. Z39.18

Page Intentionally Left Blank

TABLE OF CONTENTS

ACKNOWLEDGEMENTS ..... **iv**
EXECUTIVE SUMMARY ..... **v**
1.0 INTRODUCTION **1**
1.1 BACKGROUND 1
1.2 OBJECTIVES OF THE DEMONSTRATION 1
1.3 REGULATORY DRIVERS 2
2.0 TECHNOLOGY **3**
2.1 TECHNOLOGY DESCRIPTION 3
2.2 ADVANTAGES AND LIMITATIONS OF THE TECHNOLOGY 3
3.0 PERFORMANCE OBJECTIVES **5**
4.0 SITE DESCRIPTION **9**
4.1 SITE SELECTION 9
4.2 SITE GEOLOGY/HYDROGEOLOGY 10
4.3 CONTAMINANT DISTRIBUTION 12
5.0 TEST DESIGN **13**
5.1 CONCEPTUAL EXPERIMENTAL DESIGN 13
5.2 SITE CHARACTERIZATION 13
5.2.1 METHODS 14
5.2.2 RESULTS 16
5.2.3 DISCUSSION 19
5.3 PHASE I LABORATORY TESTING 19
5.3.1 Bioaugmentation Culture Optimization 20
5.3.2 Microcosm studies 23
5.3.3 Column Transport Experiments 24
5.3.4 Field Scale Culture Production 29
5.3.5 Phase I Recommendations 35
5.4 PHASE II FIELD-SCALE CELL TRANSPORT TESTING 35
5.4.1 METHODS 36
5.4.2 RESULTS 38
5.4.3 DISCUSSION AND RECOMMENDATIONS FROM PHASE II 41
5.5 PHASE III: BIOAUGMENTATION FIELD TRIAL AND PUSH-PULL TESTING 42
5.5.1 METHODS 42
5.5.2 RESULTS AND DISCUSSION 44
6.1 Quantitative Performance Objectives 50
6.2 Qualitative Performance Objectives 53
7.0 COST ASSESSMENT **54**
7.1 COST MODEL 54
7.2 COST DRIVERS 55
7.3 COST ANALYSIS 57
8.0 IMPLEMENTATION ISSUES **67**
9.0 REFERENCES **70**
APPENDICES **75**

LIST OF TABLES

Table 3.1. Demonstration performance objectives.....6
Table 5.1. RDX-degrading microbial strains included in the initial screening. 20
Table 5.2. Comparison of compositions of UMCD site groundwater and artificial groundwater (AGW) used during Phase I.....21
Table 5.3. Experimental conditions for the column transport experiments. 26
Table 5.5. Calculated retardation factors (R) for aerobic RDX-degrading bacteria in Umatilla sediments based on *xplA* gene copy numbers. 41
Table 5.6. Effects of fructose additions on groundwater composition and on *in situ* RDX degradation rates during aerobic and anaerobic biostimulation and aerobic bioaugmentation. 45
Table 6.1. Comparison of RDX degradation rate and time required to reach remedial action criteria for all treatments. 50
Table 6.2. Comparison of RDX degradation rate and time required to reach remedial action criteria for all treatments. 51
Table 6.3. Comparison of RDX mass degraded per mass of added fructose for all treatments ...52
Table 6.4. Characterization of groundwater microbial community during Phase III PPTs. 53
Table 6.5. Comparisons of groundwater geochemical data for all treatments 53
Table 7.1. RDX groundwater remedy optimization with bioremediation cost model with demonstration-specific cost details provided. 56
Table 7.2. Cost estimate for enhanced pump & treat only, no bioremediation (30 yrs, $K) 63
Table 7.3. Cost estimate for enhanced pump & treat with phased anaerobic biostimulation (11 years, $K) 64
Table 7.4. Cost estimate for enhanced pump & treat with phased, combined anaerobic biostimulation and aerobic bioaugmentation (11 years, $K).....65

LIST OF FIGURES

Figure 4.1. ESTCP demonstration location at the Umatilla Chemical Depot (UMCD). 9
Figure 4.2. UMCD site stratigraphy. 11
Figure 4.3. RDX concentrations in groundwater at UMCD (SCS, 2010). 12
Figure 5.1. Conceptual design for demonstration Phase III. 13
Figure 5.2. Installation of DW-1 in aerobic and anaerobic biostimulation test plot using air rotary method; sediment from screened interval shown in insets. 14
Figure 5.3. Demonstration well locations in test plots 1 and 2 15
Figure 5.4. Relative bromide concentrations measured during the May, 2012 tracer tests. 17
Figure 5.5. Tracer breakthrough curves during natural gradient tests 18
Figure 5.6. Tracer breakthrough curves during forced gradient tests. Tracer was injected into wells DW-1 and DW-2. 18
Figure 5.7. Survival of RDX-degrading strains in artificial groundwater (AGW) amended with 1 mM fructose and 1.3 mg L^{-1} RDX. Cultures were starved for 24 h in AGW without a carbon or nitrogen source. 22
Figure 5.8. Degradation of RDX by RDX-degrading strains in artificial groundwater amended with 1 mM fructose and 1.3 mg L^{-1} RDX. Cultures were starved for 24 h in artificial groundwater without a carbon or nitrogen source. 22
Figure 5.9. Growth physiology of select RDX-degrading strains in artificial groundwater amended with fructose and RDX. *Williamsia* sp. KTR4 (A) grew homogeneously while *G. polyisoprenivorans* pGKT2 cells (B) aggregated. 23
Figure 5.10. Bacterial viable cell numbers on 20% LB + 50 mg L^{-1} kanamycin agar plates (A) and cetrimide:nalidixic acid agar plates (B) from uninoculated and inoculated UMCD microcosms. Bacterial counts from the uninoculated microcosms are total bacterial counts on both types of media. Bacterial counts from the inoculated microcosms represent the individual strains: KTR9, RHA1, or I-C as indicated in each panel. 23
Figure 5.11. RDX concentrations in UMCD microcosms uninoculated (control) and inoculated with strains KTR9, RHA1, and PsIC. 24
Figure 5.12. Column experimental setup. 25
Figure 5.13. Breakthrough curves for Br^-, cells, and RDX for Column Experiment 1. The initial breakthrough results and the results of the entire experiment are shown, where C is the measured tracer or cell concentration in the column effluent; Co is the influent tracer or cell concentration. 27
Figure 5.14. Breakthrough curves for Br^-, cells, and RDX for Column Experiment 2.....28

LIST OF FIGURES (continued)

Figure 5.15. Breakthrough curves for Br^- and RDX for Column Experiment 3, where C is the measured tracer or cell concentration in the column effluent; Co is the influent tracer or cell concentration. 28

Figure 5.17. Photographs of benchtop (left) and 750-L (right) bioreactors. 31

Figure 5.18. Change in culture densities (relative to the initial OD_{550}) of pilot-scale cultures of the three RDX degrading strains (A, KTR9; B, RHA1; C, I-C) over time during incubation at 4°C (solid lines) and 37°C (dashed lines). 32

Figure 5.19. Change in cultivable cell densities of pilot-scale cultures of the three RDX degrading strains (A, KTR9; B, RHA1; C, I-C) over time during incubation at 4°C (solid lines) and 37°C (dashed lines). 33

Figure 5.22. Photographs of 20-L soda keg used for culture starvation and shipping. 36

Figure 5.21. Well layout for phase II cell transport test. 37

Figure 5.23. Cl^- breakthrough curves in the injection well (◆), DW-2, and monitoring well, 4-106 (□). 38

Figure 5.24. Viable plate counts on (A) LBKan agar plates of KTR9 (■) and RHA1 (△) from wells DW-2 (dashed lines) and 4-106 (solid lines); and (B) 5% PTYG agar plates of heterotrophic groundwater bacteria plus bioaugmentation culture from wells DW2 (■), 4106 (◆), and EW2 (▲). 39

Figure 5.25. qPCR analysis of UMCD groundwater samples from wells DW-2 (■), 4-106 (▲), and EW-2 (◆) targeting the (A) 16S rRNA genes, (B) *xplA* gene, (C) kanamycin gene, and (D) *xenB* gene. 40

Figure 5.26. Well layout for Phase III push-pull tests 42

Figure 5.27. Example push-pull test results in MW-28 showing *in situ* RDX degradation and first-order model fits following biostimulation. 46

Figure 5.28. Viable KTR9 colony counts and *xplA* gene copy numbers during aerobic bioaugmentation. Arrows refer to 1st, 2nd, and 3rd push-pull tests used to measure *in situ* RDX degradation rates. 47

Figure 5.29. Push-pull test results in DW-2 showing *in situ* RDX degradation and first-order model fits following bioaugmentation. 48

Figure 7.1. Schematic of costed remedy scenarios at UMCD. Ovals represent relative groundwater plume size. 59

LIST OF APPENDICES

APPENDIX A – SITE CHARACTERIZATION RESULTS MEMORANDUM
APPENDIX B – PHASE I RESULTS MEMORANDUM
APPENDIX C – PHASE II RESULTS MEMORANDUM
APPENDIX D – TSCA/TERA PERMIT FOR USE OF GMO
APPENDIX E – PHASE III ANALYTICAL AND FIELD DATA

LIST OF ACRONYMS

°C	Degrees Celsius
µg	Microgram
16S rRNA	16S ribosomal RNA gene
AGW	Umatilla artificial groundwater
AeroBioaug	Aerobic bioaugmentation
AnaerobBiostim	Anaerobic biostimulation
bioaug	Bioaugmentation
biostim	Biostimulation
BSM	Basal salts medium
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
CFU	Colony forming unit
DNX	Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine
EPA/USEPA	United States Environmental Protection Agency
FFS	Focused Feasibility Study
g	Gram
GMOs	Genetically-modified organisms
gpm	Gallons per minute
h	Hour(s)
HGT	Horizontal gene transfer
IC	Ion chromatography
Kan^R	Kanamycin resistance
L	Liter
MCL	Maximum contaminant level
MEDINA	Methylenedinitramine
min	Minutes(s)
mL	Milliliter
MNX	Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine
MW	Monitoring well
NCP	National Contingency Plan
NDAB	4-Nitro-2,4-diazabutanal
OD	Optical density
ORP	Oxidation reduction potential
P&T	Pump and treat
PPT	Push-pull test
PV	Pore volume
qPCR	Quantitative polymerase chain reaction
RDX	Hexahydro-1,3,5-trinitro-1,3,5-triazine
TERA	Toxic Substance Control Act Experimental Release Applications
TNT	Trinitrotoluene
TNX	Hexahydro-1,3,5-trinitroso-1,3,5-triazine
TSCA	Toxic Substance Control Act
UMCD	Umatilla Chemical Depot
USACE	United States Army Corps of Engineers
xenA	Xenobiotic reductase A gene
xenB	Xenobiotic reductase B gene
xplA	Flavodoxin cytochrome P450 gene
XplA	Flavodoxin cytochrome P450 protein
XplAB	Flavodoxin cytochrome P450 and flavodoxin reductase proteins

ACKNOWLEDGEMENTS

This report was prepared by the U.S. Army Corps of Engineers, Seattle District and the U.S. Army Engineer Research and Development Center (ERDC), Environmental Laboratory, Vicksburg, MS in partnership with Oregon State University (OSU) and CB&I Federal Services (CB&I). The research was sponsored by the Environmental Security Technology Certification Program (ESTCP), Arlington, VA, Dr. Andrea Leeson, Project Manager, under Project Number ER 201207. The principal investigator was Dr. Mandy M. Michalsen, P.E. Co-principal investigators were Drs. Fiona Crocker, Karl Indest and Carina Jung (ERDC), Drs. Mark Fuller and Paul Hatzinger (CB&I), and Professor Jonathan Istok (OSU).

The authors acknowledge the assistance of the following organizations and people for their collaborative efforts in support of this project. Dr. Hillary Eaton (Badger Technical Services, Vicksburg, MS) and Mrs. Dawn Hancock (ERDC) for their assistance with the optimization and analysis of the qPCR assays. The assistance of Dr. Jed Eberly and Mr. Matthew Carr (ERDC, Vicksburg, MS) and Mr. Michael Jung and Mr. Gary Blakeney (Badger Technical Services, Vicksburg, MS) with sample processing is greatly appreciated. Aaron King from Seattle District USACE for his exhaustive efforts during the Phase III field work, as well as Rebecca Weiss for assistance with preparation of this final report.

At the time of publication of this report, Col. Bryan S. Green was Commander of ERDC and Dr. Jeffery P. Holland was the Director.

EXECUTIVE SUMMARY

This project demonstrated an innovative application of bioaugmentation to enhance the biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in contaminated groundwater under aerobic conditions. RDX is mobile and persistent in aerobic groundwater and typically forms large, dilute plumes that are difficult and costly to remediate using conventional technologies such as pump and treat or anaerobic biostimulation. The Umatilla Chemical Depot (UMCD) in Umatilla, OR was selected as the field site for this demonstration. The principal demonstration objectives were: (1) to select and optimize RDX-degrading microbial cultures for use in aerobic bioaugmentation at the UCMD, (2) to compare *in situ* RDX biodegradation rates for aerobic bioaugmentation to those for biostimulation, and (3) to quantify and compare costs of RDX remediation in groundwater and time-to-complete at UMCD using aerobic bioaugmentation, conventional pump and treat, and both anaerobic and aerobic biostimulation without bioaugmentation. The performance objectives for this demonstration included:

(1) Aerobic bioaugmentation degrades RDX to < 2.1 $\mu g\ L^{-1}$.

(2) RDX removal rate for aerobic bioaugmentation is comparable to removal rates for aerobic and anaerobic biostimulation.

(3) RDX mass removed per mass of substrate added is enhanced for aerobic bioaugmentation compared to aerobic and anaerobic biostimulation, and

(4) Bioaugmentation culture remains viable and retains RDX-degrading capability over time *in situ*.

Several strains of RDX degrading bacteria were initially evaluated in laboratory studies (Phase I) to assess RDX degradation rates on various substrates, as well as their growth, viability and transportability under simulated field conditions. Field testing was conducted with selected strains to evaluate the transportability of RDX degrading strains (Phase II). An extensive series of field tests (Phase III) were conducted to compare the rate and extent of RDX degradation following bioaugmentation to two conventional treatments: aerobic biostimulation and anaerobic biostimulation.

Aerobic biostimulation was accomplished by five injections of 6 m^3 site groundwater containing low concentration fructose (0.25 to 1 mM) into two adjacent wells over 50 days to stimulate the growth of indigenous organisms with the ability to degrade RDX. After push-pull tests were conducted in all wells to measure RDX degradation rates, six additional, higher concentration (15 to 24 mM) fructose additions over 50 days were used to create anaerobic conditions in those same wells. Average RDX degradation rates (all wells combined) for aerobic and anaerobic biostimulation were 0.49 and 0.67 day^{-1}, respectively. Rates for aerobic biostimulation were not significantly different from zero (p values ≥ 0.060).

Three additional wells were bioaugmented by injecting 6 m^3 of site groundwater amended with RDX, tracer, fructose, and 10^8 cells mL^{-1} of *Gordonia* sp. KTR9 Kan^R (KTR9). Rates of RDX degradation were measured three times, once immediately following initial bioaugmentation with KTR9 (1st test) and twice more over a period of 130 days. The results indicated that aerobic bioaugmentation achieved a rate and extent of RDX degradation comparable to anaerobic biostimulation, while requiring substantially less added substrate. The average RDX degradation rate (all wells combined) for aerobic bioaugmentation, was 1.2 day^{-1}.

The cost-benefit analysis completed for this demonstration was based on groundwater remedy optimization work completed at UMCD. Cost estimates were developed for the following four UMCD groundwater remedy optimization scenarios:

(1) Installation of additional extraction wells for enhanced pump & treat;
(2) Enhanced pump & treat followed by anaerobic biostimulation in the remaining smaller plume footprint;
(3) Enhanced pump & treat followed by a combination of anaerobic biostimulation and aerobic bioaugmentation in the remaining smaller plume footprint; and
(4) Enhanced pump & treat followed by a combination of anaerobic and aerobic biostimulation in the smaller plume footprint.

KTR9 (and other *xplA* gene-containing microbes) are able to utilize RDX as a nitrogen source for growth and thus promote RDX degradation; however, these bacteria are not able to use (or degrade) TNT. Therefore, scenario 3 includes application of aerobic bioaugmentation for the distal RDX plume only. Anaerobic biostimulation effectively degrades both RDX and TNT and is therefore well suited for remediation of comingled explosives present near the source area. Assuming a 1.4% discount rate, the total estimated costs to implement scenarios 1 through 4 above were $11.9M, $10.3M, $10.7M, and $9.6M, respectively. Aerobic biostimulation performed poorly but scenario 4 was costed for completeness. Including aerobic bioaugmentation as part of the bioremediation strategy at UMCD has the potential to save over $1M in costs, prevent anaerobic groundwater quality impacts over a large portion of the distal RDX groundwater plume, and achieve cleanup in 15 years compared to scenario one, which is predicted to achieve cleanup in 30 years.

Aerobic bioaugmentation satisfied the performance objectives listed above and is considered the first successful demonstration of bioaugmentation for treatment of RDX-contaminated groundwater plumes. Demonstration results are being used to optimize the existing pump-and-treat groundwater remedy at UMCD by supporting incorporation of bioaugmentation into a full-scale remediation program. Cost and performance data from this demonstration concerning the utilization of aerobic bioaugmentation for full-scale RDX groundwater treatment will benefit other DoD sites with large RDX plumes as well, including Milan Army Ammunition Plant, TN, Fort Wingate, NM, former Hastings Naval Ammunition Depot, NE, former Nebraska Ordnance Plant, NE, and Massachusetts Military Reservation.

Although the aerobic bioaugmentation demonstration was considered successful, it is not possible based on demonstration results alone to know if aerobic bioaugmentation would provide sustained, more cost effective RDX removal compared to biostimulation. Therefore, as with all bioremediation remedies, a phased and flexible approach should be accounted for during design. Specific design elements of the amendment injection and circulation system would include the ability to:

- isolate aerobic and anaerobic treatment areas;
- accommodate injection of cells during bioaugmentation as well as substrate injections; and
- convert aerobic treatment areas into anaerobic treatment areas should performance data suggest the need to do so.

1.0 INTRODUCTION

1.1 BACKGROUND

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a common contaminant in soils and groundwater at military sites worldwide. RDX can be mobile and persistent in groundwater under the aerobic conditions present in many aquifers and thus tends to form large, dilute plumes. Although multiple studies have demonstrated *in situ* RDX biodegradation under anaerobic conditions, creating and maintaining anaerobic conditions across large areas is costly and technically challenging. In bioaugmentation, selected microbial cultures are injected into an aquifer to increase numbers of organisms that are efficient at degrading a particular contaminant, thereby increasing *in situ* biodegradation rates. Bioaugmentation is a well established remediation technology for anaerobic biodegradation of chlorinated solvents [1-5] but has not been previously demonstrated to enhance RDX biodegradation in contaminated groundwater. Bioaugmentation with *xplA*-containing strains *Rhodococcus rhodochrous* 11Y in soil [6] and *Rhodococcus* sp. DN22 in soil slurries [7, 8] was observed to enhance RDX removal kinetics [7, 8]; and strain DN22 was also shown to transport well through sand columns [8]. In addition, aerobic RDX degradation by these *Rhodococcus* sp. does not produce toxic nitroso end-products. This project demonstrated an innovative application of bioaugmentation to enhance *in situ* remediation of RDX-contaminated groundwater under aerobic conditions. This approach has the potential to be less costly and more easily implemented for large plumes than anaerobic biostimulation, and should avoid groundwater quality degradation caused by anaerobic processes.

1.2 OBJECTIVES OF THE DEMONSTRATION

The Umatilla Chemical Depot (UMCD) in Umatilla, OR was selected as the field site for this demonstration. RDX is widespread at UMCD in an aerobic, highly permeable groundwater aquifer. RDX concentrations range from 2 to 300 $\mu g\ L^{-1}$ over the estimated 150 ha plume. Results of this demonstration are being used to optimize the existing pump-and-treat groundwater remedy at UMCD by supporting incorporation of bioaugmentation into a full-scale remediation program.

The objectives for this demonstration were to:

(1) To select and optimize RDX-degrading, *xplA*-containing microbial cultures for use in bioaugmentation at the UCMD;
(2) To compare *in situ* RDX biodegradation rates and RDX mass removed per mass of substrate added for aerobic bioaugmentation to those for conventional anaerobic and aerobic biostimulation; and
(3) To quantify and compare costs of RDX remediation in groundwater and time-to-complete at UMCD using pump and treat, aerobic bioaugmentation, conventional anaerobic biostimulation, and aerobic biostimulation without bioaugmentation.

1.3 REGULATORY DRIVERS

There are currently no federal drinking water standards (maximum contaminant level MCL) for RDX; however, the U.S. Environmental Protection Agency (USEPA) has listed RDX on the Drinking Water Candidate Contaminant List (http://water.epa.gov/scitech/drinkingwater/dws/ccl/ccl3.cfm) and the Unregulated Contaminant Monitoring Regulation List (http://water.epa.gov/lawsregs/rulesregs/sdwa/ucmr/factsheet.cfm). In addition, the EPA has issued lifetime Health Advisory Limits (Maximum Contaminant Goal Levels) of 2 $\mu g\ L^{-1}$ for RDX. The risk-based cleanup goal based on residual carcinogenic risk of $1x10^{-6}$ is 0.8 $\mu g\ L^{-1}$ for RDX. The State of Oregon has not issued Groundwater Protection Standards for RDX. The remedial action criteria concentration established in the UMCD Record of Decision for RDX was 2.1 $\mu g\ L^{-1}$.

2.0 TECHNOLOGY

2.1 TECHNOLOGY DESCRIPTION

During bioaugmentation, selected microbial cultures are injected into an aquifer to increase the numbers of organisms that are effective at degrading a particular contaminant, thereby increasing *in situ* biodegradation rates. Although anaerobic bioaugmentation is a well-established remediation technology for chlorinated solvents [1-5], it has not been previously demonstrated for explosives like RDX in groundwater. Laboratory results [6-8] suggest that *Rhodococcus rhodochrous* 11Y and *Rhodococcus* sp. DN22 are good candidates for bioaugmentation to enhance RDX removal in groundwater. Bioaugmentation with *Rhodococcus rhodochrous* 11Y in soil [6] and *Rhodococcus* sp. DN22 in soil [7] was observed to increase RDX removal rates [7]; and strain DN22 was also shown to transport well through sand columns [8]. Also, aerobic RDX degradation by these *Rhodococcus* sp. does not produce toxic nitroso end-products. Several other aerobic actinomycete bacteria [9-13] transform RDX by the cytochrome P450 mixed function oxidase and flavodoxin reductase enzyme system, XplAB. In these isolates, the *xplA/B* genes are located on a plasmid (a mobile genetic element), and it appears that these genes and their metabolic potential have spread rapidly among bacteria on several continents [11, 12]. Transfer of the *xplA* containing plasmid to other bacteria by conjugation provides the bacterial transconjugants with the ability to degrade RDX and to use RDX as a nitrogen source for growth [14, 15]. Thus, the transfer of the *xplA*-containing plasmid from bioaugmentation cultures to indigenous microorganisms may result in RDX-degrading activity that increases or is sustained over time as *xplA* genes spread through the indigenous microbial community within the aquifer. In addition to strains with *xplA, Pseudomonas fluorescens* strain I-C is a facultative anaerobe that degrades RDX under anoxic conditions [16]; conditions that could develop locally in low permeability layers or near substrate injection wells during field implementation. Strain I-C was included during this project to create a robust bioaugmentation culture that would perform in mixed or spatially variable redox conditions in an aquifer.

2.2 ADVANTAGES AND LIMITATIONS OF THE TECHNOLOGY

Advantages of aerobic bioaugmentation include lower costs and potentially easier implementation for large aerobic RDX plumes compared to anaerobic biostimulation. An important advantage of the aerobic bioaugmentation approach is that substantially less substrate is required than for anaerobic biostimulation to accomplish comparable RDX mass removed. Moreover, this aerobic approach would result in less degradation of groundwater quality than traditional anaerobic biodegradation (e.g., sulfide production and reduction and mobilization of iron, manganese, and arsenic). Similar to other *in situ* bioremediation technologies, aerobic bioaugmentation would require substantially reduced cost and infrastructure compared to traditional pump-and-treat approaches. Finally, *in situ* aerobic transformation of RDX generates end products that are generally considered less toxic than those arising from anaerobic biostimulation or anaerobic bioaugmentation.

Potential limitations of this technology may include the following: (1) an aerobic bioaugmentation culture that has suitable transport properties and high RDX degradation rates for a given site may not be able to be developed; (2) the selected bioaugmentation culture may not retain activity in the aquifer after bioaugmentation; or (3) aerobic conditions may not be maintained during substrate additions. In contrast to anaerobic biostimulation, which can reductively transform RDX, TNT and potentially other comingled explosives present in an aquifer source area, bioaugmentation as applied in this demonstration is only effective for RDX. Initial laboratory studies were included in this demonstration to optimize both the activity and transport characteristics of the bioaugmentation culture using the UCMD site. These risks were assessed and managed using the specific quantitative performance objectives in Table 3.1.

3.0 PERFORMANCE OBJECTIVES

The performance objectives evaluate whether aerobic bioaugmentation is capable of reducing RDX concentrations to the remedial action criteria, achieving RDX degradation rates comparable to conventional anaerobic biostimulation, and reducing use/cost of added growth substrate while maintaining a better overall groundwater quality compared to anaerobic biostimulation or aerobic biostimulation without bioaugmentation. Specific project objectives as well as detailed descriptions of performance objectives, data requirements, success criteria and results are provided in Table 3.1.

A more detailed discussion of performance objectives is presented below.

Table 3.1. Demonstration performance objectives

Performance Objective	Data Requirements	Success Criteria	Results
Aerobic bioaugmentation degrades RDX to < 2.1 µg L^{-1}	Measurements of RDX groundwater concentrations during push-pull tests with suitable detection limits to confirm concentrations < 2.1 µg L^{-1}	Complete (>90%) removal by mass and/or concentration reduction to < 2.1 µg L^{-1}	No treatments met this goal during the tests; however, based on measured transformation rates, all treatments were predicted to achieve cleanup levels within less than 1 month
RDX removal rate for aerobic bioaugmentation is comparable to removal rates for aerobic and anaerobic biostimulation	Dilution –adjusted RDX concentrations during push-pull tests fit with first-order model to obtain RDX degradation rates	Rates of RDX degradation for aerobic bioaugmentation are 1) similar to or at least half the rates measured during anaerobic biostimulation, and 2) similar to or preferably larger than rates measured during aerobic biostimulation	The ratio of the average aerobic bioaugmentation RDX degradation rate to the average aerobic and anaerobic biostimulation rates was ~ 2. Bioaugmentation on average doubled the RDX degradation rate.
RDX mass removed per mass of substrate added is larger for aerobic bioaugmentation than for aerobic or anaerobic biostimulation	RDX degradation rates and substrate mass used to compute required ratios	Ratios of RDX mass removed to substrate mass added of 2 or higher for aerobic bioaugmentation compared to aerobic and anaerobic biostimulation	The ratio of mmols RDX degraded to mols fructose added for aerobic bioaugmentation, aerobic biostimulation and anaerobic biostimulation were 0.34, 0.1 and 0.01, respectively. Bioaugmentation increased the ratio by a factor of 3.4 – 34.
Bioaugmentation culture remains viable and retains RDX-degrading capability over time *in situ*	Bacterial survival will be indirectly determined by quantification of the *xplA* gene using TaqMan qPCR assay developed under ER-1609 as well as 16S rRNA for pseudomonads/biomass. RDX-degrading capability will be assessed using push-pull tests conducted approximately 1, 10 and 20 weeks following culture injection in field plot. *xplA* gene transfer to native population will also be qualitatively assessed.	Measurable RDX transformation activity in the bioaugmentation plot (see above), as well as measurable *xplA* gene copy numbers one order of magnitude higher than pre-inoculation gene copy numbers. Viable colony forming units above 300 CFU mL^{-1} of groundwater.	Although viable cell numbers and *xplA* gene copy numbers decreased over time, RDX transformation activity was sustained within the bioaugmentation test plot for the duration of the demonstration. Culture density and qPCR assessments were limited to aqueous phase; attached cells were not measured.
Minimal secondary groundwater quality degradation	pH, dissolved oxygen (O_2), oxidation-reduction potential (ORP), and ferrous iron (Fe(II)) are measured in all wells before each substrate addition.	Aerobic bioaugmentation results in pH 7-8, O_2 > 2 mg L^{-1}, ORP > 0, and Fe(II) < 2 mg L^{-1}	Aerobic conditions maintained in all wells. pH 7-8 maintained. O_2 and ORP decreased somewhat following fructose addition. Slight increase in Fe(II) observed in EW-2.

3.1 Ability to reduce RDX concentrations in groundwater to below relevant cleanup concentration.

In order for this technology to be successfully implemented at UMCD (and other sites) as part of the full-scale groundwater remedy, reduction to below site-specific RDX groundwater cleanup criteria should be achievable. At UMCD, this relevant concentration is the remedial action criteria of 2.1 $\mu g\ L^{-1}$

Data Required: RDX groundwater concentrations were measured over time during the Phase III push-pull tests with suitable detection limits.

Success Criteria: Reduction of RDX concentration to < 2.1 $\mu g\ L^{-1}$ during the Phase III push-pull test in the aerobic bioaugmentation treatment test plot wells.

3.2 Removal rates are comparable to biostimulation treatments.

For this technology to be successfully implemented at UMCD (and other sites) as part of the full-scale groundwater remedy, RDX transformation rates should be comparable to – or not significantly smaller than – those of aerobic or anaerobic biostimulation.

Data Required: First-order RDX degradation rates computed using tracer-adjusted RDX concentrations during replicate push-pull tests for aerobic bioaugmentation, aerobic biostimulation, and anaerobic biostimulation.

Success Criteria: Computed rates of RDX degradation are (1) similar to rates measured during anaerobic biostimulation, and (2) similar to or preferably larger than the rate measured during aerobic biostimulation.

3.3 Enhanced RDX mass removal per mass of substrate added for aerobic bioaugmentation compared to biostimulation.

Aerobic bioaugmentation should require significantly less growth substrate than anaerobic biostimulation to achieve the same RDX degradation rate.

Data Required: (1) Mass of RDX removed during replicate push-pull tests for aerobic bioaugmentation, aerobic biostimulation, and anaerobic biostimulation. (2) Quantities of growth substrate added to each test well.

Success Criteria: Ratio of RDX mass removed to substrate mass added at least twice as large for aerobic bioaugmentation compared to aerobic and anaerobic biostimulation.

3.4 Bioaugmentation culture remains viable and retains *in situ* RDX-degrading capability over time.

In order for this technology to be successfully implemented at UMCD (and other sites) as part of the full-scale groundwater remedy, the bioaugmentation culture must remain viable and retain RDX-degrading capability over time *in situ* for as long as needed to achieve RDX reduction to below the site-specific groundwater cleanup concentration.

Data Required: (1) Quantification of the *xplA* gene in groundwater samples from test wells using TaqMan qPCR assay developed under ER-1609 as well as quantification of

*xen*B and 16S rRNA genes for pseudomonads/biomass, respectively. (2) Quantification of colony forming units (CFUs) in groundwater samples using species-specific media. (3) RDX degradation rates from push-pull tests conducted approximately 1, 10 and 20 weeks after bioaugmentation

Success Criteria: Measurable RDX transformation activity in the bioaugmentation plot, as well as measurable *xplA* gene copy numbers one order of magnitude higher than pre-inoculation gene copy numbers. Viable colony forming units above 300 CFU per ml of groundwater.

3.5 Aerobic bioaugmentation preserves secondary groundwater quality.

Substrate additions in aerobic bioaugmentation and aerobic biostimulation test plot must be controlled to maintain aerobic conditions, thereby preventing dissolution of redox sensitive metals, as well as formation of hydrogen sulfide or methane associated with anaerobic treatments.

Data Required: Measurements of dissolved oxygen concentration, oxidation-reduction potential and ferrous iron concentration measured in the field prior to each substrate addition and push-pull test.

Success Criteria: Groundwater measurements confirm dissolved oxygen > 2 mg L^{-1}, Fe(II) < 5 mg L^{-1}, ORP > 0 mV in aerobic bioaugmentation and aerobic biostimulation wells prior to push-pull tests.

4.0 SITE DESCRIPTION

4.1 SITE SELECTION

The UMCD located near Hermiston, OR (Figure 4.1) was selected as an ideal site for this demonstration. UMCD was selected based upon facility interest and relevant site physical and geochemical characteristics including: (1) basic aquifer conditions (e.g., depth to groundwater, geochemistry, hydrology etc); (2) RDX concentrations and plume characteristics; (3) basic infrastructure (e.g., site access, presence of wells, roads, etc.) and (4) ability to leverage USACE bioremediation efforts and site-specific experience. Personnel at UMCD and site regulators were contacted concerning this research effort, and all were supportive of hosting the demonstration.

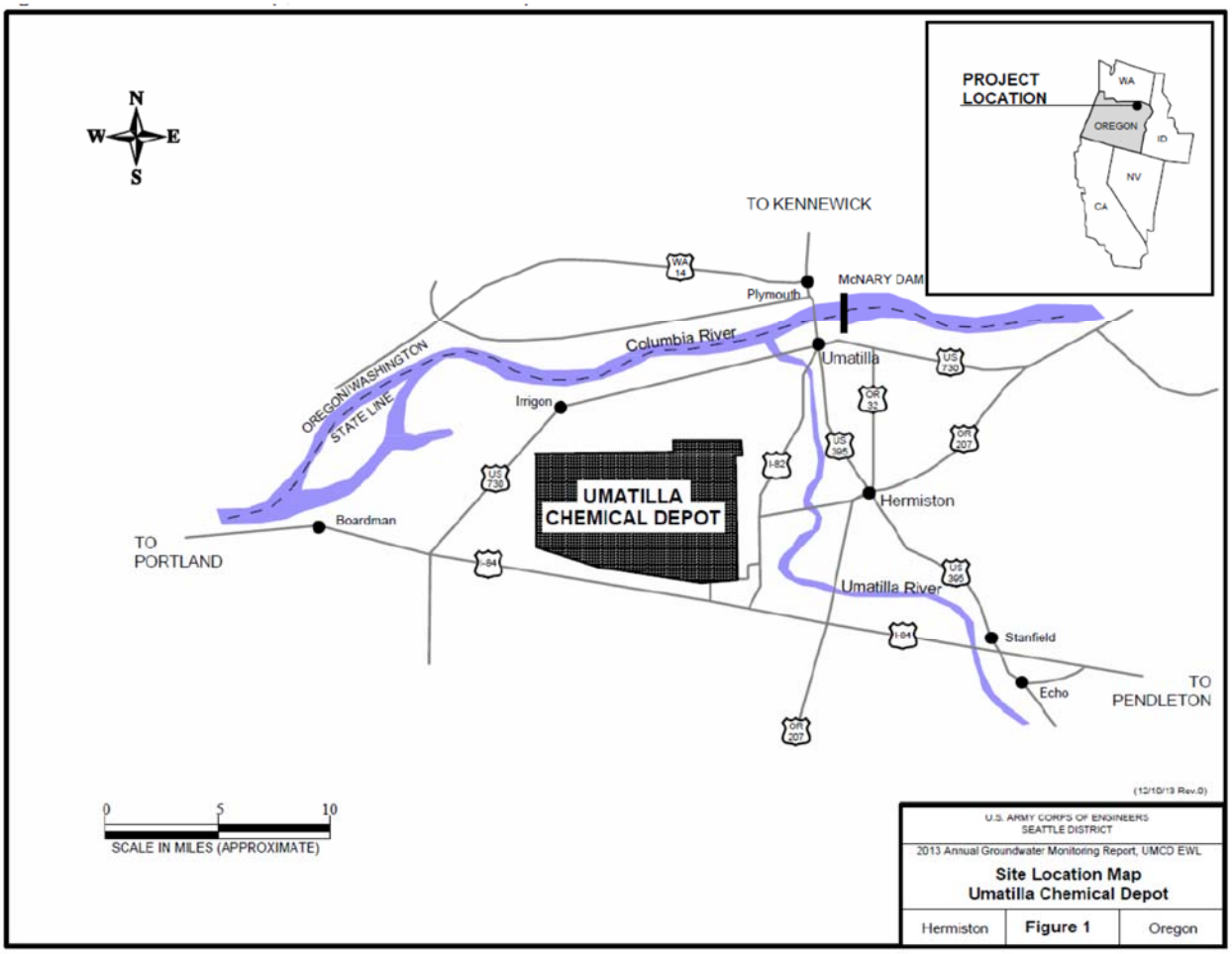


Figure 4.1. ESTCP demonstration location at the Umatilla Chemical Depot (UMCD).

4.2 SITE GEOLOGY/HYDROGEOLOGY

The unconfined aquifer at UMCD consists of alluvial deposits and the weathered surface of the Elephant Mountain Member basalt, overlain by unsaturated alluvial sand and gravel (Figure 4.2). The saturated thickness of the aquifer in the former lagoon area varies from 4 to 11 m. The nearest surface water body to the site is the Umatilla River, which is over 3.2 kilometers away. Although the aquifer permeability is very large, hydraulic gradients are very small and results in very slowly moving groundwater under ambient conditions. A large-scale aquifer recharge project was initiated near the site in October 2011, which currently involves injection of 10,000 acre-feet of water per event. This program resulted in approximately 1 m increased groundwater elevations. However, there is no evidence that the groundwater gradient, flow direction or velocity has changed appreciably as a result of these increases.

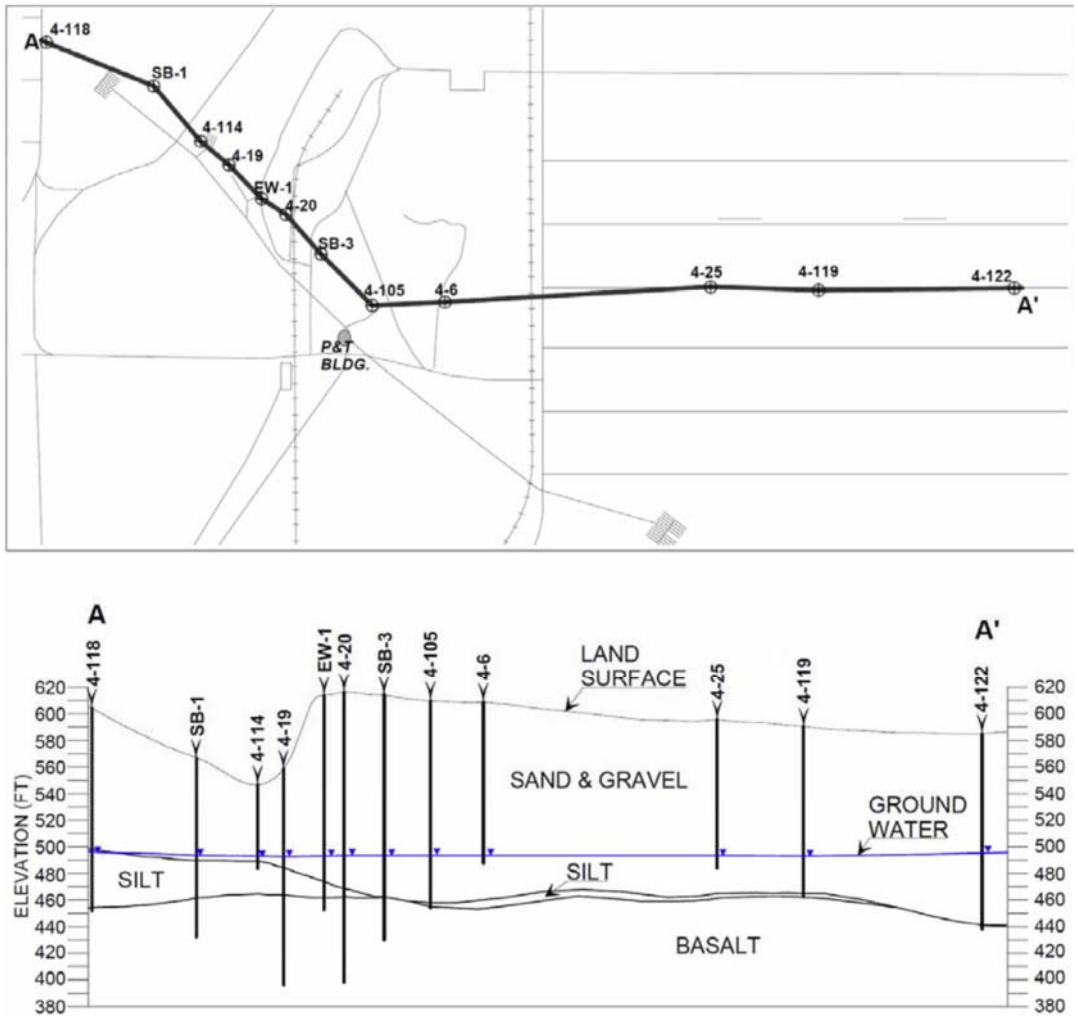


Figure 4.2. **UMCD site stratigraphy. Top: Cross-section showing well locations at Umatilla Chemical Depot, 4-114 is near the lagoon source area. Wells 4-114 and 4-105 included in the push-pull test study are screened within the unconfined aquifer. Bottom: Corresponding stratigraphy and groundwater depths. Average permeability values form the wells in the unconfined aquifer are on the order of 180 m day^{-1}, with a maximum of 830 m day^{-1}. Groundwater gradients are very low, in the range of 0.00015 m/m.**

4.3 CONTAMINANT DISTRIBUTION

The areal extent of the RDX groundwater plume above the remedial action criteria is over 150 ha (Figure 4.3). Residual soil contamination is present beneath the former wastewater infiltration lagoons. For many years, evapotranspiration and moderate precipitation resulted in minimal infiltration through the contaminated soil at the lagoons. However, following installation of the pump & treat system in the 1990s, an infiltration gallery was installed beneath the former lagoon area. The intent of this infiltration system was to percolate treated site groundwater through the contaminated soil into groundwater that would then be captured by the pump & treat extraction wells. The infiltration system operated following plant startup but was discontinued after 5 years following no apparent increase in RDX mass captured.

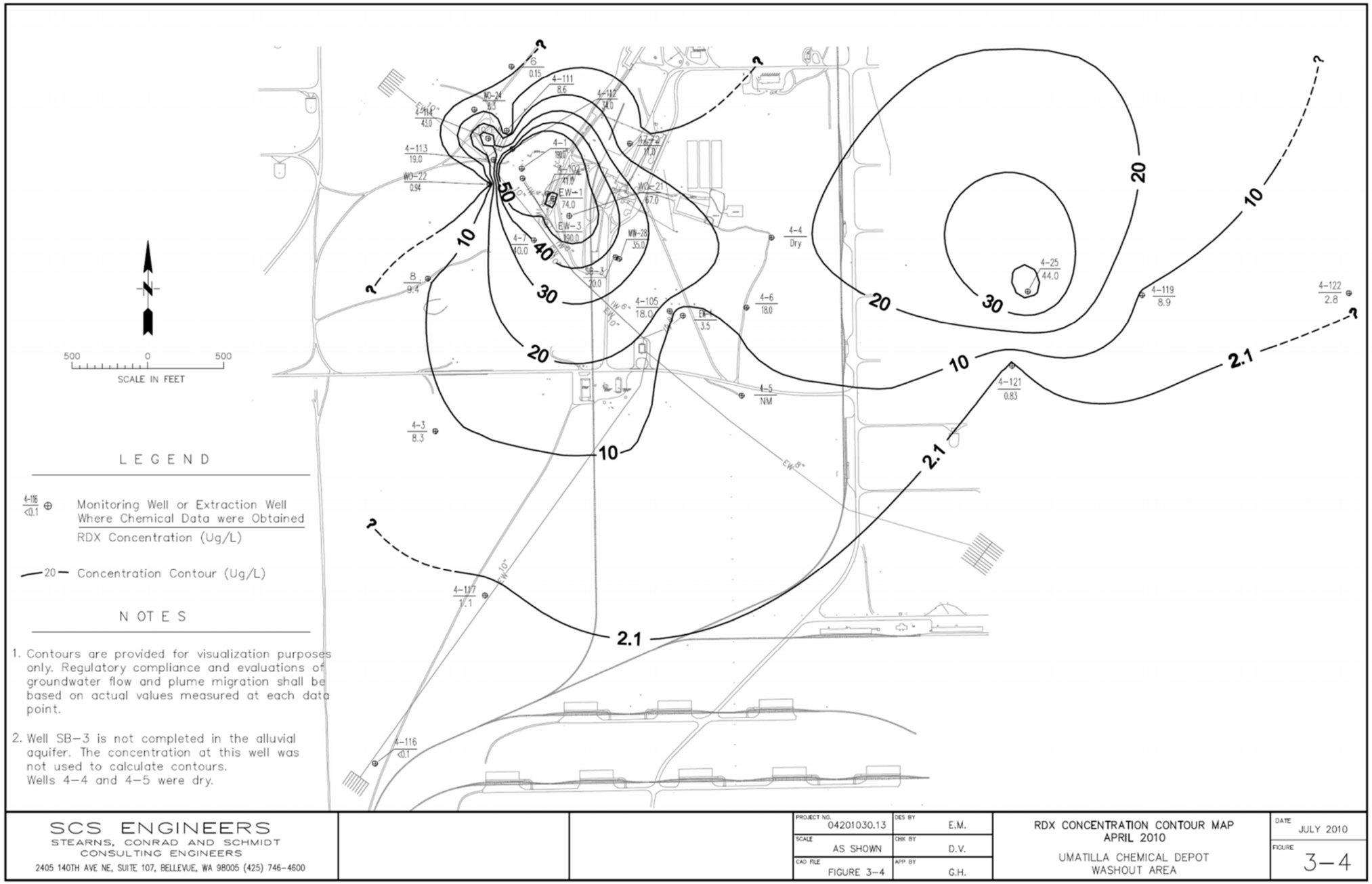


Figure 4.3. RDX concentrations in groundwater at UMCD (SCS, 2010).

5.0 TEST DESIGN

This section provides a brief overview of the field demonstration conceptual design (Section 5.1), followed by an overview of the site characterization and Phase I laboratory results (Sections 5.2 and 5.3, respectively). Detailed descriptions of field demonstration Phases II and III are included in Sections 5.4 and 5.5, respectively.

5.1 CONCEPTUAL EXPERIMENTAL DESIGN

The first phase of this demonstration included site characterization (described in Section 5.2 below), as well as a series of laboratory microcosm and column tests to select and optimize bacterial strains for use during bioaugmentation and to confirm survival, transport properties and RDX-degrading activity in UMCD aquifer material and groundwater. Phase II included a field-scale cell transport test conducted under forced-gradient conditions. Phase III included a series of push-pull tests for aerobic and anaerobic biostimulation treatments and aerobic bioaugmentation (Figure 5.1). The aerobic biostimulation with no cells added served as the bioaugmentation control.

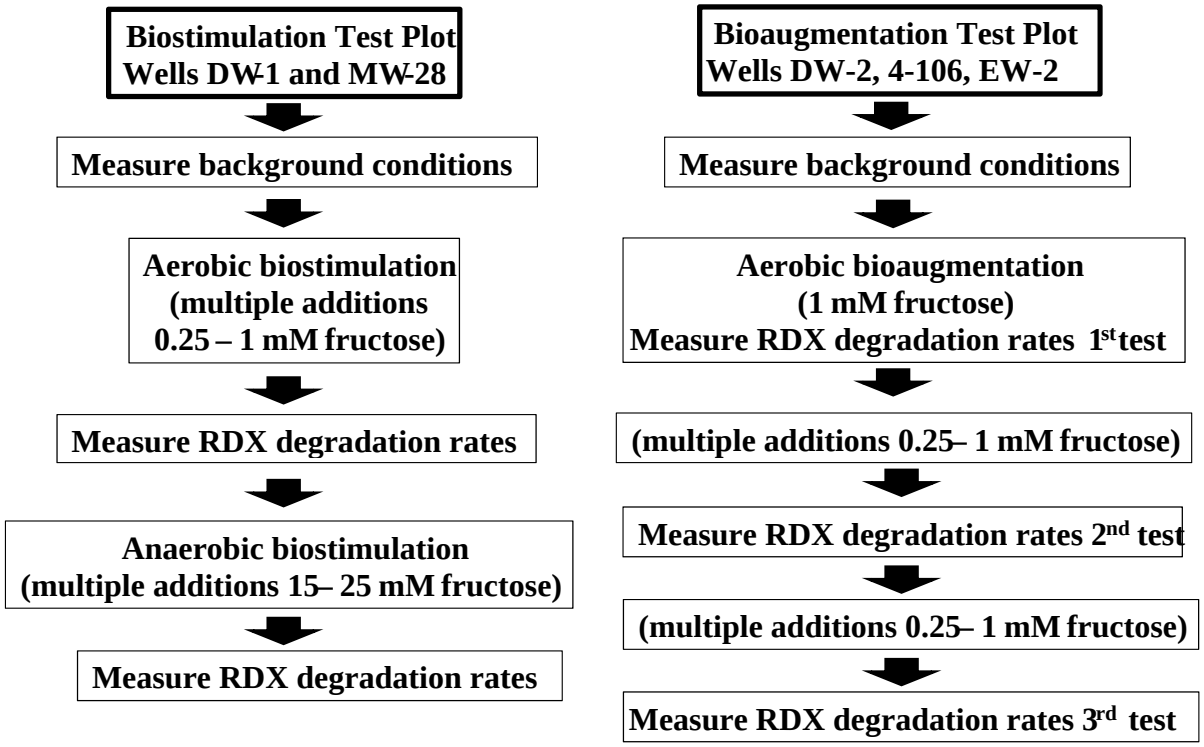


Figure 5.1. Conceptual design for demonstration Phase III.

5.2 SITE CHARACTERIZATION

Phase I site characterization work included installation of two demonstration wells (DW-1 and DW-2), as well as a series of tracer tests. Detailed methods and results (including boring logs) were presented in the Site Characterization Memorandum (Appendix A). The objectives of the tracer tests were to:

(1) Confirm the existence of hydraulically connected flow paths between two new demonstration wells and downgradient monitoring well(s);
(2) Estimate groundwater travel times and dilution factors for use in designing subsequent tracer and microbial transport tests, substrate delivery protocols, and push-pull tests; and
(3) Confirm previously estimated values for hydraulic conductivity and dispersivity in the vicinity of newly constructed demonstration wells.

5.2.1 Methods

Well Installation. Two new wells were installed for use in this project using the air rotary drilling method (Figure 5-2): Demonstration Well 1 (DW-1) and Demonstration Well 2 (DW-2). As-built drawings and boring logs for DW-1 and DW-2 are included in Appendix A. Well locations were chosen so that extraction well EW-4 could be used to control groundwater flow direction during the demonstration (Figure 5.3). Tracer tests were conducted three times during the period May-August, 2012. For each test, site groundwater (from near-by extraction wells EW-4 or EW-1) was collected in a plastic tank placed next to each demonstration well. Sufficient NaCl or KBr was added to each tank to achieve a Cl^- or Br^- tracer concentration of 100 mg L^{-1}; the tracer solution was thoroughly mixed using a recirculation pump. Each test consisted of injecting tracer solution into DW-1 and DW-2 and monitoring tracer transport by sampling the injection wells and downgradient monitoring wells MW-28, 4-106, and EW-2. At DW-1, samples were collected from monitoring well MW-28; at DW-2, samples were collected from monitoring wells 4-106 and EW-2. Well MW-28 is located 170 m from EW-4, well 4-106 is located 49 m from EW-4 and well EW-2 is located 33 m from EW-4.

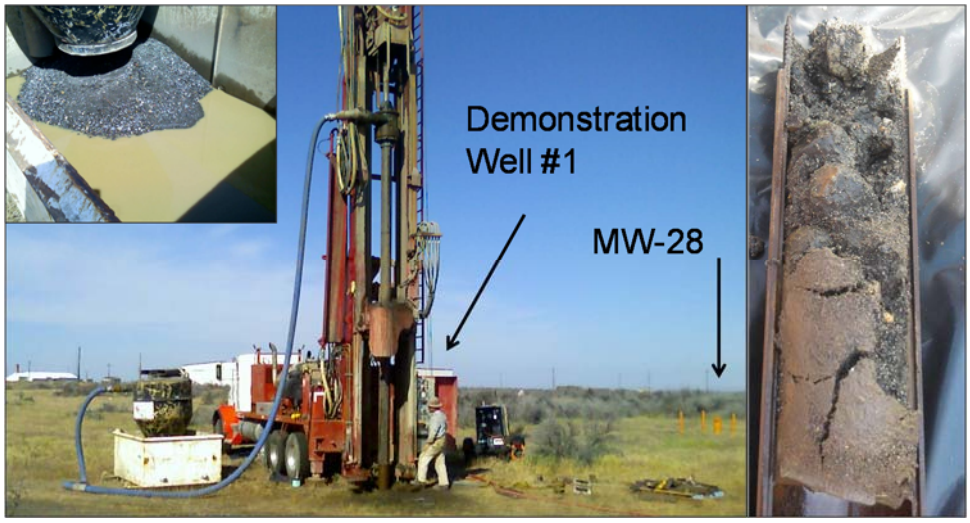


Figure 5.2. Installation of DW-1 in aerobic and anaerobic biostimulation test plot using air rotary method; sediment from screened interval shown in insets.

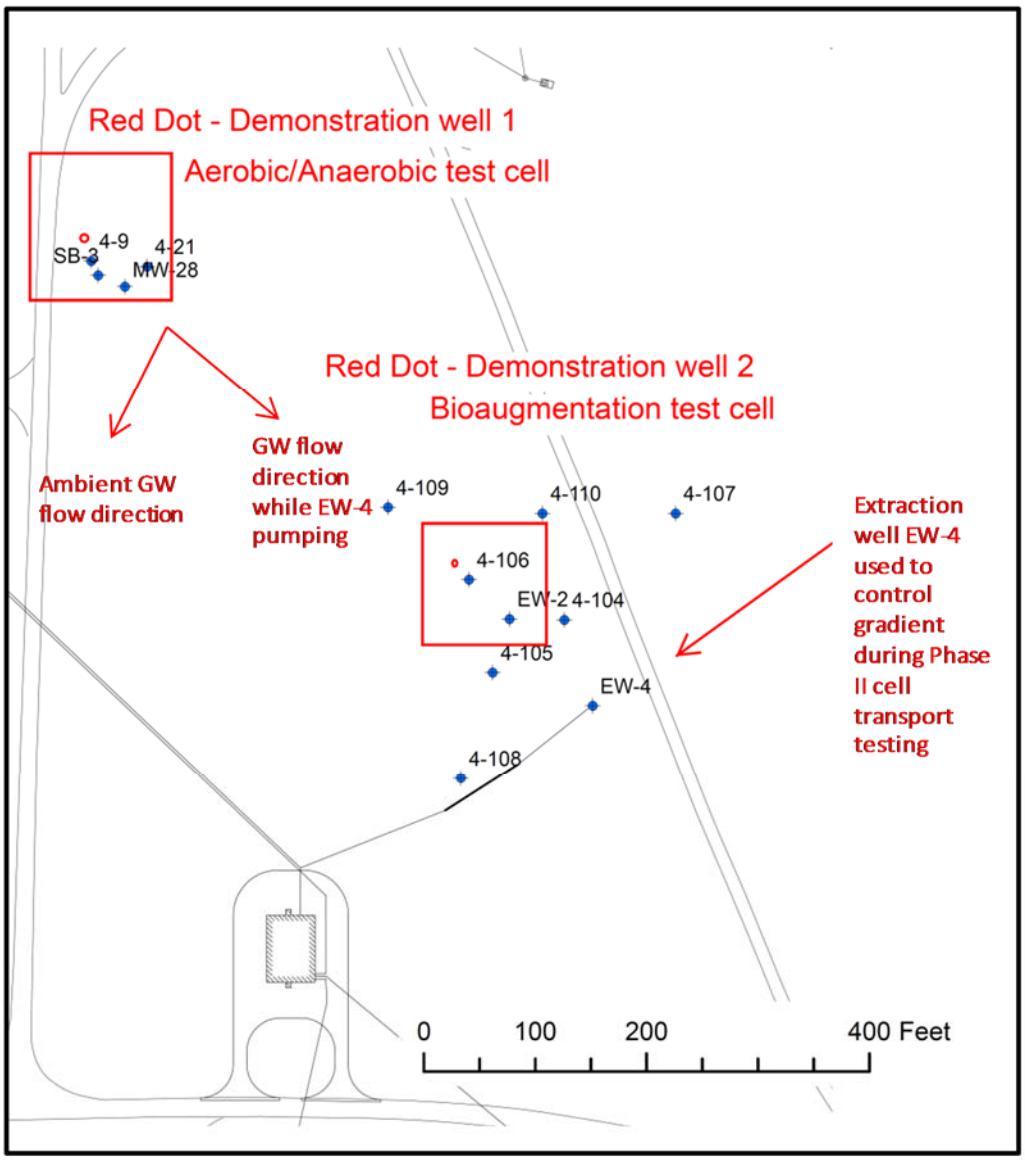


Figure 5.3. Demonstration well locations in test plots 1 and 2 are shown as red dots. Demonstration well 1 is 15 m from downgradient monitoring well MW-28. Demonstration well 2 is 3 m from downgradient monitoring well 4-106 and 18 m from well EW-2.

<u>Slow Injection Forced Gradient Tracer Test (May 2012).</u> During this test, extraction well EW-4 operated at 4,200 L per minute to force groundwater to flow from the demonstration wells toward the monitoring wells. The tracer solution was injected using a combination of siphons and pumps. For the tracer test in DW-1, the injection rate was 26 L per minute and the duration of the injection phase was 4 h. For the tracer test in DW-2, the injection rate was 15 L per minute and the duration of the injection phase was 6 h. Samples of the injected tracer solution were collected for field measurement of Br- concentration using a portable ion specific electrode and meter; duplicate samples were collected for subsequent analysis by ion chromatography. Groundwater samples

were also collected from downgradient monitoring wells using submersible pumps. Groundwater depth was measured periodically in all wells during tracer solution injection and subsequent groundwater sampling.

Borehole Dilution Tracer Test (July 2012). It was not possible to control the gradient using extraction well EW-4 during this test due to a power failure at UMCD. However, this borehole dilution test performed under natural gradient conditions provided critical information to support push-pull test design in the upcoming push-pull test design. The volume of injected tracer solution (100 mg L^{-1} Cl^- or Br^-) was 3,800 L in DW-1 and DW-2. A high-speed transfer pump was used to inject the tracer solution at 380 L min^{-1} to both wells. Samples of the injected tracer solution were collected for subsequent analysis by IC. Groundwater samples were collected from DW-1 and DW-2 using bailers and from downgradient monitoring wells were collected using submersible pumps.

Fast Injection Forced Gradient Tracer Test (August 2012). During this test, extraction well EW-4 was pumping at 4,200 L per minute. The volume of injected tracer solution (100 mg L^{-1} Cl- or Br-) was 3,800 L in DW-1 and DW-2. A high-speed transfer pump was used to inject the tracer solution at 380 L min^{-1}. Samples of the injected tracer solution were collected for subsequent analysis by IC. Groundwater samples were collected from DW-1 and DW-2 using bailers; groundwater samples from downgradient monitoring wells were collected using submersible pumps.

5.2.2 Results

Slow Injection Forced Gradient Tracer Test (May 2012). Bromide was detected in all downgradient monitoring wells sampled, confirming that hydraulically connected flow paths exist between DW-1 and well MW-28 and between DW-2 and wells 4-106 and EW-2. Results for MW-28 (located 15 m downgradient from DW-1) showed a rapid increase in Br- concentration that remained constant for the duration of the test (Figure 5.4). Estimated groundwater velocity (pore water velocity) was > 8 m day^{-1} at this location. The breakthrough curve for well 4-106 (located 3 m downgradient from DW-2) also clearly showed the arrival of the tracer solution (Figure 5.4). Estimated groundwater velocity (pore water velocity) is 2.3 m day^{-1} at this location. Surprisingly, the breakthrough curve for well EW-2 (located 18 m downgradient from DW-2), which is closer to EW-4 showed a more rapid arrival of the Br- tracer (Figure 5.4). Estimated groundwater velocity at this location is > 12 m day^{-1}. Water table depth data indicated that buildup in groundwater levels during tracer injection and subsequent groundwater sampling in all wells was < 0.02 m, which is consistent with the very large values of hydraulic conductivity that have been previously estimated at this site (240-305 ft day^{-1}).

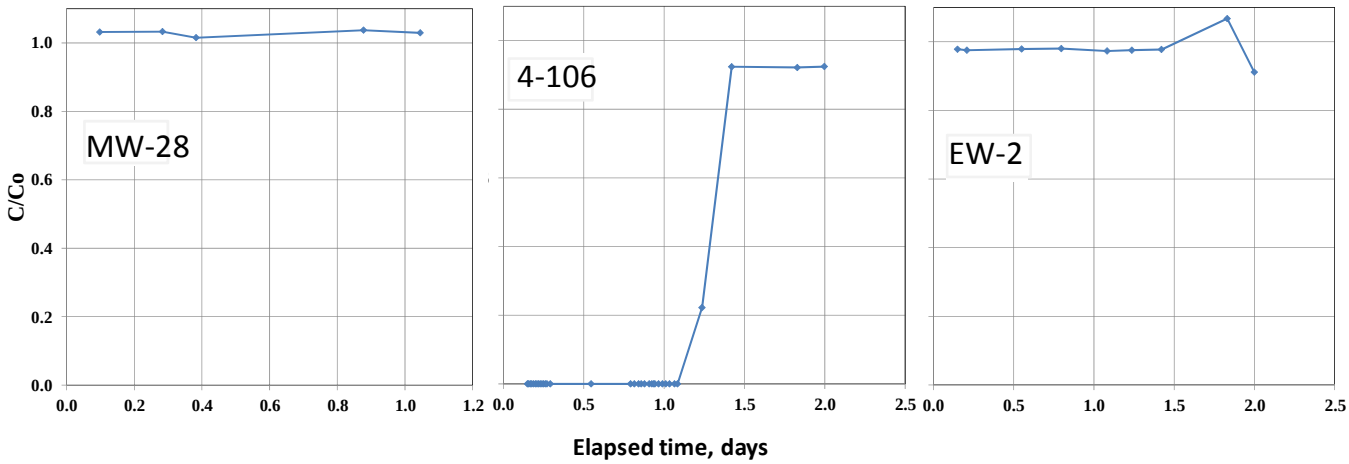


Figure 5.4. Relative bromide concentrations measured during the May, 2012 tracer tests. MW-28 is located 15 m downgradient from DW-1; 4-106, located 3 m downgradient from DW-2; EW-2, located 21 m downgradient from DW-2. C is measured bromide in a groundwater sample and Co is the average bromide concentration in the injected tracer solution. Elapsed time is measured from the midpoint of the injection phase.

Borehole Dilution Tracer Test (July 2012). Although tracer was detected in 4-106, no tracer was detected in downgradient monitoring wells MW-28 and EW-2 because the regional gradient (no pumping) is not aligned with EW-4. Breakthrough curves for DW-1 and DW-2 show the gradual dilution expected as the injected tracer is transported away from the well (Figure 5.5). Breakthrough curves for 4-106 (near DW-2) showed the tracer pulse passing through the well within 5 h after injection (Figure 5.5).

Fast Injection Forced Gradient Tracer Test (August 2012). The breakthrough curves for DW-1 shows the decline in Cl^- concentration as injected tracer is transported downgradient but surprisingly no tracer was detected in MW-28 (Figure 5.6). This in contrast to the results of tracer tests conducted in May 2012, which showed rapid tracer transport between DW-1 and MW-28. The difference is potentially due to the different tracer injection rate in the two rounds of test but is currently unresolved. Breakthrough curves in the vicinity of DW-2 showed a much more predictable response as injected tracer moved from the injection well to the downgradient monitoring wells (Figure 5.6). The peak of the tracer pulse arrived at 4-106 about 10 h after injection and at EW-2 about 27 h after injection.

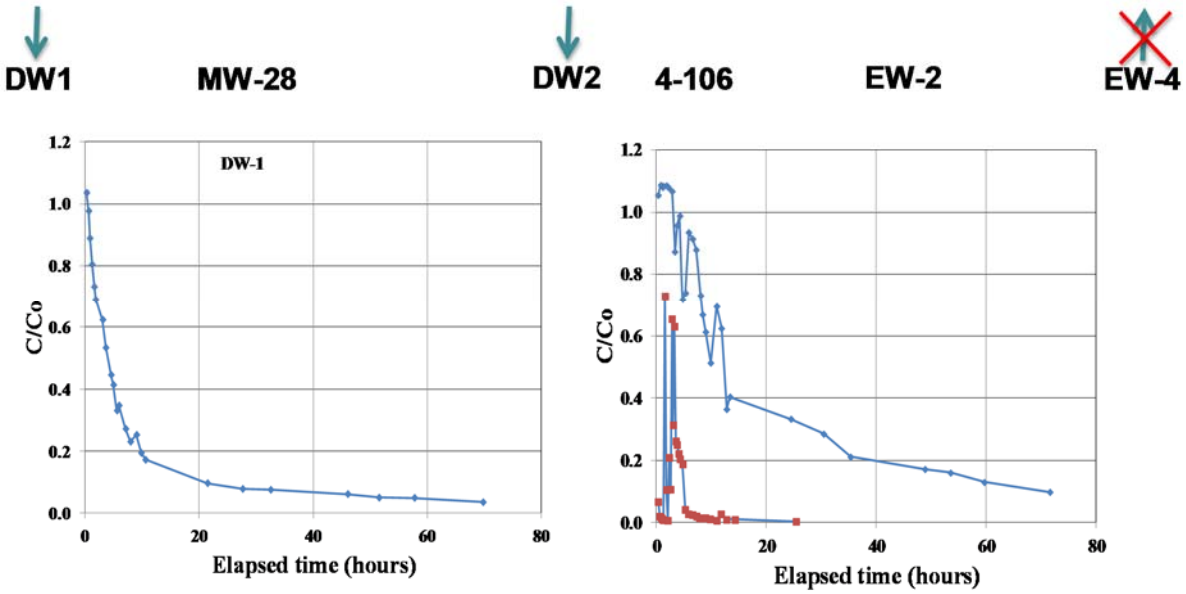


Figure 5.5. **Tracer breakthrough curves during natural gradient tests (EW-4 not pumping). Tracer was injected into wells DW-1 (left panel) and DW-2 (right panel). Tracer was observed in nearby well 4-106 (red symbols, right panel) during test.**

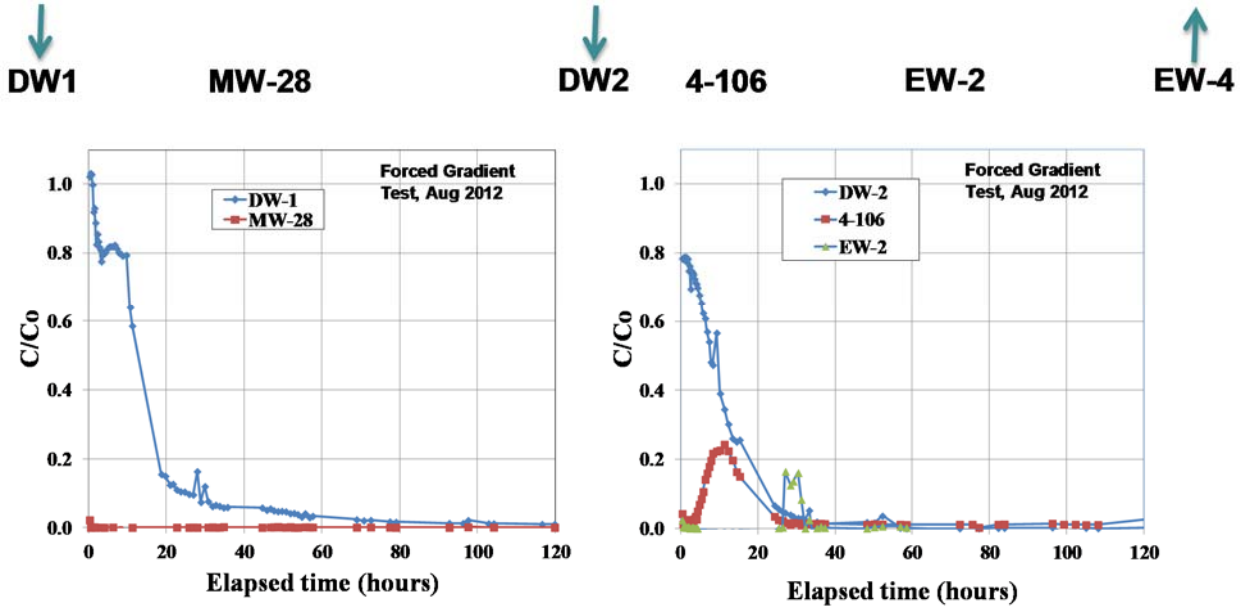


Figure 5.6. **Tracer breakthrough curves during forced gradient tests. Tracer was injected into wells DW-1 and DW-2.**

5.2.3 Discussion

Three separate rounds of tracer tests were conducted during summer 2012. Test results confirmed that hydraulically connected flow paths exist between demonstration wells DW-1 and DW-2 and their respective downgradient monitoring wells. Using a high speed transfer pump and injection rates of 380 L min^{-1} resulted in well-defined breakthrough curves and the sequential arrival of tracer at 4-106 (about 3 m from DW-2) and EW-2 (about 15 m from DW-2). These results are encouraging because they confirmed that it is possible to conduct well-to-well transport experiments using tracers, growth substrates, and microorganisms and were helpful in designing the field protocols used for those tests.

Estimated porewater seepage velocity ranged from 7.6 m day^{-1} near DW-1 to between 2.4 to 12 m day^{-1} near DW-2 based on results of the May 2012 tracer testing. Water table fluctuations during the forced gradient testing were very small confirming the very large hydraulic conductivity values (240-305 ft day^{-1}) previously estimated for this site.

The most surprising result of this study was the inconsistency between results of tracer tests conducted in DW-1. In the May tests, which used a very slow injection rates, tracer arrived at MW-28 very rapidly, while in the August tests, which used a much higher injection rate, tracer was never detected at MW-28. The explanation for these differences is unresolved.

5.3 PHASE I LABORATORY TESTING

The first phase of this demonstration (Phase I) included laboratory testing to obtain a bioaugmentation culture that could be transported and sustain the highest possible RDX-degrading activity in the UMCD aquifer. Phase I was designed to select the bacterial strains to include in the bioaugmentation culture and to recommend a "go" decision to proceed with project Phase II. Detailed methods and results of the laboratory testing were presented in the Phase I Results Memorandum (Appendix B). The specific objectives of the laboratory studies were to:

(1) Optimize the growth yields of aerobic and facultative anaerobic RDX-degrading bacterial strains;

(2) Determine cell viability and RDX degradation rates in UMCD groundwater and sediment microcosms;

(3) Determine if the selected strains could be transported through UMCD site sediments;

(4) Determine if the selected strains could survive and maintain RDX-degrading activity over several months in UMCD sediment columns; and

(5) Determine if the selected strains could be grown to the required cell densities for field-scale bioaugmentation and evaluate the strains' longevity during storage.

The success criteria for the Phase I laboratory studies were defined as the selection of a mixed bacterial culture that could:

(1) Survive in UMCD sediment and groundwater for several months;

(2) Be transported through repacked UMCD sediments; and

(3) Reduce RDX concentration to less than 2.1 μg L^{-1}.

5.3.1 Bioaugmentation Culture Optimization

The growth of seven aerobic, *xplA*-containing bacterial strains and two facultative anaerobic RDX-degrading strains (Table 5.1) was optimized by varying the type and concentration of nine carbon and four nitrogen sources. The aquifer at UMCD is aerobic with RDX concentrations between 2 and 300 μg L^{-1} and nitrate concentrations between 0.1 and 2 mM. These conditions are likely to support the activity of the *Rhodococcus*, *Gordonia*, and *Williamsia* strains that degrade RDX to 4-nitro-2,4-diazabutanal (NDAB) [7, 9, 13]. However, RDX degradation is inhibited in some of these strains by nitrate concentrations of 4 mM or higher. Furthermore, the *Rhodococcus* strains have been shown to degrade RDX under microaerophilic conditions but at considerably slower rates than in the presence of high oxygen concentrations [17]. For this reason, and because spatially variable redox conditions may be created in the aquifer following substrate additions (e.g. due to preferential flow of added substrate in the heterogeneous aquifer), we also evaluated *Pseudomonas fluorescens* I-C and *Pseudomonas putida* IIB strains. These strains are facultative anaerobes that degrade RDX via xenobiotic reductases under microaerophilic to anoxic conditions, and are not inhibited by nitrate [16].

Table 5.1. RDX-degrading microbial strains included in the initial screening.

Bacterial Strain	Genotype	Reference
Gordonia sp. KTR9	*xplA*$^{+}$, Kan^{R}	9, 15
Williamsia sp. KTR4	*xplA*$^{+}$	9
Rhodococcus sp. DN22	*xplA*$^{+}$	8
Rhodococcus rhodochrous 11Y	*xplA*$^{+}$	13
Rhodococcus jostii RHA1 [pGKT2]	*xplA*$^{+}$, Kan^{R}	15
Gordonia polyisoprenivorans DSMZ 44302 [pGKT2]	*xplA*$^{+}$, Kan^{R}	15
Nocardia sp. TW2 [pGKT2]	*xplA*$^{+}$, Kan^{R}	15
Pseudomonas fluorescens I-C	*xen*B^{+}	16
Pseudomonas putida IIB	*xen*A^{+}	16

Fructose at 50 mM and ammonium sulfate at 18 mM provided the optimal growth conditions for biomass yields necessary for large scale culture production. The nine strains were then evaluated for their ability to survive and degrade RDX in artificial UMCD groundwater (AGW; Table 5.2). Following growth, the cells were washed once with AGW and resuspended in AGW to an absorbance of 1.0 (at 600 nm). The cultures were starved in the AGW for 24 h at 15ºC (*in situ* groundwater temperature) to reduce residual nitrogen levels within the cells. After 24 h of starvation, RDX and fructose dissolved in AGW were added to achieve final concentrations of 5.5 μM (1.2 mg L^{-1}) and 1 mM, respectively. Cell viability and RDX concentrations were monitored periodically over the next 7 days.

Table 5.2. Comparison of compositions of UMCD site groundwater and artificial groundwater (AGW) used during Phase I.

Component	UMCD groundwater ($mg\ L^{-1}$)	AGW ($mg\ L^{-1}$)
pH	8	8
Nitrate as NO_3^-	48	46
Sulfate as SO_4^{2-}	24	25
Alkalinity as CO_3^{2-}	92	89
Na^+	20	54
Ca^{2+}	35	28
Cl^-	21	78
Mg^{2+}	16	16
K^+	3.5	3.5
NH_4^+	<0.5	3.5

All nine cultures survived for 7 days in AGW at 15ºC with very little loss of cell viability (Figure 5.7). Even though the cultures were grown and starved in the absence of RDX, RDX was rapidly degraded within 1-2 days by strains KTR9, KTR4, 11Y, DN22, and the transconjugant strains, RHA1::pGKT2, *G. polyisoprenivorans* pGKT2, and TW2::pGKT2 (Figure 5.8). Nitrogen starvation significantly increased *xplA* gene expression in KTR9 [18]. In comparison, RDX was slowly degraded by strains *P. fluorescens* I-C and *P. putida* II-B with about 90% and 16% degraded within 7 days, respectively (Figure 5.7). Since anoxic conditions were not specifically created (reduced headspace volume), a smaller amount of RDX was degraded by strains I-C and II-B. Based on growth yields and > 90% of the RDX degraded within 4 days, *Gordonia* sp. KTR9::Kan^R, *Rhodococcus jostii* RHA1 pGKT2, and *P. fluorescens* I-C were selected for microcosm and column transport studies. These strains are hence forth referred to as KTR9, RHA1, and I-C. Strain KTR4 performed similarly to KTR9 but its morphology was not unique (creamy, white colored) compare to the bright orange pigment of KTR9 so it was not included in further studies. Cell aggregation and adherence to glass surfaces was observed with strains 11Y, DN22, *G. polyisoprenivorans* pGKT2, and TW2::pGKT2 (Figure 5.9). Clumping of cells in the AGW could be an indication of possible problems during large-scale fermentation and/or during injection into the aquifer during bioaugmentation. Relatively low RDX degradation was observed with *P. putida* II-B. Therfore these five strains were not considered further for inclusion in the field bioaugmentation culture.

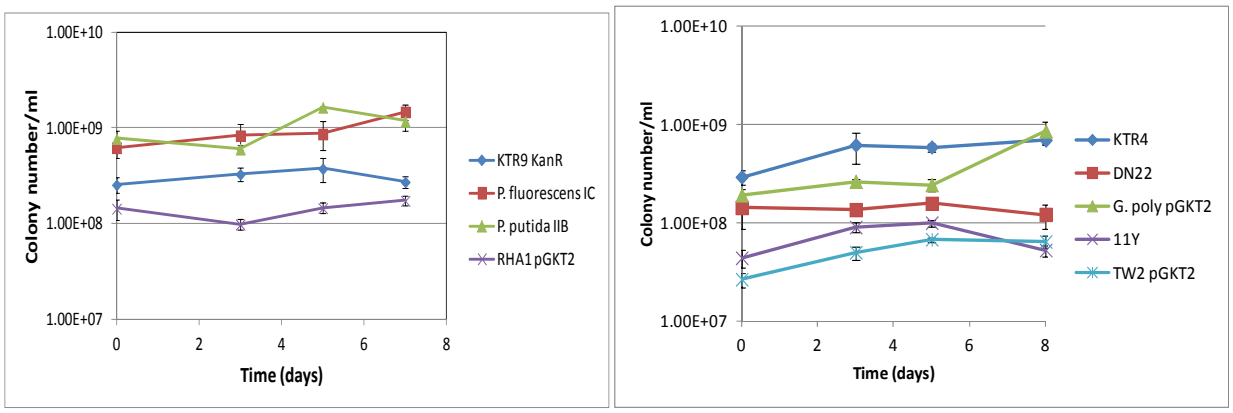


Figure 5.7. **Survival of RDX-degrading strains in artificial groundwater (AGW) amended with 1 mM fructose and 1.3 mg L^{-1} RDX. Cultures were starved for 24 h in AGW without a carbon or nitrogen source.**

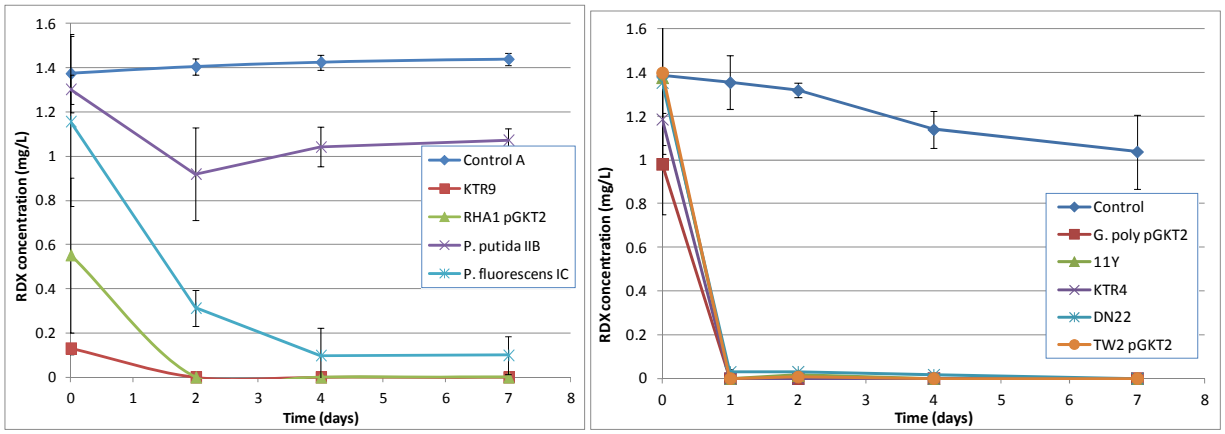


Figure 5.8. **Degradation of RDX by RDX-degrading strains in artificial groundwater amended with 1 mM fructose and 1.3 mg L^{-1} RDX. Cultures were starved for 24 h in artificial groundwater without a carbon or nitrogen source.**

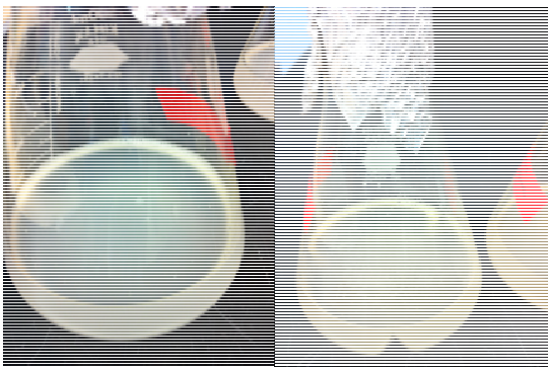


Figure 5.9. Growth physiology of select RDX-degrading strains in artificial groundwater amended with fructose and RDX. *Williamsia* sp. KTR4 (A) grew homogeneously while *G. polyisoprenivorans* pGKT2 cells (B) aggregated.

5.3.2 Microcosm studies

The selected strains were further evaluated in microcosms to assess the efficacy of this mixed culture to degrade RDX in the presence of UMCD site sediment and AGW. Microcosms were constructed in 20 mL vials, and consisted of 2 g (wet weight) of UMCD sediment (2 mm sieved) plus 1 mL of AGW amended with RDX at 5.5 µM (1.1 mg L^{-1}), 1 mM fructose (180 mg L^{-1}) and 1 x 10^6 cells mL^{-1} of each bacterial culture. Bacterial cultures were starved in AGW for 24 h at 15ºC before inoculation. Uninoculated microcosms were prepared without the addition of cells. Microcosms were incubated at 15°C and three replicates of each treatment were periodically sacrificed for analysis of RDX concentrations and cell viability. Strains KTR9, RHA1, and I-C remained viable for 7 days at 15ºC in the microcosms despite the presence of the indigenous population and the low nutrient levels (Figure 5.10). In addition, degradation of RDX occurred very quickly in the inoculated microcosms with 98% of the RDX removed to below the 2.1 µg L^{-1} site-specific objective in 1 day (Figure 5.11). Degradation of RDX by the indigenous UMCD sediment population was significantly slower with only 15% degraded in 7 days (Figure 5.11).

In this simple system, the bioaugmentation of UMCD sediment with these three RDX-degrading bacteria stimulated the rapid degradation of RDX and provided the necessary information required to proceed with column experiments to evaluate cell transport and RDX degradation under dynamic flow conditions in repacked UMCD sediment columns.

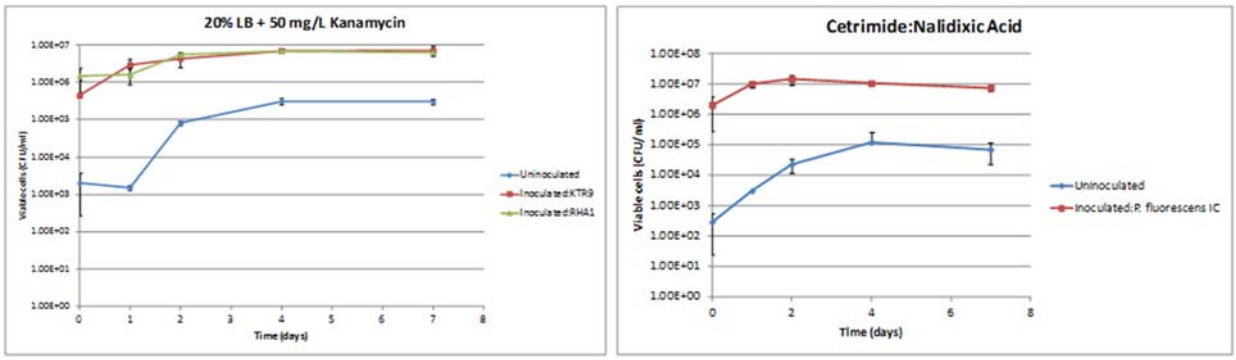


Figure 5.10. Bacterial viable cell numbers on 20% LB + 50 mg L^{-1} kanamycin agar plates (A) and cetrimide:nalidixic acid agar plates (B) from uninoculated and inoculated UMCD microcosms. Bacterial counts from the uninoculated microcosms are total bacterial counts on both types of media. Bacterial counts from the inoculated microcosms represent the individual strains: KTR9, RHA1, or I-C as indicated in each panel.

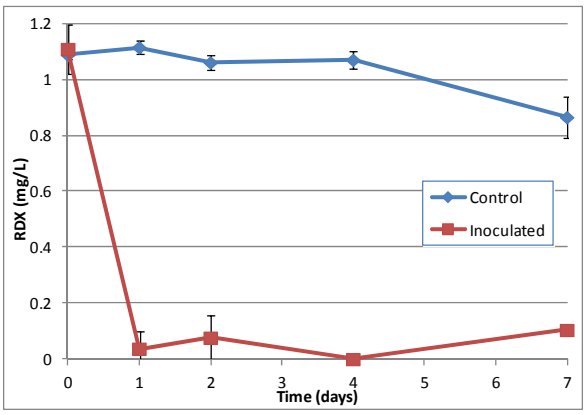


Figure 5.11. RDX concentrations in UMCD microcosms uninoculated (control) and inoculated with strains KTR9, RHA1, and PsIC.

5.3.3 Column Transport Experiments

The following is a summary of work performed, which is described in more detail in Fuller et al., 2015 [19].

Multiple factors affect bacterial cell transport in groundwater including cell surface properties [20-25], cell growth phase [26, 27], cell density [28], aquifer sediment characteristics, and groundwater chemistry [29-31]. In some instances when cells transport poorly through sediments, selection procedures have been used to obtain adhesion-deficient culture variants that still biodegrade the contaminant of interest but are more readily transported [32]. Alternatively, ionic strength adjustment to modify cell surface charge has been shown to promote cell transport through sediments [33]. This section describes column experiments that were performed to determine (a) ability of the bioaugmentation culture to transport through UMCD site sediment and (b) strain survival and RDX-degrading activity over several months in UMCD sediment columns

Column transport experiments were performed using previously described methods [34-38]. The column setup is illustrated in Figure 5.12. Three identical columns were wet-packed to a bulk density of 1.6 g cm^{-3} with freshly collected homogenized UMCD sediments. AGW was pumped into the bottom and exited at the top of the columns at seepage velocities representative of UMCD field conditions. AGW was amended with 2.2 μM (or 500 μg L^{-1}) RDX to approximate UMCD plume concentrations.

After stable flow conditions were reached and effluent RDX (C) was not significantly different from influent RDX (C_o), washed and starved cells were injected into the influent end of the column at a flow rate of 12 to 15 mL min^{-1} (equivalent to approximately 3 m day^{-1}, or field forced gradient conditions). Once the 1 pore volue (PV) of cells was injected, the influent was switched back to AGW with RDX. The strains tested, cell density for inoculation, and some operational parameters varied between the column experiments, as summarized in Table 5.3.

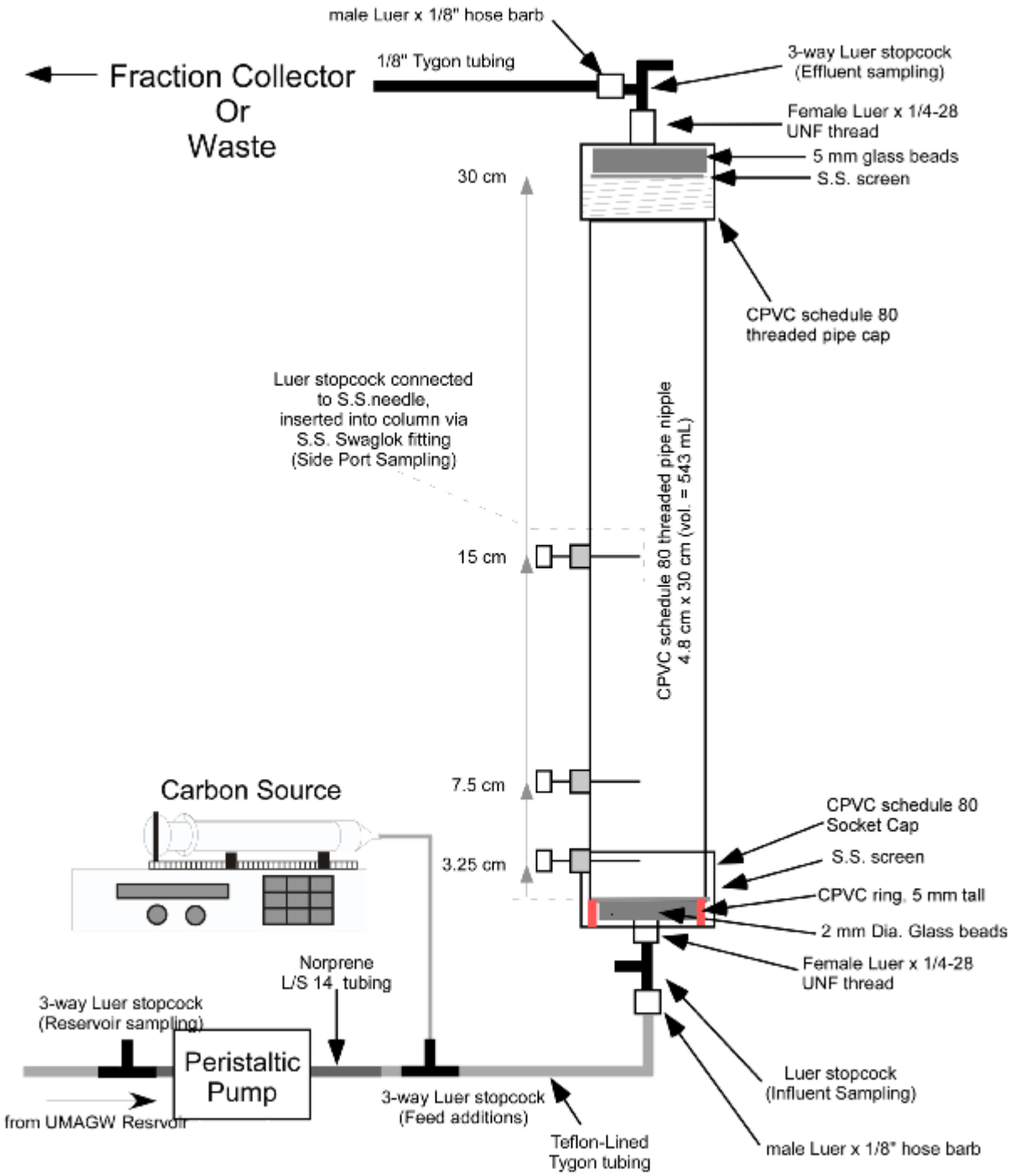


Figure 5.12. Column experimental setup.

Table 5.3. Experimental conditions for the column transport experiments.

Parameter	Experiment 1	Experiment 2	Experiment 3
Strain(s)	KTR9	KTR9, RHA1, I-C	KTR9, RHA1, I-C
Temperature (°C)	22	15	15
Injection cell density (cells mL^{-1})	$1x10^9$	$1x10^8$ each	$1x10^8$ each
Injection volume (mL)	182	190	190
Flow rate (mL min^{-1}) -high (=forced gradient) -low (=seepage velocity)	1.15 0.15	1.15 0.15	1.15 0.15
Fructose concentration (mM), periodic addition	0.1	0.1	0.1
Current elapsed time (d)	140	100	40

Each column was subjected to several cycles of fructose addition both before- and after-bioaugmentation. A concentrated fructose solution was added to the column AGW feed to achieve a final influent concentration of 0.1 mM. Fructose additions lasted until 2 PV of AGW had entered the column, when the feed was switched back to fructose-free AGW. RDX-degrading activity was followed over time in each column by measuring RDX concentrations in the influent and effluent.

After inoculation of Column 1, and passing 30 PV of RDX-containing artificial groundwater through the column, a 0.1 mM fructose injection began with concomitant rapid decrease in RDX effluent concentrations (Figure 5.13). A distinctive breakthrough curve of KTR9 cells was observed, confirming this strain is transportable in UMCD site soil. Consistent with other bacterial transport studies, the cells were transported through the soil faster than the tracer, likely the result of the pore exclusion process [36, 39]. The estimated retardation factor for cells in this experiment, determined by method of moments, was 0.77. Following this initial 2 PV injection of fructose, we pumped fructose- free AGW through the column for 70 PV and then injected a second fructose + RDX pulse and observed rapid RDX degradation. Similar results were observed following a third fructose + RDX pulse, which was completed after 120 PV. KTR9 appeared to retain good RDX degradation activity, as rapid decreases in the C/Co of RDX were observed upon each fructose addition (Figure 5.13). Furthermore, the ability to degrade RDX was maintained well even with periods of up to 2 months between fructose additions.

Column 2 was operated at expected *in situ* groundwater temperatures (15°C), and received all three strains chosen as candidates for the bioaugmentation culture. Breakthrough of the combined inoculum was approximately 40% of the injected cells based on OD_{550} measurements of the effluent (Table 5.4), but the three strains exhibited different elution from the column based on plate counts of viable cells (Figure 5.14). Effluent CFU counts dropped to low levels after the main bioaugmentation pulse, indicating that the cells would not likely be flushed under forced gradient flow regimes. Fructose was added on two separate occasions 5 and 38 days after bioaugmentation. The injected cells resulted in good RDX degradation with each fructose addition (Figure 5.14). As with Column Experiment 1, the ability to degrade RDX was maintained well even over long periods with no fructose additions.

Column 3 was operated under slow flow rate conditions (0.3 m d^{-1}) for six weeks prior to bioaugmentation. Three injections of fructose did not appear to stimulate any significant RDX degradation by the indigenous UMCD microbial community (Figure 5.15). Inoculation of Column 3 resulted in rapid RDX degradation. The inoculated cells remained viable following a starvation of period of 100 PVs. Rapid RDX degradation was observed when fructose was injected at 180 PVs (Figure 5.15).

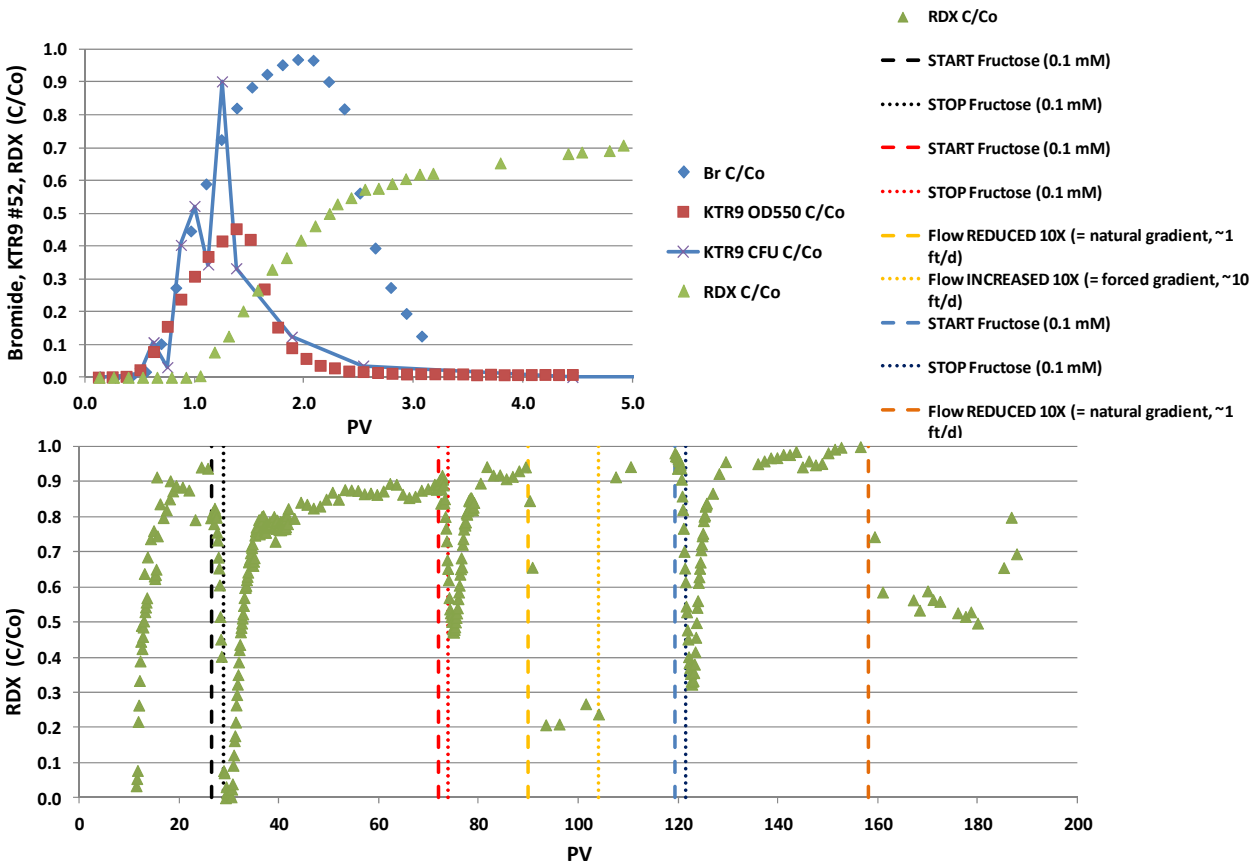


Figure 5.13. Breakthrough curves for Br^-, cells, and RDX for Column Experiment 1. The initial breakthrough results and the results of the entire experiment are shown, where C is the measured tracer or cell concentration in the column effluent; Co is the influent tracer or cell concentration.

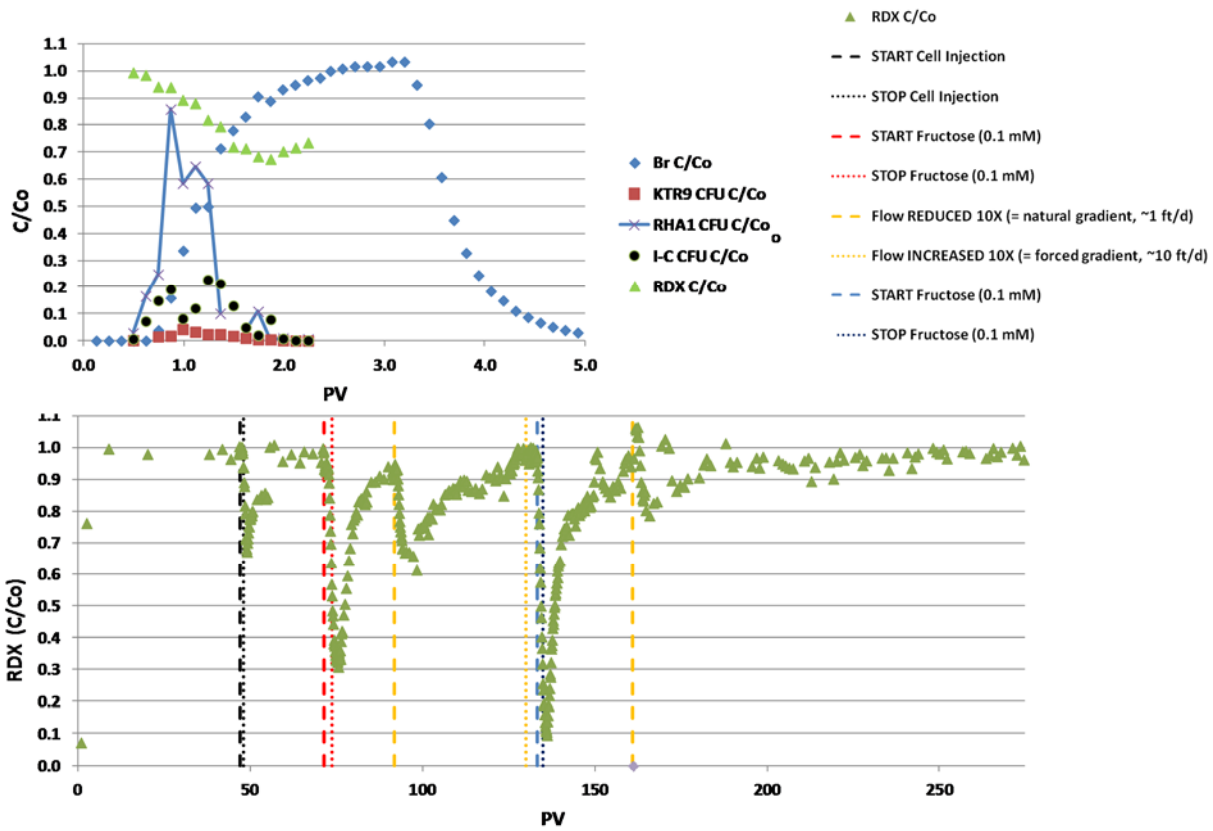


Figure 5.14. Breakthrough curves for Br^-, cells, and RDX for Column Experiment 2. The initial breakthrough results and the results of the entire experiment are shown, where C is the measured tracer or cell concentration in the column effluent; Co is the influent tracer or cell concentration.

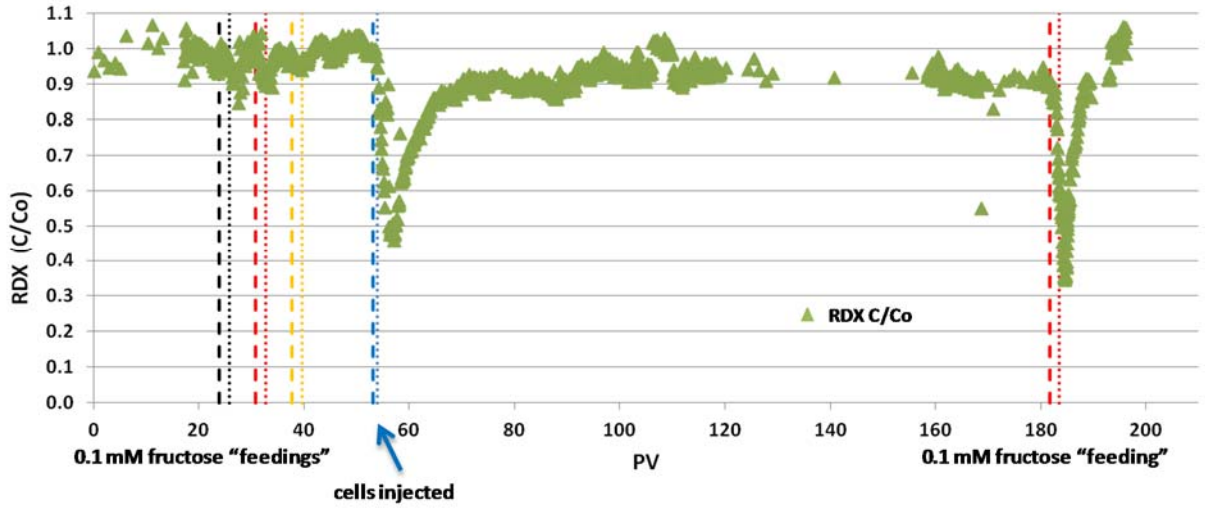


Figure 5.15. Breakthrough curves for Br^- and RDX for Column Experiment 3, where C is the measured tracer or cell concentration in the column effluent; Co is the influent tracer or cell concentration.

Table 5.4. Summary of key column transport experiment results.

Parameter	Experiment 1	Experiment 2	Experiment 3
Strain(s)	KTR9	KTR9, RHA1, I-C	KTR9, RHA1, I-C
Recovery of cells in effluent (% of injected OD)	42	40	12
Cell retardation factor	0.77	ND[a]	ND
Average RDX degradation rate with fructose (mg $L^{-1}d^{-1}$)	1.51 (n=4)	1.66 (n=2)	1.60 (n=2)
Cells in column effluent after 60 days (CFU mL^{-1})	~10^3	~10^2	~10^2

[a]ND: not determined

5.3.4 Field Scale Culture Production

The following is a summary of the work performed, which is described in more detail in Fuller et al. 2015 [19].

The long term viability and RDX degrading activity of three strains was evaluated after they were grown and concentrated to densities required for field-scale application. Starter cultures were individually grown in 3- or 7-L benchtop bioreactors (Applikon Biotechnology B.V., Schiedam, The Netherlands) (Figure 5.17). The bioreactors were continuously mixed, and positive pressure was maintained to minimize foaming. The pH and dissolved oxygen were monitored by specific probes, and residual fructose and ammonium were determined using colorimetric tests. Control of pH was achieved by automatic addition of aqueous solutions of acid (H_2SO_4) or base (NaOH). Periodic samples were removed for measurement of the cell density (OD_{600} and CFU).

Once a starter culture had reached a constant OD_{600}, it was used to inoculate a 750-L bioreactor (Abec, Allentown, PA, USA) (Figure 5.17). Growth continued in the 750-L bioreactor until the cell density (as determined by OD_{600}) multiplied by bioreactor volume reached the target number of cells for a hypothetical pilot-scale injection (e.g., 10,000 L at 5 x 10^7 cells mL^{-1}, or 5 x 10^{14} total cells). After the required cell density was achieved, the culture was passed through a custom-built cross-flow filtration unit (Kerasep™ tubular ceramic membranes, Novasep, Inc., Boothwyn, PA, USA) to remove the culture media and concentrate the biomass. The culture was further concentrated using a flow-through centrifuge (CEPA Z41, Carl Padberg Zentrigugenbau GmbH, Geroldsecker Vorstadt, Germany; 17,000 x *g* at 21°C) with final resuspension in AGW.

Subsamples (100 mL) of the concentrated cultures were transferred to duplicate 250 mL sterile glass bottles. One bottle of each culture was placed in a refrigerator at 4°C (expected shipping and long-term storage temperature during a field injection), and the other was placed in an incubator at 37°C (anticipated highest temperature the cultures would experience during shipping). Bottles were incubated without shaking. Well-mixed samples (10 mL) were removed from the bottles initially, and after 1, 2, 5, 7, 14 days, and additionally at 30, 60, and 90 days for the bottles incubated at 4°C. After passing the sample several times through a 25 gauge hypodermic needle

using a 20 mL disposable syringe to reduce cell clumping, the optical density (OD_{550}) and viable cell counts were determined (spread plating onto LB + kanamycin and R2A media).

The RDX degradation potential of the cultures over time was assayed on the same schedule (except at the 2 day time point) by combining 1 mL of a 1:100 dilution of the sample (in phosphate-buffered saline) with 9 mL of sterile AGW amended with RDX (10 mg L^{-1}) and fructose (9 mg L^{-1}). The assays with KTR9 and RHA1 were performed in 25 mL serum vials with 15 mL of headspace to maintain aerobic conditions. The assays with strain I-C were performed in 11 mL serum vials with minimal headspace to create suboxic conditions favorable to RDX degradation by this strain. An uninoculated control was set up with each batch of assays. Assays were incubated with shaking (125 rpm) at room temperature. Subsamples were removed after 24 and 48 h, passed through a 0.45 µm glass microfiber filter, and analyzed for RDX.

As part of the evaluation of bioaugmentation for RDX remediation, it is critical to confirm that large volumes of degradative strains can be produced, stored, and deployed to the field without loss of viability or activity. Similar studies have been performed for dechlorination consortia, which are now widely used for anaerobic bioaugmentation for chlorinated solvent remediation [40, 41]. After pilot-scale fermentation and concentration, the cell density (relative to the initial OD_{550}) of all three of the bioaugmentation cultures remained constant for at least 90 days at 4°C for KTR9 and strain I-C, and 60 days for RHA1 (Figure 5.18). The data for culturable cells (as CFU mL^{-1}) was similar to the OD_{550} data (Figure 5.19). Some decrease in cultivable cells was observed for all three strains incubated at 4°C during the first 14 days, followed by a period of stable CFU counts for up to 90 days for KTR9 and I-C, and 60 days for RHA1 (Figure 5.19). Viable counts on R2A agar were the same as on LB+kanamycin agar for KTR9 and RHA1 (data not shown). Cell density-normalized RDX degradation potential (defined as percent of initial RDX degraded in 24 h divided by the relative OD_{550}) remained relatively stable for KTR9, while some decrease was observed for RHA1 after 30 days (Figure 5.20). This is in agreement with nitrogen starvation inducing the RDX degrading genes in these strains. The RDX degradation assay was not performed under optimal conditions for strain I-C, but some activity was observed by this culture during the experiment (data not shown). For RHA1 and strain I-C, incubation at 37°C resulted in rapid decreases in OD_{550}, loss of viability, and reduced RDX degradation potential. KTR9 incubated at 37°C showed similar patterns in viability and RDX degradation, but less of a reduction in OD_{550}, possibly indicating that KTR9 cells were dying, but were not lysing. These testing results clearly indicated that large volumes of high density cultures could be produced in advance of a field application and stored at 4°C for at least two months without significant loss of cell density, and more importantly, RDX degradation activity.

Figure 5.17. Photographs of benchtop (left) and 750-L (right) bioreactors.

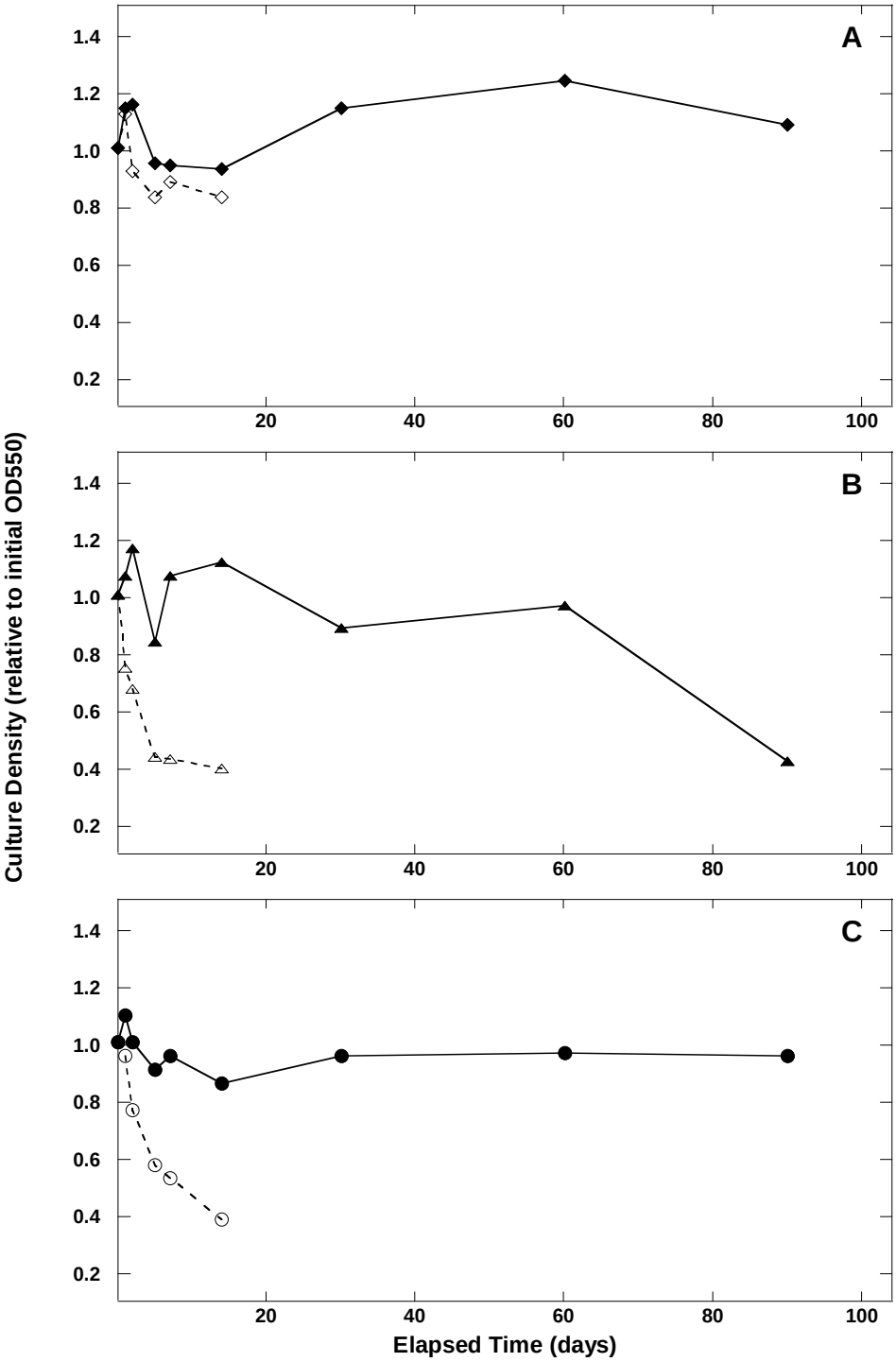


Figure 5.18. Change in culture densities (relative to the initial OD_{550}) of pilot-scale cultures of the three RDX degrading strains (A, KTR9; B, RHA1; C, I-C) over time during incubation at 4°C (solid lines) and 37°C (dashed lines).

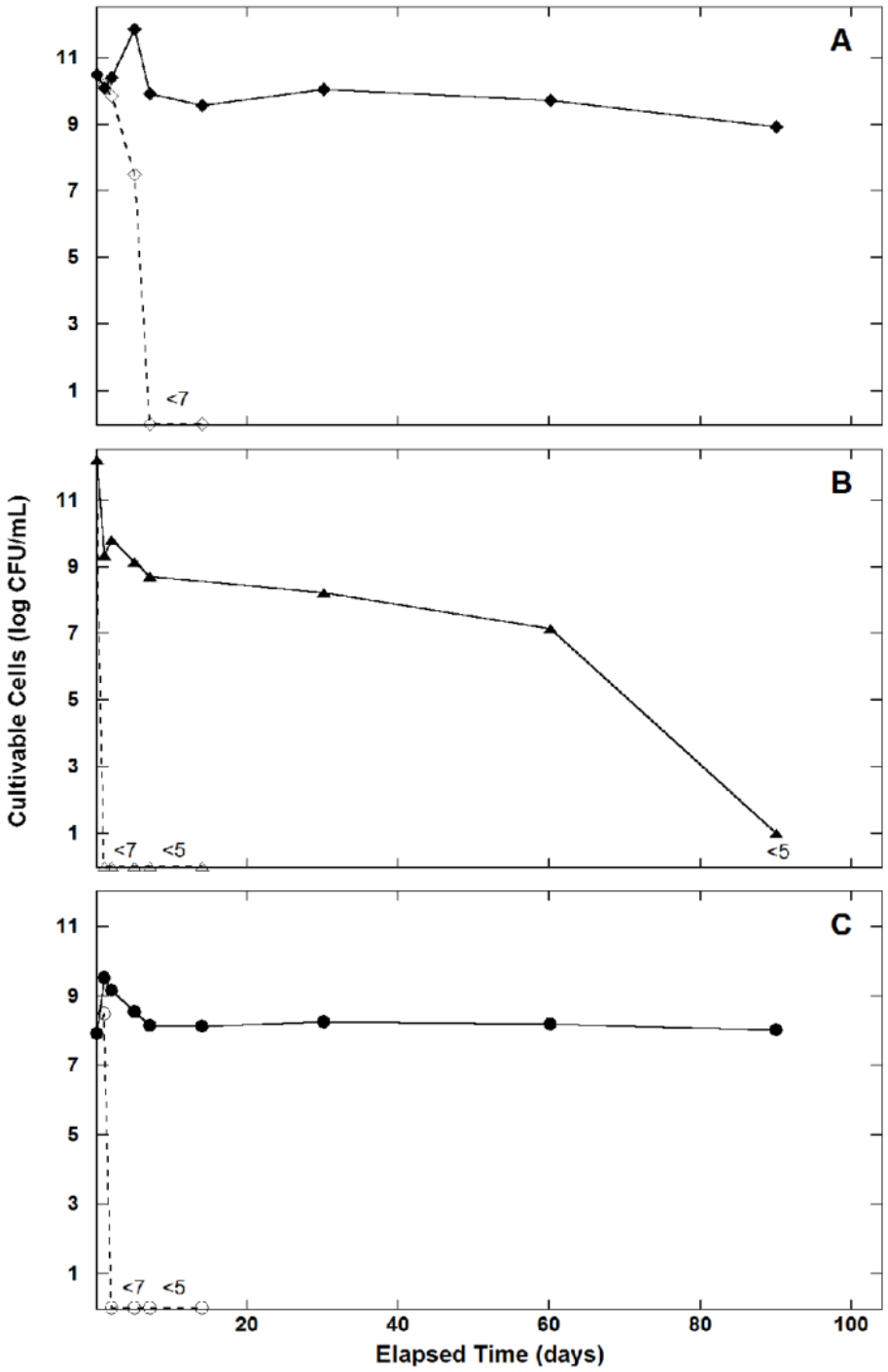


Figure 5.19. Change in cultivable cell densities of pilot-scale cultures of the three RDX degrading strains (A, KTR9; B, RHA1; C, I-C) over time during incubation at 4°C (solid lines) and 37°C (dashed lines).

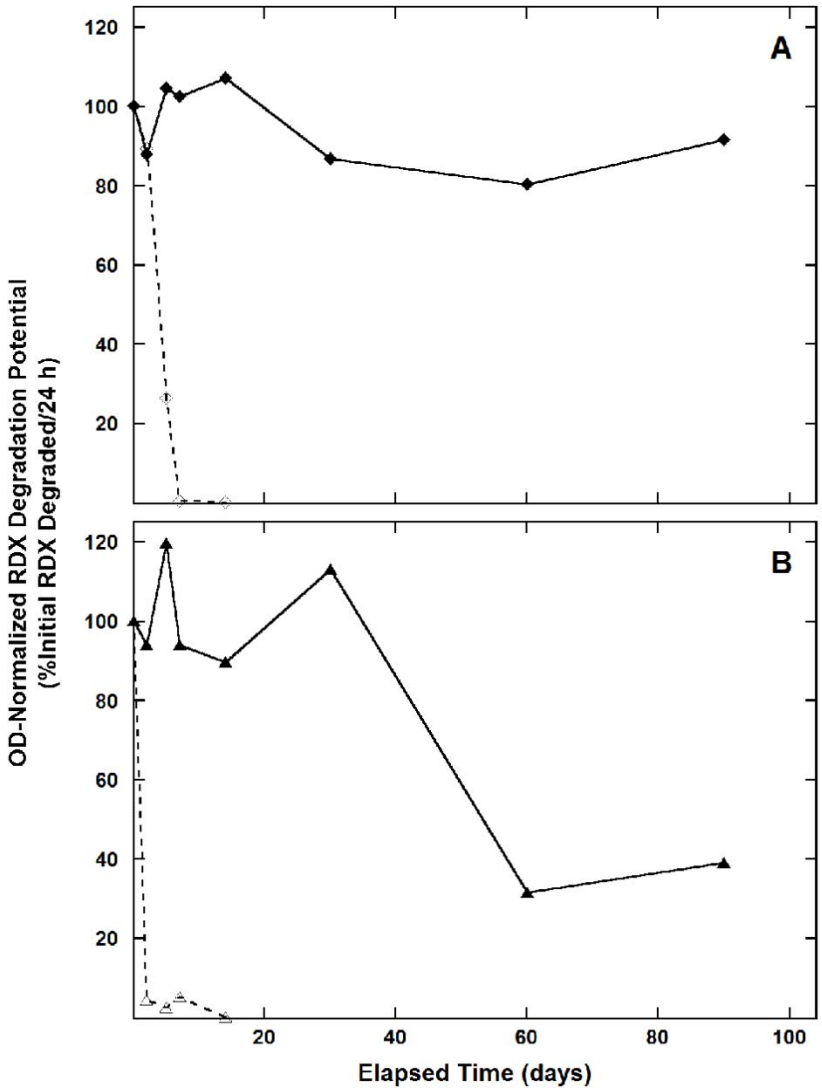


Figure 5.20. Change in OD-normalized RDX degradation potential of pilot-scale cultures of the RDX degrading strains KTR9 (A) and RHA1 (B) over time during incubation at 4°C (solid lines) and 37°C (dashed lines).

5.3.5 Phase I Recommendations

The re-packed UMCD sediment columns inoculated with the bioaugmentation culture sustained RDX degrading activity throughout the study, as rapid decreases in the C/Co of RDX were observed upon each fructose addition (Figures 5.13-15). Furthermore, the ability to degrade RDX was maintained over several months in between fructose additions. We did not observe the production of the nitroso intermediates. If the denitration intermediate, NDAB, was produced its concentration would have been too low to detect. The contribution of each strain or strains in the mixed culture to the overall rate and extent of the observed RDX degradation could not be determined.

Based on these results, there was a high degree of confidence that the selected strains could be well distributed *in situ* at UMCD, and were supportive of moving the project to Phase II. The column transport and retention data indicated that not only would the cells likely move a reasonable distance from the injection well(s), but also that the retained cells would establish themselves in the aquifer matrix and not be flushed away when the forced gradient was active. It should be noted that there are some difference between these column transport experiments and the *in situ* conditions e.g., the larger rocks, which made up a good portion of the aquifer solids, were screened out of the material used for the columns. Hence, obtaining field-scale cell transport data during demonstration Phase II would be critical to the design of Phase III. These column results also reinforced our hypothesis that once the cells were injected, they would maintain their ability to degrade RDX for at least several months or longer, and would respond to carbon source additions by quickly reducing the *in situ* RDX concentrations for at least several months. Finally, this was the first reported production of aerobic RDX degraders at a scale that is relevant for field application.

5.4 PHASE II FIELD-SCALE CELL TRANSPORT TESTING

The following is a summary of the work performed, which is presented in more detail in Crocker et al. 2015 [42].

Phase II consisted of a short duration forced-gradient cell transport test to confirm the ability to distribute the mixed culture in the Bioaugmentation Test Plot. Phase II results were critical to the design and initiation of the Phase III field demonstration in that any potential technical issues (e.g., cell shipping, cell injection, groundwater sampling, etc.,) could be identified and resolved prior to Phase III. Detailed methods and results were presented in the Phase II Results Memorandum (Appendix C). The objectives of Phase II were to:

(1) Obtain regulatory approval for injection of the genetically-modified KTR9 strain and the transconjugant strain of RHA1 in the UMCD aquifer; and

(2) Determine the transport distance and survival potential of the bacterial inoculum in the UMCD aquifer.

The success criterion for Phase II was defined as detection of the bioaugmentation culture gene biomarkers in downgradient wells at or above the quantitative polymerase chain reaction (qPCR) detection limit.

5.4.1 Methods

Culture Transport to UMCD. The three strains selected for inclusion in the bioaugmentation cultures were grown as described in section 5.3. The cultures were shipped in 20-L soda kegs sealed with top cross-bars to maintain a watertight seal (6 kegs total, 2 kegs per culture) (Figure 5.22). Kegs were packed into coolers equipped with special racks to stabilize the kegs during shipping (Figure 5.22). Ice packs were included to maintain the cultures at a cool temperature during transport to the Groundwater Research Laboratory at Oregon State University (OSU). The cultures were continuously iced during transport via automobile to the UMCD field site.

Figure 5.22. Photographs of 20-L soda keg used for culture starvation and shipping. The kegs were packed in ice prior to overnight shipment to the field in coolers.

Field Testing Methodology. The cell transport test was conducted in the field test plot located at well DW-2 (Figure 5.21) in July, 2013. The 3,000 gallon (12,000 L) UMCD groundwater solution was prepared in two large plastic tanks. Sufficient NaCl was added to achieve a chloride concentration of 125 mg L^{-1} in each tank. Concentrated cell suspensions were transferred from the kegs into the tanks (3 kegs into each tank). The test solution was mixed using two, flow-matched, high-speed transfer pumps, each pumping at 150 gpm (570 L min^{-1}). Mixing continued until the contents of each tank had been exchanged approximately 10 times (200 min). The cell transport test was conducted under "forced gradient" conditions. Extraction well EW-4 was turned on 8.6 h before injection began and pumped at an average rate of 1080 gpm (4100 L min^{-1}) during the test.

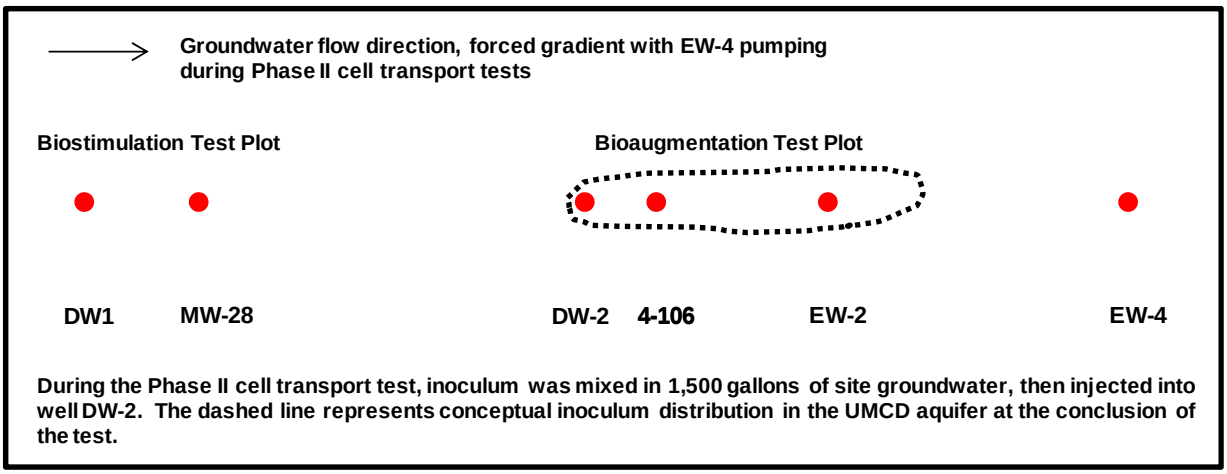


Figure 5.21. Well layout for phase II cell transport test.

The bioaugmentation inoculum solution containing the tracer and cells was injected into DW-2 at an average rate of 145 gpm (550 L min^{-1}); the duration of the injection was 21 min. During injection, samples of the inoculum solution were collected from the tanks and analyzed for tracer and microbial parameters.

Permitting and Decontamination of Field Equipment. Toxic Substance Control Act (TSCA) Experimental Release Applications (TERAs) for use of the two genetically-modified organisms (GMOs) – KTR9::Kan^R and RHA1 pGTK2 were prepared and submitted to the Environmental Protection Agency's Office of Pollution Prevention. The project received TERA application approval for both microorganisms on July 10, 2013 prior to test initiation (Appendix D). Best management practices for handling the bioaugmentation strains and decontamination of equipment were used during the field test. Prior to adding the strains to the groundwater tank, all connections and tubing associated with the mixing and injection were tested to ensure there were no leaks. Plastic sheeting was placed around the tank opening where the cultures were added, and on the ground surrounding the tank/injection area to ensure any minor spills were contained to the test area and could be disinfected prior to disposal. All personnel used appropriate personal protective equipment and other applicable safety procedures for work at CERCLA sites as described in the health and safety plan. All equipment, tanks and tubing that contacted the culture were disinfected using a 5-10% bleach solution.

Sampling Schedule. The sampling schedule was based on previous tracer tests at this test plot conducted during site characterization activities (Section 5.2) and the results of cell transport tests in laboratory columns packed with site sediment from wll DW-2. After injection of the bioaugmentation culture was completed, groundwater samples were collected from injection well DW-2 and downgradient monitoring wells 4-106 and EW-2 on the following schedule: every 0.5 h for 48 h, then every 1.5 h for 12 h, then every 2.5 h for 25 h and then approximately every 5 h for the remainder of the test. Total test duration was 120 h. Groundwater samples were collected from separate submersible pumps in each well prior to and following injection. All pumps extracted groundwater continuously at a low rate (0.35 L min^{-1}) that remained constant during the test. Samples for Cl^- analyses were collected in plastic, screw top, 15-mL plastic vials. Samples for microbial analyses were collected in sterile, polypropylene, screw top, 125-mL (viability) or

1-L plastic bottles (qPCR). All samples were stored on ice after collection and during shipping, and were stored at 4°C for up to 1 week in the laboratory.

5.4.2 Results

During the test, the Cl^- tracer was observed to reach the nearest down-gradient well, 4-106 (3 m), after 10 h (Figure 5.23). However, no tracer was detected in monitoring well EW-2 (18-m down-gradient) for the duration of the test (122 h). Similarly, KTR9 and RHA1 cells were rapidly transported to downgradient well 4-106 during the test, with cells reaching this well within 2 h (Figure 5.24). Over the next 24 h, KTR9 and RHA1 viable numbers declined by several orders of magnitude in the injection well (DW-2) and well 4-106. Viable numbers of KTR9 and RHA1 then stabilized between 4 x 10^4 and 2 x 10^5 CFU mL^{-1} for the next 5 days. However, there was no evidence that KTR9 or RHA1 reached well EW-2, since these colony types were not observed on LBkan agar plates. The lack of transport of these two strains to well EW-2 was confirmed by the relatively constant total viable cell numbers (5% PTYG) in the groundwater at EW-2 in contrast to total cell counts in wells DW-2 and 4-106 (Figure 5.24). Strain I-C could not be selectively identified apart from the indigenous population and so evidence for the transport of strain I-C was based solely on qPCR analysis of the *xenB* gene.

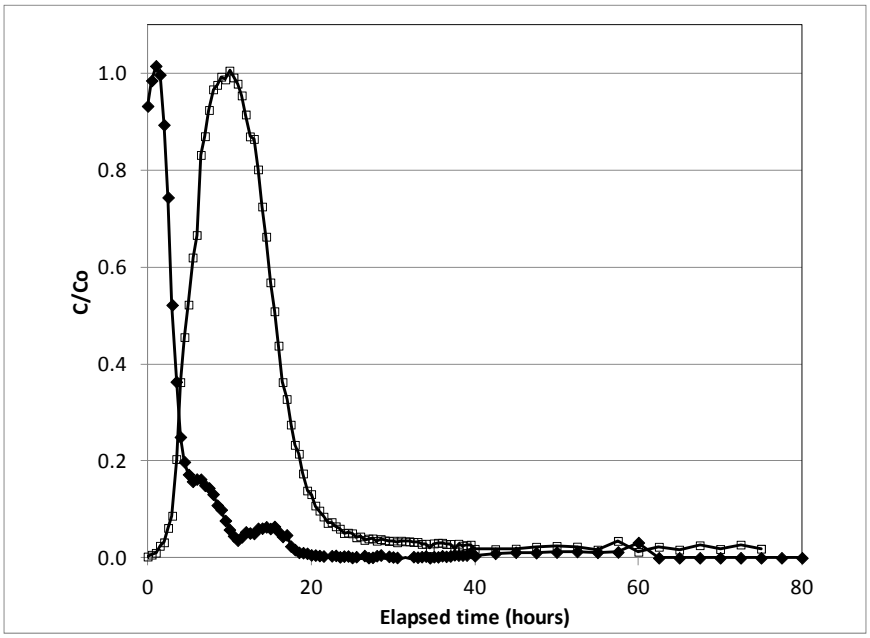


Figure 5.23. Cl^- breakthrough curves in the injection well (◆), DW-2, and monitoring well, 4-106 (□).

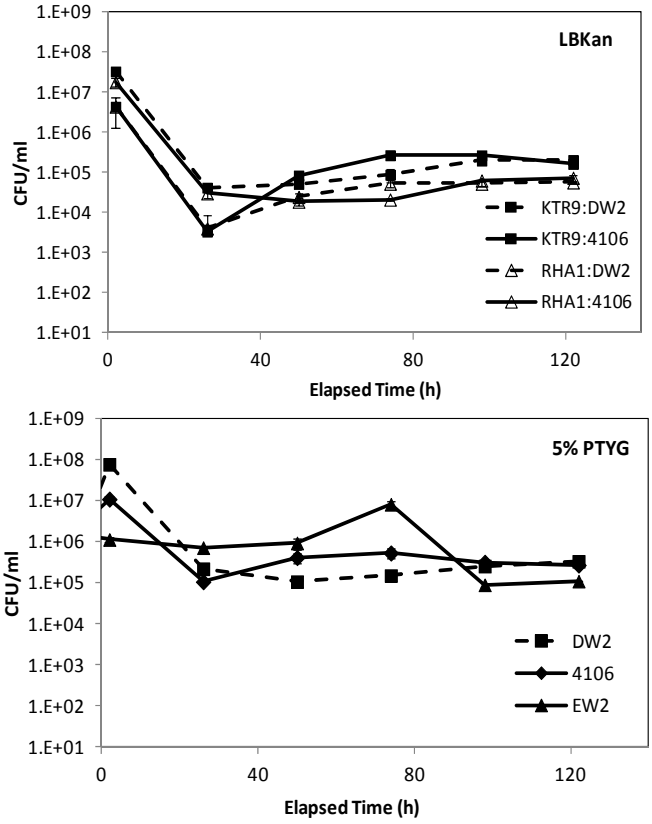


Figure 5.24. Viable plate counts on (A) LBKan agar plates of KTR9 (■) and RHA1 (△) from wells DW-2 (dashed lines) and 4-106 (solid lines); and (B) 5% PTYG agar plates of heterotrophic groundwater bacteria plus bioaugmentation culture from wells DW2 (■), 4106 (◆), and EW2 (▲).

qPCR results showed a rapid *xplA* gene copy number response in downgradient well 4-106 during the test, corroborating cell count results. Prior to bioaugmentation, background levels of *xplA*, kanamycin, and *xenB* genes were significantly lower than the estimate of the total bacterial population (16S rRNA). qPCR analysis indicated a robust groundwater population at 10^6 to 10^8 16S rRNA gene copies mL^{-1} (Figure 5.25A). In comparison, background levels of *xplA*, kanamycin, and *xenB* genes were between 10^4-10^5, 10^3-10^4, and 10^2-10^3 gene copies mL^{-1}, respectively. A significant increase of 3 orders of magnitude in the quantity of the *xplA* and kanamycin genes was observed immediately following inoculation in well DW-2 and within 2 h in well 4-106 (Figure 5.25 B and C). Over the next 24 h, the quantity of *xplA*, and kanamycin genes decreased by about 2 orders of magnitude in these two wells and then stabilized for the duration of the test. At the completion of the field demonstration the *xplA* and kanamycin genes were detected at 1 to 2 orders of magnitude above background quantities in wells DW-2 and 4-106 (Figure 5.25 B and C). In contrast, the quantity of *xplA* and kanamycin genes in well EW-2 did not increase above background levels and were approximately 1 order of magnitude lower by the end

of the study (Figure 5.25 B and C). The qPCR and viability data clearly indicated the survival and transport of KTR9 and RHA1 in wells DW-2 and 4-106, but in well EW-2.

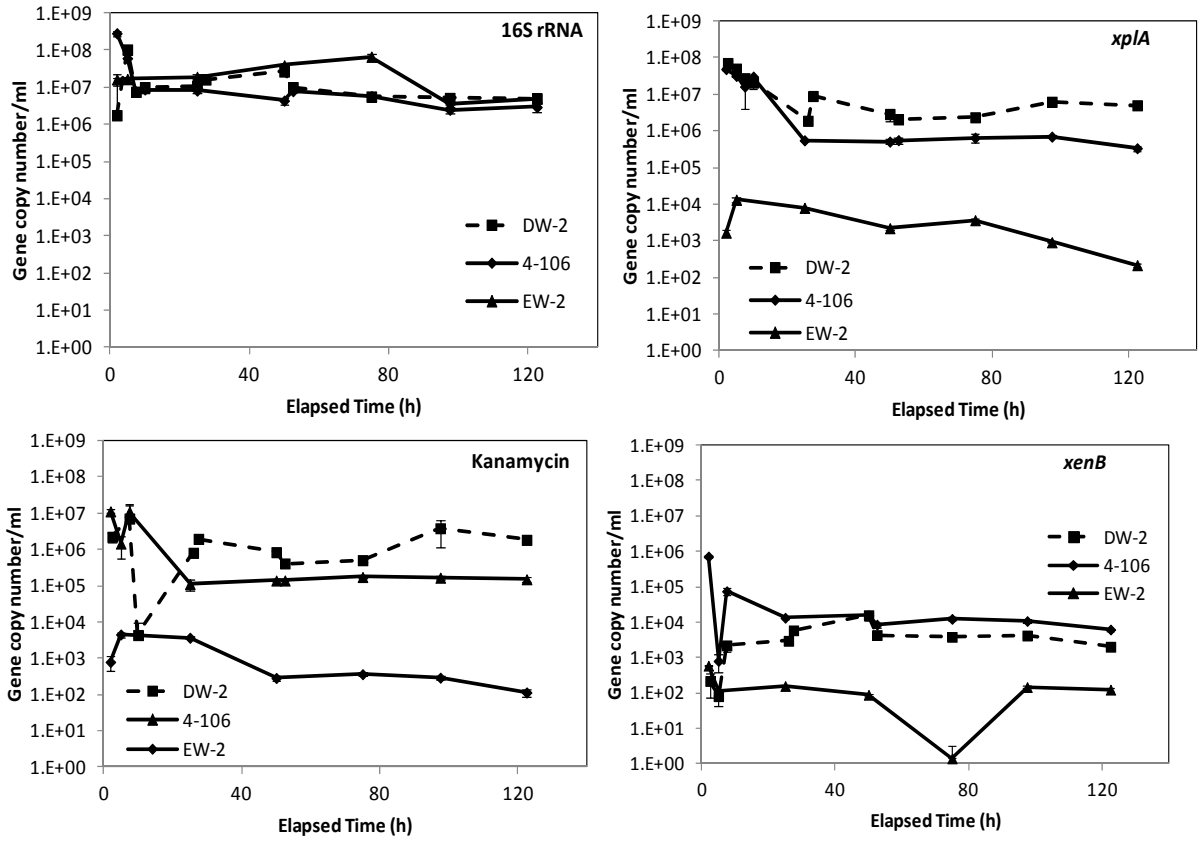


Figure 5.25. qPCR analysis of UMCD groundwater samples from wells DW-2 (■), 4-106 (▲), and EW-2 (◆) targeting the (A) 16S rRNA genes, (B) *xplA* gene, (C) kanamycin gene, and (D) *xenB* gene.

Based on the quantification of the *xenB* gene, strain I-C was transported as rapidly as KTR9 and RHA1 to downgradient well 4-106, but not to well EW-2. Quantities of the *xenB* gene in well 4-106, indicated an initial rapid increase in *xenB* copies within 2 h, followed by a transient decline leading to stable quantities one order of magnitude above background *xenB* gene levels in UMCD groundwater. (Figure 5.25D).

Microbial and tracer breakthrough curve results at downgradient well 4-106 were similar to results obtained during the laboratory column cell transport tests completed during demonstration Phase I. A clear breakthrough of Cl^- tracer by 10 h was observed in the downgradient well, 4-106 (Figure 5.23). Similar to the column tests in Phase I, transport of the tracer was slower than the transport of the cells. In addition, the column transport tests demonstrated that the three bioaugmentation strains remained viable and metabolically active up to 3 months with effluent concentrations about 10^3 CFU mL^{-1}. In comparison, the field demonstration confirmed the transport of the strains over

3 m in the subsurface as well as survival at 1 x 10^5 CFU mL^{-1} or 10^3-10^7 gene copies mL^{-1} for up to 5 days.

Table 5.5. Calculated retardation factors (R) for aerobic RDX-degrading bacteria in Umatilla sediments based on *xplA* gene copy numbers.

Experiment	Well	Distance (m)	R
Packed column	Column 1	0.3	0.77
Small scale field transport	DW-2	NA[a]	-
	4-106	3.1	0.49

[a]NA: not applicable

5.4.3 Discussion and Recommendations from Phase II

In order for this technology to be successfully implemented at UMCD (and other sites) the bioaugmentation culture must remain viable and sustain RDX-degrading capability over time *in situ*. Bacterial survival was assessed using (a) viable plate counts over time on species-specific media, (b) quantification of specific biomarker genes: *xplA*, kanamycin, and *xenB*, and (c) RDX degradation rates. The Phase II bacterial transport demonstration confirmed the ability to transport the bioaugmentation culture at the field scale and that the culture could remain viable *in situ*. The viable plate counts and qPCR data for the *xplA* and kanamycin genes confirmed the rapid transport of strains KTR9 and RHA1 to well 4-106, which was located 3 m downgradient of the injection well. Only qPCR data for the *xenB* gene was available to infer groundwater transport of strain I-C to well 4-106. Transport of halorespiring cultures up to 30 m from the injection well in recirculating systems [43] and up to 13.5 m with semi-passive groundwater circulation approaches [44] have been observed in other bioaugmentation studies. However, in this study transport of the bioaugmentation culture and the chloride tracer wasn't detected 21 m downgradient from the injection well in EW-2. Colonies similar in appearance to strains KTR9, RHA1, or I-C were not detected and only background levels of the *xplA*, kanamycin, and x*enB* genes were measured in groundwater from well EW-2. Previous tracer tests had indicated that groundwater connectivity between wells 4-106 and EW-2 may not be vertically uniform, thereby raising the possibility that the injected cells bypassed the downgradient well or that the sampling regime missed their arrival. Strains KTR9 and RHA1 survived well for up to 5 days in the UMCD aquifer with viable numbers remaining steady at approximately 10^5 CFU mL^{-1} and *xplA* gene quantities greater than 10^6 copies mL^{-1}. Strain I-C survived during the transport demonstration but at lower numbers (10^4 *xenB* copies mL^{-1}) than KTR9 and RHA1. Overall the viable plate counts on LBkan agar plates and the qPCR TaqMan assay of the *xplA* gene were the most reliable indicators of the presence of KTR9 and RHA1 in the groundwater. The qPCR assays of the 16S rRNA and kanamycin resistance genes followed the same trend as the *xplA* gene. The SybrGreen qPCR assay of the *xenB* gene was more reliable than the use of the selective medium, PIA, for detection of strain I-C even though the qPCR data gave some variable results. It was also decided to include only KTR9 in the bioaugmentation culture for the Phase III demonstration. The inclusion of RHA1 was deemed unnecessarily redundant, since both strains were transported and survived in the groundwater to a similar extent. In addition, the inability to track viable cells of strain I-C via plate counts, and the inconsistent qPCR assay with *xenB,* lead to the decision to not use strain I-C during the Phase III demonstration.

5.5 PHASE III: BIOAUGMENTATION FIELD TRIAL AND PUSH-PULL TESTING

The following is a summary of the work performed, which is described in more detail in Michalsen et al. 2015 [45]. Analytical and supportive data collected during the Phase III field testing is included in Appendix E. The objectives of Phase III were to:

(1) Determine if bioaugmentation of aerobic groundwater with *Gordonia* sp. KTR9 Kan^R could support rates and extents of RDX degradation that were comparable to stimulation of anaerobic RDX biodegradation in the same groundwater; and

(2) Determine the effects of bioaugmentation and biostimulation on groundwater chemistry.

5.5.1 Methods

Bioaugmentation Test Format. Aerobic bioaugmentation was investigated in wells DW-2, 4-106, and EW-2 (Figure 5.26). KTR9 was grown in basal salts medium amended with 50 mM fructose at 30°C and harvested by centrifugation using methods in Fuller et al. 2015 [19]. The culture was shipped to the field in stainless steel vessels with pressurized air headspace. Bioaugmentation was accomplished by injecting 6 m^3 site groundwater containing the inoculating culture (10^8 cells mL^{-1} based on qPCR determination of *xplA* gene copy numbers, see below) and 1 mM fructose into each well. DW-2 and 4-106 were bioaugmented once. EW-2 was bioaugmented twice; initially and then again one week prior to the third and final set of push-pull tests (described below). Over the 130 days of this demonstration, these wells also received five to six additional injections of 6 m^3 site groundwater containing 0.25 to 1 mM fructose into each well to stimulate growth and activity of KTR9.

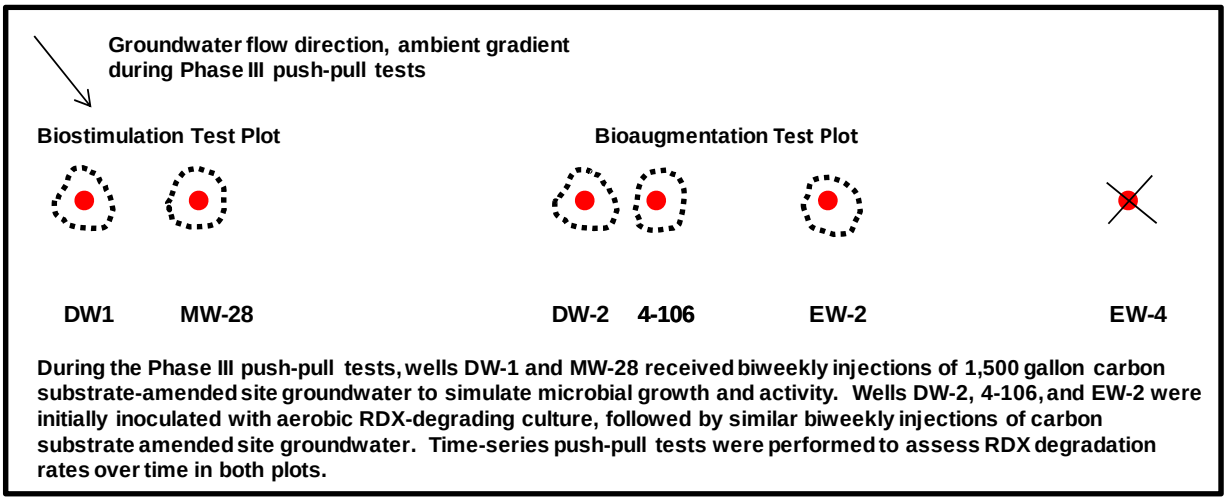


Figure 5.26. Well layout for Phase III push-pull tests

Biostimulation Test Format. Aerobic and anaerobic biostimulation were investigated in wells DW-1 and MW-28. Aerobic biostimulation was accomplished by five injections of 6 m^3 site groundwater containing 0.25 to1 mM fructose into each well over 24 days to stimulate the growth

of indigenous organisms with the ability to degrade RDX. Redox potential, dissolved oxygen and Fe(II) concentrations were measured before each fructose addition to insure that groundwater remained aerobic (dissolved oxygen > 1 mg L^{-1}). After push-pull tests were conducted in all wells to measure rates of RDX degradation, six additional, higher concentration (15 to 24 mM) fructose additions were used to create anaerobic conditions in wells DW-1 and MW-28.

Push-Pull Test (PPT) Methodology. *In situ* rates of RDX degradation in the aquifer were determined using data from single-well, push-pull tests conducted in all wells using methods similar to Michalsen et al. 2013 [46]. Briefly, 6 m^3 of site groundwater containing 1 mM Cl^-, 1-5 mM fructose, and 1 mg L^{-1} RDX were injected into each well; groundwater samples were collected from the same well for 50-120 h after injection and analyzed for Cl^- and the nitroso containing breakdown products hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). Selected samples were analyzed for viable cell counts and *xplA* gene copy numbers. RDX degradation rates were computed from dilution-adjusted RDX concentrations using the method of Istok et al. 1997 [47], which incorporates site specific RDX retardation factors reported by Michalsen et al. 2013 [46]. Rates of RDX degradation for aerobic and anaerobic biostimulation were measured once in wells DW-1 and MW-28. Rates for aerobic bioaugmentation were measured three times in wells DW-2, 4-106, and EW-2, once immediately following initial bioaugmentation with KTR9 (1st test) and twice more over a period of 130 days (2nd and 3rd tests).

Groundwater Sample Collection. Dissolved oxygen, pH and oxidation reduction potential (ORP or redox potential) were measured in the field using an in-line flow cell (MicroPurge® Flow Cell Model MP20), and Fe(II) was measured using a HACH® Kit Model IR-18C. Groundwater sampling for chloride and microbial analyses were collected and analyzed as described previously [46]. To quantify RDX concentrations, groundwater was collected in 1L HDPE amber bottles. The bottles were almost completely filled, acidified to pH 3 with HCl, closed with HDPE caps, and placed on ice after collection and during shipping. The bottles were stored at 4ºC for up to 1 week in the field prior to shipping on ice to the laboratory for analysis.

Analytical Methods. Samples for inorganic anion analysis were collected in 15 mL plastic centrifuge tubes; samples for RDX analyses were collected in 250 mL amber glass bottles, shipped on ice, and stored at 4 °C until analysis. RDX, MNX, DNX and TNX were measured using high performance liquid chromatography (HPLC) according to a modified EPA Method 8330 using a Dionex 3000 Ultimate HPLC with a Agilent Zorbax Bonus-RP column (4.6 x 75 mm, 3.5 µm particle diameter), variable wavelength detector (254 nm), and a photodiode array detector collecting peak spectral data. The mobile phase was 50:50 methanol:0.2% (v:v) trifluoroacetic acid in water at a flow rate of 1 mL min^{-1}. Inorganic anion concentrations (Br^-, sulfate, nitrate and nitrite) were determined by IC (EPA Method 300.0).

Methods for Microbial Parameters. Samples for microbial analyses were collected in 1 L sterile, plastic bottles, shipped on ice, and stored at 4ºC prior to analysis. Groundwater samples were filtered onto polyethersulfone filters (0.2 µm porosity, 73 mm diameter; Corning® disposable filtration units, Lowell, MA) in the laboratory and the filters were aseptically cut in half and each half placed into a DNA extraction tube. The PowerWater DNA Isolation Kit (MoBio Inc., Carlsbad, CA) was used to extract the DNA. The samples were homogenized in a bead beater for

1 min followed by vortex mixing for 40 s. All qPCR amplification reactions were performed using Applied Biosystems 7900HT Fast Real-time PCR system (Foster City, CA). qPCR was performed in 20 µL reaction volumes in 384-well optically clear plates. Analysis of the *xplA* genes used a QuantiTect Probe PCR Kit (Qiagen, Valencia, CA), 300 nM of the respective primers (*xplA*457_f: 5'-CGACGAGGAGGACATGAGATG-3' and *xplA*562_r; 5'-GCAGTCGCCTATACCAGGGATA-3'), 200 nM of the respective TaqMan probe (*xplA*_Tm479: 5'- [6-FAM]CCGCTGCGTCCATCGATCGC[Tamra-Q]-3') and 1 µL of template DNA. Thermal cycler conditions were 95°C for 12 min; then 40 cycles of 95°C for 30 s; 50°C for 60 s; and 72°C for 20 s. Standard curves for each qPCR assay were obtained from serial dilutions of genomic DNA isolated from strain KTR9 (kanamycin gene); or an *xplA* containing plasmid (pET11a, Celtek Genes, Franklin, TN) [6]. The quantity of gene copies per mL of groundwater was calculated according to Ritalahti, et al 2006 [48].

5.5.2 Results and Discussion for Phase III

Prior to testing, groundwater in all wells had a similar chemical composition: RDX (20 to 84 µg L^{-1}), O_2 (≥6 mg L^{-1}), NO_3^- (4 to 22 mg L^{-1}), SO_4^{2-} (≥18 mg L^{-1}), Fe(II) (0 mg L^{-1}), ORP (≥49 mV), and pH (~ 8 S.U.); viable KTR9 cell counts were near or below detection (< 10 CFU mL^{-1}) and xplA gene copy numbers were between 10^3 and 10^5 copies mL^{-1}. Fructose additions during low carbon biostimulation in DW-1 and MW-28 resulted in reduced O2 and ORP; and unchanged NO_3^-, SO_4^{2-} and Fe(II) (Table 5.6). During subsequent PPTs, injected RDX was degraded in both wells. For example, in MW-28, dilution-adjusted RDX concentrations decreased from 0.55 to 0.25 mg L^{-1} within 80 hours after injection (Figure 5.27). However, the fitted RDX degradation rate coefficients for low carbon biostimulation treatments were not statistically different from zero (0.44±0.48 day^{-1}, p=0.060; 0.54±0.75 day^{-1}, p=0.100; Table 5.6). Trace levels of anaerobic RDX degradation products MNX, DNX, and TNX were detected during low carbon biostimulation (maximum concentrations of 2.7, 6.3, and 3.2 µg L^{-1}, respectively).

Subsequent higher concentration (15 to 24 mM) fructose additions during high carbon biostimulation in DW-1 and MW-28 resulted in a slight decrease in pH; further decreases in O2, NO_3^-, and ORP, as well as production of Fe(II), but no change in xplA gene copies (Table 5.6). PPTs showed faster and more complete RDX degradation under high carbon compared to low carbon biostimulation conditions. For example, the fitted RDX degradation rate increased to 0.70±0.18 day^{-1} in MW-28 under high carbon biostimulation conditions (Table 5.6, Figure 5.27). MNX concentrations increased by an order of magnitude or more during the high carbon biostimulation compared to the low carbon biostimulation (e.g. 65 vs. 3.0 µg L^{-1}, respectively). DNX and TNX remained in the same general range. Reduced NO_3^- concentrations coupled with transient appearance of NO_2^- (1 to 2 mg L^{-1}) in some samples (data not shown), together with appearance of Fe(II), reduced SO_4^{2-}, and ORP values of <-169 mV, suggests denitrification, iron and sulfate reduction occurred during the high carbon biostimulation tests, although O2 concentrations ≥1.8 mg L^{-1} were measured during this period.

Table 5.6. Effects of fructose additions on groundwater composition and on *in situ* RDX degradation rates during aerobic and anaerobic biostimulation and aerobic bioaugmentation.

Wells		*xplA* copies[a] mL^{-1}	CFU[b] mL^{-1}	**RDX Degradation[c] Rate, day^{-1}**	RDX[d] $\mu g\ L^{-1}$	MNX[e] $\mu g\ L^{-1}$	DNX[e] $\mu g\ L^{-1}$	TNX[e] $\mu g\ L^{-1}$	O_2 [f] $mg\ L^{-1}$	NO_3^- [g] $mg\ L^{-1}$	SO_4^{2-} [g] $mg\ L^{-1}$	Fe(II) [f] $mg\ L^{-1}$	ORP [f] mV	pH [f] S.U.
DW-1	Background (no fructose)	3×10^3	-		65.0	0	0	0	6.0	22.0	18.0	0	49	8.0
	Aerobic biostimulation [1]	5×10^4	-	0.54 ± 0.75 p = 0.100	4.1	2.7	1.3	3.2	2.8	27.0	16.0	0	12	7.6
	Anaerobic biostimulation [2]	3×10^4	-	**0.63 ± 0.22 p < 0.001**	4.1	27.0	0	7.9	2.0	0	0	3.0	-169	6.8
MW-28	Background (no fructose)	2×10^4	-		84.0	0	0	0	7.0	9.0	18.0	0	64	8.1
	Low carbon biostimulation [1]	7×10^3	-	0.44 ± 0.48 p = 0.060	41.0	0.9	6.3	1.8	2.9	13.0	15.0	0	24	7.6
	high carbon biostimulation [2]	9×10^3	-	**0.70 ± 0.18 p < 0.001**	9.5	65.0	0	8.9	1.8	0	1.0	1.0	-172	6.8
	[1] Five 0.25-1 mM fructose additions between 5/22 and 7/2													
	[2] Six 15-24 mM fructose additions between 7/24 and 9/4													
DW-2	Background (no fructose/cell inj.)	7×10^5	*5×10^2*		20.0	0	0	0	-	13.0	26.0	-	-	-
	Initial post cell injection	1×10^8	5×10^6											
	Aerobic bioaugmentation 1st test [g]	4×10^7	2×10^6	**2.90 ± 1.80 p = 0.007**	6.2	1.0	11.0	0	9.5	0	18.0	-	6	8.6
	Aerobic bioaugmentation 2nd test [3]	6×10^4	1×10^3	**0.63 ± 0.36 p = 0.004**	54.0	0	0	0	2.2	0	19.0	0.1	28	7.3
	Aerobic bioaugmentation 3rd test [4]	4×10^3	ND	0.24 ± 0.51 p = 0.300	97.0	0	0	0	3.2	0	18.0	0	-63	7.5
4-106	Background (no fructose/cell inj.)	6×10^4	ND		24.0	0	5.2	0	-	18.0	26.0	-	-	-
	Initial post cell injection	2×10^8	6×10^6											
	Aerobic bioaugmentation 1st test [g]	7×10^7	2×10^6	**4.00 ± 2.90 p = 0.010**	8.1	0	12.0	0	9.5	0	24.0	-	6	8.6
	Aerobic bioaugmentation 2nd test [3]	7×10^6	*2×10^2*	**1.40 ± 1.10 p = 0.020**	51.0	0	0	0	2.5	0	17.0	0.1	-48	7.4
	Aerobic bioaugmentation 3rd test [4]	2×10^4	ND	0.15 ± 0.38 p = 0.400	131.0	0	0	0	3.1	0	20.0	0	-72	7.4
EW-2	Background (no fructose/cell inj.)	2×10^5	ND		22.0	0	0	0	-	4.0	24.0	-	-	-
	Initial post cell injection	2×10^8	1×10^7											
	Aerobic bioaugmentation 1st test [g]	9×10^7	4×10^6	**0.96 ± 0.58 p = 0.007**	9.1	0.9	15.0	0	9.5	0	21.0	-	6	8.6
	Aerobic bioaugmentation 2nd test [3]	2×10^5	*6×10^1*	0.08 ± 0.24 p = 0.500	34.0	0	0	0	2.8	0	16.0	0.7	-39	7.5
	Aerobic bioaugmentation 3rd test [4*]	1×10^6	3×10^3	**0.16 ± 0.10 p = 0.010**	174.0	27.0	0	2.3	5.6	0	23.0	0	-56	7.5
	[3] Six 0 - 0.5 mM fructose additions between 5/22 and 7/2; no additional cells added to any wells													
	[4] Six 0 - 0.5 mM fructose additions between 7/24 and 9/4; no additional cells added to DW-2 or 4-106; additional cells were added to EW2 on 9/4 prior to the 3rd PPT on 9/15													
	[a]gene copies were measured in each well immediately prior to corresponding push-pull test													
	[b] values shown in italics are near reporting limits but indicate viable cells were present													
	[c]rate coefficient ± 95% confidence interval; bold values indicate slope is significantly different from zero at α=0.05 level.													
	[d] pre-test RDX concentrations in wells represent background; all other RDX concentrations reported represent minimum achieved during associated tests													
	[e]MNX, DNX, and TNX concentrations represent maximum concentrations detected during the associated test													
	[f] values represent the average associated with each treatment phase													
	[g] values represent minimums associated with each treatment phase													
	[g] pH, O_2, ORP were collected from proxy well 4-105 located < 15 ft from bioaugmentation wells; background concentrations represent pretest/pre-cell injection values; "-" here and throughout table indicates not measured													

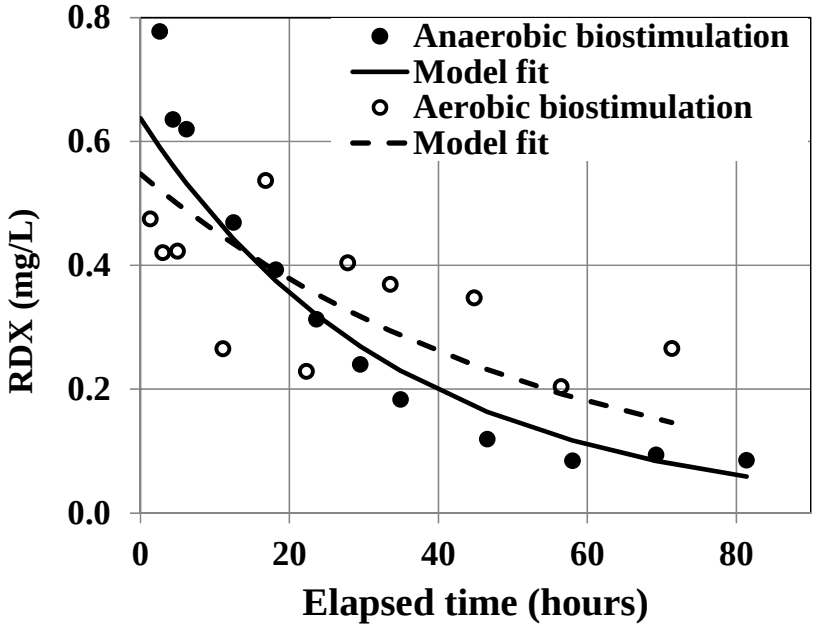


Figure 5.27. Example push-pull test results in MW-28 showing *in situ* RDX degradation and first-order model fits following biostimulation.

Bioaugmentation with the KTR9 culture in wells DW-2, 4-106, and EW-2 resulted in initial viable cell counts of 10^7 to 10^8 CFU mL^{-1} and 10^7 to 10^8 *xplA* copies mL^{-1}, which decreased during subsequent testing to below 10^4 CFU mL^{-1} and 10^5 *xplA* copies mL^{-1} (Figure 5.28). Viable cell counts and *xplA* gene copies briefly increased in EW-2 following a second bioaugmentation with KTR9 that was performed in that well just prior to the third PPT (day 130). Fructose additions to bioaugmented wells resulted in reduced O_2, NO_3^-, and ORP; SO_4^{2-} concentrations remained in the same range (Table 5.6). A single Fe(II) detection (0.5 mg L^{-1}) in wells DW-2 and 4-106 and two Fe(II) detections (1.5 and 2.5 mg L^{-1}) in well EW-2 were measured prior to the second PPT. Several subsequent substrate injections included aerobic groundwater only, with no added fructose, in an effort to supply O_2 and prevent onset of reducing conditions. Fe(II) was not detected again in bioaugmented wells during the study.

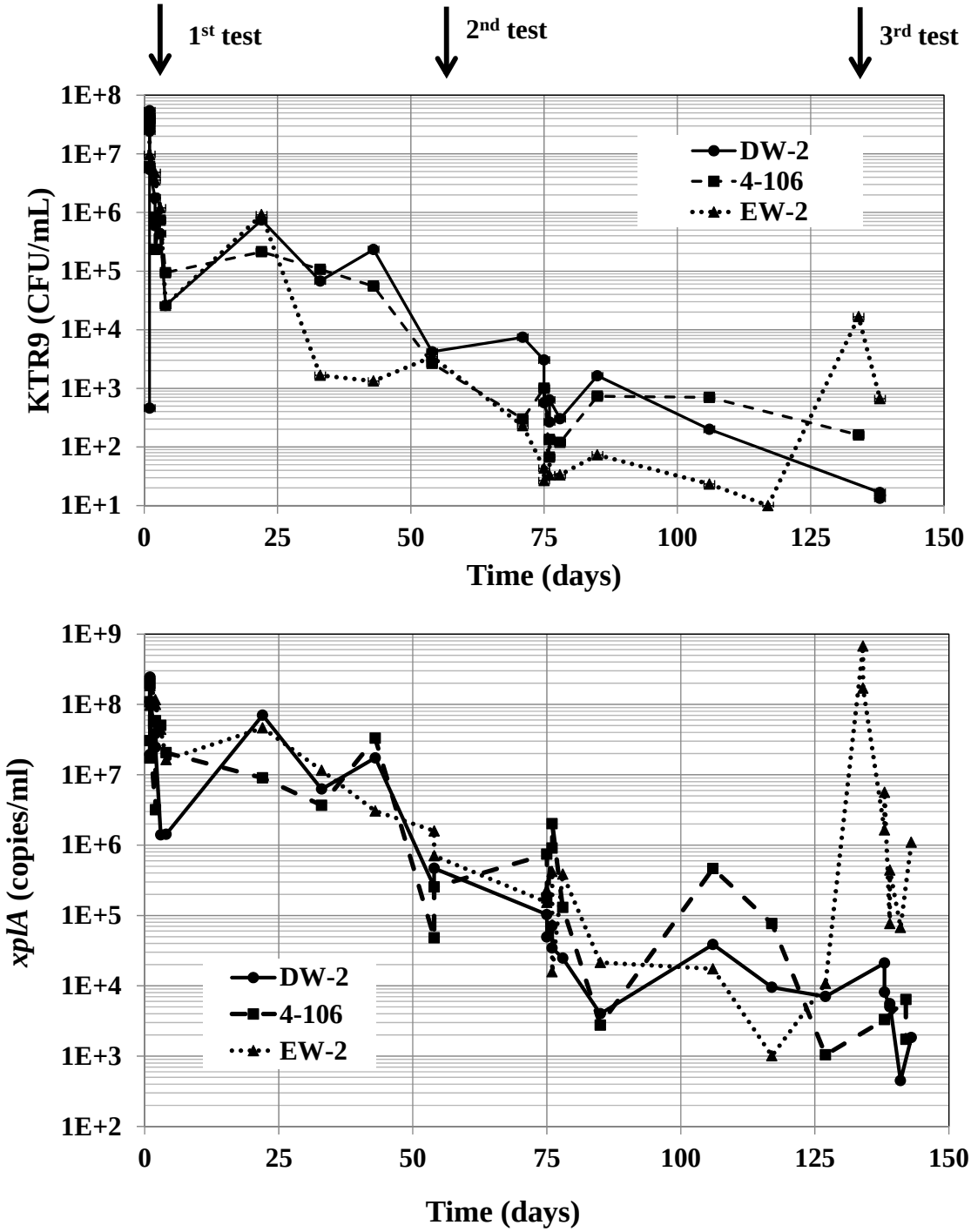


Figure 5.28. Viable KTR9 colony counts and *xplA* gene copy numbers during aerobic bioaugmentation. Arrows refer to 1st, 2nd, and 3rd push-pull tests used to measure *in situ* RDX degradation rates.

Injected RDX was degraded in all bioaugmented wells. Dilution-adjusted RDX concentrations decreased from 1 to 0.1 mg L^{-1} in the first test in well DW-2 (Figure 5.29). Initial RDX degradation rate coefficients ranged from 0.96±0.58 to 4.00±2.90 day^{-1} during the first PPT but declined over time (Table 5.6). RDX degradation rate coefficients were noticeably lower in well EW-2 compared to DW-2 and 4-106, in fact the rate coefficient during the second PPT in EW-2 was not statistically different from zero (0.08±0.24 day^{-1} p=0.500) and remained low even after this well was bioaugmented for a second time prior to the third PPT (Table 5.6). Reduced nitrate concentrations coupled with transient appearance of nitrite (1 to 2 mg L^{-1}) in some samples (data not shown), suggests denitrification also occurred during the bioaugmentation PPTs. Fe(II) was detected at low concentrations prior to the second PPT in all bioaugmented wells, suggesting iron reduction occurred during this time. Reduced fructose concentrations following Fe(II) detection resulted in increased O_2 and no Fe(II) detections prior to or during the third PPT.

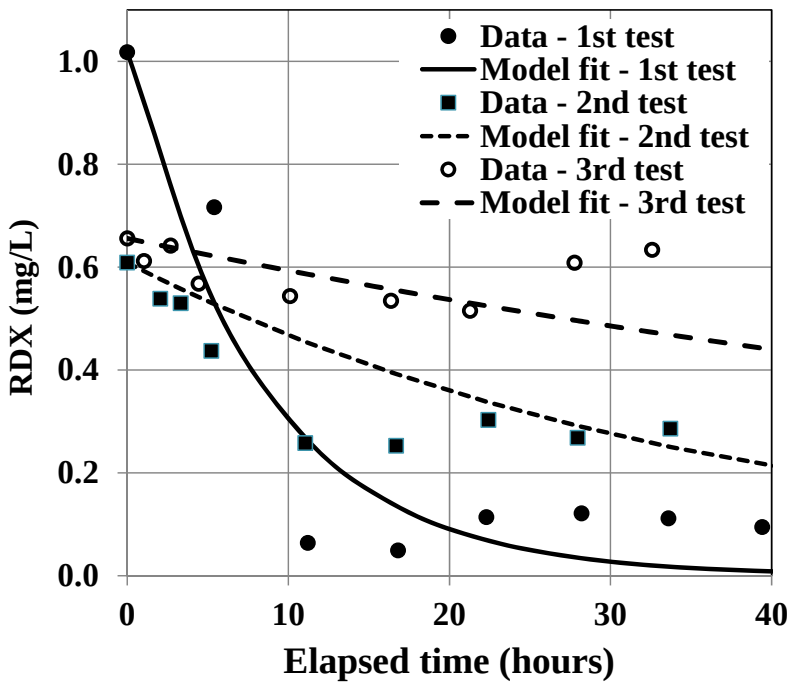


Figure 5.29. Push-pull test results in DW-2 showing *in situ* RDX degradation and first-order model fits following bioaugmentation.

Bioaugmentation yielded the highest average (all wells and tests combined) rate of RDX degradation of the three treatments (1.2 day^{-1}), approximately twice the average rate of RDX degradation for either low carbon (0.49 day^{-1}) or high carbon biostimulation (0.67 day^{-1}; Table 6.1). Low carbon biostimulation treatment resulted in RDX removal rate coefficients that were not significantly different from zero but removal efficiencies were included in this assessment for completeness. A comparison of substrate added per mass of RDX degraded showed that bioaugmentation (10^3 mol fructose/mol RDX) required 97% less growth substrate than either low

carbon (10^4 mol fructose/mol RDX) or high carbon biostimulation (10^5 mol fructose/mol RDX; Table 6.3) to achieve comparable reductions in RDX concentrations. Bioaugmentation treatment costs include the unit production cost of required KTR9 cells, which is estimated at ~ $250 for 1 L containing ~ 10^{12} cells, but this cost may decline with further process optimization, as has occurred with some commercially available dechlorinating bioaugmentation cultures.

The results of this project support the inclusion of bioaugmentation with *Gordonia* sp. KTR9 as part of the remedial action plan at UMCD. This technology may be implemented in the portion of the contaminated plume where RDX concentrations are generally less than 100 $\mu g\ mL^{-1}$ and the groundwater is aerobic.

6.0 PERFORMANCE ASSESSMENT

The performance of the technology during the demonstration included both qualitative and quantitative objectives (Table 3.1). Each objective was assessed using data gathered during the demonstration, as described below.

6.1 QUANTITATIVE PERFORMANCE OBJECTIVES

1. Ability to reduce RDX concentrations in groundwater to below relevant cleanup concentration. In order for this technology to be successfully implemented at UMCD as part of the full-scale groundwater remedy, reduction of RDX concentrations to below site-specific RDX groundwater cleanup criteria should be achievable. At UMCD, the relevant RDX concentration is the remedial action criteria of 2.1 μg L^{-1}.

Success Criteria: Reduction of RDX concentration to < 2.1 μg L^{-1} during the Phase III push-pull test in the aerobic bioaugmentation treatment test plot wells.

Results: Measured RDX concentrations during individual tests in any well did not reach 2.1 μg L^{-1} (Table 5.6) but the magnitude of concentration decreases was larger for the first two aerobic bioaugmentation tests than for aerobic or anaerobic biostimulation. This suggests that aerobic bioaugmentation could be an effective alternative to biostimulation for *in situ* treatment of RDX contaminated groundwater at the UMCD. The decrease in RDX degradation rate and the increase in time required to reach the remedial action criteria, during repeated testing over 130 days, is attributed to decreases in cell numbers as well as the onset of reducing conditions following repeated fructose additions. However, the time required to reach the remedial action criteria during full-scale implementation can be estimated using the *in situ* RDX degradation rates measured during Phase III. The results show that all three treatments can achieve the remedial action criteria but a single cell addition was sufficient to allow aerobic bioaugmentation to achieve the remedial action criteria in the shortest time for the first two tests (Table 6.1).

Table 6.1. Comparison of RDX degradation rate and time required to reach remedial action criteria for all treatments.

Treatment	[a]Average RDX degradation rate coefficient (day^{-1})	[b]Estimated time required to reach remedial action criteria (days)
Aerobic bioaugmentation		
1st test	2.60 (0.96-4.00)	1.5
2nd test	0.70 (0.08-1.40)	5.5
3rd test	0.18 (0.15-0.24)	21.0
Aerobic biostimulation	0.49 (0.44-0.54)	7.9
Anaerobic biostimulation	0.67 (0.63-0.70)	5.8

[a]The range of individual values included in average is provided in parentheses; [b]Computed using $t = -\frac{1}{k_{ave}} \ln\left(\frac{Cr}{Cb}\right)$, where C_r = 2.1 μg L^{-1} and C_b= assumed initial RDX concentration = 100 μg L^{-1}, which is representative of initial RDX concentrations when applying in field at UMCD.

2. Removal rates are comparable to anaerobic biostimulation treatment. In order for this technology to be successfully implemented at UMCD (and other sites) as part of the full-scale groundwater remedy, RDX transformation rates should be comparable to – or not significantly less than – those of either aerobic or anaerobic biostimulation.

Success Criteria: Computed rates of RDX degradation are 1) similar to or at least half the rates measured during anaerobic biostimulation, and 2) similar to or preferably larger than the rates measured during aerobic biostimulation.

Results: Aerobic bioaugmentation had the largest average (all wells and tests combined) rate of RDX degradation of all treatments; ~2x the average rate of RDX degradation for either aerobic or anaerobic biostimulation (Table 6.2).

Table 6.2. Comparison of RDX degradation rate and time required to reach remedial action criteria for all treatments.

Treatment	Average RDX degradation rate (all wells and all tests combined) (day^{-1})	(Average rate for aerobic bioaugmentation)/ (Average rate for biostimulation)
Aerobic bioaugmentation	1.20	-
Aerobic biostimulation*	0.49	2.40
Anaerobic biostimulation	0.67	1.80

* RDX removal rates were not statistically different from zero but values were included in this comparative assessment for completeness.

3. Enhanced RDX mass removal per mass of substrate added for aerobic bioaugmentation compared to biostimulation. The Phase I laboratory column studies showed rapid RDX removal in bioaugmented columns and microcosm tests that were periodically amended with low concentrations of fructose. Application of these results to field conditions means that aerobic bioaugmentation would require significantly less growth substrate than anaerobic biostimulation to achieve comparable RDX degradation.

Success Criteria: Ratios of RDX mass removed to substrate mass added of 2 or higher for aerobic bioaugmentation compared to aerobic and anaerobic biostimulation.

Results: Aerobic bioaugmentation achieved mass ratios that were approximately 34 times that of anaerobic biostimulation and approximately 10 times that of aerobic biostimulation (Table 6.3). It is important to note that even in the third aerobic bioaugmentation test – when RDX degradation rates for aerobic bioaugmentation had decreased below that for anaerobic biostimulation (Table 6.1) – still required 20 times less fructose than anaerobic biostimulation (Table 6.3).

Table 6.3. Comparison of RDX mass degraded per mass of added fructose for all treatments

Measured or Computed Values/Treatments		Aerobic bioaugmentation	Aerobic biostimulation*	Anaerobic biostimulation
RDX Transformation Rates	k_{avg}, day^{-1}	1.20	0.49	0.67
Representative initial RDX groundwater concentration at start of treatment	C_o RDX, $\mu g\ L^{-1}$	100.00	100.00	100.00
Computed final RDX groundwater concentration after 5 days of treatment	C_f RDX, $\mu g\ L^{-1}$	25.00	79.00	64.00
Computed RDX removed during 5 days of treatment, assumes 5,700 L of water treated.	millimols	1.90	0.56	0.92
Measured fructose mols during each 5,700 L push-pull test	mols	5.70	5.70	136.00
Computed mmol RDX removed per mol fructose added		**0.34**	**0.10**	**0.01**

* RDX removal rates were not statistically different from zero but values were included in this comparative assessment for completeness.

4. Bioaugmentation culture remains viable and retains *in situ* RDX-degrading capability over time. In order for this technology to be successfully implemented at UMCD (and other sites) as part of the full-scale groundwater remedy, the bioaugmentation culture must remain viable and retain RDX-degrading capability over time *in situ* for as long as needed to achieve RDX reduction to below the site-specific groundwater cleanup concentration.

Success Criteria: The rate and extent of RDX degradation observed during the second and third aerobic PPTs should be similar to the first PPT. Measureable levels of the *xplA* biomarker should be one order of magnitude higher than pre-inoculation levels and viable numbers of KTR9 should be greater than 30 CFU mL^{-1} during the 130 day demonstration.

Results: RDX transformation activity was sustained within the bioaugmentation test plot for the duration of the demonstration. *xplA* gene copy numbers and viable KTR9 cell counts were sustained for the first two PPTs but decreased below success criteria during the third PPT. Multiple injections of groundwater or groundwater plus fructose intended to maintain aerobic conditions and stimulate growth and activity of KTR9 resulted in the decreases in the microbial parameters overtime during this demonstration. This "flushing" artifact may be less problematic during full-scale implementation where injections occur over a larger scale with less frequency. RDX degradation rates decreased from the first to the third PPTs following bioaugmentation in all wells, except in well EW-2, which was bioaugmented for a second time shortly before the third PPT (Table 6.4). In well EW-2, the additional bioaugmentation increased the RDX degradation rate, viable KTR9 cell numbers and *xplA* gene copy levels during the third test (Table 6.4). Within the scope of this demonstration it was not possible to determine the viability of KTR9 cells attached to sediment particles.

Table 6.4. Microbial community characterization during Phase III push-pull tests.

Well	RDX degradation rate (day^{-1})	Average viable cells ($CFU\ mL^{-1}$)	Average 16S ($copies\ mL^{-1}$)	Average *xplA* ($copies\ mL^{-1}$)
DW-2				
Initial[b]		5×10^6	1×10^7	1×10^8
1st test	2.90	2×10^6	1×10^7	4×10^7
2nd test	0.63	1×10^3	1×10^6	6×10^4
3rd test	0.24	[a]BD	2×10^6	4×10^3
4-106				
Initial[b]		6×10^6	4×10^7	2×10^8
1st test	4.00	2×10^6	2×10^7	7×10^7
2nd test	1.40	2×10^2	3×10^6	7×10^6
3rd test	0.15	[a]BD	4×10^6	2×10^4
EW-2				
Initial[b]		1×10^7	5×10^7	2×10^8
1st test	0.96	4×10^6	3×10^7	9×10^7
2nd test	0.08	6×10^1	6×10^6	2×10^5
3rd test	0.16	3×10^3	2×10^7	1×10^6

[a]Below detection (<30 $CFU\ mL^{-1}$); [b]initial post cell injection microbial values

6.2 QUALITATIVE PERFORMANCE OBJECTIVES

5. Aerobic bioaugmentation preserves secondary groundwater quality. Aerobic bioaugmentation will maintain aerobic conditions in site groundwater, thereby preventing the accumulation of sulfide, ferrous iron, or methane, the potential dissolution of redox sensitive metals, as well as the formation of the RDX nitroso degradation products MNX, DNX, or TNX.

Success Criteria: Aquifer heterogeneity may result in localized water quality impacts but would be minimal for aerobic compared to anaerobic treatments. Success entails confirming minimal effect on secondary groundwater quality as measured using geochemical indicator data and laboratory analyses of groundwater samples for aerobic bioaugmentation compared to anaerobic biostimulation. Accumulation of RDX degradation products was also quantified.

Results: Generally aerobic conditions were maintained during aerobic bioaugmentation and aerobic biostimulation tests but reducing conditions developed during the anaerobic biostimulation tests (Table 6.5). Low concentrations of nitroso-derivatives were detected during the aerobic tests indicating some localized anaerobic zones of anaerobic activity could have contributed to RDX reduction during the aerobic tests. Higher concentrations of nitroso-derivatives were detected during the anaerobic compared to aerobic tests.

Table 6.5. Comparisons of groundwater geochemical data for all treatments

Treatment	Average pH	Average O_2 $mg\ L^{-1}$	Average ORP, mV	Average Fe(II), $mg\ L^{-1}$	[a]MNX, $\mu g\ L^{-1}$	[a]DNX, $\mu g\ L^{-1}$	[a]TNX, $\mu g\ L^{-1}$
Aerobic bioaugmentation	7.8	5.3	-25.8	0.14	27.0	15.0	2.0
Aerobic biostimulation	7.6	2.8	10.0	0.00	3.0	6.0	3.0
Anaerobic biostimulation	6.8	1.9	-171.0	2.20	65.0	0.0	9.0

[a]Maximum concentration detected during tests in all wells.

7.0 COST ASSESSMENT

This section is intended to provide remediation professionals with information to support consideration of aerobic bioaugmentation for cleanup of RDX-contaminated groundwater at a given site. The cost model and other information presented in this section are based on groundwater remedy optimization work completed at the UMCD site. At this site, an increasingly inefficient pump & treat (P&T) remedy for explosives-contaminated groundwater prompted an evaluation of bioremediation technology for remedy enhancement. A focused feasibility study (FFS) was completed to evaluate various combinations of enhanced P&T and bioremediation [49]. The preferred alternative included phased implementation of enhanced P&T to shrink the plume, followed by bioremediation in the remaining plume. At the time of the FFS completion, anaerobic biostimulation had been demonstrated at UMCD but no cost and performance information was available to support inclusion of aerobic bioaugmentation.

This demonstration provided performance and cost information to support inclusion of aerobic bioaugmentation as part of remedy optimization. Accordingly, the FFS is being amended to include an additional alternative where anaerobic biostimulation is applied in the RDX and TNT comingled source area with the potential for aerobic bioaugmentation in the distal portions of the plume. KTR9 (and other *xplA* gene-containing microbes) are able to utilize RDX as a nitrogen source for growth and thus promote RDX degradation; however, these cells are not able to use (or degrade) TNT. In fact, as discussed in Section 8.0, TNT has been shown to inhibit RDX degrading activity of KTR9. Therefore, aerobic bioaugmentation is only applied in this work to the distal RDX plume. Anaerobic biostimulation effectively degrades both RDX and TNT and is therefore well suited for remediation of comingled explosives present near the source area.

In Section 7.1, a simple cost model describing key phases and cost elements of the optimized full-scale groundwater remedy at UMCD is provided, along with costs tracked during this demonstration. In Section 7.2, technology- and site-specific cost drivers that impact viability of aerobic bioaugmentation are described. Finally in Section 7.3, cost analyses are provided for four approaches to full-scale RDX-contaminated groundwater cleanup: enhanced P&T only, enhanced P&T followed by anaerobic biostimulation, enhanced P&T followed by a combined anaerobic biostimulation and aerobic bioaugmentation, and finally enhanced P&T followed by combined anaerobic and aerobic biostimulation.

7.1 COST MODEL

The cost model presented in Table 7.1 is based on the phased remedy optimization approach applied at UMCD, which included the following key phases.

- **Expansion of the existing P&T remedy** to shrink the ~150 ha RDX groundwater plume, including the following Remedial Design and Remedial Action Construction cost elements:
  (1) UMCD project-funded anaerobic biostimulation pilot testing;
  (2) Site-specific groundwater modeling to determine number and placement of additional extraction wells;

(3) Design and construction of new extraction wells, pumps, piping and connections to the existing system; and
(4) Perform bioremediation pilot testing.

ESTCP demonstration activities most related to this project phase included the initial site characterization and laboratory treatability studies (Demonstration Phase I).

- **Application of bioremediation** to reduced plume footprint, including the following Remedial Design and Remedial Action Construction cost elements:
  (1) Site-specific groundwater modeling to determine number of injection/extraction wells (or other infrastructure) required to effectively distribute bioremediation amendment over targeted treatment area;
  (2) Simulation of bioremediation effectiveness over time by applying RDX transformation rates measured during push-pull tests to aquifer footprints in the model; and
  (3) Design and construction of injection/extraction wells, growth substrate metering system, and other related bioremediation system components.

ESTCP demonstration activities most related to this project phase included the forced-gradient cell transport testing and in situ biostimulation and bioaugmentation field treatments followed by push-pull testing (Demonstration Phases II and III).

- **Field-scale anaerobic/aerobic biostimulation** was the final stage of remedy optimization at UMCD. This included field-scale growth substrate injections to maintain anaerobic biostimulation treatments, as well as to sustain growth and RDX-degrading activity of KTR9 cells in the bioaugmented treatments.

Cost elements associated with this project phase include operations and maintenance followed by project completion activities.

7.2 COST DRIVERS

Cost elements associated with the phased remedy optimization at UMCD are generally applicable to other explosives-contaminated groundwater sites where P&T remedies have declined in performance. However, costs for ESTCP demonstration phases presented in Table 7.1 may be high based on UMCD site-specific considerations and demonstration-specific features as follows.

- **Drilling costs.** Due to drilling depths (46 m in demonstration test plot area) and presence of gravels and cobbles requiring sonic or air rotary installation methods, drilling costs at UMCD are substantial.
- **Microcosm testing.** Significant screening and optimization of select bacterial strains were required upfront during this project because aerobic bioaugmentation had not been previously demonstrated. The larger degree of upfront bacterial screening required during this demonstration increased treatability study costs. If considering aerobic bioaugmentation for RDX remediation at another site, pure culture screening would likely not be required. Instead, selection of two or three relevant strains could be evaluated in

microcosms prepared using site soil and groundwater to confirm (a) strains are able to grow and (b) strains rapidly and completely degrade RDX in solution with select growth substrates.

Table 7.1. RDX groundwater remedy optimization with bioremediation cost model with demonstration-specific cost details and amounts provided.

Cost Element	Sub Element	Tracked during ESTCP demonstration	Demonstration Totals
Remedial Design, Phase I	Field-Scale Anaerobic Biostimulation Testing		-
	Phase I – Design Expanded Groundwater Extraction System		-
	Install 2x Injection Well Pilot; Laboratory Treatability Studies	Installation of 2, 4" demonstration wells to 46 m bgs, including field oversight $75K	~$440K
		Forced gradient, well-to-well tracer testing, $50K	
		Field borehole Dilution Test, $50K	
		Pure culture screening and optimization of Bioaugmentation Culture (Lab), $125K	
		Cell transport column testing (Lab), $90K	
		Reporting, $50K	
Remedial Action Construction. Phase I	Expanded Groundwater Extraction System Construction		-
	Anaerobic Biostimulation in Lagoon Source Area		-
	1 Monitoring Event yr^{-1} during RA		-
	Operation and Maintenance		-
Remedial Design, Phase II	Phase II – Design modifications to existing, expanded P&T system to include Biostimulation and/or Bioaugmentation in Plume including GW model simulations	Prepare field-scale quantity of cells for shipment to field, $50K	~$ 383K
		Field-scale, forced-gradient cell transport testing, $180K	
		Analytical costs including qPCR and viable cell counts, $103K	
		Reporting, $50K	
		Prepare field-scale quantity of cells for bioaugmentation, $50K	~$468K
		Growth substrate injections in field 4+months, $80K	
		Complete 13 push-pull tests to measure RDX removal effectiveness, $150K	
		Analytical costs including qPCR and viable cell counts, $138K	
		Reporting, $50K	
Remedial Action Construction	Comingled Plume Biodegradation – Anaerobic Injection Well and trenching Construction		-
	RDX Plume Biodegradation – Aerobic Injection Well and trenching Construction		-
Operation and Maintenance	Comingled Explosives Plume Biodegradation – Aerobic Substrate Injections		-
	RDX Plume Biodegradation – Aerobic Substrate Injections		-
	Semiannual Monitoring		-
	Five Year Reviews		-
Completion Activities	Site Close-Out Documentation		-
	Administrative Land Use Controls		-
Non Discounted Cost			**$1,291K**

- **Column Testing.** It can be very helpful to conduct column tests using repacked aquifer material and actual or simulated site groundwater to evaluate cell transport and RDX-degrading activity for selected microbial strains. Such laboratory tests provide a controlled environment where cell viability, transport and performance overtime can be evaluated. The costs of column testing at a site will depend on (a) contracting costs associated with acquiring technical services for collection of sediment and groundwater quantities needed

for column testing, and (b) number of columns and duration of the tests, which determines number of samples for analysis.

- **Analytical Costs.** Analytical costs were included in the cost of laboratory treatability and field testing. For this demonstration, a genetically modified/kanamycin resistant strain of KTR9 was used, which allowed efficient enumeration of viable cells on selective kanamycin-containing plates. The cost for determining viable cell numbers from groundwater samples is estimated to be around $250 per sample based on the availability of general laboratory supplies and 4 h ($50/h) of labor for a laboratory technician. Quantitative PCR analysis of groundwater samples is the preferred method for monitoring the presence of bioaugmentation cultures, since a selective agar plate medium isn't always available for colony discrimination between the indigenous and inoculated strains. The cost of the *xplA* qPCR assay is estimated to be about $500 per sample since a senior technician trained in molecular biology is required along with specific reagent kits and analytical instruments. Kits for DNA extraction and reagents and instruments for the TaqMan qPCR assay are available commercially from a variety of life science companies. The primer and probe sequences for the *xplA* TaqMan assay are available from the authors and can be synthesized commercially or by a post-secondary institution that offers this capability.
- **Field Cell Transport and Performance Testing.** Cell transport properties determined during column testing provide valuable information to support go/no-go decision making for viability of aerobic bioaugmentation at a site. However, confirmation of cell transport using optimally-placed desired injection/extraction wells (or other approaches) relevant to a site is desirable, since repacked sediment columns may not be representative of sediment or hydraulic heterogeneities that exist *in situ*. A typical cell density desired *in situ* for bioaugmentation is $1x10^6$ cells mL^{-1}. Reporting limits for *xplA* gene copy numbers by qPCR are in the $1x10^3$ range. Accordingly, users may plan to conduct a field tracer test to confirm (a) hydraulic connectivity between the injection and downgradient monitoring locations targeted during the test.
- **Cost of Cells.** The cost of the bioaugmentation culture production is based on prevailing rates for production of specialty bacterial cultures. This cost was in the range of $250-$300 per L for this project, but could be reduced if/when these cultures become more widely used.

Other key cost drivers include the choice of implementation strategy. For example, if the remedial objective includes preventing discharge from a site, it could be possible to install a biobarrier where targeted cell density is reached and maintained over time through injection of low concentration growth substrate. This approach would require microbial and substrate distribution within a considerably smaller targeted zone compared to the entire contaminated groundwater footprint. This could substantially reduce microbial costs (although the same amount of substrate would be required but would just be extended over a longer treatment time). At UMCD, the objective is to achieve mass reduction within the plume within 3 – 5 years, which requires distribution of cells at 10^6 cell mL^{-1} density within a large portion of the plume.

7.3 COST ANALYSIS

A description of the site was provided in Sections 4.1 (site location), 4.2 (description of site geology/hydrogeology) and 4.3 (contaminant distribution). Approximately 85 million gallons of explosives-contaminated wastewater were infiltrated through unlined washout lagoons to UMCD site soil between the mid-1950s and 1965 [49]. Explosives-laden wastewater percolated through the unsaturated alluvium beneath the lagoons to groundwater, creating the groundwater plume. The remedial action plan for UMCD groundwater included design, installation, and operation of a groundwater extraction, treatment, and re-infiltration system that began operation in 1996.

A portion of the treated groundwater was infiltrated through the washout lagoons in an effort to flush the remaining explosives contamination from soil into the groundwater, which could then be captured and treated by the pump and treat system. This *in situ* soil-flushing component was completed in 2000. Following years of operation, the pump and treat system reached diminishing removal efficiency, leaving 45 ha over 20 $\mu g\ L^{-1}$ RDX and over 150 ha over the 2.1 $\mu g\ L^{-1}$ RDX cleanup level (Figure 4.3). The saturated thickness of the aquifer across the plume footprint varies depending on location; an average saturated thickness of 5.5 m was used in estimating groundwater quantities.

Figure 7.1 provides a schematic of the explosives-contaminated groundwater plume at Umatilla and four potential optimized remediation scenarios. Within this section, estimated operating costs of each scenario are included for evaluation and comparison. The four potential optimization scenarios include:

(1) Expanded pump and treat system;
(2) Expanded pump and treat system plus RDX plume treatment using anaerobic biostimulation;
(3) Expanded pump and treat system plus comingled RDX/TNT plume treatment using anaerobic biostimulation and RDX plume treatment using aerobic bioaugmentation; and
(4) Expanded pump and treat system plus comingled RDX/TNT plume treatment using anaerobic biostimulation and RDX plume treatment using aerobic biostimulation.

Tables 7.2, 7.3, 7.4 and 7.5 thereafter summarize the expense assumptions associated with each of the four optimization scenarios. Cost tables include both non-discounted costs and discounted costs based on the OMB defined real 30-Year 2016 interest rate of 1.4% (www.whitehouse.gov/omb/circulars/a094/a94_appx-c.html). Program management costs were not included.

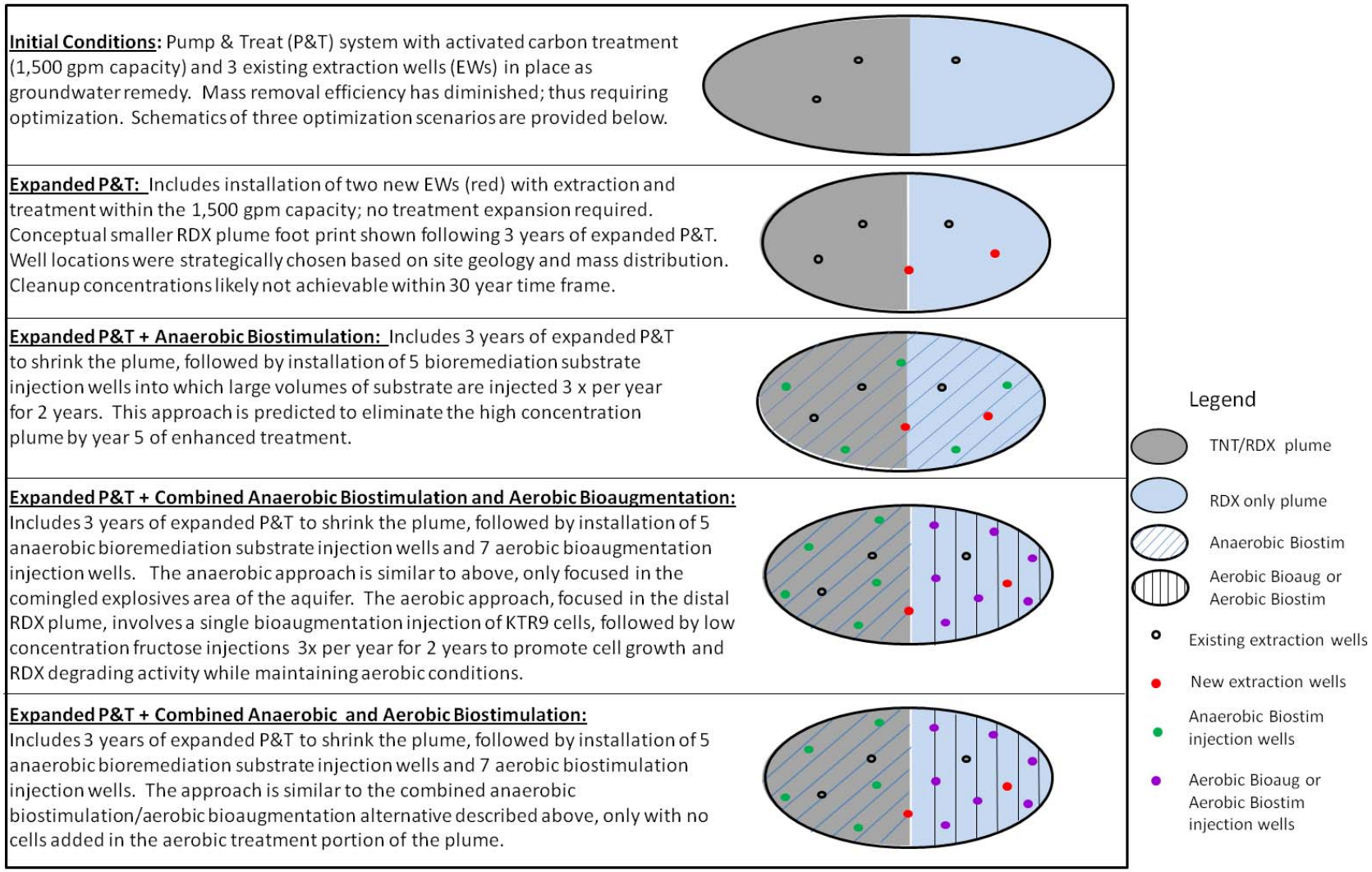


Figure 7.1. Schematic of costed remedy scenarios at UMCD. Ovals represent relative groundwater plume size.

Scenario 1 - Expanded P&T

The expanded pump and treat (P&T) system would include installation of two new extraction wells (Figure 7.1) anticipated to reduce the 20 µg L^{-1} RDX plume contour to 24 ha within three years of operation. Costs for this scenario (Table 7.2) included remedial design and construction of the upgraded facility, thirty years of operation and maintenance (O&M), including replacement and disposal of activated carbon over this timeframe. Monitoring, five-year review and site closure costs were also included. The addition of two extraction wells would not require an upgrade of the current granular activated carbon (GAC) treatment system sized for 1,500 gpm. Groundwater model simulation results predict remediation complete in 30 years, which may be an underestimate. Simplified groundwater models effectively simulate aquifer hydraulics and dissolved-phase contaminant movement but are subject to limitations. Model simulation results are appropriate for comparing performance of different pumping scenarios and treatment approaches; however, results must be considered in relative terms, interpreted considering model limitations and site-specific knowledge. Measured groundwater concentrations – not simulated concentrations – are the basis for establishing site closure. As an example, the groundwater model referenced in the UMCD 1994 Record of Decision predicted cleanup of UMCD site groundwater within 10 years, which of course did not occur.

Scenario 2 - Expanded P&T + Anaerobic Biostimulation

The second optimization scenario would depend on the expanded P&T system to reduce the plume size to 24 ha over three years. At the same time, bioremediation would occur in the lagoon source area by injecting a growth substrate (fructose) along with extracted groundwater into the lagoon area. An estimated 1 million pounds of fructose, at a unit cost of \$0.24 lb^{-1}, was included for anaerobic biostimulation in the lagoon source area during initial remedial design. Following the three years of expanded P&T with lagoon biostimulation, fructose solution would be injected throughout the 20 µg L^{-1} RDX plume using injection/extraction wells. Five bioremediation substrate injection wells would be installed to ensure sufficient distribution of substrate within the plume (Figure 7.1). Bioremediation amendment injection and groundwater re-circulation would be completed in 120-day cycles: extraction/injection for 30 days followed by 90 days of no pumping. An estimated 7.6 million pounds of fructose was included for anaerobic biostimulation of the plume within the 2 year bioremediation period. Quantity of carbon substrate for anaerobic biostimulation was based on achieving a 24 mM aquifer concentration of fructose.

Costs for this scenario (Table 7.3) included pilot testing, remedial design and construction costs of the enhanced P&T facility, as well as remedial design and construction costs of bioremediation infrastructure and bioremediation substrate. Circulation of the bioremediation substrate would require use of P&T infrastructure. Therefore, O&M costs for P&T were included during the bioremediation period as well as 5 years following bioremediation, during which time extraction wells may be operated for polishing. Monitoring, five-year review and site closure costs were also included. Groundwater model simulation results predict remediation complete in 15 years.

Scenario 3 - Expanded P&T + Combined Anaerobic Biostimulation and Aerobic Bioaugmentation

The third optimization scenario is similar to the second scenario for the first three years of expanded P&T and lagoon bioremediation. Thereafter the RDX/TNT comingled plume would be treated using a similar anaerobic biostimulation approach discussed in scenario 2, whereas the remainder of the RDX-only plume would be treated using aerobic bioaugmentation. For the purposes of this example, it was assumed that the anaerobic and aerobic fractions of the plume were 50% of the entire bioremediation footprint. An estimated 3.8 million pounds of fructose, at a unit cost of \$0.24 lb^{-1}, was included for anaerobic biostimulation in the RDX/TNT comingled portion of the plume. The remaining half of the plume would be treated at a lower carbon dose of 1 mM in order to maintain aerobic conditions throughout the aquifer, totaling 0.40 million pounds of fructose for two years. In addition, microbes would be injected in the RDX-only plume to achieve a cell density of 10^6 cells mL^{-1} concentration within the initial injection footprint. Based on results of this demonstration and our expanded cell transport test [42], we assumed for costing purposes that we would be able to transport cells to achieve targeted 10^6 cells mL^{-1} concentration over one quarter of the targeted aerobic bioaugmentation treatment area, or 3 ha total. As in scenario 3, bioremediation amendment injection and groundwater re-circulation would occur in 120-day cycles: extraction/injection for 30 days followed by 90 days of no pumping over two years. We further assumed that the cells injected only once during the first injection/recirculation cycle would grow, attach/detach and ultimately colonize the entire 12 ha aerobic bioaugmentation treatment area. A total of twelve bioremediation substrate injection wells would be installed (seven within the aerobic footprint and five within the anaerobic footprint) to ensure sufficient distribution of substrate within the plume (Figure 7.1).

Costs for this scenario (Table 7.4) included pilot testing, remedial design and construction costs of the enhanced P&T facility, as well as remedial design and construction costs of bioremediation infrastructure, bioremediation substrate and microbes. Circulation of the bioremediation substrate would require use of P&T infrastructure. Therefore, O&M costs for P&T were included during the bioremediation period as well as 5 years following bioremediation, during which time extraction wells may be operated for polishing. Monitoring, five-year review and site closure costs were also included. Groundwater model simulation results predict remediation complete in 15 years.

Scenario 4 - Expanded P&T + Combined Anaerobic and Aerobic Biostimulation

The fourth optimization scenario is similar to the second scenario for the first three years of expanded P&T and lagoon bioremediation. Thereafter the RDX/TNT comingled plume would be treated using a similar anaerobic biostimulation approach discussed in scenarios 2 and 3, whereas the remainder of the RDX-only plume would be treated using aerobic biostimulation. For the purposes of this example, it was assumed that the anaerobic and aerobic fractions of the plume were 50% of the entire bioremediation footprint. An estimated 3.8 million pounds of fructose, at a unit cost of \$0.24 lb^{-1}, was included for anaerobic biostimulation in the RDX/TNT comingled portion of the plume. The remaining half of the plume would be treated via aerobic biostimulation at a lower carbon dose of 1 mM, totaling 0.56 million pounds of fructose for two years. A total of twelve bioremediation substrate injection wells would be installed (seven within the aerobic footprint and five within the anaerobic footprint) to ensure sufficient distribution of substrate within the plume.

Costs for this scenario (Table 7.5) included pilot testing, remedial design and construction costs of the enhanced P&T facility, as well as remedial design and construction costs of bioremediation

infrastructure and bioremediation substrate. Circulation of the bioremediation substrate would require use of P&T infrastructure. Therefore, O&M costs for P&T were included during the bioremediation period as well as 5 years following bioremediation, during which time extraction wells may be operated for polishing. Monitoring, five-year review and site closure costs were also included. Groundwater model simulation results predict remediation complete in 15 years.

Table 7.2. Cost estimate for enhanced pump & treat only with no bioremediation: Scenario 1 (30 yrs, $K)

Cost Element	Sub Element	0	1	2	3	4	5	6-29	30	Total Cost
Remedial Design	Design expanded pump & treat facility	175								175
Remedial Action Construction	Construct expanded pump & treat facility		2,000							2,000
Operation and Maintenance	Operation and Maintenance		302	302	302	302	302	302	302	9,060
	1 Monitoring Event/yr in yrs 1-26, 2/yr in yrs 27-29		90	90	90	90	90	90	90	2,700
	Five Year Reviews						19	19[a]		95
Completion Activities	Site Close-Out Documentation								6	6
	Administrative Land Use Controls								155	155
	Non-Discounted Cost ($K)	175	2,392	392	392	392	411	9,484[b]	553	**14,191**
	1.4% Discount Rate ($K)	175	2359	381	376	371	383	7,470[b]	364	**11,880**

[a]Five Year Review cost every five years. [b]Sum for years 6-9 is shown

Table 7.3. Cost estimate for enhanced pump & treat with phased anaerobic biostimulation: Scenario 2 (15 yrs, $K)

Cost Element	Sub Element	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Total Cost
Remedial Design	Lagoon Area Anaerobic Bio Testing	99																99
	Install 2 x Injection Well Pilot	180																180
	Field Scale Pilot Injection Using New Injection Well	179																179
	Phase I - Design Expanded Groundwater Extraction System	175																175
Remedial Action Construction	Expanded Groundwater Extraction System Construction		2,000															2,000
Operation and Maintenance and Pre-Design	Anaerobic Biostimulation in Lagoon Source Area	173	173															346
	1 Monitoring Event/yr		90	90	90	90	90											450
	Operation and Maintenance		302	302	302	302	302	302	302	302	302	302						3,020
Remedial Design	Phase II - Transition to Bioremediation in Plume including GW model simulations			190														190
Remedial Action Construction	Construct additional bioremediation wells, one additional extraction well, bioremediation amendment injections				1,400	900	900											3,200
Operation and Maintenance	1 Monitoring Event/yr 6-12, 2/yr 12-14							90	90	90	90	90	90	90	90	90	90	900
	Five Year Reviews						19					19						38
Completion Activities	Site Close-Out Documentation																6	6
	Administrative Land Use Controls																155	155
	Non Discounted Cost (K)	806	2,565	582	1,792	1,292	1,311	392	392	392	392	411	90	90	90	90	251	**10,938**
	1.4% Discount Rate (K)	806	2,530	566	1,719	1,222	1,223	361	356	351	346	358	77	76	75	74	204	**10,342**

Table 7.4. Cost estimate for enhanced pump & treat with phased, combined anaerobic biostimulation and aerobic bioaugmentation: Scenario 3 (15 yrs, $K)

Cost Element	Sub Element	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Total Cost
Remedial Design	Lagoon Area Anaerobic Bio Testing	99																99
	Install 2 x Injection Well Pilot	180																180
	Field Scale Pilot Injection Using New Injection Well	179																179
	Phase I - Design Expanded Groundwater Extraction System	175																175
Remedial Action Construction	Expanded Groundwater Extraction System Construction		2,000															2,000
Operation and Maintenance and Pre-Design	Anaerobic Biostimulation in Lagoon Source Area	173	173															346
	1 Monitoring Event/yr		90	90	90	90	90											450
	Operation and Maintenance		302	302	302	302	302	302	302	302	302	302						3,020
Remedial Design	Phase II - Transition to Bioremediation in Plume including GW model simulations			190														190
Remedial Action Construction	Construct additional bioremediation wells, one additional extraction well, Anaerobic bioremediation amendment injections				1,400	450	450											2,300
	Aerobic bioaugmentation microbes and amendment injections					1,160	67											1,227
Operation and Maintenance	1 Monitoring Event/yr 6-12, 2/yr 12-14							90	90	90	90	90	90	90	90	90	90	900
	Five Year Reviews						19					19						38
Completion Activities	Site Close-Out Documentation																6	6
	Administrative Land Use Controls																155	155
	Non Discounted Cost (K)	806	2,565	582	1,792	2,002	928	392	392	392	392	411	90	90	90	90	251	**11,265**
	1.4% Discount Rate (K)	806	2,530	566	1,719	1,894	866	361	356	351	346	358	77	76	75	74	204	**10,657**

Table 7.5. Cost estimate for enhanced pump & treat with phased, combined anaerobic and aerobic biostimulation: Scenario 4 (15 yrs, $K)

Cost Element	Sub Element	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Total Cost
Remedial Design	Lagoon Area Anaerobic Bio Testing	99																99
	Install 2 x Injection Well Pilot	180																180
	Field Scale Pilot Injection Using New Injection Well	179																179
	Phase I - Design Expanded Groundwater Extraction System	175																175
Remedial Action Construction	Expanded Groundwater Extraction System Construction		2,000															2,000
Operation and Maintenance and Pre-Design	Anaerobic Biostimulation in Lagoon Source Area	173	173															346
	1 Monitoring Event/yr		90	90	90	90	90											450
	Operation and Maintenance		302	302	302	302	302	302	302	302	302	302						3,020
Remedial Design	Phase II - Transition to Bioremediation in Plume including GW model simulations			190														190
Remedial Action Construction	Construct additional bioremediation wells, one additional extraction well, Anaerobic bioremediation amendment injections				1,400	450	450											2,300
	Aerobic bioremediation amendment injections					67	67											134
Operation and Maintenance	1 Monitoring Event/yr 6-12, 2/yr 12-14							90	90	90	90	90	90	90	90	90	90	900
	Five Year Reviews						19					19						38
Completion Activities	Site Close-Out Documentation																6	6
	Administrative Land Use Controls																155	155
	Non Discounted Cost (K)	806	2,565	582	1,792	909	928	392	392	392	392	411	90	90	90	90	251	**10,172**
	1.4% Discount Rate (K)	806	2,530	566	1,719	860	866	361	356	351	346	358	77	76	75	74	204	**9,623**

8.0 IMPLEMENTATION ISSUES

The results of this demonstration show that aerobic bioaugmentation is possible and effective for treatment of RDX-contaminated groundwater at UMCD. Future implementation of the technology requires that the necessary permitting regulations are met, end user concerns are addressed, and lessons learned during the demonstration are implemented at full-scale.

Implementation issues that were encountered during the project include the impact of permeability on cell and carbon substrate transport, the importance of treatability studies, and the difference in observed conditions when converting between bench scale column tests to field scale push pull testing (such as differences in redox conditions). Also, there were issues with cell contamination during large-scale culture production.

8.1 Regulations

Toxic Substance Control Act (TSCA) Experimental Release Applications (TERAs) for use of the two genetically-modified bacteria, *Gordonia* sp. KTR9 pGKT2::Kan^r and *Rhodococcus jostii* RHA1 pGKT2::Kan^r, were approved by the Environmental Protection Agency's Office of Pollution Prevention prior to the field demonstrations. The genetic modifications were included so that another means of detection of the inoculated strains, i.e. selective plate counts on kanamycin containing agar medium, compared to qPCR analysis of the *xplA* gene was possible during the demonstrations. The viable plate counting is not necessary for field monitoring of the inoculated strains since the qPCR assay was accurate and reliable. In addition, the use of genetically-modified organisms in a full-scale bioaugmentation operation is not envisioned and is unnecessary, and so only permits required for inoculation of wild-type bacterial strains may be required.

8.2 End User Concerns

The primary end-users of this technology are expected to be industrial or military clients that have a history of munitions manufacturing, testing, or training at their facility that has led to contamination with RDX. Additional stakeholders with interest in this technology demonstration include the EPA and DoD.

One issue that may negatively affect the performance objectives of this project is inhibition of RDX degradation by nitrate, ammonium or TNT present in the groundwater. The inhibition by inorganic nitrogen appears to be strain specific [7, 15, 50] and so groundwater concentrations should be evaluated prior to selection of the bioaugmentation strain(s). At UMCD the concentration of nitrate and ammonium are lower than inhibitory concentrations for RDX degradation by strain KTR9. Within the source zone, the TNT concentration ($2.8 < TNT < 70$ µg L^{-1}) are more than adequate to inhibit the growth of strain KTR9 (LD50 = 5 µg mL^{-1}, data not shown). In general, Gram positive soil bacterial isolates have been found to be more sensitive to TNT than Gram negative isolates [51, 52]. Concentrations of TNT around 10 to 20 mg L^{-1} resulted in a 50% inhibition of cell growth for Gram-positive isolates [51, 52]. Similarly, the growth of KTR9 was inhibited by TNT at concentrations greater than 5 µg mL^{-1} (Crocker, unpublished). The degradation of RDX by purified XplAB proteins was inhibited by 80% in the presence of an

equimolar amount of TNT (28 mg L^{-1}) [53]. Furthermore, RDX (7.5 mg L^{-1}) degradation by *Rhodococcus* strain YH1 (via XplA) was inhibited by TNT (7.5 mg L^{-1}) and RDX degradation only occurred after the TNT had been completely transformed [54]. While these studies used much higher concentrations of TNT than are present in UMCD groundwater, they indicate that a 1:1 molar ratio of TNT:RDX is sufficient to inhibit XplA activity. For this reason, bioaugmentation with KTR9 will be limited to aquifers with significantly lower concentrations of TNT than RDX.

8.3 Lessons Learned

In bioaugmentation, the main concern is an ability to effectively distribute the inoculated cells and to preserve survival and activity of the inoculated cells for the time period necessary to meet treatment goals. This project successfully demonstrated the rapid transport of the mixed bioaugmentation culture a minimum of 3 m from the injection well. Despite preliminary site tracer testing that indicated hydraulic connectivity of all three wells in this study, the transport of the tracer and cells to the next downgradient well (EW2 at 21 m) could not be detected. In order to overcome this limitation in Phase III all three wells were inoculated to create the bioaugmentation zone. Scenario 3 is based on this premise that multiple inoculation wells would be required for effective distribution of the inoculum in the required bioaugmentation zone. A subsequent cell transport test at UMCD (to be discussed elsewhere; [42]) showed that with a 10-fold increase in the injection volume, cells could be transported up to 23 m downgradient of the injection well. Thus, similar injection volumes at this site would create the bioaugmentation zone required to treat the aerobic and dilute RDX portion of the plume at UMCD.

The long-term laboratory column studies confirmed that bioaugmented cells retained viability and RDX-degrading activity over a field-relevant timeframe. In contrast, decreases in RDX-degrading activity in the bioaugmented wells during Phase III was concomitant with decreases in viable cell counts and *xplA* copy numbers, which may have been caused by repeated high-flow substrate injections. In the preceding column study (Section 5.3.3) intended to simulate push-pull test (PPT) conditions [19], the maximum seepage velocity was a notable difference amid many similarities. The PPTs and column study both contained approximately 10^7 *xplA* copies mL^{-1} following bioaugmentation, had similar pore volumes exchanged (182 and 160 pore volumes in the column and prior to the third PPT, respectively) and approximately 10^4 and 10^5 *xplA* copies mL^{-1} present in column effluent and site groundwater, respectively, immediately prior to measuring RDX degradation rates. However, the estimated first order RDX transformation rate coefficient in the column (~ 0.5 day^{-1} first order estimated based on published data) was twice as large as the average transformation rate measured during the third PPT (0.18 day^{-1}). At the conclusion of the column study, the *xplA* copy numbers ranged from 10^7 mL^{-1} near the column inlet to 10^5 mL^{-1} near the outlet. Attached cells were not assessed following PPTs. However, we hypothesize that the repeated high-flow substrate injections (~6,000 L each) in the PPT wells, which produced a maximum seepage velocity of 520 m day^{-1} during injection compared to the maximum seepage velocity of 0.37 m day^{-1} in the column study, likely washed bioaugmented cells and substrate away from the PPT volumes and into the aquifer, thereby reducing the total number of cells that were able to attach and grow within the test volume prior to measuring RDX transformation rates during the second and subsequent PPTs.

Decreases in RDX degradation rates were also concomitant with decreases in dissolved oxygen and ORP. Anaerobic conditions have been shown to inhibit RDX-degrading activity in *Gordonia* sp. strain KTR9 used in this study (Crocker, unpublished), suggesting reducing conditions may have further decreased KTR9's ability to degrade RDX in the UMCD aquifer during these tests. Field scale implementation (Scenario 3) would limit the carbon substrate amendment to three times per year instead of the biweekly injections conducted in this demonstration. It is expected that KTR9 will remain viable during the ~ 3 month time between "feedings" and will be stimulated to degrade RDX with subsequent substrate additions. KTR9 maintained viability in situ at UMCD for approximately 3 months without substrate feedings (to be discussed elsewhere; [42]).

In summary, anaerobic biostimulation has been demonstrated to rapidly reduce and sustain reductions in RDX concentrations for years following amendment (fructose) injections in the UMCD aquifer [55] but aerobic biostimulation had not been considered. Column testing results (Section 5.3) showed negligible RDX removal during aerobic biostimulation and aerobic biostimulation rates were not different from zero in this study (p values ≥ 0.060), supporting our hypothesis that bioaugmentation with aerobic RDX degraders would be required to support aerobic RDX remediation of the UMCD aquifer. Based on these results, we recommend that the full-scale bioremediation design include an amendment injection and circulation system that is able to:

- isolate aerobic and anaerobic treatment areas,
- accommodate injection of cells during bioaugmentation as well as substrate injections, and
- convert aerobic treatment areas into anaerobic treatment areas should treatment performance suggest the need to do so.

As with all full-scale bioremediation programs, flexibility and adaptive management will be required to cost-effectively implement combined anaerobic and aerobic biostimulation/bioaugmentation groundwater remedies at RDX-contaminated sites. As observed in the aerobic bioaugmentation treatments, it is easy to add a little too much of a readily-degradable carbon source to wells and reversing those effects can be difficult. Therefore, when implementing an aerobic biostimulation program in the field, it is wise to start with low substrate concentrations then increase as needed based on results.

9.0 REFERENCES

1. Schaefer, C.E., D.R. Lippincott, and R.J. Steffan, *Field-scale evaluation of bioaugmentation dosage for treating chlorinated ethenes.* Ground Water Monitoring & Remediation, 2010. **30**(3): p. 113-124.

2. Vainberg, S., C.W. Condee, and R.J. Steffan, *Large-scale production of Dehalococcoides sp. containing cultures for bioaugmentation.* Journal of Industrial Microbiology and Biotechnology, 2009. **36**: p. 1189-1197.

3. Schaefer, C.E., et al., *Bioaugmentation for chlorinated ethenes using Dehalococcoides sp.: Comparison between batch and column experiments.* Chemosphere, 2009. **75**(2): p. 141-148.

4. Steffan, R.J. and S. Vainberg, *Chapter 3: Culturing and Handling Bioaugmentation Cultures*, in *Bioaugmentation for Groundwater Remediation*, SERDP/ESTCP, Editor. 2011, *In Press.*

5. Aziz, C., R. Wymore, and R. Steffan, *Chapter 4: Methods of Bioaugmentation*, in *Bioaugmentation for Groundwater Remediation*, SERDP/ESTCP, Editor. 2011, *In Press*.

6. Indest, K.J., F.H. Crocker, and R. Athow, *A TaqMan polymerase chain reaction method for monitoring RDX-degrading bacteria based on the xplA functional gene.* Journal of Microbiological Methods, 2007. **68**: p. 267-274.

7. Coleman, N.V., D.R. Nelson, and T. Duxbury, *Aerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) as a nitrogen source by a Rhodococcus sp., strain DN22.* Soil Biology and Biochemistry, 1998. **30**: p. 1159-1167.

8. Priestley, J.T., N.V. Coleman, and T. Duxbury, *Growth rate and nutrient limitation affect the transport of Rhodococcus sp. strain DN22 through sand* Biodegradation, 2006. **17**(6): p. 571-576.

9. Thompson, K.T., F.H. Crocker, and H.L. Fredrickson, *Mineralization of the cyclic nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine by Gordonia and Williamsia spp.* Applied and Environmental Microbiology, 2005. **71**(8265-8272).

10. Ronen, Z., et al., *Metabolism of the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in a contaminated vadose zone.* Chemosphere, 2008. **73**(9): p. 1492-1498.

11. Seth-Smith, H.M.B., et al., *The explosives-degrading cytochrome P450 system is highly concerved amont strains of Rhodococcus spp.* Applied and Environmental Microbiology, 2008. **74**: p. 4550-4552.

12. Andeer, P., et al., *Lateral Transfer of Genes for RDX Degradation.* Applied and Environmental Microbiology, 2009. **75**: p. 3258-3262.

13. Seth-Smith, H.M.B., et al., *Cloning, sequencing, and characterization of the hexahydro-1,3,5-trinitro-1,3,5-triazine degradation gene cluster from Rhodococcus rhodochrous.* Applied and Environmental Microbiology, 2002. **68**: p. 4764-4771.

14. Indest, K.J., et al., *Functional characterization of pGKT2, a 182 kb plasmid containing the xplAB genes involved in the degradation of RDX by Gordonia sp. KTR9.* Applied and Environmental Microbiology, 2010. **76**(19): p. 6329-6337.

15. Jung, C.M., et al., *Horizontal gene transfer (HGT) as a mechanism of disseminating RDX-degrading activity amont Actinomycete bacteria.* Journal of Applied Microbiology, 2011. **110**: p. 1449-1459.

16. Fuller, M.E., et al., *Transformation of RDX and other energetic compounds by xenobiotic reductases XenA and XenB.* Applied Microbial and Cell Physiology, 2009. **84**: p. 535-544.

17. Fuller, M.E., J. Hawari, and N. Perreault, *Microaerophilic degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by three Rhodococcus strains.* Letters In Applied Microbiology, 2010. **51**(3): p. 313-318.

18. Zhu, S.-H., et al., *The essential role of nitrogen limitation in expression of xplA and degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in Gordonia sp. strain KTR9.* Applied Microbiology and Biotechnology, 2014. **99**(1): p. 459-467.

19. Fuller, M.E., et al., *Laboratory evaluation of bioaugmentation for aerobic treatment of RDX in groundwater.* Biodegradation, 2015. **26**(1): p. 77-89.

20. Huysman, F. and W. Verstraete, *Water-facilitated transport of bacteria in unsaturated soil columns: Influence of cell surface hydrophobicity and soil properties.* Soil Biology and Biochemistry, 1993. **25**(1): p. 83-90.

21. Rijnaarts, H., et al., *Bacterial deposition in porous media: effects of cell-coating, substratum hydrophobicity, and electrolyte concentration.* Environmental Science & Technology, 1996. **30**(10): p. 2877-2883.

22. Baygents, J., et al., *Variations of surface charge density in monoclonal bacterial populations: Implications for transport through porous media.* Environmental Science & Technology, 1998. **32**: p. 1596-1603.

23. Bolster, C., et al., *Effect of intra-population variability of the long-distance transport of bacteria.* Ground Water, 2000. **38**(3): p. 370-375.

24. Williams, V. and M. Fletcher, *Pseudomonas fluorescens adhesion and transport through porous media are affected by lipopolysaccharide composition.* Applied and Environmental Microbiology, 1996. **62**(1): p. 100 - 104.

25. Rosenberg, M., D. Gutnick, and E. Rosenberg, *Adherence of bacteria to hydrocarbons--a simple method for measuring cell-surface hydrophobicity.* FEMS Microbiology Letters, 1980. **9**: p. 29-33.

26. Heise, S. and G. Gust, *Influence of the physiological status of bacteria on their transport into permeable sediments.* Marine Ecology Progress Series, 1999. **190**: p. 141-153.

27. Weiss, T.H., et al., *Effect of bacterial cell shape on transport of bacteria in porous media.* Environmental Science & Technology, 1995. **29**(7): p. 1737-1740.

28. Schaefer, C.E., D.R. Lippincott, and R.J. Steffan, *Field-scale evaluation of bioaugmentation dosage for treating chlorinated ethenes.* Ground Water Monitoring and Remediation, 2010. **30**: p. 113-124.

29. Appenzeller, B., et al., *Influence of phosphate on bacterial adhesion onto iron oxyhydroxide in drinking water.* Environmental Science & Technology, 2002. **36**(4): p. 646-652.

30. Johnson, W. and B. Logan, *Enhanced transport of bacteria in porous media by sediment-phase and aqueous-phase natural organic matter.* Water Research, 1996. **30**(4): p. 923-931.

31. Mills, A., et al., *Effect of solution ionic strength and iron coatings on mineral grains on the sorption of bacterial cells to quartz sand.* Applied and Environmental Microbiology, 1994. **60**(9): p. 3300-3306.

32. Streger, S.H., et al., *Enhancing transport of Hydrogenophaga flava ENV735 for bioaugmentation of aquifers contaminated with methyl tert-butyl ether.* Applied and Environmental Microbiology, 2002. **68**(11): p. 5571-5579.

33. Brown, D. and P. Jaffé, *Effects of nonionic surfactants on bacterial transport through porous media.* Environmental Science & Technology, 2001. **35**(19): p. 3877-3883.

34. Mailloux, B.J. and M.E. Fuller, *Determination of in situ bacterial growth rates in aquifers and aquifer sediments.* Applied and Environmental Microbiology, 2003. **69**(7): p. 3798-3808.

35. DeFlaun, M.F., et al., *Comparison of methods for monitoring bacterial transport in the subsurface.* Journal of Microbiological Methods, 2001. **47**(2): p. 219-231.

36. Dong, H., et al., *Simultaneous transport of two bacterial strains in intact cores from Oyster, Virginia: Biological effects and numerical modeling.* Applied and Environmental Microbiology, 2002. **68**(5): p. 2120-2132.

37. Fuller, M.E., et al., *Examining bacterial transport in intact cores from Oyster, Virginia: Effect of sedimentary facies type on bacterial breakthrough and retention.* Water Resources Research, 2000. **36**(9): p. 2417-2431.

38. Schaefer, C.E., et al., *Bioaugmentation for chlorinated ethenes using Dehalococcoides sp.: Comparison between batch and column experiments.* Chemosphere, 2009. **75**: p. 141-148.

39. Ginn, T.R., *travel time approach to exclusion on transport in porous media.* Water Resources, 2002. **38**(4): p. 12-1.

40. Steffan, R.J. and S. Vainberg, *Production and Handling of Dehalococcoides Bioaugmentation Cultures*, in *Bioaugmentation for Groundwater Remediation*, H.F. Stroo, A. Leeson, and C.H. Ward, Editors. 2013, Springer: New York. p. 89-115.

41. Vainberg, S., C.W. Condee, and R.J. Steffan, *Large-scale production of Dehalococcoides sp. containing cultures for bioaugmentation.* Journal of Industrial Microbiology and Biotechnology, 2009. **36**: p. 1189-1197.

42. Crocker, F.H., et al., *Evaluation of Microbial Transport during Aerobic Bioaugmentation of an RDX-Contaminated Aquifer.* Biodegradation, 2015. **26**(6): p.443-451.

43. Major, D.W., et al., *Field demonstration of successful bioaugmentation to achieve dechlorination of tetrachloroethene to ethene.* Environmental Science and Technology, 2002. **36**(5106-5116).

44. Trotsky, J., et al., *A low-cost, passive approach for bacterial growth and distribution for large-scale implementation of bioaugmentation. Final Report. ESTCP, Arlington, VA, USA. http://www.serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/ER-200513. Accessed September 25, 2014.* 2010.

45. Michalsen, M.M., et al., *Evaluation of Biostimulation and Bioaugmentation to Stimulate Hexahydro-1,3,5-trinitor-1,3,5-triazine Degradation in an Aerobic Groundwater Aquifer.* Environmental Science & Technology, 2016. **50**(14): p. 7625-32.

46. Michalsen, M., et al., *Push-Pull Tests for Estimating RDX and TNT Degradation Rates in Groundwater.* Ground Water Monitoring & Remediation, 2013. **33**(3): p. 61-68.

47. Istok, J.D., et al., *Single-Well, "Push-Pull" Test for In Situ Determination of Microbial Activities.* Ground Water, 1997. **35**(4): p. 619-631.

48. Ritalahti, K.M., et al., *Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes simultaneously monitors multiple Dehalococcoides strains.* Applied and Environmental Microbiology, 2006. **72**: p. 2765-2774.

49. USACE, *Focused Feasibility Study for Groundwater at the Explosives Washout Lagoon (EWL) Area, Operable Unit 3 (OU3), at the Umatilla Chemical Depot, Umatilla, OR.*

2012: Prepared for ARMY/BRACD by USACE Seattle District, 16 May 2011. Finalized Dec 2012.

50. Bernstein, A., et al., *Isolation and characterization of RDX-degrading Rhodococcus species from a contaminated aquifer.* Biodegradation, 2011. **22**(5): p. 997-1005.

51. Fuller, M.E. and J.J. Manning, *Aerobic Gram-Positive and Gram-Negative Bacteria Exhibit Differential Sensitivity to and Transformation of 2,4,6-Trinitrotoluene (TNT).* Current Opinions in Microbilogy, 1997. **35**: p. 77-83.

52. Fuller, M.E. and J.F.J. Manning, *Evidence for differential effects of 2,4,6-trinitrotoluene and other munitions compounds on specific subpopulations of soil microbial communities.* Environmental Toxicology and Chemistry, 1998. **17**: p. 2185-2195.

53. Jackson, R.G., et al., *Exploring the biochemical properties and remediation applications of the unusual explosives-degrading P450 system XplA/B.* Proceedings of National Academy of Sciences USA, 2007. **104**: p. 16822-168277.

54. Nejidat, A., et al., *Effect of organic and inorganic nitrogenous compounds on RDX degradation and cytochrome P-450 expression in Rhodococcus strain YH1.* Biodegradation, 2008. **19**(3): p. 313-320.

55. Michalsen, M.M., et al. *A Pilot to Full-Scale Success Story: Combined Anaerobic Biostimulation and Aerobic Bioaugmentation for Explosives-Contaminated Groundwater Cleanup, May 20, 2015.* in *Battelle Bioremediation Conference*. 2015. Miami, FL.

APPENDICES

APPENDIX A

SITE CHARACTERIZATION RESULTS MEMORANDUM

Bioaugmentation for Aerobic Bioremediation of RDX-Contaminated Groundwater

ESTCP PROJECT ER-201207
Site Characterization Memorandum

Prepared by:

Mandy Michalsen
Seattle District, U.S. Army Corps of Engineers

Fiona Crocker, Karl Indest, Carina Jung
U.S. Army Engineer Research Development Center

Paul Hatzinger, Mark Fuller
Shaw Environmental Infrastructure, Inc.

Jack Istok
Oregon State University

October 15, 2012

TABLE OF CONTENTS

1. Introduction and Objectives 1
2. Methods 2
2.1 Slow Injection Forced Gradient Tracer Test (May 2012) 3
2.2 Borehole Dilution Tracer Test (July 2012) 3
2.3 Fast Injection Forced Gradient Tracer Test (August 2012) 3
3. Results 4
3.1 Slow Injection Forced Gradient Tracer Test (May 2012) 4
3.2 Borehole Dilution Tracer Test (July 2012) 4
3.3 Fast Injection Forced Gradient Tracer Test (August 2012) 4
4. Conclusions 5
5. Project Schedule 6

ATTACHMENT 1 Schedule
ATTACHMENT 2 Boring logs

1. Introduction and Objectives

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a common contaminant in soils and groundwater at military sites worldwide. RDX can be mobile and persistent in groundwater under the aerobic conditions present in many aquifers and thus tends to form large, dilute plumes. Although multiple studies have demonstrated in situ RDX biodegradation under anaerobic conditions, creating and maintaining anaerobic conditions across large areas is costly and technically challenging. This project will demonstrate an innovative application of bioaugmentation to enhance RDX biodegradation in contaminated groundwater under aerobic conditions. The project will provide field data to support both a technical evaluation and a cost-benefit analysis of this approach.

During this ESTCP demonstration, a mixed microbial culture capable of aerobically biodegrading RDX will be injected into an aerobic RDX plume. The amount of growth substrate required to degrade RDX by this culture is much smaller than would be needed to create and sustain conditions required for anaerobic RDX biodegradation (with or without bioaugmentation), thus reducing the costs and technical complexities of remediating large aerobic RDX plumes. Moreover, the RDX-degrading activity of the bioaugmentation culture is attributed primarily to *xplA* genes located on plasmids (mobile genetic elements), thus providing the potential for transfer of RDX-degrading activity from injected cultures to indigenous microorganisms, which are likely well adapted to site-specific conditions, and thus potentially capable of sustaining and spreading RDX-degrading activity throughout the subsurface.

This demonstration will be performed in three phases. Phase I consists of field site characterization and laboratory testing to select a suitable bioaugmentation culture and to optimize conditions that facilitate growth, RDX-degrading activity, and cell transport under field conditions at the UMCD. Phase II consists of initial field tests including short duration push-pull tests to measure *in situ* RDX retardation factors and a forced-gradient cell transport test to confirm ability to distribute cells over the targeted test area. Phase III consists of a field-scale (~ 100 m^3) bioaugmentation demonstration with subsequent triplicate push-pull tests to obtain field-scale performance data on bioaugmentation culture transport, viability, and *xplA* gene transfer, as well as RDX degradation rates. The demonstration will be conducted in two field plots, one for aerobic bioaugmentation only and the other for sequential evaluation of aerobic and anaerobic biostimulation. The initial aerobic biostimulation test will provide an independent evaluation of RDX degradation performance under aerobic biostimulation conditions and will serve as the aerobic bioaugmentation control. Although presence of increased biomass from the aerobic test may expedite time to achieve anaerobic

conditions during the subsequent anaerobic biostimulation, RDX degradation performance will not be evaluated until the end of the two month aquifer conditioning period at which time the community composition and aquifer geochemistry will be representative of the anaerobic biostimulation treatment and will be suitable for performance comparison to aerobic treatments. RDX degradation rates and mass removed per mass of substrate added for the aerobic bioaugmentation treatment will be compared with non-bioaugmented aerobic and anaerobic biostimulation treatments and will form the basis of a cost-benefit analysis for the innovative aerobic bioaugmentation approach for *in situ* treatment of RDX in groundwater.

Site characterization work (Task 1a) included installation of two demonstration wells, as well as a series of tracer tests completed using both wells. The objectives of the tracer tests were

- To confirm the existence of hydraulically connected flow paths between two new demonstration wells and downgradient monitoring well(s),
- To estimate groundwater travel times and dilution factors for use in designing subsequent tracer and microbial transport tests, substrate delivery protocols, and push-pull tests, and
- To confirm previously estimated values for hydraulic conductivity and dispersivity in the vicinity of newly constructed demonstration wells.

This document describes well installation, tracer testing methodology and results. The current project schedule and demonstration well boring logs are included in Attachments 1 and 2, respectively.

2. Methods

Two new wells were installed for use in this project using the air rotary drilling method: Demonstration Well 1 (DW-1) and Demonstration Well 2 (DW-2). As-built drawings or DW-1 and DW-2 are presented in Figures 1 and 2, respectively. Boring logs are included in Attachment 2. Well locations were chosen so that extraction well EW-4 could be used to control groundwater flow direction during the demonstration (Figure 3).

Tracer tests were conducted three times during the period May-August, 2012. For each test, site groundwater (from near-by extraction wells EW-4 or EW-1) was collected in a plastic tank placed next to each demonstration well. Sufficient NaCl or KBr was added to each tank to achieve a Cl- or Br- tracer concentration of ~ 100 mg/L; the tracer solution was thoroughly mixed using compressed air or a recirculation pump to vigorously agitate the water prior to injection. Each test consisted of injecting tracer solution into DW-1 and DW-2 and monitoring tracer transport by sampling the injection wells and downgradient monitoring wells MW-28, 4-106, and EW-2. At DW-1, samples

were collected from monitoring well MW-28; at DW-2, samples were collected from monitoring wells 4-106 and EW-2. Well MW-28 is located ~ 560 ft from EW-4, well 4-106 is located ~ 160 ft from EW-4 and well EW-2 is located ~ 108 ft from EW-4.

2.1 Slow Injection Forced Gradient Tracer Test (May 2012)

During this test, extraction well EW-4 operated at ~ 1,100 gallons per minute to force groundwater to flow from the demonstration wells toward the monitoring wells. The tracer solution was injected using a combination of siphons and pumps. For the tracer test in DW-1, the injection rate was ~ 7 gallons per minute and the duration of the injection phase was ~ 4 hours. For the tracer test in DW-2, the injection rate was ~ 4 gallons per minute and the duration of the injection phase was ~ 6 hours. Samples of the injected tracer solution were collected for field measurement of Br- concentration using a portable ion specific electrode and meter; duplicate samples were collected for subsequent analysis by ion chromatography. Groundwater samples were also collected from downgradient monitoring wells using submersible pumps. Groundwater depth was also measured periodically in all wells during tracer solution injection and subsequent groundwater sampling.

2.2 Borehole Dilution Tracer Test (July 2012)

It was not possible to control the gradient using extraction well EW-4 during this test due to a power failure at UMCD. However, this borehole dilution test performed under natural gradient conditions provides critical information to support push-pull test design in the upcoming push-pull test design. The volume of injected tracer solution (~ 100 mg/L Cl- or Br-) was 1000 gallons in DW-1 and DW-2. A high-speed transfer pump was used to inject the tracer solution at ~ 100 gallons per minute to both wells. Samples of the injected tracer solution were collected for subsequent analysis by ion chromatography. Groundwater samples were collected from DW-1 and DW-2 using bailers; groundwater samples from downgradient monitoring wells were collected using submersible pumps.

2.3 Fast Injection Forced Gradient Tracer Test (August 2012)

During these tests, extraction well EW-4 was pumping at ~ 1,100 gallons per minute. The volume of injected tracer solution (~ 100 mg/L Cl- or Br-) was 1000 gallons in DW-1 and DW-2. A high-speed transfer pump was used to inject the tracer solution at ~ 100 gallons per minute. Samples of the injected tracer solution were collected for subsequent analysis by ion chromatography. Groundwater samples were collected from DW-1 and DW-2 using bailers; groundwater samples from downgradient monitoring wells were collected using submersible pumps.

3. Results

3.1 Slow Injection Forced Gradient Tracer Test (May 2012)

Br- was detected in all downgradient monitoring wells sampled, confirming that hydraulically connected flow paths exist between DW-1 and well MW-28 and between DW-2 and wells 4-106 and EW-2. Results for MW-28 (located ~ 50 ft downgradient from DW-1) showed a rapid increase in Br- concentration that remained constant for the duration of the test (Figure 4). Estimated groundwater velocity (pore water velocity) was > 25 ft/day at this location.

The breakthrough curve for well 4-106 (located ~ 10 ft downgradient from DW-2) also clearly showed the arrival of the tracer solution (Figure 5). Estimated groundwater velocity (pore water velocity) is ~ 7.7 ft/day at this location. Surprisingly, the breakthrough curve for well EW-2 (located ~ 60 ft downgradient from DW-2), which is closer to EW-4 showed a more rapid arrival of the Br- tracer (Figure 6). Estimated groundwater velocity at this location is > 40 ft/day.

Water table depth data indicated that buildup in groundwater levels during tracer injection and subsequent groundwater sampling in all wells was < 0.05 ft, which is consistent with the very large values of hydraulic conductivity that have been previously estimated at this site (800-1000 ft/day).

3.2 Borehole Dilution Tracer Test (July 2012)

Although tracer was detected in 4-106, no tracer was detected in downgradient monitoring wells MW-28 and EW-2 because the regional gradient (no pumping) is not aligned with EW-4. Breakthrough curves for DW-1 and DW-2 show the gradual dilution expected as the injected tracer is transported away from the well (Figures 7 and 8). Breakthrough curves for 4-106 (near DW-2) shows the tracer pulse passing through the well within 5 hours after injection (Figure 9).

3.3 Fast Injection Forced Gradient Tracer Test (August 2012)

The breakthrough curves for DW-1 shows the decline in Cl- concentration as injected tracer is transported downgradient but surprisingly no tracer was detected in MW-28 (Figure 10). This in contrast to the results of tracer tests conducted in May, 2012, which showed rapid tracer transport between DW-1 and MW-28. The difference is potentially

due to the different tracer injection rate in the two rounds of test but is currently unresolved.

Breakthrough curves in the vicinity of DW-2 showed a much more predictable response as injected tracer moved from the injection well to the downgradient monitoring wells (Figure 11). The peak of the tracer pulse arrived at 4-106 about 10 hours after injection and at EW-2 about 27 hours after injection.

4. Conclusions

Three separate rounds of tracer tests were conducted during the summer of 2012. Test results confirmed that hydraulically connected flow paths exist between demonstration wells DW-1 and DW-2 and monitoring wells located downgradient. Using a high speed transfer pump and injection rates of ~ 100 gallons per minute resulted in well defined breakthrough curves and the sequential arrival of tracer at 4-106 (about 10 ft from DW-2) and EW-2 (about 50 ft from DW-2). These results are encouraging because they suggest that it will be possible to conduct well-to-well transport experiments using tracers, growth substrates, and microorganisms and will be helpful in designing efficient field protocols for those tests.

Estimated porewater seepage velocity ranged from ~ 25 ft/day near DW-1 to between ~ 8 and 40 ft/day near DW-2 based on results of the May 2012 tracer testing. Water table fluctuations during the forced gradient testing were very small confirming the very large hydraulic conductivity values (800-1000 ft/day) previously estimated for this site. Results of the borehole dilution tests are currently being evaluated with local groundwater elevations measured in July 2012 to estimate groundwater flow directions and seepage velocities with no pumping, similar to what would be expected during future push-pull testing. Porewater seepage velocities under forced and natural gradient conditions – integrated with RDX degradation rates and cell transport properties determined during laboratory treatability study – are keys to designing successful Phase II and III field tests. The demonstration plan will link results of groundwater tracer testing and laboratory treatability tests with proposed field testing methods.

The most surprising result of this study was the inconsistency between results of tracer tests conducted in DW-1. In the May tests, which used a very slow injection rates, tracer arrived at MW-28 very rapidly, while in the August tests, which used a much higher injection rate, tracer was never detected at MW-28. The explanation for these differences is unresolved.

5. Project Schedule

The current project schedule, which identifies work sequence and schedule for all project phases and phase subtasks, is included as Attachment 1.

FIGURES

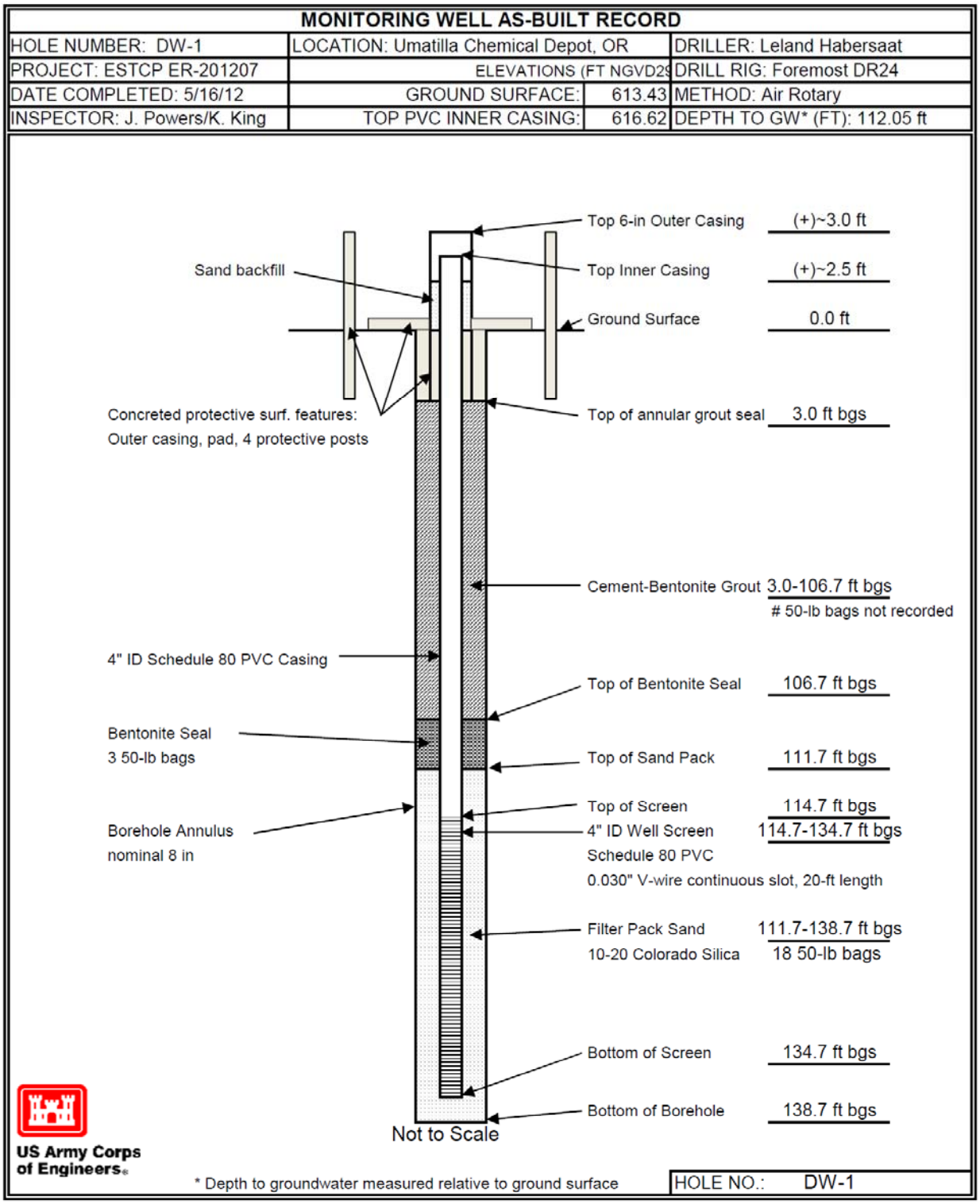


Figure 1. As-built drawing for demonstration well-1 (DW-1)

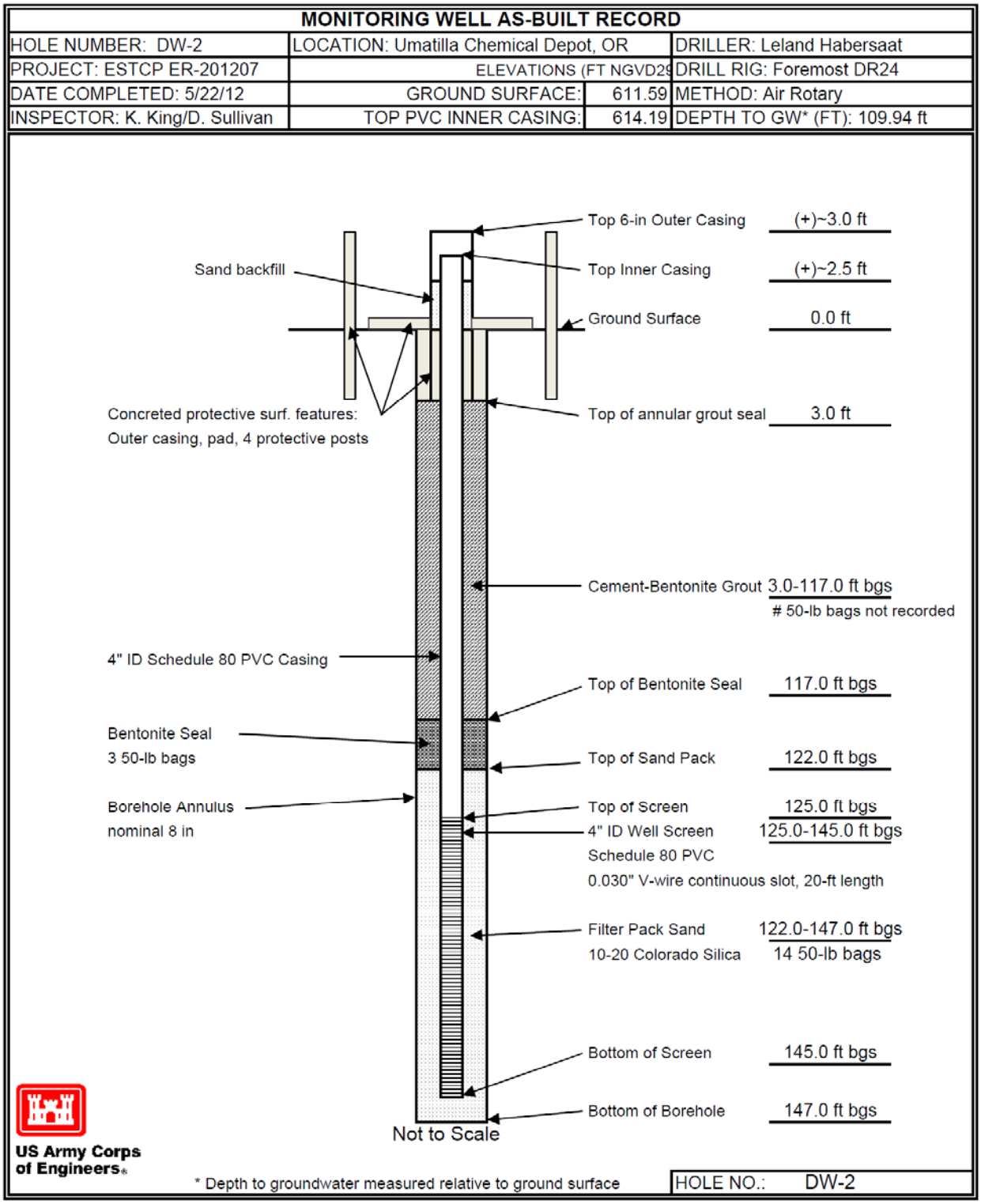


Figure 2. Boring log for demonstration well-2 (DW-2)

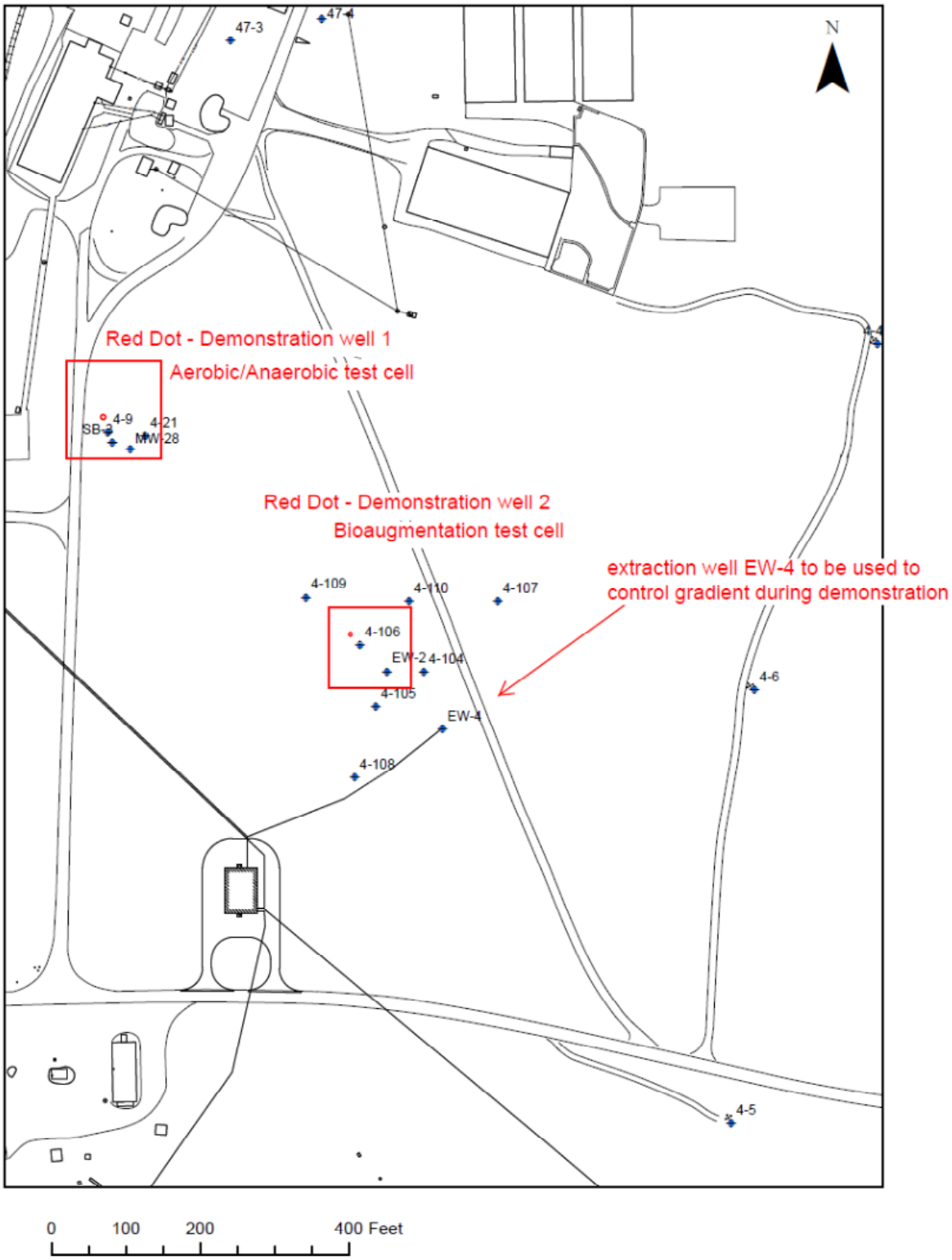


Figure 3. Demonstration well locations in test plots 1 and 2 are shown as red circles. Demonstration well 1 is ~ 50 ft from downgradient monitoring well MW-28. Demonstration well 2 is ~ 10ft from downgradient monitoring well 4-106 and ~ 60 ft from well EW-2.

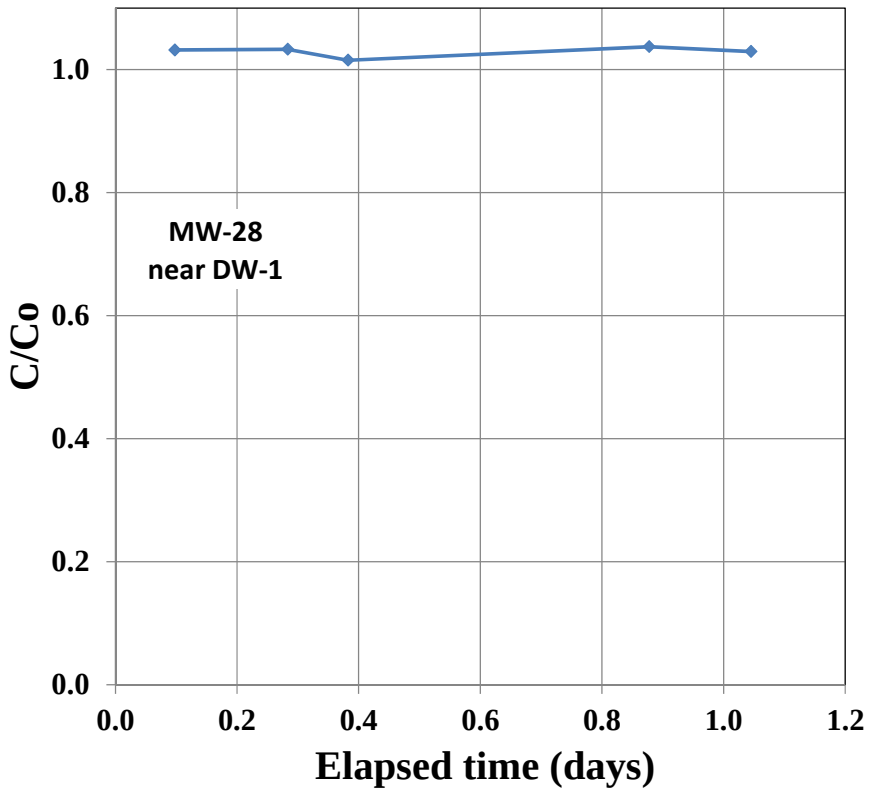


Figure 4. Relative Br- concentrations measured in well MW-28 during May, 2012 tracer tests. MW-28 is located ~ 50 ft downgradient from DW-1. C is measured Br- in a groundwater sample and Co is the average measured Br- concentration in the injected tracer solution. Elapsed time is measured from the midpoint of the injection phase.

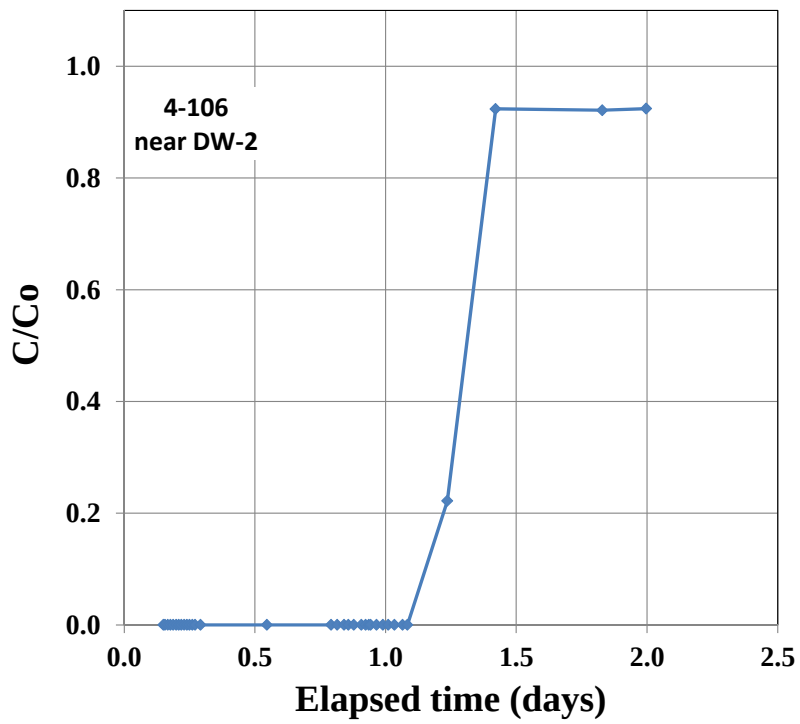


Figure 5. Relative Br- concentrations measured in well 4-106, located ~ 10 ft downgradient from DW-2. C is measured Br- in a groundwater sample and Co is the average measured Br- concentration in the injected tracer solution. Elapsed time is measured from the midpoint of the injection phase.

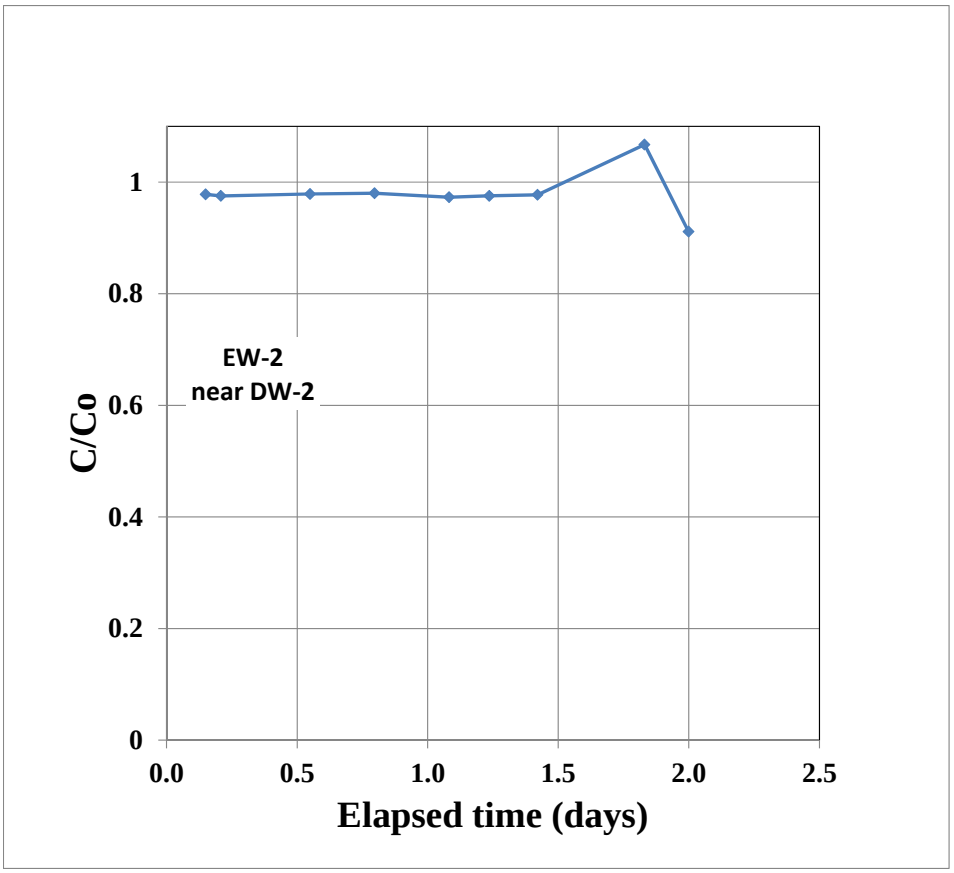


Figure 6. Relative Br- concentrations measured in well EW-2, located ~ 70 ft downgradient from DW-2. C is measured Br- in a groundwater sample and Co is the average measured Br- concentration in the injected tracer solution. Elapsed time is measured from the midpoint of the injection phase.

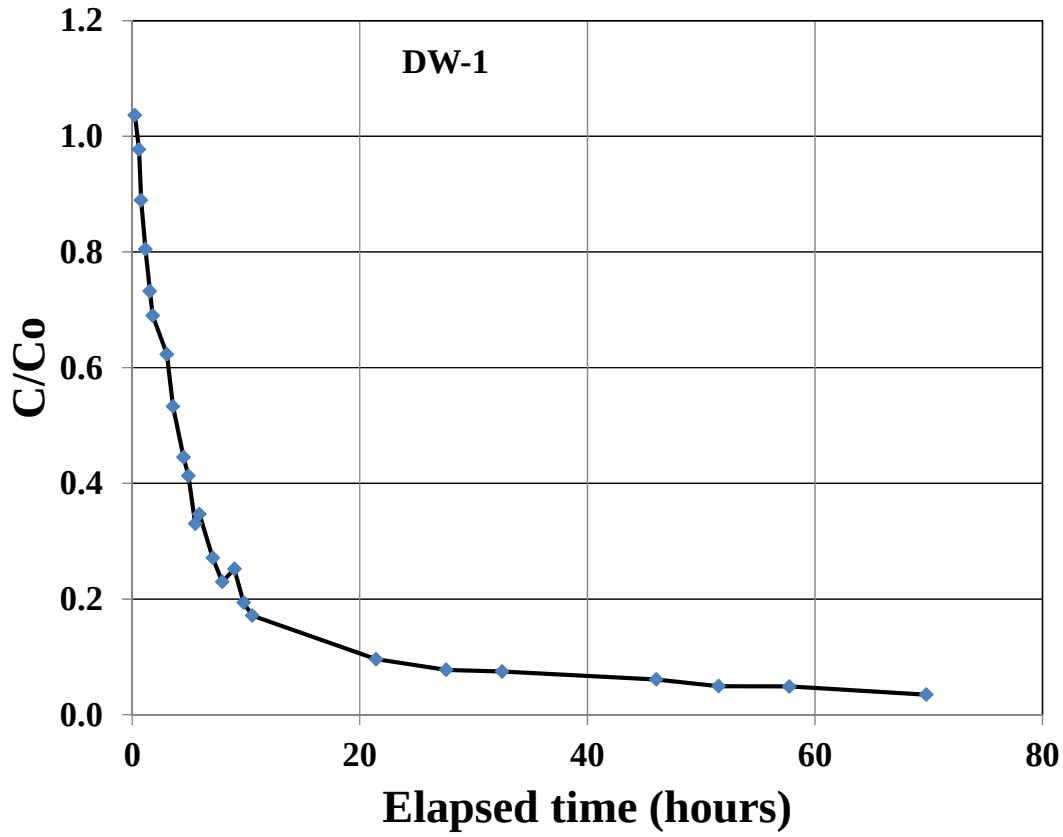


Figure 7. Relative Cl- concentrations measured in well DW-1. C is measured Cl- in a groundwater sample and Co is the average measured Cl- concentration in the injected tracer solution. Elapsed time is measured from the midpoint of the injection phase.

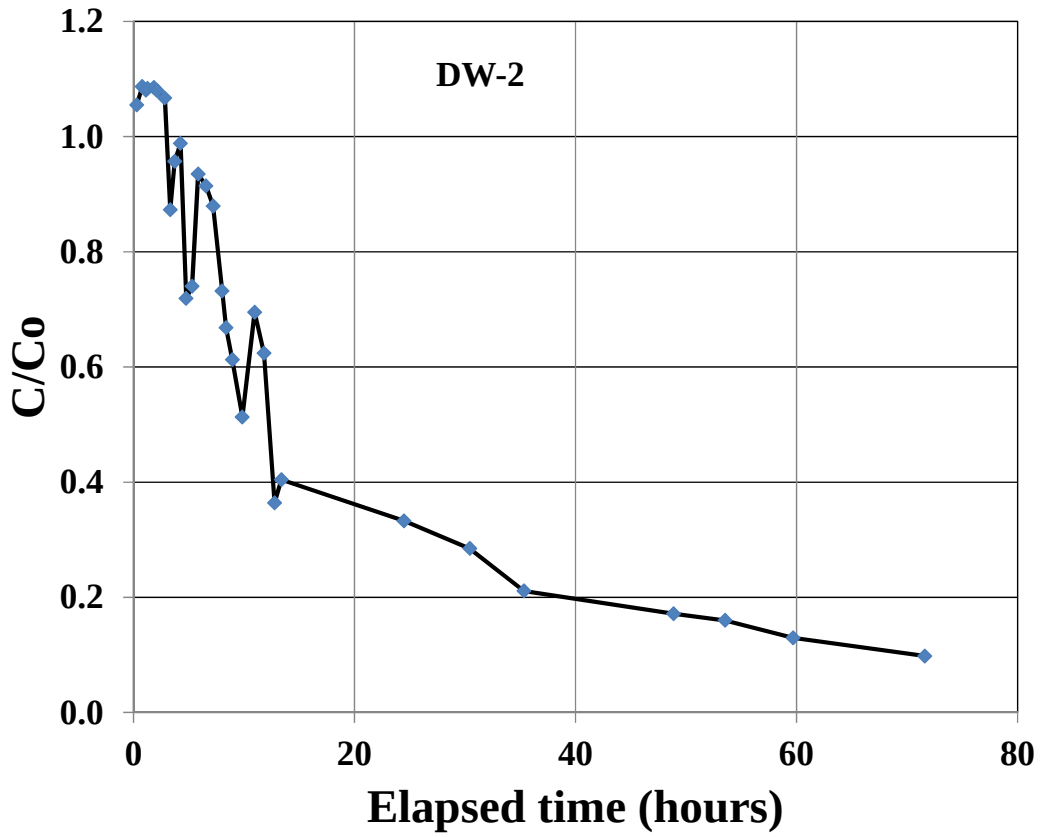


Figure 8. Relative Cl- concentrations measured in well DW-2. C is measured Cl- in a groundwater sample and Co is the average measured Cl- concentration in the injected tracer solution. Elapsed time is measured from the midpoint of the injection phase.

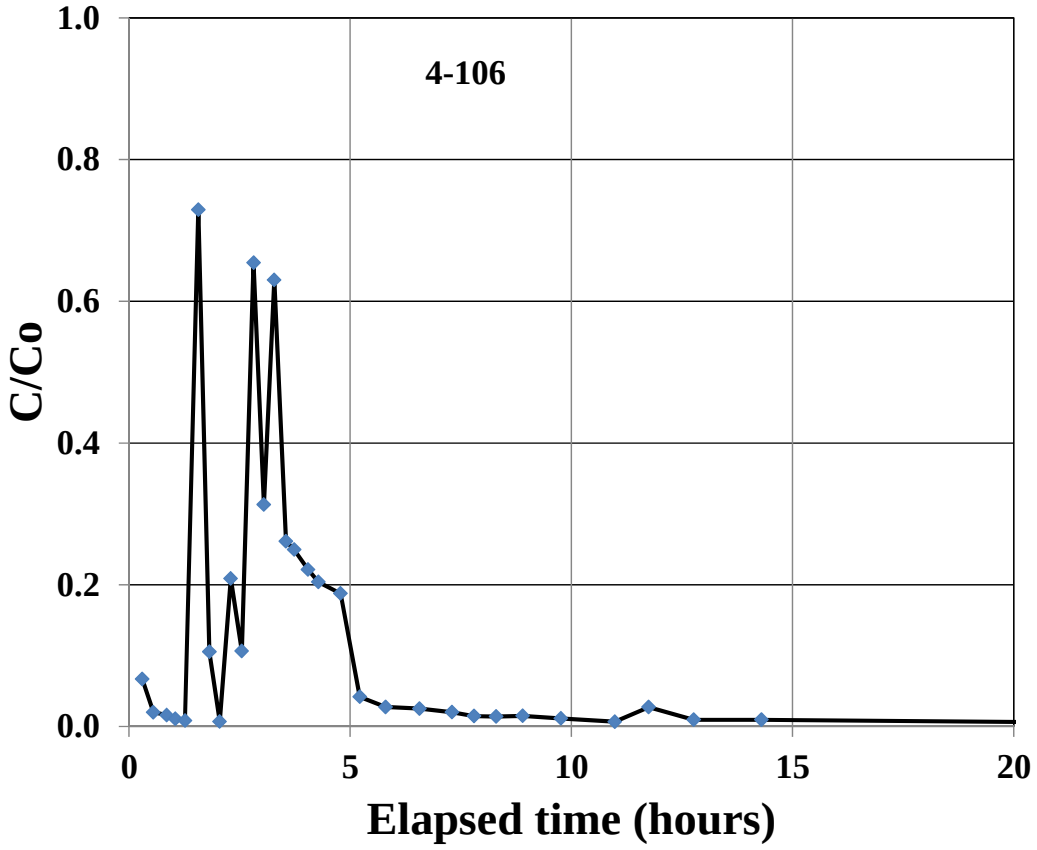


Figure 9. Relative Cl- concentrations measured in well 4-106. C is measured Cl- in a groundwater sample and Co is the average measured Cl- concentration in the injected tracer solution. Elapsed time is measured from the midpoint of the injection phase.

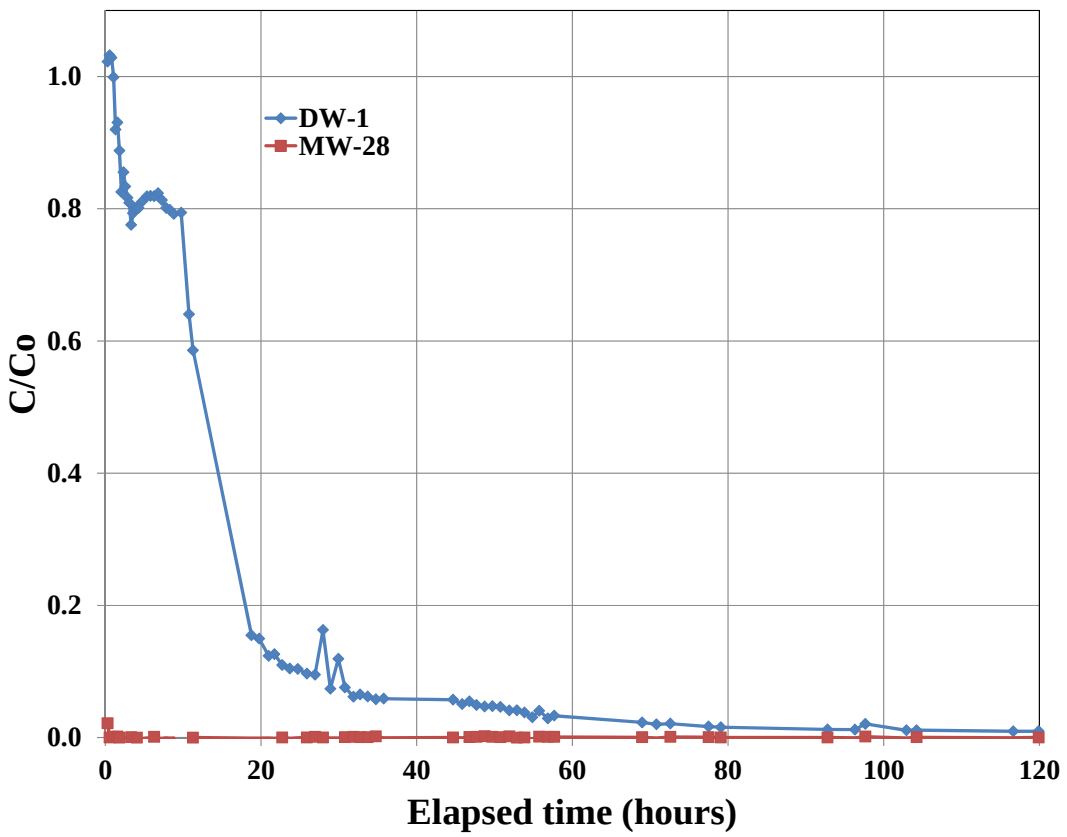


Figure 10. Relative Cl- concentrations measured in injection well DW-1 and downgradient monitoring well MW-28. C is measured Cl- in a groundwater sample and Co is the average measured Cl- concentration in the injected tracer solution. Elapsed time is measured from the midpoint of the injection phase.

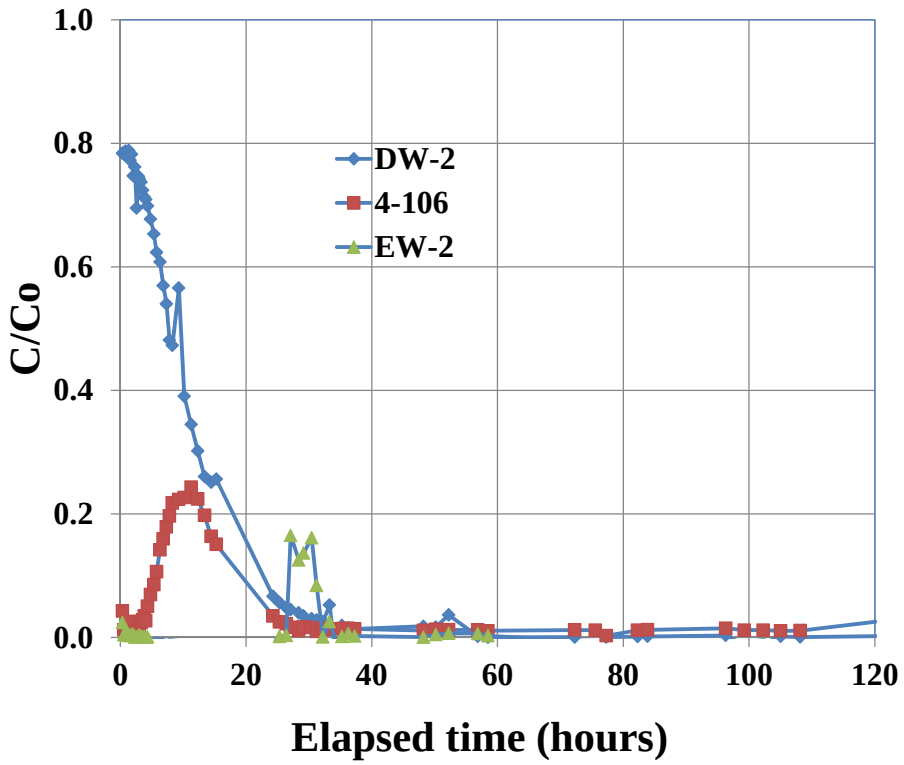


Figure 11. Relative Cl- concentrations measured in injection well DW-1 and downgradient monitoring wells 4-106 and EW-2. C is measured Cl- in a groundwater sample and Co is the average measured Cl- concentration in the injected tracer solution. Elapsed time is measured from the midpoint of the injection phase.

ATTACHMENT 1

Project Schedule

ID	Task Name	Start	Finish	Duration
3	**Project Funding Received**	Thu 3/1/12	Thu 3/1/12	0 days
4	**Start of Project**	Thu 3/1/12	Thu 5/24/12	61 days
5	a. SOW Development for SHAW	Thu 3/1/12	Fri 3/9/12	7 days
6	Contract Development/ Award for SHAW	Mon 3/12/12	Fri 5/4/12	40 days
7	b. SOW Development for OSU	Thu 3/1/12	Fri 3/9/12	7 days
8	Contract Development/ Award for OSU	Mon 3/12/12	Fri 5/4/12	40 days
9	c. SOW Development for Drillers	Thu 3/1/12	Fri 3/9/12	7 days
10	Contract Development/Award for Drillers	Mon 3/12/12	Fri 5/4/12	40 days
11	d. MIPR to ERDC from NWS	Thu 3/1/12	Wed 3/7/12	5 days
12	Draft Phase I Laboratory Studies Work Plan. Part 1: Culture Optimization and C	Thu 3/1/12	Wed 5/2/12	45 days
13	ESTCP Review, discussion, complete revisions	Thu 5/3/12	Wed 5/23/12	15 days
14	MS 1: Phase I Laboratory Studies Work Plan.	Thu 5/24/12	Thu 5/24/12	1 day
15	**Phase I: Site characterization and bioaugmentation culture optimization**	Mon 5/7/12	Wed 7/10/13	308 days
16	Task 1a. Site characterization	Mon 5/7/12	Fri 6/1/12	20 days
17	MS2: Site characterization memorandum	Mon 6/4/12	Fri 10/12/12	95 days
18	Task 1b. Bioaugmentation culture optimization (pure cultures in growth media)	Mon 6/4/12	Wed 10/31/12	108 days
19	Task 1b. Bioaugmentation culture optimization (site soil/slurry microcosms)	Thu 11/1/12	Fri 2/15/13	77 days
20	Midpoint of microcosms - informs culture selection for column transport optimiz	Thu 11/1/12	Wed 11/28/12	20 days
21	Task 1c. Cell transport optimization (adhesion assays)	Fri 5/25/12	Thu 11/22/12	130 days
22	Task 1c. Cell transport optimization (column transport)	Thu 11/29/12	Wed 2/20/13	60 days
23	Midpoint of cell transport column - confirms culture selection for production opti	Thu 11/29/12	Wed 1/9/13	30 days
24	Task 1d. Culture production optimization	Thu 1/10/13	Wed 3/6/13	40 days
25	MS3: Phase I Memo (treatability study report)	Thu 3/7/13	Wed 4/3/13	20 days
26	MS4: Draft Field Demonstration Work Plan	Thu 3/7/13	Wed 5/1/13	40 days
27	MS5: Final Field Demonstration Work Plan	Thu 5/2/13	Wed 6/26/13	40 days
28	Field Demonstration - Mob Prep	Thu 6/27/13	Wed 7/10/13	10 days
29	MS6: Phase II Field Demonstration Start	Wed 7/10/13	Wed 7/10/13	0 days
30	**Phase II: Demonstrate field-scale transport of RDX-degrading culture and perform treatment control push-pull tests**	Thu 4/4/13	Wed 1/29/14	215 days
31	Task 2a. Determine RDX retardation factors	Thu 7/11/13	Wed 7/17/13	5 days
32	Task 2b. Culture production for field transport tests	Thu 4/4/13	Wed 6/26/13	60 days
33	Task 2c. Field-scale culture transport test	Thu 7/18/13	Wed 7/24/13	5 days
34	Laboratory turnaround time (8330 and molecular)	Thu 7/25/13	Wed 9/4/13	30 days
35	Data analysis	Thu 9/5/13	Wed 10/16/13	30 days
36	Phase II Draft Memo	Thu 10/17/13	Wed 11/6/13	15 days
37	MS7: Phase II Memo	Wed 11/6/13	Wed 11/6/13	0 days
38	Update and Finalize Demonstration Work Plan for Phase III	Thu 11/7/13	Wed 1/8/14	45 days
39	Phase III Mob Prep	Thu 1/9/14	Wed 1/29/14	15 days
40	MS8: Phase III Field Demonstration Start	Wed 1/29/14	Wed 1/29/14	0 days
41	**Phase III: Demonstrate transport of aerobic RDX degrading bioaugmentation culture, RDX degrading activity and xplA gene transfer to indigenous microorganisms at field-scale**	Thu 1/30/14	Fri 4/24/15	322 days
42	Task 3a. Field-scale culture production, aquifer conditioning and bioaugmentati	Thu 1/30/14	Wed 5/21/14	80 days
43	Task 3b. (1) Triplicate push-pull tests for determination of in situ RDX degradati	Thu 5/22/14	Thu 6/5/14	11 days
44	Task 3b. (2) Triplicate push-pull tests for determination of in situ RDX degradati	Fri 6/6/14	Thu 7/31/14	40 days
45	Laboratory turnaround time	Fri 8/1/14	Thu 9/25/14	40 days
46	Data analysis	Fri 9/26/14	Thu 10/23/14	20 days
47	MS9: Draft Final Technical Report	Fri 10/24/14	Thu 12/18/14	40 days
48	MS10: Final Technical Report	Fri 12/19/14	Thu 2/26/15	50 days
49	MS11: Draft Final Cost and Performance Report	Fri 10/24/14	Thu 12/18/14	40 days
50	MS12: Final Cost and Performance Report	Fri 12/19/14	Thu 2/26/15	50 days
51	MS13: Draft Guidance Document	Fri 2/27/15	Thu 4/23/15	40 days
52	MS14: Final Guidance Document	Fri 2/27/15	Thu 4/23/15	40 days
53	Final Project Debrief Conference Call	Fri 4/24/15	Fri 4/24/15	1 day
54	Project Complete	Thu 4/23/15	Thu 4/23/15	0 days

ATTACHMENT 2

Hole No. DW-1

DRILLING LOG

DIVISION: Seattle District

INSTALLATION: UMCD

SHEET 1 OF 9 SHEETS

1. PROJECT: ESTCP ER-201207
2. LOCATION (Coordinates or Station): UMCD "400 AREA"
3. DRILLING AGENCY: Jensen Drilling
4. HOLE NO. (As shown on drawing title and file number): DW-1
5. NAME OF DRILLER: Leland Habersaat
6. DIRECTION OF HOLE: [x] VERTICAL [ ] INCLINED _____ DEG. FROM VERT.
7. THICKNESS OF OVERBURDEN: 138' 8" 3 5/8"
8. DEPTH DRILLED INTO ROCK: —
9. TOTAL DEPTH OF HOLE: 138' 8" 3 5/8"
10. SIZE AND TYPE OF BIT: downhole hammer for 8" CASING
11. DATUM FOR ELEVATION SHOWN (TBM or MSL): NGVD 1929
12. MANUFACTURES'S DESIGNATION OF DRILL: Foremost DR24 Air Rotary
13. TOTAL NO. OF OVER-BURDEN SAMPLES TAKEN: DISTURBED: ; UNDISTURBED:
14. TOTAL NUMBER CORE BOXES: 3 coolers
15. ELEVATION GROUND WATER: ~112'
16. DATE HOLE: STARTED 5/11/12; COMPLETED 5/10/12
17. ELEVATION TOP OF HOLE:
18. TOTAL CORE RECOVERY FOR BORING: %
19. SIGNATURE OF INSPECTOR: Jeff Powers; Katie King

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth weathering, etc., if significant) g
614.0 (approx.) NGVD 29	0		SP, brown SAND, med. grained dry, loose		run 1	"DW-1" stands for Demonstration Well #1
	1.0					well is located 50 ft northwest of MW-28
						5/11/12 0955 hrs begin drilling
	2.0					
	3.0					8" diam temporary steel casing, welded joints depths as noted.
	4.0			N/A	N/A	soil cuttings are logged except where split spoon samples collected, as noted.
	5.0		GW, brown sandy GRAVEL, well graded gravel up to 1" diam rounded (65%), med. to coarse-grained sand (35%), little to no fines, dry loose to medium-loose			
	6.0					
	7.0					
	8.0		same, GW			
	9.0					
	10.0					

ENG FORM 1836 MAR 71 PREVIOUS EDITIONS ARE OBSOLETE. (modified by GCA 1/94)

PROJECT: ESTCP ER-201207 HOLE NO. DW-1

DRILLING LOG (Cont Sheet)	ELEVATION TOP OF HOLE ~614.0' NGVD 29	Hole No. DW-1
1. PROJECT ESTCP ER-201207	2. INSTALLATION UMCD	SHEET 2 OF 9 SHEETS

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth of weathering, etc., if significant) g
	10.0					currently using potable water down casing for dust suppression
	11.0		GW, gray sandy GRAVEL, dry, loose to medium			
	12.0		~70% grav, rnd to sub-rounded up to 1" diam			
			~30% medium to coarse sand			
	13.0					
	14.0			N/A	N/A	
	15.0		same, GW			
	16.0					
	17.0					
	18.0					finish advancing casing to 19.33 @ 1033 hrs:
	19.0					← casing weld @ 19.33'
	20.0		Same, GW		Run 2	begin casing advancement @ 1237 hrs
	21.0					
	22.0					
	23.0					
	24.0					
	25.0			N/A	N/A	
	26.0					
	27.0					
	28.0					

ENG FORM JUN 67 1836-A (ER 1110-1-1801)

PROJECT ESTCP ER-201207

HOLE NO. DW-1

DRILLING LOG (Cont Sheet)	ELEVATION TOP OF HOLE	Hole No. DW-1
1. PROJECT ESTCP ER-201207	2. INSTALLATION UMCD	SHEET 3 OF 9 SHEETS

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth of weathering, etc., if significant) g
	28.0		GW			* over drill original hole post sheared casing retreival on 5/14
	29.0					
	30.0					
	31.0					
	32.0		GW, gray sandy GRAVEL, well-graded. rounded to 1" diam (~70%)			
	33.0		med. to coarse sand (~30%) little to no fines loose to med-loose	N/A	N/A	
	34.0					
	35.0					
	36.0					
	37.0		* 5/14 at 1427 "			
	38.0					
	39.0					
	40.0					finish advancing casing to 40.0' @ 1330 hrs
					Run3	casing weld @ 40'
	41.0					begin casing advancement @ 1455
	42.0					
	43.0		GW gray sandy GRAVEL, well-graded med. to coarse sand, gravel up to 1" No fines ~70% grav ~30% sand	N/A	N/A	5/11/12 @ 1505 hrs
	44.0					drill rod sheared off - begin downtime to figure solution 44' casing in ground ~39' drill rod down-hole
	45.0					
	46.0					

DRILLING LOG (Cont Sheet)	ELEVATION TOP OF HOLE	Hole No. DW-1
1. PROJECT ESTCP ER-201207	2. INSTALLATION UMCD	SHEET 4 OF 9 SHEETS

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth of weathering, etc., if significant) g
	46.0					
	47.0					
	48.0					
	49.0					
	50.0					
	51.0		GW, gray sandy GRAVEL, well-graded med to coarse sand, gravel up to 2"	N/A	N/A	
	52.0		NO FINES ~70% gravel ~30% sand			
	53.0					
	54.0					
	55.0					
	56.0				Run4	• 1439 on 5/14 position next 20ft section of casing at 56' bgs
	57.0					• 1544 4" threaded in place - begin welding outer 8" casing in place
	58.0		apparent cobbles	N/A	N/A	• 1646 welding complete - start drilling! encounter apparent boulder at ~57' bgs
	59.0		GW as above			
	60.0					first 20' casing
	61.0					second 21'
	62.0					third 19' 5½"
	63.0					4th 21' 5/8"
	64.0					81.51'

10' 9" left when stop work 5/14 at 530

at 70.8'

ENG FORM JUN 67 1836-A (ER 1110-1-1801)

PROJECT ESTCP ER-201207

HOLE NO. DW-1

DRILLING LOG (Cont Sheet)	ELEVATION TOP OF HOLE	Hole No. DW-1
1. PROJECT ESTCP ER-201207	2. INSTALLATION UMCD	SHEET 5 OF 9 SHEETS

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth of weathering, etc., if significant) g
	64.0					
	65.0					
	66.0					
	67.0					
	68.0					
	69.0					
	70.0					5/14/12 End of Day @ 70'8" 1730
	71.0		GW, same		Run 5	Started up @ 0727 5/15/12 KK
	72.0					5th casing: 19' 3" length
	73.0					
	74.0					
	75.0					~@ 0755 finish casing advancement (w/5' stick up)
	76.0					0830 - welding
	77.0					0917 Start casing advancement
	78.0					
	79.0					
	80.0					
	81.0					
	82.0					

DRILLING LOG (Cont Sheet)	ELEVATION TOP OF HOLE	Hole No. DW-1
1. PROJECT ESTCP ER-201207	2. INSTALLATION UMCD	SHEET 6 OF 9 SHEETS

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth of weathering, etc., if significant) g
	82.0					
	83.0					
	84.0					
	85.0					
	86.0					
	87.0					
	88.0					
	89.0					
	90.0					
	91.0					
	92.0					
	93.0					
	94.0					
	95.0					
	96.0		Same, GW		Run6	0950 finish advancing casing ↳ welding Begin casing advance
	97.0					Casing: 21'0.5"
	98.0					
	99.0					
	100.0					

ENG FORM JUN 67 1836-A (ER 1110-1-1801)

PROJECT ESTCP ER-201207

HOLE NO. DW-1

DRILLING LOG (Cont Sheet)	ELEVATION TOP OF HOLE	Hole No.

1. PROJECT	2. INSTALLATION	SHEET 7 OF 9 SHEETS
ESTCP ER-201207	UMCD	

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth of weathering, etc., if significant) g
	100.0					
	101.0					
	102.0					
	103.0					
	104.0					
	105.0					
	106.0					
	107.0					
	108.0					
	109.0					
	110.0		Fine gray sandy gravels; medium graded → gravel up 1" → appears to be more sand & fine gravels (~35%) medium to coarse sand Trace fines?			@1150 → advanced 10' of casing.
	111.0					brown / tan water → Trace fines
112' ▽ @5/16/12 0745	112.0					
	113.0					@1154 clump hopper - overfull ? cuttings were building up in the cyclone
114' ▽ @5/15/12 1335	114.0					
	115.0					
	116.0					
	117.0					
	118.0			Run 7	117.5' Split Spoon Sample	@1216 stop advancing casing

ENG FORM JUN 67 1836-A (ER 1110-1-1801)

PROJECT	HOLE NO.
ESTCP ER-201207	DW-1

DRILLING LOG (Cont Sheet)	ELEVATION TOP OF HOLE	Hole No. DW-1
1. PROJECT ESTCP ER-201207	2. INSTALLATION UMCD	SHEET 8 OF 9 SHEETS

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth of weathering, etc., if significant) g
	118.0		GW gray sandy gravel, wet, gravel = mostly fine w/ some coarse to 1", sand → fine to coarse (well graded) Trace fines - brown/tan water subrounded mixed lithologies.	55% @ 1645 Began casing advance	10"/18"	@1220 start to do split spoon sample -Driller hasn't noticed groundwater, @ 117', should have reached it, split spoon sample to test if we have hit gw. -Driller has been using alot of water. Hence drill string was pulled out water measured @ 114' @ 1335 casing length: 20' 11" @127' Driller noted picking up more water. -Hopper dumped again @1610, fine material from 117.5' - 138'8" placed in 3 coolers on 5/15/12 3 5/16/12 Welding 1610-1645 begin casing advance. 1645
	119.0					
	120.0					
	121.0					
	122.0					
	123.0					
	124.0					
	125.0					
	126.0					
	127.0					
	128.0					
	129.0					
	130.0					
	131.0					
	132.0					
	133.0					
	134.0					
	135.0					
	136.0					

ENG FORM JUN 67 1836-A (ER 1110-1-1801)

PROJECT ESTCP ER-201207 | HOLE NO. DW-1

DRILLING LOG (Cont Sheet)	ELEVATION TOP OF HOLE	Hole No. DW-1
1. PROJECT EST CP ER-201207	2. INSTALLATION UMCD	SHEET 9 OF 9 SHEETS

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth of weathering, etc., if significant) g
136.0						
137.0			TD: 138' 8" 5/8"			
138.0						Finish casing advance to 138.8" 5/8" @ 1725
139.0						* collect 1st cooler sample
140.0						End of Day 5/15/12 @ ~ 1745
141.0						5/16/12
142.0						↳ no need to drill further, well screen
143.0						will be installed
144.0						from 115' - 135'
145.0						- Filled 2 more coolers w/fine material @ 0745

Hole No. DW-2

DRILLING LOG	DIVISION Seattle District	INSTALLATION UMCD	SHEET 1 OF 9 SHEETS

1. PROJECT ESTCP ER-201207	10. SIZE AND TYPE OF BIT Downhole hammer for 8" casing
2. LOCATION (Coordinates or Station) UMCD "400 AREA"	11. DATUM FOR ELEVATION SHOWN (TBM or MSL) NGVD 1929
3. DRILLING AGENCY Jensen Drilling	12. MANUFACTURES'S DESIGNATION OF DRILL Foremost DR24 Air Rotary
4. HOLE NO. (As shown on drawing title and file number) DW-2	13. TOTAL NO. OF OVER-BURDEN SAMPLES TAKEN — DISTURBED: — UNDISTURBED: —
5. NAME OF DRILLER Leland Habersaat	14. TOTAL NUMBER CORE BOXES 3 coolers
6. DIRECTION OF HOLE ☑ VERTICAL ☐ INCLINED ____ DEG. FROM VERT.	15. ELEVATION GROUND WATER
7. THICKNESS OF OVERBURDEN 147'	16. DATE HOLE STARTED 5/18/12 COMPLETED 5/21/12
8. DEPTH DRILLED INTO ROCK —	17. ELEVATION TOP OF HOLE
9. TOTAL DEPTH OF HOLE 147'	18. TOTAL CORE RECOVERY FOR BORING %
	19. SIGNATURE OF INSPECTOR Katie King (Kate King) / Dave Sullivan

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth weathering, etc., if significant) g
611.5 (approx)	0		SP, brown sand, med grained dry, loose	Little return	Run1	"DW-2" stands for Demonstration well #2
	1.0					well is located 10 ft northwest of 4-106
						1110 - Drilling begins
	2.0					- Drilling w/ water
						1st casing length: 20'6"
	3.0					1110-1140: Driller discovers issue w/ hammer not unloading air pressure @ 1200 decides to drill in 1st casing → 1216 casing in ground; begin troubleshoot; 1235-1337
	4.0		Gray sandy fine gravels <1"			
	5.0					- Found debris in hammer, should work now
	6.0					
	7.0					
	8.0					
	9.0					
	10.0					

DRILLING LOG (Cont Sheet)	ELEVATION TOP OF HOLE ~611.5'	Hole No. DW-2
1. PROJECT ESTCP ER-201207	2. INSTALLATION UMCD	SHEET 2 OF 9 SHEETS

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth of weathering, etc., if significant) g
	10.0					
	11.0					
	12.0					
	13.0					
	14.0					
	15.0					
	16.0					
	17.0					
	18.0					
	19.0					
	20.0					1216 end advance 1426-1505 Weld casing 1505 begin advance casing 19'1"
	21.0		Darker brown water - more gray		Run 2	
	22.0		GW, Dark gray gravel w/ sand & w/trace silt; angular chips → subangular to sub round, to round gravels <1" mixed lithologies			
	23.0					
	24.0					
	25.0		More sand → sandy gravels			
	26.0					
	27.0					
	28.0					

DRILLING LOG (Cont Sheet)	ELEVATION TOP OF HOLE	Hole No. DW-2
1. PROJECT ESTCP ER-201207	2. INSTALLATION UMCD	SHEET 3 OF 9 SHEETS

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth of weathering, etc., if significant) g
	28.0					
	29.0					
	30.0					
	31.0					
	32.0					
	33.0					
	34.0					
	35.0		Finer gravels < 0.75"			
	36.0					
	37.0					
	38.0					
	39.0		GW continued 90% gravels 10% sand no fines			1535 Finished advancing casing @ 39' 7"
	40.0				Run3	Weld: 1605 – 1630 Begin advancing casing 1637
	41.0		V. little sand → all coarse			length: 20' 10"
	42.0					
	43.0					
	44.0					
	45.0					
	46.0					

ENG FORM JUN 67 1836-A (ER 1110-1-1801)

PROJECT ESTCP ER-201207

HOLE NO. DW-2

DRILLING LOG (Cont Sheet)	ELEVATION TOP OF HOLE	Hole No. DW-2
1. PROJECT ESTCP ER-201207	2. INSTALLATION UMCD	SHEET 4 OF 9 SHEETS

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth of weathering, etc., if significant) g
	46.0					
	47.0					
	48.0					
	49.0					
	50.0		increasing % of coarse sand 70% gravel 30% sand			
	51.0					
	52.0					
	53.0					
	54.0					
	55.0		More fines gravelly sand → medium to coarse			
	56.0		trace silt? dark brown/gray to tan			* End of Day → ~4'5" stick up
	57.0		increasing gravel sizes → 1.5", rounded to subround			
	58.0					
	59.0		less gravels → more medium sand 50% g 50% s			
	60.0					Finished advancing casing @1700 60'5"
	61.0				Run4	We id: 1726-1800, End 5/18/12 Begin 5/19/12 @ 0750
	62.0		GW again → ~70% g, ~30% s			Casing 20'10"
	63.0					
	64.0					

DRILLING LOG (Cont Sheet)	ELEVATION TOP OF HOLE	Hole No. DW-2
1. PROJECT ESTCP ER-201207	2. INSTALLATION UM CD	SHEET 5 OF 9 SHEETS

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth of weathering, etc., if significant) g
	64.0					
	65.0					
	66.0					
	67.0					
	68.0					
	69.0					
	70.0		GW continued, medium to fine gravels with coarse to fine sand; round to subround Drk gray → many basalt peices; mixed lithologies, ~80% g ~20% S gravels: <1"			
	71.0					
	72.0					
	73.0					
	74.0					
	75.0		GW continued.			
	76.0					
	77.0					
	78.0					
	79.0					
	80.0					
	81.0					End casing advance 0830 81'3" wtld: 0856-0938
					Run5	Begin casing advance 0940 length: 20' 10"
	82.0					

ENG FORM JUN 67 1836-A (ER 1110-1-1801)

PROJECT ESTCP ER-201207 | HOLE NO. DW-2

DRILLING LOG (Cont Sheet)	ELEVATION TOP OF HOLE	Hole No. DW-2
1. PROJECT ESTCP ER-201207	2. INSTALLATION UMCD	SHEET 6 OF 9 SHEETS

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth of weathering, etc., if significant) g
	82.0					
	83.0					
	84.0					
	85.0					
	86.0					
	87.0					
			GW continued...			
	88.0					
	89.0					
	90.0					
	91.0					
	92.0					
	93.0					
	94.0					
	95.0					
	96.0					
			same..GW			
	97.0					
	98.0		GW, medium to fine gravel, gray, coarse sand, round to subround <0.75" gravels, loose	- - - - -		~4' of stick up
	99.0					
	100.0					

DRILLING LOG (Cont Sheet)	ELEVATION TOP OF HOLE	Hole No. DW-2
1. PROJECT ESTCP ER-201207	2. INSTALLATION VMCD	SHEET 7 OF 9 SHEETS

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth of weathering, etc., if significant) g
	100.0					
	101.0					
	102.0					1004: End advance 102'1" weld: 1035-1120 Begin advance: 1120 casing length: 19'4"
			continue w/ finer gravels, coarse sand		Run 6	
	103.0					
	104.0					
	105.0					* Better return this run
	106.0					
	107.0		most gravels <0.75" many less <0.5"			Alot of water Brown-tan water
	108.0					
	109.0					
5/19/12 109'8" @1415	110.0		~ Becomes wet			
	111.0					
	112.0					
	113.0					
5/21/12 114.4' @0930	114.0					
	115.0					
	116.0					
	117.0				Split Spoon Sample @1400	End @ 1147 117'1" @ 1147 = Bottom of casing, 4'4" stick up Weld: 1515-1600, End of Day
	118.0					

ENG FORM JUN 67 1836-A (ER 1110-1-1801)

PROJECT ESTCP ER-201207

HOLE NO. DW-2 5/19/12 @ 1600

DRILLING LOG (Cont Sheet)	ELEVATION TOP OF HOLE	Hole No. DW-2

1. PROJECT	2. INSTALLATION	SHEET 8 OF 9 SHEETS
ESTCP ER-201207	VMCD	

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth of weathering, etc., if significant) g
	118.0		SP poorly graded, fine gravelly, m-c SAND		RUN 7	21 May 12 0745 St. Drilling
	119.0		40% Gravel, poorly graded 60% fines - almost all sand			easy drilling. wet/saturated
	120.0		mostly fine gr (~80%), <0.5"			
	121.0					
	122.0					
	123.0		sand - M-C, sub ang, [illegible] ~60-70% coarse			
	124.0		gravel - mostly [illegible]			
	125.0		[illegible]			
	126.0		rounded - [illegible]			
	127.0					
	128.0		More medium gr			
	129.0		med gr - 50% to 0.75" fine gr - 30%			
	130.0		sand 70%, 80% coarse			
	131.0		back to m-c sand w/ fine gravel (25%)			
	132.0					
	133.0					
	134.0					
	135.0		more sand - ~75% + fines - ~5%			
	136.0		SP-SM poorly graded fine gravelly m-c SAND w/ silt			

ENG FORM JUN 67 1836-A (ER 1110-1-1801)

PROJECT: ESTCP ER-201207

HOLE NO.: DW-2

DRILLING LOG (Cont Sheet)	ELEVATION TOP OF HOLE	Hole No. DW-2
1. PROJECT ESTCP ER-201207	2. INSTALLATION UMCD	SHEET 9 OF 9 SHEETS

ELEVATION a	DEPTH b	LEGEND c	CLASSIFICATION OF MATERIALS (Description) d	% CORE RECOVERY e	BOX OR SAMPLE NO. f	REMARKS (Drilling time, water loss, depth of weathering, etc., if significant) g
	136					saturated
	137				Run 8	Stopped @ 1215 start @ 1220 saturated
	138					
	139					
	140					
	141		SW-SM well graded f-c SAND w/ f gr (<0.5") and silt (10%)			
	142					
	143					
	144					
	145		SM well graded silty SAND w/ gravel			S: 70% M: 20% G: 10%
	146					
	147		BOH 147' bgs			stopped @ 1240 21May12
	148					
	149					
	150					
	151					
	152					
	153					
	154					

APPENDIX B

PHASE I RESULTS MEMORANDUM

Bioaugmentation for Aerobic Bioremediation of RDX-Contaminated Groundwater

ESTCP PROJECT ER-201207
Phase I Results Memorandum

Prepared by:

Mandy Michalsen, PI
Seattle District, U.S. Army Corps of Engineers

Fiona Crocker, Karl Indest, Carina Jung
U.S. Army Engineer Research Development Center

Paul Hatzinger, Mark Fuller
CB&I

Jack Istok
Oregon State University

May 1, 2013

TABLE OF CONTENTS

1. INTRODUCTION 1
1.1 Project Background 1
1.2 Document Purpose and Organization 2
2. METHODS 2
2.1 Bioaugmentation Culture Optimization 3
2.1.1 Cell Suspension studies 3
2.1.2 Microcosm studies 4
2.1.3 Cell "tagging" for detecting xplA gene transfer 5
2.2 Cell Transport and Production Optimization 6
2.2.1 Cell adhesion assay 6
2.2.2 Column Transport Experiments 7
2.2.3 Bioaugmentation culture production optimization 8
3. RESULTS 9
3.1 Cell Suspension Studies 9
3.2 Microcosm Tests 10
3.3 Cell "tagging" for monitoring xplA gene transfer 10
3.4 Cell Adhesion Testing 11
3.5 Column Transport Testing 11
3.6 Bioaugmentation culture production optimization 12
4. DISCUSSION AND "GO" DECISION RECOMMENDATION 13
5. NEXT STEPS 16
6. REFERENCES 17

LIST OF TABLES

Table 1. List of all RDX-degrading microbial strains included in the initial screening. 21
Table 2. List of carbon sources and nitrogen sources screened in initial tests. 22
Table 3. Composition of the artificial groundwater (AGW) used during all treatability study experiments. 23
Table 4. Experimental conditions for the column transport experiments. 24
Table 5. Summary of adhesion assay results for the various strains screened. 25
Table 6. Summary of key column transport experiment results. 26

LIST OF FIGURES

Figure 1. Schematic showing cell suspension and microcosm study phasing 28
Figure 2. Illustration of the column experiment setup. 29
Figure 3. Growth curves of RDX-degrading bacterial strains on various carbon and nitrogen sources. 30
Figure 4. Maximum growth yields of RDX-degrading bacterial strains on select carbon and nitrogen sources during initial screening. 31
Figure 5. Growth curves of selected RDX-degrading bacterial strains on optimal carbon and nitrogen sources. 32
Figure 6. Survival of RDX-degrading strains in artificial groundwater. 33
Figure 7. Degradation of RDX by RDX-degrading strains in artificial groundwater. 34
Figure 8. Growth physiology of select RDX-degrading strains in AGW amended with fructose and RDX. 35
Figure 9. Bacterial viable cell numbers during microcosm 36
Figure10. RDX concentrations in UMCD microcosms 37
Figure 11. Quantitation of *xplA* (A), *xenB* (B), kanamycin (C), and 16S rRNA (D) genes in microcosms 38
Figure 12. Gel electrophoresis of PCR confirmation of KTR9 ZSGreen transcongucates 39
Figure 13. Visual shortwave UV light exposure of KTR9 wildtype and KTR9 ZSGreen 40
Figure 14. Breakthrough curves for bromide, cells, and RDX for Column Experiment 1. 41
Figure15. Breakthrough curves for bromide, cells, and RDX for Column Experiment 2. 42

ATTACHMENT 1 Schedule

ACRONYM LIST

RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine
UMCD	Umatilla Chemical Depot
AGW	Umatilla artificial groundwater
CFU	colony forming unit
qPCR	Quantitative polymerase chain reaction
BSM	basal salts medium
ml	milliliter
°C	degrees Celsius
g	gram
L	liter
µg	Microgram
PV	Pore volume
HGT	Horizontal gene transfer

1. INTRODUCTION

1.1 Project Background

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a common contaminant in soils and groundwater at military sites worldwide. RDX can be mobile and persistent in groundwater under the aerobic conditions present in many aquifers and thus tends to form large, dilute plumes. Although multiple studies have demonstrated *in situ* RDX biodegradation under anaerobic conditions, creating and maintaining anaerobic conditions across large areas is costly and technically challenging. This project will demonstrate an innovative application of bioaugmentation to enhance RDX biodegradation in contaminated groundwater under aerobic conditions. The project will provide field data to support both a technical evaluation and a cost-benefit analysis of this approach.

During this ESTCP demonstration, a mixed microbial culture capable of aerobically biodegrading RDX will be injected into an aerobic RDX plume. The amount of growth substrate required to degrade RDX by this culture is much smaller than would be needed to create and sustain conditions required for anaerobic RDX biodegradation (with or without bioaugmentation), thus reducing the costs and technical complexities of remediating large aerobic RDX plumes. Moreover, the RDX-degrading activity of the bioaugmentation culture is attributed primarily to *xplA* genes located on plasmids (mobile genetic elements), thus providing the potential for transfer of RDX-degrading activity from injected cultures to indigenous microorganisms, which are likely well adapted to site-specific conditions, and thus potentially capable of sustaining and spreading RDX-degrading activity throughout the subsurface.

The Umatilla Chemical Depot (UMCD) was selected as an ideal site for this demonstration. At UMCD, RDX is widespread in an aerobic, highly permeable groundwater aquifer. RDX concentrations range from 2 to 300 µg/L over the ~200 acre plume. This demonstration will be performed in three phases. Phase I consists of field site characterization and laboratory testing to select a suitable bioaugmentation culture and to optimize conditions that facilitate growth, RDX-degrading activity, and cell transport under field conditions at the UMCD. Phase II consists of initial field tests including a forced-gradient cell and tracer transport test to confirm ability to distribute cells at the field scale. Phase III consists of field-scale bioaugmentation demonstration with subsequent push-pull tests to obtain field-scale performance data on bioaugmentation culture transport, viability, and *xplA* gene transfer, as well as RDX degradation rates over time. The demonstration will be conducted in two field plots, one for aerobic bioaugmentation only and the other for sequential evaluation of aerobic

and anaerobic biostimulation. The initial aerobic biostimulation test will provide an independent evaluation of RDX degradation performance under aerobic biostimulation conditions and will serve as the aerobic bioaugmentation control. Although presence of increased biomass from the aerobic test may expedite time to achieve anaerobic conditions during the subsequent anaerobic biostimulation, RDX degradation performance will not be evaluated until the end of the two month aquifer conditioning period at which time the community composition and aquifer geochemistry will be representative of the anaerobic biostimulation treatment and will be suitable for performance comparison to aerobic treatments. RDX degradation rates and mass removed per mass of substrate added for the aerobic bioaugmentation treatment will be compared with non-bioaugmented aerobic and anaerobic biostimulation treatments and will form the basis of a cost-benefit analysis for the innovative aerobic bioaugmentation approach for *in situ* treatment of RDX in groundwater.

1.2 Document Purpose and Organization

Phase I success criteria is defined as selection of bacterial culture combination that can survive, grow and be optimally transported through UMCD site soil and groundwater and reduce RDX concentrations to less than 2.1 µg/L[1].

This memorandum synthesizes laboratory treatability testing methods and results, which meet all Phase I success criteria. We therefore recommend a "go" decision to proceed with project Phases II and III. Laboratory and analytical methods are presented in Section 2, results are presented in Section 3, followed by discussion presented in Section 4. The project schedule, which identifies work sequence and schedule of all project phases and phase subtasks, is included as Attachment 1. The Demonstration Plan, which includes a detailed description of the field demonstration, is being developed in parallel with this memorandum and will be submitted for review by May 28, 2013.

2. METHODS

This section summarizes laboratory testing methodology for the bioaugmentation culture optimization work, cell transport and production optimization, as well as analytical methods.

[1] This is a site-specific quantitative performance objective equal to the remedial action criteria for RDX in groundwater established in the UMCD Explosives Washout Lagoon Groundwater Operable Unit, Record of Decision 1994.

2.1 Bioaugmentation Culture Optimization

The goal of this task was to obtain a bioaugmentation culture that will sustain the highest possible RDX-degrading activity in the spatially variable geochemical conditions in the RDX contaminated aquifer at UMCD. The growth and RDX-degrading activity of each candidate microbial strain (Table 1) was optimized by varying the type and concentration of growth substrate, nitrogen source, and micronutrients. The aquifer at UMCD is aerobic with RDX concentrations between 2 and 300 µg/L and nitrate concentrations between 0.1 and 2 mM. These conditions are likely to support the activity of the *Rhodococcus*, *Gordonia*, and *Williamsia* strains that degrade RDX to NDAB [1-3]. However, RDX degradation is inhibited in some of these strains by nitrate concentrations of ~ 4 mM or higher. Furthermore, the *Rhodococcus* strains have been shown to degrade RDX under microaerophilic conditions but at considerably slower rates than in the presence of high oxygen concentrations [4]. For this reason, and because spatially variable redox conditions may be created in the aquifer following substrate additions (e.g. due to preferential flow of added substrate in the heterogeneous aquifer), we also evaluated *Pseudomonas fluorescens* I-C and *Pseudomonas putida* IIB strains, which are facultative anaerobes that degrade RDX via xenobiotic reductases under microaerophilic to anoxic conditions, and are not inhibited by nitrate [5].

2.1.1 Cell Suspension studies

Optimization of Cell Growth. Cell suspension studies were conducted to identify optimal growth and nitrogen sources that would support maximum biomass yields and which strains to carry forward to subsequent demonstration phases. See Figure 1 for a schematic of the cell suspension task phasing. Individual cultures (Table 1) were grown as cell suspensions in a nitrogen-free basal salts medium (BSM) solution (in g/L: $K_2HPO_4.3H_2O$, 4.25; $NaH_2PO_4.\ H_2O$, 1; $MgSO_4.H_2O$, 0.20; $FeSO_4.7H_2O$, 0.012; $MnSO_4.H_2O$, 0.003; $ZnSO_4.7H_2O$, 0.003; $CoSO_4.7H_2O$, 0.001. pH 7.0). Initially, growth of the strains on 9 different carbon sources, some at 2 or 3 different concentrations, and 4 nitrogen sources (Table 2) was examined. In order to include all of these combinations at one time, a screening test in 96 well microtitre plates (maximum volume 200 µl) was performed. Strains were initially grown for 1 to 2 days in BSM amended with 20 mM succinate and 18 mM ammonium sulfate. Cultures were washed once with BSM and then inoculated into the various media. Microtiter plates were grown statically at 30°C and the growth was followed by measuring the optical density (600 nm) every hour for 48 hours. The carbon and nitrogen combinations were then reduced to 3 or 4 and the growth of the cultures was followed in a larger volume (50 ml) with agitation to provide better aeration than in the microtitre plates. These growth curves were then used to identify the carbon and nitrogen source that provided the maximum growth yield.

Cell Survival and RDX Kinetics. The goal of this task was to identify 1 or more strains with the ability to survive in UMCD groundwater and degrade RDX. Bacterial cultures were grown in BSM containing 50 mM fructose as the carbon source and 18 mM ammonium sulfate as the nitrogen source. Cultures were grown for 1 to 2 days at 30°C and then the cell suspensions were washed once and resuspended in artificial groundwater (AGW; Table 3). Cultures were starved for 24 h at 15°C to reduce residual nitrogen levels and then resuspended to an absorbance of 1.0 (600 nm). The flasks for the pseudomonad cultures were 75% filled and sealed with rubber stoppers to produce lower oxygen conditions (oxygen was not measured). After 24 h, RDX and fructose were added to a final concentration of approximately 5.5 µM (1.2 mg/L) and 1 mM, respectively. Cultures were incubated at 15°C with shaking at 120 rpm. RDX concentrations were determined by HPLC analysis following extraction with an equal volume of dichloromethane and concentration with acetonitrile. Cell viability was measured as optical density (600 nm) and with viable plate counts on 5% PTYG agar plates. Results were used to downselect aerobic RDX-degrading strains and one of the *Pseudomonas* strain.

2.1.2 Microcosm studies

The strains selected based on Task 2.1.1 results were further evaluated in microcosm studies conducted using UMCD sediment and AGW. A bioaugmentation culture was prepared by individually growing and starving strains *Gordonia* KTR9 Kan^{R}, *Rhodococcus jostii* RHA1 pGKT2, and *P. fluorescens* I-C (hence forth referred to as KTR9 Kan^{R}, RHA1 pGKT2, and strain IC) as described in Section 2.1.1. Microcosms consisted of 2 g of UMCD sediment (2 mm sieved) plus 1 ml of AGW. The AGW was amended with RDX at 1.1 mg L^{-1} (5.5 µM) and 1 mM fructose (180 mg L^{-1}) and 1 x 10^{6} cells ml^{-1} of each bacterial culture One set of microcosms was amended with only the AGW + soil + RDX + fructose solution (uninoculated). Microcosms were incubated at 15°C and periodically sacrificed for analysis of RDX concentrations (3 replicate microcosms per time point). One gram of soil was mixed with 10 ml of dichloromethane by vortex mixing for 1 min. The solvent was recovered after centrifugation and subsequently dried under a stream of nitrogen. The extract was then resuspended in 250 µl of acetonitrile and analyzed via HPLC. An additional 3 replicate vials per time point were used to quantify viable cell numbers and the number of copies of 16S rRNA and functional genes via quantitative polymerase chain reaction (qPCR). Colony forming units (CFUs) per mL were determined from agar plate counts on LB + 50 mg L^{-1} kanamycin or cetrimide:nalidixic acid agar following dilution in phosphate buffered saline.

Quantitative PCR was used to estimate the total bacterial population (16S rRNA), strains KTR9 Kan^{R}, RHA1 pGKT2 (*xplA* and *Kan*), and strain IC (*xenB*). DNA was extracted from dried microcosm soil using the MoBio PowerSoil kit with the following changes. After

the addition of the initial lysis solution (C1) samples were incubated for 2 hours at 70°C, followed by 2 rounds of bead beating at 5 m/s for 17 s with a 5 min rest period in between. The remaining steps were as indicated by the manufacturer. Three replicate samples of 0.3 g were extracted from each microcosm and subsequently pooled using Microcon 100 columns. All qPCR amplification reactions were performed using Applied Biosystems' 7900HT Fast Real-time PCR system (Foster City, CA). PCR was carried out in 20 µL reaction volumes in 394-well optically clear plates. Analysis of the kanamycin and *xenB* genes used the SYBR green format and a SYBR Green PCR Master Mix, 300 nM each primer with 0.5 µL template DNA. Thermal cycler conditions were 95°C for 10 min; then 40 cycles of 95°C for 15 s; 60°C for 60 s plus dissociation stage. Analysis of the 16S rRNA and *xplA* genes followed the TaqMan format using the Quantitect PCR Probe Mix, 300 nM each primer, 200 nM probe and 0.5 µL of template DNA Thermal cycler conditions were 95°C for 12 min; then 40 cycles of 95°C for 30 s; 50°C for 60 s; and 72°C for 20 s. Standard curves for each qPCR assay were obtained from serial dilutions of genomic DNA isolated from strain KTR9 Kan^{R} (*xplA* and kan) and strain IC (16S rRNA and *xenB*)

2.1.3 Cell "tagging" for detecting xplA gene transfer

To qualitatively detect the transfer of the *xplA* gene from the inoculated strain KTR9 Kan^{R} to indigenous UMCD microbes, a dual labeling technique was proposed to track the movement of the *xplA*-containing plasmid while retaining the ability to differentiate between inoculated donors and potential *in situ* recipients. Our goal was to "tag" the plasmid pGKT2 of strain KTR9 Kan^{R} with a red fluorescent tracking protein (DS-RedExpress2) and "tag" the chromosome (non-transferable element) of KTR9 Kan^{R} with a separate green fluorescent protein (ZSGreen) [6, 7].

The ZSGreen encoding gene, flanked by ~ 0.6-kb regions specific to the KTR9 Kan^{R} chromosome (non-essential coding sequence, KTR9_2685) was synthesized by Celtek Biosciences, LLC (Nashville, TN, USA), into a pCR2.1 vector (Invitrogen, Carlsbad, CA, USA). The ZSGreen construct containing the KTR9 Kan^{R} flanking regions was liberated from the plasmid by digestion with BamHI (New England BioLabs, Inc., Ipswich, MA, USA) and gel purified with a Wizard SV Gel and PCR Clean-up System Kit (Promega, Madison, WI, USA). The purified ~ 2.0-kb fragment was ligated into a BamHI-cut mobilizable vector, pK18mobsacB and transformed into One Shot Top10 Chemically Competent Escherichia coli cells (Invitrogen) that were selected for on Luria–Bertani (LB) plates with 50 µg ml-1 kanamycin. Recombinant pK18mobsacB was introduced from Top10 E. coli cells into Gordonia sp. KTR9 Kan^{R} based on the conjugation strategy presented by van der Geize et al 2001 [8]. Double crossover recombinant strains with the insertion of the ZSGreen marker from pK18mobsacB into the chromosome of KTR9 were detected by PCR. The ZSGreen gene and various locations of the pK18mobsacB

plasmids were targeted to confirm their presence or, in the case of pK18mobsacB, its absence. The fluorescence of resulting ZSGreen recombinant KTR9 strains was evaluated via visual inspection of colonies and culture media under shortwave UV light and measurement of fluorescent intensity with excitation/emission wavelengths at 480/509.

The KTR9 Kan^R strain tagged with green fluorescent protein was further subjected to attempts to label plasmid pGKT2 with a red fluorescent encoding gene. The DS-RedExpress2 encoding gene, flanked by ~ 0.6-kb regions specific to pGKT2 (KTR9_4820), was synthesized by Celtek Biosciences, LLC (Nashville, TN, USA), into a pCR2.1 vector (Invitrogen, Carlsbad, CA, USA). The DS-RedExpress2 construct containing the KTR9 flanking regions was liberated from the plasmid by digestion with BamHI (New England BioLabs, Inc., Ipswich, MA, USA) and gel purified with a Wizard SV Gel and PCR Clean-up System Kit (Promega, Madison, WI, USA). The purified ~ 2.9-kb fragment was ligated into a BamHI-cut mobilizable vector, pK18mobsacB and transformed into One Shot Top10 Chemically Competent *Escherichia coli* cells (Invitrogen) that were selected for on Luria–Bertani (LB) plates with 50 µg ml^{-1} kanamycin. Recombinant pK18mobsacB was introduced from Top10 *E. coli* cells into *Gordonia* sp. KTR9 Kan^R based on the conjugation strategy presented by van der Geize et al 2001. Double crossover recombinants with the DS-RedExpress2 marker from pK18mobsacB inserted into pGKT2 were detected by PCR. PCR targeting the DS-RedExpress2 construct and various locations of the pK18mobsacB plasmids was used to confirm their presence or, in the case of pK18mobsacB, its absence.

2.2 Cell Transport and Production Optimization

Multiple factors affect bacterial cell transport in groundwater including cell surface properties [9-14], cell growth phase [15, 16], cell density [17], aquifer sediment characteristics, and groundwater chemistry [18-20]. In some instances when cells transport poorly through sediments, selection procedures have been used to obtain adhesion-deficient culture variants that still biodegrade the contaminant of interest but are more readily transported [21]. Alternatively, ionic strength adjustment to modify cell surface charge has been shown to promote cell transport through sediments [22]. This section describes laboratory testing conducted to determine (a) ability of selected strains to transport through UMCD site sediment and (b) optimal production procedures required to grown, concentrate and ship cells during the field demonstration.

2.2.1 Cell adhesion assay

The cell adhesion assay of DeFlaun et al. 1990 [23] was used to evaluate the adhesion of the selected strains to UMCD sediment. UMCD site sediment was air dried, homogenized, and packed loosely into 20 mL syringe barrels and sealed with aluminum

foil. The packed syringes were sterilized by autoclaving on three consecutive days. The strains were grown and starved as described above. Cell supsensions (approximately 10^7 CFU/mL) were added to saturate the sand in the syringes of UMCD sediment and allowed to stabilize for 5 minutes. The columns were then drained and the cells in the effluent were enumerated. The adhesion value was calculated as the percent of the initial cells that were retained within the sand, with a lower percent indicating less adhesion to the UMCD site sediment.

2.2.2 Column Transport Experiments

Column experiments were performed using the selected strain(s) to evaluate cell transport and RDX degradation under dynamic flow conditions in actual UMCD sediments.

Column transport experiments were performed using previously described methods [24-28]. The column setup is illustrated in Figure 2. Three identical columns were wet-packed to a bulk density of ~1.6 g/cm^3 with freshly collected homogenized UMCD sediments. The pore volume (PV) of each of the columns was approximately 200 mL. AGW was pumped into the bottom and exited at the top of the columns at seepage velocities representative of UMCD field conditions. The effluent was directed into a fraction collector and all fraction volumes were recorded. Side ports permitted porewater sampling at various distances along the column as needed. AGW was amended with a nominal concentration of 2.2 µM (or 500 µg/L) RDX to approximate UMCD plume concentrations.

Tracer testing with bromide (100 mg/L) was performed to determine the effective porosity and actual PV of each column, as well as to assure no short-circuiting was occurring. The influent and effluent dissolved oxygen (DO, via Chemetrics colormetric assay) and pH (via probe) were monitored on a regular basis, as were influent and effluent alkalinity (as $CaCO_3$, Method 310.1) and anions (e.g., nitrate, sulfate, etc., via IC, Method 300.0). Influent and effluent RDX was monitored via high performance liquid chromatography (HPLC, see below).

The selected strains were grown in BSM plus fructose (9 g/L, 50 mM) at ~30°C overnight, or until a maximum the culture density was achieved. Cells were then pelleted by centrifugation (20 to 60 minutes, 3400 rpm, 15°C), the growth medium was decanted, and the cells were washed twice with AGW, and then resuspended in AGW. Washed cells were then starved for 48 to 72 hours at 15°C, then pelleted and washed as described above. Final resuspension was done in AGW to achieve the desired injection cell densities for each strain in 1 PV of AGW, based on OD_{600} measurement.

After stable flow conditions were reached and effluent RDX (C) was not significantly different from influent RDX (C_0), cells were injected into the influent end of the column at a flow rate of 12 to 15 mL/min (equivalent to approximately 10 ft/d, or field forced gradient conditions). The column inoculum was continuously stirred during the injection to keep cells in suspension. Once the 1 PV of cells was injected, the influent was switched back to AGW with RDX. The strains tested, cell density for inoculation, and some operational parameters were varied between the column experiments, as summarized in Table 4.

Following inoculation, each column was subjected to several cycles of fructose addition (0.1 mM final concentration, added over 2 PV time period), with periods of no fructose addition in between. RDX-degrading activity was followed over time in each column by measuring RDX concentrations in the influent and effluent. Additionally, one column experiment was conducted such that several fructose addition cycles were completed prior to bioaugmentation to evaluate RDX-degrading activity by the indigenous UMCD microbial community.

RDX was analyzed according to a modified EPA Method 8330. Aqueous samples were passed through a 0.45 µm glass microfiber filter into a 2 ml autosampler glass vial and sealed with a screw-on septa cap. Analysis was performed using a using a Dionex 3000 Ultimate HPLC with a Agilent Zorbax Bonus-RP column (4.6 x 75 mm, 3.5 µm particle dimater), variable wavelength detector (254 nm), and a photodiode array detector collecting peak spectral data. The mobile phase was 40:60 methanol:0.2% (v:v) trifluoroacetic acid in water at a flow rate of 1 ml/min. The column temperature was 33°C. The practical quantitation limit was approximately 10 µg/L.

Measured tracer and cell effluent concentrations (as OD) were plotted vs time to obtain cell breakthrough curves for analysis. Effective porosity, pore water velocity, and dispersitvity were computed from tracer breakthrough curves using the method of moments [29]. Cell retardation factors were also computed using the method of moments assuming that cell attachment to sediment surfaces was a reversible, equilibrium process (similar to the retardation factor used to represent reversible, equilibrium sorption of a solute in the advection-dispersion equation)

2.2.3 Bioaugmentation culture production optimization

Production of a sufficient quantity of bioaugmentation culture for use in field testing requires knowledge of each strain's physiology, as well as an understanding of the conditions that will yield the most viable cells for field testing. In addition, understanding the effects of post-growth processing (e.g., washing, starving, storage) and transportation from the laboratory to the field on cell activity is critical. During this

task, the growth, processing, and shipping procedures for selected cultures are being optimized.

A preliminary cell yield experiment was performed with KTR9 Kan^R and fructose as the carbon substrate (identified as the best growth substrate during the strain selection experiments described above). Cells were grown in a 3-L bioreactor with vigorous stirring to minimize cell clumping and a high aeration rate to support maximum growth rate. Periodic samples were removed to check the optical density (OD). At the end of the experiment, plating on non-selective (R2A) and selective (LB+kanamycin) media was done to measure the viable CFU in the culture. In addition, glucose and sucrose were tested for their ability to support the growth of RHA1 pGKT2, KTR9 Kan^R, and strain IC. These compounds are similar to fructose, but relatively cheaper in cost and also somewhat easier to use during larger scale fermentation operations.

3. RESULTS

3.1 Cell Suspension Studies

A rapid screening of various combinations of carbon and nitrogen sources indicated that citrate, glutamate, benzoate, and glycerol were generally poor carbon sources for most of the 9 RDX-degrading strains (Figure 3). Ethanol (0.5, 1% v:v), succinate (50 mM), fructose (50 mM), and occasionally sucrose (50 mM) produced the highest growth yields for most of the strains (Figure 4), although a long lag period was observed with ethanol (Figure 3). With most strains, the biomass yields after 48 h were similar on 18 mM ammonium, nitrate, ammonium plus nitrate, or 1% (w:v) yeast extract as nitrogen sources (Figures 3, 4). Based on these results, 50 mM fructose or ethanol as carbon sources and 18 mM ammonium as the nitrogen source, or 1% yeast extract as a carbon and nitrogen source were selected for further growth studies using larger culture volumes. Growth yields were highest with yeast extract or fructose/ammonium (Figure 5), while yields with ethanol were strain specific. With most strains, a long lag period was observed before ethanol was used, which indicated the possibility of toxicity. The combination of 50 mM fructose and 18 mM ammonium consistently yielded good growth among the RDX-degrading strains, and so this medium was selected as the optimal growth medium.

All 9 cultures survived for 7 days in AGW at 15°C with very little loss of cell viability (Figure 6). Even though the cultures were grown and starved in the absence of RDX, RDX was rapidly degraded within 1-2 days by strains KTR9 Kan^R, KTR4, 11Y, DN22, and the transconjugant strains, RHA1 pGKT2, *G. polyisoprenivorans* pGKT2, and TW2 pGKT2 (Figure 7). In comparison, RDX was slowly degraded by strains *P. fluorescens* IC and *P. putida* IIB with about 90% and 16% degraded within 7 days, respectively (Figure 7).

However, cell aggregation and adherence to glass surfaces was observed with strains 11Y, DN22, *G. polyisoprenivorans* pGKT2, and TW2 pGKT2 (Figure 8). Since clumping of cells in the AGW could be an indication of possible attachment to aquifer sediments, these strains were not chosen for the bioaugmentation culture. Based on growth yields and RDX kinetics, *Gordonia* sp. KTR9 Kan^R, *Rhodococcus jostii* RHA1 pGKT2, and *P. fluorescens* I-C (hence forth referred to as KTR9 Kan^R, RHA1 pGKT2, and strain IC) were selected for microcosm and column transport studies.

3.2 Microcosm Tests

Viable cell numbers indicate that the target concentrations for each bacterial culture were achieved (Figure 9). The three RDX-degrading bacterial cultures remained viable during the study. Initially, the indigenous microbial population in the UMCD sediments was at significantly lower viable numbers than the inoculated strains. With time, the indigenous population grew but the maximum population size remained significantly lower than the population size observed in the inoculated microcosms (Figure 9). On the LB plus kanamycin agar plates, strains KTR9 Kan^R and RHA1 pGKT2 were individually enumerated due to the cells forming orange and white colonies, respectively. No orange colonies were observed on these plates with samples from the uninoculated microcosms. Water soluble and/or fluorescent pigments produced on the cetrimide:nalidixic acid agar is used to discriminate certain *Pseudomonas* strains. However, samples from the inoculated or uninoculated microcosms led to the growth of similar colony types these agar plates so that strain IC could not be differentiated from the indigenous population. Yet, viable counts from the inoculated microcosms were consistently higher than samples from the uninoculated microcosms, which indicated that strain IC survived in the microcosms (Figure 9). Degradation of RDX was rapid in the inoculated microcosms, with essentially all of the RDX consumed after 1 day (Figure 10). In the uninoculated microcosms RDX loss was significantly slower with a lag period of 4 days before an approximate 15% reduction in the concentration of RDX. In this simple system, it appears that inoculation with RDX-degrading bacteria stimulates the rapid degradation of RDX.

3.3 Cell "tagging" for monitoring xplA gene transfer

The progress of the inoculated strains in the inoculated microcosms was also assessed using qPCR to estimate trends in functional and 16S rRNA gene copy number. In the uninoculated microcosms, the quantity of *xplA*, *xenB,* and kanamycin genes were generally low and stable for 7 days (Figure 11). In comparison, the quantity of 16S rRNA gene copies in these microcosms increased from approximately $1x10^3$ to $1x10^5$ gene copies g^{-1} in 2 days and then remained at this level. These results indicate that growth of

the indigenous population was stimulated by the addition of fructose. However, populations containing the *xplA*, *xenB,* and kanamycin genes that can be found in the inoculated strains were not present in UMCD sediments or were not stimulated. In inoculated microcosms, the quantity of these 4 genes transiently increased by 1 order of magnitude by day 2 and then either remained stable (*xplA* and 16S rRNA) or declined (*xenB* and kanamycin) (Figure 11). In general, the initial gene copy numbers were 2 orders of magnitude higher than quantified in the uninoculated microcosms, which reflected the addition of the bioaugmentation culture to the sediment. The variation in copy number was high for analysis of the xenB and kanamycin genes so that it was difficult to interpret the final data points.

Transfer of the ZSGreen marker into the KTR9 Kan^R chromosome was detected by polymerase chain reaction (PCR) targeting the ZSGreen gene and various locations of the pK18mobsacB plasmid to confirm their presence or, in the case of pK18mobsacB, its absence. Several clones of KTR9 Kan^R tagged with the ZSGreen gene were obtained as shown by PCR products of the expected size for the gene (Figure 12, lanes 1-5). The absence of the vector plasmid, pK18mobsacB was confirmed by a lack of amplification with vector specific primers (Figure 12, lanes 6-10). A green-tagged clone of KTR9 Kan^R was grown in liquid broth and shown to emit green light when exposed to shortwave UV light (Figure 13). Specifically, one clone of green-tagged KTR9 Kan^R emitted green fluorescent light that was approximately 3 times greater than observed by a culture of the wild-type KTR9 Kan^R strain (944 ± 15 vs. 304 ± 16). Attempts to mark the pGKT2 plasmid of this clone with DS-RedExpress 2 were unsuccessful, despite several attempts. Previous attempts to knockout genes on pGKT2 in the wild-type resulted in spontaneous loss of the pGKT2 plasmid. These results suggested that attempts to modify this plasmid with non-essential, non-selectable elements may be difficult [30], and so efforts to label KTR9 Kan^R with differential fluorescent proteins were ended.

3.4 Cell Adhesion Testing

A total of 5 strains were tested using methods described in Section 2.2.1. Preliminary results showed the cells were quite adhesive, with average percent adhesion results ranging from 90-100% (Table 5). However, cell adhesion did not impede cell transport in repacked UMCD sediment columns, as described in Section 3.5 below.

3.5 Column Transport Testing

Column Transport Experiment 1. Cells were readily transported through UMCD aquifer sediment packed in laboratory columns (Figure 14). After inoculation, the fast flow rate was maintained and a distinct breakthrough of KTR9 Kan^R was observed, with approximately 42% of the injected cells recovered in the effluent after approximately 10

PV (Table 6). The breakthrough curve for the conservative bromide tracer was analyzed to determine the effective porosity of the packed sediment (0.34) and the retardation factor for the cells (1.39). This result indicated that transport of this strain would likely be transported well in the UMCD aquifer, as opposed to being strongly retained near the point of injection.

Effluent concentrations of viable cell counts dropped to very low levels (e.g., <0.01% of injected concentrations), indicating that the injected cells were well retained in the UMCD sediment and would likely not be flushed from the soil even under forced gradient conditions. Fructose was added on four separate occassions 3, 8, 23, and 82 days after bioaugmentation. KTR9 Kan^R retained good RDX degradation activity, as rapid decreases in the C/C_o of RDX were observed upon each fructose addition (Figure 14). Furthermore, the ability to degrade RDX was maintained well even with periods of up to 2 months between fructose additions.

Column Transport Experiment 2. Column 2 was operated at expected *in situ* groundwater temperatures (~15°C), and received a combination of three strains. Breakthrough of the comingled inoculum was approximately 40% of the injected cells based on OD_{550} measurements of the effluent (Table 6), but the three strains exhibited different elution from the column based on plate counts of viable cells (Figure 15). Effluent CFU counts dropped to low levels after the main bioaugmentation pulse, indicating that the cells would not likely be flushed under forced gradient flow regimes. Fructose was added on two separate occasions 5 and 38 days after bioaugmentation. The injected cells exhibited good RDX degradation with each fructose addition (Figure 15), although it was not clear which strain(s) were contributing to the observed RDX loss. As with Column Experiment 1, the ability to degrade RDX was maintained well even with long periods with no fructose additions.

Column Transport Experiment 3. Column 3 was operated under slow flow rate conditions (approximately 1 ft/d) for six weeks prior to bioaugmentation. Three injections of fructose did not appear to stimulate any signficant RDX degradation by the indigenous UMCD microbial community (Figure 16). Inoculation of Column Experiment 2 is pending; relevant results will be reported at a later date.

3.6 Bioaugmentation culture production optimization

Glucose and sucrose were tested for their ability to support the growth of all three organisms. These compounds are similar to fructose, but relatively cheaper in cost and also somewhat easier to use during larger scale fermentation operations. However,

neither sugar was able to be utilized by all three strains. Therefore, fermentation optimization proceeded using fructose.

A preliminary cell yield experiment was performed with KTR9 Kan^R. Cells were grown in a 3-L bioreactor with vigorous stirring to minimize cell clumping and a high aeration rate to support maximum growth rate. Fructose was added at concentrations of 15 to 17.5 g/L, and additional ammonium was added as needed. Periodic samples were removed to check the optical density (OD_{600}) and biomass dry weight. The growth curve for KTR9 Kan^R is shown in Figure 17. An OD_{550} of 35 was achieved, which is in the range required for the eventual field inoculums preparation. The yield was calculated to be 0.54 g biomass per g fructose, or 1 x 10^{11} cells per g fructose (assume 1 x 10^{13} g/cell).

At the end of the experiment, plating on non-selective (R2A) and selective (LB+kanamycin) media yielded similar counts of viable CFU in the final culture (6.8 x 10^9 vs. 8.3 x 10^9 CFU/ml, respectively), and indicated that the kanamycin resistance marker was maintained in strain KTR9 Kan^R.

4. DISCUSSION AND "GO" DECISION RECOMMENDATION

The nine RDX-degrading strains investigated in Phase I grew well in a basal salts medium with 50 mM fructose and 18 mM ammonium as carbon and nitrogen sources, respectively. In addition, these 9 strains remained viable and active following 24 h of starvation in AGW. Following amendment of these cultures with 1 mM fructose and about 1 mg L^{-1} RDX, RDX was rapidly degraded in 1 to 2 days by the 4 wild-type and 3 transconjugant actinomycete strains. Interestingly, these strains were able to degrade RDX and remain active in the presence of significantly lower carbon and nitrogen levels than normally used in previous studies [1-3]. Despite these similarities, several of the actinomycete cultures aggregated and also adhered to glass surfaces when incubated in the AGW. This could indicate that these cells may not transport well in the UMCD subsurface, even though all strains produced similar cell adhesion results (Task 2.2.1). The two *Pseudomonas* strains degraded RDX at a slightly slower rate which may have been due to the fact that the cultures were not incubated under strict anaerobic conditions. These two strains optimally degrade RDX under microaerophilic or anaerobic conditions. Based on these results, we decided to examine a mixed inoculum in microcosm and column trials. KTR9 Kan^R and RHA1 pGKT2 were chosen from the set of actinomycetes as they did not aggregate and grow quickly. Strain IC was selected as the facultative anaerobe to be included in the inoculum since it degraded RDX at a faster rate than strain IIB.

UMCD microcosms inoculated with these 3 strains at equal cell densities were very promising. Each strain remained viable in the microcosms despite the presence of the

indigenous population and the low nutrient levels. In addition, degradation of RDX occurred very quickly in the inoculated microcosms with 98% of the RDX removed within 1 day. We did not observe the production of the nitroso intermediates in the microcosms and the method of extraction was unsuitable to detect the denitration intermediate, 2,4-diazabutanal (NDAB). Thus, at this time it in unclear as to which strain or strains in the mixed culture are responsible for the degradation of RDX. This may not be a vital piece of information, since the mixed culture did in fact lower the RDX concentration below the target level of 2 µg ml^{-1}. Furthermore, inoculation of these strains into UMCD sediment columns was also successful with regards to survival and RDX degradation over several months (Task 2.2.2) In uninoculated microcosms, significant RDX loss did not occur until after day 4 and it is uncertain whether abiotic or biotic degradation was being observed. In addition, the qPCR data successfully showed that the inoculated strains were present within the inoculated microcosm treatments and at significantly higher levels than in the uninoculated microcosms. Some issues with variability among replicates were observed with regards to each primer set that will be addressed before the field trial this year.

Previously, we demonstrated the *in vitro* ability of strain KTR9 Kan^R to transfer the *xplAB*-containing plasmid, pGKT2, and as a result, the ability to degrade RDX to other actinomycetes [31]. This observation is important for bioaugmentation with KTR9 Kan^R since it presents a unique opportunity to demonstrate horizontal gene transfer (HGT) of *xplAB* and the ability to degrade RDX *in situ.* It is expected that bioaugmentation with KTR9 Kan^R could have a multiplicative effect on RDX degradation, with KTR9 Kan^R serving as the primary mediator for RDX degradation. However, perhaps more significant is the potential for KTR9 Kan^R to serve as a secondary/tertiary mediator by seeding the *in situ* microbial population, via HGT, with the ability of degrade RDX. Unfortunately, we were unsuccessful in dual labeling KTR9 Kan^R with a green fluorescent protein (chromosome tag) and a red fluorescent protein (plasmid pGKT2). Therefore we will rely on a strain of KTR9 Kan^R genetically modified to contain the kanamycin gene to detect the transfer of the *xplA* gene from the inoculated bacteria to the indigenous microbes. The pGKT2 plasmid of KTR9 Kan^R was modified with a kanamycin resistance gene using an approach similar to insertion of the green and red fluorescent marker genes in Task 2.1.3 [31]. Potential transconjugants will be selected from groundwater samples cultured on minimal medium with RDX as a sole nitrogen source and 50 mg/ml kanamycin or LB plus kanamycin agar plates. Kanamycin resistant colonies will be visually discriminated from KTR9 Kan^R on the basis of colony morphology and putative recipients will be screened via PCR for the presence of *xplAB* and other pGKT2 plasmid sequences.

Following the ESTCP In Progress Review Brief in February, EPA Region 10 indicated that our project would be required to submit a Toxic Substances Control Act (TSCA) Environmental Release Application (TERA) for approval to use genetically modified organisms during *in situ* testing (http://www.epa.gov/oppt/biotech/index.htm). We

have submitted these forms for strains KTR9 Kan^R, containing a kanamycin gene inserted into plasmid pGKT2, and the transconjugant strain of RHA1 containing pGKT2. We are working with HQ EPA to provide additional information as needed and anticipate no impact to our project schedule at this point. If approved, we will move forward with production of RHA1 pGKT2, strain IC, KTR9 Kan^R as the inoculum for inclusion in subsequent field tests. If not approved, we will include KTR9 wild type (without the kanamycin resistance marker) and strain IC in our selected inoculum. Using KTR9 wild type in the inoculums would not permit us to evaluate gene transfer *in situ*; however, it would meet all demonstration performance objectives (Attachment 2). Further, should this technology be adopted and applied at other sites, wild type strains would likely be used so that regulatory hurdles could be avoided. We are hopeful that the TERA application will be approved for this demonstration so that we can evaluate HGT *in situ* but are set to accomplish all project objectives with slightly modified inoculum as a contingency.

Based on the results to date, we have a high degree of confidence that the selected strains can be distributed quite well *in situ* at UMCD. The column trasnsport and retention data indicate that we will not only be able to get the cells to move a reasonable distance from the injection well(s), but also that the retained cells will establish themselves in the aquifer matrix and will not be flushed away when the forced gradient is active. It should be noted that there may be some difference between these column transport experiments and the *in situ* conditions e.g., the larger rocks, which made up a good portion of the aquifer solids, were screened out of the material used for the columns. Hence, obtaining field-scale cell transport data during demonstration Phase II is critical. Our results also reinforce our belief that once the cells are injected, they will maintain their ability to degrade RDX, and will respond to carbon source additions by quickly starting to reduce the *in situ* RDX concentrations. We expect the cells to remain active and able to be stimulated for several months or longer.

Phase I success criteria was defined as selection of bacterial culture combination that can survive, grow and be optimally transported through UMCD site soil and groundwater and reduce RDX concentrations to less than 2.1 µg/L[2].

Based on our Phase I results, which demonstrated (a) KTR9 Kan^R and mixed inoculum can be successfully transported through UMCD soil over laboratory column relevant scales, and (b) KTR9 Kan^R and mixed inoculum can retain RDX degrading activity in site soil following periods of no carbon/growth substrate being provided, we recommend a "go" decision to proceed with field Phases II and III of the demonstration.

[2] This is a site-specific quantitative performance objective equal to the remedial action criteria for RDX in groundwater established in the UMCD Explosives Washout Lagoon Groundwater Operable Unit, Record of Decision 1994.

5. NEXT STEPS

Next steps on this project include the following.

- The TSCA TERA application for injection of genetically modified organisms (GMOs) KTR9 Kan^R and RHA1 pGKT2 was submitted in April and is currently in review by Headquarters EPA. We are in the process of answering the review committee's questions and anticipate go/no-go decision from them by May 30. Because growth and production of wild type KTR9 will be identical to that of KTR9 Kan^R, we are proceeding with cell production optimization work with this GMO strain, as well as RHA1 pGKT2. If we receive a "no-go" on our TSCA TERA application, we will proceed with production of wild type KTR9 and strain IC only. This will not impact our project schedule.
- Complete analysis of the Column 2 breakthrough curve data to determine retardation factors for KTR9 Kan^R, strain IC, and RHA1 pGKT2 in the mixed inoculums.
- Continue to refine qPCR methods for *xenB* and kanamycin resistance gene primers.
- Complete the draft Demonstration Plan including this additional information and submit to ESTCP for review by May 28.

6. REFERENCES

1. Seth-Smith, H.M.B., et al., *Cloning, sequencing, and characterization of the hexahydro-1,3,5-trinitro-1,3,5-triazine degradation gene cluster from Rhodococcus rhodochrous.* Applied and Environmental Microbiology, 2002. **68**: p. 4764-4771.
2. Thompson, K.T., F.H. Crocker, and H.L. Fredrickson, *Mineralization of the cyclic nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine by Gordonia and Williamsia spp.* Applied and Environmental Microbiology, 2005. **71**(8265-8272).
3. Coleman, N.V., D.R. Nelson, and T. Duxbury, *Aerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) as a nitrogen source by a Rhodococcus sp., strain DN22.* Soil Biology & Biochemistry, 1998. **30**(8-9): p. 1159-1167.
4. Fuller, M.E., J. Hawari, and N. Perreault, *Microaerophilic degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by three Rhodococcus strains.* Letters In Applied Microbiology, 2010. **51**(3): p. 313-318.
5. Fuller, M.E., et al., *Transformation of RDX and other energetic compounds by xenobiotic reductases XenA and XenB.* Applied Microbiology and Biotechnology, 2009. **84**(3): p. 535-544.
6. Shaner, N.C., P.A. Steinbach, and R.Y. Tsien, eds. *A guide to choosing fluorescent proteins*. 2005. 905-909.
7. Sørensen, S.J., et al., *Direct detection and quantification of horizontal gene transfer by using flow cytometry and gfp as a reporter gene.* Current Microbiology, 2003. **47**: p. 129-133.
8. van der Geize, R., et al., *Unmarked gene deletion mutagenesis of kstD, encoding 3-ketosteroid D1-dehydrogenase, in Rhodococcus erythropolis SQ1 using sacB as a counter-selectable marker.* FEMS Microbiology Letters, 2001. **205**: p. 197-202.
9. Huysman, F. and W. Verstraete, *Water-facilitated transport of bacteria in unsaturated soil columns: Influence of cell surface hydrophobicity and soil properties.* Soil Biology and Biochemistry, 1993. **25**(1): p. 83-90.
10. Rijnaarts, H., et al., *Bacterial deposition in porous media: effects of cell-coating, substratum hydrophobicity, and electrolyte concentration.* Environmental Science & Technology, 1996. **30**(10): p. 2877-2883.
11. Baygents, J., et al., *Variations of surface charge density in monoclonal bacterial populations: Implications for transport through porous media.* Environmental Science & Technology, 1998. **32**: p. 1596-1603.
12. Bolster, C., et al., *Effect of intra-population variability of the long-distance transport of bacteria.* Ground Water, 2000. **38**(3): p. 370-375.
13. Williams, V. and M. Fletcher, *Pseudomonas fluorescens adhesion and transport through porous media are affected by lipopolysaccharide composition.* Applied and Environmental Microbiology, 1996. **62**(1): p. 100 - 104.

14. Rosenberg, M., D. Gutnick, and E. Rosenberg, *Adherence of bacteria to hydrocarbons--a simple method for measuring cell-surface hydrophobicity.* FEMS Microbiology Letters, 1980. **9**: p. 29-33.
15. Heise, S. and G. Gust, *Influence of the physiological status of bacteria on their transport into permeable sediments.* Marine Ecology Progress Series, 1999. **190**: p. 141-153.
16. Weiss, T.H., et al., *Effect of bacterial cell shape on transport of bacteria in porous media.* Environmental Science & Technology, 1995. **29**(7): p. 1737-1740.
17. Schaefer, C.E., D.R. Lippincott, and R.J. Steffan, *Field-scale evaluation of bioaugmentation dosage for treating chlorinated ethenes.* Ground Water Monitoring and Remediation, 2010. **30**: p. 113-124.
18. Appenzeller, B., et al., *Influence of phosphate on bacterial adhesion onto iron oxyhydroxide in drinking water.* Environmental Science & Technology, 2002. **36**(4): p. 646-652.
19. Johnson, W. and B. Logan, *Enhanced transport of bacteria in porous media by sediment-phase and aqueous-phase natural organic matter.* Water Research, 1996. **30**(4): p. 923-931.
20. Mills, A., et al., *Effect of solution ionic strength and iron coatings on mineral grains on the sorption of bacterial cells to quartz sand.* Applied and Environmental Microbiology, 1994. **60**(9): p. 3300-3306.
21. Streger, S.H., et al., *Enhancing transport of Hydrogenophaga flava ENV735 for bioaugmentation of aquifers contaminated with methyl tert-butyl ether.* Applied and Environmental Microbiology, 2002. **68**(11): p. 5571-5579.
22. Brown, D. and P. Jaffé, *Effects of nonionic surfactants on bacterial transport through porous media.* Environmental Science & Technology, 2001. **35**(19): p. 3877-3883.
23. DeFlaun, M., et al., *Development of an adhesion assay and characterization of an adhesion-deficient mutant of Pseudomonas fluorescens.* Applied and Environmental Microbiology, 1990. **56**(1): p. 112-119.
24. Mailloux, B.J. and M.E. Fuller, *Determination of in situ bacterial growth rates in aquifers and aquifer sediments.* Applied and Environmental Microbiology, 2003. **69**(7): p. 3798-3808.
25. DeFlaun, M.F., et al., *Comparison of methods for monitoring bacterial transport in the subsurface.* Journal of Microbiological Methods, 2001. **47**(2): p. 219-231.
26. Dong, H., et al., *Simultaneous transport of two bacterial strains in intact cores from Oyster, Virginia: Biological effects and numerical modeling.* Applied and Environmental Microbiology, 2002. **68**(5): p. 2120-2132.
27. Fuller, M.E., et al., *Examining bacterial transport in intact cores from Oyster, Virginia: Effect of sedimentary facies type on bacterial breakthrough and retention.* Water Resources Research, 2000. **36**(9): p. 2417-2431.
28. Schaefer, C.E., et al., *Bioaugmentation for chlorinated ethenes using Dehalococcoides sp.: Comparison between batch and column experiments.* Chemosphere, 2009. **75**: p. 141-148.
29. Yu, C., A.W. Warrick, and M.H. Conklin, *A moment method for analyzing breakthrough curves of step inputs.* Water Resources Research, 1999. **35**(11): p. 3567-3572.

30. Indest, K.J., et al., *Functional characterization of pGKT2, a 182 kb plasmid containing the xplAB genes involved in the degradation of RDX by Gordonia sp. KTR9.* Applied and Environmental Microbiology, 2010. **76**(19): p. 6329-6337.
31. Jung, C.M., et al., *Horizontal gene transfer (HGT) as a mechanism of disseminating RDX-degrading activity amont Actinomycete bacteria.* Journal of Applied Microbiology, 2011. **110**: p. 1449-1459.
32. Fuller, M.E., et al., *Transformation of RDX and other energetic compounds by xenobiotic reductases XenA and XenB.* Applied Microbial and Cell Physiology, 2009. **84**: p. 535-544.

TABLES

Table 1. List of all RDX-degrading microbial strains included in the initial screening. Ultimately, strains Gordonia KTR9 KanR, Rhodococcus jostii RHA1 pGKT2, and P. fluorescens I-C selected as the optimal inoculum for bioaugmentation at UMCD.

Bacterial Strain	**Genotype**	**Reference**
Gordonia sp. KTR9	*xplA*$^{+}$, Kan^{R}	[2, 31]
Williamsia sp. KTR4	*xplA*$^{+}$	[2]
Rhodococcus sp. DN22	*xplA*$^{+}$	[3]
Rhodococcus rhodochrous 11Y	*xplA*$^{+}$	[1]
Rhodococcus jostii RHA1 [pGKT2]	*xplA*$^{+}$, Kan^{R}	[31]
Gordonia polyisoprenivorans DSMZ 44302 [pGKT2]	*xplA*$^{+}$, Kan^{R}	[31]
Nocardia sp. TW2 [pGKT2]	*xplA*$^{+}$, Kan^{R}	[31]
Pseudomonas fluorescens IC	*xen*B^{+}	[32]
Pseudomonas putida IIB	*xen*A^{+}	[32]

Table 2. List of carbon sources and nitrogen sources screened in initial tests.

Concentrations used in mM are shown in parentheses. Fructose and ammonium sulfate were ideal growth and nitrogen substrates, respectively, for most strains and thus selected for inclusion in batch and microcosm testing.

Carbon substrate (mM)	Nitrogen substrate (mM)
Glycerol (10)	Ammonium sulfate (18)
Glycerol (20)	Ammonium sulfate (18)
Glycerol (50)	Ammonium sulfate (18)
Fructose (50)	Ammonium sulfate (18)
Galactose (50)	Ammonium sulfate (18)
Sucrose (50)	Ammonium sulfate (18)
Succinate (20)	Ammonium sulfate (18)
Succinate (50)	Ammonium sulfate (18)
0.5%(v:v) Ethanol (85.8)	Ammonium sulfate (18)
0.75%(v:v) Ethanol (128.6)	Ammonium sulfate (18)
1% (v:v) Ethanol (171.5)	Ammonium sulfate (18)
Glutamate (50)	Ammonium sulfate (18)
Citrate (50)	Ammonium sulfate (18)
Benzoate (50)	Ammonium sulfate (18)
Succinate (20)	Sodium nitrate (18)
Succinate (20)	Ammonium nitrate (18)
Succinate (20)	0.1 % (w/v) Yeast extract
Succinate (20)	0.5 % (w/v) Yeast extract
Succinate (20)	1 % (w/v) Yeast extract

Table 3. Composition of the artificial groundwater (AGW) used during all treatability study experiments.

Component	UMCD groundwater (mg/L)	AGW (mg/L)
pH	8	8
Nitrate as NO_3	48	46
Sulfate as SO_4	24	25
Alkalinity as CO_3	92	89
Na^+	20	54
Ca^{2+j}	35	28
Cl^-	21	78
Mg^{2+}	16	16
K^+	3.5	3.5
NH_4^+	<0.5	3.5

Table 4. Experimental conditions for the column transport experiments.

Parameter	Experiment 1	Experiment 2	Experiment 3
Strain(s)	KTR9 Kan^R	KTR9 Kan^R, RHA1 pGKT2, *Ps. fluorescens* I-C	KTR9 Kan^R, RHA1 pGKT2, *Ps. fluorescens* I-C
Temperature (°C)	22	15	15
Injection cell density (cells/mL)	~$1x10^9$	~$1x10^8$ each strain	~$1x10^8$ each strain
Injection volume (mL)	182	190	190
Flow rate (mL/min) -high (=forced gradient) -low (=seepage velocity)	 1.15 0.15	 1.15 0.15	 1.15 0.15
Fructose concentration (mM), periodic addition	0.1	0.1	0.1
Current elapsed time (d)	140	100	40

Table 5. Summary of adhesion assay results for the various strains screened.

	%Adhesion		
	AVG	**SD**	**n**
Starved cells			
Gordonia KTR9-#52	99		1
Arthrobacter RHA1-T	100	0	2
Ps. fluorescens I-C	94	1	2
Unstarved cells			
Gordonia KTR9-#52	100		1
Nocardia TW2-T	100	0	4
Arthrobacter RHA1-T	99	1	4
Gordonia polyisoprenivorans-T	98	1	8
Gordonia KTR9	100	0	8
Rhodococcus DN22	92	10	9
Rhodococcus 11Y	100	0	11
Ps. putida II-B	93	12	6
Ps. fluorescens I-C	96	4	5

"T" designates transconjugate strains

Table 6. Summary of key column transport experiment results.

Parameter	Experiment 1	Experiment 2
Strain(s)	KTR9 Kan^R	KTR9 Kan^R, RHA1 pGKT2, *Ps. fluorescens* I-C
Recovery of cells in effluent (% of injected OD)	42	40
Cell retardation factor	1.39	pending data analysis
Average RDX degradation rate with fructose (mg/L/d)	1.51 (n=4)	1.66 (n=2)
Cells in column effluent after 60 days (CFU/mL)	$\sim10^3$	$\sim10^2$

FIGURES

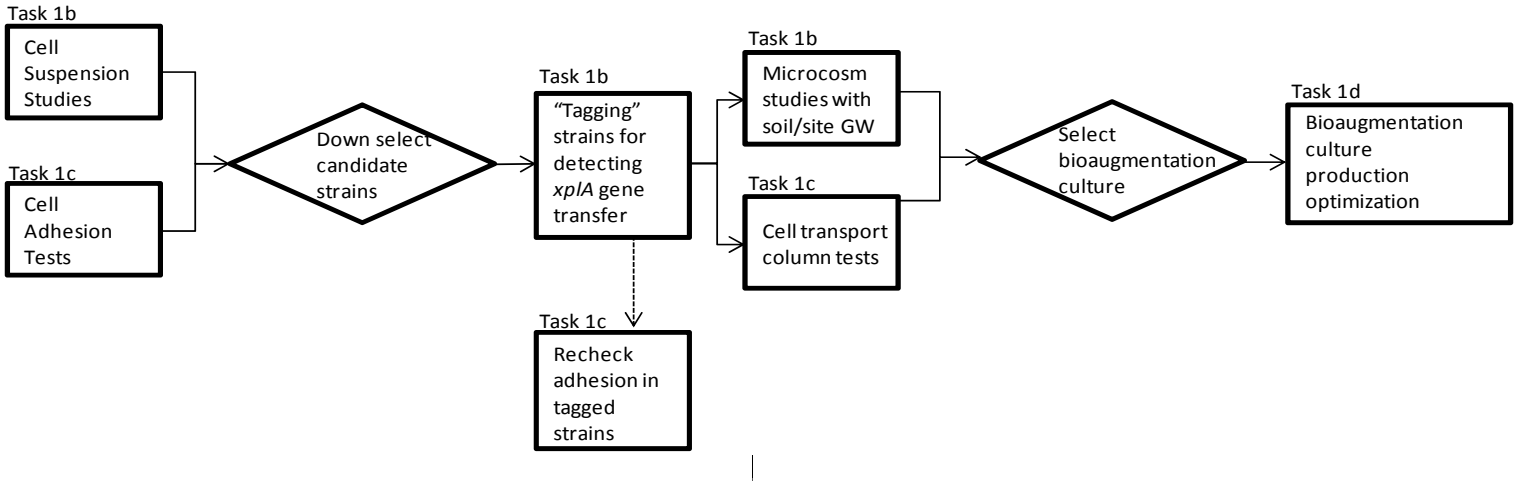


Figure 1. Schematic showing cell suspension and microcosm study phasing

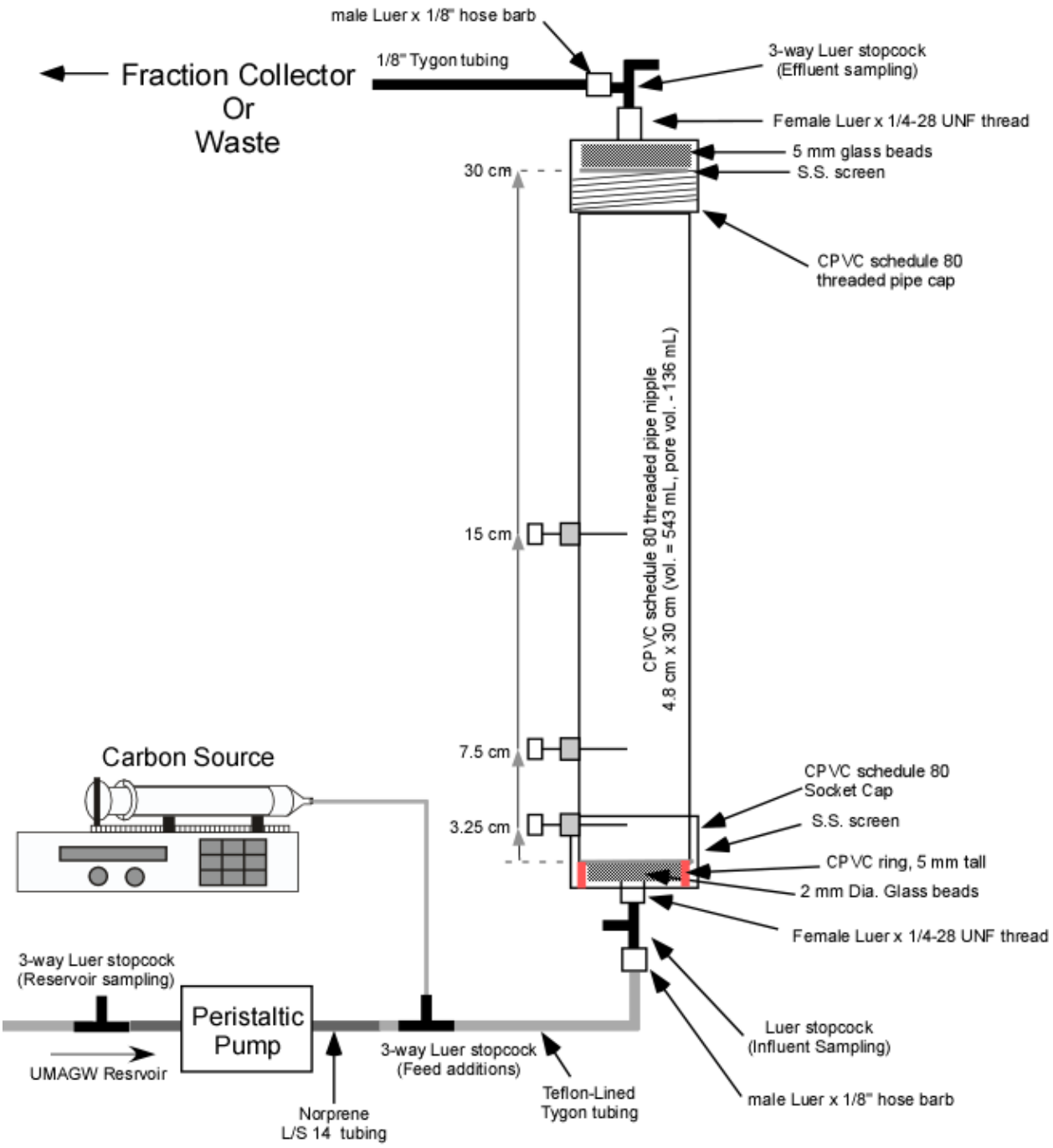


Figure 2. Illustration of the column experiment setup.

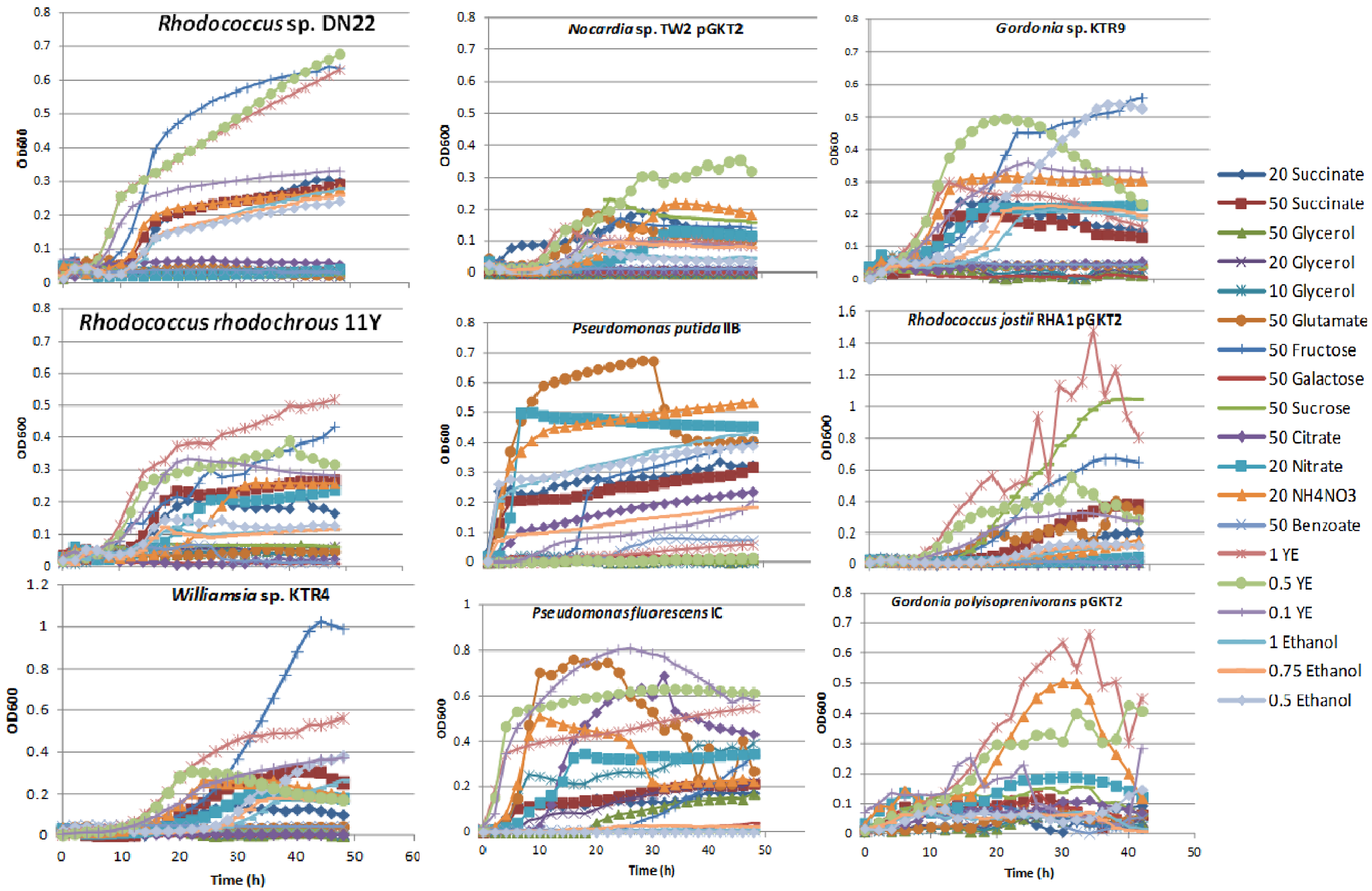
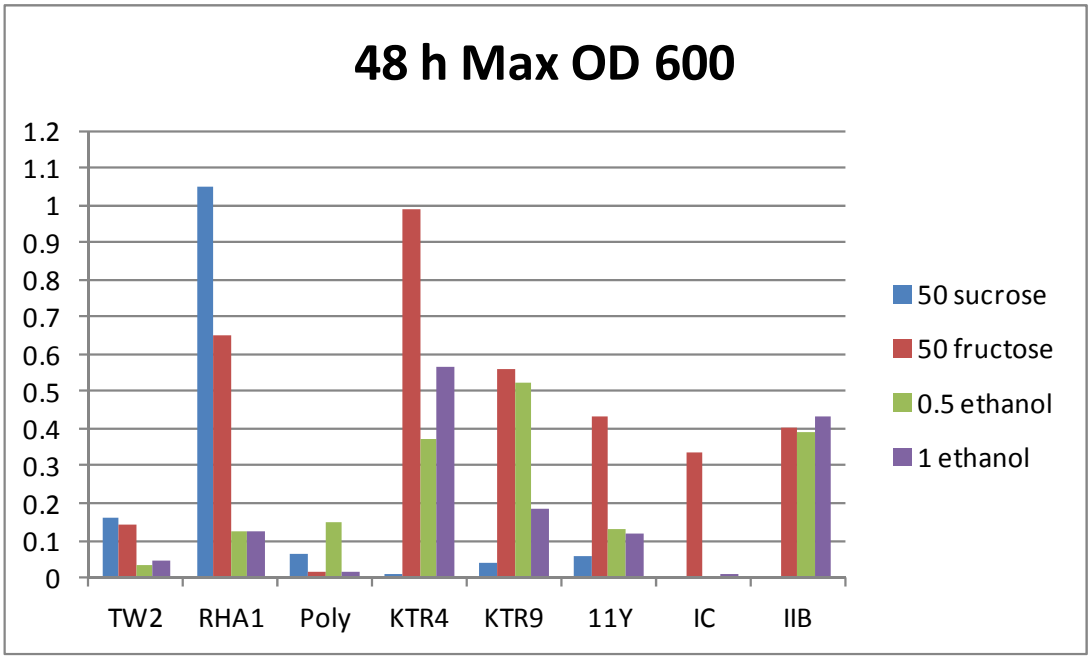


Figure 3. Growth curves of RDX-degrading bacterial strains on various carbon and nitrogen sources.
Initial screening was performed in 96 well microtiter plates with a 0.2 ml culture volume. Carbon/nitrogen sources are indicated, along with respective mM concentrations, to right of plots.



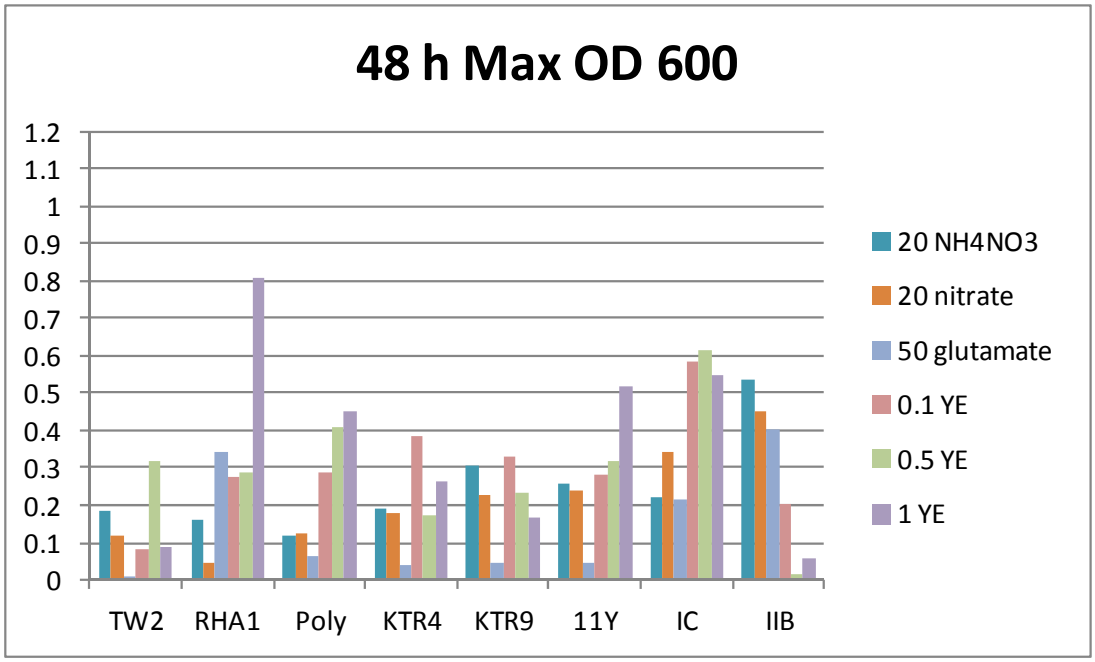


Figure 4. Maximum growth yields of RDX-degrading bacterial strains on select carbon and nitrogen sources during initial screening.
Growth yields were determined at 48 h from measured optical density values (n=3).

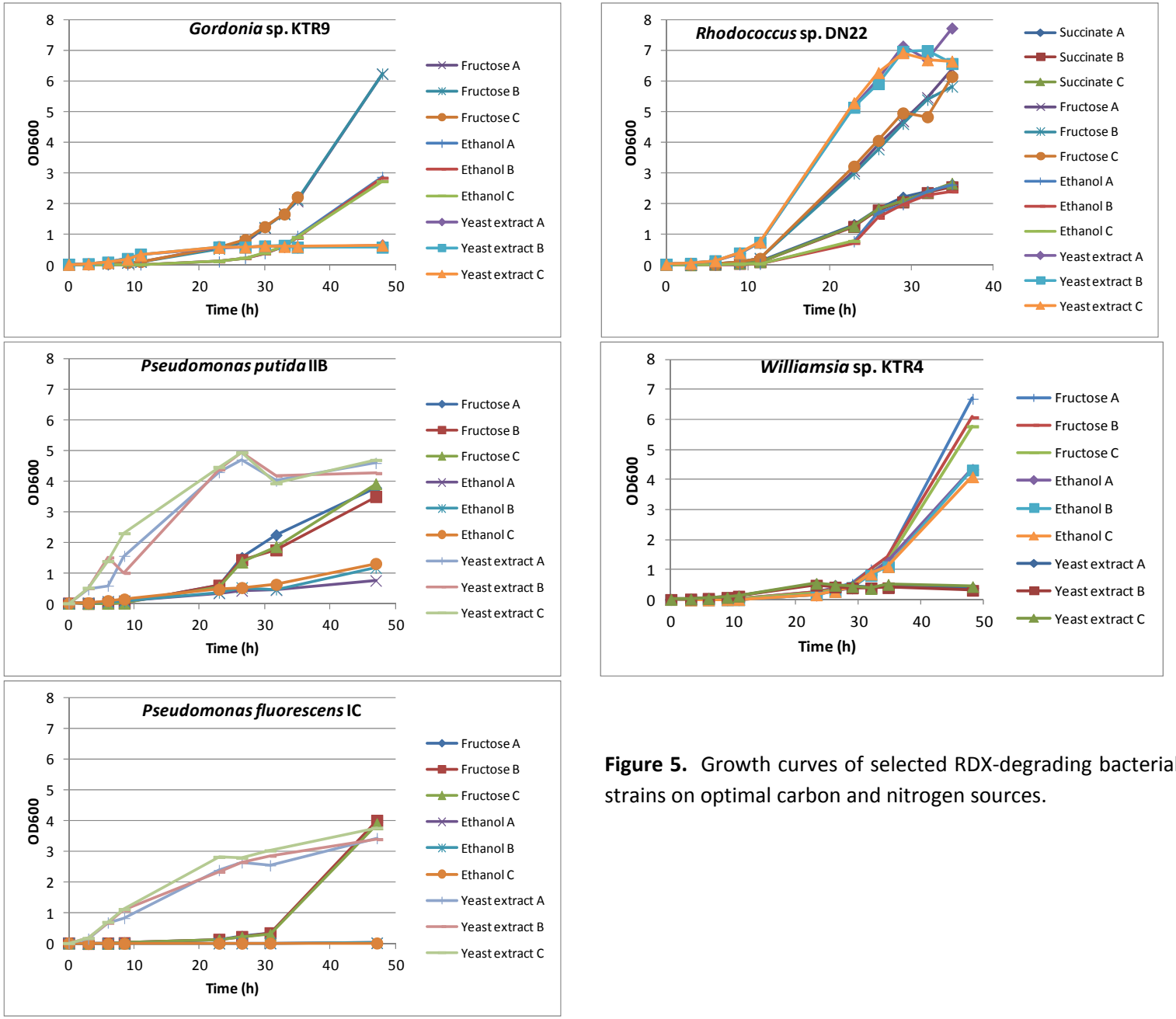
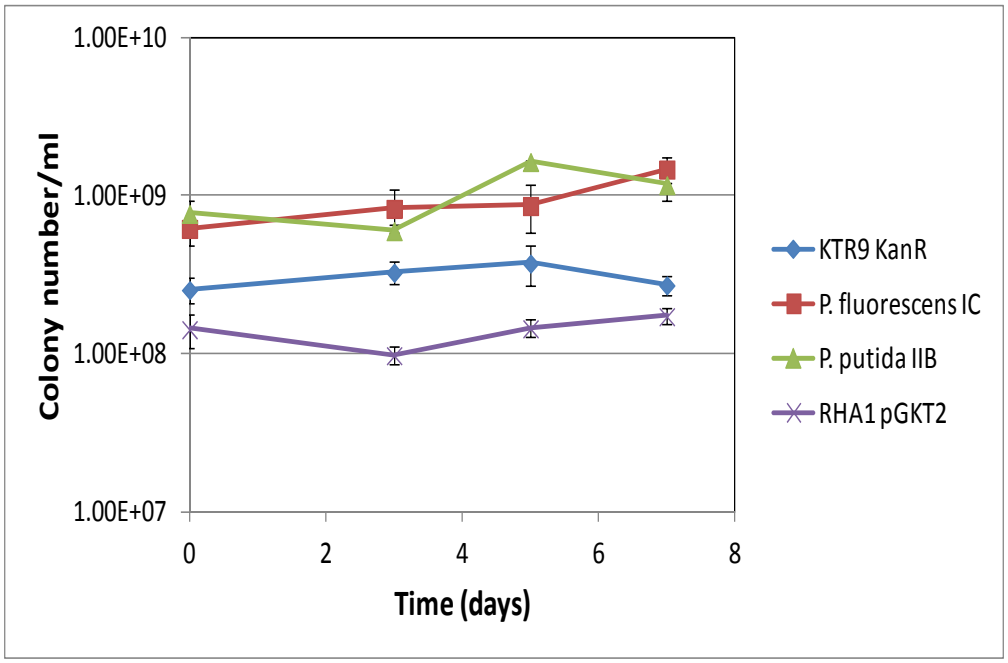


Figure 5. Growth curves of selected RDX-degrading bacterial strains on optimal carbon and nitrogen sources.



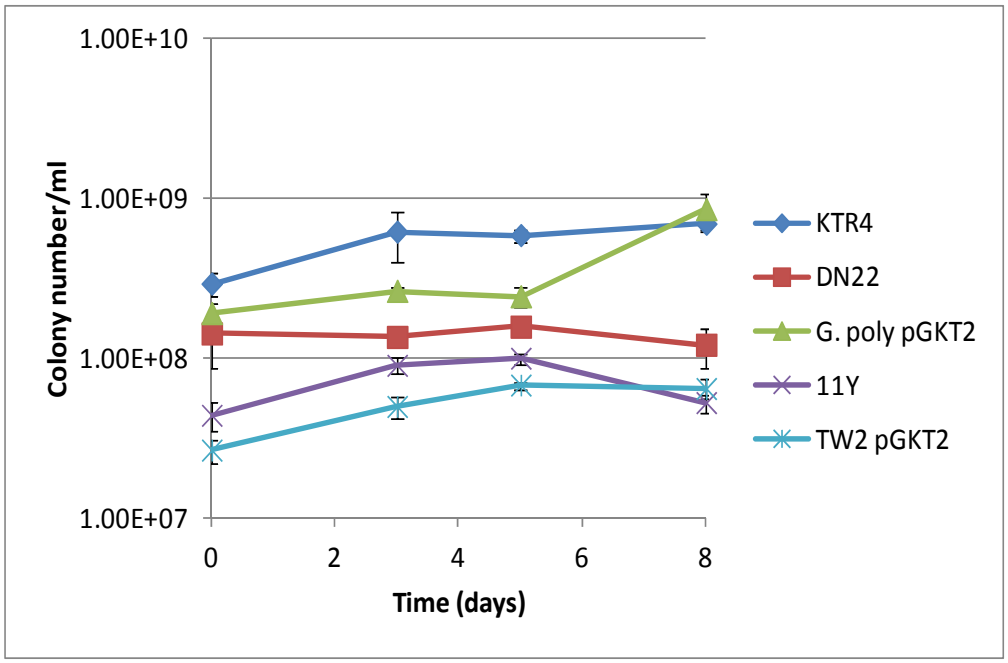
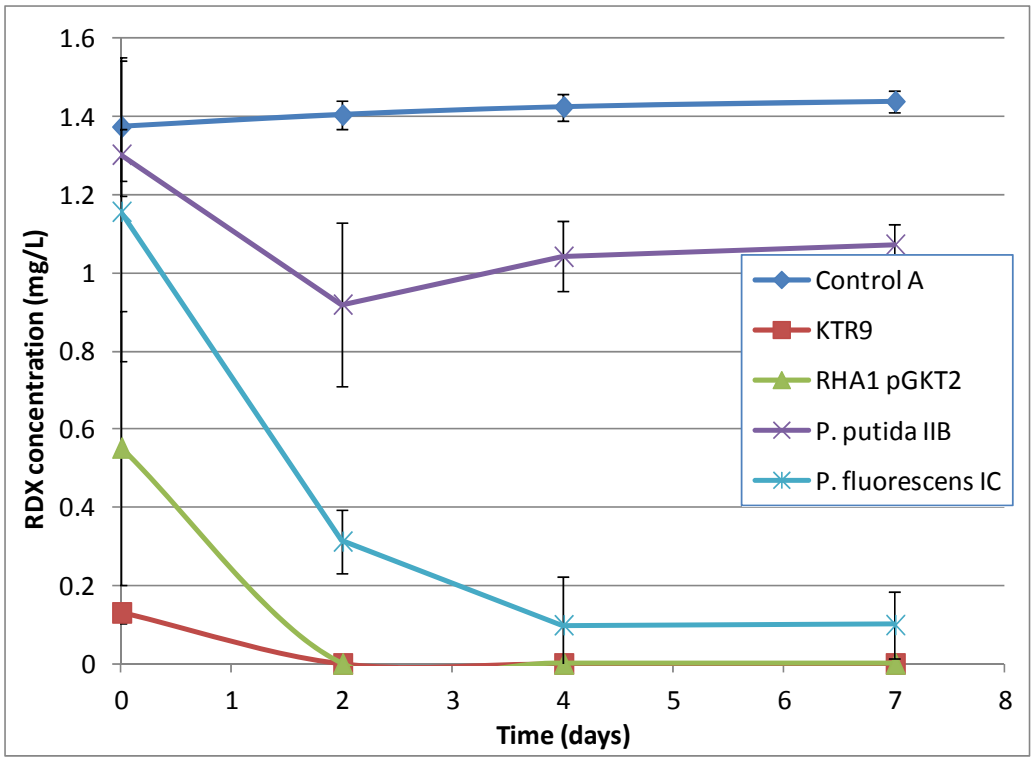


Figure 6. Survival of RDX-degrading strains in artificial groundwater.
Fructose (10 mM) and RDX (1.3 mg/L) were added to solution. Cultures were starved for 24 h in AGW without a carbon or nitrogen source.



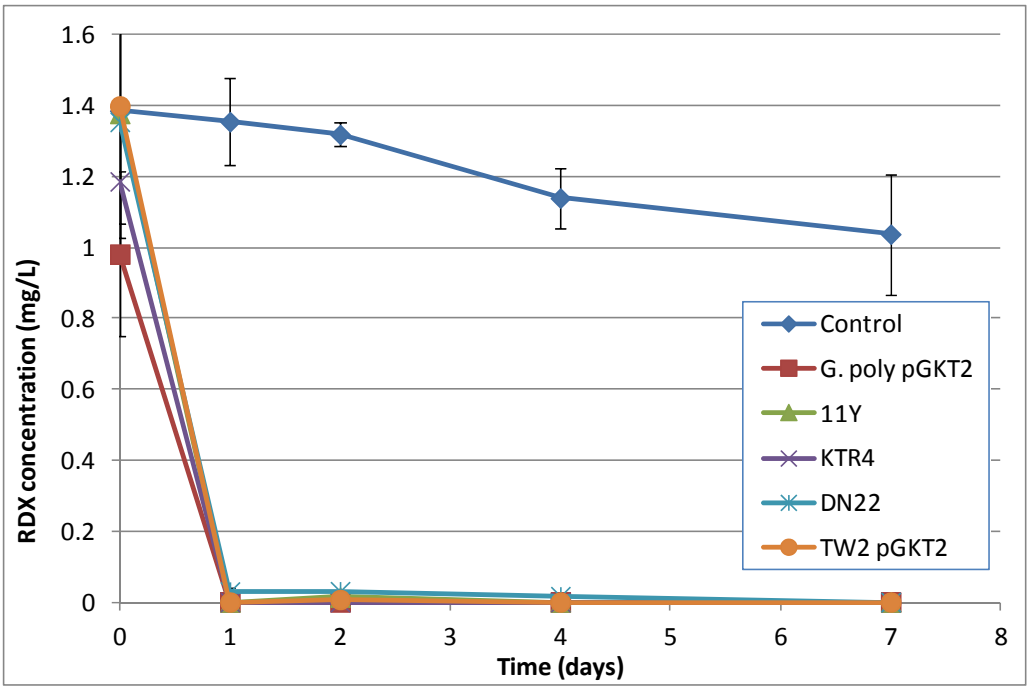


Figure 7. Degradation of RDX by RDX-degrading strains in artificial groundwater. Fructose (10 mM) and RDX (1.3 mg/L) were added to solution. Cultures were starved for 24 h in AGW without a carbon or nitrogen source.

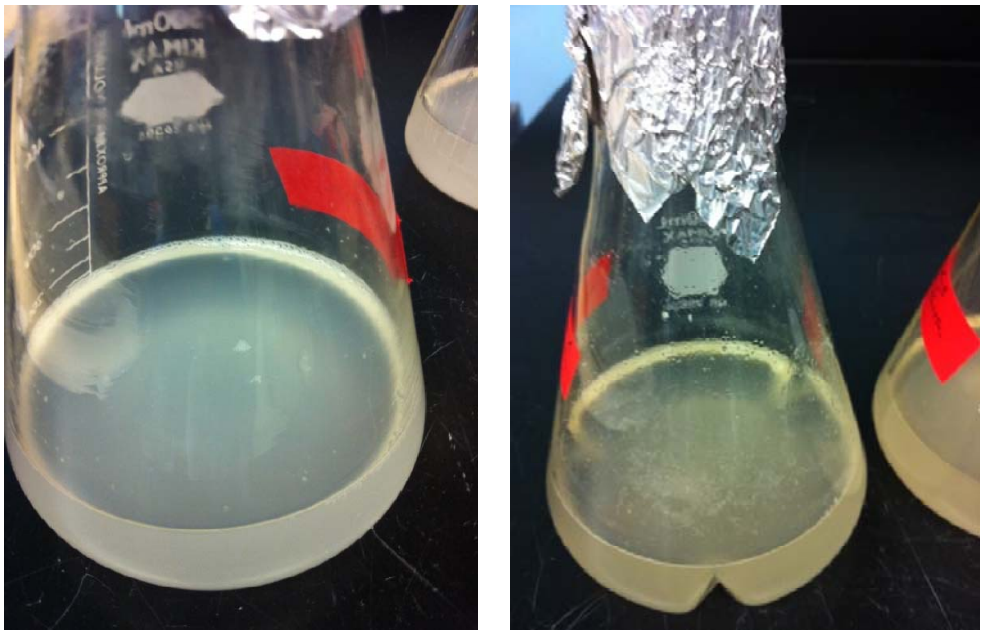
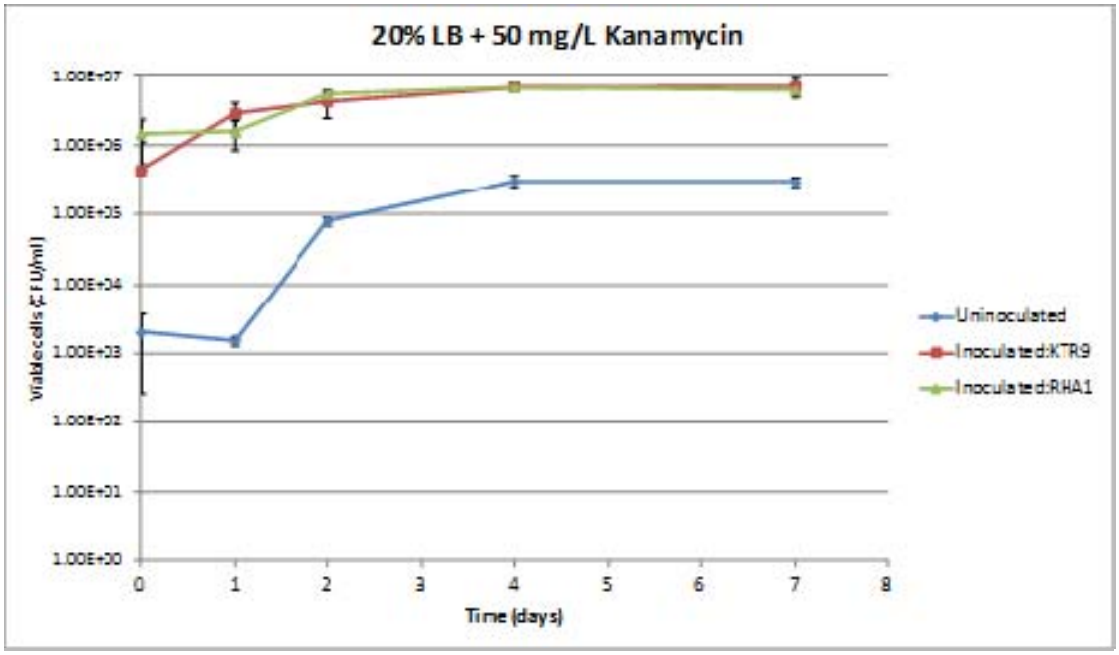


Figure 8. Growth physiology of select RDX-degrading strains in AGW amended with fructose and RDX.
Williamsia sp. KTR4 (A) grew homogeneously while G. polyisoprenivorans pGKT2 cells (B) aggregated.



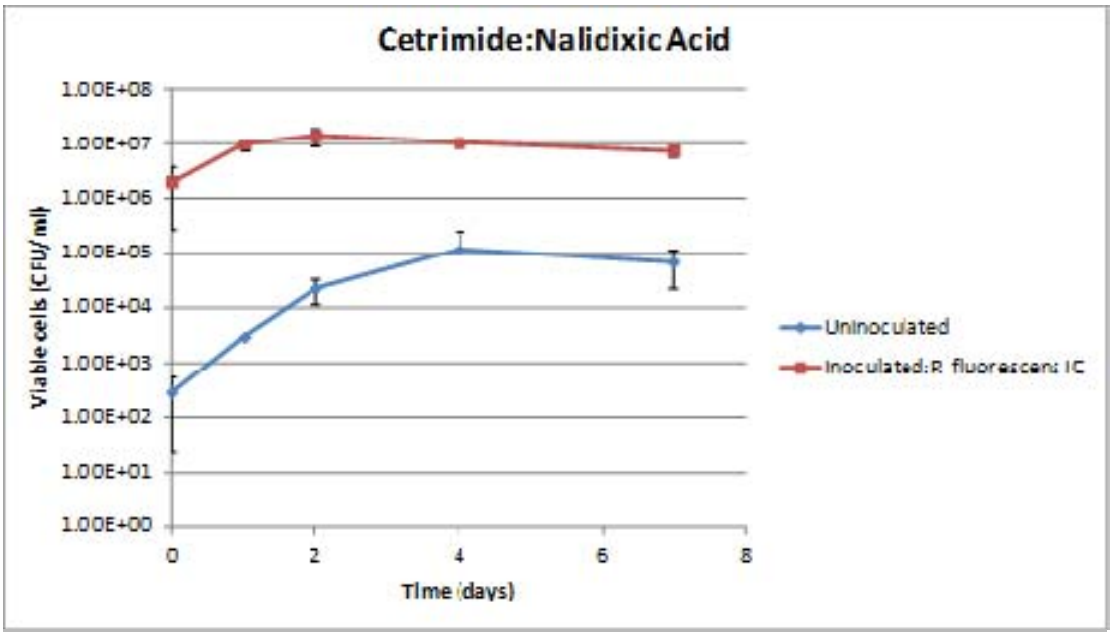


Figure 9. Bacterial viable cell numbers during microcosm on 20% LB + 50 mg/L kanamycin agar plates (A) and cetrimide:nalidixic acid agar plates (B) from uninoculated and inoculated UMCD microcosms. Bacterial counts from the uninoculated microcosms are total bacterial counts on both types of media. Bacterial counts from the inoculated microcosms represent the individual strains: KTR9 Kan^R, RHA1 pGKT2, or IC as indicated in each panel.

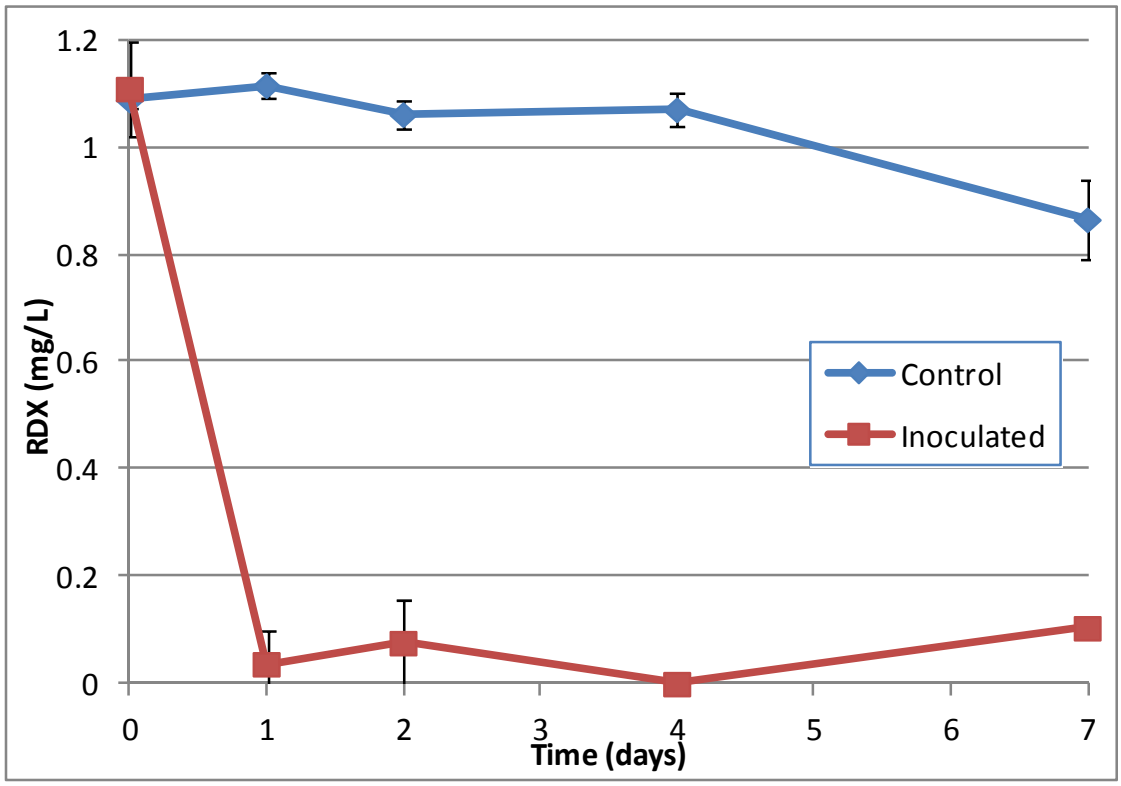


Figure10. RDX concentrations in UMCD microcosms
in both uninoculated (control) and inoculated with strains KTR9 Kan^R, RHA1 pGKT2, and *P. fluorescens* I-C.

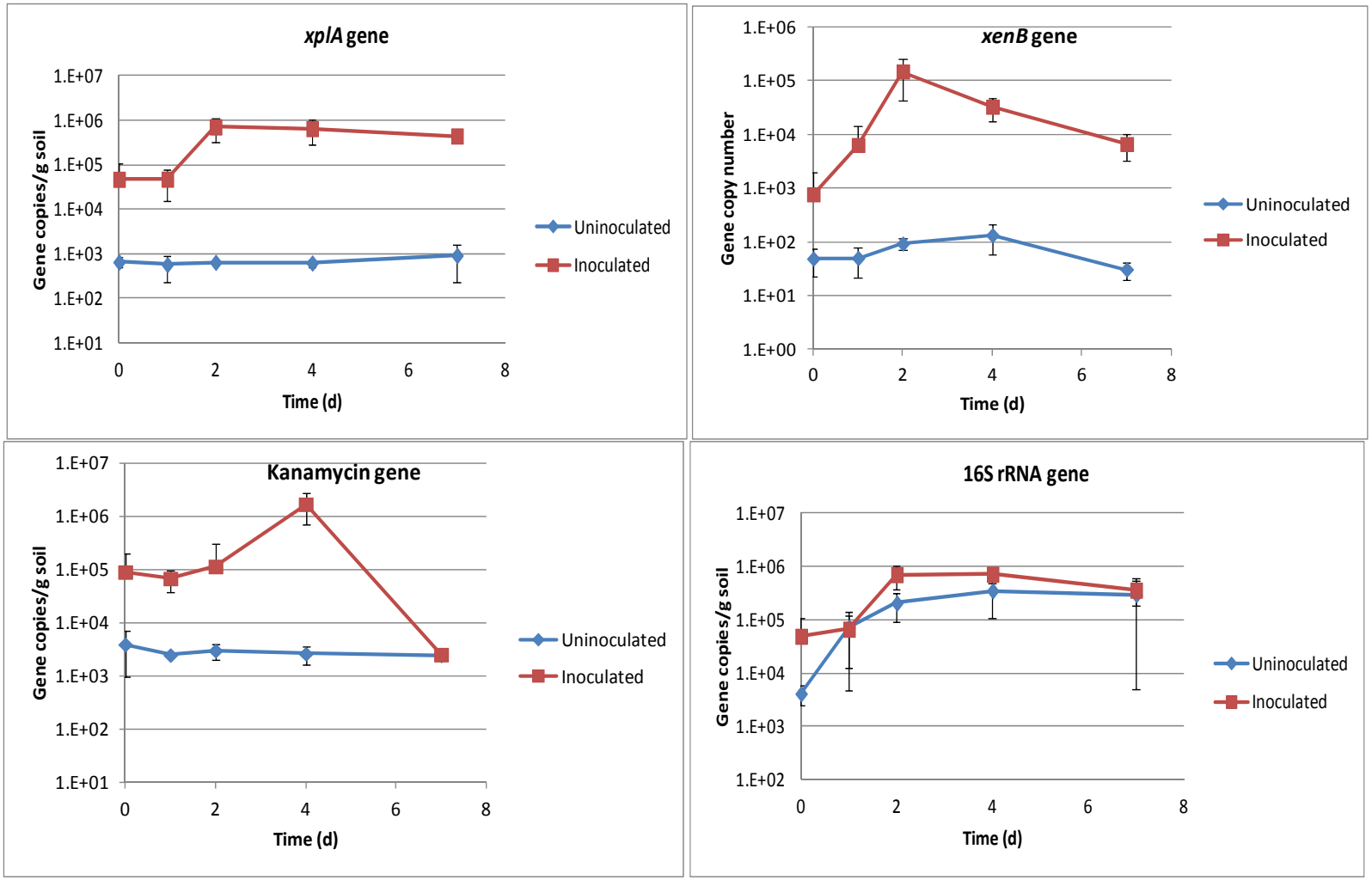


Figure 11. Quantitation of *xplA* (A), *xenB* (B), kanamycin (C), and 16S rRNA (D) genes in microcosms both uninoculated (control) and microcosms inoculated with strains KTR9 Kan^{R}, RHA1 pGKT2, and *P. fluorescens* I-C. The quantity of *xenB* gene has not been normalized to copy number/g soil due to some non-specific binding.

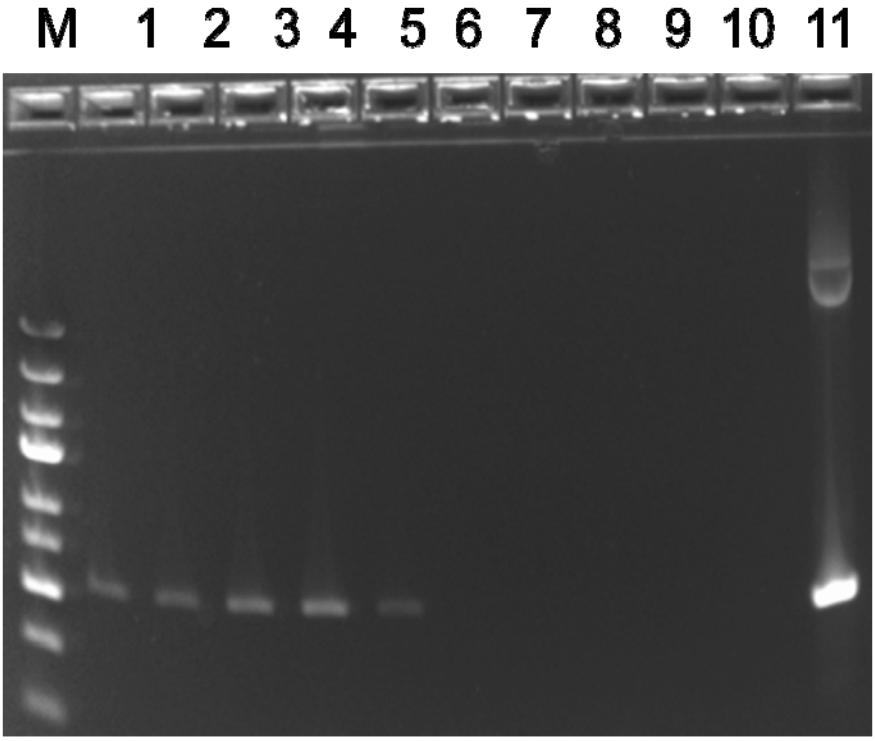


Figure 12. Gel electrophoresis of PCR confirmation of KTR9 ZSGreen transcongucates Lanes 1-5: ZSGreen (GFP) positive KTR9 clones; Lanes 6-10: pK18mobsacB negative; Lanes 11: positive control for pK18mobsacB plasmid.

Figure 13. Visual shortwave UV light exposure of KTR9 wildtype and KTR9 ZSGreen transconjugant illustrating fluorescence.

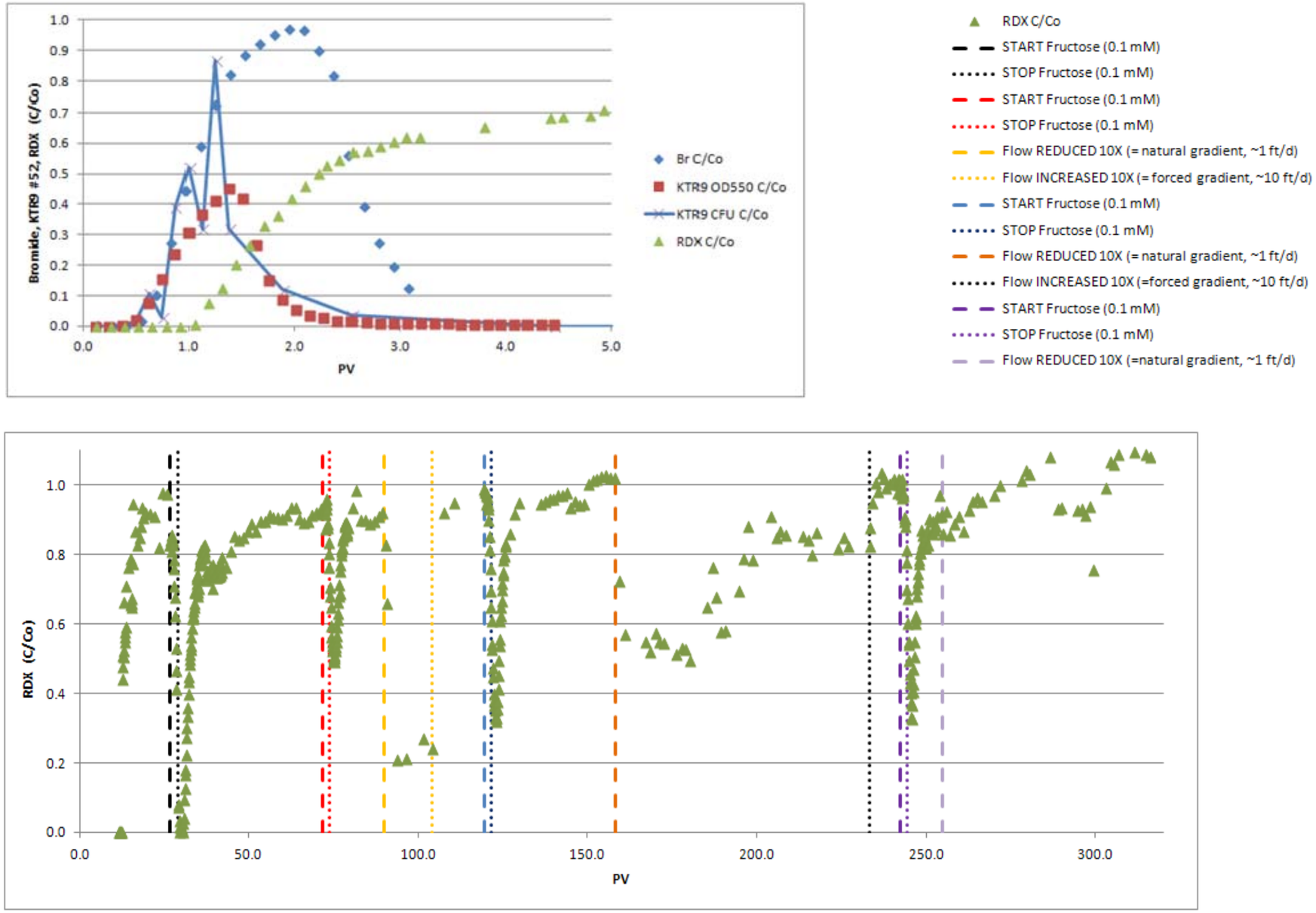


Figure 14. Breakthrough curves for bromide, cells, and RDX for Column Experiment 1.
The initial breakthrough results and the results of the entire experiment are shown, where C is the measured tracer or cell concentration in the column effluent; Co is the influent tracer or cell concentration.

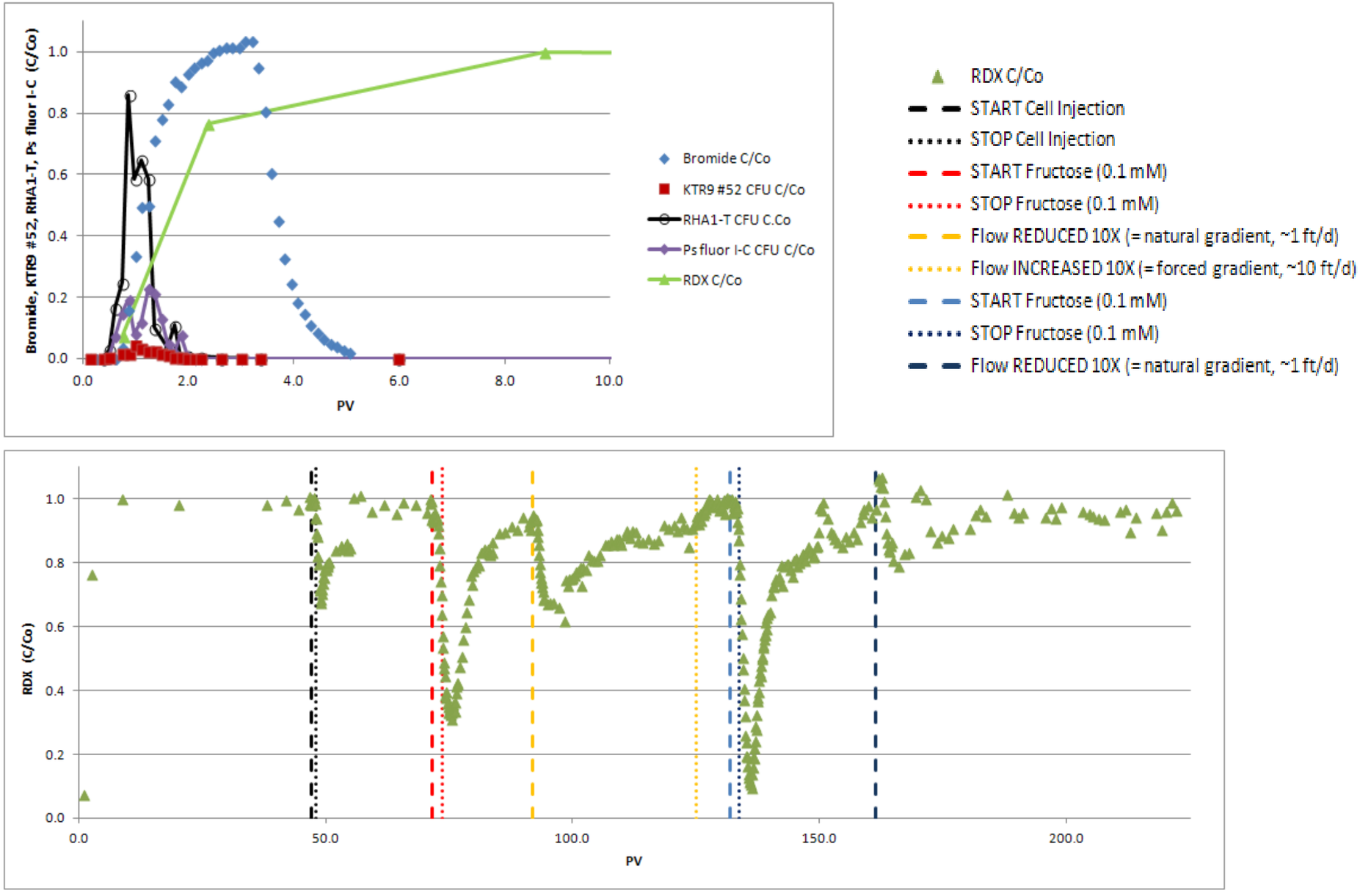


Figure15. Breakthrough curves for bromide, cells, and RDX for Column Experiment 2.
The initial breakthrough results and the results of the entire experiment are shown, where C is the measured tracer or cell concentration in the column effluent; Co is the influent tracer or cell concentration.

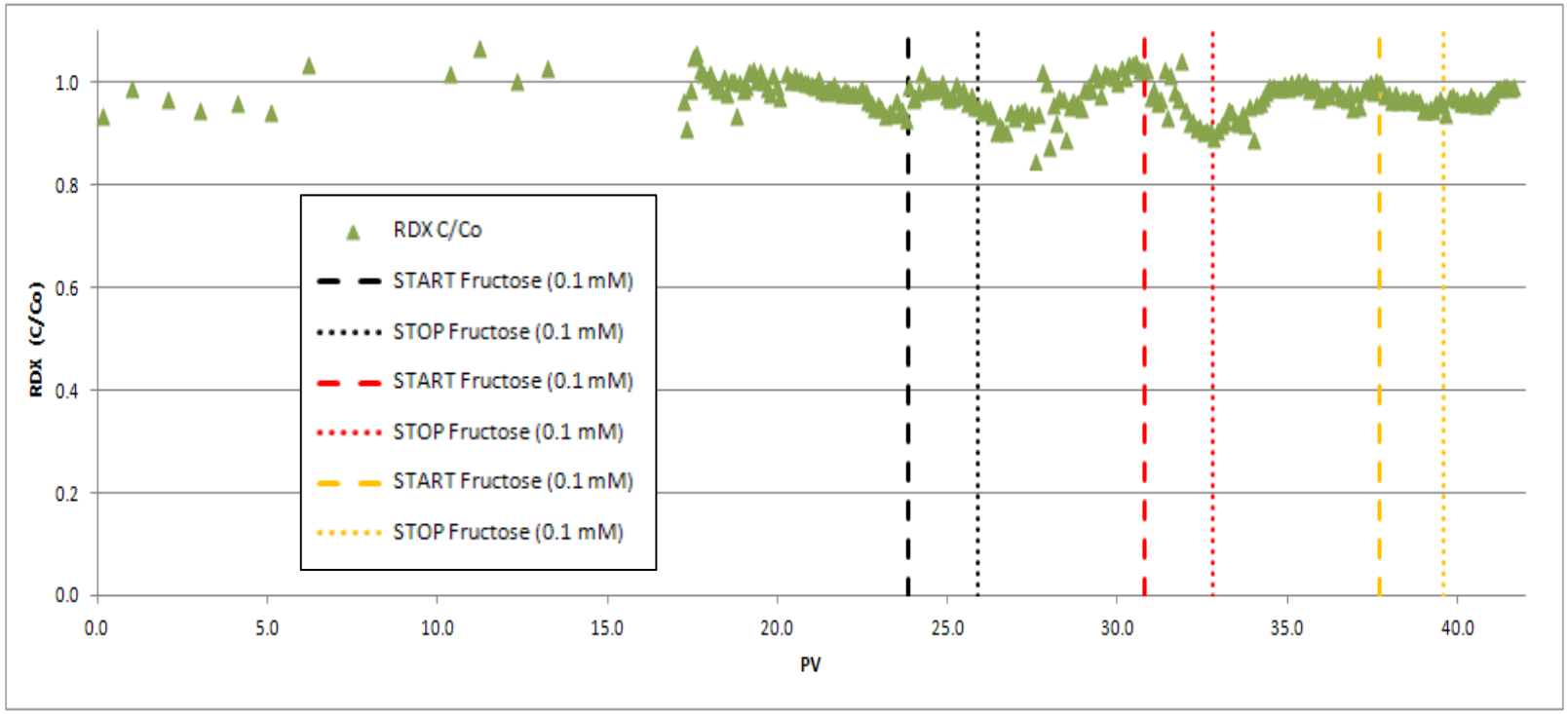


Figure16. Breakthrough curves for bromide and RDX for Column Experiment 3, where C is the measured tracer or cell concentration in the column effluent; Co is the influent tracer or cell concentration. Column Experiment 3 has not yet been inoculated.

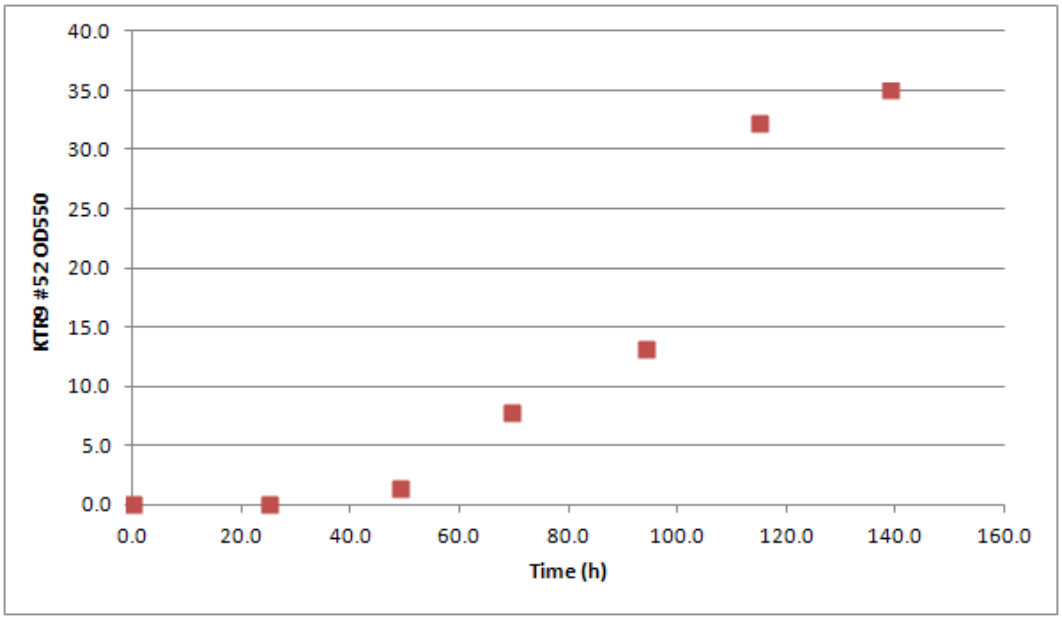
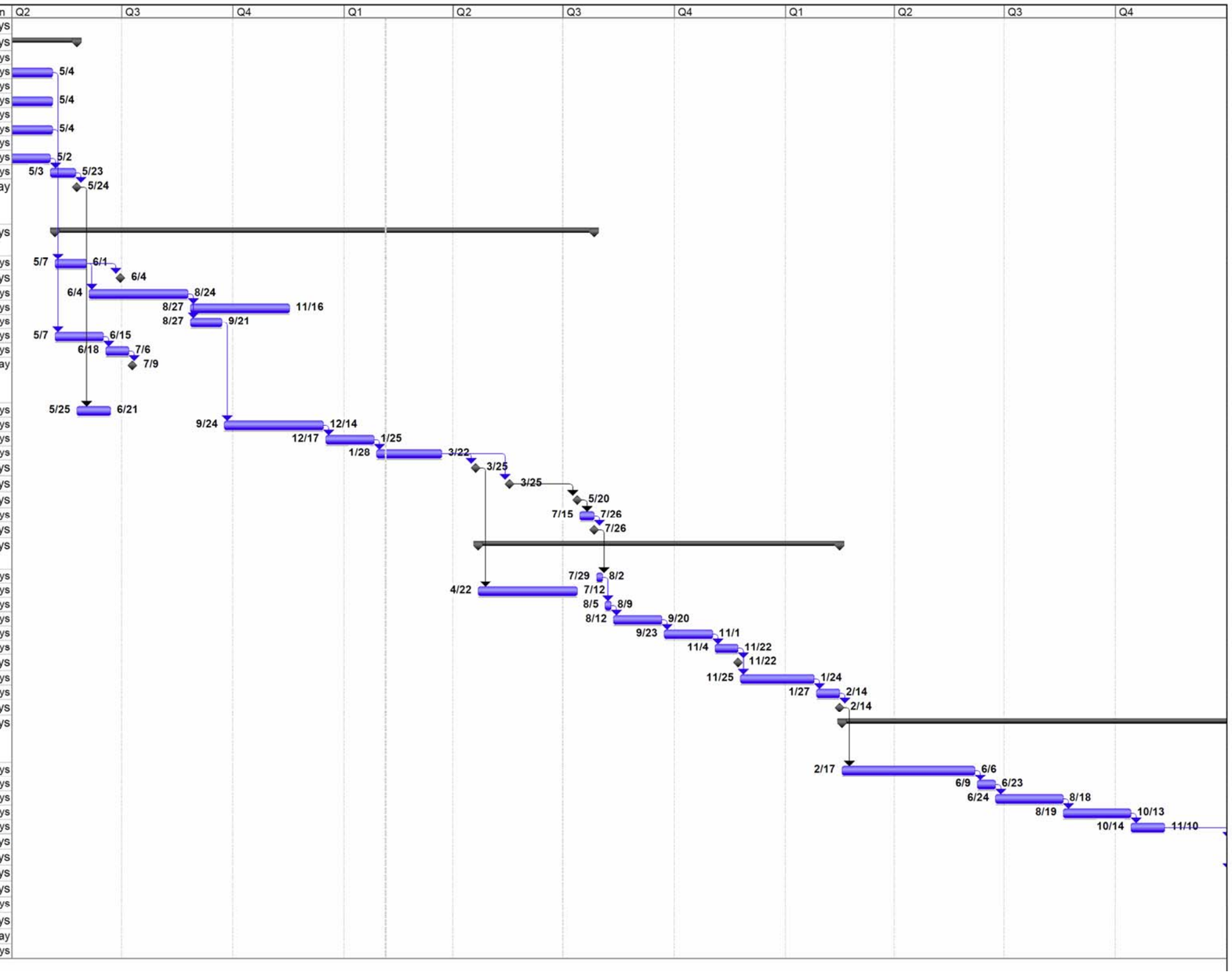


Figure17. KTR9 optical density versus time during initial bench-scale reactor growth yield experiment.

ATTACHMENT 1

Project Schedule

ID	Task Name	Start	Finish	Duration
3	**Project Funding Received**	Thu 3/1/12	Thu 3/1/12	0 days
4	**Start of Project**	Thu 3/1/12	Thu 5/24/12	61 days
5	a. SOW Development for SHAW	Thu 3/1/12	Fri 3/9/12	7 days
6	Contract Development/ Award for SHAW	Mon 3/12/12	Fri 5/4/12	40 days
7	b. SOW Development for OSU	Thu 3/1/12	Fri 3/9/12	7 days
8	Contract Development/ Award for OSU	Mon 3/12/12	Fri 5/4/12	40 days
9	c. SOW Development for Drillers	Thu 3/1/12	Fri 3/9/12	7 days
10	Contract Development/Award for Drillers	Mon 3/12/12	Fri 5/4/12	40 days
11	d. MIPR to ERDC from NWS	Thu 3/1/12	Wed 3/7/12	5 days
12	Draft Phase I Laboratory Studies Work Plan. Part 1: Culture	Thu 3/1/12	Wed 5/2/12	45 days
13	ESTCP Review, discussion, complete revisions	Thu 5/3/12	Wed 5/23/12	15 days
14	MS 1: Phase I Laboratory Studies Work Plan. Part 1: Culture Optimization and Cell Adhesion Assays	Thu 5/24/12	Thu 5/24/12	1 day
15	**Phase I: Site characterization and bioaugmentation culture optimization**	Mon 5/7/12	Fri 7/26/13	320 days
16	Task 1a. Site characterization	Mon 5/7/12	Fri 6/1/12	20 days
17	MS2: Site characterization memorandum	Mon 6/4/12	Fri 6/29/12	20 days
18	Task 1b. Bioaugmentation culture optimization (pure culture	Mon 6/4/12	Fri 8/24/12	60 days
19	Task 1b. Bioaugmentation culture optimization (site soil/slun	Mon 8/27/12	Fri 11/16/12	60 days
20	Midpoint of microcosms - informs culture selection for colum	Mon 8/27/12	Fri 9/21/12	20 days
21	Draft Phase I Laboratory Studies Work Plan. Part 2: Column	Mon 5/7/12	Fri 6/15/12	30 days
22	ESTCP Review, discussion, complete revisions	Mon 6/18/12	Fri 7/6/12	15 days
23	MS3: Phase I Laboratory Studies Work Plan. Part 2: Column Transport Tests and Culture Production Optimization	Mon 7/9/12	Mon 7/9/12	1 day
24	Task 1c. Cell transport optimization (adhesion assays)	Fri 5/25/12	Thu 6/21/12	20 days
25	Task 1c. Cell transport optimization (column transport)	Mon 9/24/12	Fri 12/14/12	60 days
26	Midpoint of cell transport column - confirms culture selection	Mon 12/17/12	Fri 1/25/13	30 days
27	Task 1d. Culture production optimization	Mon 1/28/13	Fri 3/22/13	40 days
28	MS4: Phase I Memo (treatability study report)	Mon 3/25/13	Fri 4/19/13	20 days
29	MS5: Draft Field Demonstration Work Plan	Mon 3/25/13	Fri 5/17/13	40 days
30	MS6: Final Field Demonstration Work Plan	Mon 5/20/13	Fri 7/12/13	40 days
31	Field Demonstration - Mob Prep	Mon 7/15/13	Fri 7/26/13	10 days
32	MS7: Phase II Field Demonstration Start	Fri 7/26/13	Fri 7/26/13	0 days
33	**Phase II: Demonstrate field-scale transport of RDX-degrading culture and perform treatment**	Mon 4/22/13	Fri 2/14/14	215 days
34	Task 2a. Determine RDX retardation factors	Mon 7/29/13	Fri 8/2/13	5 days
35	Task 2b. Culture production for field transport tests	Mon 4/22/13	Fri 7/12/13	60 days
36	Task 2c. Field-scale culture transport test	Mon 8/5/13	Fri 8/9/13	5 days
37	Laboratory turnaround time (8330 and molecular)	Mon 8/12/13	Fri 9/20/13	30 days
38	Data analysis	Mon 9/23/13	Fri 11/1/13	30 days
39	Phase II Draft Memo	Mon 11/4/13	Fri 11/22/13	15 days
40	MS8: Phase II Memo	Fri 11/22/13	Fri 11/22/13	0 days
41	Update and Finalize Demonstration Work Plan for Phase III	Mon 11/25/13	Fri 1/24/14	45 days
42	Phase III Mob Prep	Mon 1/27/14	Fri 2/14/14	15 days
43	MS9: Phase III Field Demonstration Start	Fri 2/14/14	Fri 2/14/14	0 days
44	**Phase III: Demonstrate transport of aerobic RDX degrading bioaugmentation culture, RDX degrading activity and xplA gene transfer to indigenous**	Mon 2/17/14	Tue 5/12/15	322 days
45	Task 3a. Field-scale culture production, aquifer conditioning	Mon 2/17/14	Fri 6/6/14	80 days
46	Task 3b. (1) Triplicate push-pull tests for determination of in	Mon 6/9/14	Mon 6/23/14	11 days
47	Task 3b. (2) Triplicate push-pull tests for determination of in	Tue 6/24/14	Mon 8/18/14	40 days
48	Laboratory turnaround time	Tue 8/19/14	Mon 10/13/14	40 days
49	Data analysis	Tue 10/14/14	Mon 11/10/14	20 days
50	MS10: Draft Final Technical Report	Tue 11/11/14	Mon 1/5/15	40 days
51	MS11: Final Technical Report	Tue 1/6/15	Mon 3/16/15	50 days
52	MS12: Draft Final Cost and Performance Report	Tue 11/11/14	Mon 1/5/15	40 days
53	MS13: Final Cost and Performance Report	Tue 1/6/15	Mon 3/16/15	50 days
54	MS14: Draft Guidance Document	Tue 3/17/15	Mon 5/11/15	40 days
55	MS15: Final Guidance Document	Tue 3/17/15	Mon 5/11/15	40 days
56	Final Project Debrief Conference Call	Tue 5/12/15	Tue 5/12/15	1 day
57	Project Complete	Mon 5/11/15	Mon 5/11/15	0 days



APPENDIX C

PHASE II RESULTS MEMORANDUM

Bioaugmentation for Aerobic Bioremediation of RDX-Contaminated Groundwater

ESTCP PROJECT ER-201207
Phase II Results Memorandum

Prepared by:

Mandy Michalsen, PI
Seattle District, U.S. Army Corps of Engineers

Fiona Crocker, Karl Indest, Carina Jung
U.S. Army Engineer Research Development Center

Paul Hatzinger, Mark Fuller
CB&I Federal Services

Jack Istok
Oregon State University

December 10, 2013

TABLE OF CONTENTS

1. INTRODUCTION 2
1.1 Project Background 2
1.2 Document Purpose and Organization 3
2. METHODS 3
2.1 Growth and Shipment of Bioaugmentation Culture to Demonstration Site 3
2.2 Longer-Term Culture Viability and RDX Degrading Activity Assessment 4
2.3 Preparing the Bioaugmentation Culture for Injection 5
2.4 Cell Transport Test Format and Sampling Plan 5
2.5 Microbial Characterization Methods 6
2.6 Analtyical Methods 7
2.7 Permitting and Decontamination of Field Equipment 7
3. RESULTS 8
4. DISCUSSION 10
5. RECOMMENDATIONS FOR PHASE III 12
6. REFERENCES 12

LIST OF TABLES

Table 1. Composition of the artificial groundwater (AGW) 14
Table 2. Gene copy number quantified in cultures prepared for shipment 14

LIST OF FIGURES

Figure 2.1. Photographs of benchtop (left) and 750-L (right) bioreactors. 16
Figure 2.2. Photographs of the 20-L keg used for culture starvation and shipping. 17
Figure 3.1. Change in relative culture optical densities over time at 4°C and 37°C. 19
Figure 3.2. Change in viable cell densities over time at 4°C and 37°C. 20
Figure 3.3. Change in OD-normalized RDX degradation activity overtime. 21
Figure 3.4. Growth of KTR9 KanR, RHA1 pGKT2 and groundwater bacteria on LB + kanamycin agar (a) and UMCD groundwater bacteria prior to inoculation on 5% PTYG agar (b). 22
Figure 3.5. Growth of strain I-C on Pseudomonas isolation agar plates showing active fluorescence (a) and UMCD groundwater from well DW2 at 2 h with very few colonies that are fluorescent (b). 23
Figure 3.6. Viable plate counts of KTR9 KanR (a) and RHA1 pGKT2 (b) on LB+kanamycin agar plates and heterotrophic groundwater bacteria plus inoculum strains on 5% PTYG agar plates (c). 24
Figure 3.7. Quantitative PCR analysis of UMCD groundwater samples from 3 wells targeting the 16S rRNA genes (a), *xplA* gene (b), Kanamycin gene (c), and *xenB* gene (d). 25
Figure 3.8. Breakthrough curves for tracer, KTR9 KanR and RHA1 pGKT2 cell counts, and *xplA* gene copy numbers in wells DW-2 (a) and 4-106 (b). 26

ATTACHMENT 1 Current Project Schedule

ATTACHMENT 2 Toxic Substance Control Act Exerimental Release Application

ACRONYM LIST

RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine
UMCD	Umatilla Chemical Depot
AGW	Umatilla artificial groundwater
CFU	colony forming unit
qPCR	quantitative polymerase chain reaction
BSM	basal salts medium
mL	milliliter
°C	degrees Celsius
g	gram
L	liter
µg	microgram
PV	pore volume
OD_{600}, OD_{550}	optical density at 600 nm or 550 nm, respectively
DO	dissolved oxygen

1. INTRODUCTION

1.1 Project Background

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a common contaminant in soils and groundwater at military sites worldwide. RDX can be mobile and persistent in groundwater under the aerobic conditions present in many aquifers and thus tends to form large, dilute plumes. Although multiple studies have demonstrated *in situ* RDX biodegradation under anaerobic conditions, creating and maintaining anaerobic conditions across large areas is costly and technically challenging. This project will demonstrate an innovative application of bioaugmentation to enhance RDX biodegradation in contaminated groundwater under aerobic conditions. The project will provide field data to support both a technical evaluation and a cost-benefit analysis of this approach.

During this ESTCP demonstration, a mixed microbial culture capable of aerobically biodegrading RDX will be injected into an aerobic RDX plume. The amount of growth substrate required to degrade RDX by this culture is much smaller than would be needed to create and sustain conditions required for anaerobic RDX biodegradation (with or without bioaugmentation), thus reducing the costs and technical complexities of remediating large aerobic RDX plumes. Moreover, the RDX-degrading activity of the bioaugmentation culture is attributed primarily to *xplA* genes located on plasmids (mobile genetic elements), thus providing the potential for transfer of RDX-degrading activity from injected cultures to indigenous microorganisms, which are likely well adapted to site-specific conditions, and thus potentially capable of sustaining and spreading RDX-degrading activity throughout the subsurface.

The Umatilla Chemical Depot (UMCD) was selected as an ideal site for this demonstration. At UMCD, RDX is widespread in an aerobic, highly permeable groundwater aquifer. RDX concentrations range from 2 to 300 µg/L over the ~200 acre plume. This demonstration will be performed in three phases. Phase I consisted of field site characterization and laboratory testing to select a suitable bioaugmentation culture and to optimize conditions that facilitate growth, RDX-degrading activity, and cell transport under field conditions at the UMCD. Phase II consists of initial field tests including a forced-gradient cell and tracer transport test to confirm ability to distribute cells at the field scale. Phase III will consist of a field-scale bioaugmentation demonstration with subsequent push-pull tests to obtain field-scale performance data on bioaugmentation culture transport, viability, and RDX degradation rates over time. The demonstration will be conducted in two field plots, one for aerobic bioaugmentation only and the other for sequential evaluation of aerobic and anaerobic biostimulation. The initial aerobic biostimulation test will provide an independent

evaluation of RDX degradation performance under aerobic biostimulation conditions and will serve as the aerobic bioaugmentation control. Although presence of increased biomass from the aerobic test may expedite time to achieve anaerobic conditions during the subsequent anaerobic biostimulation, RDX degradation performance will not be evaluated until the end of the two month aquifer conditioning period at which time the community composition and aquifer geochemistry will be representative of the anaerobic biostimulation treatment and will be suitable for performance comparison to aerobic treatments. RDX degradation rates and mass removed per mass of substrate added for the aerobic bioaugmentation treatment will be compared with non-bioaugmented aerobic and anaerobic biostimulation treatments and will form the basis of a cost-benefit analysis for the innovative aerobic bioaugmentation approach for *in situ* treatment of RDX in groundwater.

1.2 Document Purpose and Organization

This memorandum presents results of bioaugmentation culture production and cell transport field testing, which meet the **Phase II success criteria of detection of bioaugmentation culture biomarkers at or above the quantitative-PCR detection limit in the downgradient well.** Laboratory, field and analytical methods are presented in Section 2, results are presented in Section 3, followed by discussion in Section 4. The project schedule, which identifies work sequence and schedule of all project phases and phase subtasks, is included as Attachment 1.

2. METHODS

This section summarizes bioaugmentation culture production, field cell transport testing, as well as microbial community and standard analytical methods.

2.1 Growth and Shipment of Bioaugmentation Culture to Demonstration Site

Pilot-scale volumes of the following three strains were prepared by CB&I in Lawrenceville, NJ: a transconjugant strain of *Rhodococcus jostii* RHA1, which contains the conjugative plasmid pGKT2::Km^r from the donor bacterium *Gordonia* sp. KTR9 [1], referred to in this document as RHA1 pGTK2; *Gordonia* sp. KTR9 pGKT2::Km^r, which contains intergeneric DNA in the form of an introduced kanamycin resistance gene [1], referred to in this document as KTR9 KanR; and *Pseudomonas fluorescens* I-C, referred to in this document as Strain I-C. Starter cultures were initially grown from verified pure cultures in basal salts medium (BSM) solution (in g/L: $K_2HPO_4.3H_2O$, 4.25; $NaH_2PO_4.\ H_2O$, 1; $MgSO_4.H_2O$, 0.20; $FeSO_4.7H_2O$, 0.012; $MnSO_4.H_2O$, 0.003; $ZnSO_4.7H_2O$, 0.003; $CoSO_4.7H_2O$, 0.001. pH 7.0) on fructose (50 mM) and ammonium sulfate (20 mM) in 3- or 7-L benchtop bioreactors (Applikon Biotechnology B.V., Schiedam, The Netherlands)

(Figure 2.1). Bioreactors were continuously mixed, and the pH, dissolved oxygen (DO), and other process parameters (e.g., residual fructose, ammonium, etc.) were monitored. Control of pH was achieved by automatic addition of aqueous solutions of acid (H_2SO_4) or base (NaOH). Periodic samples were removed for measurement of OD_{600} and viable cells. Once the starter culture had reached the maximum OD_{600}, it was used to inoculate a 750-L bioreactor (Abec, Allentown, PA, USA) (Figure 2.1). Growth continued in the 750-L bioreactor until the cell density (as determined by OD_{600}) multiplied by bioreactor volume yielded the required number of cells of each of three strains (e.g., $5x10^{14}$ cells).

After the required cell density in the 750-L reactor was achieved, the culture was passed through a custom-built cross-flow filtration unit (Kerasep™ tubular ceramic membranes, Novasep, Inc., Boothwyn, PA, USA) to remove the culture media and collect the biomass. The cells were further concentrated using a flow-through centrifuge (CEPA Z41, Carl Padberg Zentrigugenbau GmbH, Geroldsecker Vorstadt, Germany; 17,000 x *g*, 21°C) with final resuspension in approximately 18 L of artificial groundwater (AGW; Table 1) and split between two, 20-L soda kegs (Figure 2.2). A subsample of each culture was removed for measurement of total and viable cell densities via qPCR and spread plating, respectively. Kegs were stored at 4^oC for 2 to 4 weeks prior to being shipped.

The three bioaugmentation cultures were shipped in 20-L soda kegs (6 kegs total, 2 kegs per culture). Kegs were sealed with top cross-bars to maintain a watertight seal. Kegs were packed into coolers equipped with special racks to stabilize the kegs during shipping (Figure 2.2). Ice packs were included to maintain the cultures at a cool temperature during transport to the Groundwater Research Laboratory at Oregon State University. The cultures were continuously iced during transport via car to the UMCD field site.

2.2 Longer-Term Culture Viability and RDX Degrading Activity Assessment

Samples of each of the bioaugmentation cultures prepared above (100 mL) were placed in sterilized 250 mL amber glass bottles. One bottle of each culture was placed at 4°C (expected shipping and long-term storage temperature), and the other was placed at 37°C (worst-case scenario shipping temperature). Well-mixed samples (10 mL) were removed from the bottles initially, and after 1, 2, 5, 7, 14 days for the bottles incubated at both temperatures, and additonally at 30, 60, and 90 days for the bottles incubated at 4°C. After passing the sample several times through a 25 gauge hypodermic needle to reduce cell clumping, the optical density (OD_{550}) and viable cell counts were determined (spread plating onto LB+kanamycin and R2A media), and a subsample was frozen for qPCR.

An RDX degradation assay was set up at each timepoint by making a 1:100 dilution of the sample with phosphate buffered saline, and adding 1 mL of the diluted sample into 9 mL of sterile AGW amended with RDX (10 mg/L) and fructose (9 mg/L). The assays with KTR9 KanR and RHA1 pGTK2 were performed in 25 mL serum vials with 15 mL of headspace to maintain aerobic conditions. The assays with *Ps. fluorescens* I-C were performed in 11 mL serum vials with minimal headspace to create more anoxic conditions. An uninoculated control was set up with each batch of assays. Assays were incubated with shaking (125 rpm) at room temperature. Samples of the assays were removed after 24 and 48 h, passed through a 0.45 µm glass microfiber filter, and analyzed for RDX concentrations.

2.3 Preparing the Bioaugmentation Culture for Injection

The cell transport test was conducted in the field test plot located at well DW-2 (Figure 2.3) in July, 2013. The 3,000 gallon (11,364 L) UMCD groundwater solution was prepared in two large plastic tanks. Sufficient NaCl was added to achieve a Cl^- concentration of 125 mg/L in each tank. Concentrated cell suspensions prepared in the laboratory were transferred from the kegs into the tanks (3 kegs into each tank). The test solution was mixed using two, flow-matched, high-speed transfer pumps, each pumping at a rate of 150 gpm (568 L/min). Mixing continued until the contents of each tank had been exchanged approximately 10 times (~200 minutes).

2.4 Cell Transport Test Format and Sampling Plan

The cell transport test was conducted under "forced gradient" conditions; extraction well EW-4 was turned on 8.6 hours before injection began and pumped at an average rate of 1080 gpm (4091 L/min) during the test. The mixed inoculum solution containing the tracer and cells was injected into DW-2 at an average rate of 145 gpm (549 L/min); the duration of the injection was 21 minutes. During injection, samples of the inoculum solution were collected from the tanks and analyzed for tracer and microbial parameters.

The sampling schedule was based on previous tracer tests at this test plot conducted during Phase I and the results of cell transport tests in laboratory columns packed with site sediment from DW-2. After injection of the inoculum was completed, groundwater samples were collected from injection well DW-2 and downgradient monitoring wells 4-106 and EW-2 on the following schedule: every 0.5 hours for 48 hours, then every 1.5 hours for 12 hours, then every 2.5 hours for 25 hours and then approximately every 5 hours for the remainder of the test. Total test duration was 120 hours. Groundwater samples were collected from separate submersible pumps in each well both prior to and following injection. All pumps extracted groundwater continuously at a small rate (~ 0.35 L/minute) that remained constant during the test. Samples for Cl^- analyses were

collected in plastic, screw top, 15 mL plastic vials. Samples for microbial analyses were collected in sterile, polypropylene, screw top, 125 mL (viability) or 1 L plastic bottles (qPCR). All samples were stored on ice after collection, during shipping, and until laboratory analyses.

2.5 Microbial Characterization Methods

Cell viability of the bioaugmentation culture strains was determined using several types of growth media. Total heterotrophic bacterial counts in the groundwater (indigenous plus inoculated strains) were determined on 5% PTYG agar plates [2]. Viability of strains KTR9 KanR and RHA1 pGKT2 was determined on LB plus kanamycin (50 mg/L) agar plates, while viability of Strain I-C was determined *Pseudomonas* isolation agar (PIA). Groundwater samples were diluted in buffered peptone water (in g/L: peptone, 0.1 ; NaCl, 8.5 ; K_2HPO_4, 0.3; NaH_2PO_4, 0.6; pH 7.0) and appropriate dilutions were spread onto the surface of each type of agar. Plates were incubated at 30°C and counted after 5 days.

Quantitative PCR (qPCR) was used as a rapid, culture-independent approach to track the transport of the bioaugmentation culture. Groundwater samples (200-1200 mL) were filtered onto polyethersulfone filters (0.2 µm porosity, 73 mm diameter) in the laboratory and the filters were aseptically cut in half and placed into 2 DNA extraction tubes. The PowerWater DNA extraction kit (MoBio Inc., Carlsbad, CA) was used to extract the DNA. The samples were homogenized in a bead beater for 1 min followed by vortex mixing for 40 s. All qPCR amplification reactions were performed using Applied Biosystems 7900HT Fast Real-time PCR system (Foster City, CA). Quantitative PCR was performed in 20 µL reaction volumes in 384-well optically clear plates. Analysis of the kanamycin and *xenB* genes used a SYBR Green PCR Master Mix, 300 nM of the respective primers, and 1 µL of template DNA. Thermal cycler conditions were 95°C for 10 min; then 40 cycles of 95°C for 15 s; 60°C for 60 s; followed by a final dissociation stage. Analysis of the 16S rRNA and *xplA* genes used a Quantitect PCR Probe Mix, 300 nM of the respective primers, 200 nM of the respective TaqMan probe and 1 µL of template DNA. Thermal cycler conditions were 95°C for 12 min; then 40 cycles of 95°C for 30 s; 50°C for 60 s; and 72°C for 20 s. Standard curves for each qPCR assay were obtained from serial dilutions of genomic DNA isolated from strain KTR9 KanR (kanamycin gene) and Strain I-C (16S rRNA and *xenB*), or an *xplA* containing plasmid (pET11a, Celtek Genes, Franklin, TN). The quantity of gene copies per mL of groundwater was calculated as follows.

$$\frac{[(gene\ copy\ number\ per\ reaction)\ \times\ (total\ volume\ of\ extracted\ DNA\ \{\mu L\})]}{[(reaction\ volume\ \{\mu L\})\ \times\ (total\ volume\ of\ groundwater\ filtered\ \{ml\})]}$$

2.6 Analtyical Methods

Chloride anion concentrations were determined by external calibration using a Dionex (Sunnyvale, CA) model ICS 2000 ion chromatograph equipped with an electrical conductivity detector and a Dionex IonPac AS18 analytical column.

2.7 Permitting and Decontamination of Field Equipment

Toxic Substance Control Act (TSCA) Experimental Release Applications (TERAs) for use of the two genetically-modified organisms (GMOs) – KTR9 KanR and RHA1 pGTK2 were prepared and submitted to the Environmental Protection Agency's Office of Pollution Prevention. The project received TERA application approval for both microorganisms on July 10, 2013 prior to test initiation (Attachment 2). Best management practices for handling the bioaugmentation strains and decontamination of equipment were used during the field test. Prior to adding the strains to the groundwater tank, all connections and tubing associated with the mixing and injection were tested to ensure there were no leaks. Plastic sheeting was placed around the tank opening where the cultures were added, and on the ground surrounding the tank/injection area to ensure any minor spills were contained to the test area and could be disinfected prior to disposal. All personnel used appropriate personal protective equipment and other applicable safety procedures for work at CERCLA sites as described in the health and safety plan. All equipment, tanks and tubing that contacted the culture were disinfected using a 5-10% bleach solution.

3. RESULTS

KTR9 KanR, RHA1 pGTK2, and Strain I-C retained viability and RDX-degrading activity at 4 ^{o}C during storage. Changes in the culture OD, viable cells, and OD-normalized RDX degradation capacity of the pilot-scale bioaugmentation cultures are presented in Figures 3.1 through 3.3. All three cultures quickly decayed in terms of culture density, cultivable cells, and RDX degrading activity when incubated at 37°C. Storage at 4°C maintained culture density for at least 90 days for all three cultures (Figure 3.1). Some decrease in cultivable cells was observed for all three strains incubated at 4°C during the first 14 days, followed by a period of stable CFU counts for all strains until following day 60 when RHA2 pGTK2 counts declined (Figure 3.2). OD-normalized RDX activity also remained relatively stable for KTR9 KanR over the study period, while some decrease was observed for RHA1 pGTK2 after 30 days (Figure 3.3). The RDX degradation assay was not optimized for Strain I-C (e.g. the assay was not performed under anoxic conditions), but some activity was observed by the culture incubated at 4°C over the duration of the experiment (Figure 3.3).

Plating groundwater samples on kanamycin-containing agar plates permitted clear distinction of biouaugmentation culture during test. On LB plus kanamycin plates and 5% PTYG plates, KTR9 KanR colonies were orange and RHA1 pGKT2 colonies were beige (Figure 3.4a). Groundwater samples collected prior to injection did not contain bacterial strains similar in colony morphology (Figure 3.4b) to strains KTR9 KanR and RHA1 pGKT2 (Figure 3.4a), thus permitting clear colony distinction. After mixing the concentrated cell suspensions into 11,000 L of groundwater the concentration of viable KTR9 KanR and RHA1 pGKT2 cells was 1.7 x 10^9 and 6.4 x 10^8 CFU/mL, respectively. On PIA plates, Strain I-C colonies produced a fluorescent greenish-yellow pigment (Figure 3.5a). However, none to very few fluorescing colonies were identified in samples collected from groundwater post inoculation (Figure 3.5b). The reason for the inability to detect fluorescent colonies isolated from the inoculated UMCD groundwater is unknown. Thus, data to support groundwater transport of Strain I-C could only be derived from qPCR data of the *xenB* gene.

KTR9 KanR and RHA1 pGKT2 cells were rapidly transported to downgradient well 4-106 during the test. Strains KTR9 KanR and RHA1 pGKT2 were not present in UMCD groundwater prior to inoculation (indicated as -10 h on Figure 3.6). Transport of these two strains was rapid, with cells reaching well 4-106 within 2 hours (Figure 3.6a,b). Over the next 24 hours, KTR9 KanR and RHA1 pGKT2 viable numbers declined by several orders of magnitude in the injection well (DW-2) and well 4-106. Viable numbers of KTR9 KanR and RHA1 pGKT2 then stabilized between 4 x 10^4 and 2 x 10^5 CFU/mL for the next 5 days. Total cell numbers (indigenous plus bioaugmented strains) as determined on 5% PTYG agar followed a similar trend to the viability trends observed with KTR9 KanR and RHA1 pGKT2 (Figure 3.6c). However, there was no evidence that KTR9 KanR or RHA1 pGKT2 reached well EW-2, since these colony types were not observed on

LB+kanamycin agar plates. The lack of transport of these two strains to well EW-2 was confirmed by the relatively constant total viable cell numbers (5% PTYG) in the groundwater at EW-2 in contrast to total cell counts in wells DW-2 and 4-106 (Figure 3.6).

Quantitative PCR results showed rapid *xplA* gene copy number response in downgradient well 4-106 during the test, corroborating cell count results. Prior to bioaugmentation, background levels of *xplA*, kanamycin, and *xenB* genes were significantly lower than the estimate of the total bacterial population (16S rRNA) (Figure 3.7). Quantitative PCR analysis indicated a robust groundwater population at 10^6 to 10^8 16S rRNA gene copies/mL (Figure 3.7a). In comparison, background levels of *xplA*, kanamycin, and *xenB* genes were between 10^4-10^5, 10^3-10^4, and 10^2-10^3 gene copies/mL, respectively. The 11,000 L bioaugmentation solution contained approximately 10^8 *xplA* gene copies/mL representing KTR9 KanR plus RHA1 pGKT2 cells (Figure 3.7a,b). Levels of Strain I-C in the injection solution were measured at 10^2-10^3 *xenB* gene copies/mL (Figure 3.7c). This is considerably lower than the expected 10^8-10^9 gene copies/mL expected based on the 5 x 10^{11} *xenB* gene copies/mL measured in the concentrated suspension of Strain I-C before it was shipped to the site (Table 2). The reason for the lower quantity of *xenB* genes, and thus Strain I-C, measured in the bioaugmentation solution is unclear at this moment. We are also unsure why measurable copy numbers for *xplA* and the kanamycyin resistance gene were detected in the Strain I-C culture.

Injection of the bioaugmentation inoculum into well DW-2 was followed by an increase in 16S rRNA gene copies of about 1 order of magnitude (Figure 3.7a). Overall, the differences in quantity of 16S rRNA genes prior to bioaugmentation and at the end of the demonstration were less significant than quantity differences for the three biomarker genes. A significant increase of 3 orders of magnitude in the quantity of the *xplA* and kanamycin genes was observed immediately following inoculation (Figure 3.7b, c). An analogous increase in the quantity of these two genes was also observed at well 4-106 within 2 h of injection (Figure 3.7b, c). Over the next 24 h, the quantity of *xplA*, and kanamycin genes decreased by about 2 orders of magnitude in wells DW-2 and 4-106. Biomarker gene copy number then stabilized at significantly higher levels than background quantities of these genes. At the completion of the field demonstration *xplA* and kanamycin genes were detected at 1 to 2 orders of magnitude above background quantities in wells DW-2 and 4-106 (Figure 3.7b, c). In contrast, the quantity of *xplA* and kanamycin genes in well EW-2 did not increase above background levels and were approximately 1 order of magnitude lower by the end of the study (Figure 3.7b, c). The qPCR and viability data clearly indicates the survival and transport of KTR9 KanR and RHA1 pGKT2 in wells DW-2 and 4-106, but not well EW-2.

Quanties of the *xenB* gene in groundwater remained fairly stable in wells DW-2 and EW-2 during test (Figure 3.7d). In contrast, *xenB* genes quantities in groundwater increased

by 3 orders of magnitude in downgradient well 4-106 within 2 h of bioaugmentation (Figure 3.7d). As observed with the *xplA* and kanamycin resistance genes, the trend in *xenB* gene copy numbers within this well was similar. The initial rapid increase in *xenB* gene copies was followed by a transient decline leading to stable quantities that were 1 order of magnitude above background *xenB* gene levels in UMCD groundwater. Based on the quantitification of the *xenB* gene, Strain I-C was transported as rapidly as the other two bioaugmentation strains to downgradient well 4-106.

Microbial and tracer breakthrough curve results at downgradient well 4-106 were similar to results obtained during the laboratory column cell transport tests completed during demonstration Phase I. A clear breakthrough of chloride tracer by 10 h was observed in the downgradient well, 4-106 (Figure 3.8b). Similar to previous column tests (Phase I report), transport of the tracer was slower than the transport of the cells. In addition, the column transport tests demonstrated that the three bioaugmentation strains remained viable and metabolically active over 3 months with effluent concentrations about 10^3 CFU/mL (Phase I report). In comparison, the field demonstration confirmed the transport of the three strains over 3 m in the subsurface as well as survival at 10^5 CFU/mL or 10^3-10^7 gene copies/mL for the 5 days. The proportion of Strain I-C (10^3-10^4 gene copies/mL) that was transported or survived in the UMCD groundwater was 2-3 orders of magnitude lower than strains KTR9 KanR and RHA1 pGKT2 (10^6-10^7 gene copies/mL).

4. DISCUSSION

Transport of strains KTR9 KanR, RHA1 pGKT2, and Strain I-C in the UMCD aquifer.
In order for this technology to be successfully implemented at UMCD (and other sites) the bioaugmentation culture must remain viable and retain RDX-degrading capability over time *in situ*. Bacterial survival was assessed using (a) viable plate counts over time on species-specific media, (b) quantification of specific biomarker genes: *xplA*, kanamycin, and *xenB*, and (c) RDX degradation rates Several lines of evidence from three different approaches substantiate this requirement. (2) The three bioaugmentation cultures, prepared and starved in AGW for shipment, survived and maintained RDX activity for 90 days at 4^oC. (2) Viable cells of each strain were recovered from column effluent for the duration of the x day trial, which indicates that the cells probably colonized the sediment within the column. Also, strains KTR9 KanR, RHA1 pGKT2, and I-C degraded RDX after stimulation with the electron donor fructose. Fructose did not stimulate RDX degradation by the indigenous sediment bacteria (Phase I report). (3) Both viable plate counts and qPCR results demonstrated the survival and presence of the bioaugmentation culture in UMCD groundwater at wells DW-2 and 4-106. Measurement of *in situ* RDX-degrading capability by the bioaugmentation culture was not the focus of the cell transport test. This capability will be assessed during the Phase III portion of this field demonstration.

The objective of this Phase II study was to confirm the ability to transport the bioaugmentation culture at the field scale. The viable plate counts and qPCR data for the *xplA* and kanamycin genes confirmed the rapid transport of strains KTR9 KanR and RHA1 pGKT2 to well 4-106, which was located 3 m downgradient of the injection well. Only qPCR data for the *xenB* gene was available to infer groundwater transport of Strain I-C to well 4-106. Similarly, groundwater transport and survival of bioaugmentation cultures over distances up to 12 m have been observed to be unretarded at some sites [3] to significantly slower than the tracer at other sites . In the latter case, physical straining or cell death was the suspected cause, since the strain had been selected for low adhesion to the aquifer sediments [4]. In contrast, transport of neither the bioaugmentation culture nor the chloride tracer was detected 21 m downgradient from the injection well in EW-2. Colonies similar in appearance to strains KTR9 KanR, RHA1 pGKT2, or I-C were not detected, and only background levels of *xplA*, kanamycin, and *xenB* genes were determined in groundwater from well EW-2. Previous tracer tests have indicated groundwater connectivity between wells 4-106 and EW-2 may not be vertically uniform, thereby raising the possibility that the injected cells bypassed the downgradient well or that the sampling regime missed their arrival.

Strains KTR9 KanR and RHA1 pGKT2 survived well for up to 5 days in the UMCD aquifer with viable numbers remaining steady at approximately 10^5 CFU/mL and *xplA* gene quantities greater than 10^6 copies/mL. Apparently Strain I-C survived during the field trial but at a significantly lower cell density. Even though measurements on the concentrated cell suspension of Strain I-C indicated a large viable and active cell suspension (Figures 3.1, 3.3, Table 2) there were difficulties in detecting both viable cells and *xenB* genes in the bioaugmentation solution prior to injection and in the groundwater samples collected post-injection. Possible contamination of the Strain I-C culture is a possibility based on the detection of *xplA* and kanamycin genes in the pre-shipment samples (Table 2). However, this should not have interfered with the ability to detect *xenB* genes or I-C cells due to the specificity and selectivity of the assays. The reason for our inability to accurately quantify Strain I-C is not known at this time.

Large-Scale Culture Production and Culture Longevity Testing

This is the first reported production of aerobic RDX degraders for field-relevant bioaugmentation. The processing, shipment, and pre-injection storage procedures resulted in a successful injection and transport of three RDX-degrading strains into the UMCD aquifer. Furthermore, the longevity testing results clearly indicate that these cultures can be produced in advance of plamnned field application and stored at 4°C for at two to three months without significant loss of cell density, and more importantly, RDX degradation activity.

5. RECOMMENDATIONS FOR PHASE III

Next steps on this project include the following.

- Overall the viable plate counts on LB + kanamycin agar plates and the qPCR TaqMan assay of the *xplA* gene were the most reliable indicators of the presence of KTR9 KanR and RHA1 pGKT2 in the groundwater. The qPCR assays of the 16S rRNA and kanamycin resistance genes followed the same trend as the *xplA* gene. The SybrGreen qPCR assay of the *xenB* gene was more reliable than the use of the selective medium, PIA, for detection of Strain I-C even though the qPCR data gave some variable results. For Phase III, it is suggested that the qPCR assays of *xplA* and *xenB* genes will be sufficient to quantify the bioaugmentation culture in the field. This will also increase throughput of sample processing and data analysis with the increased number of samples.
- A second recommendation is to include only KTR9 KanR in the bioaugmentation culture. The inclusion of RHA1 pGKT2 is no longer required since both strains survive in the groundwater to the same extent. Commercial production of the inoculum would preferably use non-genetically modified bacteria to minimize environmental regulations with organism releases in the field, therefore restricting commercial use to the wild-type KTR9 strain. In addition, we were unable to account for viable cells of Strain I-C and the qPCR assay with xenB was inconsistent. For these reasons, we will limit the Phase III field demonstration to strain KTR9 KanR.

6. REFERENCES

1. Jung, C.M., et al., *Horizontal gene transfer (HGT) as a mechanism of disseminating RDX-degrading activity amont Actinomycete bacteria.* Journal of Applied Microbiology, 2011. 110: p. 1449-1459.
2. Balkwill, D.L. and W.C. Ghiorse, *Characterization of subsurface bacteria associated with two shallow aquifers in Oklahoma.* Applied and Environmental Microbiology, 1985. 50: p. 580-588.
3. Trotsky, J., et al., *Low-Cost, Passive Approach for Bacterial Growth and Distribution for Large-Scale Implementation of Bioaugmentation.* ESTCP Project ER-200513, TR-2354-ENV Final Report, 2010. Dec. 2010.
4. Steffan, R.J., et al., *Field-Scale Evaluation of In Situ Bioaugmentation for Remedies of Chlorinated Solvents in Groundwater.* Environmental Science & Technology, 1999. 33: p. 2771-2781.

TABLES

Table 1. Composition of the artificial groundwater (AGW) used during bioaugmentation culture preparation

Component		UMCD groundwater (mg/L)	AGW (mg/L)
pH		8	8
Nitrate as NO_3		48	46
Sulfate as SO_4		24	25
Alkalinity as CO_3		92	89
Na^+		20	54
Ca^{2+j}		35	28
Cl^-		21	78
Mg^{2+}		16	16
K^+	3.5	3.5	
NH_4^+	<0.5	3.5	

Table 2. Gene copy number quantified in cultures prepared for shipment

	Avg gene copies/ml			
Culture	16S rRNA	*xplA*	kan	*xenB*
RHA1 pGKT2 keg 1	2.73E+13	2.96E+13	1.03E+13	4.46E+07
RHA1 pGKT2 keg 2	2.22E+13	2.59E+13	9.84E+12	4.06E+07
KTR9 KanR	1.91E+14	5.29E+12	1.32E+12	4.64E+07
P. fluorescens I-C	6.73E+14	1E+11	1.48E+10	5.01E+11

FIGURES

Figure 2.1. Photographs of benchtop etleft) and 750-L (right) bioreactors.

Figure 2.2. Photographs of the 20-L keg used for culture starvation and shipping.

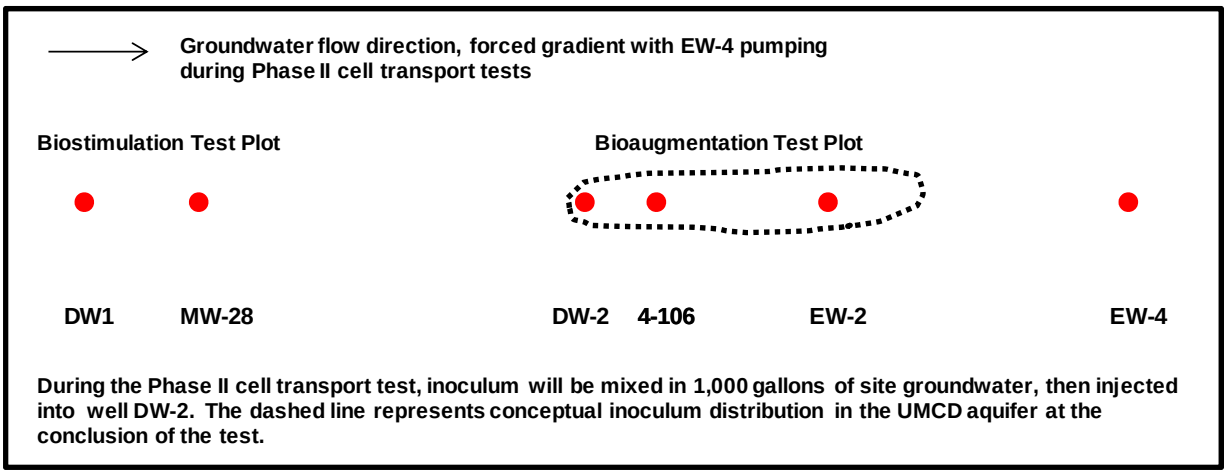


Figure 2.3. Well layout in test plots.

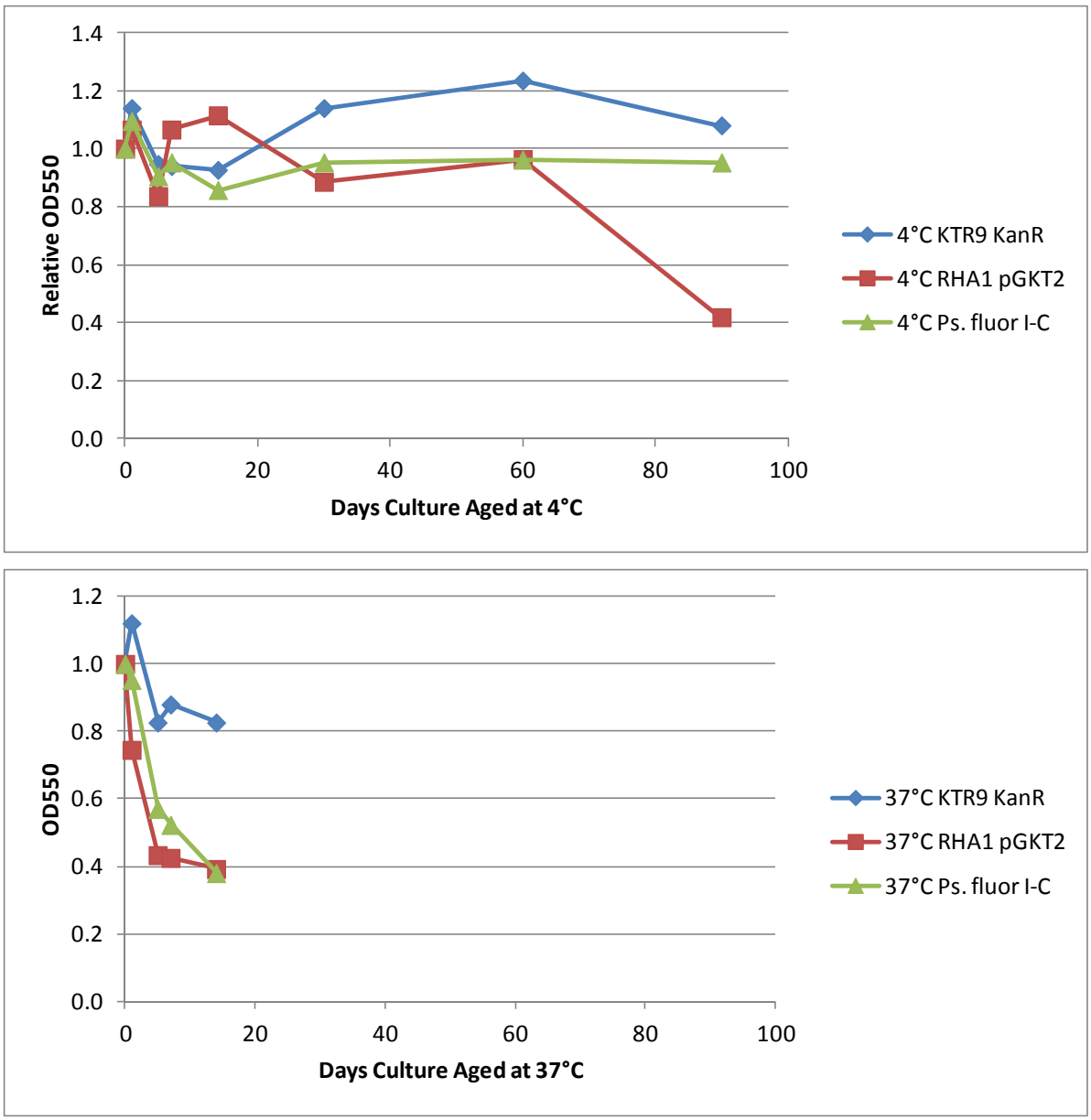


Figure 3.1. Change in relative culture optical densities over time at 4°C and 37°C.

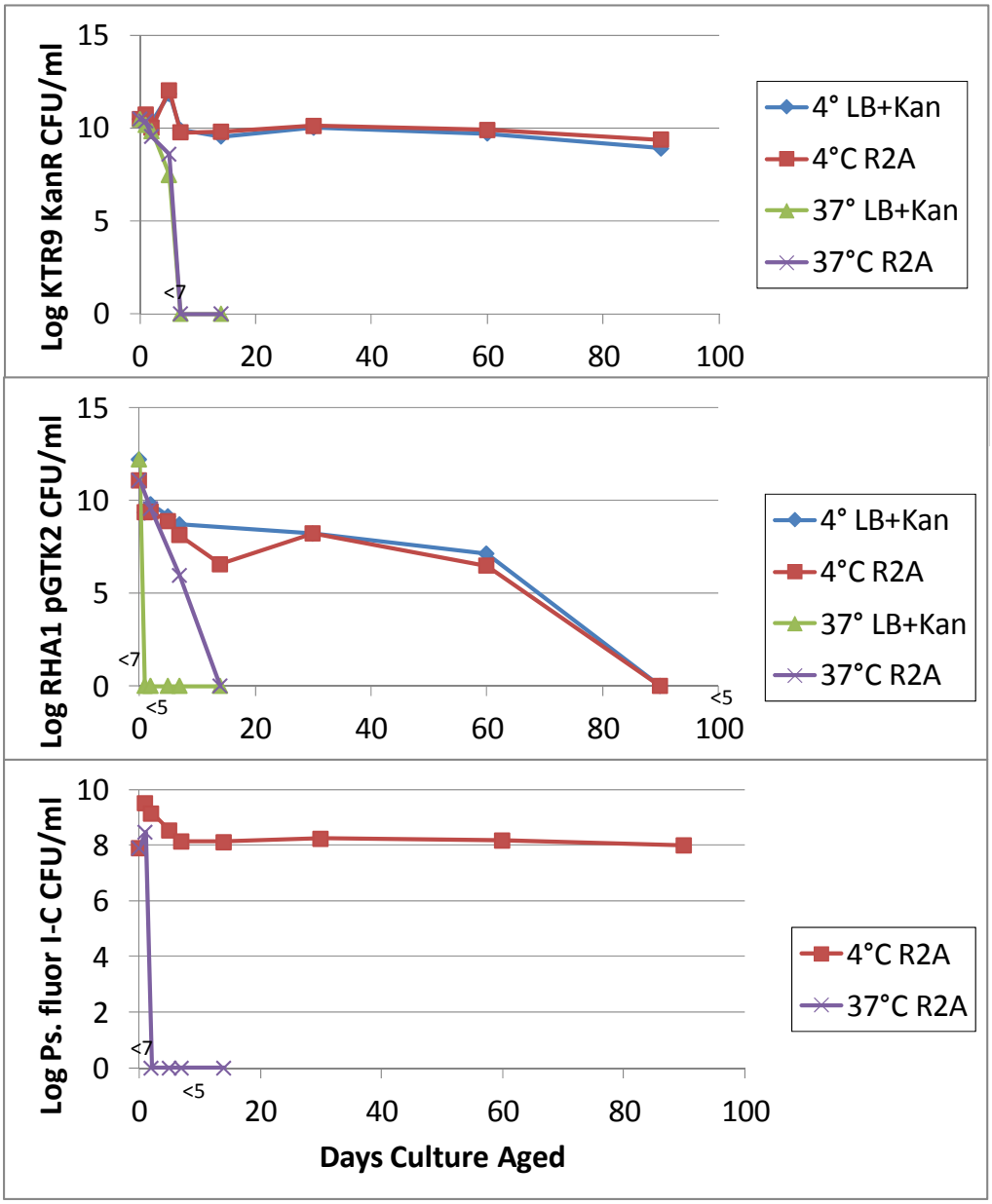


Figure 3.2. Change in viable cell densities over time at 4°C and 37°C.

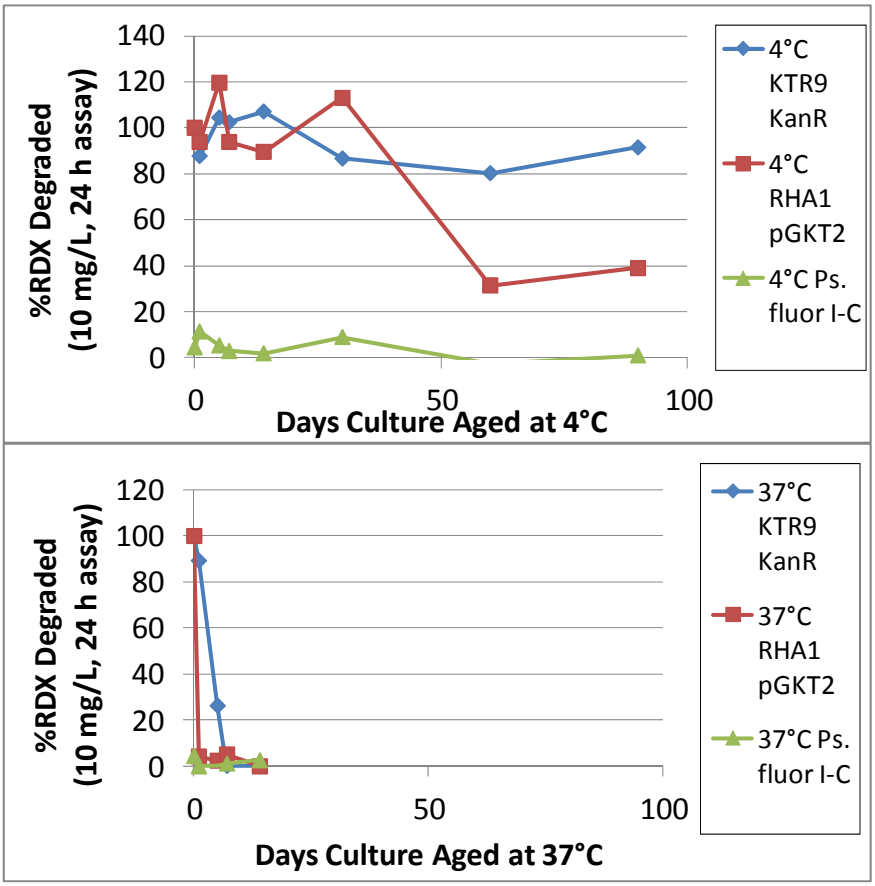


Figure 3.3. Change in OD-normalized RDX degradation activity overtime.

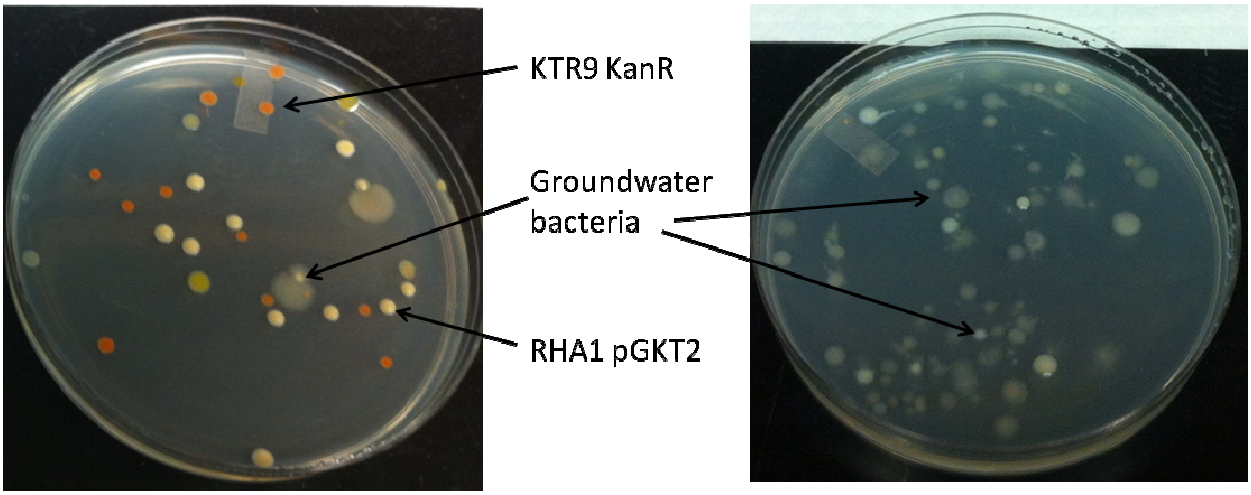


Figure 3.4. Growth of KTR9 KanR, RHA1 pGKT2 and groundwater bacteria on LB + kanamycin agar (a) and UMCD groundwater bacteria prior to inoculation on 5% PTYG agar (b).

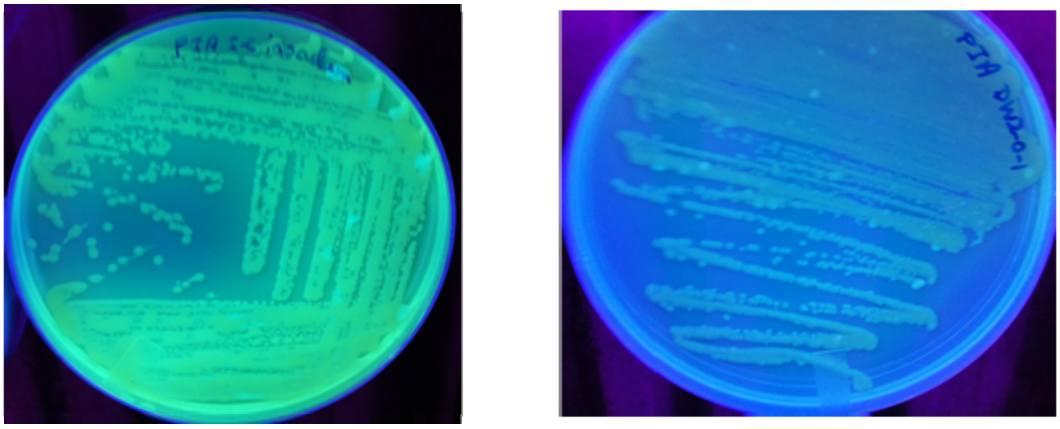


Figure 3.5. Growth of Strain I-C on Pseudomonas isolation agar plates showing active fluorescence (a) and UMCD groundwater from well DW2 at 2 h with very few colonies that are fluorescent (b).

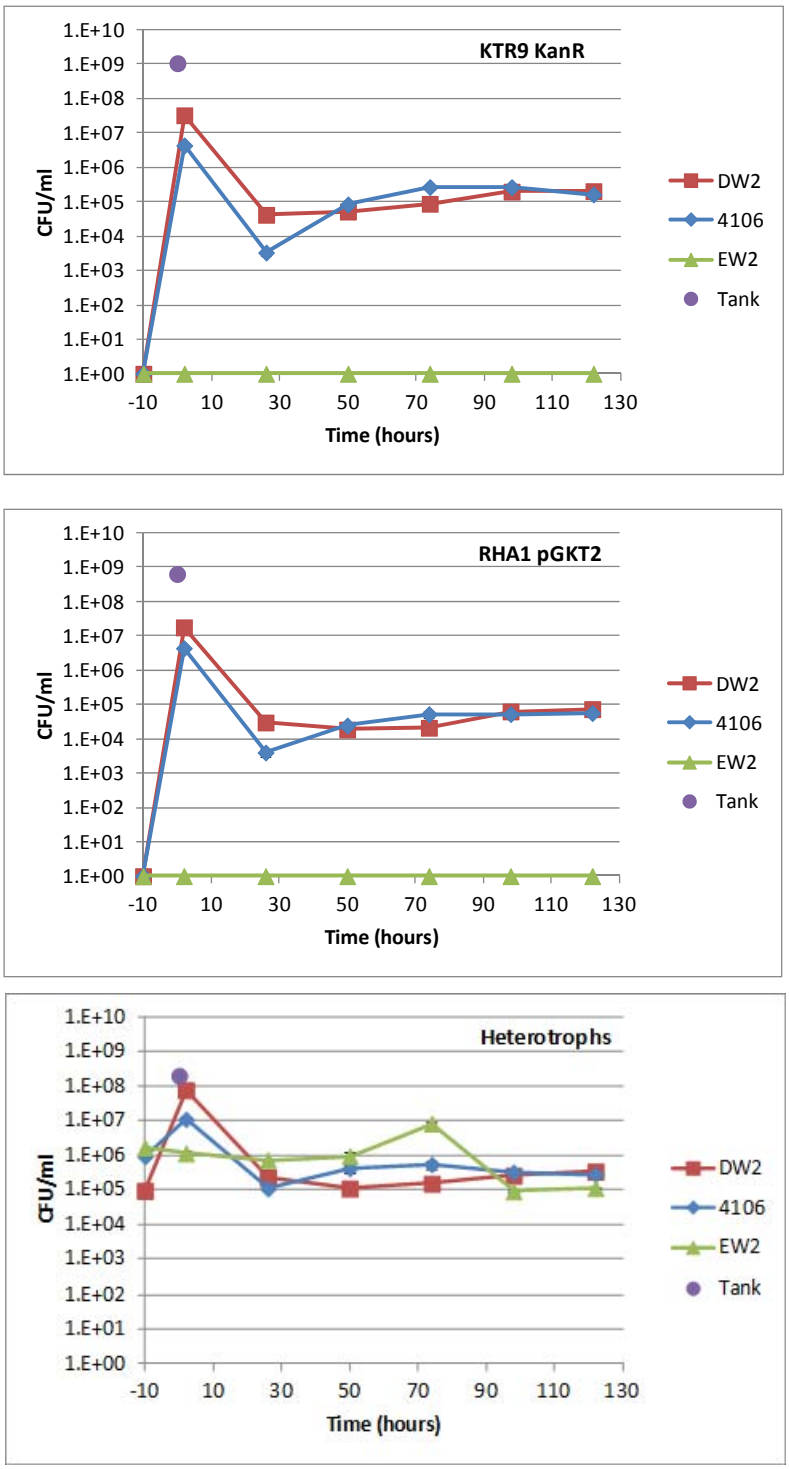


Figure 3.6. Viable plate counts of KTR9 KanR (a) and RHA1 pGKT2 (b) on LB+kanamycin agar plates and heterotrophic groundwater bacteria plus inoculum strains on 5% PTYG agar plates (c).

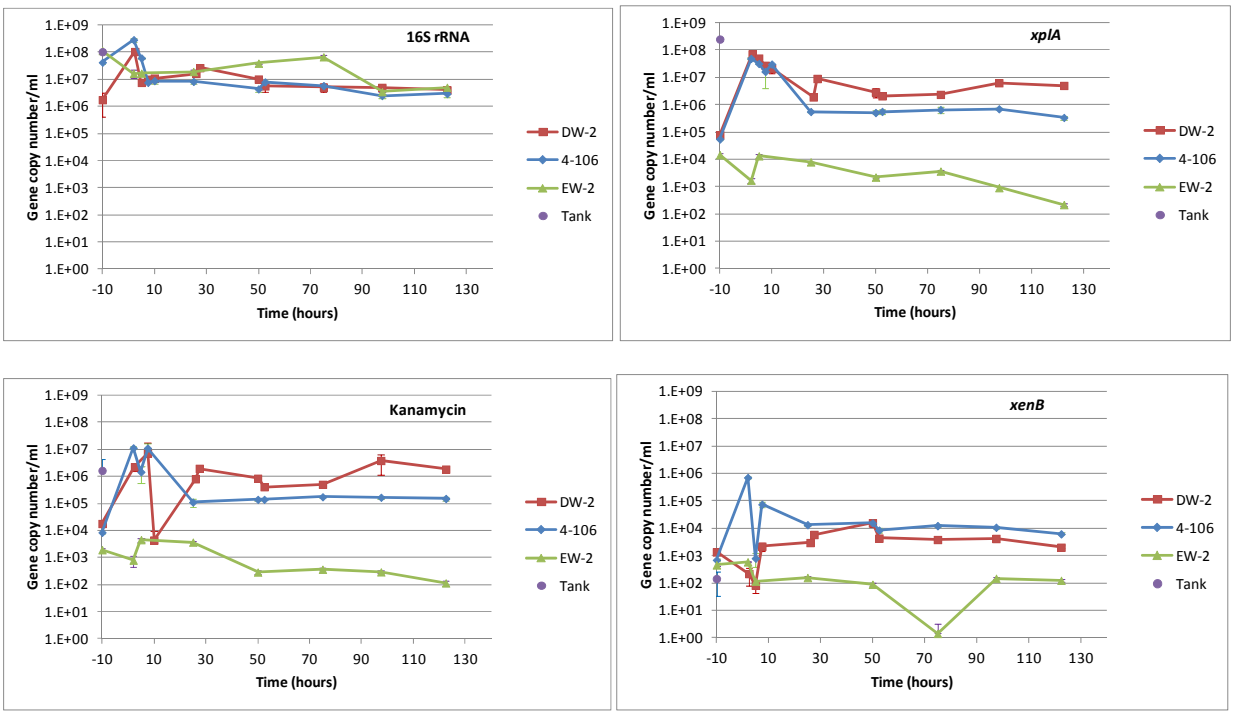
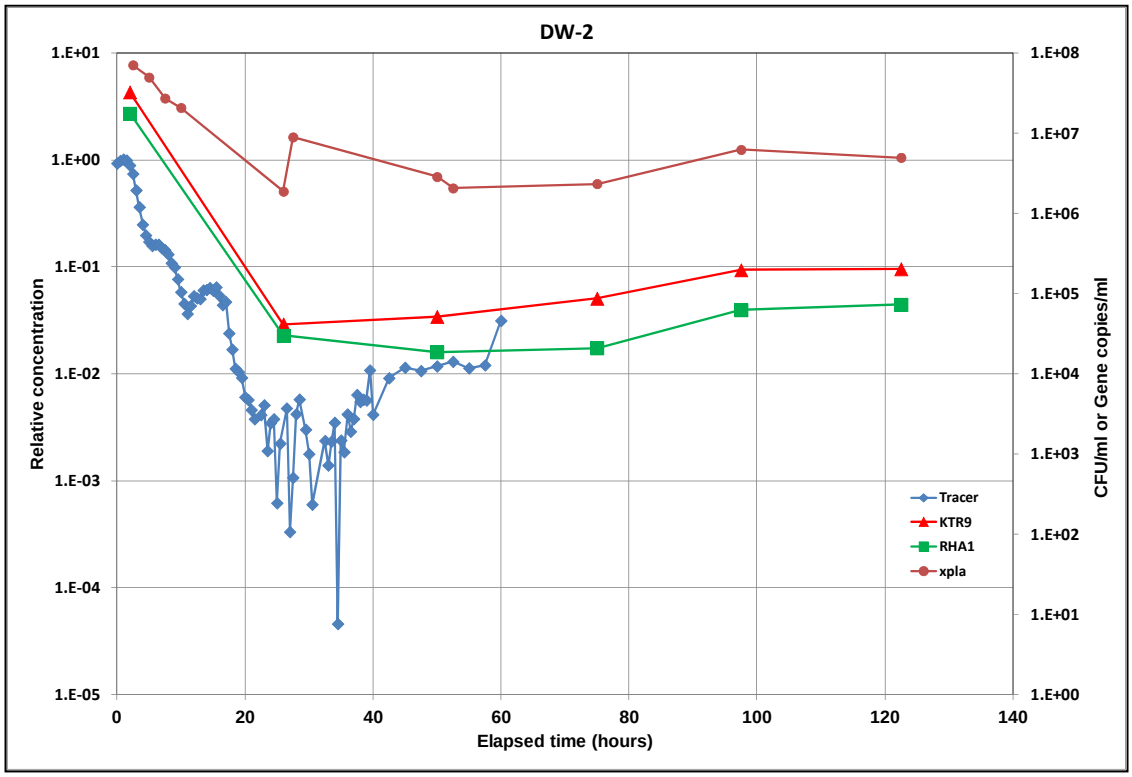


Figure 3.7. Quantitative PCR analysis of UMCD groundwater samples from 3 wells targeting the 16S rRNA genes (a), *xplA* gene (b), Kanamycin gene (c), and *xenB* gene (d).



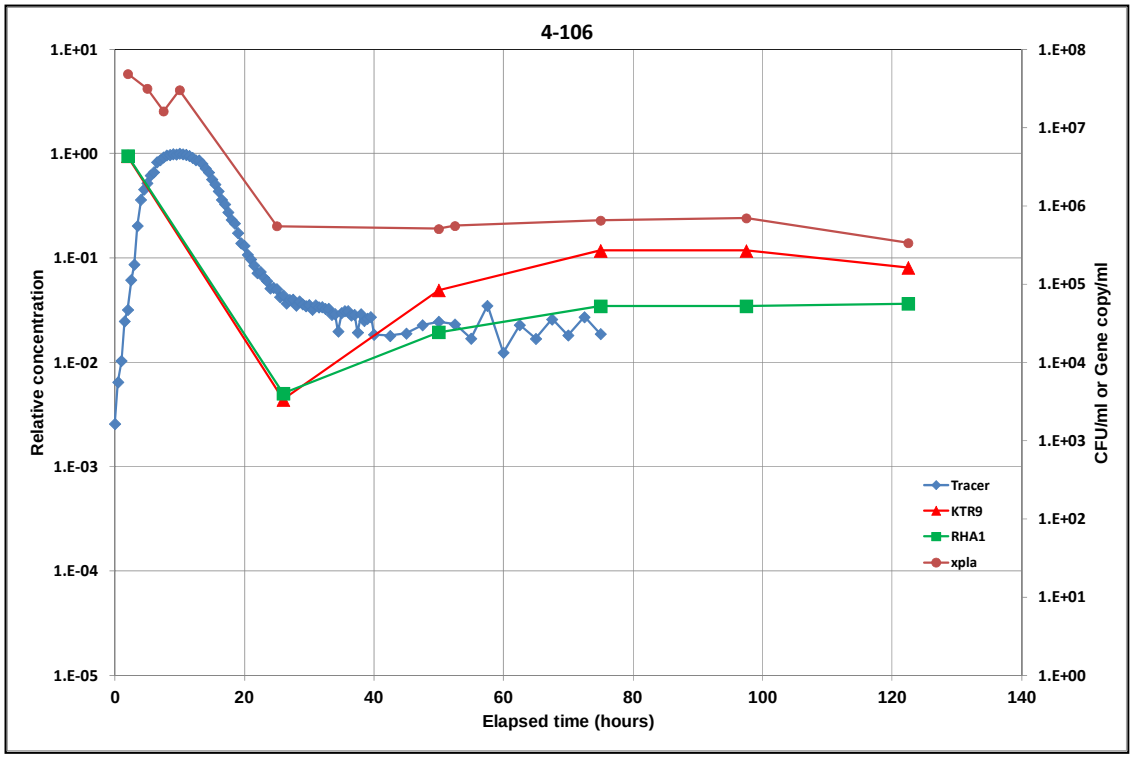
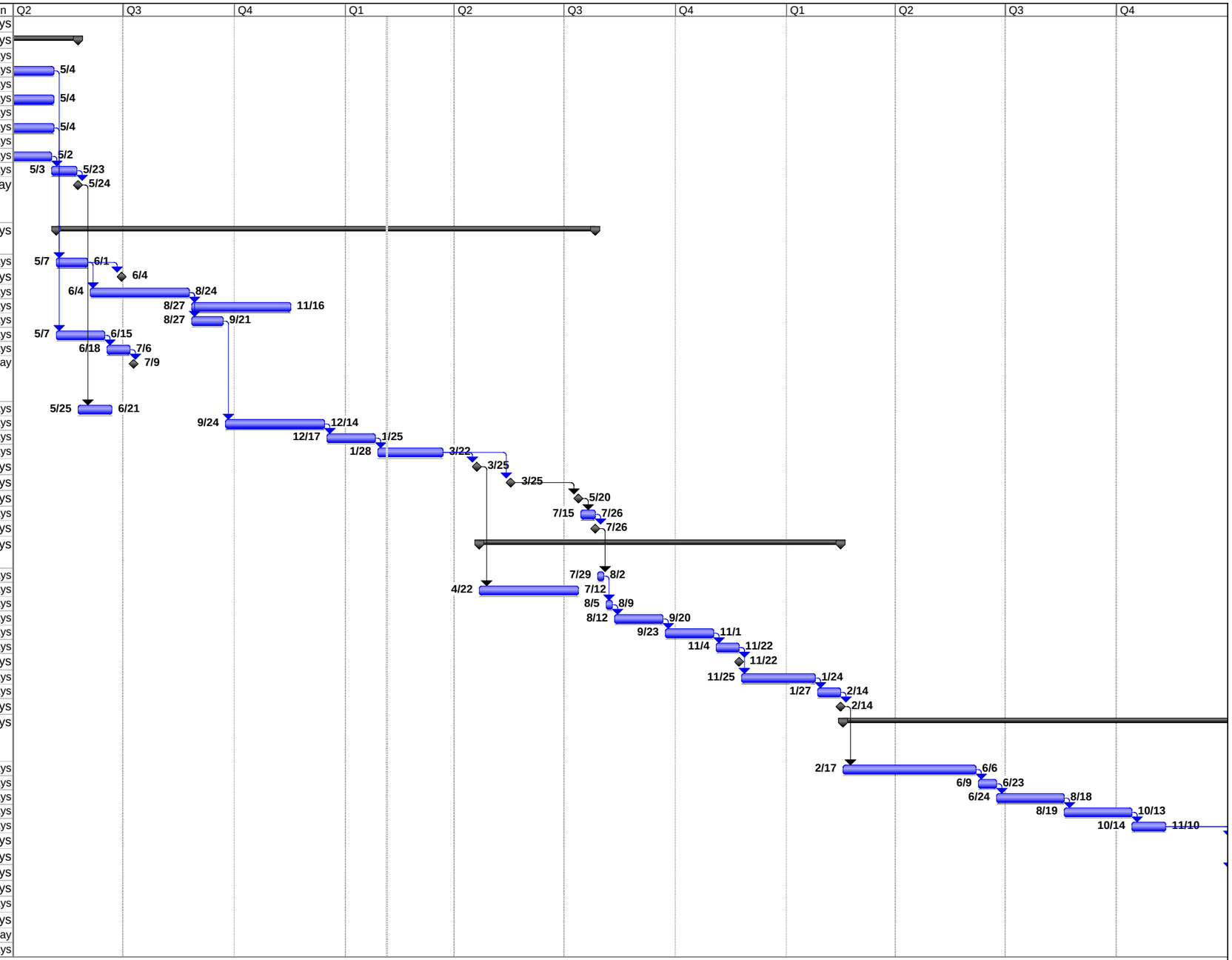


Figure 3.8. Breakthrough curves for tracer, KTR9 KanR and RHA1 pGKT2 cell counts, and *xplA* gene copy numbers in wells DW-2 (a) and 4-106 (b).

ATTACHMENT 1

Current Project Schedule

ID	Task Name	Start	Finish	Duration
3	**Project Funding Received**	Thu 3/1/12	Thu 3/1/12	0 days
4	**Start of Project**	Thu 3/1/12	Thu 5/24/12	61 days
5	a. SOW Development for SHAW	Thu 3/1/12	Fri 3/9/12	7 days
6	Contract Development/ Award for SHAW	Mon 3/12/12	Fri 5/4/12	40 days
7	b. SOW Development for OSU	Thu 3/1/12	Fri 3/9/12	7 days
8	Contract Development/ Award for OSU	Mon 3/12/12	Fri 5/4/12	40 days
9	c. SOW Development for Drillers	Thu 3/1/12	Fri 3/9/12	7 days
10	Contract Development/Award for Drillers	Mon 3/12/12	Fri 5/4/12	40 days
11	d. MIPR to ERDC from NWS	Thu 3/1/12	Wed 3/7/12	5 days
12	Draft Phase I Laboratory Studies Work Plan. Part 1: Culture	Thu 3/1/12	Wed 5/2/12	45 days
13	ESTCP Review, discussion, complete revisions	Thu 5/3/12	Wed 5/23/12	15 days
14	MS 1: Phase I Laboratory Studies Work Plan. Part 1: Culture Optimization and Cell Adhesion Assays	Thu 5/24/12	Thu 5/24/12	1 day
15	**Phase I: Site characterization and bioaugmentation culture optimization**	Mon 5/7/12	Fri 7/26/13	320 days
16	Task 1a. Site characterization	Mon 5/7/12	Fri 6/1/12	20 days
17	MS2: Site characterization memorandum	Mon 6/4/12	Fri 6/29/12	20 days
18	Task 1b. Bioaugmentation culture optimization (pure culture	Mon 6/4/12	Fri 8/24/12	60 days
19	Task 1b. Bioaugmentation culture optimization (site soil/slur	Mon 8/27/12	Fri 11/16/12	60 days
20	Midpoint of microcosms - informs culture selection for colum	Mon 8/27/12	Fri 9/21/12	20 days
21	Draft Phase I Laboratory Studies Work Plan. Part 2: Column	Mon 5/7/12	Fri 6/15/12	30 days
22	ESTCP Review, discussion, complete revisions	Mon 6/18/12	Fri 7/6/12	15 days
23	MS3: Phase I Laboratory Studies Work Plan. Part 2: Column Transport Tests and Culture Production Optimization	Mon 7/9/12	Mon 7/9/12	1 day
24	Task 1c. Cell transport optimization (adhesion assays)	Fri 5/25/12	Thu 6/21/12	20 days
25	Task 1c. Cell transport optimization (column transport)	Mon 9/24/12	Fri 12/14/12	60 days
26	Midpoint of cell transport column - confirms culture selection	Mon 12/17/12	Fri 1/25/13	30 days
27	Task 1d. Culture production optimization	Mon 1/28/13	Fri 3/22/13	40 days
28	MS4: Phase I Memo (treatability study report)	Mon 3/25/13	Fri 4/19/13	20 days
29	MS5: Draft Field Demonstration Work Plan	Mon 3/25/13	Fri 5/17/13	40 days
30	MS6: Final Field Demonstration Work Plan	Mon 5/20/13	Fri 7/12/13	40 days
31	Field Demonstration - Mob Prep	Mon 7/15/13	Fri 7/26/13	10 days
32	MS7: Phase II Field Demonstration Start	Fri 7/26/13	Fri 7/26/13	0 days
33	**Phase II: Demonstrate field-scale transport of RDX-degrading culture and perform treatment**	Mon 4/22/13	Fri 2/14/14	215 days
34	Task 2a. Determine RDX retardation factors	Mon 7/29/13	Fri 8/2/13	5 days
35	Task 2b. Culture production for field transport tests	Mon 4/22/13	Fri 7/12/13	60 days
36	Task 2c. Field-scale culture transport test	Mon 8/5/13	Fri 8/9/13	5 days
37	Laboratory turnaround time (8330 and molecular)	Mon 8/12/13	Fri 9/20/13	30 days
38	Data analysis	Mon 9/23/13	Fri 11/1/13	30 days
39	Phase II Draft Memo	Mon 11/4/13	Fri 11/22/13	15 days
40	MS8: Phase II Memo	Fri 11/22/13	Fri 11/22/13	0 days
41	Update and Finalize Demonstration Work Plan for Phase III	Mon 11/25/13	Fri 1/24/14	45 days
42	Phase III Mob Prep	Mon 1/27/14	Fri 2/14/14	15 days
43	MS9: Phase III Field Demonstration Start	Fri 2/14/14	Fri 2/14/14	0 days
44	**Phase III: Demonstrate transport of aerobic RDX degrading bioaugmentation culture, RDX degrading activity and xplA gene transfer to indigenous**	Mon 2/17/14	Tue 5/12/15	322 days
45	Task 3a. Field-scale culture production, aquifer conditioning	Mon 2/17/14	Fri 6/6/14	80 days
46	Task 3b. (1) Triplicate push-pull tests for determination of in	Mon 6/9/14	Mon 6/23/14	11 days
47	Task 3b. (2) Triplicate push-pull tests for determination of in	Tue 6/24/14	Mon 8/18/14	40 days
48	Laboratory turnaround time	Tue 8/19/14	Mon 10/13/14	40 days
49	Data analysis	Tue 10/14/14	Mon 11/10/14	20 days
50	MS10: Draft Final Technical Report	Tue 11/11/14	Mon 1/5/15	40 days
51	MS11: Final Technical Report	Tue 1/6/15	Mon 3/16/15	50 days
52	MS12: Draft Final Cost and Performance Report	Tue 11/11/14	Mon 1/5/15	40 days
53	MS13: Final Cost and Performance Report	Tue 1/6/15	Mon 3/16/15	50 days
54	MS14: Draft Guidance Document	Tue 3/17/15	Mon 5/11/15	40 days
55	MS15: Final Guidance Document	Tue 3/17/15	Mon 5/11/15	40 days
56	Final Project Debrief Conference Call	Tue 5/12/15	Tue 5/12/15	1 day
57	Project Complete	Mon 5/11/15	Mon 5/11/15	0 days



Project: Project1_071411
Date: Mon 2/4/13

Task | Progress | Milestone | Summary | Rolled Up Task | Rolled Up Milestone | Rolled Up Progress | Split | External Tasks | Project Summary | Group By Summary | Deadline

ATTACHMENT 2

Toxic Substance Control Act Experimental Release Application

Cover Letter

Thank you for considering this TERA application. We look forward to receiving your decision soon. Please feel free to contact me directly if you have any questions or need additional information.

Regards,

Fiona

Fiona H. Crocker, Ph.D.
U.S. Army Engineer Research and Development Center
3909 Halls Ferry Rd.
Vicksburg, MS 39180
601-634-4673

EPA Biotech Form

Biotech Form Report Number	KTR9130412664121970	Mark (X) if anything is CBI ☐

I. SUBMITTER IDENTIFICATION INFORMATION — CBI ☐

First Name	Fiona	Last Name	Crocker
Position	Microbiologist	Company	U.S. Army Engineer Research and
Mailing Address (Number & Street)	3909 Halls Ferry Road		
City	Vicksburg	State	MS
Postal Code	39180		
e-mail	fiona.h.crocker@usace.army.mil	Telephone (include area code)	601-634-4673

Ia. JOINT SUBMITTER -- If you are submitting this notice as part of a joint submission, mark (X) ☐ CBI ☐

First Name		Last Name	
Position		Company	
Mailing Address (Number & Street)			
City		State	
Postal Code			
e-mail		Telephone (include area code)	

II. TECHNICAL CONTACT IDENTIFICATION INFORMATION — CBI ☐

First Name	Mandy	Last Name	Michalsen
Position	Engineer	Company	USACE Seattle District
Mailing Address (Number & Street)	4735 East Marginal Way South		
City	Seattle	State	WA
Postal Code	98134		
e-mail	mandy.m.michalsen@usace.army.mil	Telephone (include area code)	206-764-3324

III. TYPE OF SUBMISSION (Check One)

- ☐ MCAN (Microbial Commercial Activity Notice)
- ☒ TERA (TSCA Experimental Release Application)
- ☐ Tier I Exemption
- ☐ Tier II Exemption
- ☐ Biotech TME (Test Market Exemption)

IV. TEXT / COMMENTS — CBI ☐

We prepared this application using the "points to consider" format. Please find the PDF file "TERA_KTR9.pdf" with all required information attached.

LIST OF ATTACHMENTS

#	Attachment Name	Attachment Filename	Number of Pages	Associated Section Number	CBI
001	TERA_KTR9_12Apr2013	TERA_KTR9.pdf	14		

Mark (X) this box if the data continues on the next page. ☐

ATTACHMENT HEADER SHEET

Attachment Number 001

Attachment Name

TERA_KTR9_12Apr2013

Associated PMN Section Number

N/A

Does not contain CBI

Report Number

KTR9130412664121970

Toxic Substances Control Act Experimental Release Application

PROJECT BACKGROUND

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a common contaminant in soils and groundwater at military sites worldwide. RDX can be mobile and persistent in groundwater under the aerobic conditions present in many aquifers and thus tends to form large, dilute plumes. Although multiple studies have demonstrated *in situ* RDX biodegradation under *anaerobic* conditions, creating and maintaining anaerobic conditions across large areas is costly and technically challenging. The Environmental Security Technology Certification Program (ESTCP) has provided funds to conduct a field demonstration of an innovative application of bioaugmentation to enhance RDX biodegradation in contaminated groundwater under *aerobic* conditions. Project ER-201207 will provide field data to support both a technical evaluation and a cost-benefit analysis of this approach.

The Umatilla Chemical Depot (UMCD) has been identified as an ideal site for the proposed demonstration. At UMCD, RDX is widespread in an aerobic, highly permeable groundwater aquifer. RDX concentrations range from 2 to 300 µg/L over the ~300 acre plume. During this ESTCP demonstration, a mixed microbial culture capable of aerobically biodegrading RDX will be injected into the aerobic RDX plume. In July, 2013 a forced-gradient culture transport experiment will be conducted by injecting a tracer/culture (~10^6 cells ml^{-1}) solution in the bioaugmentation well with extraction pumping and sampling from a downgradient well. This test will confirm that field test parameters determined in 2012 are suitable for distributing cells at the field-scale. In 2014, a field-scale demonstration of the application of the bioaugmentation plot (~100 m^3) will be compared with aerobic and anaerobic biostimulation plots. The demonstration will use low concentration (~10 mM fructose) substrate injections in the aerobic biostimulation and aerobic bioaugmentation field plots and culture injection in the bioaugmentation plot only, followed by high concentration (50-100 mM corn syrup or ethanol) substrate injections in the anaerobic biostimulation field plot. *In situ* RDX degradation rates will be determined by triplicate push-pull tests in injection wells and down gradient wells within the treatment plots twice over a ~ 3 month period. The field-scale demonstration assess bioaugmentation culture transport, viability and *xplA* gene transfer, RDX degradation rates over time, as well as data needed to perform a cost-benefit analysis for implementation of the aerobic bioaugmentation approach for RDX treatment at UMCD.

This application pertains to one of the microbial strains *Gordonia* sp. KTR9, which contains intergeneric DNA in the form of an introduced kanamycin resistance gene to be used in the bioaugmentation culture in the ESTCP demonstration project ER-201207. We are requesting approval to introduce this intergeneric microorganism into the groundwater at UMCD during field trials in July, 2013 and 2014.

IV.A RECIPIENT ORGANISM CHARACTERIZATION

A.1. Taxonomy: General

A.2. Taxonomy: Specific issues

Gordonia sp. KTR9 was isolated from surface soil enrichments originating from the Naval Air Warfare Center Weapons Division, China Lake using a mineral salts medium with RDX as the nitrogen source (21). Soil at China Lake was collected from Site 8, an area that receives surface water runoff and is therefore an active fluvial deposit. This area may have been contaminated with explosive residues in the past; however, no explosive residues were detected at the time of sampling. The surface soil is characterized as a sandy loam soil (78% sand, 6% silt, 16% clay) with a pH of 8.4, an organic carbon content of < 0.1%, and a NO_3-N content of 6 mg kg^{-1}. Strain KTR9 was observed to be Gram-positive, non-motile, rods; it is oxidase-negative, and catalase-positive by standard microbiological techniques. It grows under aerobic conditions in minimal media with ammonium, nitrate, or nitrite as nitrogen sources and can utilize sucrose, fructose, succinate, ethanol, and galactose as carbon sources for growth. KTR9 colonies on a variety of agar media (trypticase-soy, nutrient, Luria-Bertani) are orange, convex, and 0.5-1 mm in diameter. KTR9 was determined to be naturally sensitive to the following types and concentrations of antibiotics: 1 µg/ml Amp, 2 µg/ml Gen, 15 µg/ml Strep, 2µg/ml Kan. Total cellular fatty acids were analyzed by MIDI Laboratories using the Sherlock Microbial Identification system (Microbial ID, Inc., Newark, DE). Comparisons of the total cellular fatty acid profile of KTR9 to the environmental database matched most closely to *Gordonia sputi* with a similarity index of 0.903. The nearly complete 16S rRNA gene sequence (1488 bp) of strain KTR9 was sequenced and compared to selected species within the suborder *Corynebacterineae*, including *Rhodococcus*, *Nocardia*, *Gordonia*, and *Williamsia* species. The nucleotide sequence of the 16S rRNA gene of KTR9 (GenBank accession number DQ068383) was 99% similar to that of *Gordonia terrae*. Since this submission, the complete genome sequence of KTR9 has been determined and annotated (3). The genome sequence is available from GenBank under accession numbers CP002907 (KTR9), CP002908 (pGKT1), CP002112 (pGKT2), and CP002909 (pGKT3).

The KTR9 genome consists of a 5.4-Mbp circular chromosome and three circular plasmids: pGKT1 (89 kbp), pGKT2 (182 kbp), and pGKT3 (172 kbp) (3). KTR9 is similar to other actinomycetes that degrade RDX (4) in that it utilizes XplA, a cytochrome P450, to catalyze the denitration of RDX yielding 4-nitro-2,4-diazabutanal (NDAB), nitrite, ammonium, and carbon dioxide. In KTR9, the *xplAB* genetic locus resides on a 182-kb plasmid, pGKT2, and is sufficient for the degradation of RDX in actinobacteria (14). The *xplAB* genes show a high level of conservation (>98%) at the amino acid sequence level, despite that the *xplAB* complex has been independently isolated from disparate geographical locations. Horizontal gene transfer (HGT) of *xplAB* has been implied from the analysis of DNA sequencing data of this gene locus and associated flanking region (1).

IV.B. SUBJECT ORGANISM CHARACTERIZATION

B.1. Taxonomies of the Subject and Donor Microorganisms

B.2. Final Construct

B.3. Construction of the Subject Microorganism

B.4. Properties of the Subject Microorganism

A detailed description of the creation of *Gordonia* sp. KTR9 pGKT2::Km^r is summarized below as previously described (18). A predicted nonessential coding region of the *Gordonia* sp. KTR9 plasmid pGKT2, found on ORF pGKT2_4819 (Genbank Acc. No. CP002112), with no significant matches in the Genbank database, was targeted for homologous recombination and insertion of a kanamycin resistance (*aph*) marker (NP_478145.1). This kanamycin gene encodes an aminoglycoside 3'-phosphotransferase (APH) that is identical to one found in *Corynebacterium diphtheriae* but is also found on various transposons and plasmids which are thought to have originated as a self-defense mechanism used by microorganisms that produce the antibiotics (Blast search result). This site is approx. 40 kb downstream from the *xplAB* gene complex. The *aph* gene, flanked by 1-kb regions specific to pGKT2, was synthesized by Celtek Biosciences, LLC (Nashville, TN, USA), into a pCR2.1 vector (Invitrogen, Carlsbad, CA, USA). The Km^r construct containing the KTR9 flanking regions (2.9 kb) was liberated from the plasmid by digestion with BamHI (New England BioLabs, Inc., Ipswich, MA, USA) and gel purified with a Wizard SV Gel and PCR Clean-up System Kit (Promega, Madison, WI, USA). The purified 2.9-kb fragment was ligated into a BamHI-cut mobilizable vector, pK18mobsacB (20,22) and transformed into One Shot Top10 Chemically Competent *Escherichia coli* cells (Invitrogen) that were selected for on Luria–Bertani (LB) plates with 50 $\mu g\ ml^{-1}$ kanamycin. Recombinant pK18mobsacB was introduced from Top10 *E. coli* cells into *Gordonia* sp. KTR9 based on the conjugation strategy presented by van der Geize et al (2001). Double crossover transconjugants of the Km^r marker from pK18mobsacB into pGKT2 via homologous flanking regions were selected for on LB agar plates with 50 $\mu g\ ml^{-1}$ kanamycin and 10% sucrose. Each step was screened by polymerase chain reaction (PCR) targeting the Km^r construct and various locations of the pGKT2 and pK18mobsacB plasmids to confirm their presence or, in the case of pK18mobsacB, its absence.

The physiology of KTR9 was characterized with respect to the rates of RDX degradation when RDX was supplied as a sole nitrogen source (13,14). KTR9 degraded 180 µM RDX within 48 h when RDX served as the only added nitrogen source. Additional physiological studies examining the effects of exogenous inorganic nitrogen sources on RDX degradation in strain KTR9 revealed that ammonium, nitrite, and nitrate each (4 mM) inhibited RDX degradation by up to 79%. Parallel transcriptome studies indicated that nitrogen availability was a major determinant of RDX degradation and *xplA* gene expression in KTR9, with RDX degradation/*xplA* gene expression highest under nitrogen limiting conditions.

.

IV.C. POTENTIAL HUMAN HEALTH EFFECTS OF THE SUBJECT MICROORGANISM

C.1. Pathogenicity of Subject Microorganism

C.2. Toxicity and Immunological Effects of Subject Microorganism or Its Products

Gordonia sp. KTR9 is not known to be a pathogen. In general, *Gordonia* species are metabolically diverse and possess the metabolic capabilities to degrade a variety of xenobiotic compounds (2,11). These species are also widely distributed in natural environments with many species isolated from soil, water, marine sediments, plants, animals, insects, and industrial applications. In very limited cases have some members of the *Gordonia* species been associated

with human disease, often in the setting of intravascular or catheter-related infections. *Gordonia* infections in humans are rare and usually affect immunocompromised patients (16,17,19). The complete genome sequence of *Gordonia bronchialis* type strain, an isolate associated with human disease, is available at the NCBI genome database (15). Currently, no known microbial virulence factors have been described for these organisms.

IV.D. PREDICTED ENVIRONMENTAL EFFECTS AND FATE OF SUBJECT MICROORGANISM

D.1. Ecological Effects

Gordonia species are metabolically diverse and possess the metabolic capabilities to degrade a variety of xenobiotic compounds (2,11). These species are also widely distributed in natural environments with many species isolated from soil, water, marine sediments, plants, animals, insects, and industrial applications. Thus, *Gordonia* species are considered to have important roles in nutrient cycling, a potential to be used for the beneficial remediation of pollutants, and for the production of metabolites of biotechnological importance.

There are no studies with KTR9 regarding pathogenicity, virulence, or infectivity to mammals, fish, insects, invertebrates, and plants. In addition, they have not been tested for the production of toxins to these organisms or prokaryotes. *Gordonia* are known to metabolize a variety of nitrogen and sulfur containing organic compounds and so probably have a role in the cycling of such organic compounds in soil and water. However, we have not examined the effects of KTR9 on nitrogen, sulfur, or phosphorus cycling, or CO_2-fixation.

The *Gordonia* sp. KTR9 recipient strain is known to utilize several carbohydrates, ethanol, and acetone for growth. Strain KTR9 was first isolated for its ability to use RDX as a sole nitrogen source for growth (21). It is unable to use other explosives, such as HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) or CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane), as nitrogen sources for growth. Strain KTR9 has been shown to degrade RDX by the highly conserved enzymes, XplA and XplB. The genes encoding these enzymes have been shown to be globally distributed among strains of *Rhodococcus, Gordonia, Williamsia, and Microbacterium*, which have been isolated from RDX contaminated soils and groundwater (1). The degradation of RDX by XplA and XplB proceeds by the enzymatic removal of 2 nitro-groups from the molecule and subsequent abiotic ring cleavage (7). End products of the reaction include 4-nitro-2,4-diazabutanal (NDAB), nitrite, formaldehyde, and ammonium. The nitrite and ammonium are used as nitrogen sources for growth while the formaldehyde is subsequently transformed to carbon dioxide by the strains. The NDAB accumulates in cultures of these actinomycetes and accounts for 64% of the carbon mass balance. NDAB is transformed by soil microorganisms, including a *Methylobacterium* species (9) and *Phanerochaete chrysosporium* (8)so that it is unlikely to accumulate in the natural environment. Furthermore, NDAB has been shown to be non-toxic via a luminescence bioassay (Fournier et al. 2004). At UMCD, RDX is present at concentrations ranging from 2 to 300 $\mu g\ L^{-1}$ over the ~300 acre plume. During the cell transport field test that will occur in July, 2013, the expected concentration of formaldehyde produced by the subject strain is about 0.27 to 40.5 $\mu g\ L^{-1}$. In 2014, the field-scale demonstration of the bioaugmentation with the subject strain will

use an RDX concentration of 1000 μg L^{-1} and thus approximately 135 μg L^{-1} of formaldehyde is expected to be produced. These formaldehyde concentrations are well below the 10 mg L^{-1}, 5 mg L^{-1}, and 1 mg L^{-1} drinking water limits set by EPA for 1 day, 10 day, and lifetime exposure, respectively (Agency for Toxic Substances and Disease Registry (ATSDR). 2008. Toxicological profile for Formaldehyde. (*Draft for Public Comment*). Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service). Based on these results the addition of the subject microorganism to the groundwater at UMCD is not expected to produce toxic end products of RDX biotransformation. Based on these results the addition of the subject microorganism to the groundwater at UMCD is not expected to produce toxic end products of RDX biotransformation.

D.2. Survival and Fate

The subject microorganism was grown at 30°C in a basal salts medium containing 50 mM fructose as the carbon source and 18 mM ammonium as the nitrogen source. The cell suspension was washed and resuspended in artificial groundwater (AGW) and then starved at 15°C to reduce residual nitrogen levels. After 24 h of starvation, RDX and fructose were added to a final concentration of approximately 5.5 μM and 1 mM, respectively. Triplicate cultures were incubated at 15 °C with shaking at 120 rpm. Cell viability and RDX concentrations were determined periodically for 7 days. Cell viability was determined by plate counts on agar media, while RDX concentrations were determined by HPLC (5).

The sediment microcosms consisted of 2 g of Umatilla sediment (2 mm sieved) plus 1 ml of artificial groundwater (AGW). The AGW was amended with RDX at 1.1 mg L^{-1} + 1 mM fructose (1.8 mg L^{-1}) and 1 x 10^6 cells ml^{-1} of each bacterial culture (*Gordonia* sp. KTR9 pGKT2:Km^+; *Rhodococcus jostii* RHA1 pGKT2:Km^+; *Pseudomonas fluorescens* IC). The bacterial cultures were grown and starved as described above. One set of microcosms was amended with only the AGW + RDX + fructose solution (uninoculated). Microcosms were incubated at 15°C and periodically sacrificed for analysis of RDX concentrations and viable cell numbers.

The subject microorganism survived for 7 days in the AGW broth studies and degradation of the RDX was complete within 1-2 days. Initial cell densities were between 1.4 and 2.5 x 10^8 CFU ml^{-1} and had increased slightly to between 1.7 and 2.7 x 10^8 CFU ml^{-1} by day 7. Similarly, the subject microorganism remained viable during a 7 day incubation in UMCD sediment microcosms at 15°C. Initial cell densities were between 4.5 and 15 x 10^5 CFU ml^{-1} and increased to approximately 6.8 x 10^6 CFU ml^{-1} by day 7. Degradation of RDX was rapid in the inoculated microcosms, with approximately 96% of the RDX being consumed after 1 day. In the uninoculated microcosms RDX loss was significantly slower with a lag period of 4 days and a loss of only 21% by day 7.

Subsequent to these studies, UMCD sediment columns were designed to test the transport and activity of both subject microorganisms. The columns were set up as described previously (6,10). Two identical columns were wet-packed to a bulk density of ~1.6 g cm^{-3} with the UMCD sediment and AGW containing RDX at 1 mg L^{-1} was pumped into the bottom and exited at the top of the columns. Once stable conditions were reached (e.g., influent RDX = effluent RDX), the column was injected with the bacterial inoculum plus 0.1 mM fructose, followed by a continuous flow of AGW. The starved inoculum (prepared as above) was composed of the 3

microorganisms, indicated above in the sediment microcosms, each at a cell density of 1 x 10^8 cells ml^{-1}. The effluent concentrations of RDX and cell densities have been followed for up to 6 weeks with 2 separate additions of fructose following the initial fructose addition. Effluent concentrations of cells stabilized at approximately 10^3 CFU ml^{-1} and RDX concentrations decreased with each addition of fructose, with rates of RDX loss between 1.58 and 1.73 $mgL^{-1}d^{-1}$ for each substrate addition. The effluent concentration of cells along with the rates of RDX degradation indicate that a viable population of the 3 inoculated microorganisms exists within the column sediment matrix, but this concentration has not been determined.

We have not examined the ability of either the recipient or subject microorganisms in soil or aquatic media other than media from UMCD.

With regards to conditions favorable for growth of the subject microorganism, it can be grown in minimal medium with 4 mM ammonium and 20 mM succinate with no added vitamins or growth factors necessary. A complete temperature range for growth has not been determined for KTR9, but growth between 15 and 37°C is possible.

We will use standard plating methods to detect viable numbers of the subject microorganism at the UMCD field site. The agar medium will be 20% LB (tryptone, 2 g L^{-1}; glucose, 1 g L^{-1}; and sodium chloride, 1 g L^{-1}) + 50 mgL-1 kanamycin. The detection limits on this medium should be approximately 300 CFU ml^{-1}. In addition, we will use quantitative polymerase chain reaction (qPCR) to detect the presence of the RDX biodegradative gene, *xplA,* in the groundwater. The qPCR assay is based on a TaqMan™ format using *xplA* primers and probes designed in our SERDP project ER-1609 (12). The detection limit of this assay is approximately 300 gene copies L^{-1} of filtered groundwater.

We do not have any information regarding the potential for conjugal transfer of the pGKT2:Km^R plasmid from either subject microorganism to indigenous bacteria in soil or water. However, in filter paper matings in the laboratory *Gordonia polyisoprenivorans*, *Rhodococcus jostii* RHA1, and *Nocardia* sp. TW2 acquired pGKT2:Km^R at frequencies of 5×10^{-4}, 4×10^{-5}, and 7×10^{-6} transconjugants per recipient, respectively (18). Conjugal transfer to 14 closely related actinomycete strains, an *E. coli* strain, and 2 *Pseudomonas* strains did not occur (18).

IV.E. PREDICTED PRODUCTION VOLUME, BYPRODUCTS, USE, AND CONSUMER EXPOSURE

E.1. Information on production volume

The total production volume for this project is not expected to exceed 100 L per stain, depending on achievable cell densities per batch. Estimated maximum cell densities of 10^{10} cells/ml are expected based on initial data. The total cells of each strain that are being produced for use during the field testing has been set at 10^{14} per year. Production time is expected to be 1 week per batch per strain, and one batch per year of each strain will be produced.

E.2. Information on Byproducts

No specified proteins, DNA, or other byproducts are being produced during this project. Cells are grown under standard fermentation conditions. Cells are separated from culture broth and

waste products during concentration steps. Any wastes liquid and biomass is disposed of according to local ordinances (e.g., sterilized and directed into a municipal sanitary sewer).

E.3. Use Information and Consumer Exposure
Cultures are being used solely for field demonstration (pilot scale) research and evaluation purposes, and are not being produced for any commercial or consumer use.

IV.F. PREDICTED RELEASES DUE TO MANUFACTURING OF THE SUBJECT MICROORGANISM, AND WORKER AND CONSUMER EXPOSURES TO THE SUBJECT MICROORGANISM

F.1. Industrial Sites Controlled by the Submitter
Culture production is being done at a small pilot scale for research and development purposes only. No commercial production of industrial-scale amounts is being performed, therefore this work does not fall under the pre-manufacture notice (PMN) requirements.

F.2. Industrial Sites Controlled by Others
No culture production is being performed by anyone other than the submitter.

IV.G. INFORMATION APPLICABLE TO FIELD TESTS OF THE SUBJECT MICROORGANISM

G.1. Objectives

RDX is a common contaminant in soils and groundwater at military sites worldwide. RDX can be mobile and persistent in groundwater under the aerobic conditions present in many aquifers and thus tends to form large, dilute plumes. Although multiple studies have demonstrated *in situ* RDX biodegradation under *anaerobic* conditions, creating and maintaining anaerobic conditions across large areas is costly and technically challenging. The Environmental Security Technology Certification Program (ESTCP) has provided funds to conduct a field demonstration of an innovative application of bioaugmentation to enhance RDX biodegradation in contaminated groundwater under *aerobic* conditions. The primary objective is to demonstrate that aerobic bioaugmentation with *Gordonia* sp. KTR9 promotes aerobic RDX degradation in groundwater. Project ER-201207 will provide field data to support both a technical evaluation and a cost-benefit analysis of this approach.

G.2. Nature of the site

The Umatilla Chemical Depot (UMCD) has been identified as an ideal demonstration site. Explosives-contaminated sludge and liquid wastes generated at the UMCD Washout Plant in Hermiston, OR were discharged to unlined lagoons, where infiltration through ~ 50 ft of unsaturated site soil to groundwater resulted in a ~ 300 acre RDX groundwater plume. Maximum RDX and TNT concentrations within the plume have been ~ 200 µg/L and 70 µg/L, respectively, in recent years. A groundwater pump and treat facility with activated carbon adsorption began full-time operations at the site in 1997. Treatment efficiency of the pump and treat remedy has

significantly declined over the years, which has prompted consideration of bioremediation technology for its potential to optimize the existing remedy.

The unconfined aquifer at UMCD consists of alluvial deposits and the weathered surface of the Elephant Mountain Member basalt, overlain by approximately 50 to 120 feet of unsaturated alluvial sand and gravel. The saturated thickness of the aquifer in the former lagoon area is approximately 15 to 35 feet. The nearest surface water body to site is the Umatilla River, which is over 2 miles away. Groundwater gradients are very small in this highly permeable aquifer, which translates to very slow moving groundwater under ambient conditions. An aquifer recharge program was initiated near the site in October 2011, which currently involves injection of ~ 10,000 acre-feet of water. This program resulted in ~ 3 ft increased groundwater elevations. However, there is no evidence that the groundwater gradient and flow direction has appreciably changed appreciably as a result.

G.3. Field test design

This demonstration will be performed in three phases. Phase I consists of field site characterization and laboratory testing to select a suitable bioaugmentation culture and to optimize conditions that facilitate growth, RDX-degrading activity, and cell transport under field conditions at the UMCD. Phase II consists of a forced-gradient cell transport test to confirm ability to distribute cells at field-scale (~ 60 feet). Phase III consists of a bioaugmentation demonstration with subsequent push-pull tests to obtain field-scale performance data on bioaugmentation culture transport, viability, and xplA gene transfer, as well as RDX degradation rates. The demonstration will be conducted in two field plots, one for aerobic bioaugmentation only and the other for sequential evaluation of aerobic and anaerobic biostimulation (no cells injected). RDX degradation rates and mass removed per mass of substrate added for the aerobic bioaugmentation treatment will be compared with non-bioaugmented aerobic and anaerobic biostimulation treatments and will form the basis of a cost-benefit analysis for the innovative aerobic bioaugmentation approach for in situ treatment of RDX in groundwater.

Bioaugmentation tests will be conducted during demonstration Phases II and III as follows.

Phase II: Forced Gradient Cell Transport Test. Site groundwater will be pumped into a plastic tank next to the demonstration well (Demonstration Well 2 (DW2), Figure 1). Sufficient NaCl or KBr will be added to each tank to achieve a Cl- or Br- tracer concentration of ~ 100 mg/L in 3800 L. Next, the *Gordonia* sp. KTR9 culture will be extruded from the leak-proof 20 L stainless steel soda kegs into the plastic tank. The bioaugmentation solution will then be thoroughly mixed using a recirculation pump to vigorously agitate the water. Extraction well EW-4 will be used to control the groundwater gradient/flow direction during the cell transport test. The bioaugmentation solution will be injected into DW2 using the same pump and tubing used during mixing, followed by monitoring for cell "breakthrough curves" at down gradient monitoring wells 4-106 and EW-2 (Figure 1) for an estimated period of 10 days. This test will conclude Summer 2013.

Phase III: Natural Gradient Bioaugmentation Test. Site groundwater (~3800 L) will be pumped into a plastic tank next to the demonstration wells DW2, 4-102 and EW2 (Figure 1). Next, the

Gordonia sp. KTR9 culture will be extruded from the leak-proof 20 L stainless steel soda kegs into the plastic tanks. The bioaugmentation solution will then be thoroughly mixed using a recirculation pump to vigorously agitate the water. The bioaugmentation solution will be injected into DW2 using the same pump and tubing used during mixing, followed by a push-pull tests to quantify RDX degradation rate in the "bioaugmented" test wells. The "bioaugmented" wells will then receive periodic low concentration growth substrate (1 mM fructose) on a biweekly basis for up to 3 months. A final series of push-pull tests will be conducted to determine RDX degradation rates in these wells 3 months following bioaugmentation. This bioaugmentation test will conclude Summer 2014.

G.4. On-site containment practices

Cultures will be delivered to the field in leak-proof 20 L stainless steel soda kegs. Kegs will be shipped to the field in sealed plastic coolers via a common carrier in accordance with all applicable regulations. All equipment, tanks and tubing that contact the culture will be disinfected using an appropriate sterilizing agent (e.g. bleach or hydrogen peroxide) prior to disposal. All personnel will use appropriate personal protective equipment and other applicable safety procedures for work at CERCLA sites.

Testing will occur in a small demonstration test plot < 200 ft long. The nearest surface water body (Umatilla River) is over 2 miles away so no surface water interception of test water is not expected. The ambient groundwater gradient is very small and groundwater seepage velocities are very low; rapid transport of groundwater from the test plot is not expected. The existing groundwater pump & treat system could be utilized to control the gradient during/following the bioaugmentation demonstration if needed.

G.5. Application methods

Cultures will be extruded from the 20 L soda kegs into plastic tanks filled with site groundwater. The bioaugmentation solution will be mixed via recirculating pump. The solution will then be injected into the site well using this same pump and tubing.

G.6. Termination and mitigation procedures

Soda kegs will be placed back in their plastic bags and sealed in plastic coolers and shipped in accordance with applicable regulations. All equipment, tanks and tubing that contact the culture will be disinfected using an appropriate sterilizing agent (e.g. bleach or hydrogen peroxide). Prior to adding the strain to the groundwater tank, all connections and tubing associated with the mixing and injection will be tested to ensure there are no leaks. Plastic sheeting will be placed around the tank opening where the culture will be added, and on the ground surrounding the tank/injection area to ensure any minor spills are contained to the test area and can be disinfected prior to disposal of materials.

G.7. Monitoring endpoints & procedures for isolating subject Microorganism

Groundwater samples will be collected and analyzed using microbial culturing and molecular methods during both Phase II and III bioaugmentation tests. Monitoring endpoints from Phase III will indicate cell counts per unit of site groundwater present at the conclusion of the test. Testing will occur in a small demonstration test plot < 200 ft long. The nearest surface water body (Umatilla River) is over 2 miles away so no interception of test water is expected. The ambient groundwater gradient is very small and groundwater seepage velocities are very low; rapid transport of groundwater from the test plot is not expected. The existing groundwater pump & treat system could be utilized to control the gradient during/following the bioaugmentation demonstration if needed.

G.8. Sampling procedure

Groundwater samples will be collected from wells DW2, 4-102 and EW2 during Phases II and III of the demonstration using low-flow groundwater sampling procedures and submersible pumps. During Phase II, samples will be collected multiple times per day over a ~ 10 day period. During Phase III, high-resolution time series sampling will be conducted during the initial push-pull test completed immediately following biaugmentation (~ hourly sampling over ~ 36 hour time period). Biweekly sampling will occur during the ~ 3 month growth substrate "feeding" period, which begins when the initial push-pull test concludes. High-resolution time series sampling (~ hourly sampling over ~ 36 hour time period) will be conducted during the final push-pull test completed following the 3 month "feeding" period. The test will conclude following completion of this final push-pull test. All equipment, tanks and tubing that contact the culture will be disinfected using an appropriate sterilizing agent (e.g. bleach or hydrogen peroxide) prior to disposal. All personnel will use appropriate personal protective equipment and other applicable safety procedures for work at CERCLA sites. The kanamycin resistance gene permits this strain to be quantified on kanamycin-containing auger plates. Copies of the xplA gene will be tracked using quantitative PCR. All samples will be immediately placed on ice and shipped via overnight shipping for laboratory processing. Colony forming units and gene copy numbers over time in site groundwater samples will be utilized to determine strain survival *in situ*. This information will be used to interpret RDX transformation results overtime.

G.9. Record keeping & reporting test results

All record keeping will be in accordance with the project demonstration work plan. Results will be report to the ESTCP program, the UMCD project team including EPA, as well as published in peer reviewed journal articles.

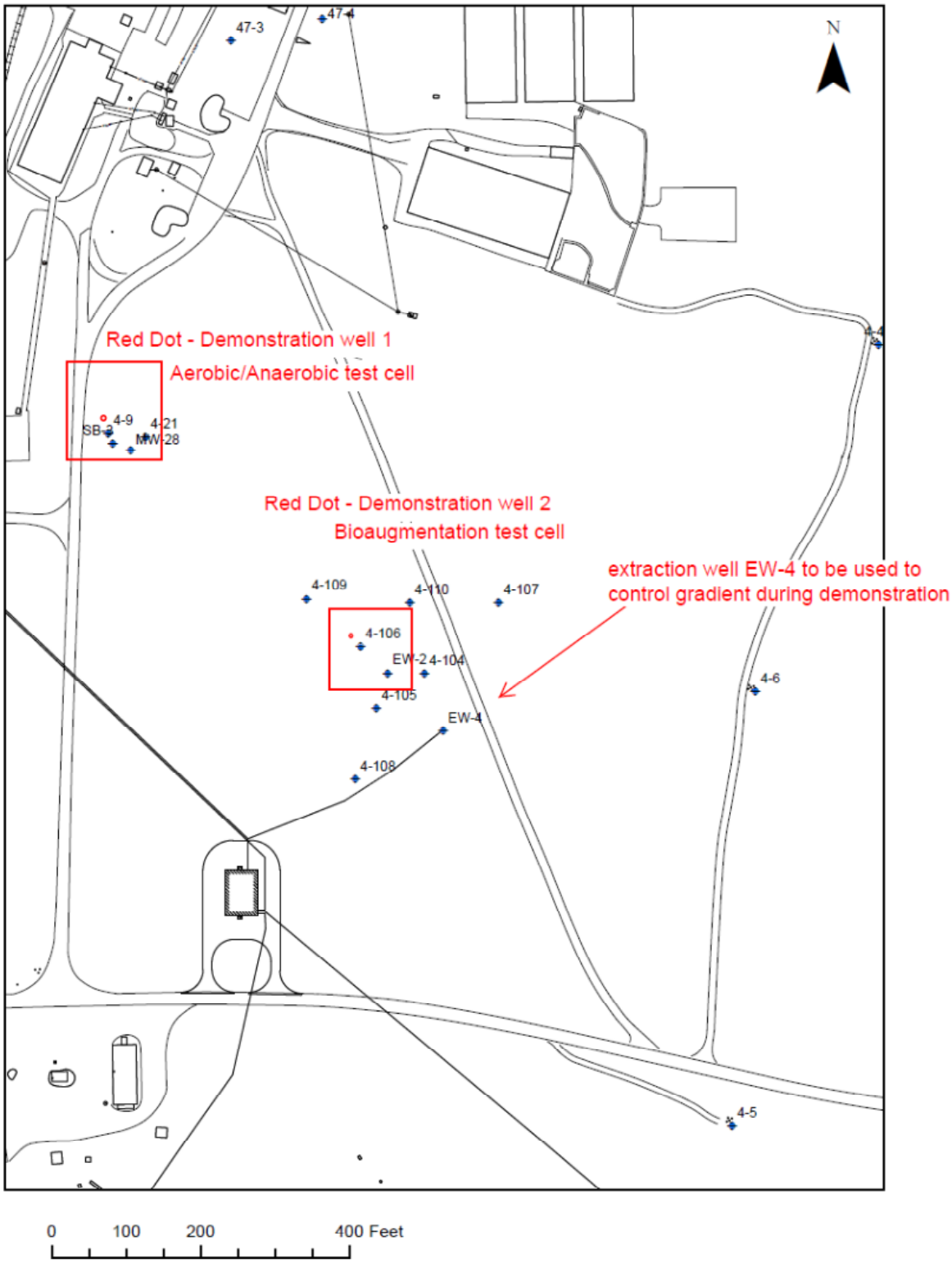


Figure 1. Demonstration well locations in test plots 1 and 2 are shown as red circles. Bioaugmentation would occur in test plot 2 only. Demonstration well 2 is ~ 10ft from downgradient monitoring well 4-106 and ~ 60 ft from well EW-2.

Reference List

1. **Andeer, P. F., D. A. Stahl, N. C. Bruce, and S. E. Strand**. 2009. Lateral transfer of genes for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degradation. Appl.Environ.Microbiol. **75**:3258-3262. doi:AEM.02396-08 [pii];10.1128/AEM.02396-08 [doi].

2. **Arenskotter, M., D. Broker, and A. Steinbuchel**. 2004. Biology of the metabolically diverse genus Gordonia. Appl.Environ.Microbiol. **70**:3195-3204. doi:10.1128/AEM.70.6.3195-3204.2004 [doi];70/6/3195 [pii].

3. **Chen, H. P., S. H. Zhu, I. Casabon, S. J. Hallam, F. H. Crocker, W. W. Mohn, K. J. Indest, and L. D. Eltis**. 2012. Genomic and transcriptomic studies of an RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine)-degrading actinobacterium. Appl.Environ.Microbiol. **78**:7798-7800. doi:AEM.02120-12 [pii];10.1128/AEM.02120-12 [doi].

4. **Crocker, F. H., K. J. Indest, and H. L. Fredrickson**. 2006. Biodegradation of the cyclic nitramine explosives RDX, HMX, and CL-20. Appl.Microbiol.Biotechnol. **73**:274-290. doi:10.1007/s00253-006-0588-y [doi].

5. **Crocker, F. H., K. T. Thompson, J. E. Szecsody, and H. L. Fredrickson**. 2005. Biotic and abiotic degradation of CL-20 and RDX in soils. J.Environ.Qual. **34**:2208-2216. doi:34/6/2208 [pii];10.2134/jeq2005.0032 [doi].

6. **Dong, H., R. Rothmel, T. C. Onstott, M. E. Fuller, M. F. DeFlaun, S. H. Streger, R. Dunlap, and M. Fletcher**. 2002. Simultaneous transport of two bacterial strains in intact cores from Oyster, Virginia: biological effects and numerical modeling. Appl.Environ.Microbiol. **68**:2120-2132.

7. **Fournier, D., A. Halasz, J. Spain, P. Fiurasek, and J. Hawari**. 2002. Determination of key metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine with Rhodococcus sp. strain DN22. Appl.Environ.Microbiol. **68**:166-172.

8. **Fournier, D., A. Halasz, J. Spain, R. J. Spanggord, J. C. Bottaro, and J. Hawari**. 2004. Biodegradation of the hexahydro-1,3,5-trinitro-1,3,5-triazine ring cleavage product 4-nitro-2,4-diazabutanal by Phanerochaete chrysosporium. Appl.Environ.Microbiol. **70**:1123-1128.

9. **Fournier, D., S. Trott, J. Hawari, and J. Spain**. 2005. Metabolism of the aliphatic nitramine 4-nitro-2,4-diazabutanal by Methylobacterium sp. strain JS178. Appl.Environ.Microbiol. **71**:4199-4202. doi:71/8/4199 [pii];10.1128/AEM.71.8.4199-4202.2005 [doi].

10. **Fuller, M. E., S. H. Streger, R. K. Rothmel, B. J. Mailloux, J. A. Hall, T. C. Onstott, J. K. Fredrickson, D. L. Balkwill, and M. F. DeFlaun**. 2000. Development of a vital fluorescent staining method for monitoring bacterial transport in subsurface environments. Appl.Environ.Microbiol. **66**:4486-4496.

11. **Gurtler, V., B. C. Mayall, and R. Seviour**. 2004. Can whole genome analysis refine the taxonomy of the genus Rhodococcus? FEMS Microbiol.Rev. **28**:377-403.

12. **Indest, K. J., F. H. Crocker, and R. Athow**. 2007. A TaqMan polymerase chain reaction method for monitoring RDX-degrading bacteria based on the xplA functional gene. J.Microbiol.Methods **68**:267-274. doi:S0167-7012(06)00250-8 [pii];10.1016/j.mimet.2006.08.008 [doi].

13. **Indest, K. J., D. E. Hancock, C. M. Jung, J. O. Eberly, W. W. Mohn, L. D. Eltis, and F. H. Crocker**. 2013. Role of nitrogen limitation in transformation of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) by Gordonia sp. strain KTR9. Appl.Environ.Microbiol. **79**:1746-1750. doi:AEM.03905-12 [pii];10.1128/AEM.03905-12 [doi].

14. **Indest, K. J., C. M. Jung, H. P. Chen, D. Hancock, C. Florizone, L. D. Eltis, and F. H. Crocker**. 2010. Functional characterization of pGKT2, a 182-kilobase plasmid containing the xplAB genes, which are involved in the degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine by Gordonia sp. strain KTR9. Appl.Environ.Microbiol. **76**:6329-6337. doi:AEM.01217-10 [pii];10.1128/AEM.01217-10 [doi].

15. **Ivanova, N., J. Sikorski, M. Jando, A. Lapidus, M. Nolan, S. Lucas, T. G. Del Rio, H. Tice, A. Copeland, J. F. Cheng, F. Chen, D. Bruce, L. Goodwin, S. Pitluck, K. Mavromatis, G. Ovchinnikova, A. Pati, A. Chen, K. Palaniappan, M. Land, L. Hauser, Y. J. Chang, C. D. Jeffries, P. Chain, E. Saunders, C. Han, J. C. Detter, T. Brettin, M. Rohde, M. Goker, J. Bristow, J. A. Eisen, V. Markowitz, P. Hugenholtz, H. P. Klenk, and N. C. Kyrpides**. 2010. Complete genome sequence of Gordonia bronchialis type strain (3410). Stand.Genomic Sci. **2**:19-28. doi:10.4056/sigs.611106 [doi].

16. **Jannat-Khah, D. P., E. S. Halsey, B. A. Lasker, A. G. Steigerwalt, H. P. Hinrikson, and J. M. Brown**. 2009. Gordonia araii infection associated with an orthopedic device and review of the literature on medical device-associated Gordonia infections. J.Clin.Microbiol. **47**:499-502. doi:JCM.01504-08 [pii];10.1128/JCM.01504-08 [doi].

17. **Johnson, J. A., A. B. Onderdonk, L. A. Cosimi, S. Yawetz, B. A. Lasker, S. J. Bolcen, J. M. Brown, and F. M. Marty**. 2011. Gordonia bronchialis bacteremia and pleural infection: case report and review of the literature. J.Clin.Microbiol. **49**:1662-1666. doi:JCM.02121-10 [pii];10.1128/JCM.02121-10 [doi].

18. **Jung, C. M., F. H. Crocker, J. O. Eberly, and K. J. Indest**. 2011. Horizontal gene transfer (HGT) as a mechanism of disseminating RDX-degrading activity among Actinomycete bacteria. J.Appl.Microbiol. **110**:1449-1459. doi:10.1111/j.1365-2672.2011.04995.x [doi].

19. **Kageyama, A., S. Iida, K. Yazawa, T. Kudo, S. Suzuki, T. Koga, H. Saito, H. Inagawa, A. Wada, R. M. Kroppenstedt, and Y. Mikami**. 2006. Gordonia araii sp. nov. and Gordonia effusa sp. nov., isolated from patients in Japan. Int.J.Syst.Evol.Microbiol. **56**:1817-1821. doi:56/8/1817 [pii];10.1099/ijs.0.64067-0 [doi].

20. **Schafer, A., A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, and A. Puhler**. 1994. Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium glutamicum. Gene **145**:69-73.

21. **Thompson, K. T., F. H. Crocker, and H. L. Fredrickson**. 2005. Mineralization of the cyclic nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine by Gordonia and Williamsia spp. Appl.Environ.Microbiol. **71**:8265-8272. doi:71/12/8265 [pii];10.1128/AEM.71.12.8265-8272.2005 [doi].

22. **van der Geize, R., G. I. Hessels, G. R. van, P. van der Meijden, and L. Dijkhuizen**. 2001. Unmarked gene deletion mutagenesis of kstD, encoding 3-ketosteroid Delta1-dehydrogenase, in Rhodococcus erythropolis SQ1 using sacB as counter-selectable marker. FEMS Microbiol.Lett. **205**:197-202. doi:S0378109701004645 [pii].

Cover Letter

Thank you for considering this TERA application. We look forward to receiving your decision soon. Please feel free to contact me directly if you have any questions or need additional information.

Regards,

Fiona

Fiona H. Crocker, Ph.D.
U.S. Army Engineer Research and Development Center
3909 Halls Ferry Rd.
Vicksburg, MS 39180
601-634-4673

EPA Biotech Form

Biotech Form Report Number	RHA1130412320404292	Mark (X) if anything is CBI ☐

I. SUBMITTER IDENTIFICATION INFORMATION — CBI ☐

First Name	Fiona	Last Name	Crocker
Position	Microbiologist	Company	U.S. Army Engineer Research and
Mailing Address (Number & Street)	3909 Halls Ferry Road		
City	Vicksburg	State	MS
Postal Code	39180		
e-mail	fiona.h.crocker@usace.army.mil	Telephone (include area code)	601-634-4673

Ia. JOINT SUBMITTER -- If you are submitting this notice as part of a joint submission, mark (X) ☐ CBI ☐

First Name		Last Name	
Position		Company	
Mailing Address (Number & Street)			
City		State	
Postal Code			
e-mail		Telephone (include area code)	

II. TECHNICAL CONTACT IDENTIFICATION INFORMATION — CBI ☐

First Name	Mandy	Last Name	Michalsen
Position	Engineer	Company	U.S. Army Corps of Engineers
Mailing Address (Number & Street)	4735 East Marginal Way South		
City	Seattle	State	WA
Postal Code	98134		
e-mail	mandy.m.michalsen@usace.army.mil	Telephone (include area code)	206-764-3324

III. TYPE OF SUBMISSION (Check One)

- ☐ MCAN (Microbial Commercial Activity Notice)
- ☒ TERA (TSCA Experimental Release Application)
- ☐ Tier I Exemption
- ☐ Tier II Exemption
- ☐ Biotech TME (Test Market Exemption)

IV. TEXT / COMMENTS — CBI ☐

We prepared this application using the "points to consider" format. Please find the PDF file "TERA_RHA1.pdf" with all required information attached.

LIST OF ATTACHMENTS

#	Attachment Name	Attachment Filename	Number of Pages	Associated Section Number	CBI
001	TERA_RHA1	TERA_RHA1.pdf	0		

Mark (X) this box if the data continues on the next page. ☐

ATTACHMENT HEADER SHEET

Attachment Number 001

Attachment Name

TERA_RHA1

Associated PMN Section Number

N/A

Does not contain CBI

Report Number

RHA1130412320404292

Toxic Substances Control Act Experimental Release Application

PROJECT BACKGROUND

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a common contaminant in soils and groundwater at military sites worldwide. RDX can be mobile and persistent in groundwater under the aerobic conditions present in many aquifers and thus tends to form large, dilute plumes. Although multiple studies have demonstrated *in situ* RDX biodegradation under *anaerobic* conditions, creating and maintaining anaerobic conditions across large areas is costly and technically challenging. The Environmental Security Technology Certification Program (ESTCP) has provided funds to conduct a field demonstration of an innovative application of bioaugmentation to enhance RDX biodegradation in contaminated groundwater under *aerobic* conditions. Project ER-201207 will provide field data to support both a technical evaluation and a cost-benefit analysis of this approach.

The Umatilla Chemical Depot (UMCD) has been identified as an ideal site for the proposed demonstration. At UMCD, RDX is widespread in an aerobic, highly permeable groundwater aquifer. RDX concentrations range from 2 to 300 µg/L over the ~300 acre plume. During this ESTCP demonstration, a mixed microbial culture capable of aerobically biodegrading RDX will be injected into the aerobic RDX plume. In July, 2013 a forced-gradient culture transport experiment will be conducted by injecting a tracer/culture (~10^6 cells ml^{-1}) solution in the bioaugmentation well with extraction pumping and sampling from a downgradient well. This test will confirm that field test parameters determined in 2012 are suitable for distributing cells at the field-scale. In 2014, a field-scale demonstration of the application of the bioaugmentation plot (~100 m^3) will be compared with aerobic and anaerobic biostimulation plots. The demonstration will use low concentration (~10 mM fructose) substrate injections in the aerobic biostimulation and aerobic bioaugmentation field plots and culture injection in the bioaugmentation plot only, followed by high concentration (50-100 mM corn syrup or ethanol) substrate injections in the anaerobic biostimulation field plot. *In situ* RDX degradation rates will be determined by triplicate push-pull tests in injection wells and down gradient wells within the treatment plots twice over a ~ 3 month period. The field-scale demonstration assess bioaugmentation culture transport, viability and *xplA* gene transfer, RDX degradation rates over time, as well as data needed to perform a cost-benefit analysis for implementation of the aerobic bioaugmentation approach for RDX treatment at UMCD.

This application pertains to one of the microbial strains that are proposed to be used in the bioaugmentation culture in the ESTCP demonstration project ER-201207. The intergeneric microorganism *Rhodococcus jostii* RHA1 pGKT2:Km^+ contains a conjugative plasmid, pGKT2, which encodes genes for the degradation of RDX. The recipient *Rhodococcus jostii* RHA1 is a soil bacterium originally isolated from herbicide contaminated soil. We are requesting approval to introduce this intergeneric microorganism into the groundwater at UMCD during field trials in July, 2013 and 2014.

IV.A RECIPIENT ORGANISM CHARACTERIZATION

Rhodococcus jostii RHA1 was isolated from soil contaminated with the insecticide, γ-hexachlorocyclohexane (18) and is best known for its ability to degrade aromatic hydrocarbons, steroids, and polychlorinated biphenyl compounds (15). On the basis of its cell morphology, fatty acid composition, and other physiological traits, strain RHA1 was assigned to the genus *Rhodococcus* (18). Rhodococci are generally Gram-positive, non-motile, aerobic bacteria with a rod/cocci life cycle. On the basis of polyphasic taxonomic data this genus is placed in the sub-order *Corynebacterineae* in the family *Nocardiaceae* within in the phylum *Actinobacteria*. The phylogenetic analysis of 16S rRNA genes from *Rhodococcus* species placed RHA1 within a cluster containing *Rhodococcus jostii* (9), however is was not until 2008 that RHA1 was designated as a species of *R. jostii* (14). Genome sequencing of RHA1 (NCBI accession nos. NC_008268, NC_008269, NC_008270, and NC_0082710) has provided insights into the metabolic versatility of this strain and its genetic relationships with other closely related actinomycetes (15).

IV.B. SUBJECT ORGANISM CHARACTERIZATION

B.1. Taxonomies of the Subject and Donor Microorganisms

B.2. Final Construct

B.3. Construction of the Subject Microorganism

B.4. Properties of the Subject Microorganism

The subject organism is a transconjugant strain of *R. jostii* RHA1 that contains the conjugative plasmid pGKT2:Km^+ from the donor bacterium, *Gordonia* sp. KTR9 (13). *Gordonia* sp. KTR9 was isolated from surface soil enrichments originating from the Naval Air Warfare Center Weapons Division, China Lake using a mineral salts medium with RDX as the nitrogen source (19). The total cellular fatty acid profile of KTR9 and nearly complete 16S rRNA gene sequence (GenBank accession number DQ068383)) indicated that strain KTR9 is a strain within the genus *Gordonia* (19). The wild type strain of KTR9 naturally contains plasmid pGKT2 which encodes genes for the aerobic denitration of RDX, *xplAB*, in actinobacteria (12). As the donor strain, pGKT2 in KTR9 was genetically modified to contain an active kanamycin resistance gene (NP_478145.1), and is the focus of a companion TERA application (13). The kanamycin marker was inserted in pGKT2 as a selective marker to select for recipient transconjugants of RHA1 as well as a marker for identification during the field release.

The subject microorganism was constructed by first constructing the intergeneric donor strain, KTR9 pGKT2:Km^+ and subsequently mating this strain with the recipient strain. The intergeneric plasmid, pGKT2:Km^+, in the donor and subject bacterial strains is summarized below as previously described (13). A predicted nonessential coding region of the *Gordonia* sp. KTR9 plasmid pGKT2, found on ORF pGKT2_4819 (Genbank Acc. No. CP002112), with no significant matches in the Genbank database, was targeted for homologous recombination and insertion of a kanamycin resistance (*aph*) marker (NP_478145.1). This kanamycin gene encodes an aminoglycoside 3'-phosphotransferase (APH) that is identical to one found in *Corynebacterium diphtheriae* but is also found on various transposons and plasmids which are thought to have originated as a self-defense mechanism used by microorganisms that produce the

antibiotics (Blast search result). The insertion site is approx. 40 kb downstream from the *xplAB* gene complex and was confirmed by polymerase chain reaction (PCR) analysis targeting various genetic loci on pGKT2 (13) and kanamycin resistance in both the donor and subject bacterial strains.

The approach for constructing the donor strain was as follows. The *aph* gene, flanked by 1-kb regions specific to pGKT2, was synthesized by Celtek Biosciences, LLC (Nashville, TN, USA), into a pCR2.1 vector (Invitrogen, Carlsbad, CA, USA). The *aph* construct containing the KTR9 flanking regions (2.9 kb) was liberated from the plasmid by digestion with BamHI (New England BioLabs, Inc., Ipswich, MA, USA) and gel purified with a Wizard SV Gel and PCR Clean-up System Kit (Promega, Madison, WI, USA). The purified 2.9-kb fragment was ligated into a BamHI-cut mobilizable vector, pK18mobsacB (17,20) and transformed into One Shot Top10 Chemically Competent *Escherichia coli* cells (Invitrogen) that were selected for on Luria–Bertani (LB) plates with 50 μg ml^{-1} kanamycin. Recombinant pK18mobsacB was introduced from Top10 *E. coli* cells into *Gordonia* sp. KTR9 based on the conjugation strategy presented by van der Geize et al (2001). Double crossover transconjugants of the Km^r marker from pK18mobsacB into pGKT2 via homologous flanking regions were selected for on LB agar plates with 50 g ml^{-1} kanamycin and 10% sucrose. Each step was screened by PCR targeting the Km^r construct and various locations of the pGKT2 and pK18mobsacB plasmids to confirm their presence or, in the case of pK18mobsacB, its absence.

To construct the subject bacterial strain, conjugation reactions between the kanamycin resistant, orange-pigmented *Gordonia* sp. KTR9 donor strain and recipient RHA1 strain were carried out by the filter mating method of Lessard et al. (2004). Resultant transconjugants that were capable of growth on LBP (per L: 10 g peptone, 10 g NaCl, 5 g yeast extract) with 100 μg ml^{-1} kanamycin were then screened by PCR targeting various locations of pGKT2 to confirm its presence. Transconjugants were also screened for an ability to degrade RDX in minimal or rich medium. Recipient strains were chosen based on colony morphology, ability to grow at 30°C on LBP medium, sensitivity to kanamycin, and inability to utilize RDX as a sole nitrogen source. *R. jostii* RHA1 acquired pGKT2:Km^+ at a frequency of 4×10^5 transconjugants per recipient. Transfer of pGKT2:Km^+ into strain RHA1 was verified by pulsed field gel electrophoresis, in conjunction with Southern analysis, using DNA probes that were pGKT2-specific. The relative stability of pGKT2:Km^+ in the subject transconjugant strain showed less than a 10% plasmid loss by 100 generations (13).

The physiology of the subject microorganism *R. jostii* sp. RHA1 pGKT2:Km^+ was characterized with respect to the recipient RHA1 and donor KTR9 strains (13). Growth of *R. jostii* RHA1 pGKT2:Km^+ was similar to its respective wild-type strain in rich LBP medium. The subject microorganism grew in mineral salts medium with RDX as the sole source of nitrogen, while the wild-type strain showed no signs of growth over 96 h. RDX was completely degraded by *R. jostii* RHA1 pGKT2:Km^+ within 24 h, while the donor strain, KTR9 pGKT2:Km^+ degraded RDX within 48 h. There was no significant difference in the RDX degradation abilities between the subject and donor strains when the rates of RDX degradation were normalized for growth rate (13). RHA1 transconjugants were grown in MSM with either RDX as a sole source of nitrogen or in concert with an alternative nitrogen source in the form of KNO_3, KNO_2 or $(NH_4)_2SO_4$. The inhibitory effect of inorganic nitrogen on RDX degradation for the subject microorganism was

similar to that of *Gordonia* sp. KTR9 (13). After 96 h, incomplete degradation of RDX (approx. 50%) was observed by *R. jostii* RHA1 pGKT2:Km^+ when NO_3 and NO_2 were added, and less than 10% RDX degradation occurred in the presence of NH_4.

IV.C. POTENTIAL HUMAN HEALTH EFFECTS OF THE SUBJECT MICROORGANISM

C.1. Pathogenicity of Subject Microorganism

C.2. Toxicity and Immunological Effects of Subject Microorganism or Its Products

R. jostii RHA1 pGKT2:Km^+ is not known to be a pathogen and no instances of pathogenicity could be found in the literature. Very few genera of *Rhodococcus* have been recognized as pathogens of young horses, immune compromised individuals, insects, and plants (23). For example, *Rhodococcus equi* is a veterinary pathogen that can cause morbidity in patients that are immune compromised and are occupationally and recreationally exposed to farming, livestock, and dry soil environments. *R. equi* harbors a number of virulence determinants as extrachromosomal elements known as Vaps (21). These genes/proteins are unique to *R. equi* with no homologs present in the *R. jostii* RHA1 pGKT2:Km^+ strain that will be used in this study. Furthermore, the exact taxonomic designation of *R. equi* as a *Rhodococcus* species is unclear since its 16S rRNA gene is more closely related to the 16S rRNA genes of *Nocardia* species than to other *Rhodococcus* species (9).

IV.D. PREDICTED ENVIRONMENTAL EFFECTS AND FATE OF SUBJECT MICROORGANISM

D.1. Ecological Effects

Many *Rhodococcus* species are metabolically diverse and possess the metabolic capability to degrade a variety of xenobiotic compounds (2,9). They are also widely distributed in natural environments with many species isolated from soil, water, marine sediments, plants, animals, insects, and industrial applications. The *R. jostii* RHA1 recipient strain is known to utilize a diverse group of compounds (15) including aromatics, carbohydrates, nitriles, and steroids as carbon and energy sources for growth. It also has the ability to cometabolize polychlorinated biphenyls when growing on aromatic or biphenyl substrates. Several of the catabolic pathways are encoded on the 3 plasmids within RHA1. Similar to soil pseudomonads, RHA1 is predicted to have evolved its nutritional diversity in response to the complex chemical structure of plant compounds and the competitive advantage of utilizing several substrates simultaneously. Thus, *Rhodococcus* species are considered to have important roles in nutrient cycling, the potential to be used for the beneficial remediation of pollutants, and for the production of metabolites of biotechnological importance.

To the best of our knowledge, strain RHA1 has not been tested for pathogenicity, virulence, or infectivity to mammals, fish, insects, invertebrates, and plants. In addition, we do not know if

toxins towards these organisms or prokaryotes are produced by RHA1. Similarly, the metabolic versatility of rhodococci and their frequent isolation from soil suggests that they may have a role in nutrient cycling. RHA1 has recently been shown to contain peroxidases that are active in degrading lignin (16) along with numerous genes for steroid catabolism (15), indicating a role in the metabolism of plant residues. We are unaware of any studies that examine a role of RHA1 in nitrogen, sulfur, or phosphorus cycling, or CO_2-fixation, or the effect of RHA1 on microbial community diversity and activity.

The subject bacterial strain was constructed to permit it to degrade RDX and utilize the RDX as a sole source of nitrogen for growth. The metabolic pathway created by the insertion of the *xplAB* genes proceeds via the enzymatic removal of 2 nitro-groups from the molecule and subsequent abiotic ring cleavage (5). End products of the reaction include 4-nitro-2,4-diazabutanal (NDAB), nitrite, formaldehyde, and ammonium. The nitrite and ammonium are used as nitrogen sources for growth while the formaldehyde is subsequently transformed to carbon dioxide. The NDAB accumulates in the culture and accounts for 64% of the carbon mass balance. However, NDAB is transformed by soil microorganisms, including a *Methylobacterium* species (7) and *Phanerochaete chrysosporium* (Fournier et al. 2004), so that it is unlikely to accumulate in the natural environment. Furthermore, NDAB has been shown to be non-toxic via a luminescence bioassay (6) The *xplA* gene is highly conserved among globally distributed species of *Rhodococcus, Gordonia, Williamsia, and Microbacterium*, which have been isolated from RDX contaminated soils and groundwater (1). At UMCD, RDX is present at concentrations ranging from 2 to 300 $\mu g\ L^{-1}$ over the ~300 acre plume. During the cell transport field test that will occur in July, 2013, the expected concentration of formaldehyde produced by the subject strain is about 0.27 to 40.5 $\mu g\ L^{-1}$. In 2014, the field-scale demonstration of the bioaugmentation with the subject strain will use an RDX concentration of 1000 $\mu g\ L^{-1}$ and thus approximately 135 $\mu g\ L^{-1}$ of formaldehyde is expected to be produced. These formaldehyde concentrations are well below the 10 $mg\ L^{-1}$, 5 $mg\ L^{-1}$, and 1 $mg\ L^{-1}$ drinking water limits set by EPA for 1 day, 10 day, and lifetime exposure, respectively (Agency for Toxic Substances and Disease Registry (ATSDR). 2008. Toxicological profile for Formaldehyde. (*Draft for Public Comment*). Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.) Based on these results the addition of the subject microorganism to the groundwater at UMCD is not expected to produce toxic end products of RDX biotransformation.

D.2. Survival and Fate

The survival and activity of the subject microorganism has been determined in artificial groundwater flask studies and in microcosms containing UMCD sediment and the artificial groundwater. The artificial groundwater was synthesized to simulate the groundwater at the UMCD site and was based on actual chemical analysis of groundwater collected from 6 wells in January, 2010. The UMCD sediment was collected in May, 2012 from 2 demonstration wells used for tracer tests in May and July, 2012. The subject microorganism was grown at 30°C in a basal salts medium containing 50 mM fructose as the carbon source and 18 mM ammonium as the nitrogen source. The cell suspension was washed and resuspended in artificial groundwater (AGW) and then starved at 15°C to reduce residual nitrogen levels. After 24 h of starvation, RDX and fructose were added to a final concentration of approximately 5.5 µM and 1 mM, respectively. Triplicate cultures were incubated at 15 ^{o}C with shaking at 120 rpm. Cell viability

and RDX concentrations were determined periodically for 7 days. Cell viability was determined by plate counts on agar media, while RDX concentrations were determined by HPLC (3).

The sediment microcosms consisted of 2 g of Umatilla sediment (2 mm sieved) plus 1 ml of artificial groundwater (AGW). The AGW was amended with RDX at 1.1 mg L^{-1} + 1 mM fructose (1.8 mg L^{-1}) and 1 x 10^6 cells ml^{-1} of each bacterial culture (*Gordonia* sp. KTR9 pGKT2:Km^+; *Rhodococcus jostii* RHA1 pGKT2:Km^+; *Pseudomonas fluorescens* IC). The bacterial cultures were grown and starved as described above. One set of microcosms was amended with only the AGW + RDX + fructose solution (uninoculated). Microcosms were incubated at 15°C and periodically sacrificed for analysis of RDX concentrations and viable cell numbers.

The subject microorganism survived for 7 days in the AGW broth studies and degradation of the RDX was complete within 1-2 days. Initial cell densities were between 1.4 and 2.5 x 10^8 CFU ml^{-1} and had increased slightly to between 1.7 and 2.7 x 10^8 CFU ml^{-1} by day 7. Similarly, the subject microorganism remained viable during a 7 day incubation in UMCD sediment microcosms at 15°C. Initial cell densities were between 4.5 and 15 x 10^5 CFU ml^{-1} and increased to approximately 6.8 x 10^6 CFU ml^{-1} by day 7. Degradation of RDX was rapid in the inoculated microcosms, with approximately 96% of the RDX being consumed after 1 day. In the uninoculated microcosms RDX loss was significantly slower with a lag period of 4 days and a loss of only 21% by day 7.

Subsequent to these studies, UMCD sediment columns were designed to test the transport and activity of both subject microorganisms. The columns were set up as described previously (4,8). Two identical columns were wet-packed to a bulk density of ~1.6 g cm^{-3} with the UMCD sediment and AGW containing RDX at 1 mg L^{-1} was pumped into the bottom and exited at the top of the columns. Once stable conditions were reached (e.g., influent RDX = effluent RDX), the column was injected with the bacterial inoculum plus 0.1 mM fructose, followed by a continuous flow of AGW. The starved inoculum (prepared as above) was composed of the 3 microorganisms, indicated above in the sediment microcosms, each at a cell density of 1 x 10^8 cells ml^{-1}. The effluent concentrations of RDX and cell densities have been followed for up to 6 weeks with 2 separate additions of fructose following the initial fructose addition. Effluent concentrations of cells stabilized at approximately 10^3 CFU ml^{-1} and RDX concentrations decreased with each addition of fructose, with rates of RDX loss between 1.58 and 1.73 $mgL^{-1}d^{-1}$ for each substrate addition. The effluent concentration of cells along with the rates of RDX degradation indicate that a viable population of the 3 inoculated microorganisms exists within the column sediment matrix, but this concentration has not been determined.

We have not examined the ability of either the recipient or subject microorganisms in soil or aquatic media other than media from UMCD. A review of the literature identified two studies examining the survival of RHA1 in a field soil collected from an agricultural station in Japan (10). Specifically, RHA1 grew by up to 2 to 4 orders of magnitude in the soil microcosms amended with or without biphenyl. The field soil had a pH of 6.6 and total carbon content of 9.7 g kg^{-1} (22).

With regards to conditions favorable for growth of the subject microorganism, RHA1 was exposed to dessication (20% humidity) and starvation stress over a period of 2 weeks during

which cell viability decreased by 80% (14). The recipient and subject strain can be grown in 1/5 dilution of a rich broth medium LB (10), and both can be grown in minimal medium with 4 mM ammonium and 20 mM succinate with no added vitamins or growth factors necessary.

We will use standard plating methods to detect viable numbers of the subject microorganism at the UMCD field site. The agar medium will be 20% LB (tryptone, 2 g L^{-1}; glucose, 1 g L^{-1}; and sodium chloride, 1 g L^{-1}) + 50 mg L^{-1} kanamycin. The detection limits on this medium should be approximately 300 CFU ml^{-1}. In addition, we will use a quantitative polymerase chain reaction (qPCR) to detect the presence of the RDX biodegradative gene, *xplA,* in the groundwater. The qPCR assay is based on a TaqMan™ format using *xplA* primers and probes designed in our SERDP project ER-1609 (11). The detection limit of this assay is approximately 300 gene copies L^{-1} of filtered groundwater.

We do not have any information regarding the potential for conjugal transfer of the pGKT2:Km^R plasmid from the subject microorganism to indigenous bacteria in soil or water. However, in filter paper matings in the laboratory *Gordonia polyisoprenivorans*, *Rhodococcus jostii* RHA1, and *Nocardia* sp. TW2 acquired pGKT2:Km^+ at frequencies of 5×10^{-4}, 4×10^{-5}, and 7×10^{-6} transconjugants per recipient, respectively (13). Conjugal transfer to 14 closely related actinomycete strains, an *E. coli* strain, and 2 *Pseudomonas* strains did not occur (13).

IV.E. PREDICTED PRODUCTION VOLUME, BYPRODUCTS, USE, AND CONSUMER EXPOSURE

E.1. Information on production volume

The total production volume for this project is not expected to exceed 100 L per stain, depending on achievable cell densities per batch. Estimated maximum cell densities of 10^{10} cells/ml are expected based on initial data. The total cells of each strain that are being produced for use during the field testing has been set at 10^{14} per year. Production time is expected to be 1 week per batch per strain, and one batch per year of each strain will be produced.

E.2. Information on Byproducts

No specified proteins, DNA, or other byproducts are being produced during this project. Cells are grown under standard fermentation conditions. Cells are separated from culture broth and waste products during concentration steps. Any wastes liquid and biomass is disposed of according to local ordinances (e.g., sterilized and directed into a municipal sanitary sewer).

E.3. Use Information and Consumer Exposure

Cultures are being used solely for field demonstration (pilot scale) research and evaluation purposes, and are not being produced for any commercial or consumer use.

IV.F. PREDICTED RELEASES DUE TO MANUFACTURING OF THE SUBJECT MICROORGANISM, AND WORKER AND CONSUMER EXPOSURES TO THE SUBJECT MICROORGANISM

F.1. Industrial Sites Controlled by the Submitter

Culture production is being done at a small pilot scale for research and development purposes only. No commercial production of industrial-scale amounts is being performed, therefore this work does not fall under the pre-manufacture notice (PMN) requirements.

F.2. Industrial Sites Controlled by Others
No culture production is being performed by anyone other than the submitter.

IV.G. INFORMATION APPLICABLE TO FIELD TESTS OF THE SUBJECT MICROORGANISM

G.1. Objectives

RDX is a common contaminant in soils and groundwater at military sites worldwide. RDX can be mobile and persistent in groundwater under the aerobic conditions present in many aquifers and thus tends to form large, dilute plumes. Although multiple studies have demonstrated *in situ* RDX biodegradation under *anaerobic* conditions, creating and maintaining anaerobic conditions across large areas is costly and technically challenging. The Environmental Security Technology Certification Program (ESTCP) has provided funds to conduct a field demonstration of an innovative application of bioaugmentation to enhance RDX biodegradation in contaminated groundwater under *aerobic* conditions. The primary objective is to demonstrate that aerobic bioaugmentation with *Rhodococcus jostii* RHA1 promotes aerobic RDX degradation in groundwater. Project ER-201207 will provide field data to support both a technical evaluation and a cost-benefit analysis of this approach.

G.2. Nature of the site

The Umatilla Chemical Depot (UMCD) has been identified as an ideal demonstration site. Explosives-contaminated sludge and liquid wastes generated at the UMCD Washout Plant in Hermiston, OR were discharged to unlined lagoons, where infiltration through ~ 50 ft of unsaturated site soil to groundwater resulted in a ~ 300 acre RDX groundwater plume. Maximum RDX and TNT concentrations within the plume have been ~ 200 µg/L and 70 µg/L, respectively, in recent years. A groundwater pump and treat facility with activated carbon adsorption began full-time operations at the site in 1997. Treatment efficiency of the pump and treat remedy has significantly declined over the years, which has prompted consideration of bioremediation technology for its potential to optimize the existing remedy.

The unconfined aquifer at UMCD consists of alluvial deposits and the weathered surface of the Elephant Mountain Member basalt, overlain by approximately 50 to 120 feet of unsaturated alluvial sand and gravel. The saturated thickness of the aquifer in the former lagoon area is approximately 15 to 35 feet. The nearest surface water body to site is the Umatilla River, which is over 2 miles away. Groundwater gradients are very small in this highly permeable aquifer, which translates to very slow moving groundwater under ambient conditions. An aquifer recharge program was initiated near the site in October 2011, which currently involves injection of ~ 10,000 acre-feet of water. This program resulted in ~ 3 ft increased groundwater elevations.

However, there is no evidence that the groundwater gradient and flow direction has appreciably changed appreciably as a result.

G.3. Field test design

This demonstration will be performed in three phases. Phase I consists of field site characterization and laboratory testing to select a suitable bioaugmentation culture and to optimize conditions that facilitate growth, RDX-degrading activity, and cell transport under field conditions at the UMCD. Phase II consists of a forced-gradient cell transport test to confirm ability to distribute cells at field-scale (~ 60 feet). Phase III consists of a bioaugmentation demonstration with subsequent push-pull tests to obtain field-scale performance data on bioaugmentation culture transport, viability, and xplA gene transfer, as well as RDX degradation rates. The demonstration will be conducted in two field plots, one for aerobic bioaugmentation only and the other for sequential evaluation of aerobic and anaerobic biostimulation (no cells injected). RDX degradation rates and mass removed per mass of substrate added for the aerobic bioaugmentation treatment will be compared with non-bioaugmented aerobic and anaerobic biostimulation treatments and will form the basis of a cost-benefit analysis for the innovative aerobic bioaugmentation approach for in situ treatment of RDX in groundwater.

Bioaugmentation tests will be conducted during demonstration Phases II and III as follows.

Phase II: Forced Gradient Cell Transport Test. Site groundwater will be pumped into a plastic tank next to the demonstration well (Demonstration Well 2 (DW2), Figure 1). Sufficient NaCl or KBr will be added to each tank to achieve a Cl- or Br- tracer concentration of ~ 100 mg/L in 3800 L. Next, the *Rhodococcus jostii* RHA1 culture will be extruded from the leak-proof 20 L stainless steel soda kegs into the plastic tank. The bioaugmentation solution will then be thoroughly mixed using a recirculation pump to vigorously agitate the water. Extraction well EW-4 will be used to control the groundwater gradient/flow direction during the cell transport test. The bioaugmentation solution will be injected into DW2 using the same pump/tubing used during mixing, followed by monitoring for cell "breakthrough curves" at down gradient monitoring wells 4-106 and EW-2 (Figure 1) for an estimated period of 10 days. This test will conclude Summer 2013.

Phase III: Natural Gradient Bioaugmentation Test. Site groundwater (~3800 L) will be pumped into a plastic tank next to the demonstration wells DW2, 4-102 and EW2 (Figure 1). Next, the *Rhodococcus jostii* RHA1 culture will be extruded from the leak-proof 20 L stainless steel soda kegs into the plastic tanks. The bioaugmentation solution will then be thoroughly mixed using a recirculation pump to vigorously agitate the water. The bioaugmentation solution will be injected into DW2 using the same pump and tubing used during mixing, followed by a push-pull tests to quantify RDX degradation rate in the "bioaugmented" test wells. The "bioaugmented" wells will then receive periodic low concentration growth substrate (1 mM fructose) on a biweekly basis for up to 3 months. A final series of push-pull tests will then be conducted to determine RDX degradation rates in these wells 3 months following bioaugmentation. This bioaugmentation test will conclude Summer 2014.

G.4. On-site containment practices

Cultures will be delivered to the field in leak-proof 20 L stainless steel soda kegs. Kegs will be shipped to the field in sealed plastic coolers via a common carrier in accordance with all applicable regulations. All equipment, tanks and tubing that contact the culture will be disinfected using an appropriate sterilizing agent (e.g. bleach or hydrogen peroxide) prior to disposal. All personnel will use appropriate personal protective equipment and other applicable safety procedures for work at CERCLA sites.

Testing will occur in a small demonstration test plot < 200 ft long. The nearest surface water body (Umatilla River) is over 2 miles away so no surface water interception of test water is not expected. The ambient groundwater gradient is very small and groundwater seepage velocities are very low; rapid transport of groundwater from the test plot is not expected. The existing groundwater pump & treat system could be utilized to control the gradient during/following the bioaugmentation demonstration if needed.

G.5. Application methods

Cultures will be extruded from the 20 L soda kegs into plastic tanks filled with site groundwater. The bioaugmentation solution will be mixed via recirculating pump. The solution will then be injected into the site well using this same pump and tubing.

G.6. Termination and mitigation procedures

Soda kegs will be placed back in their plastic bags and sealed in plastic coolers and shipped in accordance with applicable regulations. All equipment, tanks and tubing that contact the culture will be disinfected using an appropriate sterilizing agent (e.g. bleach or hydrogen peroxide). Prior to adding the strain to the groundwater tank, all connections and tubing associated with the mixing and injection will be tested to ensure there are no leaks. Plastic sheeting will be placed around the tank opening where the culture will be added, and on the ground surrounding the tank/injection area to ensure any minor spills are contained to the test area and can be disinfected prior to disposal of materials.

G.7. Monitoring endpoints & procedures for isolating subject Microorganism

Groundwater samples will be collected and analyzed using microbial culturing and molecular methods during both Phase II and III bioaugmentation tests. Monitoring endpoints from Phase III will indicate cell counts per unit of site groundwater present at the conclusion of the test. Testing will occur in a small demonstration test plot < 200 ft long. The nearest surface water body (Umatilla River) is over 2 miles away so no interception of test water is expected. The ambient groundwater gradient is very small and groundwater seepage velocities are very low; rapid transport of groundwater from the test plot is not expected. The existing groundwater pump & treat system could be utilized to control the gradient during/following the bioaugmentation demonstration if needed.

G.8. Sampling procedure

Groundwater samples will be collected from wells DW2, 4-102 and EW2 during Phases II and III of the demonstration using low-flow groundwater sampling procedures and submersible pumps. During Phase II, samples will be collected multiple times per day over a ~ 10 day period. During Phase III, high-resolution time series sampling will be conducted during the initial push-pull test completed immediately following biaugmentation (~ hourly sampling over ~ 36 hour time period). Biweekly sampling will occur during the ~ 3 month growth substrate "feeding" period, which begins when the initial push-pull test concludes. High-resolution time series sampling (~ hourly sampling over ~ 36 hour time period) will be conducted during the final push-pull test completed following the 3 month "feeding" period. The test will conclude following completion of this final push-pull test. All equipment, tanks and tubing that contact the culture will be disinfected using an appropriate sterilizing agent (e.g. bleach or hydrogen peroxide) prior to disposal. All personnel will use appropriate personal protective equipment and other applicable safety procedures for work at CERCLA sites. The kanamycin resistance gene permits this strain to be quantified on kanamycin-containing auger plates. Copies of the xplA gene will be tracked using quantitative PCR. All samples will be immediately placed on ice and shipped via overnight shipping for laboratory processing. Colony forming units and gene copy numbers over time in site groundwater samples will be utilized to determine strain survival *in situ*. This information will be used to interpret RDX transformation results overtime.

G.9. Record keeping & reporting test results

All tests will be performed in accordance with the project demonstration work plan. Results will be report to the ESTCP program, the UMCD project team including EPA, as well as in peer reviewed journal articles.

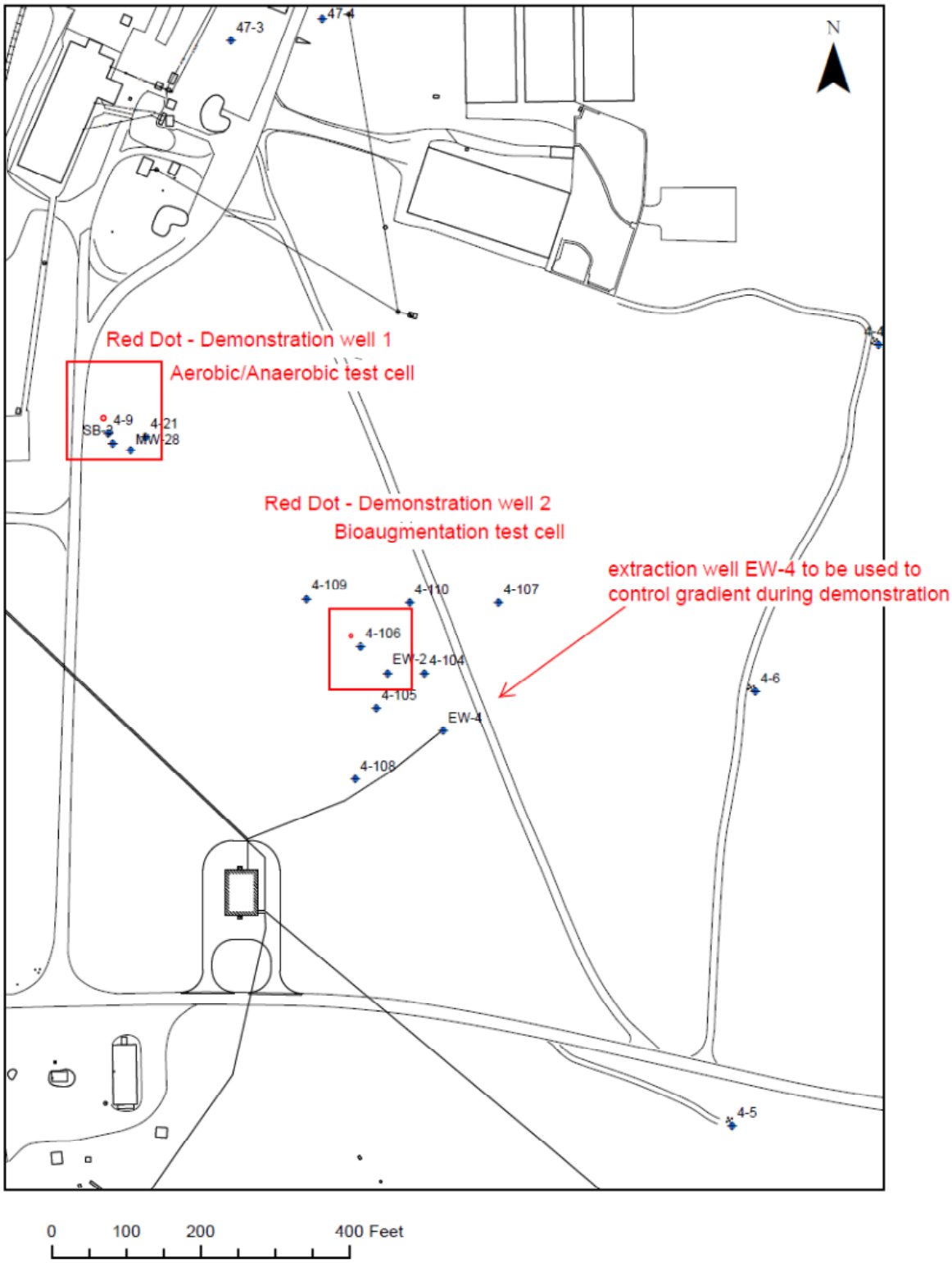


Figure 1. Demonstration well locations in test plots 1 and 2 are shown as red circles. Bioaugmentation would occur in test plot 2 only. Demonstration well 2 is ~ 10ft from downgradient monitoring well 4-106 and ~ 60 ft from well EW-2.

Reference List

1. **Andeer, P. F., D. A. Stahl, N. C. Bruce, and S. E. Strand**. 2009. Lateral transfer of genes for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degradation. Appl.Environ.Microbiol. **75**:3258-3262. doi:AEM.02396-08 [pii];10.1128/AEM.02396-08 [doi].

2. **Arenskotter, M., D. Broker, and A. Steinbuchel**. 2004. Biology of the metabolically diverse genus Gordonia. Appl.Environ.Microbiol. **70**:3195-3204. doi:10.1128/AEM.70.6.3195-3204.2004 [doi];70/6/3195 [pii].

3. **Crocker, F. H., K. T. Thompson, J. E. Szecsody, and H. L. Fredrickson**. 2005. Biotic and abiotic degradation of CL-20 and RDX in soils. J.Environ.Qual. **34**:2208-2216. doi:34/6/2208 [pii];10.2134/jeq2005.0032 [doi].

4. **Dong, H., R. Rothmel, T. C. Onstott, M. E. Fuller, M. F. DeFlaun, S. H. Streger, R. Dunlap, and M. Fletcher**. 2002. Simultaneous transport of two bacterial strains in intact cores from Oyster, Virginia: biological effects and numerical modeling. Appl.Environ.Microbiol. **68**:2120-2132.

5. **Fournier, D., A. Halasz, J. Spain, P. Fiurasek, and J. Hawari**. 2002. Determination of key metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine with Rhodococcus sp. strain DN22. Appl.Environ.Microbiol. **68**:166-172.

6. **Fournier, D., A. Halasz, J. Spain, R. J. Spanggord, J. C. Bottaro, and J. Hawari**. 2004. Biodegradation of the hexahydro-1,3,5-trinitro-1,3,5-triazine ring cleavage product 4-nitro-2,4-diazabutanal by Phanerochaete chrysosporium. Appl.Environ.Microbiol. **70**:1123-1128.

7. **Fournier, D., S. Trott, J. Hawari, and J. Spain**. 2005. Metabolism of the aliphatic nitramine 4-nitro-2,4-diazabutanal by Methylobacterium sp. strain JS178. Appl.Environ.Microbiol. **71**:4199-4202. doi:71/8/4199 [pii];10.1128/AEM.71.8.4199-4202.2005 [doi].

8. **Fuller, M. E., S. H. Streger, R. K. Rothmel, B. J. Mailloux, J. A. Hall, T. C. Onstott, J. K. Fredrickson, D. L. Balkwill, and M. F. DeFlaun**. 2000. Development of a vital fluorescent staining method for monitoring bacterial transport in subsurface environments. Appl.Environ.Microbiol. **66**:4486-4496.

9. **Gurtler, V., B. C. Mayall, and R. Seviour**. 2004. Can whole genome analysis refine the taxonomy of the genus Rhodococcus? FEMS Microbiol.Rev. **28**:377-403.

10. **Iino, T., Y. Wang, K. Miyauchi, D. Kasai, E. Masai, T. Fujii, N. Ogawa, and M. Fukuda**. 2012. Specific gene responses of Rhodococcus jostii RHA1 during growth in soil. Appl.Environ.Microbiol. **78**:6954-6962. doi:AEM.00164-12 [pii];10.1128/AEM.00164-12 [doi].

11. **Indest, K. J., F. H. Crocker, and R. Athow**. 2007. A TaqMan polymerase chain reaction method for monitoring RDX-degrading bacteria based on the xplA functional gene.

J.Microbiol.Methods **68**:267-274. doi:S0167-7012(06)00250-8 [pii];10.1016/j.mimet.2006.08.008 [doi].

12. **Indest, K. J., C. M. Jung, H. P. Chen, D. Hancock, C. Florizone, L. D. Eltis, and F. H. Crocker**. 2010. Functional characterization of pGKT2, a 182-kilobase plasmid containing the xplAB genes, which are involved in the degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine by Gordonia sp. strain KTR9. Appl.Environ.Microbiol. **76**:6329-6337. doi:AEM.01217-10 [pii];10.1128/AEM.01217-10 [doi].

13. **Jung, C. M., F. H. Crocker, J. O. Eberly, and K. J. Indest**. 2011. Horizontal gene transfer (HGT) as a mechanism of disseminating RDX-degrading activity among Actinomycete bacteria. J.Appl.Microbiol. **110**:1449-1459. doi:10.1111/j.1365-2672.2011.04995.x [doi].

14. **LeBlanc, J. C., E. R. Goncalves, and W. W. Mohn**. 2008. Global response to desiccation stress in the soil actinomycete Rhodococcus jostii RHA1. Appl.Environ.Microbiol. **74**:2627-2636. doi:AEM.02711-07 [pii];10.1128/AEM.02711-07 [doi].

15. **McLeod, M. P., R. L. Warren, W. W. Hsiao, N. Araki, M. Myhre, C. Fernandes, D. Miyazawa, W. Wong, A. L. Lillquist, D. Wang, M. Dosanjh, H. Hara, A. Petrescu, R. D. Morin, G. Yang, J. M. Stott, J. E. Schein, H. Shin, D. Smailus, A. S. Siddiqui, M. A. Marra, S. J. Jones, R. Holt, F. S. Brinkman, K. Miyauchi, M. Fukuda, J. E. Davies, W. W. Mohn, and L. D. Eltis**. 2006. The complete genome of Rhodococcus sp. RHA1 provides insights into a catabolic powerhouse. Proc.Natl.Acad.Sci.U.S.A **103**:15582-15587. doi:0607048103 [pii];10.1073/pnas.0607048103 [doi].

16. **Roberts, J. N., R. Singh, J. C. Grigg, M. E. Murphy, T. D. Bugg, and L. D. Eltis**. 2011. Characterization of dye-decolorizing peroxidases from Rhodococcus jostii RHA1. Biochemistry **50**:5108-5119. doi:10.1021/bi200427h [doi].

17. **Schafer, A., A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, and A. Puhler**. 1994. Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium glutamicum. Gene **145**:69-73.

18. **Seto, M., K. Kimbara, M. Shimura, T. Hatta, M. Fukuda, and K. Yano**. 1995. A Novel Transformation of Polychlorinated Biphenyls by Rhodococcus sp. Strain RHA1. Appl.Environ.Microbiol. **61**:3353-3358.

19. **Thompson, K. T., F. H. Crocker, and H. L. Fredrickson**. 2005. Mineralization of the cyclic nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine by Gordonia and Williamsia spp. Appl.Environ.Microbiol. **71**:8265-8272. doi:71/12/8265 [pii];10.1128/AEM.71.12.8265-8272.2005 [doi].

20. **van der Geize, R., G. I. Hessels, G. R. van, P. van der Meijden, and L. Dijkhuizen**. 2001. Unmarked gene deletion mutagenesis of kstD, encoding 3-ketosteroid Delta1-dehydrogenase, in Rhodococcus erythropolis SQ1 using sacB as counter-selectable marker. FEMS Microbiol.Lett. **205**:197-202. doi:S0378109701004645 [pii].

21. **von, B. K. and A. Haas**. 2009. Molecular and infection biology of the horse pathogen Rhodococcus equi. FEMS Microbiol.Rev. **33**:870-891. doi:FMR181 [pii];10.1111/j.1574-6976.2009.00181.x [doi].

22. **Wang, Y., J. Shimodaira, T. Miyasaka, S. Morimoto, T. Oomori, N. Ogawa, M. Fukuda, and T. Fujii**. 2008. Detection of bphAa gene expression of Rhodococcus sp. strain RHA1 in soil using a new method of RNA preparation from soil. Biosci.Biotechnol.Biochem **72**:694-701. doi:JST.JSTAGE/bbb/70493 [pii].

23. **Yamshchikov, A. V., A. Schuetz, and G. M. Lyon**. 2010. Rhodococcus equi infection. Lancet Infect.Dis. **10**:350-359. doi:S1473-3099(10)70068-2 [pii];10.1016/S1473-3099(10)70068-2 [doi].

APPENDIX D

TSCA/TERA PERMIT FOR USE OF GMO

TSCA NON-CONFIDENTIAL BUSINESS INFORMATION

DOCUMENT DESCRIPTION	DOCUMENT CONTROL NUMBER	DATE RECEIVED
Commun E	R-13-1, R-13-2	

COMMENTS:

DOES NOT CONTAIN CBI

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

ML48674

CONTAINS NO CBI

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

Mandy Michalsen
Environmental Engineer
U.S. Army Corps of Engineers
4735 East Marginal Way
Seattle, WA 98134

JUL 10 2013

Re: TERAs R-13-0001 and R-13-0002

Dear Ms. Michalsen:

This letter responds to the above-referenced TSCA Experimental Release Applications (TERAs), received by the Environmental Protection Agency (EPA) on April 24, 2013. The application was approved on June 17, 2013.

Pursuant 40 CFR 725.270(c)(1), a TERA approval issued by EPA is legally binding. Pursuant 40 CFR 725.270(c)(2), when EPA approves a TERA, the submitter must conduct the research and development activity only as described in the TERA and in accordance with any requirements and conditions prescribed by EPA in the approval of the TERA.

As a condition of EPA approval of the above referenced TERAs, quarterly progress reports including descriptions of field methods and preliminary findings for the activities described in the TERA must be provided to the EPA. Microbial data submitted shall include polymerase chain reaction (PCR) results and colony forming units (CFUs) per milliliter of groundwater. In addition to the quarterly reports, detailed reports including all field, laboratory, and microbial analysis methods and results must be provided upon completion of Phase II and again upon completion of Phase III, as described in the TERAs.

In addition to the required reporting of Phase II and III data above, EPA recommends monitoring of metabolites of concern to assess on-site subsurface concentrations and the potential for aerobically-generated metabolites that migrate beyond the injection well locations: 4-nitro-2,4-diazabutanal (NDAB) and formaldehyde could be monitored. If TNT is present at the test area, then monitoring for 2-amino-4,6-dinitrotoluene, 2-nitro-4,6-diaminotoluene, and methylenedinitramine (MEDINA) may also be of value. The periodicity of metabolite sampling should allow an evaluation of the potential for effects on ecological and human receptors at current and/or future sites. Finally, data-gathering to assess the potential for spread of the introduced bacteria beyond the injection site should be assessed. This data may be beneficial in addressing concerns that may arise in future field tests.

Thank you for your cooperation. If you have any questions or comments, please contact Ken Moss, the Program Manager for these TERAs, at (202) 564-9232.

Sincerely,

Greg Schweer, Chief
New Chemicals Management Branch
Chemical Control Division (7405 M)

CONTAINS NO
CBI

APPENDIX E

PHASE III ANALYTICAL AND FIELD DATA

Bottle ID	Date and Time	DAYS	RDX	HMX	MNX	DNX	TNX	pH	DissO2	ORP	Fe(II)	Temp	xplA	16S	CFU/mL	Fructose (mM)
DW1-BKG	5/1/14 10:20	0.00	0.0647[a]										3012.77[f]			
DW1-BKG-052214	5/22/14 12:34	21.09	0.10					7.99[b]	5.99[c]	49[d]	0[e]	32.38				1.00
DW1-BKG-060214	6/2/14 14:18	32.17	0.05					7.61	0.41	-151.00	0.00	24.46	109433.27			1.00
DW1-BKG-061214	6/12/14 11:32	42.05	0.05					7.54	0.49	-16.80	0.00	24.20				0.00
DW1-BKG-062314	6/23/14 12:20	53.08	0.10					7.49	1.12	70.10	0.00	25.20	19358.03			0.25
DW1-BKG-070214	7/2/14 11:51	62.06	0.08					7.49	4.11	79.00	0.00	24.00				0.00
DW1-PPT2-BKG	7/10/14 15:21	70.21	0.12	0.02	0.00	0.00	0.00	7.71	4.49	44.00	0.00	27.93	47332.27[f]			0.50
DW1-PPT2-INJ1	7/10/14 17:14	70.29	0.57	0.05	0.00	0.00	0.00									
DW1-PPT2-INJ2	7/10/14 17:15	70.29	0.59	0.04	0.00	0.00	0.00									
DW1-PPT2-INJ3	7/10/14 17:15	70.29	0.58													
DW1-PPT2-1	7/10/14 19:00	70.36	0.39	0.05	6.86E-04	5.76E-04	1.06E-03									
DW1-PPT2-2	7/10/14 20:00	70.40	0.21	0.03	1.32E-03	5.66E-04	1.74E-03									
DW1-PPT2-3	7/10/14 20:59	70.44	0.11	0.02	2.08E-03	8.85E-04	2.26E-03									
DW1-PPT2-4	7/11/14 0:02	70.57	0.07													
DW1-PPT2-5	7/11/14 3:06	70.70	0.05	0.02	1.98E-03	6.52E-04	2.26E-03									
DW1-PPT2-6	7/11/14 5:59	70.82	0.03													
DW1-PPT2-7	7/11/14 9:02	70.95	0.04													
DW1-PPT2-8	7/11/14 12:01	71.07	0.04	0.02	2.66E-03	1.30E-03	3.23E-03									
DW1-PPT2-9	7/11/14 17:58	71.32	0.04													
DW1-PPT2-10	7/12/14 0:02	71.57	0.06	0.00	0.00	0.00	0.00									
DW1-PPT2-11	7/12/14 7:51	71.90	0.04													
DW1-PPT2-12	7/12/14 13:58	72.15	0.06													
DW1-PPT2-13	7/12/14 20:43	72.43	0.29	0.02	0.00	0.00	0.00									
DW1-PPT2-14	7/13/14 8:20	72.92	0.02													
DW1-PPT2-15	7/13/14 19:40	73.39	0.03	0.00	0.00	0.00	0.00									
DW1-BKG-072414	7/24/14 9:10	83.95	0.03					6.89	1.67	-165.00	1.00	21.59	3158.46	9.64E+05		15.00
DW1-BKG-080414	8/5/14 9:33	95.97	0.03					6.90	2.01	-155.00	2.50	23.15				15.00
DW1-BKG-081414	8/14/14 9:20	104.96	0.03					6.76	1.60	-154.00	3.50	23.56	1483.24	4.84E+06		15.00
DW1-BKG-081414	8/14/2014 9:20	104.96	0.03					6.76	1.60	-154.00	3.50	23.56				15.00
DW1-BKG-082514	8/25/2014 11:49	116.06	0.06					6.66	3.05	-164.00	3.50	24.61				24.00
DW1-BKG-090414	9/4/2014 11:46	126.06	0.02					6.72	3.30	-155.00	2.00	21.21				24.00
DW1-PPT3-BKG	9/11/2014 13:14	133.12	0.00	0.00	0.00	0.00	0.00	6.84	0.42	-239.00	5.00	21.60	25513.94[h]	1.26E+07		24.00
DW1-PPT3-INJ1	9/11/2014 16:24	133.25	0.67	0.04	0.00	0.00	0.00									
DW1-PPT3-INJ2	9/11/2014 16:22	133.25	0.67	0.04	0.00	0.00	0.00									
DW1-PPT3-INJ3	9/11/2014 16:16	133.25	0.68													
DW1-PPT3-1	9/11/2014 18:54	133.36	0.58	0.04	0.00	0.00	0.00									
DW1-PPT3-2	9/11/2014 19:49	133.40	0.55	0.04	0.00	0.00	0.00									
DW1-PPT3-3	9/11/2014 20:47	133.44	0.49													
DW1-PPT3-4	9/12/2014 0:08	133.57	0.35													
DW1-PPT3-5	9/12/2014 3:07	133.70	0.28	0.03	0.00	0.00	0.01									
DW1-PPT3-6	9/12/2014 6:02	133.82	0.23													
DW1-PPT3-7	9/12/2014 9:06	133.95	0.19													
DW1-PPT3-8	9/12/2014 11:58	134.07	0.15													
DW1-PPT3-9	9/12/2014 18:04	134.32	0.11													
DW1-PPT3-10	9/13/2014 0:07	134.57	0.08	0.02	0.03	0.00	0.00									
DW1-PPT3-11	9/13/2014 6:06	134.82	0.11													
DW1-PPT3-12	9/13/2014 12:25	135.09	0.04													
DW1-PPT3-13	9/13/2014 19:49	135.40	0.04													
DW1-PPT3-14	9/14/2014 8:09	135.91	0.02													
DW1-PPT3-15	9/14/2014 14:38	136.18	0.02	0.03	0.00	0.00	0.01									

Ref Table 5.6 Aerobic biostimulation RDX value is minimum of highlighted selection

Ref Table 5.6 Anaerobic biostimulation RDX value is minimum of highlighted selection

Ref Table 5.6 Aerobic biostimulation associated values (i.e. pH S.U., O2, ORP, FE(II)) are averagese of highlighted section

Ref Table 5.6 Anaerobic biostimulation associated values (i.e. pH S.U., O2, ORP, FE(II)) are averages of highlighted section

a. Ref table 5.6 Background RDX Value
b. Ref Table 5.6 Background pH S.U.
c. Ref table 5.6 Background O_2
d. Ref table 5.6 Background ORP
e. Ref table 5.6 Background FE (II)
f. Ref table 5.6 Background xplA copies
g. Ref table 5.6 aerobic biostim xplA copies
h. Ref table 5.6 anaerobic biostim xplA copies

Bottle ID	Date and Time Collected	DAYS	RDX	HMX	MNX	DNX	TNX	pH	DissO2	ORP	Fe(II)	Temp	xplA	16S	CFU/mL	Fructose (mM)	Total moles*
MW28-BKG-050114	5/1/14 11:18 AM	0.00	0.0835[a]										15587.79			1.00	5.68
MW28-BKG-052214	5/22/14 1:16 PM	21.08	0.09					8.14[b]	7.04[c]	64[d]	0[e]	30.90				1.00	5.68
MW28-BKG-060214	6/2/14 3:19 PM	32.17	0.09					7.60	2.12	-78.00	0.00	25.71	15178.50			1.00	5.68
MW28-BKG-061214	6/12/14 10:45 AM	41.98	0.09					7.64	2.13	8.00	0.00	23.00				0.00	0.00
MW28-BKG-062314	6/23/14 1:53 PM	53.11	0.09					7.33	2.83	92.20	0.00	24.80	20665.93			0.25	1.42
MW28-BKG-070214	7/2/14 11:05 AM	61.99	0.09					7.47	1.80	79.00	0.00	24.17				0.00	0.00
MW28-PPT2-BKG	7/10/14 3:59 PM	70.20	0.10	0.06	0.00	0.01	0.00	7.62	1.31	-24.00	0.00	23.96	6867.29			0.50	2.84
MW28-PPT2-INJ1	7/10/14 6:02 PM	70.28	0.55	0.04	0.00	0.00	0.00										
MW28-PPT2-INJ2	7/10/14 6:02 PM	70.28	0.56	0.05	0.00	0.00	0.00										
MW28-PPT2-INJ3	7/10/14 6:03 PM	70.28	0.53	0.05													
MW28-PPT2-1	7/10/14 7:02 PM	70.32	0.55	0.05	0.00	0.00	0.00										
MW28-PPT2-2	7/10/14 7:55 PM	70.36	0.48	0.04	0.00	0.00	0.00										
MW28-PPT2-3	7/10/14 8:57 PM	70.40	0.41	0.06	0.00	0.00	0.00										
MW28-PPT2-4	7/11/14 12:10 AM	70.54	0.20	0.02													
MW28-PPT2-5	7/11/14 3:11 AM	70.66	0.18	0.04	5.79E-04	4.51E-03	1.16E-03										
MW28-PPT2-6	7/11/14 6:04 AM	70.78	0.16	0.05	5.62E-04	5.63E-03	1.51E-03										
MW28-PPT2-7	7/11/14 8:59 AM	70.90	0.14	0.03													
MW28-PPT2-8	7/11/14 11:59 AM	71.03	0.11	0.03													
MW28-PPT2-9	7/11/14 5:55 PM	71.28	0.10	0.03													
MW28-PPT2-10	7/12/14 12:04 AM	71.53	0.07	0.03	0.00	0.01	0.00										
MW28-PPT2-11	7/12/14 7:53 AM	71.86	0.08	0.02													
MW28-PPT2-12	7/12/14 2:04 PM	72.12	0.07	0.02	0.00	0.01	0.00										
MW28-PPT2-13	7/12/14 8:40 PM	72.39	0.08	0.03													
MW28-PPT2-14	7/13/14 8:22 AM	72.88	0.10	0.02													
MW28-PPT2-15	7/13/14 7:40 PM	73.35	0.06	0.02	0.00	0.00	0.00										
MW28-BKG-072414	7/24/14 8:26 AM	83.88	0.08					7.13	1.75	-47.00	0.00	21.84	2937.25		1.68E+05	15.00	85.23
MW28-BKG-080514	8/5/14 8:45 AM	95.89	0.14					7.43	1.32	-68.00	0.00	22.44				15.00	85.23
MW28-BKG-081414	8/14/14 10:07 AM	104.95	0.06	0.03				7.08	2.00	-173.00	0.50	23.75	8875.44		1.26E+06	15.00	85.23
MW28-BKG-082514	8/25/14 12:45 PM	116.06	0.06	0.03				6.75	2.38	-222.00	1.00	25.28				24.00	136.36
MW28-BKG-090414	9/4/14 8:38 AM	125.89	0.04					6.08	2.33	-238.00	2.50	21.30				24.00	136.36
MW28-PPT3-BKG	9/11/14 12:19 PM	133.04	0.01		0.00		0.00	6.52	0.86	-284.00	4.50	20.40	9254.24		9.29E+06	24.00	136.36
MW28-PPT3-INJ1	9/11/14 5:08 PM	133.24	0.63	0.10	0.00		0.00										
MW28-PPT3-INJ2	9/11/14 5:11 PM	133.25	0.64	0.10	0.00		0.00										
MW28-PPT3-INJ3	9/11/14 5:14 PM	133.25	0.64	0.10													
MW28-PPT3-1	9/11/14 7:00 PM	133.32	0.72	0.14	0.00		0.00										
MW28-PPT3-2	9/11/14 7:56 PM	133.36	0.61	0.98	0.00		0.00										
MW28-PPT3-3	9/11/14 8:53 PM	133.40	0.57	0.04													
MW28-PPT3-4	9/12/14 11:12 AM	134.00	0.37	0.05													
MW28-PPT3-5	9/12/14 3:12 AM	133.66	0.27	0.04	0.04		0.00										
MW28-PPT3-6	9/12/14 6:04 AM	133.78	0.18	0.04													
MW28-PPT3-7	9/12/14 9:10 AM	133.91	0.12	0.05													
MW28-PPT3-8	9/12/14 12:00 PM	134.03	0.09														
MW28-PPT3-9	9/12/14 6:08 PM	134.28	0.05	0.07													
MW28-PPT3-10	9/13/14 12:09 AM	134.54	0.03	0.07	0.03		0.01										
MW28-PPT3-11	9/13/14 6:04 AM	134.78	0.02														
MW28-PPT3-12	9/13/14 12:27 PM	135.05	0.02														
MW28-PPT3-13	9/13/14 7:52 PM	135.36	0.37	0.04													
MW28-PPT3-14	9/14/14 8:11 AM	135.87	0.05	0.03													
MW28-PPT3-15	9/14/14 2:47 PM	136.15	0.53	0.05	0.06		0.00										

Ref Table 5.6 Aerobic biostimulation RDX value is minimum of highlighted selection

Ref Table 5.6 Anaerobic biostimulation RDX value is minimum of highlighted selection

Ref Table 5.6 Aerobic biostimulation associated values (i.e. pH S.U., O2, ORP, FE(II)) are averagese of highlighted section

Ref Table 5.6 Anaerobic biostimulation associated values (i.e. pH S.U., O2, ORP, FE(II)) are averages of highlighted section

*Converts X mM into moles added when 1500 gallons are injected (factor of 5.68)
a. Ref table 5.6 Background RDX Value
b. Ref Table 5.6 Background pH S.U.
c. Ref table 5.6 Background O2
d. Ref table 5.6 Background ORP
e. Ref table 5.6 Background FE (II)
f. Ref table 5.6 Background xplA copies
g. Ref table 5.6 aerobic biostim xplA copies
h. Ref table 5.6 anaerobic biostim xplA copies

Bottle ID	Date and Time Collected	DAYS	RDX	HMX	MNX	DNX	TNX	pH	DissO2	ORP	Fe(II)	Temp	xplA	16S	CFU/mL	Fructose (mM)	Total moles*
DW2-BKG-050114	5/1/14 14:36	0.00	0.019[a]										709762.24[d]	2.10E+06	460[b]		
DW2-INJ-No Cells			1.02	0.05	9.21E-04	0.03											
DW2-INJ-Early			1.15										2.44E+08	1.18E+07	2.43E+07	1.00	5.68
DW2-INJ-Late			1.13										1.94E+07	3.24E+06	5.43E+07		
DW2-1	5/1/14 23:01	0.35	0.65	0.04	1.02E-03	1.06E-02							108996163.44[e]	1.49E+07	5400000[c]		
DW2-2	5/2/14 2:04	0.48	0.04	0.03	0.00	8.20E-03											
DW2-3	5/2/14 5:01	0.60	0.02	0.02	0.00	8.09E-03											
DW2-4	5/2/14 7:55	0.72	0.04	0.02	0.00	0.00							3.87E+07	1.20E+07	1.77E+06		
DW2-5	5/2/14 11:01	0.85	0.03	0.01	0.00	3.00E-03											
DW2-6	5/2/14 13:51	0.97	0.03	0.01	0.00	4.49E-03											
DW2-7	5/2/14 16:55	1.10	0.02	0.01	0.00	5.60E-03											
DW2-8	5/2/14 20:00	1.23	0.01	4.03E-03	0.00	7.87E-04							2.47E+07	2.67E+07	5.97E+05		
DW2-9	5/3/14 1:49	1.47															
DW2-10	5/3/14 7:56	1.72															
DW2-11	5/3/14 13:55	1.97											1.40E+06	4.78E+06	4.33E+05		
DW2-12	5/3/14 19:51	2.22	0.01	2.19E-03	0.00	0.00											
DW2-13	5/4/14 3:47	2.55															
DW2-14	5/4/14 11:57	2.89															
DW2-15	5/4/14 19:58	3.22											1.43E+06	1.06E+07	2.60E+04		
DW-2-BKG-052214	5/22/14 9:34	20.79	0.04					7.29	3.17	275.00	0.00	25.68	7.04E+07	7.04E+06	7.43E+05	1.00	5.68
DW-2-BKG-060214	6/2/14 11:34	31.87	0.01					7.34	0.48	-237.00	0.50	22.94	6.26E+06	1.66E+07	6.77E+04	0.50	2.84
DW-2-BKG-061214	6/12/14 9:42	41.80	0.01					7.52	0.18	-90.40	0.00	21.20	1.74E+07	2.49E+06	3.05E+05	0.00	0.00
DW-2-BKG-02314	6/23/14 12:23	52.91	0.02					7.33	0.33	56.30	0.00	24.70	2.54E+05	1.34E+06	4.20E+03	0.25	1.42
DW2-BKG-070214	7/2/14 10:08	61.81	0.03					7.15	3.42	85.00	0.00	23.47				0.00	0.00
DW2-PPT2-BKG	7/10/14 11:26	69.87	0.05	0.08				7.09	5.53	79.00	0.00	25.46	4.68E+05	1.86E+06	7.43E+03	0.50	2.84
DW2-PPT2-INJ1	7/14/14 7:57	73.72	0.61	0.08													
DW2-PPT2-INJ2	7/14/14 7:59	73.72	0.61	0.08	0.00												
DW2-PPT2-INJ3	7/14/14 8:15	73.74	0.61	0.06													
DW2-PPT2-1	7/14/14 9:20	73.78	0.56	0.06	0.00								1.03E+05	7.49E+05	3.03E+03		
DW2-PPT2-2	7/14/14 10:00	73.81	0.55	0.05	0.00												
DW2-PPT2-3	7/14/14 11:00	73.85	0.46	0.03													
DW2-PPT2-4	7/14/14 14:04	73.98	0.26	0.02									4.93E+04	7.10E+05	5.67E+02		
DW2-PPT2-5	7/14/14 17:02	74.10	0.23	0.02	0.00												
DW2-PPT2-6	7/14/14 20:03	74.23	0.19	0.02													
DW2-PPT2-7	7/14/14 22:58	74.35	0.16	0.01													
DW2-PPT2-8	7/15/14 2:00	74.48	0.14	0.01									7.09E+04	9.05E+05	2.67E+02		
DW2-PPT2-9	7/15/14 7:56	74.72	0.14	0.09													
DW2-PPT2-10	7/15/14 14:02	74.98	0.09	0.07	0.00												
DW2-PPT2-11	7/15/14 20:10	75.23	0.07	0.06									3.44E+04	1.95E+06	6.33E+02		
DW2-PPT2-12	7/16/14 2:04	75.48	0.06	0.06													
DW2-PPT2-13	7/16/14 10:02	75.81	0.06														
DW2-PPT2-14	7/16/14 17:57	76.14	0.06														
DW2-PPT2-15	7/17/14 2:03	76.48	0.05		0.00								2.47E+04	1.66E+06	3.03E+02		
DW2-BKG-072414	7/24/14 12:04	83.89	0.06					7.48	6.27	-30.00	0.00	22.23	4.00E+03	1.35E+06	1.63E+03		
DW2-BKG-080414	8/4/14 18:49	95.18	0.05					7.73	4.42	-16.00	0.00	23.50				0.50	2.84
DW2-BKG-081414	8/14/14 10:52	104.84	0.07					7.47	3.08	-69.00	0.00	21.42	3.87E+04	3.09E+05	2.00E+02		
DW2-BKG-082514	8/25/2014 13:35	115.96	0.09					7.48	1.19	-127.00	0.00	23.10	9.51E+03	2.87E+05			
DW2-BKG-090414	9/4/2014 9:39	125.79	0.08					7.24	2.04	-110.00	0.00	21.63	7.05E+03	1.07E+05		0.50	2.84
DW2-PPT3-BKG	9/11/2014 10:53	132.85	0.09	0.00	0.00		0.00	7.44	2.38	-25.00	0.00	20.88	2.10E+04	4.90E+05	0.00E+00	5.00	28.41
DW2-PPT3-INJ1	9/11/2014 18:10	133.15	0.75	0.04	0.00		0.00										
DW2-PPT3-INJ2	9/11/2014 18:12	133.15	0.75	0.03	0.00		0.00										
DW2-PPT3-INJ3	9/11/2014 18:16	133.15	0.66														
DW2-PPT3-1	9/11/2014 19:14	133.19	0.63	0.04	0.00		0.00						8.12E+03	4.13E+05	1.67E+01		
DW2-PPT3-2	9/11/2014 20:06	133.23	0.63	0.04	0.00		0.00										
DW2-PPT3-3	9/11/2014 21:01	133.27	0.58														
DW2-PPT3-4	9/12/2014 0:00	133.39	0.53										5.04E+03	3.00E+06	1.33E+01		
DW2-PPT3-5	9/12/2014 3:18	133.53	0.49	0.02	0.00		0.00										
DW2-PPT3-6	9/12/2014 5:53	133.64	0.44														
DW2-PPT3-7	9/12/2014 9:18	133.78	0.37														
DW2-PPT3-8	9/12/2014 11:50	133.88	0.28										5.55E+03	4.43E+06	0.00E+00		
DW2-PPT3-9	9/12/2014 17:56	134.14	0.21														
DW2-PPT3-10	9/13/2014 0:00	134.39	0.16	0.01	0.00		0.00										
DW2-PPT3-11	9/13/2014 5:55	134.64	0.14										4.49E+02	4.00E+05	0.00E+00		
DW2-PPT3-12	9/13/2014 12:15	134.90	0.11														
DW2-PPT3-13	9/13/2014 19:41	135.21	0.11														
DW2-PPT3-14	9/14/2014 8:00	135.72	0.11														
DW2-PPT3-15	9/14/2014 15:10	136.02	0.10	0.00	0.00		0.00						1.84E+03	3.11E+06	0.00E+00		

Ref Table 5.6 Bioaug 1 RDX value is minimum of highlighted selection

Ref Table 5.6 Bioaug 2 RDX value is minimum of highlighted selection

Ref Table 5.6 Bioaug 3 RDX value is minimum of highlighted selection

Ref Table 5.6 Bioaug 2 associated values (i.e. pH S.U., O2, ORP, FE(II)) are averagesof highlighted section

Ref Table 5.6 Bioaug 3 associated values (i.e. pH S.U., O2, ORP, FE(II)) are averages of highlighted section

Ref Table 5.6 Bioaug 1 associated CFU or xplA copies value is average of highlighted section

Ref Table 5.6 Bioaug 2 associated CFU or xplA copies value is average of highlighted section

Ref table 5.6 Bioaug 3 xplA copies is average of highlighted section

*Converts X mM into moles added when 1500 gallons are injected (factor of 5.68)
a. Ref table 5.6 Background RDX Value
b. Ref table 5.6 Background CFU value
c. Ref table 5.6 Bioaug 1, initial post-cell injection CFU value
d. Ref table 5.6 background xplA copies
e. Ref table 5.6 Bioaug 1, initial post-cell injection xplA copies

Bottle ID	Date and Time	DAYS	RDX	HMX	MNX	DNX	TNX	pH	DissO2	ORP	Fe(II)	Temp	xplA	16S	CFU/mL	Fructose (mM)
4106-BKG	5/1/14 3:32	0.00	0.024[a]	8.25E-04	0.00	5.17E-03							58275.14[b]	356501.78	0.00	
4106-INJ-No Cells			1.15	0.06	1.88E-03	0.03										
4106-INJ-EARLY													1.29E+09	70715247.77	3.30E+07	
4106-INJ-LATE													4.00E+08	43720970.71	3.90E+07	
4106-1	5/5/14 14:00	4.44	0.21	0.03	0.00	8.68E-03							196217176.86[d]	43499875.52	6066666.67[c]	
4106-2	5/5/14 17:00	4.56	0.18	0.03	0.00	0.00										
4106-3	5/5/14 19:57	4.68	0.12	0.02	0.00	5.06E-03										
4106-4	5/5/14 22:57	4.81	0.12	0.02	0.00	1.20E-02							1.09E+08	11137589.94	8.33E+05	
4106-5	5/6/14 2:00	4.94	0.11	0.03	0.00	2.28E-03										
4106-6	5/6/14 4:55	5.06	0.07	0.02	0.00	5.72E-03										
4106-7	5/6/14 7:56	5.18	0.04	0.01	0.00	0.00										
4106-8	5/6/14 11:00	5.31	0.04	0.01	0.00	0.00							1.72E+07	25978071.70	2.33E+05	
4106-9	5/6/14 17:00	5.56														
4106-10	5/6/14 10:54	5.31														
4106-11	5/7/14 6:44	6.13											3.06E+07	9726584.91	7.37E+05	
4106-12	5/7/14 12:34	6.38	0.01													
4106-13	5/7/14 18:55	6.64														
4106-14	5/8/14 0:00	6.85														
4106-15	5/8/14 20:10	7.69											3.18E+06	8146402.46	9.40E+04	
4106-BKG-052214	5/22/14 10:57	21.31	0.03					7.52	2.23	-110.00	0.00	28.54	5.85E+07	25412661.79	2.13E+05	1.00
4106-BKG-060214	6/2/14 12:24	32.37	0.01					7.40	0.31	-258.00	0.50	24.16	5.04E+07	8842395.24	1.07E+05	0.50
4106-BKG-061214	6/12/14 8:53	42.22	0.02					7.63	0.77	-65.90	0.00	22.20	2.06E+07	6755617.38	5.53E+04	0.00
4106-BKG-062314	6/23/14 11:34	53.33	0.03					7.49	0.89	47.30	0.00	25.20	9.00E+06	1443974.50	2.67E+03	0.25
4106-BKG-070214	7/3/14 9:22	63.24	0.04					7.30	5.06	57.00	0.00	25.28				0.00
4106-PPT2-BKG	7/10/14 12:05	70.36	0.05	0.02	0.00	0.00	0.00	7.30	5.97	43.00	0.00	27.81	3.68E+06	1296633.87	3.00E+02	0.50
4106-PPT2-INJ1	7/10/14 13:28	70.41	0.51	0.04	0.00	0.00	0.00									
4106-PPT2-INJ2	7/10/14 13:35	70.42	0.49	0.04	0.00	0.00	0.00									
4106-PPT2-INJ3	7/10/14 13:55	70.43	0.51													
4106-PPT2-1	7/10/14 19:12	70.65	0.32										3.31E+07	2061497.46	1.00E+03	
4106-PPT2-2	7/10/14 20:10	70.69	0.27	0.02	0.00	0.00	0.00									
4106-PPT2-3	7/10/14 21:02	70.73	0.22													
4106-PPT2-4	7/10/14 23:52	70.85	0.14										4.80E+04	10591433.11	0.00	
4106-PPT2-5	7/11/14 2:54	70.97	0.11	0.01	0.00	0.00	0.00									
4106-PPT2-6	7/11/14 5:47	71.09	0.16													
4106-PPT2-7	7/11/14 8:50	71.22	0.06													
4106-PPT2-8	7/11/14 11:54	71.35	0.07										2.54E+05	661579.93	80.00	
4106-PPT2-9	7/11/14 18:03	71.60	0.07													
4106-PPT2-10	7/11/14 23:57	71.85	0.06	0.00	0.00	0.00	0.00									
4106-PPT2-11	7/12/14 7:44	72.18	0.06										7.42E+05	383779.70	6.00	
4106-PPT2-12	7/12/14 13:54	72.43	0.05													
4106-PPT2-13	7/12/14 20:34	72.71	0.06													
4106-PPT2-14	7/13/14 8:16	73.20	0.06													
4106-PPT2-15	7/13/14 19:46	73.68	0.05	0.00	0.00	0.00	0.00						1.78E+05	1443555.50	120.00	
4106-BKG-0702414	7/24/14 11:25	84.33	0.05					7.36	5.02	-41.00	0.00	23.07	9.09E+05	280249.82	7.33E+02	
4106-BKG-080414	8/4/14 16:30	95.54	0.06					7.54	3.22	-37.00	0.00	26.18				0.50
4106-BKG-081414	8/14/14 11:31	105.33	0.08					7.34	2.16	-79.00	0.00	24.65	2.01E+06	9.25E+05	4.67E+02	
4106-BKG-082514	8/25/14 14:10	116.44	0.07					7.38	3.20	-91.00	0.00	25.71	1.29E+05	2.74E+05	0.00	
4106-BKG-090414	9/4/14 10:14	126.28	0.07					7.28	2.66	-99.00	0.00	21.95	2.77E+03	1.66E+04		0.50
4106-PPT3-BKG	9/11/14 9:35	133.25	0.06	0.00	0.00	0.00	0.00	7.40	2.59	-85.00	0.00	22.00	4.64E+05	6.14E+05	1.60E+02	5.00
4106-PPT3-INJ1	9/15/14 8:25	137.20		0.03	0.00	0.00	0.00									
4106-PPT3-INJ2	9/15/14 8:55	137.22		0.03	0.00	0.00	0.00									
4106-PPT3-INJ3	9/15/14 9:45	137.26	0.61													
4106-PPT3-1	9/15/14 12:01	137.35	0.59	0.03	0.00	0.00	0.00						7.64E+04	2254678.49	0.00E+00	
4106-PPT3-2	9/15/14 13:04	137.40	0.58	0.03	0.00	0.00	0.00									
4106-PPT3-3	9/15/14 14:00	137.44	0.57													
4106-PPT3-4	9/15/14 17:00	137.56	0.56	0.03	0.00	0.00	0.00						1.04E+03	406000.00	0.00E+00	
4106-PPT3-5	9/15/14 19:51	137.68	0.53													
4106-PPT3-6	9/15/14 23:03	137.81	0.13													
4106-PPT3-7	9/16/14 1:51	137.93	0.50													
4106-PPT3-8	9/16/14 5:04	138.06	0.49										3.30E+03	4350000.00	0.00E+00	
4106-PPT3-9	9/16/14 11:03	138.31	0.45													
4106-PPT3-10	9/16/14 16:59	138.56	0.42	0.01	0.00	0.00	0.00									
4106-PPT3-11	9/16/14 23:01	138.81	0.37										6.40E+03	12800000.00	0.00E+00	
4106-PPT3-12	9/17/14 5:11	139.07	0.35													
4106-PPT3-13	9/17/14 13:00	139.39	0.30													
4106-PPT3-14	9/17/14 19:57	139.68	0.27													
4106-PPT3-15	9/18/14 6:57	140.14	0.20	0.00	0.00	0.00	0.00						1.73E+03	105000.00	0.00E+00	

Ref Table 5.6 Bioaug 1 RDX value is minimum of highlighted selection

Ref Table 5.6 Bioaug 2 RDX value is minimum of highlighted selection

Ref Table 5.6 Bioaug 3 RDX value is minimum of highlighted selection

Ref Table 5.6 Bioaug 2 associated values (i.e. pH S.U., O2, ORP, FE(II)) are averagesof highlighted section

Ref Table 5.6 Bioaug 3 associated values (i.e. pH S.U., O2, ORP, FE(II)) are averages of highlighted section

Ref Table 5.6 Bioaug 1 associated CFU or xplA copies value is average of highlighted section

Ref Table 5.6 Bioaug 2 associated CFU or xplA copies value is average of highlighted section

Ref table 5.6 Bioaug 3 xplA copies is average of highlighted section

*Converts X mM into moles added when 1500 gallons are injected (factor of 5.68)
a. Ref table 5.6 Background RDX Value
b. Ref table 5.6 background xplA copies
c. Ref table 5.6 Bioaug 1, initial post-cell injection CFU value
d. Ref table 5.6 Bioaug 1, initial post-cell injection xplA copies

Bottle ID	Date and Time	DAYS	RDX	HMX	MNX	DNX	TNX	pH	DissO2	ORP	Fe(II)	Temp	xplA	16S	CFU/mL	Fructose (mM)
EW2-BKG-050114	5/1/14 3:58	0.00	0.0217[a]										223195.21[b]	4.96E+05	**0.00E+00**	
EW2-INJ-No Cells			0.89	0.05	1.03E-03											
EW2-INJ-Early													9.76E+07	9.98E+06	4.00E+07	
EW2-INJ-Late													1.10E+08	9.76E+06	4.27E+07	
EW2-1	5/1/14 23:16	0.80	4.89E-01	3.36E-02	8.87E-04	1.53E-02							184228002.13[d]	4.53E+07	9733333.33[c]	
EW2-2	5/2/14 2:11	0.93														
EW2-3	5/2/14 5:05	1.05	1.60E-01	2.39E-02	0.00	1.29E-02										
EW2-4	5/2/14 8:00	1.17											1.16E+08	2.54E+07	4.80E+06	
EW2-5	5/2/14 11:06	1.30	1.98E-01	2.40E-02	0.00	1.21E-02										
EW2-6	5/2/14 13:55	1.41														
EW2-7	5/2/14 17:00	1.54	9.88E-02	1.59E-02	0.00	5.89E-03										
EW2-8	5/2/14 20:08	1.67											9.97E+07	3.76E+07	3.57E+06	
EW2-9	5/3/14 1:54	1.91	9.88E-02	1.59E-02	0.00	5.89E-03										
EW2-10	5/3/14 5:01	2.04	8.20E-02	1.65E-02	0.00	5.96E-03										
EW2-11	5/3/14 14:00	2.42											4.39E+07	3.93E+07	1.20E+06	
EW2-12	5/3/14 19:55	2.66														
EW2-13	5/3/14 4:00	2.00	1.23E-02	8.92E-03	0.00	2.86E-03										
EW2-14	5/4/14 12:00	3.33														
EW2-15	5/4/14 20:10	3.68											1.64E+07	2.67E+07	2.60E+04	
EW2-BKG-052214	5/22/14 11:40	21.32	0.02					7.61	0.65	15.00	0.00	32.28	4.64E+07	4.83E+06	1.67E+03	1.00
EW2-BKG-060214	6/2/14 13:20	32.39	0.01					7.65	0.19	-259.00	1.50	20.62	1.16E+07	2.63E+07	1.33E+03	0.50
EW2-BKG-061214	6/12/14 8:01	42.17	0.01					7.61	0.24	-200.50	2.50	19.90	3.06E+06	5.45E+06	9.13E+05	0.00
EW2-BKG-062314	6/23/14 10:49	53.29	0.02					7.51	3.31	56.60	0.00	22.60	1.59E+06	7.81E+05	3.43E+03	0.25
EW2-BKG-070214	7/3/14 8:40	63.20	0.02					7.35	6.08	103.00	0.00	20.20				0.00
EW2-PPT2-BKG	7/10/14 12:53	70.37	0.02	0.00	0.00	0.00	0.00	7.53	6.50	49.00	0.00	25.79	7.05E+05	3.39E+05	2.30E+02	0.50
EW2-PPT2-INJ1	7/14/14 8:49	74.20	0.69	0.05	0.00	0.00	0.00									
EW2-PPT2-INJ2	7/14/14 8:52	74.20	0.69	0.05	0.00	0.00	0.00									
EW2-PPT2-INJ3	7/14/14 8:53	74.20	0.69													
EW2-PPT2-1	7/14/14 9:29	74.23	0.68	0.06	0.00	0.00	0.00						1.54E+05	8.59E+05	4.33E+01	
EW2-PPT2-2	7/14/14 10:04	74.25	0.68	0.05	0.00	0.00	0.00									
EW2-PPT2-3	7/14/14 11:03	74.30	0.65	0.08												
EW2-PPT2-4	7/14/14 14:00	74.42	0.53	0.06									2.30E+05	5.64E+05	2.67E+01	
EW2-PPT2-5	7/14/14 16:57	74.54	0.40	0.03	0.00	0.00	0.00									
EW2-PPT2-6	7/14/14 20:02	74.67	0.33	0.04												
EW2-PPT2-7	7/14/14 22:54	74.79	0.28	0.03												
EW2-PPT2-8	7/15/14 1:58	74.92	0.24	0.03									4.22E+05	5.07E+06	2.00E+02	
EW2-PPT2-9	7/15/14 7:54	75.16	0.17	0.02												
EW2-PPT2-10	7/15/14 14:00	75.42	0.12	0.01	0.00		0.00									
EW2-PPT2-11	7/15/14 20:06	75.67	0.10	0.01	0.00	0.00	0.00						1.58E+04	2.21E+07	3.33E+01	
EW2-PPT2-12	7/16/14 2:02	75.92	0.08													
EW2-PPT2-13	7/16/14 9:57	76.25	0.06													
EW2-PPT2-14	7/16/14 17:50	76.58	0.06	0.01	0.00	0.00	0.00									
EW2-PPT2-15	7/17/14 1:57	76.92	0.05	0.00	0.00		0.00						3.87E+05	9.03E+05	1.30E+01	
EW2-BKG-072414	7/24/14 10:32	84.27	0.04					7.46	6.60	-45.00	0.00	21.65	2.14E+04	1.06E+07	7.33E+01	
EW2-BKG-080414	8/4/14 14:59	95.46	0.44					7.55	7.39	27.00	0.00	23.98				0.50
EW2-BKG-081414	8/14/14 11:31	105.31	0.03					7.58	6.33	-28.00	0.00	22.85	1.74E+04	5.08E+05	2.33E+01	
EW2-BKG-082514	8/25/14 14:52	116.45	0.06					7.73	6.98	-66.00	0.00	23.18	1.01E+03	5.38E+03	2.00E+01	
EW2-BKG-090414	9/4/14 10:54	126.29	0.05					7.46	6.07	-72.00	0.00	21.62	1.08E+04	1.07E+05		0.50
EW2-PPT3-BKG	9/11/14 8:35	133.19		0.00	0.00	0.00	0.00	7.06	0.40	-152.00	0.00	19.38	1.64E+06	6.41E+05	1.67E+04	5.00
EW2-PPT3-INJ1	9/15/14 10:37	137.28		3.62E-02	0.00	0.00	0.00						6.75E+08	5.51E+07		
EW2-PPT3-INJ2	9/15/14 10:40	137.28		3.77E-02	0.00	0.00	0.00						1.70E+08	8.94E+06		
EW2-PPT3-INJ3	9/15/14 10:43	137.28	0.64													
EW2-PPT3-1	9/15/14 12:11	137.34	0.64	3.41E-02	0.00	0.00	0.00						5.63E+06	8.30E+06	6.67E+02	
EW2-PPT3-2	9/15/14 13:00	137.38	0.64	3.82E-02	0.00	0.00	0.00									
EW2-PPT3-3	9/15/14 13:57	137.42	0.62													
EW2-PPT3-4	9/15/14 16:58	137.54	0.52										7.64E+04	8.65E+05	0.00E+00	
EW2-PPT3-5	9/15/14 19:57	137.67	0.50	3.06E-02	0.00	0.00	0.00									
EW2-PPT3-6	9/15/14 22:59	137.79	0.44												6.67E+03	
EW2-PPT3-7	9/16/14 1:55	137.91	0.43	3.33E-02	0.00	0.00	0.00									
EW2-PPT3-8	9/16/14 5:00	138.04	0.43										4.42E+05	8.48E+06	1.33E+04	
EW2-PPT3-9	9/16/14 10:56	138.29	0.41													
EW2-PPT3-10	9/16/14 16:57	138.54	0.36	3.57E-03	0.00	0.00	0.00									
EW2-PPT3-11	9/16/14 22:54	138.79	0.31										6.77E+04	1.64E+07	0.00	
EW2-PPT3-12	9/17/14 5:08	139.05	0.26													
EW2-PPT3-13	9/17/14 12:56	139.37	0.24	2.44E-02	2.66E-02	0.00	0.00									
EW2-PPT3-14	9/17/14 19:53	139.66	0.20													
EW2-PPT3-15	9/18/14 6:45	140.12	0.17	8.41E-03	0.00	0.00	0.00						1.10E+06	4.47E+07	0.00	

Ref Table 5.6 Bioaug 1 RDX value is minimum of highlighted selection

Ref Table 5.6 Bioaug 2 RDX value is minimum of highlighted selection

Ref Table 5.6 Bioaug 3 RDX value is minimum of highlighted selection

Ref Table 5.6 Bioaug 2 associated values (i.e. pH S.U., O2, ORP, FE(II)) are averagesof highlighted section

Ref Table 5.6 Bioaug 3 associated values (i.e. pH S.U., O2, ORP, FE(II)) are averages of highlighted section

Ref Table 5.6 Bioaug 1 associated CFU or xplA copies value is average of highlighted section

Ref Table 5.6 Bioaug 2 associated CFU or xplA copies value is average of highlighted section

Ref table 5.6 Bioaug 3 associated CFU or xplA copies value is average of highlighted section

a. Ref table 5.6 Background RDX Value
b. Ref table 5.6 background xplA copies
c. Ref table 5.6 Bioaug 1, initial post-cell injection CFU value
d. Ref table 5.6 Bioaug 1, initial post-cell injection xplA copies