ESTCP
Cost and Performance Report

(ER-201207)

Bioaugmentation for Aerobic Bioremediation of RDX-Contaminated Groundwater

September 2015

This document has been cleared for public release;
Distribution Statement A

ESTCP

ENVIRONMENTAL SECURITY
TECHNOLOGY CERTIFICATION PROGRAM

U.S. Department of Defense

Page Intentionally Left Blank

Page Intentionally Left Blank

COST & PERFORMANCE REPORT

Project: ER-201207

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

Page Intentionally Left Blank

ACRONYMS AND ABBREVIATIONS

°C	Degrees Celsius
µg	Microgram(s)
AGW	Umatilla artificial groundwater
CB&I	CB&I Federal Services
CFU	Colony forming unit
DNX	Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine
DoD	U.S. Department of Defense
ERDC	U.S. Army Engineer Research and Development Center
ESTCP	Environmental Security Technology Certification Program
Fe(II)	ferrous iron
FFS	Focused Feasibility Study
ha	Hectare
g	Gram
GMOs	Genetically-modified organisms
gpm	Gallons per minute
h	Hour(s)
HGT	Horizontal gene transfer
hr	Hour(s)
I-C	*Pseudomonas fluorescens* strain I-C
IC	Ion chromatography
KanR	Kanamycin resistance
KTR9	*Gordonia* sp. KTR9 Kan^R
L	Liter(s)
lb	Pound(s)
m	Meter(s)
m^3	Cubic meter(s)
mg	Milligram(s)
MCL	Maximum contaminant level
MEDINA	Methylenedinitramine
min	Minutes(s)
mL	Milliliter(s)
mM	Millimolar(s)

mmol	Millimole
MNX	Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine
MW	Monitoring well
NCP	National Contingency Plan
NDAB	4-Nitro-2,4-diazabutanal
O&M	Operation and maintenance
O_2	dissolved oxygen
OD	Optical density
ORP	Oxidation reduction potential
OSU	Oregon State University
P&T	Pump and treat
PPT	Push-pull test
PV	Pore volume
qPCR	Quantitative polymerase chain reaction
RA	Remedial action
RDX	Hexahydro-1,3,5-trinitro-1,3,5-triazine
RHA1	*Rhodococcus jostii* RHA1 pGKT2
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
U.S.	United States
USACE	U.S. Army Corps of Engineers
USEPA	U.S. Environmental Protection Agency
xenB	xenobiotic reductase B gene
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
yr	year(s)

ACKNOWLEDGEMENTS

This report was prepared by the U.S. Army Corps of Engineers (USACE) 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 quantitative polymerase chain reaction (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; Aaron King from USACE Seattle District for his exhaustive efforts during the Phase III field work; and Ms. Rebecca Weiss from the U.S. Army 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.

Page Intentionally Left Blank

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 (P&T) or anaerobic biostimulation. The Umatilla Chemical Depot (UMCD) in Umatilla, OR, was selected as the field site for this demonstration.

OBJECTIVES

The principal demonstration objectives were: (1) to select and optimize RDX-degrading microbial cultures for use in aerobic bioaugmentation at the UMCD, (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 P&T, and both anaerobic and aerobic biostimulation without bioaugmentation. The performance objectives were as follows:

(1) Aerobic bioaugmentation degrades RDX to <2.1 micrograms per liter $(\mu g\ L)^{-1}$,

(2) RDX removal rate for aerobic bioaugmentation would be comparable to removal rates for aerobic and anaerobic biostimulation,

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

(4) Bioaugmentation culture would remain viable and retain RDX-degrading capability over time *in situ*.

TECHNOLOGY DESCRIPTION

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.

Biostimulation was accomplished by five injections of 6 cubic meters (m^3) of site groundwater containing 0.25–1 millimolar (mM) fructose into two adjacent wells over 24 days to stimulate the growth of indigenous organisms with the ability to degrade RDX. After push-pull tests (PPTs) were conducted in all wells to measure RDX degradation rates, six additional, higher concentrations (15–24 mM) fructose additions 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.

Three additional wells were bioaugmented by injecting 6 m^3 of site groundwater amended with RDX, tracer, fructose, and 10^8 cells milliliter $(mL)^{-1}$ of *Gordonia* sp. KTR9 Kan^R (KTR9).

Rates of RDX degradation were measured three times—once immediately following initial bioaugmentation with KTR9 (first test) and twice more over a period of 130 days. The results indicated that aerobic bioaugmentation achieved a rate and extent of RDX degradation larger than aerobic biostimulation and 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 P&T,

(2) Enhanced P&T followed by anaerobic biostimulation in the remaining smaller plume footprint,

(3) Enhanced P&T followed by a combination of anaerobic biostimulation and aerobic bioaugmentation in the remaining smaller plume footprint, and

(4) Enhanced P&T followed by a combination of anaerobic biostimulation and aerobic biostimulation in the remaining smaller plume footprint.

KEY RESULTS

KTR9 (and other flavodoxin cytochrome P450 gene [*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) trinitrotoluene (TNT). Therefore, Scenarios 3 and 4 include application of aerobic bioaugmentation or aerobic biostimulation 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 1–4 were approximately $11.9M, $10.3M, $10.7M, and $9.6M, respectively. By including aerobic bioaugmentation as part of the bioremediation strategy at UMCD, this has the potential to save over $1M in costs, preserve aerobic groundwater quality over a large portion of the distal RDX groundwater plume, and achieve cleanup in 15 years compared to Scenario 2, which is predicted to achieve cleanup in 30 years.

Aerobic bioaugmentation satisfied the performance objectives 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 P&T 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 U.S. Department of Defense (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.

IMPLEMENTATION

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 should 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.

Page Intentionally Left Blank

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 flavodoxin cytochrome P450 gene (*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]; 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 micrograms per liter $(\mu g\ L)^{-1}$ over the estimated 80 hectare (ha) plume. Results of this demonstration are being used to optimize the existing pump-and-treat (P&T) groundwater remedy at UMCD by supporting incorporation of bioaugmentation into a full-scale remediation program. The objectives for this demonstration were to:

(1) Select and optimize RDX-degrading, *xplA*-containing microbial cultures for use in bioaugmentation at the UMCD,

(2) 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) Quantify and compare costs of RDX remediation in groundwater and time-to-complete at UMCD using P&T, 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) for RDX; however, the U.S. Environmental Protection Agency (USEPA) has listed RDX on the Drinking Water Candidate Contaminant List[1] and the Unregulated Contaminant Monitoring Regulation List.[2] In addition, the USEPA has issued lifetime Health Advisory Limits (Maximum Contaminant Level Goal) of 2 μg L^{-1} for RDX. The risk-based cleanup goal based on residual carcinogenic risk of 1 x 10^{-6} is 0.8 μg L^{-1} for RDX. The State of Oregon has not issued Groundwater Protection Standards for RDX. The remedial action (RA) criteria concentration established in the UMCD Record of Decision for RDX was 2.1 μg L^{-1}.

[1] http://water.epa.gov/scitech/drinkingwater/dws/ccl/ccl3.cfm
[2] http://water.epa.gov/lawsregs/rulesregs/sdwa/ucmr/factsheet.cfm

2.0 TECHNOLOGY

2.1 TECHNOLOGY DESCRIPTION

Bioaugmentation involves injection of selected microbial cultures into an aquifer to increase effective *in situ* biological degradation of a particular contaminant. 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] suggested that *Rhodococcus rhodochrous* 11Y and *Rhodococcus* sp. DN22 are good candidates for aerobic 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]; 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 *xplAB* genes are located on a plasmid (a mobile genetic element), and it appears that these genes and their metabolic potential have spread among bacteria on several continents [11, 12]. In addition to strains with *xplA, Pseudomonas fluorescens* strain I-C is a facultative anaerobe that degrades RDX under anoxic conditions [14]—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 P&T 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, trinitrotoluene (TNT) or other comingled explosives, the culture developed for this bioaugmentation 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 UMCD site. These risks were assessed and managed using the specific quantitative performance objectives shown in Table 3.1.

Page Intentionally Left Blank

3.0 PERFORMANCE OBJECTIVES

The performance objectives evaluate whether aerobic bioaugmentation is capable of reducing RDX concentrations to the RA 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. Performance objectives, data requirements, success criteria, and results are provided in Table 3.1.

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 (PPTs) with suitable detection limits to confirm concentrations <2.1 µg L^{-1}	Complete (>90%) removal by mass 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 in <1 month.
RDX removal rate for aerobic bioaugmentation would be comparable to removal rates for aerobic and anaerobic biostimulation	Dilution-adjusted RDX concentrations during PPTs 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 of, 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 would be larger for aerobic bioaugmentation than for aerobic or anaerobic biostimulation	RDX degradation rates and substrate mass will be 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 millimoles (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 remained viable and retains RDX-degrading capability over time *in situ*	Bacterial survival will be indirectly determined by *xplA* gene counts using TaqMan quantitative polymerase chain reaction (qPCR) assay developed under ER-1609. RDX-degrading capability will be assessed over time using PPTs.	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 (CFUs) above 300 CFU milliliters $(mL)^{-1}$ 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 milligrams (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.

mmol – millimole

Page Intentionally Left Blank

4.0 SITE DESCRIPTION

4.1 SITE SELECTION

The UMCD located near Hermiston, OR, 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 the U.S. Army Corps of Engineers (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.

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. The saturated thickness of the aquifer in the former lagoon area is approximately 4 to 11 meters (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.

4.3 CONTAMINANT DISTRIBUTION

The areal extent of the RDX groundwater plume above the RA criteria is over 150 ha (Figure 4.1). 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 P&T 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 P&T extraction wells. The infiltration system operated following plant startup but was discontinued after five years following no apparent increase in RDX mass captured.

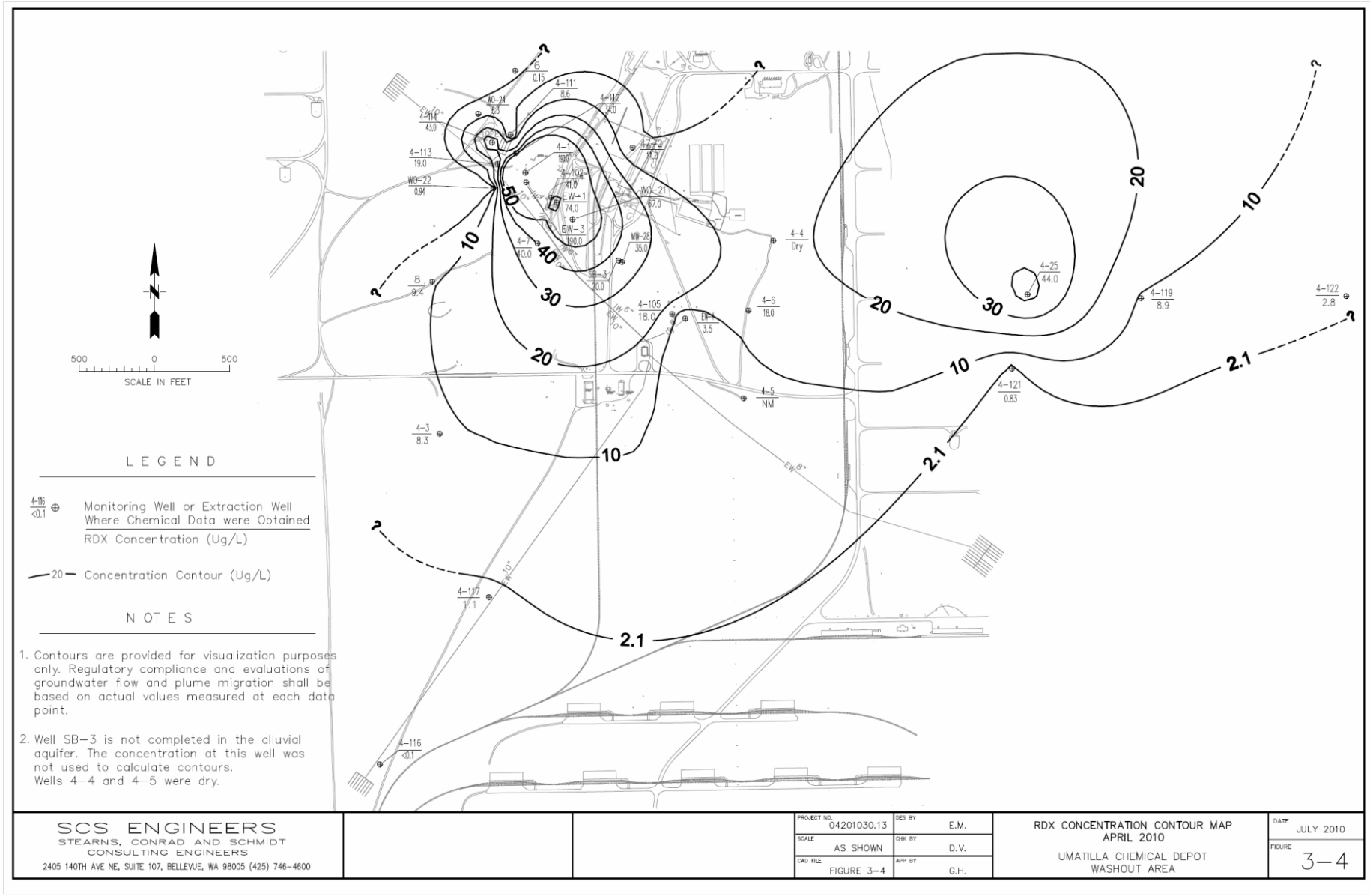

Figure 4.1. RDX Concentrations in Groundwater at UMCD (SCS, 2010).

5.0 TEST DESIGN

This section provides a brief overview of the field demonstration conceptual design (Section 5.1), followed by a baseline characterization and an overview of Phase I laboratory results (Sections 5.2 and 5.3, respectively). Descriptions of field demonstration Phases II and III are included in Sections 5.4 and 5.5, respectively. Additional detailed methods and results are presented in the ER-201207 Final Report.

5.1 CONCEPTUAL EXPERIMENTAL DESIGN

Phase I of this demonstration included site characterization, 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 (PPTs) for aerobic and anaerobic biostimulation treatments and aerobic bioaugmentation (Figure 5.1). The aerobic biostimulation with no cells added served as the bioaugmentation control.

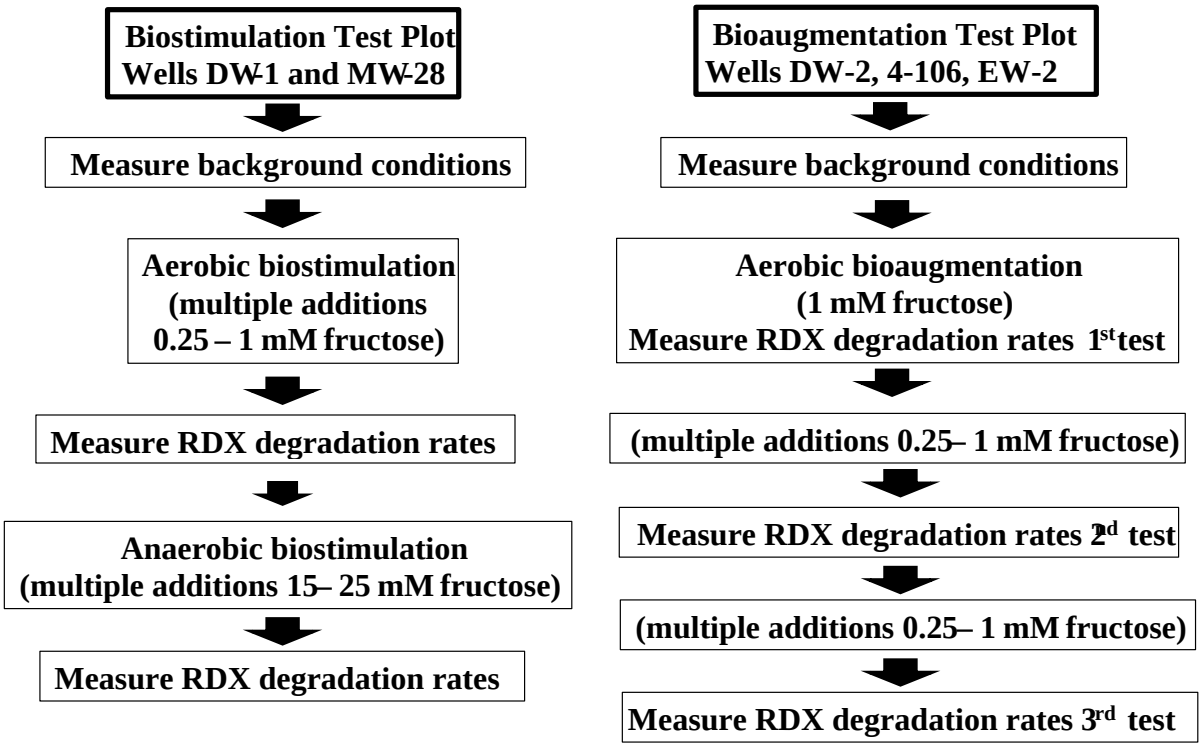

Figure 5.1. Conceptual Design for Demonstration Phase III.

5.2 BASELINE CHARACTERIZATION

Baseline characterization included installation of two demonstration wells (DW-1 and DW-2), followed by a series of forced and natural gradient tracer tests. Boring logs and detailed results of the site characterization activities are included in the ER-201207 Final Report.

In summary, tracer testing confirmed hydraulic connectivity of the test wells and confirmed suitability of planned injection volumes and test durations for subsequent field demonstration phases.

5.3 PHASE I LABORATORY TESTING SUMMARY

Laboratory testing was completed to obtain a bioaugmentation culture that could be transported and sustain the highest possible RDX-degrading activity in the UMCD aquifer. Detailed methods and results of the laboratory testing were presented in the Phase I Results Memorandum (ER-201207 Final Report, 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 survive in UMCD sediment and groundwater for several months, be transported through repacked UMCD sediments, and reduce RDX concentration to <2.1 $\mu g\ L^{-1}$.

Pure culture screening and microcosm testing. Results of initial pure culture screening resulted in selection of the following three strains for inclusion in the bioaugmentation inoculum: *Gordonia* sp. KTR9 Kan^R, *Rhodococcus jostii* RHA1 pGKT2, and *Pseudomonas fluorescens* I-C, henceforth referred to as KTR9, RHA1, and Strain I-C, respectively. 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 Umatilla artificial groundwater (AGW). Microcosms were incubated at 15 degrees Celsius (°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 seven days at 15°C in the microcosms despite the presence of the indigenous population and the low nutrient levels. RDX degradation occurred very quickly in the inoculated microcosms with 98% of the RDX removed to below the 2.1 $\mu g\ L^{-1}$ site-specific objective in one day. Degradation of RDX by the indigenous UMCD sediment population was significantly slower with only 15% degraded in seven days.

Column testing. The re-packed UMCD sediment columns were used to simulate aquifer conditions and seepage velocities. The bioaugmentation culture (10^9 cells milliliter $[mL]^{-1}$ each strain) was injected into the column followed by UMCD AGW containing 0.5 milligrams (mg) L^{-1} RDX. Aerobic biostimulation (0.1 millimolar [mM] fructose additions) before bioaugmentation resulted in negligible RDX degradation. Fructose additions (0.1 mM) following bioaugmentation stimulated rapid RDX degradation, and the ability to stimulate biodegradation upon fructose addition was sustained following a long period of starvation (Figure 5.2).

Furthermore, all three strains were able to be grown to culture densities required for field-scale application and could be stored at 4°C for several months without significant loss of viability or RDX degradation activity [15]. Accordingly, the demonstration proceeded to Phase II field testing.

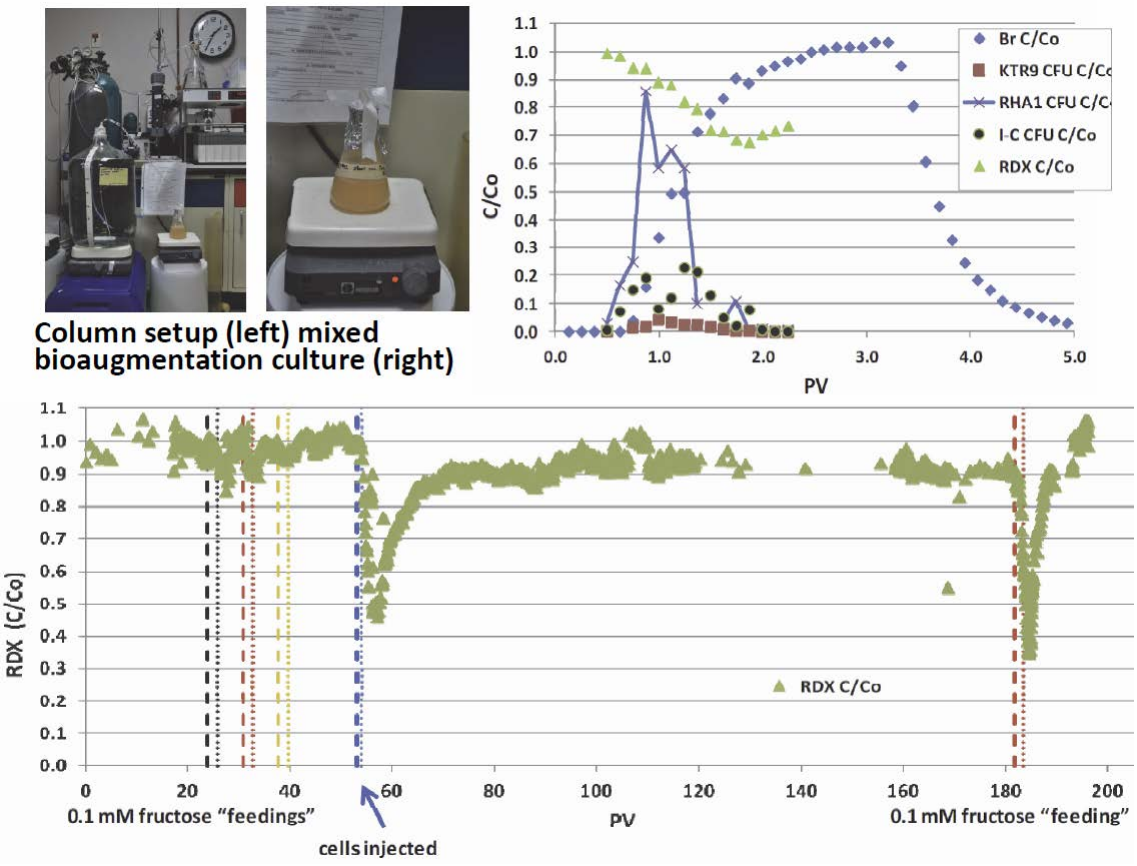

Figure 5.2. Breakthrough Curves for Br^-, Cell and RDX Concentrations for Column Experiment 3, where C is the Measured Tracer or Cell Concentration in the Column Effluent; Co is the Influent Tracer or Cell Concentration.

5.4 PHASE II FIELD-SCALE CELL TRANSPORT TESTING SUMMARY

The following is a summary of the work performed during Phase II, which is presented in more detail in Crocker et al. 2015 [16]. Phase II consisted of a short duration forced-gradient cell transport test to confirm the ability to distribute the mixed culture of KTR9, RHA1, and I-C in the Bioaugmentation Test Plot (Figure 5.3). Enhanced degradation of RDX was not evaluated in this demonstration. Detailed methods and results were presented in the Phase II Results Memorandum (ER-201207 Final Report, 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.

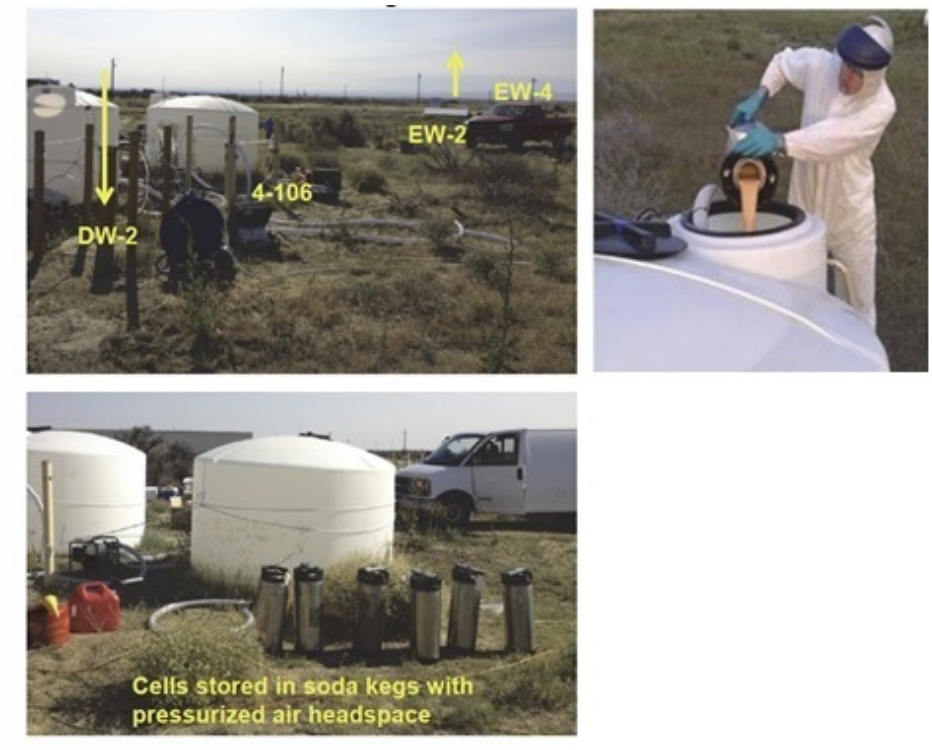

Figure 5.3. Clockwise from left: Phase II field test wells; adding cells to injection solution; inoculum staged prior to mixing.

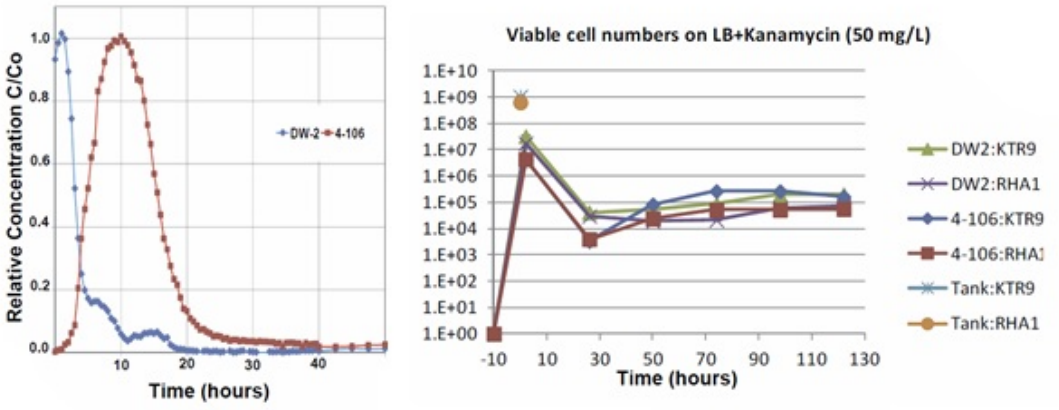

Figure 5.4. CL^- breakthrough curves in the injection well DW-2 (blue), and well 4-106 (red) (left). Viable plate counts on LBKan agar plates of KTR9 and RHA1 from wells DW-2, 4-106 and the injection tank (right).

Similar to the re-packed column tests, transport of strains KTR9, RHA1, and I-C was faster than the transport of the tracer. The field demonstration confirmed the rapid transport of the strains to downgradient Well 4-106 (3 m). However, the transport of cells and the tracer to downgradient Well EW-2 (21 m) was not observed. Strains KTR9 and RHA1 remained detectable in the test wells for up to five days at levels around 10^5 colony forming unit (CFU) mL^{-1} or 10^5–10^7 *xplA* gene copies mL^{-1}. Strain I-C survived during the transport demonstration but at lower numbers (10^4 xenobiotic reductase B gene [*xenB*] gene copies mL^{-1}) than KTR9 and RHA1. Subsequent field-scale cell transport testing confirmed the ability to transport KTR9 cells over 30 m in the aquifer; follow-on sampling confirmed cells remained viable more than two months after this field-scale cell transport test [16].

5.5 PHASE III: BIOAUGMENTATION FIELD TRIAL AND PPT SUMMARY

The following is a summary of the work performed during Phase III, which is described in more detail in Michalsen et al. 2015 [17]. Analytical and supportive data collected during the Phase III field testing is included in Appendix E of the ER-201207 Final Report. 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.

Average aerobic and anaerobic biostimulation rates were 0.49 and 0.67 day-1, respectively. RDX degradation rates were measured three times during the bioaugmentation tests—once immediately following the initial bioaugmentation and twice more over a 130-day period. Aerobic bioaugmentation removed RDX faster than aerobic biostimulation and comparable to anaerobic biostimulation, and required 34 times less added substrate. The average RDX degradation rate for aerobic bioaugmentation (all wells and all test combined) was 1.2 day-1. However, the RDX transformation rate in the bioaugmentation wells declined over time (Figure 5.4). The RDX transformation rate decline in the bioaugmented wells is attributed to decreases in cell numbers as well as the onset of reducing conditions following repeated fructose additions. The results show that all three treatments can achieve the RA criteria, but a single cell addition was sufficient to allow aerobic bioaugmentation to achieve the RA criteria in the shortest time for the first two tests (Table 6.1).

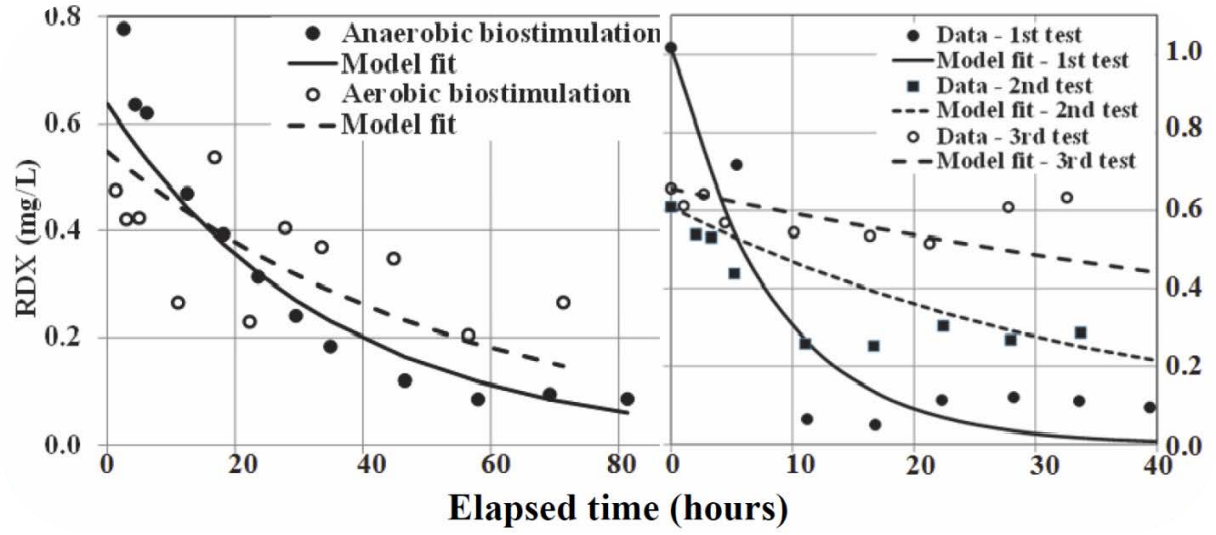

Figure 5.5. Example PPT Results in Biostimulation Well MW-28 (left) and Bioaugmentation Well DW-2 (right) Showing *In Situ* RDX Degradation and First-Order Model Fits Following Treatments.

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 RA criteria of 2.1 µg L^{-1}.

   Success criteria: Reduction of RDX concentration to <2.1 µg L^{-1} during the Phase III PPT 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 of ER-201207 Final Report) 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 RA criteria, during repeated testing over 130 days, is attributed to decreases in cell numbers and viability as well as the onset of reducing conditions following repeated fructose additions. However, the time required to reach the RA 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 RA criteria, but a single cell addition was sufficient to allow aerobic bioaugmentation to achieve the RA 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 RA Criteria for all Treatments.

Treatment	[a]Average RDX degradation rate coefficient (day^{-1})	[b]Time required to reach RA criteria (days)
Aerobic bioaugmentation		
1st test	2.6 (0.96–4.0)	1.5
2nd test	0.70 (0.08–1.4)	5.5
3rd test	0.18 (0.15–0.24)	21
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 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 of, 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—approximately 2 times 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 RA 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	1.2	-
Aerobic biostimulation	0.49	2.4
Anaerobic biostimulation	0.67	1.8

3. **Find 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 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}	1.2	0.49	0.67
Representative initial RDX groundwater concentration at start of treatment	C_o RDX, µg L^{-1}	100	100	100
Computed final RDX groundwater concentration after 5 days of treatment	C_f RDX, µg L^{-1}	25	79	64
Computed RDX removed during 5 days of treatment, assumes 5,700 L of water treated	millimols	1.9	0.56	0.92
Measured fructose mols during each 5,700 L PPT	mols	5.7	5.7	136
Computed mmol RDX removed per mol fructose added		**0.34**	**0.10**	**0.01**

4. <u>Bioaugmentation culture remains viable and retains *in situ* RDX-degrading capability over time.</u> 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.

<u>Success criteria</u>: 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.

<u>Results</u>: 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 over time 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 PPTs.

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	4.0	2×10^6	2×10^7	7×10^7
2nd test	1.4	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 (Fe(II)), or methane; the potential dissolution of redox sensitive metals; as well as the formation of the RDX nitroso degradation products hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), or hexahydro-1,3,5-trinitroso-1,3,5-triazine (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	3	2	2
Aerobic biostimulation	7.6	2.8	10	0	3	6	3
Anaerobic biostimulation	6.8	1.9	-171	2.2	65	0	9

[a]Maximum concentration detected during tests in all wells; mV – millivolt(s); O_2 – dissolved oxygen

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 are based on groundwater remedy optimization work completed at the UMCD site. At this site, an increasingly inefficient 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 [18]. 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 and aerobic bioaugmentation is applied to 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 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 RA 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) Performance of 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 RA 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 PPTs 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 PPTs (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 operation and maintenance (O&M) 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 the 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 (1) strains are able to grow and (2) 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 two 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), $160K	
		Cell transport column testing (Lab), $100K	
		Reporting, $50K	
RA Construction. Phase I	Expanded Groundwater Extraction System Construction		-
	Anaerobic Biostimulation in Lagoon Source Area		-
	1 Monitoring Event year $(yr)^{-1}$ during RA		-
	O&M		-
Remedial Design, Phase II	Phase II – Design modifications to existing, expanded P&T system to include biostimulation or bioaugmentation in plume including groundwater model simulations	Prepare field-scale quantity of cells for shipment to field, $50K	~$ 383K
		Field-scale, forced-gradient cell transport testing, $100K	
		Analytical costs including qPCR and viable cell counts, $100K	
		Reporting, $50K	
		Prepare field-scale quantity of cells for bioaugmentation, $50K	~$468K
		Growth substrate injections in field 4+ months, $80K	
		Complete 13 PPTs to measure RDX removal effectiveness, $100K	
		Analytical costs including qPCR and viable cell counts, $100K	
		Reporting, $50K	
RA Construction	Comingled Plume Biodegradation – Anaerobic Injection Well and trenching Construction		-
	RDX Plume Biodegradation – Aerobic Injection Well and trenching Construction		-
O&M	Comingled Explosives Plume Biodegradation – Aerobic Substrate Injections		-
	RDX Plume Biodegradation – Aerobic Substrate Injections		-
	Semiannual Monitoring		-
	Five-Year Reviews		-
Completion Activities	Site Closeout Documentation		-
	Administrative Land Use Controls		-
Non-Discounted Cost			**$1,291K**

bgs – below ground surface

- **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 over time can be evaluated. The costs of column testing at a site will depend on (1) contracting costs associated with acquiring technical services for collection of sediment and groundwater quantities needed for column testing, and (2) the number of columns and duration of the tests, which determines the 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/sample based on the availability of general laboratory supplies and 4 hours (hr) ($50/hr) 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 is not always available for colony discrimination between the indigenous and inoculated strains. The cost of the *xplA* qPCR assay is estimated to be about $500/sample since a senior technician trained in molecular biology is required along with specific reagent kits and analytical instruments. Kits for deoxyribonucleic acid (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 1×10^6 cells mL^{-1}. Reporting limits for *xplA* gene copy numbers by qPCR are in the 1×10^3 range. Accordingly, users may plan to conduct a field tracer test to confirm 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/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 [18]. Explosives-laden wastewater percolated through the unsaturated alluvium beneath the lagoons to groundwater, creating the groundwater plume. The RA 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 P&T system. This *in situ* soil-flushing component was completed in 2000. Following years of operation, the P&T system reached diminishing removal efficiency, leaving 45 ha over 20 µg L^{-1} RDX and over 150 ha over the 2.1 µg L^{-1} RDX cleanup level (Figure 4.1). 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 P&T system;

(2) Expanded P&T system plus RDX plume treatment using anaerobic biostimulation;

(3) Expanded P&T system plus comingled RDX/TNT plume treatment using anaerobic biostimulation and RDX plume treatment using aerobic bioaugmentation; and

(4) Expanded P&T 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 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 Office of Management and Budget (OMB) defined real 30-Year 2016 interest rate of 1.4%.[3] Program management costs were not included.

[3] www.whitehouse.gov/omb/circulars/a094/a94_appx-c.html.

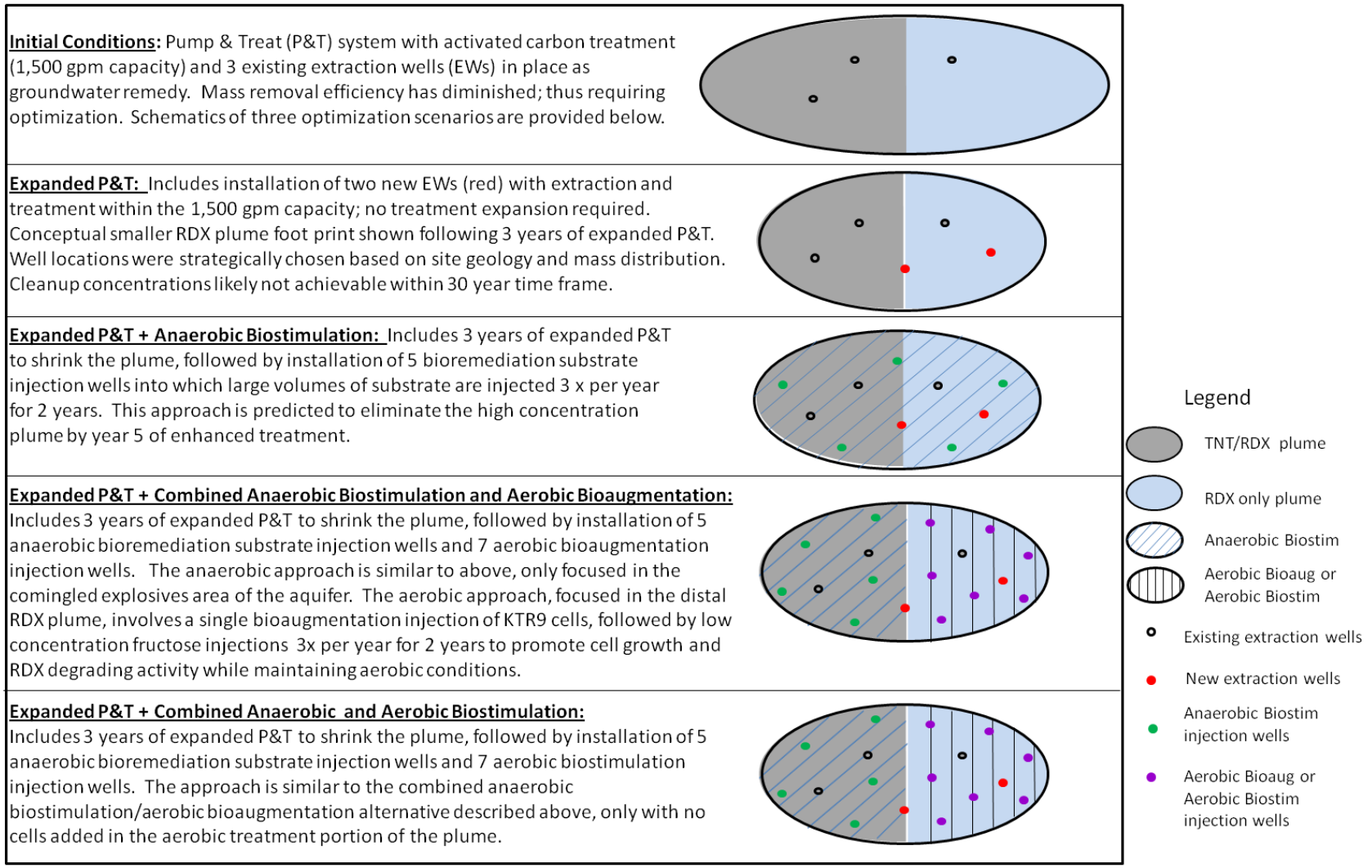

Figure 7.1. Schematic of Costed Remedy Scenarios at UMCD. Ovals Represent Relative Groundwater Plume Size.

Scenario 1 – Expanded P&T

The expanded 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, and 30 years of 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 gallons per minute (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 (lbs) 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 lbs of fructose was included for anaerobic biostimulation of the plume within the two-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 five 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 lbs 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 lbs 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 expanded cell transport test [16], it was assumed for costing purposes that cells would be transported 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. It was 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 12 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 five 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 lbs 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 lbs of fructose for two years. A total of 12 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 five 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 P&T Only, With No Bioremediation: Scenario 1 (30 Years [yrs], $K)

Cost Element	Sub Element	0	1	2	3	4	5	6–29	30	Total Cost
Remedial Design	Design expanded P&T facility	175								175
RA Construction	Construct expanded P&T facility		2,000							2,000
O&M	O&M		302	302	302	302	302	302	302	9,060
	1 Monitoring Event/yr in years 1–26, 2/yr in years 27–29		90	90	90	90	90	90	90	2,700
	Five-Year Reviews						19	19[a]		95
Completion Activities	Site Closeout 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	2,359	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 P&T 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 2x Injection Well Pilot	180																180
	Field Scale Pilot Injection Using New Injection Well	179																179
	Phase I – Design Expanded Groundwater Extraction System	175																175
RA Construction	Expanded Groundwater Extraction System Construction		2,000															2,000
O&M and Pre-Design	Anaerobic Biostimulation in Lagoon Source Area	173	173															346
	1 Monitoring Event/yr		90	90	90	90	90											450
	O&M		302	302	302	302	302	302	302	302	302	302						3,020
Remedial Design	Phase II – Transition to Bioremediation in Plume including groundwater model simulations			190														190
RA Construction	Construct additional bioremediation wells, one additional extraction well, bioremediation amendment injections				1,400	900	900											3,200
O&M	1 Monitoring Event/yr in years 6–12, 2/yr in years 12–14							90	90	90	90	90	90	90	90	90	90	900
	Five-Year Reviews						19					19						38
Completion Activities	Site Closeout 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 P&T 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 2x Injection Well Pilot	180																180
	Field Scale Pilot Injection Using New Injection Well	179																179
	Phase I – Design Expanded Groundwater Extraction System	175																175
RA Construction	Expanded Groundwater Extraction System Construction		2,000															2,000
O&M and Pre-Design	Anaerobic Biostimulation in Lagoon Source Area	173	173															346
	1 Monitoring Event/yr		90	90	90	90	90											450
	O&M		302	302	302	302	302	302	302	302	302	302						3,020
Remedial Design	Phase II – Transition to Bioremediation in Plume including groundwater model simulations			190														190
RA 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
O&M	1 Monitoring Event/yr in years 6–12, 2/yr in years 12–14							90	90	90	90	90	90	90	90	90	90	900
	Five-Year Reviews						19					19						38
Completion Activities	Site Closeout 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 P&T 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 2x Injection Well Pilot	180																180
	Field Scale Pilot Injection Using New Injection Well	179																179
	Phase I – Design Expanded Groundwater Extraction System	175																175
RA Construction	Expanded Groundwater Extraction System Construction		2,000															2,000
O&M and Pre-Design	Anaerobic Biostimulation in Lagoon Source Area	173	173															346
	1 Monitoring Event/yr		90	90	90	90	90											450
	O&M		302	302	302	302	302	302	302	302	302	302						3,020
Remedial Design	Phase II – Transition to Bioremediation in Plume including groundwater model simulations			190														190
RA 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
O&M	1 Monitoring Event/yr in years 6–12, 2/yr in years 12–14							90	90	90	90	90	90	90	90	90	90	900
	Five-Year Reviews						19					19						38
Completion Activities	Site Closeout 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 PPTs (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 USEPA'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 USEPA 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, 19, 20], 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 [21, 22]. Concentrations of TNT around 10–20 mg L^{-1} resulted in a 50% inhibition of cell growth for Gram-positive isolates [21, 22]. 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}) [23]. Furthermore, RDX (7.5 mg L^{-1}) degradation by *Rhodococcus* strain YH1 (via flavodoxin cytochrome P450 protein [*XplA*]) was inhibited by TNT

(7.5 mg L^{-1}), and RDX degradation only occurred after the TNT had been completely transformed [24]. 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 (EW-2 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 [16]) 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 the 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) intended to simulate PPT conditions [25], 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, the hypothesis is 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. This reduced 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 (O_2) and ORP. Anaerobic conditions have been shown to inhibit RDX-degrading activity in *Gordonia* sp. strain KTR9 used in this study [16], 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 approximately three-month time between "feedings" and will be stimulated to degrade RDX with subsequent substrate additions. KTR9 maintained viability *in situ* at UMCD for approximately three months without substrate feedings (to be discussed elsewhere [16]).

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 [26] 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 $\geq$ 0.060), supporting the hypothesis that bioaugmentation with aerobic RDX degraders would be required to support aerobic RDX remediation of the UMCD aquifer. Based on these results, it is recommended 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 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, one should start with low substrate concentrations, then increase as needed based on results.

Page Intentionally Left Blank

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

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

17. 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.

18. 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.

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

20. 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.

21. 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.

22. 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.

23. 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.

24. 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.

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

26. 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.

ESTCP Office
4800 Mark Center Drive
Suite 17D08
Alexandria, VA 22350-3605
(571) 372-6565 (Phone)
E-mail: estcp@estcp.org
www.serdp-estcp.org