FINAL REPORT

Groundwater Chemistry and Microbial Ecology Effects on Explosives Biodegradation

SERDP Project ER-1378

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List of Abbreviations

BLAST	basic local alignment search tool
BSA	bovine serum albumin
BSM	basal salts medium
^{12}C	carbon (normal isotope signature)
^{13}C	stable carbon isotope
$CsCl_2$	cesium chloride
CW	cheese whey
2,4-DNT / 2,6-DNT	2,4- and 2,6-dinitrotoluene
2A-DNT/4A-DNT	2-amino-4,6- and 4-amino-2,6-dinitrotoluene
DGGE	denaturing gradient gel electrophoresis
Dicumarol	3,3'-methylene-bis(4-hydroxycoumarin)
DNA	deoxyribonucleic acid
DNX	hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine
DO	dissolved oxygen
DoD	Department of Defense
EPA	Environmental Protection Agency
EOD	explosive ordnance disposal
GW	groundwater
HA	health advisory
HMX	octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
HPLC	high performance liquid chromatography
K_{ow}	soil organic water partition coefficient
MMR	Massachusetts Military Reservation
MNX	hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine
N	nitrogen
^{15}N	stable nitrogen isotope
NCBI	National Center for Biotechnology Information
NDAB	4-nitro-2,4-diazabutanal
NG	nitroglycerine (glycerol trinitrate)
NH_4	ammonia
NH_4Cl	ammonium chloride
NOP	Nebraska Ordnance Plant
OB/OD	open burn/open detonation
ORP	oxidation-reduction potential
PBS	phosphate-buffered saline
PCB	polychlorinated biphenyl
PCD	Pueblo Chemical Depot
PCR	polymerase chain reaction
PETN	pentaerythritoltetranitrate
PVDF	polyvinylidine fluoride
qPCR	quantitative polymerase chain reaction
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine
SAB	Scientific Advisory Board

SERDP	Strategic Environmental Research and Development Program
SIP	stable isotope probing
tetryl	2,4,6-trinitrophenylmethylnitramine
TKN	Total Kjeldahl Nitrogen
TNT	2,4,6-trinitrotoluene
TNX	hexahydro-1,3,5-trinitroso-1,3,5-triazine
tRFLP	terminal restriction fragment length polymorphism
UXO	unexploded ordnance
WVOW	West Virginia Ordnance Works
YE	yeast extract

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I. EXECUTIVE SUMMARY

The overall goal of this project was to gain a better understanding of the microbial ecology of explosives compound biodegradation in groundwater. Deciphering which organisms are involved with explosives degradation under various *in situ* conditions could lead to better diagnostic and monitoring tools for bioremediation of energetics based on biomarkers, as well as lead to better conceptual and predictive models.

Initially, the scope of this project included most of the major explosive compounds that have been detected in soil and groundwater at military installations – TNT, RDX, HMX, DNT, etc. However, the scope was narrowed to RDX in the second half of the project because this is the compound of greatest concern in groundwater due to its mobility and recalcitrance.

This research coupled chemical analyses to monitor RDX degradation, and developed and applied the molecular techniques of polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and stable isotope probing (SIP) to assess the microbial community. Individual members of the microbial community were identified based on recovered 16S rRNA gene sequences. Through analysis of samples from laboratory enrichments, model aquifers, and actual bioremediation field demonstrations, the following conclusions can be drawn from the data generated during this project:

1) RDX is amenable to biological degradation in groundwater when nutrients are added. Under some circumstances, the RDX can be used as the sole or supplemental nitrogen sources, as well as a carbon source. In general, RDX was not readily degraded as the sole nitrogen source under the conditions tested.

2) RDX was amenable to degradation in the presence of both complex (cheese whey, yeast extract) and defined (glucose, succinate, ± ammonium) nutrient sources.

3) RDX degradation is more labile in groundwater under anoxic/anaerobic (low redox) conditions than under aerobic conditions. Aerobic degradation was not generally observed in groundwater, and aerobic RDX-degraders were not readily isolated or detected using molecular methods.

4) RDX was amenable to degradation at typical groundwater temperatures of 15°C.

5) Organisms detected in samples actively degrading RDX were generally not closely related to bacterial strains that have been previously described as being able to degrade RDX. The exception would be sequences identified as belonging to genera *Clostridium* and *Pseudomonas*, several strains of which have been shown to degrade RDX.

6) Several nitrogen-fixing genera not previously associated with explosive compound degradation in general, or RDX degradation in particular, were detected in multiple samples. These genera included *Azospira*, *Azospirillum*, and *Pleomorphomonas*.

7) The putative RDX-degrading genes (*xenA*, *xenB*, *xplA*, *onr*, *hydA*, *nerA*) were not detected in any samples with the exception of one of the Picatinny Arsenal model aquifer effluent samples. Given the wide range of samples screened (including many samples that were actively degrading RDX), these results seem to indicate that gene probing methods based on these specific genes are not likely relevant at this time.

8) The application of stable isotope probing (SIP), based on molecular analysis of nucleic acids that become enriched in ^{13}C and/or ^{15}N as organisms degraded stable isotope-labeled RDX, confirmed that bacterial genera other than those previously identified as RDX-degrading genera were present in samples exhibiting RDX degradation.

At the conclusion of this research it appears that no single "biomarker" organism could be associated with RDX degradation in groundwater, at least under the anoxic/anaerobic conditions tested. However, the application of SIP to more directly probe which organisms are interacting with RDX (and/or RDX breakdown products) holds great promise to obtain more specific information and narrow down the list of "organisms of interest." SIP should also lead to insight into which bacterial genera may be best to study further in terms of developing bioremediation technologies for RDX in groundwater.

II. PROJECT OBJECTIVES

The objective of this project was to develop a better understanding of how environmental conditions affect the biotransformation and biodegradation of explosive compounds in groundwater, and to examine how these variables affect the composition and functioning of the indigenous microbial communities with respect to explosive compound biodegradation.

To achieve this objective, the following tasks were performed:

- Develop molecular methods to monitor the microbial communities associated with explosive compound biotransformation.
  - -Obtain explosive-degrading bacterial strains
  - -Identify putative explosive-degrading genes
  - -Develop protocols for polymerase chain reaction (PCR), quantitative PCR (qPCR) and denaturing gradient gel electrophoresis (DGGE)

- Determine how groundwater chemistry (e.g., presence of alternative electron acceptors, presence or absence of an inorganic nitrogen source) affects the transformation and degradation of nitramine explosives <u>and</u> the microbial community or sub-populations involved with explosive compound degradation.
  - -Obtain subsurface sediment and groundwater from explosive-contaminated sites
  - -Perform microcosm enrichment experiments
  - -Perform model aquifer studies (done in collaboration with ESTCP Project ER-1425)
  - -collect and analyze field samples

- Relate changes in the microbial community to changes in the abundance of genes coding for enzymes that have been shown to be involved in explosive compound transformation and degradation (based on previous research) using specific DNA probes developed during the project.

The technical approach flow chart is illustrated in Figure I-1, and the major questions addressed by this project are presented in Figure I-2.

Figure I-1. Technical approach for this project.

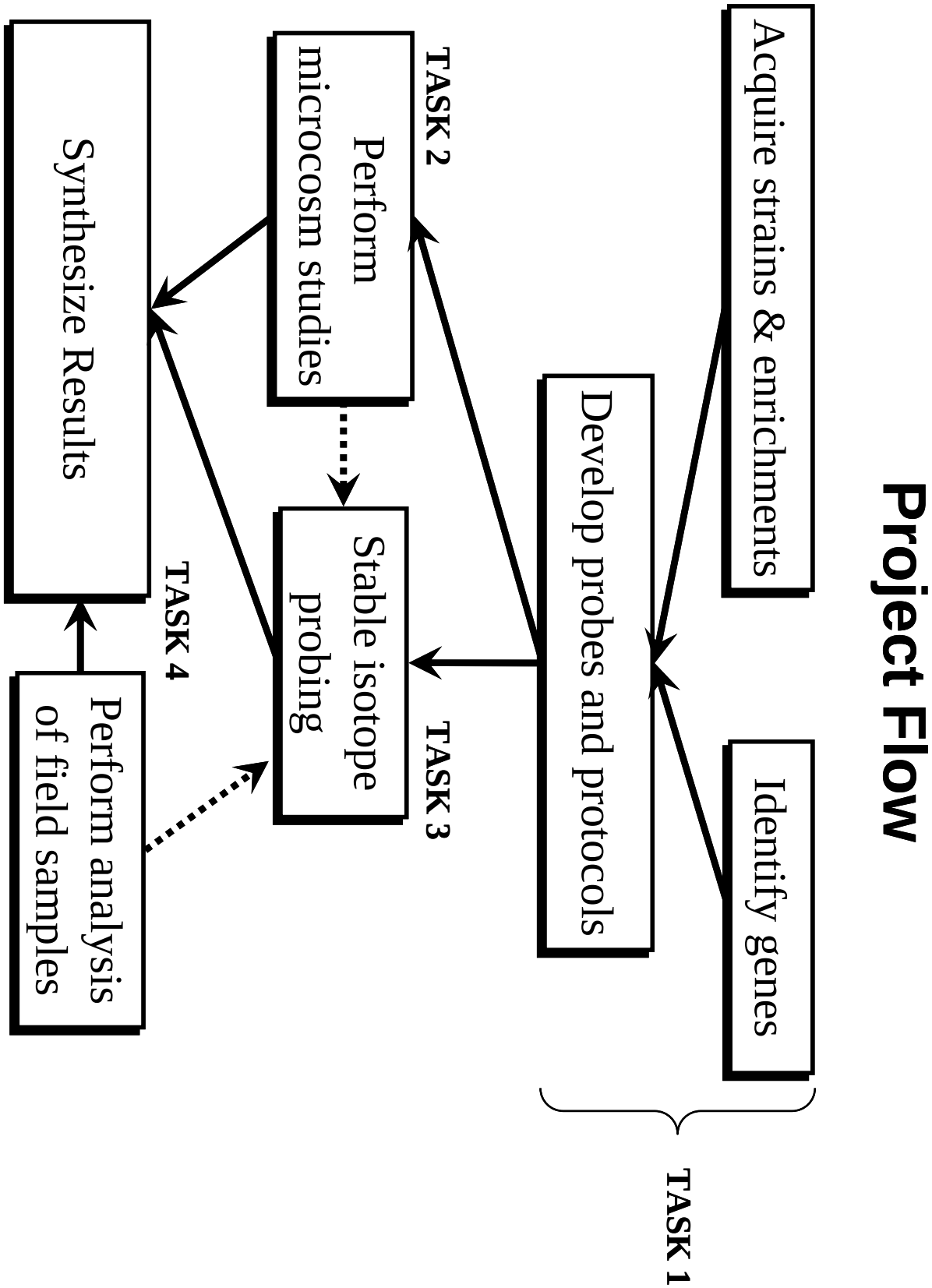
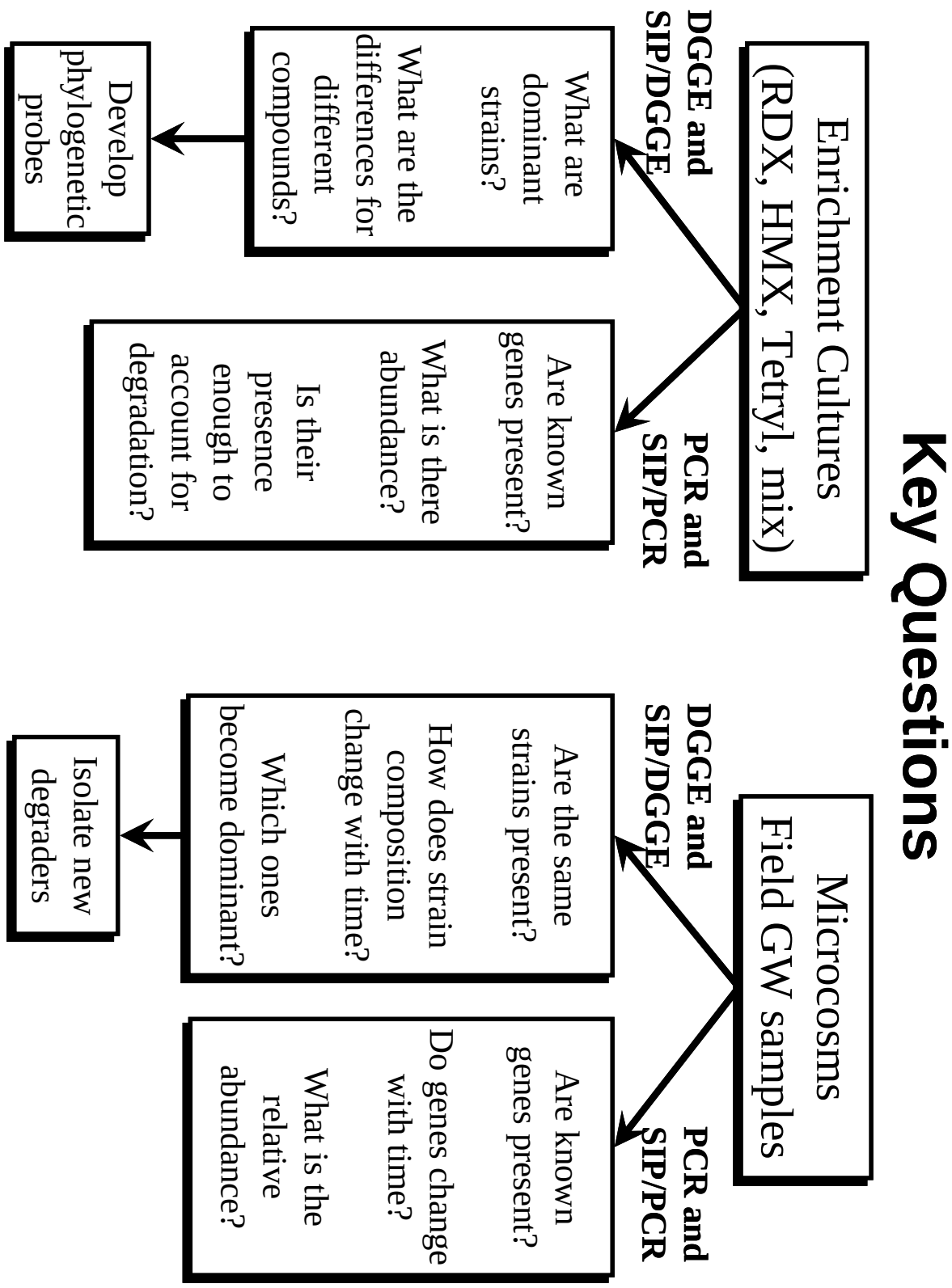


Figure I-2. Summary of the major questions addressed during this project.



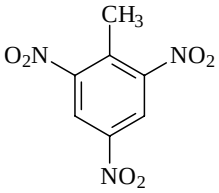
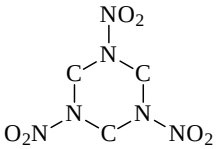
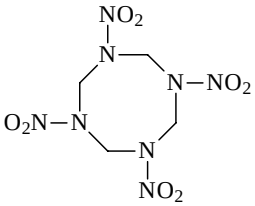
III. PROJECT BACKGROUND

Contamination at Department of Defense Installations. Past and ongoing Department of Defense (DoD) activities have the potential to adversely affect soil, sediment and groundwater at DoD facilities. Those activities, including ammunition production, load/pack/assembly lines, live fire military training, and open burn/open detonation operations that have resulted in contamination of the environment with explosive compounds. The primary contaminants at these sites that will be the focus of this research are presented in Table II-1. Although contamination at ammunition production facilities is usually characterized by small areas with relatively high concentrations, firing and impact range contamination is usually lower in concentration and exhibits a large spatial heterogeneity (18, 29). The munitions that are tested at DoD impact ranges contain a number of different explosive compounds. For example, a 60-mm mortar round contains TNT in the primer, 2,4-DNT and 2,6-DNT in the propellant charge, TNT and RDX in the filler, and RDX and HMX in the fuse. After full- or partial- detonation of a munition round, residues of these materials can remain in the impact area (41). In sandy soils with little organic matter or clay content, such as those present at the Massachusetts Military Reservation (MMR; Cape Cod, MA, USA), transport of TNT, RDX, and HMX to the vadose zone and ultimately to groundwater is possible. Recent reports of groundwater contamination at MMR with RDX confirm this assumption. These explosive-related compounds have been observed to be recalcitrant in many environments, leading to the potential for long-term contamination at sites where they are released. The contamination of groundwater underlying these facilities is particularly problematic because the explosive residues have the potential to adversely impact local drinking water supplies, and few studies have evaluated intrinsic biodegradation of explosives in this environment.

Some of the difficulties that must be addressed before this problem can be effectively dealt with include the large number and size of areas that are contaminated and the heterogeneity of the contamination, both in terms of spatial distribution and contaminant concentrations. For instance, MMR's training areas cover over 144,000 acres, with multiple target areas. These target areas are of greatest concern from a health and groundwater protection point of view, but even the extent of contamination within these types of areas is hard to clearly delineate. The presence of unexploded ordnance (UXO) likely serves as widely distributed point sources for continued contamination. Additionally, the process used to remediate ranges needs to be compatible with the continuance of training activities at contaminated sites.

Regulatory Environment. Although there are currently no federal drinking water standards for the aforementioned explosives, the U.S. Environmental Protection Agency (EPA) has established health advisory (HA) levels for TNT, HMX, RDX, 2,4-DNT, and 2,6-DNT in drinking water (http://www.epa.gov/waterscience/drinking/standards/dwstandards.pdf). 2,4-DNT, and 2,6-DNT have been also listed on the Unregulated Contaminant Monitoring Regulation list, and RDX will be added when appropriate analytical methods have been established. The drinking water HA level for lifetime exposure to HMX is 400 μg/L, while the levels for RDX, and TNT are both 2 μg/L. Based on standards for other drinking water contaminants, it is likely that the eventual Maximum Contaminant Levels for these compounds will be similar values. The health advisory levels for RDX and TNT reflect the potential threat that these compounds pose to humans and other organisms.

Table II-1. Explosive compounds found in the soil, sediment, and groundwater at DoD installations that that were examined during this research.

Abbreviation	Chemical Name	Structure	Properties
Nitroaromatics			
TNT	2,4,6-trinitrotoluene		Solubility: 120 mg/L log K_{OW}: 1.97
Nitramines			
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine		Solubility: 35 mg/L log K_{OW}: 0.85
HMX	octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine		Solubility: 5 mg/L log K_{OW}: 0.15

Chronic occupational exposure of humans to TNT, and controlled exposures of laboratory animals to TNT or RDX have resulted in similar adverse effects: liver damage, blood damage (caused by methemoglobinemia and associated cyanosis), anemia, cataracts, allergenic dermatitis, discoloration of hair and skin, and nausea (6, 11). Most of the explosive compounds examined are toxic and/or mutagenic at concentrations considerably below their respective solubility limits. These health effects lend urgency to research efforts focused on preventing new groundwater contamination with RDX, HMX, and TNT, and on treating existing contamination.

***Biodegradation of Explosive Compounds*.** The biodegradation of DNT, TNT, and to a lesser degree RDX, HMX, nitroglycerin, and tetryl (2,4,6-trinitrophenylmethylnitramine), has been studied extensively (see ref (5, 13) for review). The biotransformation of explosives is usually a reductive process requiring the presence of an exogenous electron donor (or cosubstrate) as illustrated in Figure II-1. For explosives such as RDX, multiple degradation pathways have been observed, as depicted in Figure II-2. Explosives compounds have also been shown to serve as nitrogen sources for some microbes. The extent of transformation and/or degradation is therefore dependent upon the type and concentration of the cosubstrate, the prevailing redox conditions and presence of alternate electron acceptors, and other yet-unidentified biogeochemical parameters.

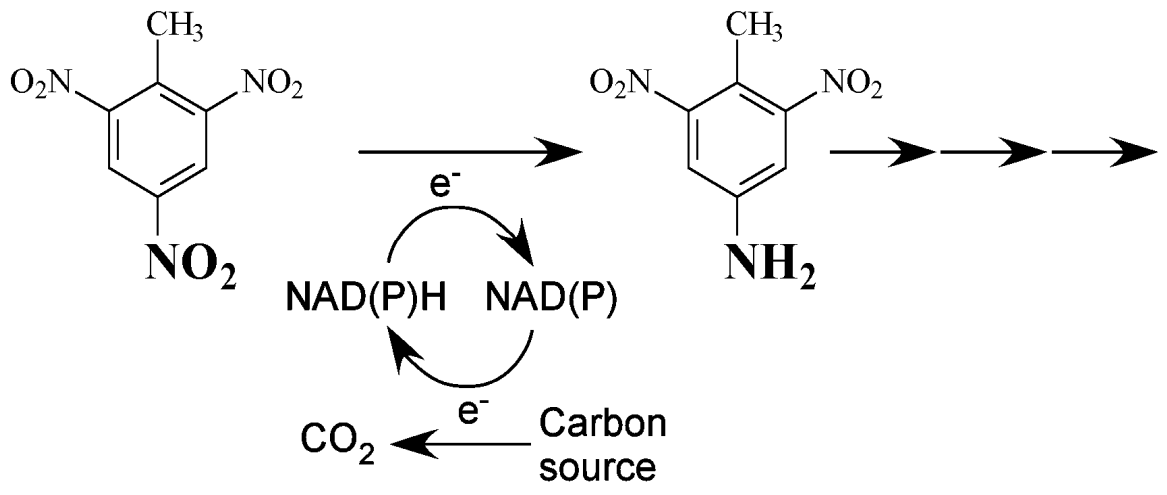


Figure II-1. Generalized biotransformation pathway for explosive compounds using the initial reduction of TNT to 4-amino-2,6-dinitrotoluene as an example.

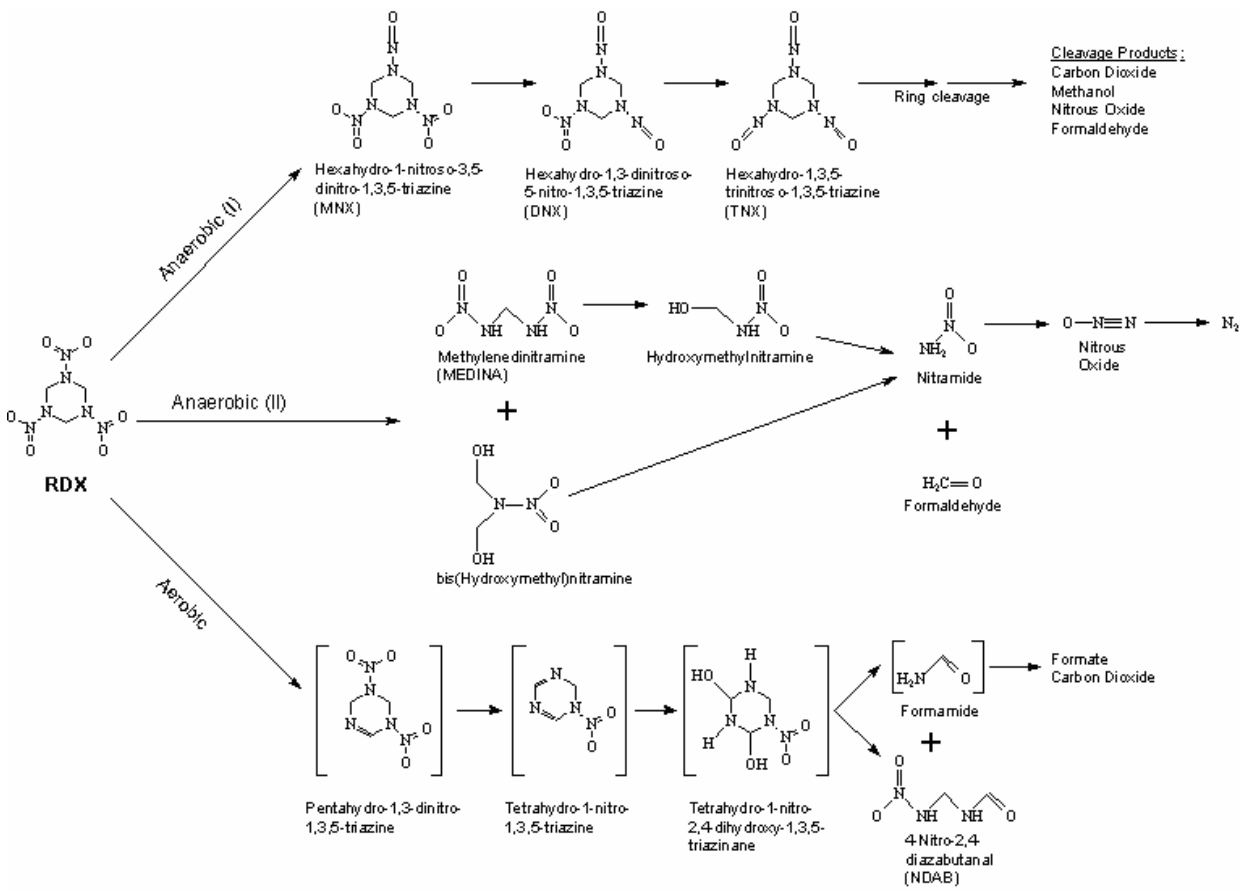


Figure II-2. RDX biodegradation pathways.

The current literature indicates that bacterial transformation and mineralization of nitroaromatic (e.g. TNT, tetryl) and nitramine (e.g. RDX, HMX) explosives is widespread across many bacterial genera (10, 12, 13). Some of the bacterial genera for which explosives-transforming genes and/or enzymes have been identified include *Enterobacter*, *Morganella*, *Agrobacterium*, *Pseudomonas*, *Rhodococcus*, *Heliobacter*, and *Clostridium*. Those bacterial strains that have demonstrated RDX-degrading abilities in pure culture are listed in Table II-2. The activities of these organisms represent a broad range of the known explosive transformation pathways, some of which can be expected to be present and operative in indigenous microbial communities when biostimulation (e.g., electron donor addition) is performed to bioremediate explosives.

Table II-2. Bacterial strains with known abilities to degrade RDX.

Organism	Inhibition by Ammonium	RDX as C or N Source	Enzymes and Catabolic Genes
Anaerobic Degradation			
Acetobacterium plausodum	Y	(Y)	Unknown
Acetobacterium malicum	Unknown	Unknown	Unknown
Clostridium acetobutylicum	Unknown	Neither	Unknown
Clostridium kluyveri	Unknown	Unknown	Flavoenzyme, diaphorase
Clostridium bifermentans	Unknown	N	Unknown
Clostridium celerecrescens	Unknown	N	Unknown
Clostridium saccharolyticum	Unknown	N	Unknown
Clostridium butyricum	Unknown	N	Unknown
Citrobacter freundii NS2	Unknown	Unknown	Unknown
Desulfovibrio desulfuricans	Unknown	N	Unknown
Enterobacter cloacae	Unknown	N	Type I Nitroreductase
Klebsiella pneumoniae SCZ-1	Unknown	Neither	Unknown
Morganella morganii B2	Unknown	Unknown	Type I Nitroreductase
Providencia rettgeri B1	Unknown	Unknown	Unknown
Serratia marcescens	Unknown	Unknown	Unknown
Aerobic Degradation			
Burkholderia sp. BL	Unknown	N	Unknown
Gordonia and *Williamsia* spp.	Unknown	C and N	Unknown
Rhodococcus sp. DN 22	Y	N	Cytochrome P450
Rhodococcus rhodochrous 11Y	Unknown	N	Cytochrome P450-like
Rhodococcus strain A	Unknown	Unknown	Unknown
Rhizobium rhizogenes BL	Unknown	N	Unknown
Stenotrophomonas maltophilia	Unknown	N	Unknown
Unidentified coryneform bacteria	Unknown	N	Unknown

Molecular monitoring of microbial communities. Monitoring and evaluating complex natural assemblages of microorganisms remains one of the most challenging tasks for assessing the progress or effectiveness of biological treatment processes. Soil microbial communities, for example, can be composed of more than 10^4 different species of bacteria, most of which can not be cultured. In the 1980s, several research laboratories began evaluating molecular biology methods, including gene probing, for detecting specific microorganisms in environmental

samples. This led to techniques for extracting DNA from environmental samples to eliminate the bias caused by culturing (28, 38), and ultimately to the first application of PCR with DNA isolated from environmental samples (37).

One of the most promising techniques to arise during this era for identifying the most numerically dominant microbes in an environmental sample, even if they are not culturable, is denaturing gradient gel electrophoresis (DGGE) (8, 27). DGGE relies on the PCR amplification of target DNA sequences using at least one primer that has a long attached sequence of up to 40 guanidine and cytosine residues (GC clamp). The resulting amplified DNA, with its GC clamp, is passed via electrophoresis through a polyacrylamide gel containing denaturing solution (typically urea and formamide) in a linear gradient from low to high concentration. As the double stranded DNA passes through the gel it is denatured into a single strand, melted form. The melted DNA ceases to migrate through the gel. DNA with higher G+C content denatures at a higher denaturant concentration than those with low G+C content and thus moves farther through the gel, and even a single base pair difference can be detected with this technique. The technique is especially useful for separating and analyzing 16S rDNA genes which have highly variable regions that provide phylogenetic identification of microorganisms. When applied to complex communities, the technique produces a bar code effect with each band representing a phylogenetic group (phylotype); often a single bacterial species. The DNA bands can be isolated and cloned or sequenced directly to determine what organisms are present in a sample. Furthermore, the most numerically dominant organisms typically produce more amplified product and a darker band on the gel. Thus, the method provides a semi-quantitative and qualitative assessment of the microbial community.

The advent of cost-effective automated DNA sequencing has made the use of DGGE very accessible to a wide range of environmental microbiology laboratories. It has been used to evaluate uncultured microbes in cyanobacterial mats, ammonia oxidizing bacteria in soil, bacterioplankton in estuaries, bacteria in grassland soils, and a wide array of other applications. Notably, the technique also has been used to evaluate microbial communities associated with biodegradation systems (1, 16, 24). A recent ecotoxicology study utilized DGGE to analyze the microbial community in soil in response to exposure to RDX (19). Under the experimental conditions (i.e., unsaturated soil, no biostimulation), significant RDX was degraded, but no changes in the numerically dominant members of the soil bacterial community were observed.

Another molecular biology technique that has increased in popularity since the early 1980s, and has become more important in recent years as a substitute for classical methods of analyzing microbial populations, is DNA hybridization or gene probing. In the last several years nucleic acid-based methods have been used for monitoring the performance of *in situ* bioremediation and for assessing "degradative potential" for successful bioremediation before deploying a field system. The methods typically have involved cloning and characterizing a particular catabolic gene, then using the cloned DNA as a probe to detect that gene in environmental samples. The probes are most commonly used to screen individual colonies (colony hybridization), or to screen DNA isolated directly from an environmental sample (DNA extraction/hybridization). In each case, the screening allows one to estimate the relative abundance of a particular gene within a population, and thereby to make predictions about an environment’s ability to respond to a particular contaminant.

Many degradative genes have been cloned, and are used as gene probes to identify specific degradative populations and to monitor changes in their abundance. For example, the complex *bphABCD* (biphenyl dioxygenase, dihydrodiol dehydrogenase, 3-phenylcatechol dioxygenase, and 2-hydroxy-6-phenylhexa-2,4-dienoate hydrolase) was used as a gene probe to analyze and compare polychlorinated biphenyl (PCB)-contaminated soils and an uncontaminated garden soil (43). The study indicated that more than 80% of the colonies from PCB-contaminated samples hybridized to one of the probes as compared to <1% of the isolates from uncontaminated soil. In another set of early experiments, a mercury-contaminated pond was studied by hybridizing mercury-resistance genes to community DNA and measuring biologically induced mercury reduction (2). The microbial population of the contaminated sample was shown to be significantly enriched (72-fold) in genetic sequences hybridizing to the mercury-resistance transposon Tn 501 compared to uncontaminated sample.

The metabolic diversity of microorganisms, however, has led to the evolution of many divergent and convergent degradative pathways that catalyze functionally similar reactions. Specific gene probes for one pathway may or may not detect organisms (genes) that perform a similar reaction. For example, in some cases native degradative organisms that have evolved in response to certain conditions, such as pollution, may have degradative genes that are not detected by hybridization to degradative genes of another "laboratory" organism (20, 21). It is sometimes valuable, therefore to develop catabolic gene probes and gene probes that detect a group or groups of organisms (e.g., phylogenetic probes) rather than genes for a specific degradative pathway.

Numerous studies have employed PCR technology to improve the sensitivity of gene probes. In one example, PCR and a PCR product gene probe were used to detect a 3-chlorobenzoate degrading bacterium in contaminated aquifer sediment 14.5 months after the original injection of the organisms into the aquifer (42). The frequency of detection of the introduced organism was greater by PCR than by the 3-chlorobenzoate most-probable number enumeration and correlated well with the results obtained from the 3-chlorobenzoate enrichment method. In a similar application, Hendrickson and colleagues (14) analyzed *Dehalococcoides* rDNA sequences from several chlorinated solvent contaminated sites and developed a set of probes and PCR primers that can be used to analyze suites and predict the utility of biostimulation and natural attenuation or the need to perform bioaugmentation.

IV. PROJECT ACCOMPLISHMENTS SUMMARY

This project focused on the following tasks:

Task 1 – Identify and obtain explosive-transforming bacterial strains, develop probes and primers.

Task 2 – Develop standard protocols for DNA extraction, PCR, and DGGE analyses.

Task 3 – Conduct batch microcosm experiments to examine the effects of groundwater parameters on explosives biotransformation and microbial community structure.

Task 4 – Develop, evaluate and apply stable isotope probing to identify the explosive-degrading organisms in microbial communities in enrichment cultures and field samples.

The key activities, developments, and findings of this project are as given below.

1. BACTERIAL STRAINS AND PRIMERS.

Explosive-degrading strains have been obtained, and primers have been developed to amplify the reported explosive-degrading genes in these bacteria for future utilization in screening putative remediation sites for their presence. The work has focused on genes that have appeared in the published literature and are associated with the degradation of at least one specific explosive compound.

1.1 IDENTIFYING GENES OF INTEREST AND DEVELOPING PRIMERS

The list of strains and genes used for the project is presented in Table 1.1-1. It was determined that the gene originally designated as *rdxA* gene was in fact similar P450-like gene *xplA*. The name of *rdxA* was therefore been changed to *xplA*. Experiments with some strains were discontinued later in the project.

A revised listing of the primers employed is presented in Table 1.1-2.

Table 1.1-1. Bacterial strains and/or genes that were selected for study for this project.

Strain	Gene(s)	Transforms/Degrades	Reference
Pseudomonas putida II-B	*xenA*	TNT, NG	Blehert et al., 1999
Pseudomonas fluorescens I-C	*xenB*	TNT, NG	Blehert et al., 1999
Enterobacter cloacae PB2	*onr*	NG, PETN, RDX	French et al., 1998
Enterobacter cloacae ATCC 43560	*(onr)*	NG, RDX	Pudge et al., 2003
Clostridium acetobutylicum	*hydA*	TNT, RDX	Watrous et al., 2003
	nitA, nitB	TNT	Kutty and Bennett, 2005
Agrobacterium radiobacter	*nerA*	NG	Snape et al. 1997
Rhodococcus DN22	*(xplA)*	RDX	Coleman et al., 2002
Rhodococcus rhodocrous 11Y	*xplA*	RDX	Seth-Smith at., 2002
Escherichia coli	*nfsA*	TNT	Yin at al., 2005
Rhodobacter capsulatus B10	*nprA, nprB*	2,4-DNP	Perez-Reinado et al., 2005
Pseudomonas pseudoalcaligenes JS52	*nbz*	NB	Park and Kim, 2000

Table 1.1-2. Primers designed to amplify genes associated with explosive biotransformation.

Strain	Gene	Forward Primer (5'-3')	T_m[1]	Reverse Primer (5'-3')	T_m	Expected Fragment (bp)
Bacteria-specific	*16S RNA*	ACTCCTACGGGAGGCAGCAG	67.8	ATTACCGCGGCTGCTGG	67.3	257
Pseudomonas putida II-B	xenA	AGCACTCCAACAAGCGTAC	60.5	ACCGACACCAGGTCCAACT	64.5	400
Pseudomonas fluorescens I-C	xenB	TTGCTGGAAGTGACTGATG	60.0	TGCCATAGAACAGCTCAGG	61.6	397
Enterobacter clocae PB2	onr	TTCTGCCTCTGCCCTG	61.4	ACTTTCTGACGGAAGGCTT	60.5	507
Enterobacter cloacae ATCC 43560	(onr)	TTCTGCCTCTGCCCTG	61.4	ACTTTCTGACGGAAGGCTT	60.5	507
Clostridium acetobutylicum ATCC 824	hydA	AAGGATTGTGGCAATTTTGGA	65.1	CAGCAACAGGACAAGCGATTA	65.0	499
Agrobacterium radiobacter	nerA	TTATAAGCGGGAAGCGATTG	63.6	GATGGAGCCACCGTAATCAT	63.7	400
Clostridium acetobutylicum ATCC 824	NitA	ATGAATAATACAATAGATACAATGAAAAATCATAG	54.8	TTTAGTTTTTAGTCCTTGTTTATTAATAGCGCC	59.4	738
Clostridium acetobutylicum ATCC 824	NitB	ATGATAGATTTAAAAACTAGAAGAAGCATAAG	53.6	TTTAGAATATTTGTCGTAATGAAGTTTATTTAAAG	56.1	522
Rhodococcus DN22	xplA	CTACGGACAGGGTGAACTG	59.8	TCCTGTTGCAGTCGCCTAT	63.4	396
Pseudomonas pseudoalcaligenes	nbzA	AAGCGTGGTTTTCTGGAT	59.7	CAAATGAAACTGGCCCAT	60.9	398
E. coli	nfsA	GTGTCGTTGATACGGCAATG	63.9	TTTTCATGCACCAAAATGGA	63.7	225
NOTES:						
The forward universal primers (A) were synthesized with 40 bp GC clamp as previously described. Base pair length does not take into account the GC clamp.						

Table 1.2-1. In-house 16S rRNA sequences of known explosive-degrading strains.

Organism ID	Sequence (5' - 3', based on univeral primer set PRBA338F-GC and PRUN518R)	bp	GenBank Accession #
Pseudomonas putida II-B	ACTCCTACGGGAGGCAGCAGTGGGGATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAA GAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACC GACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAAT	196	EF219419
Pseudomonas fluorescens I-C	TCCTACGGGAGGCAGCAGTGGGGATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGA AGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTTGACGTTACCGA CAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAAT	194	EF219420
Enterobacter cloacae ATCC 43560	ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGA AGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGAGGTTAATAACCTCAGCAATTGACGTTA CCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT	197	EF219421
Agrobacterium radiobacter	ACTCCTACGGGAGGCAGCAGTGGGGATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAT GAAGGCCTTAGGGTTGTAAAGCTCTTTCACCGATGAAGATAATGACGGTAGTCGGAGAAGAAGCCCCGGCTAACTT CGTGCCAGCAGCCGCGGTAAT	171	EF219422
Rhodococcus sp. DN22	GGAGGCAGCAGTGGGGATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCT TCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACG TGCCAGCAGCCGCGGTAAT	167	EF219423
Rhodococcus rhodocrous 11Y	CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGA CGGCCTTCGGGTTGTAAACCTCTTTCAGTACCGACGAAGCGCAAGTGACGGTAGGTACAGAAGAAGCACCGGCCA ACTACGTGCCAGCAGCCGCGGTAAT	173	EF219424

Table 1.2-2. Identification of known degradative strains based on in-house DGGE and sequencing.

Strain	Band	ID from public database based on in-house rRNA sequence	% Identity
Pseudomonas putida II-B	1	*Pseudomonas putida* and other *Pseudomonas* spp.	99
	2	*Pseudomonas putida* and other *Pseudomonas* spp.	99
Pseudomonas fluorescens I-C	1	*Pseudomonas putida* and other *Pseudomonas* spp.	99
	2	*Pseudomonas putida* and other *Pseudomonas* spp.	99
Enterobacter cloacae ATCC 43560	1	*Enterobacter cloacae*, Uncultured gamma proteobacterium, *Pantoea* sp.	99
	2	*Enterobacter cloacae*, Uncultured gamma proteobacterium, *Pantoea* sp.	100
	3	*Enterobacter cloacae*, Uncultured gamma proteobacterium, *Pantoea* sp.	99
	4	*Enterobacter cloacae*, Uncultured gamma proteobacterium, *Pantoea* sp.	100
Rhodococcus sp. 11Y	1	*Rhodococcus sp., Rhodococcus ruber*	100
Rhodococcus sp. DN22	1	*Rhodococcus erythropolis, Lechevalieria aerocolonigenes, Lentzea albidocapillata,*	99
		Saccharothrix sp.	99
Agrobacterium radiobacter	1A	*Agrobacterium tumefaciens* (*Rhizobium radiobacter*), Uncultured *Agrobacterium* sp.,	99
		Rhizobium sp., *Rhizobium massiliae*, *Sinorhizobium* sp., *Devosia riboflavina*	99
	1B	*Agrobacterium tumefaciens* (*Rhizobium radiobacter*), Uncultured *Agrobacterium* sp.,	99
		Rhizobium sp., *Rhizobium massiliae*, *Sinorhizobium* sp., *Devosia riboflavina*	99
	2	*Agrobacterium tumefaciens* (*Rhizobium radiobacter*), Uncultured *Agrobacterium* sp.,	99
		Rhizobium sp., *Rhizobium massiliae*, *Sinorhizobium* sp., *Devosia riboflavina*	99

1.2 SEQUENCING OF KNOWN DEGRADATIVE STRAINS

The sequences of the 16S rRNA genes from known degradative bacterial strains were determined after amplification using our universal primer set. These sequences were required for molecular analyses and production of phylogenetic trees showing relationship between the known degraders and the sequences we isolated from diverse samples. Some of this information was not available in the public sequence databases. Therefore, it was decided to sequence these organisms in-house.

1.2.1 METHODS

DGGE analysis was performed (see section 2.3.4 below), and multiple DGGE bands obtained from each strain were excised, purified, and sequenced. As an additional sequencing method validation step, the sequences were entered into a 'BLAST-N' query in the National Institute of Health's National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov/) for identification.

1.2.2 RESULTS

DGGE analysis of the strains was performed and bands for each strain were sequenced and identified. Sequence data is presented in Table 1.2-1. The sequences matched with closely-related strains (Table 1.2-2), which validated the in-house sequencing procedures. Additionally, as a service to the research community, we have uploaded the new strain 16S rRNA sequence data to the NCBI database so that others can use the data.

1.3 DEGRADATION OF RDX BY KNOWN DEGRADATIVE STRAINS

As mentioned above in Section 1.1, the substrate range of several of the known explosive-degrading strains had not been previously evaluated and tested, especially with respect to RDX degradation. This work was done to fill in this knowledge gap.

1.3.1 METHODS

Strains were screened in three types of liquid cultures consisting of basal salts medium (BSM), RDX, and a carbon source. Briefly, glass 15 mL serum vials were combusted at 550º C overnight to burn off any trace carbon or nitrogen. Once cooled they were capped with Teflon®-lined butyl rubber septa and autoclaved. Two sets of liquid culture screens were performed in BSM with 5 mg/L RDX as the sole nitrogen source. Each strain was grown both with and without 1 g/L succinate or glucose. Vials were incubated under aerobic/anoxic conditions (vials allowed to go anoxic due to carbon utilization) and anaerobic conditions (vials prepared in an anaerobic glove bag and sampled anaerobically). The third set was in BSM with a full amendment of carbon (1 g/L succinate or glucose) and nitrogen (NH_4) incubated under aerobic/anoxic conditions with 3 mg/L RDX. Cells of each strain were scraped from R2A agar plates and diluted in 1 mL of sterile phosphate-buffered saline (PBS), vortexed for 30 seconds, and 100 µL was used to inoculate the serum vials. Negative controls were prepared in the same manner for every type of enrichment and were inoculated with 100 µL sterile PBS. Over several weeks samples were collected and analyzed for RDX and breakdown products via HPLC. During sampling, 1 mL of sterile nitrogen was injected through the septa and 1 mL was withdrawn with a sterile syringe and needle. Strains that were found to be degrading RDX were

supplemented with additional RDX, carbon, and inoculum; non-degrading cultures were amended with additional carbon and inoculum.

HPLC analysis for explosives in all experiments was performed according to a modified EPA Method 8330 using a Hewlett-Packard 1100 HPLC equipped with a Allure C18 column (Bellefonte, PA, USA) and a UV detector (230 nm). The mobile phase was 50:50 methanol:water at a flow rate of 0.9 mL/min. The column temperature was 25°C. The lower detection limit was approximately 25 µg/L for RDX and 50 µg/L for the RDX breakdown products.

1.3.2 RESULTS

Results of these screening experiments are presented in Table 1.3-1. Under aerobic/anoxic conditions with RDX as the sole N source, only *Rhodococcus sp.* DN22 and *Rhodococcus rhodocrous* 11Y degraded RDX. Under anaerobic conditions with RDX as the sole N source, *Rhodococcus sp.* DN22 and *Rhodococcus rhodocrous* 11Y also degraded RDX. With a supply of both carbon and nitrogen, both *Pseudomonas fluorescens* I-C and *Pseudomonas putida* II-B degraded RDX. Degradation of RDX by *Agrobacterium radiobacter* and *Enterobacter cloacae* ATCC 43560 were not observed under any of the test conditions.

Table 1.3-1. Screening of known degradative strains for RDX degradation under various conditions.

	RDX Degradation			
		N Source = RDX		N Source = NH_4
Sample ID	**C Source**	**Aerobic/Anoxic**	**Anaerobic**	**Aerobic/Anoxic**
Rhodococcus rhodocrous 11Y	none	No	**Yes**	
	succinate	**Yes**	**(Yes)**	No
	glucose	**Yes**	**(Yes)**	**Yes**
Rhodococcus sp. DN22	none	No	**Yes**	
	succinate	**Yes**	**Yes**	**Yes**
Agrobacterium radiobacter	none	No	No	
	succinate	No	No	No
Pseudomonas putida II-B	none	No	No	
	succinate	No	No	**Yes**
Pseudomonas fluorescens I-C	none	No	**(Yes)**	
	succinate	No	No	**Yes**
Enterobacter cloacae ATCC 43560	none	No	No	
	succinate	No	No	No
(Yes) indicates partial degradation observed.				

With full carbon and nitrogen amendment under aerobic/anoxic conditions, the results indicated that *Rhodococcus sp.* DN22, *Rhodococcus rhodocrous* 11Y, *Pseudomonas fluorescens* I-C and *Pseudomonas putida* II-B degraded RDX, the latter two organisms likely using the compound as a terminal electron acceptor when anoxic conditions prevail.

Although *Enterobacter cloacae* ATCC 43560 was reported in the literature to degrade RDX, no degradation by this organism was demonstrated under any of the test conditions. This is likely due to the absence of yeast extract in the test media, which was shown to be necessary for

degradation (31), but which was excluded in these experiments because we did not want to add alternate nitrogen or carbon sources.

The observation of anaerobic degradation by the rhodococci and the two pseudomonads, both of which have not been reported previously, were followed up with additional experiments, resulting in the preparation and submission of two manuscripts for publication. These follow-on experiments are easier understood in the context of the whole manuscripts, which are included in Appendix 1.

1.4 SELECTION OF UNIVERSAL PRIMER SET FOR DGGE

The initial work performed during this project employed the *Bacteria*-specific primers PRBA338F-GC and PRUN518R (25). These were selected from the pool of primers used by other researchers and reported in the literature. Some additional experimental results indicated that this primer set may have undesirable bias (data not shown), which could lead to less than accurate results for both the laboratory and field samples analyzed during this project. Specifically, there was a concern that the universal primer set initially selected might not detect the broad range of bacterial species expected in the samples. Therefore, an experiment was conducted to find the most broadly applicable of the three primer sets by testing them against a range of gram-negative and gram-positive bacteria. Tests were also conducted to see how they work for detection of bacteria in groundwater from Picatinny Arsenal.

1.4.1 METHODS

Two additional *Bacteria*-specific primer sets were obtained based on their reported ability to amplify bacteria from environmental samples: 1) 1114F and 1492R (17, 32) and 2) EUB933F and EUB1387 (22, 39). These primer sets are further characterized in Table 1.4-1.

A range of bacterial strains across multiple genera (listed in Table 1.4-2) were grown on R2A agar, with the exception of the *Mycobacterium* sp., which was grown in liquid BSM amended with propane. Bacteria in uncontaminated Picatinny Arsenal groundwater, obtained from well 40MW-4, were used unconcentrated (1X) and after a 200-fold concentration. The concentration was performed by distributing 100 mL of groundwater to two 50 mL polypropylene tubes and concentrating particulate matter by centrifugation (3400 rpm, 20 min, 22°C). The supernatant was decanted and the process was repeated with an additional 100 mL of groundwater. After the second centrifugation step, 5 mL of the supernatant was left in each tube. The pellet was resuspended in one of the tubes and its contents were transferred to the second tube. The combined sample was centrifuged again, and all except 1 mL of the supernatant was removed. Unconcentrated and 200X groundwater samples were stored at 4°C until use.

To achieve cell lysis, a loopful of plate grown bacterium was added to 0.3 g glass beads (0.1 mm diameter) and 0.3 mL sterile water for bead beating. Cells in a 1 mL sample of the liquid *Mycobacterium* culture were concentrated by centrifugation, and a loopful of the resulting pellet was used in the bead beating mixture. For the Picatinny Arsenal samples, 300 µl of the unconcentrated and 200X groundwater was added directly to 0.3 g glass beads. 2 µl of bead beating supernatant (define contents) was used in each PCR reaction. To account for the lower melting temperature of the 1492R primer, a gradient PCR protocol (range: 48° to 59°C) was

employed, and PCR reactions were conducted with annealing temperatures set approximately 5 to 7°C below the melting temperatures of each primer set.

1.4.2 RESULTS

The results are depicted in Figure 1.4-1 and summarized in Table 1.4-2. Primer set A (EUB933F and EUB1387) and B (1114F and 1492R) only resulted in successful amplification of DNA from three of the thirteen bacterial strains tested, and only one strain was common to both sets of primers. In addition, primer set B yielded very weak results overall. In contrast, primer set C (PRBA338F-GC and PRUN518R) produced the expected amplicons with twelve of the thirteen test strains. The only strain not yielding an amplicon with these primers was *Arthrobacter globiformis*. The results demonstrate that the universal primer set C, the set initially selected at the beginning of this project, was not inherently or highly biased or selective, and should provide good species coverage when used to amplify 16S sequences from microcosm and field samples.

Table 1.4-1. Universal primers evaluated for use during DGGE.

Primer ID	Primer Name	Sequence (5' ---> 3')	T_m[2]	Expected Fragment (bp)	Refs
A	EUBf933	CACAAGCGGTGGAGCATGTG	70.2	533	2,7
	EUB r1387	GCCCGGGAACGTATTCACC	68.5		
B	1114F	GCAACGAGCGCAACCC	67.0	453	1,6
	1492R	GGTTACCTTGTTACGACTT	53.1		
C	PRBA338F	ACTCCTACGGGAGGCAGCAG	67.8	257	3
	PRUN518R	ATTACCGCGGCTGCTGG	67.3		

NOTES:

Each of the forward primers were synthesized with 40 bp GC clamp as previously described (not included in base pair length)

Table 1.4-2. Evaluation of bias/selectivity of universal primers.

Band ID	Sample	PCR Amplification with Primer Set: A	B	C
1	*Enterobacter cloacae*	-	-	+
2	*Pseudomonas putida* IIB	-	-	+
3	*Rhodococcus* sp. DN22	-	-	+
4	*Agrobacterium radiobacter*	-	+	+
5	*Acinetobacter johnsoni*	+	+	+
6	*Alcaligenes eutropuhus*	-	+	+
7	*Arthrobacter globiformis*	+	-	-
8	*Bacillus cereus*	-	-	+
9	Unidentified gram-positive strain	-	-	+
10	Unclassified strain (THF[1])	-	-	+
11	*Sphingomonas capsulata*	-	-	+
12	*Mycobacterium* sp.	-	-	+
13	*Escherichia coli* BL21	+	-	+
14	Negative control	-	-	-
15	Picatinny Arsenal GW	-	-	-
16	200X Picatinny Arsenal GW	-	-	-

[1]Unclassified strain maintained in the lab; known to degrade tetrahydrofuran.

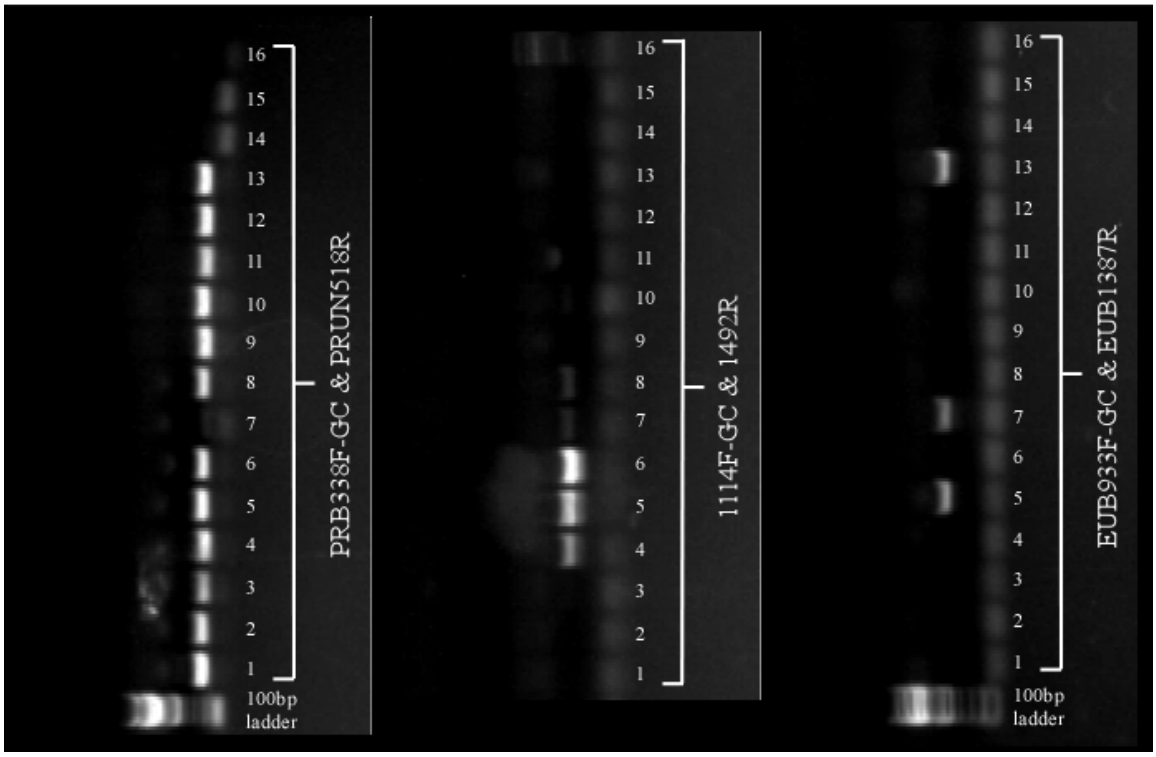


Figure 1.4-1. Evaluation of three universal DGGE primer sets for bias/selectivity issues.

2. DEVELOPMENT OF MOLECULAR PROTOCOLS.

General protocols for DNA extraction, DGGE and measuring abundance of genes involved in explosives degradation have been developed and are being optimized.

2.1 PROCEDURES TO ADDRESS AND MINIMIZE CONTAMINATION

Extreme care needs to be taken when performing molecular analyses, especially when using universal rRNA primers and dealing with very low amounts of DNA template in a given sample. There is one report in the literature that seems to indicate that there are common identifications

of DGGE bands recovered from diverse sample types that in fact are likely contaminants (40). All applicable measures to minimize and/or control for these widespread contaminants were observed so that accurate and reliable data could be obtained.

In order to minimize contamination of samples during processing and analysis, several procedures were established.

1) Lab space, pipettes, tips and ultra pure reagents were acquired and dedicated specifically for setting up PCR reactions ONLY.
2) Only 'PCR Certified' 200 µL PCR and 1.5 mL tubes were used.
3) Only 'PCR Certified' aerosol barrier pipette tips were used for pipetting PCR reagents.
4) All tubes, water, glassware and reagents that were not affected by UV radiation were exposed to ultraviolet radiation for at least 2 hours in a UV box before use.
5) The glass beads used in the bead beating process were baked at 550°C for 18 to 24 h.
6) Sterile water was made in-house by filtering Nanopure water through a 0.2 µm filter, autoclaving it and then exposing it to ultraviolet light at least 2 hours. Also, only certified RT-PCR grade water was used for diluting reagents.

Some contaminated negative controls have been sequenced and the results are presented in Table 2.1-1. It is worthy to note that recombinant Taq used in PCR is produced in *Escherichia coli*, and many of the contaminating sequences were identified as *Escherichia* and other related species. It is possible that the Taq polymerase enzyme used for PCR was contaminated.

Table 2.1-1. Identification of contaminants that appeared in negative controls.

Sample ID	Details	Sequence ID	% Identity
Contaminant, negative control 030106 band 1	Original sequencing	uncultured *Enterobacteriaceae* bacterium	97
		Escherichia sp., *Shigella* sp., *Paracoccus* sp.	97
	Re-sequencing	thermal spring bacterium	90
		Escherichia coli	90
	Re-amplify and re-sequence	*Shigella* sp., *Escherichia* sp.	96
Contaminant, negative control 030106 band 2		uncultured *Enterobacteriaceae* bacterium	90
		Shigella sp., *Escherichia* sp.	92
Contaminant, negative control 063006		bacterium JB17, uncultured bacterium, *Pseudomonas* sp.	90
Contaminant, negative control 070706		uncultured bacterium, uncultured gamma proteobacterium	95
		Erwinia papayae	95
		Photorhabdus asymbiotica, *Klebsiella oxytoca*	97
		Shigella boydii, *Escherichia albertii*	94

2.1.1 METHODS

We explored different sources of Taq DNA Polymerase from Stratagene and Sigma in an attempt to eliminate or minimize contamination in the negative controls. The different enzymes were subjected to the same PCR conditions as previously described.

2.1.2 RESULTS

Stratagene's *PfuUltra*™ II Fusion HS DNA polymerase was acquired and tested. Contamination was still detected in some of the negative control PCR reactions with this enzyme. Ultra pure

MTP *Taq* DNA polymerase from Sigma, which is tested and certified to be free from 16S rRNA contamination, also amplified contamination in some of the negative controls.

Even with all the current precautions (dedicated lab areas and equipment for molecular work, use of ultrapure buffers, etc.) contamination continued to be an issue, especially when using the universal bacterial primers. However, our analyses made judicious use of appropriate negative controls, and the results from these controls were used to "subtract out" the contamination in a given set of samples. It should be noted that the contamination was always a single easily definable band. Additionally, we were able to sequence and identify this product when necessary.

2.2 DNA EXTRACTION FROM GROUNDWATER

A key component of molecular analysis of environmental samples is the optimization of DNA extraction protocols for efficiently and reproducibly recovering representative DNA samples. Initial results indicated extraction protocols commonly reported for use with environmental samples have limitations, and that these protocols needed to be optimized for samples used in this project. After testing several DNA extraction methods to be used with the various types of project samples, general protocols were developed for soils and enrichments.

2.2.1 METHODS

We determined that filtration of larger sample volumes (1 L or greater) are likely necessary, especially for low-biomass environmental samples. Sterivex™-GV Sterile Vented 0.22 µm Filter Units (Millipore, Cat no. SVGV L10 RC, Figure 2.2-1) were used for this purpose. These are small, in-line filter units used for sterilizing aqueous solutions that can be used in connection with syringes, peristaltic pumps or pressure vessels. Based on literature references (7, 15, 36) we have tested these filters for collection of biomass from groundwater. A flowchart of the process is presented in Figure 2.2-2.

Picatinny groundwater (well ID 157MW-5) was collected in 1 L jars, shipped on ice and stored at 15ºC until use. For the filtering process, the jars were shaken vigorously and placed on a stir plate with a stir bar to keep any particulate matter in suspension during filtration. Sterile tubing connected the jar of groundwater through the peristaltic pump to the inlet of the Sterivex filter. A stopcock was also placed at the outlet, which was connected to tubing to direct the effluent into a waste collection basin. After priming the lines with ~50 mL of groundwater (to flush out air), 2 L of groundwater was filtered at a flow rate of 100 mL/min. Three filters, with a total of 6 L of groundwater, were prepared. After the filtration process was completed, 120 mL of air was introduced into the Sterivex inlet though a 0.2 µm syringe filter to expel the residual water. The cartridge was then filled with 1.8 mL of sterile lysis buffer (0.75M sucrose, 40 mM EDTA, 50 mM TRIS, pH 8.3) and frozen at -70ºC until cell lysis and DNA extraction.

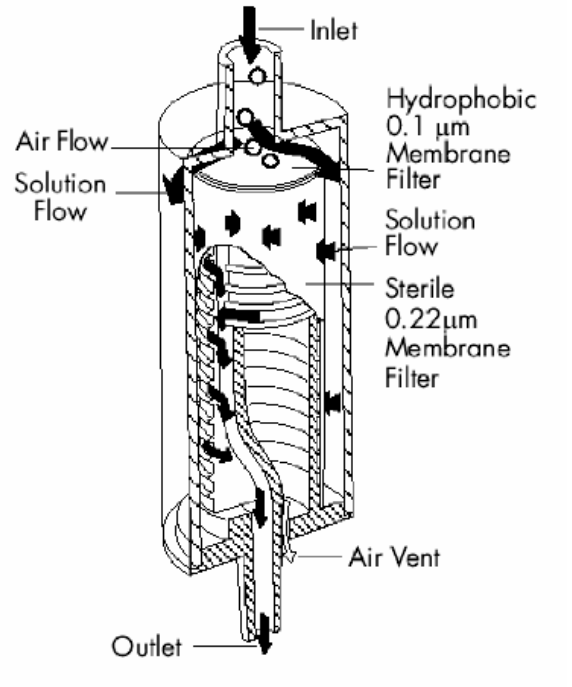


Figure 2.2-1. Photographs and illustration of the Sterivex in-line filter units (top) and the Microcon microfuge filtration devices (bottom).

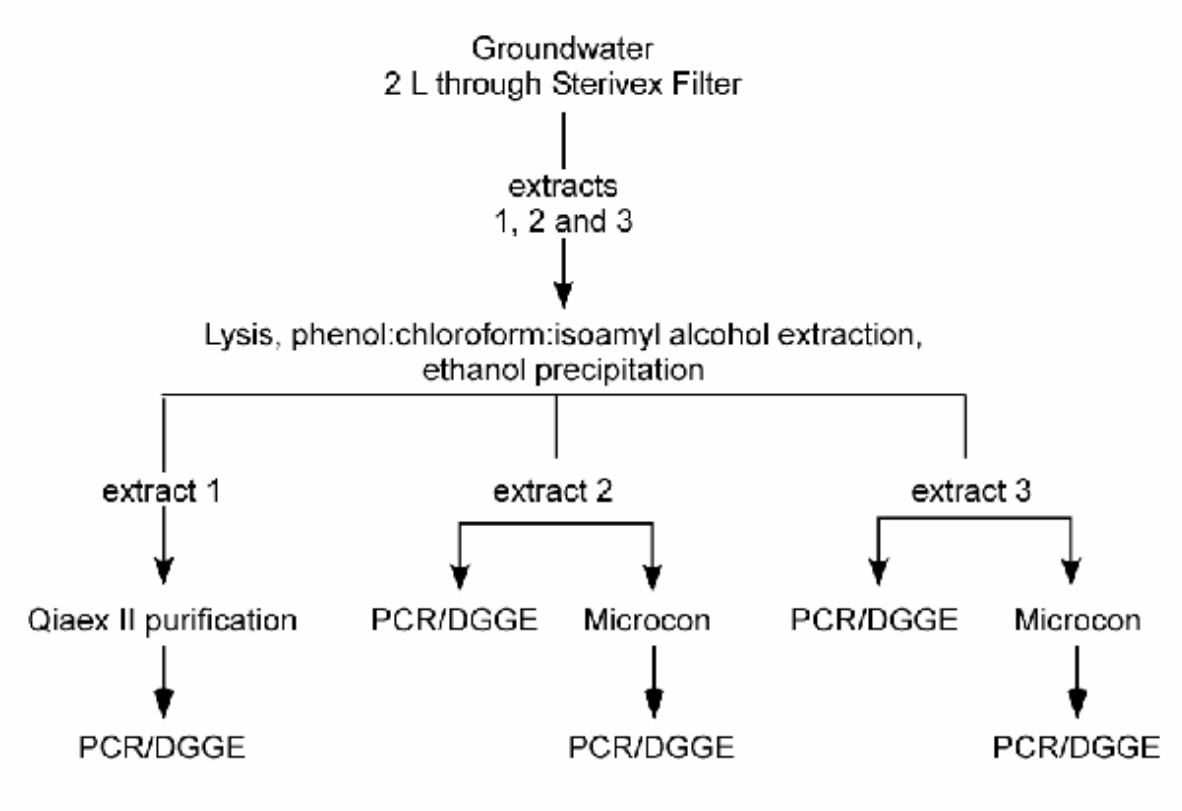


Figure 2.2-2. Flowchart for testing of Sterivex and Microcon microfuge filtration devices.

Cell lysis was carried out in the filter cartridge by adding 40 µL of 50 mg/mL lysozyme and the filters were rotated at ~40 rpm at 37ºC using a modified rock tumbling device. After 1 hour, 20 µL of 50 µg/µL proteinase K and 100 µL 20% (wt:vol) SDS was added and the filters were rotated at 40 rpm at 55ºC for 2 hours. The lysate was removed from the filters using a sterile syringe. The filters were rinsed with 1 mL of the lysis buffer and rotated at 40 rpm at 55ºC for 5 minutes. The rinse and lysate were combined and then divided into six 500 µL aliquots in 1.5 mL tubes for DNA purification. Lysates were extracted twice with 25:24:1 phenol:chloroform:isoamyl alcohol and then the DNA was precipitated with 0.3 M sodium acetate, pH 5.2 and 95% ethanol overnight at -70ºC . Pellets were rinsed twice with 70% ethanol and resuspended in the same 50 µL of sterile ultra pure TE, pH 8.0. This was done by resuspending one pellet in the TE, transferring the TE to the next tube and repeating for all six aliquots to combine all DNA back into one sample. Afterwards, 8 µL of sterile TE was placed into each of the original precipitation tubes to suspend and preserve any trace DNA left behind in the tube. All DNA extracts were quantified using Quant-iT DNA Assay Kit (Molecular Probes) and stored at -20ºC.

The first groundwater extract was subjected to a second purification step to try to minimize PCR inhibitors using a QIAEX II kit from Qiagen as per the manufacturer's protocol. This is a pH dependent particle adsorption method using glass beads. Both the original extract and the combined 8 µL suspensions were purified and the eluted volumes were combined, quantified and amplified for DGGE analysis using touchdown PCR (from 65º to 55ºC) as described below (see Section 2.3.2.1),with the final extension time extended to 10 minutes. A small amount of the second and third groundwater extracts were amplified as described after the ethanol precipitation. The rest of these extracts (combined with the 8 µL residuals) were further purified and concentrated using Microcon® Ultracel YM-100 centrifugal filter devices from Millipore (Figure 2.2-1, Cat no. 42412) with sterile ultra pure TE, pH 8.0, down to ~30 µL. These concentrated extracts were then amplified as described as well.

Further experimentation with the PCR parameters indicated that inhibition could be overcome by dilution of the template and adding bovine serum albumin (BSA) to the reactions. The final reaction mixtures were 5 µL of 3.5 to 4.5 ng/µL DNA extract with 2 µL of 10 mg/mL purified BSA in 100 µL final reaction volume. All other PCR reagents remained the same as previously described using the standard *Taq* polymerase.

2.2.2 RESULTS

Table 2.2-1 shows the DNA yields obtained after ethanol precipitation. All three extracts produced a quantifiable 5 to 7.5 ng/µL of DNA (quantified in duplicate and both values were within 0.2 ng/µL of each other). PCR inhibitors were co-precipitated with the DNA as evidenced by the inability to amplify the DNA (data not shown). This is quite common with environmental samples, and further experimentation with template dilution and adding BSA overcame this inhibition.

Table 2.2-1. Reproducibility of DNA extraction from groundwater.

Extract #	Volume Filtered (L)	Date Filtered	DNA Yield (ng/µL) Average	SD
1	2	11/9/2006	5.16	0.01
2	2	11/28/2006	7.57	0.08
3	2	11/28/2006	5.29	0.14

2.2.3 STANDARD OPERATING PROCEDURE FOR DNA COLLECTION FROM GROUNDWATER

Results from all the testing and optimization work resulted in production of a standard protocol that was subsequently employed for DNA collection from groundwater during 2007 and 2008. The standard DNA collection protocol is included in Appendix 3.

2.3 OPTIMIZATION OF MOLECULAR TECHNIQUES

The observation of uniform migration of a single DGGE band in environmental samples was a concern. To examine the validity of the DGGE technique (and for identification purposes as later described), bacterial strains isolated from groundwater (section 3.2 below) were analyzed using DGGE to see if the PCR products migrated different distances.

2.3.1 EXAMINING DGGE RESOLUTION

2.3.1.1 METHODS

To test DGGE gel resolution (i.e., the ability of the DGGE to separate distinct bands), PCR products from several samples were combined and run together on a DGGE gel. Three mixtures (7 µL each of each of three PCR products) were combined. The combinations were as follows: Mix 1 = isolate 1 + isolate 2 + isolate 3; Mix 2 = isolate 4 + isolate 5 + isolate 6; Mix 3 = isolate 12 + isolate 13 + isolate 15.

2.3.1.2 RESULTS

The DGGE results are presented in Figure 2.3.1-1. Bands from the individual strains migrated at different rates, but multiple bands were observed for most of the strains. These results indicate that the DGGE gel methodology was working.

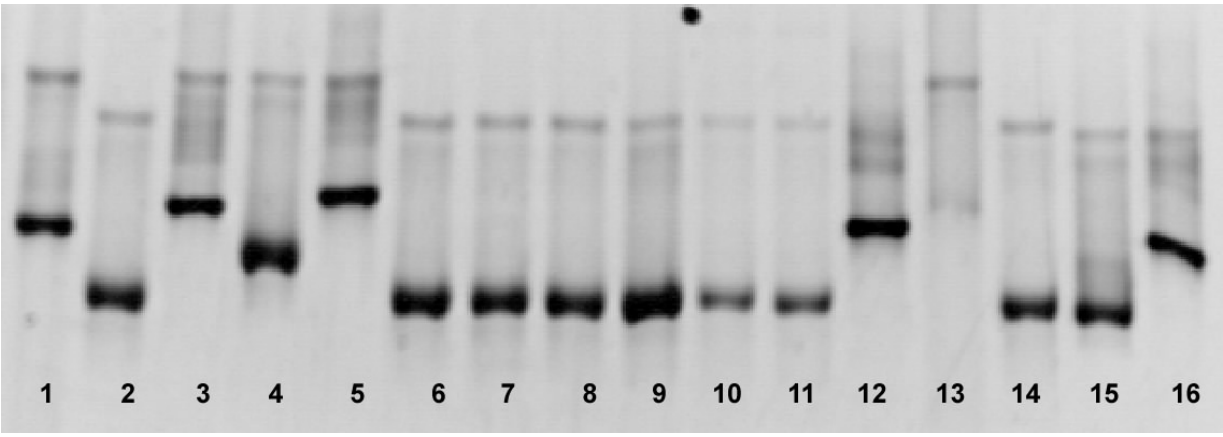


Figure 2.3.1-1. DGGE of bacterial isolates from Picatinny Arsenal groundwater and Picatinny columns. Individual colonies grown from groundwater and column effluent samples were PCR amplified and separated on a 20%-70% DGGE gel at 180 volts for 3 hours.

Lanes 1-5: Colonies isolated from Picatinny groundwater plated on yeast extract (YE) plates.
Lanes 6-9: Colonies isolated from Picatinny groundwater plated on cheese whey (CW) plates.
Lanes 10-11: Colonies isolated from Column 1 plated on CW plates.
Lanes 12-14: Colonies isolated from Column 1 plated on YE plates.
Lanes 15-16: Colonies isolated from Column 3 plated on CW plates.

Figure 2.3.1-2 illustrates the DGGE separation of the mixtures of PCR products. The PCR products within each of the mixtures resolved well, which further validated the DGGE protocol. If the multiple banding was due to temperature fluctuations in the gel and not due to multiple PCR products, we would not expect to see a reproducible separation of multiple bands. In close examination of these results one can still see the secondary bands resolved high in the lane

indicating that it is likely these are indeed different PCR products and not artifacts of inconsistent denaturing.

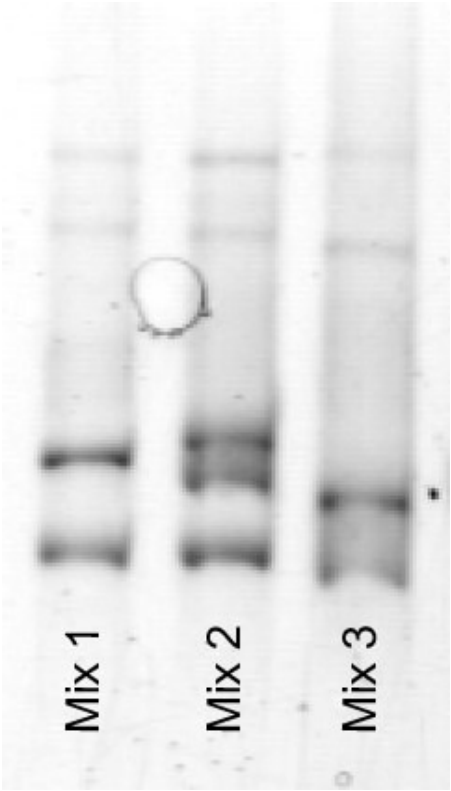


Figure 2.3.1-2. DGGE of three mixtures of PCR products.

Mix 1 = lanes 1, 2, 3.
Mix 2 = lanes 4, 5, 6.
Mix 3 = lanes 12, 13, 15.

These results provide some general insights into the use of DGGE for characterization of microbial communities. While banding patterns may be used as a "first approximation" of microbial community differences between samples, sequencing of DGGE bands is crucial to obtaining relevant and accurate results. In the data presented here, a pure culture of a single strain yielded multiple bands, which could have been interpreted as meaning there were multiple strains in the sample. Only sequencing revealed that all the bands for a given strain had similar sequences.

2.3.2 OPTIMIZING PCR REACTION CONDITIONS

Multiple banding had no observable effects with respect to the identification of the given organism from which the sequence was derived, since all the bands still yielded the same identification (sections 1.2 and 3.2). However, the elimination of multiple banding is needed if assessment of the banding patterns derived from mixed cultures that will be encountered in enrichments and environmental samples is desired.

2.3.2.1 METHODS

The double banding was assumed to be the result of the production of two DNA products during PCR reactions which differed by a consistent number of base pairs. A colleague at Rutgers University indicated that using primers without the GC-clamp eliminated this banding pattern in their DGGE gels, and it was recommended that we try this. The forward primer (PRBA338F-GC) without the GC-clamp was procured. Three strains were selected for PCR to test the new primer using the same reaction conditions as previously described.

A procedure known as "touchdown" PCR was implemented and used for all DGGE PCR reactions. Cycling for the touchdown PCR is as follows: one 5 minute pre-heat at 94ºC; 10 single cycles of three temperatures (melting-annealing-extension) with only the annealing step dropping one degree each cycle: 95ºC for 1 minute, 65ºC down to 56ºC for 30 seconds, 72ºC for 1 minute; 20 cycles of 95ºC for 1 minute, 55ºC for 30 seconds, 72ºC for 1 minute; 72ºC for an additional 5 minutes before cooling to 6ºC.

Another possible cause of the double banding pattern was the DNA polymerase enzyme. The standard *Taq* was thought to be lacking in purity, specificity and proofreading capabilities needed for amplification fidelity when using these universal primers. Therefore, two additional DNA polymerase enzymes were examined: 1) Stratagene's *PfuUltra*™ II Fusion HS DNA polymerase (a proofreading high-fidelity enzyme), and 2) Sigma's MTP *Taq* DNA polymerase (an ultrapure recombinant *Taq* that is tested specifically for 16S rRNA contamination). During evaluation of these two alternate enzymes, the touchdown PCR was used with the final extension time increased to 10 minutes.

2.3.2.2 RESULTS

The forward DGGE primer without the GC clamp continued to yield the two bands (Figure 2.3.2-1). This indicated that the double banding may be the result of mispriming of the DNA polymerase at a conserved region elsewhere on the rRNA gene. This could explain why it continued to be approximately the same distance apart from the major band obtained from each pure culture.

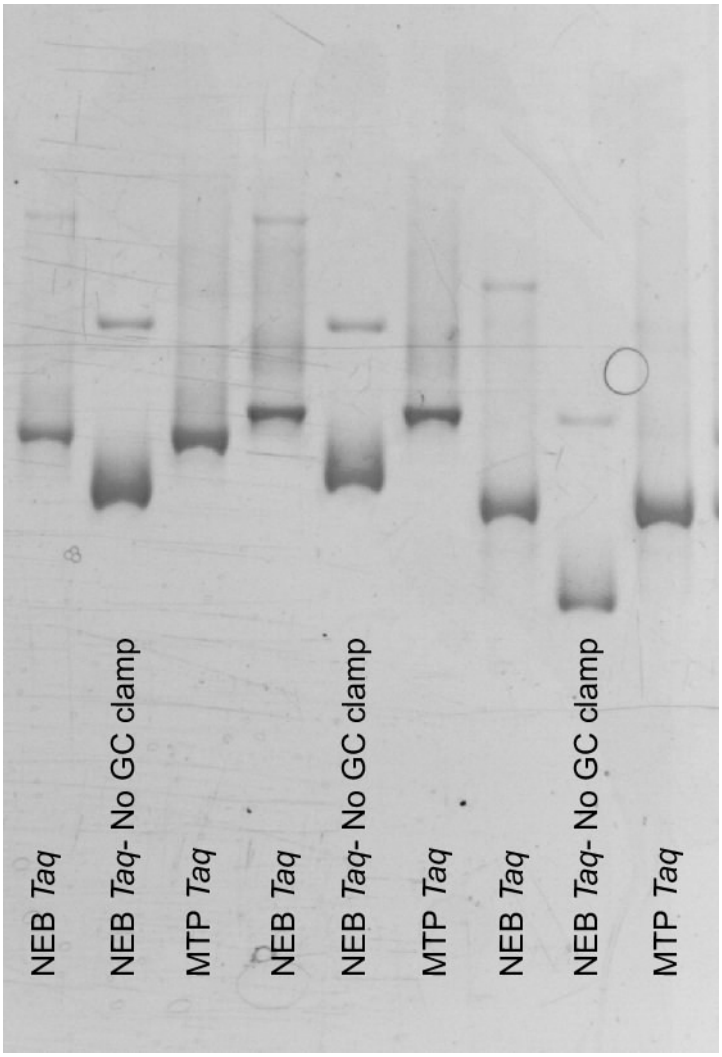


Figure 2.3.2-1. Enzyme and primer testing. NEB *Taq* DNA polymerase is the enzyme that has been used to date. MTP *Taq* DNA polymerase is the new enzyme being tested.

The Sigma MTP enzyme did not appear to produce the second band in initial tests (Figure 2.3.2-1), however, it did produce a faint second band in a second experiment (Figure 2.3.2-2). The Stratagene *PfuUltra*™ II Fusion HS DNA polymerase produced a single band when tested with a column isolate (Figure 2.3.2-2). The major difference between the two enzymes was that the *PfuUltra*™ was designed to have superior fidelity and proofreading capabilities over the MTP *Taq*. The benefit of the MTP *Taq* was that it was ultra pure and tested specifically for 16S rRNA contamination with universal primers. However, since contamination can be introduced very easily at any point, and negative controls using this MTP *Taq* enzyme have been shown to sometimes be contaminated, this purity becomes less of an important benefit. The high fidelity and proofreading were more important in producing viable DGGE gel images for analysis. Therefore, all subsequent work was performed using the Stratagene *PfuUltra*™ II Fusion HS DNA polymerase.

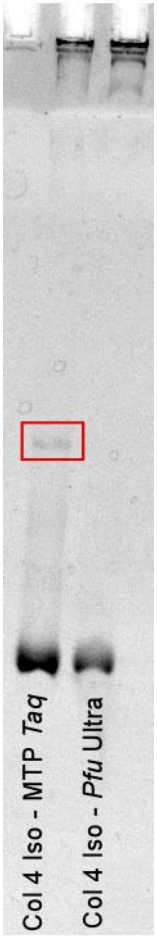


Figure 2.3.2-2. DGGE gel of PCR products produced during testing of Stratagene's *PfuUltra*™ II DNA polymerase.

2.3.3 SEQUENCING QUALITY CONTROL

Quality control regarding the reproducibility of our internal PCR reactions and the external DNA sequencing performed at Rutgers was evaluated.

2.3.3.1 METHODS

Various samples from pure cultures in duplicate and/or triplicate underwent PCR amplification, followed by DGGE and sequencing. Additionally, single samples were split and replicates were submitted to Rutgers for sequencing. The sequences obtained were aligned and analyzed for sequence similarity.

2.3.3.2 RESULTS

All sequences obtained from the replicate experiments were analyzed and compared via alignment using a simple alignment tool. All replicates of a given strain matched 96% to 100% (Table 2.3.3-1), with the lowest scores being from the sequences from different in-house PCR reactions. All sequencing replicates sequenced by Rutgers scored greater than 97% similarity.

Table 2.3.3-1. Reproducibility of internal PCR/DGGE and external DNA sequencing.

Sample ID	PCR/DGGE Replicates	Sequencing Replicates		% Sequence Similarity
		different runs of PCR/DGGE	same run of PCR/DGGE	
Ps. putida II-B		2		97
Ps. putida II-B	2			96
Ps. putida II-B	2			99
Ps. putida II-B	2			97
E. coli		2		98
E. coli	2			100
E. coli	2			99
Enterobacter cloacae ATCC 43560			3	100
Isolate 2			3	100
Isolate 6			3	99-100
Isolate 12			3	100

2.3.4 PCR AND DGGE STANDARD OPERATING PROCEDURE

Results from all the testing and optimization work resulted in production of a standard DGGE protocol that was subsequently employed for the majority of the sample analyses performed during 2007 and 2008. The standard DGGE protocol is included in Appendix 3.

2.3.5 STANDARD PROCEDURES FOR PHYLOGENTIC ANALYSIS OF DATA

Sequences obtained using DGGE were examined by treeing methods using a web-based program called CLUSTAL W (version 1.83, Kyoto University Bioinformatics Center, http://align.genome.jp/). Standard processing initially generated an aligned sequence file, which was then reprocessed to generate a phyllip tree. The standard CLUSTAL settings are given in Figure 2.3.5-1. The tree files were turned into visual phylogenetic tree diagrams using the web-based program Phylodendron (version 0.8d, Indiana University Biology Department, http://iubio.bio.indiana.edu/treeapp/treeprint-form.html). The standard settings for creating tree diagrams are shown in Figure 2.3.5-2. Identification of sequences obtained from DGGE analyses was performed using the Basic Local Alignment Search Tool (BLAST-N) program at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

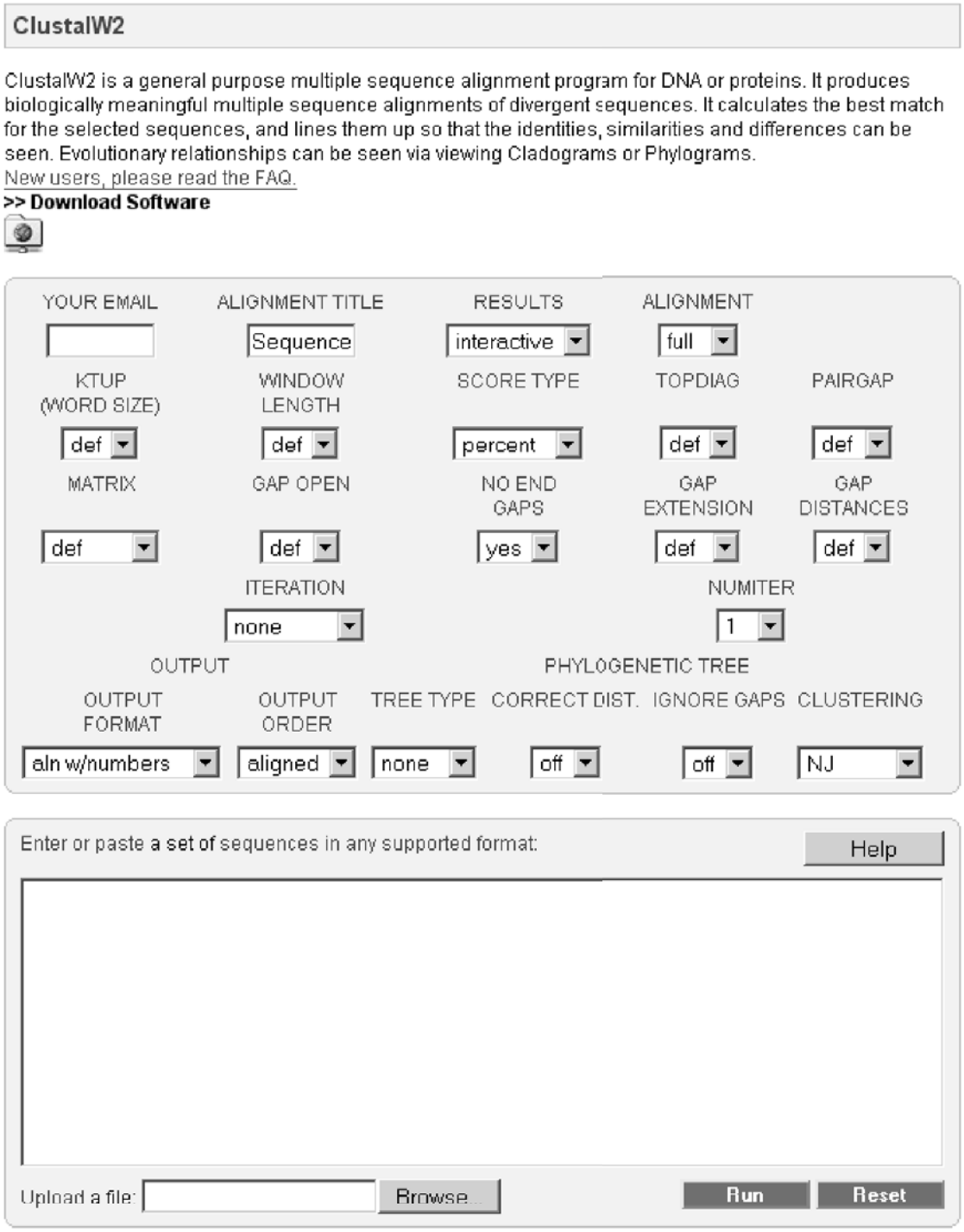


Figure 2.3.5-1. Standard settings used with the CLUSTAL program.

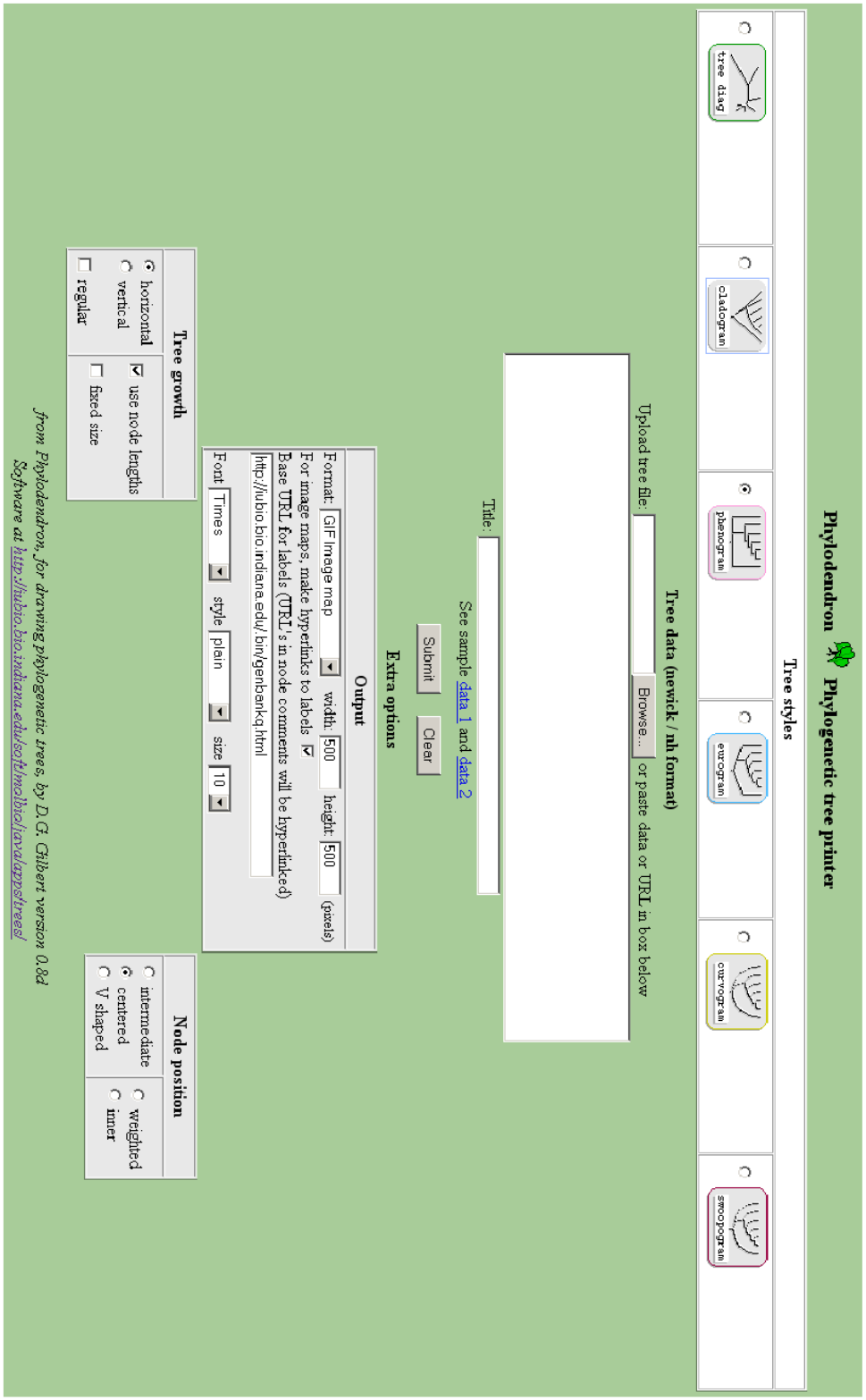


Figure 2.3.5-2. Standard settings used with the Phylodendron program.

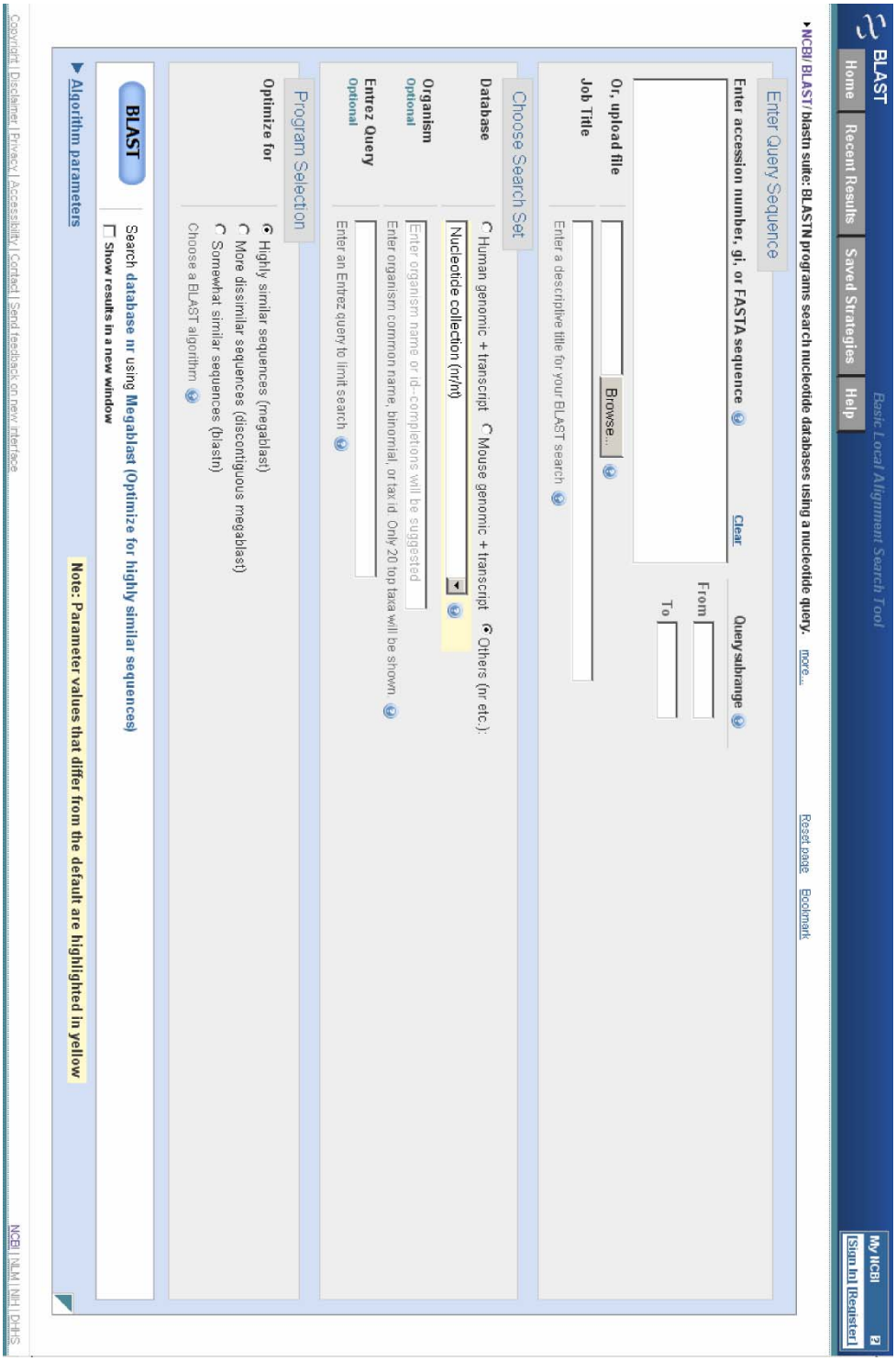


Figure 2.3.5-3. Standard settings used with the BLAST-N program.

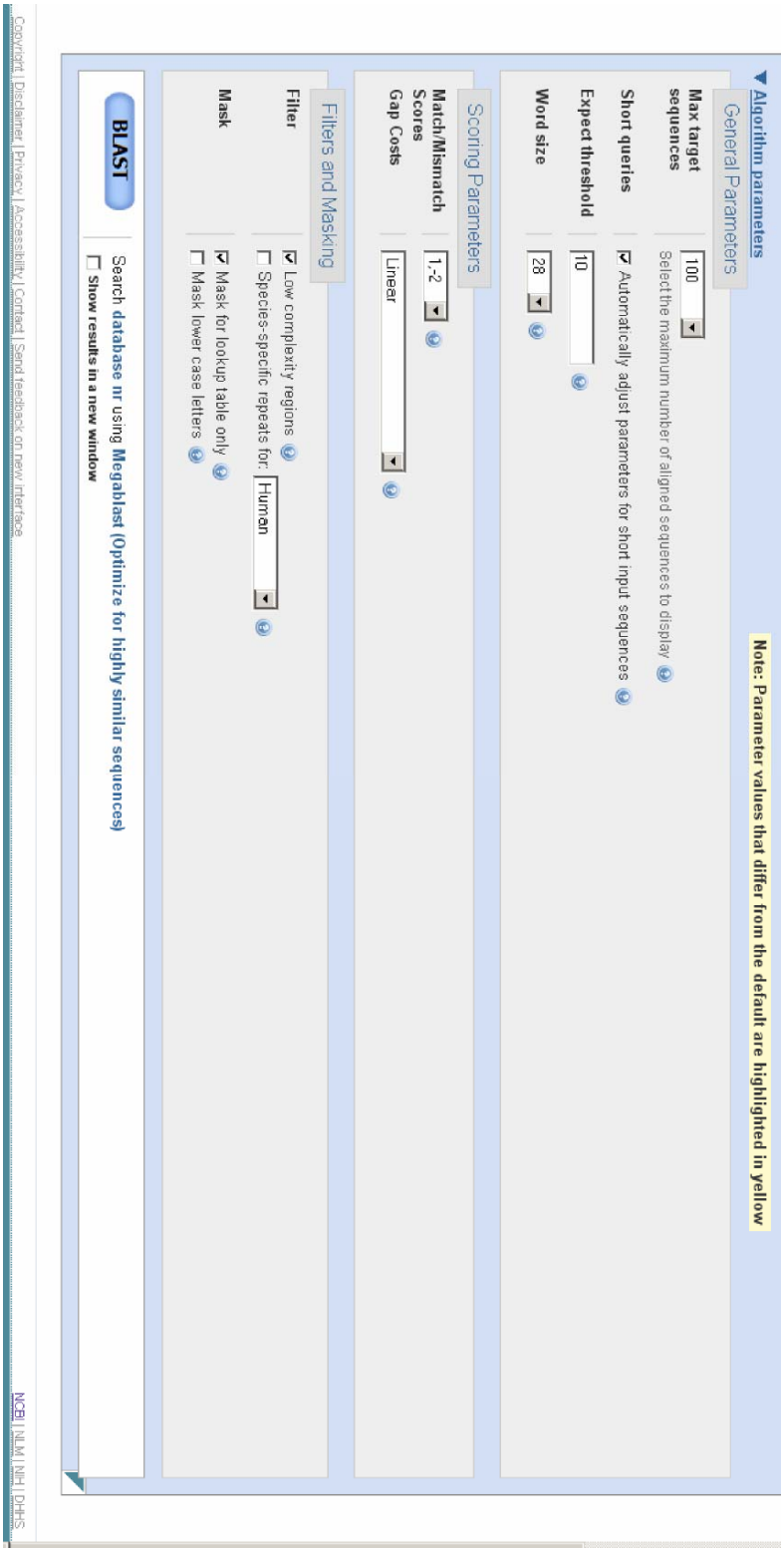


Figure 2.3.5-3 (cont). Standard settings used with the BLAST-N program.

3. APPLICATION OF MOLECULAR PROTOCOLS.

The analytical methods described above were applied over the course of this project to various laboratory (batch enrichments, columns) and field groundwater samples.

3.1 WEST VIRGINIA ORDNANCE WORKS (WVOW)

3.1.1 METHODS

Samples of subsurface sediment were collected from contaminated and uncontaminated areas at the West Virginia Ordnance Works (Table 3.1-1). Groundwater samples were collected into sterile 1 L amber glass bottles using low-flow bladder pumps attached to new tubing for each well. Sediment samples were collected using a GeoProbe rig. Continuous cores were retrieved in plastic sleeves in 2.5' sections. Each section was capped at both ends and shipped on ice within one day of collection. Groundwater and sediment cores were stored at 4°C until processed or used for experiments. Selected sediment cores were aseptically removed from the plastic casing and transferred into sterile 2 L large mouth I-Chem glass jars.

A portion of the sediment (4 x 100 g, wet wt) was immediately archived by freezing at -80°C. Archiving of the *in situ* groundwater microbial community was performed by filtration of 1 L of water through Millipore Duropore PVDF membrane filters (0.22 µm pore size, 47 mm diameter), which were then frozen in sterile 50 mL polypropylene centrifuge tubes at -80°C. Two filters were prepared from each groundwater sample.

Due to the limited amount of groundwater collected, recipes for artificial groundwater were developed (Table 3.1-2), matching all the major groundwater parameters of the actual groundwater.

Microcosms were prepared to examine the effect of the carbon source, presence of utilizable nitrogen, and explosive compounds on explosive compound degradation and the microbial community. The treatments in this experiment are presented in Table 3.1-3. A total of 120 vials (60 vials per sediment, duplicate vials per treatment) were needed to prepare all of the required treatment combinations. Microcosms were divided into sets due to the large quantity of bottles. The preparation and sampling of each set was staged as follows: microcosms prepared from contaminated sediments were prepared two days after those prepared with clean sediment. Microcosms prepared with no added explosives were prepared six days after those containing contaminated sediments. A photograph of the microcosms is presented in Figure 3.1-1.

All three sets were prepared as follows: Wet sediment (10 g) was weighed into each bottle (160 mL serum bottles with teflon lined butyl rubber stoppers) using sterile utensils, 15 mL of artificial groundwater was added, and the bottles stored at 4°C for approximately seven weeks. Artificial groundwater (85 mL) containing one of the explosive compounds was added, as well as NH_4Cl (7.3 mg/L final concentration), where indicated. Nominal initial explosive concentrations of TNT, RDX, and HMX were 10, 5, and 0.5 mg/L, respectively; actual concentrations were determined by HPLC analysis. Bottles were incubated for an additional four weeks at 4°C. Immediately prior to the addition of the carbon sources, an initial sample was removed from the first of each replicate in an aerobic environment. After the cosubstrates were

added, the microcosms were placed in boxes and incubated statically at 15°C. Each box of vials was manually shaken three times a week.

All microcosms prepared with explosives were sampled in a Coy anaerobic chamber under nitrogen headspace (anaerobic chamber hereafter), with the exception of one replicate of each control. One replicate of microcosms with no explosives added was sampled in the anaerobic chamber, while the second replicate was sampled aerobically. Subsamples were processed as follows: one replicate for probe analysis (pH, oxidation-reduction potential (ORP), and dissolved oxygen (DO)) was analyzed inside the anaerobic chamber, and the other replicate was analyzed outside the anaerobic chamber.

Subsamples for explosives and molecular analyses were processed outside the anaerobic chamber. While mixing the contents of a bottle vigorously on a stir plate, 2 x 1.5 mL samples were removed to duplicate labeled eppendorf tubes, one of which was archived at -80°C and the second one processed for explosives and molecular analyses. An additional 3 mL was removed to a 15 mL conical centrifuge tube for pH, ORP, and DO analyses. Vials were then closed with new aluminum crimp seals and Teflon®-lined butyl rubber stoppers. Eppendorf and centrifuge tubes were sealed and stored at -80°C until processing and analysis.

Subsamples in the eppendorf tubes were spun for 2 min at 14,000 rpm to pellet the microorganisms and solid matter. An aliquot (0.650 mL) was transferred into a 2 mL autosampler vial for HPLC analysis, and 0.650 mL of remaining supernatant was disposed (a total of 1.3 mL removed). The residual pelleted sample and residual supernatant was placed at -80°C for later molecular analyses using the methods described in Appendix 3.

An additional set of samples (14 mL slurry; ~1.5 g sediment) were collected after 87 days and sent to Microbial Insights (Knoxville, TN, USA) for DGGE analysis and sequencing. Bands from their DGGE gel were obtained and further analyzed in-house after amplification using the TOPO cloning system (Invitrogen Corporation, Carlsbad, CA, USA).

3.1.2 RESULTS

3.1.2.1 PROCESS PARAMETERS

The DO remained low in all of the microcosms amended with electron donor, indicating that the low oxygen conditions required for explosives degradation were generated. ORP was more variable, as has been seen in previous work. The pH of the microcosms was generally above 6.0, except for those amended with crude and pure soybean oil, which exhibited a general decrease in pH to around 5.0. No clear correlations between the presence of explosives or of utilizable nitrogen on pH have been observed.

3.1.2.2 EXPLOSIVES DEGRADATION

Degradation of TNT, RDX, and HMX (and related breakdown products) under the different conditions tested are presented in Figures 3.1-2 through 3.1-7. A summary of the observed results follows:

TNT degradation was generally more extensive in the contaminated soil microcosms than in the clean soil microcosms, even in the control bottles (no added electron donor). Re-spiking with

TNT after 86 days resulted in increased aqueous TNT concentrations in all the clean soil microcosms, but no increase was observed in the contaminated soil microcosms amended with crude soybean oil or EOS, probably due to a higher degradation capacity.

The breakdown products of TNT were also monitored. The detected compounds included: 2-amino-4,6-dinitrotoluene (2A-DNT), 2,4-dinitrotoluene (2,4-DNT), and 2,6-dinitrotoluene (2,6-DNT); 4A-DNT was not detected. Variability between replicates was observed. Lactate stimulated the greatest production of 2A-DNT and 2,6-DNT in all the microcosms. The presence of utilizable nitrogen reduced the production of both compounds, with a greater reduction observed in the clean site microcosms compared to the contaminated site microcosms. The concentrations of both 2A-DNT and 2,6-DNT appeared to be decreasing by Day 85 of incubation, except in the contaminated site microcosms amended with lactate, where concentrations appeared to be increasing. The levels of 2,4-DNT in the clean site microcosms was near the background concentration. Crude soybean oil and pure soybean oil stimulated production of 2,4-DNT in the contaminated site microcosms, whereas lactate and EOS did not.

Degradation of HMX was only evident in the contaminated soil microcosms, with no or little degradation observed in the clean soil microcosms. The microcosms that were amended with nitrogen (as NH_4) exhibited somewhat slower and less extensive degradation than the unamended microcosms. This may reflect more aggressive use of the explosives as both electron acceptors and nitrogen sources when no exogenous nitrogen is present. Degradation of RDX was most apparent in the clean soil microcosms when lactate was the electron donor, while both lactate and EOS supported good RDX biodegradation in the contaminated soil microcosms. The microcosms that were amended with nitrogen (as NH_4) exhibited somewhat slower and less extensive degradation than the unamended microcosms. This may reflect more aggressive use of the explosives as both electron acceptors and nitrogen sources when no exogenous nitrogen is present. Concentrations of aqueous RDX in contaminated soil microcosms amended with lactate, soybean oil, and EOS did not increase greatly after the spiking, indicating that these samples had a very high degradation capacity that was able to effectively cope with and degrade the added RDX.

The detection of the RDX breakdown products MNX, DNX, and TNX was variable, as was the detection of the compounds in replicate microcosms. MNX, DNX, and TNX were detected in the clean site microcosms, even though the degradation of RDX was minimal. In the contaminated site microcosms, MNX and DNX initially appeared then disappeared in the EOS amended bottles, whereas there was a slower and continuous production of MNX and DNX in the bottles which received the other electron donors. TNX dynamics were similar, but the difference in behavior observed with EOS compared to the other amendments was not as evident. The presence of utilizable nitrogen had little influence on the production and degradation of any of the RDX breakdown products.

Table 3.1-1. Field sample information for WVOW site sediment cores.

Area	Core ID	Depth	Comments
YWRGW-044	1	0.0-2.5'	
	2	2.5-5.0'	
	3	5.0-7.5'	
	4	7.5-10.0'	
	5	10.0-12.5'	
	6	12.5-15.0'	
	7	15.0-17.5'	Saturated; sandy with clayey portions
	8	17.5-20.0'	Saturated; fine sand
TNTGW-016	1	0.0-2.5'	
	2	2.5-5.0'	
	3	5.0-7.5'	
	4	7.5-10.0'	
	5	10.0-12.5'	Saturated; medium fine sand
	6	12.5-15.0'	Saturated; fine sand
	7	15.0-17.5'	Saturated; fine sand
	8	17.5-20.0'	Saturated; sandy/clayey

Table 3.1-2. Recipes for the two WVOW artificial groundwaters.

	Clean Well		**Contaminated Well**	
	YWRGW-044		TNTGW-016	
	Field GW	AGW	Field GW	AGW
pH	5.5	5.5	5.5	5.5
Chloride	8.0	6.5	5.0	6.5
Nitrate	1.0	1.1	3.0	2.9
Sulfate as SO4-	19.0	20.3	63.0	64.7
Alk. as CO3	7.0	7.1	11.5	11.4
Na+	4.5	7.7	4.5	5.5
Ca2+	5.0	4.7	15.0	14.8
Mg2+	2.0	2.0	8.0	7.9
K+	0.5	0.5	3.0	3.1
Mn2+	0.0	0.0	1.5	1.6
Salt		**mg/L**		**mg/L**
$NaHCO_3$		10.0		16.0
$NaNO_3$		1.5		4.0
Na_2SO_4		2.0		0.0
$MgSO_4$-$7H_2O$		20.0		80.0
$CaCl_2$-$2H_2O$		0.0		7.5
$CaSO_4$-$2H_2O$		20.0		55.0
KCl		1.0		6.0
NaCl		10.0		0.0
$MnSO_4$-H_20		0.0		5.0
pH adjusted with 1.0 N HCl				

Table 3.1-3. Microcosm enrichment setup for WVOW sediments.

Sediments	Carbon source	Added N	Explosive
YWRGW-044	Control (none)	None	TNT
TNTGW-016	Lactate	7.3 mg/L	RDX
	EOS		HMX
	Crude soybean oil		
	Wesson soybean oil		

Figure 3.1-1. Photograph of the WVOW microcosm enrichments.

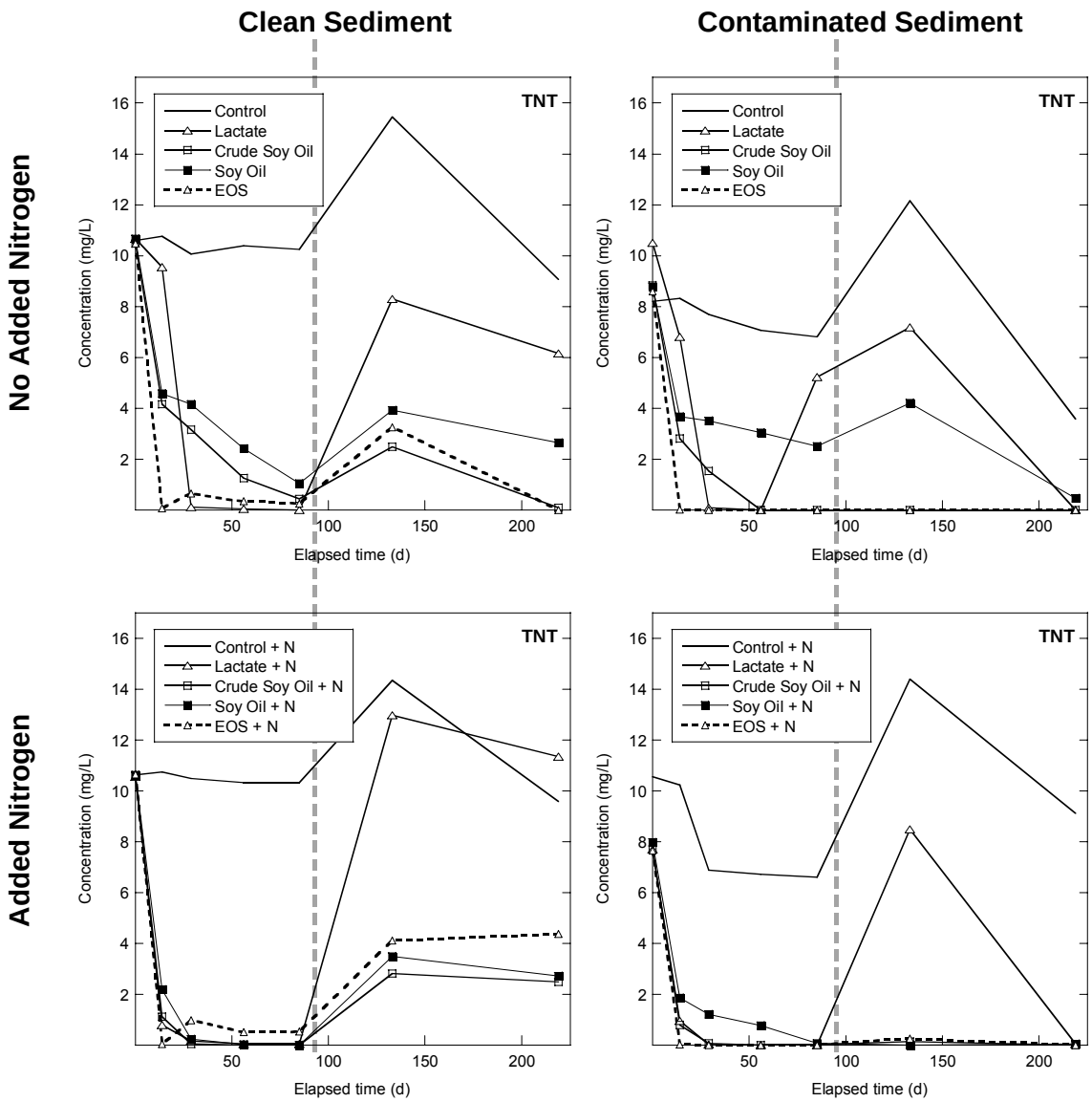


Figure 3.1-2. Degradation of TNT in WVOW microcosm enrichments. The vertical dashed line indicates when the microcosms were respiked with the explosive and/or other amendments.

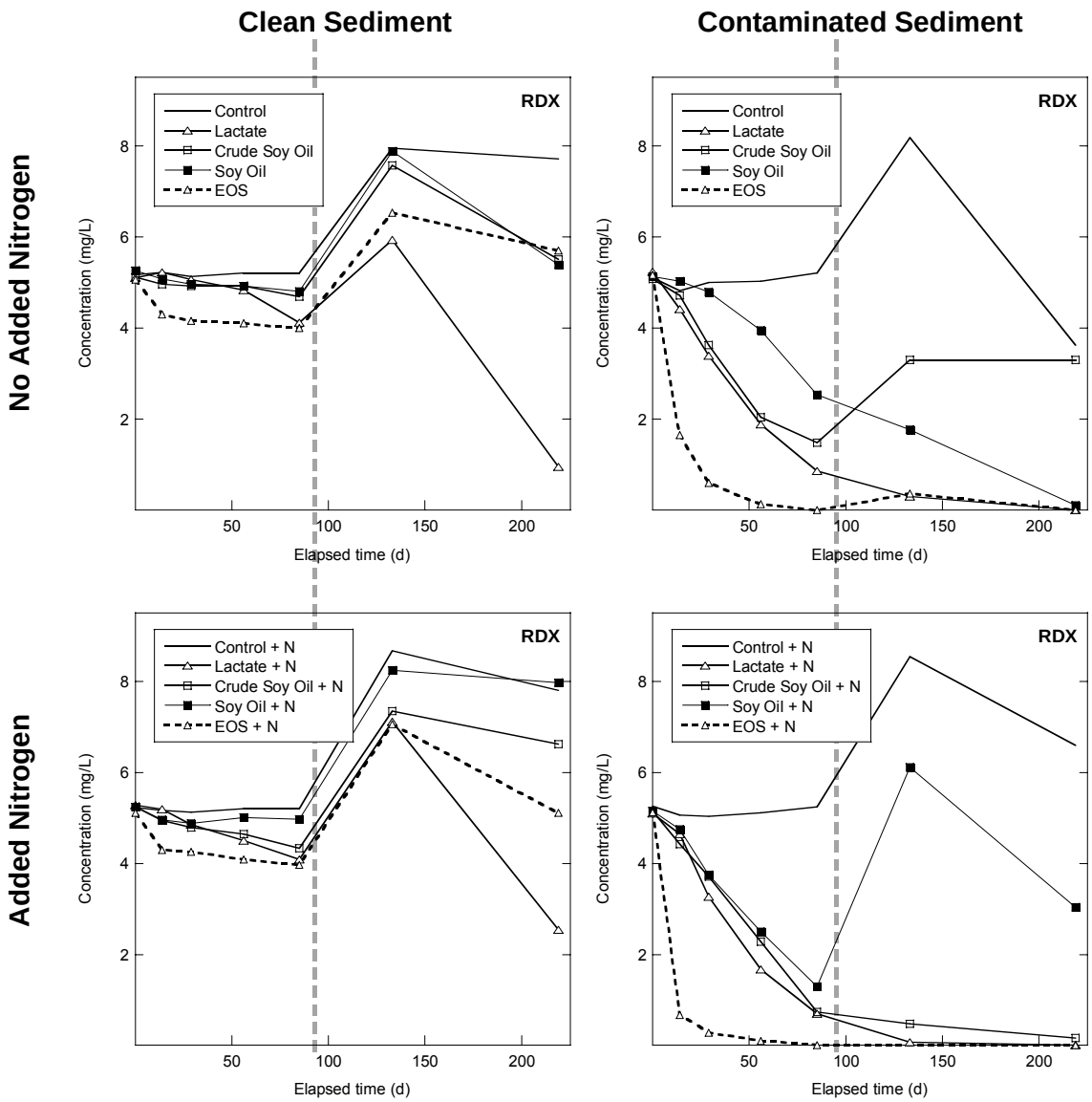


Figure 3.1-3. Degradation of RDX in WVOW microcosm enrichments. The vertical dashed line indicates when the microcosms were respiked with the explosive and/or other amendments.

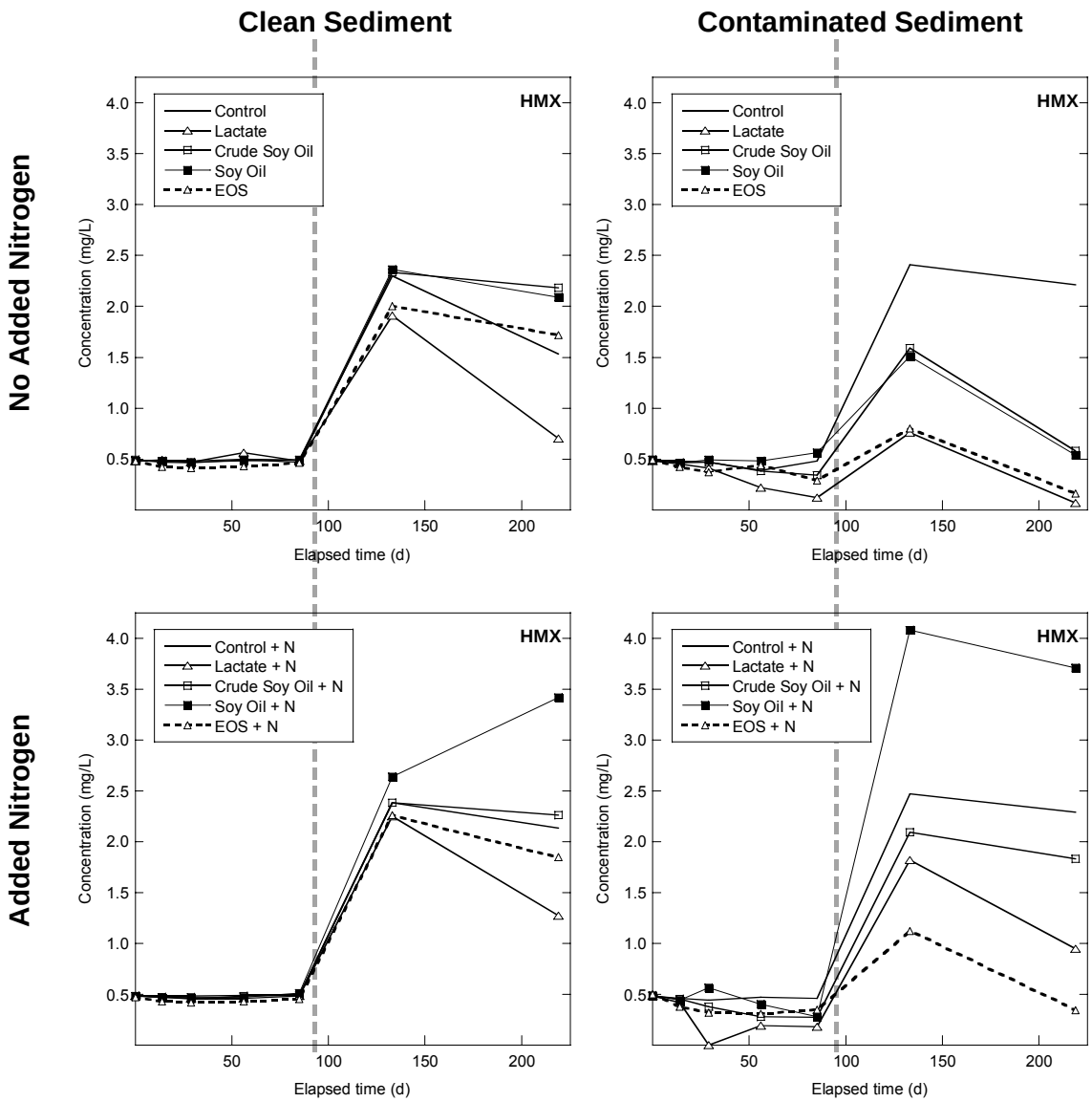


Figure 3.1-4. Degradation of HMX in WVOW microcosm enrichments. The vertical dashed line indicates when the microcosms were respiked with the explosive and/or other amendments.

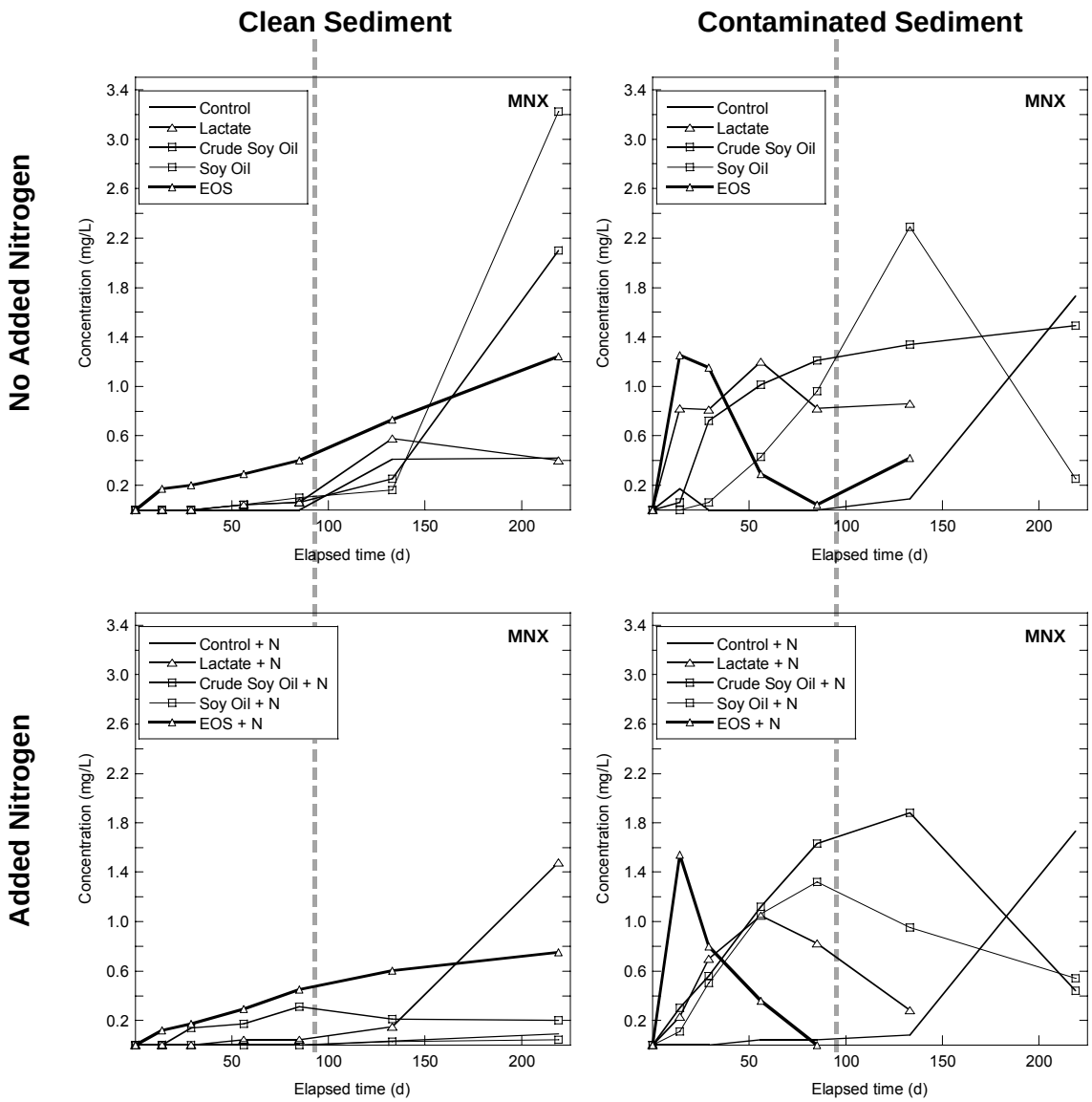


Figure 3.1-5. Concentrations of MNX in WVOW microcosm enrichments. The vertical dashed line indicates when the microcosms were respiked with the explosive and/or other amendments.

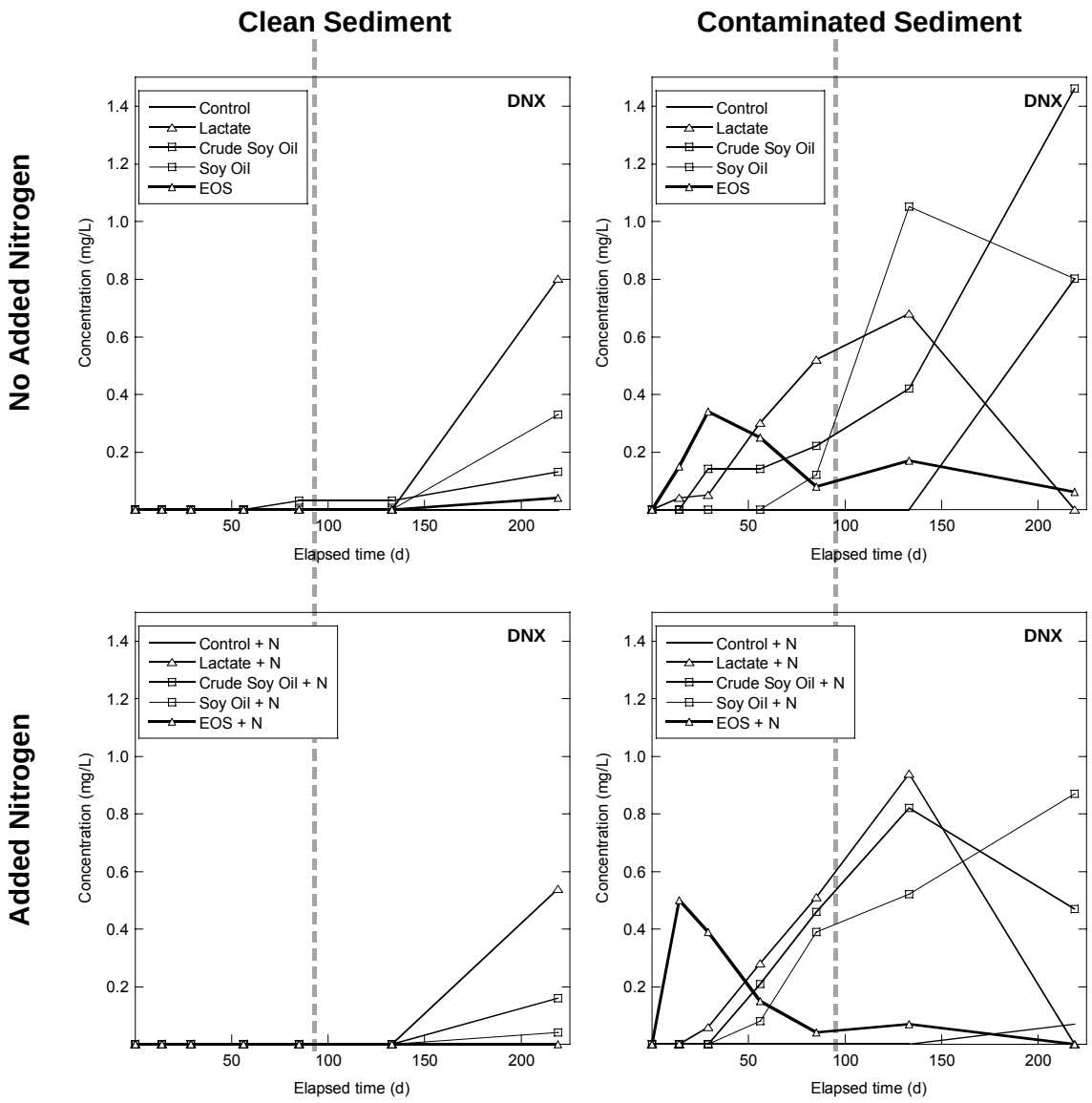


Figure 3.1-6. Concentrations of DNX in WVOW microcosm enrichments. The vertical dashed line indicates when the microcosms were respiked with the explosive and/or other amendments.

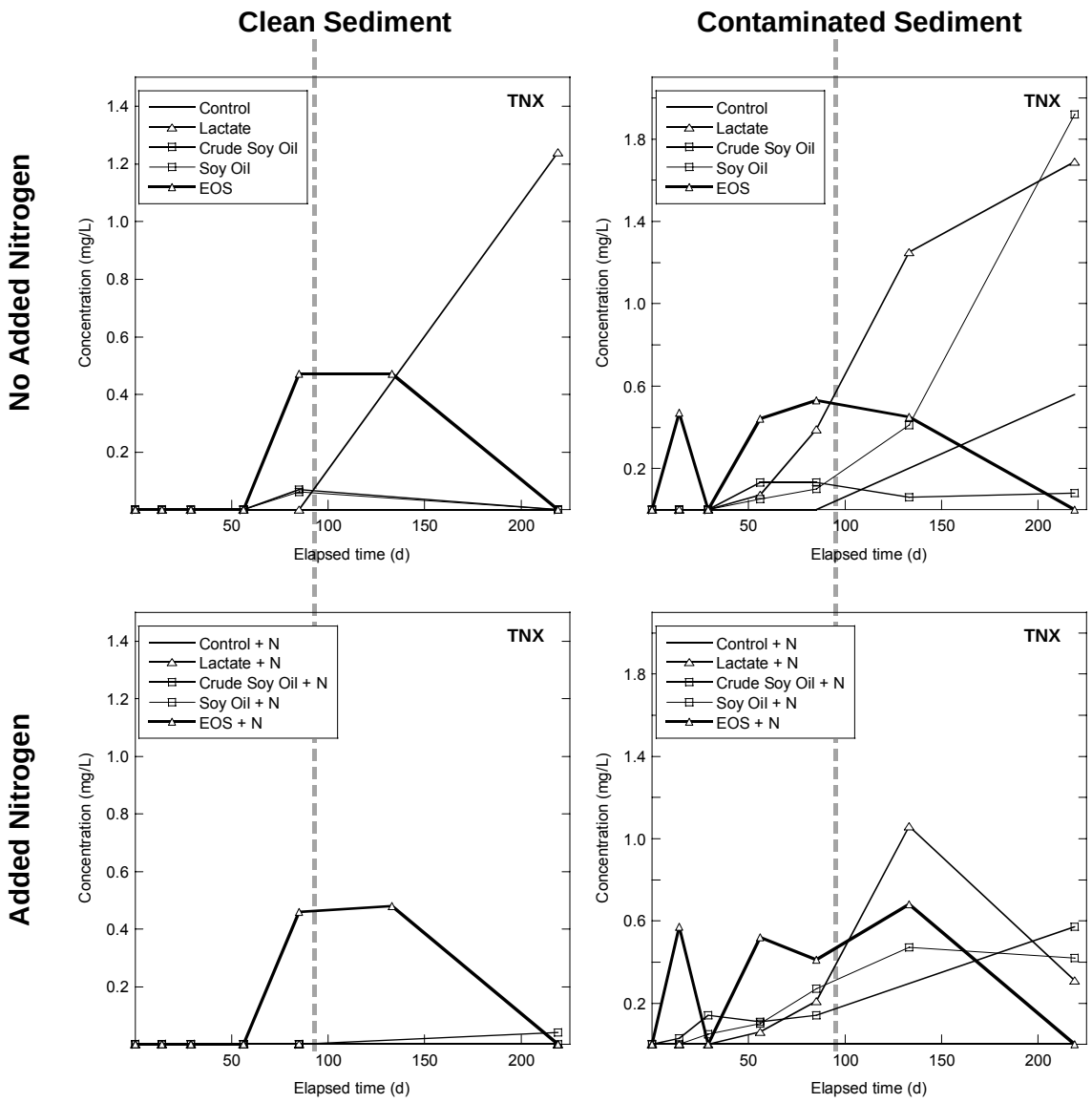


Figure 3.1-7. Concentrations of TNX in WVOW microcosm enrichments. The vertical dashed line indicates when the microcosms were respiked with the explosive and/or other amendments.

3.1.2.2 MICROBIAL COMMUNITY ANALYSES

The observed differences in the degradation of TNT, RDX, and HMX were indicative of the presence of different microbial communities in the microcosms. Initial DGGE analysis performed by Microbial Insights did indeed demonstrate differential banding patterns for samples taken from the various enrichments (Figure 3.1-8). A summary of the gel results include:

-The sediment from the uncontaminated and contaminated areas of WVOW had different DGGE banding patterns, although some bands appeared in both. This indicates different indigenous microbial communities, possibly as a result of the explosives contamination.

-When no electron donor is added and after incubation for 87 days with TNT, the banding patterns changed very little (but became fainter) compared to the initial sediment. In contrast, the banding patterns changed substantially in the presence of RDX after 87 days. This indicates that RDX may directly alter the microbial community to a greater extent than TNT even in the absence of an electron donor to stimulate biodegradation.

-The addition of lactate and soybean oil resulted in different banding patterns compared to that of the initial sediment. The patterns were different depending on the source of the sediment and whether the explosive present was TNT or RDX. A few bands that appeared in the soybean oil enrichments were possibly the same. This indicates that the specific microorganisms that are enriched depends on the original source of the sediment, the electron donor added, and the explosive present.

These results even at the level of simply comparing banding patterns indicate quite significant changes/differences in the microbial population based on the explosive residue exposure history, the explosives currently present, and the presence and type of electron donor.

Microbial Insights was able to identify and excise 72 individual bands from the DGGE gel (noted by "*" or letters). Of these, they were only able to get usable sequences from three bands (labeled B, C, and D in Figure 3.1-8). We requested and received 47 band extracts from the WVOW DGGE gel from Microbial Insights. A subset of 21 extracts was selected for TOPO cloning, which yielded 10 valid sequence results. Identifications of the 10 new sequences, as well as the three sequences provided by Microbial Insights (bands B, C, D) with isolates in GeneBank were made (Table 3.1-4). Most of the identifications are of environmental bacteria. A phylogenetic tree relating these sequences to those of known explosive-degrading bacterial strains is presented in Figure 3.1-9.

Based on guidance from the SAB and the SERDP Program Office, the focus of the project was redirected more towards understanding RDX degradation. Therefore, additional in-house analysis of selected samples from the RDX-containing enrichments was performed. The samples analyzed corresponded to the time at which a given treatment had degraded greater than 50% of the initial RDX. Since the clean soil microcosms did not exhibit significant RDX degradation, only samples from contaminated soil microcosms were analyzed.

A total of 40 samples were selected for additional DNA extraction and molecular analysis. Ten of the samples yielded amplifiable DNA, and a total of 17 16S rRNA sequences were recovered from the DGGE gel and analyzed. A phylogenetic tree and a table presenting the results are given in Figure 3.1-10 and Table 3.1-5. Descriptions of the various genera detected during all the analyses are presented in Table 3.1-6.

These subsequent analyses revealed a wide range of genera, but there was no apparent pattern with respect to the electron donor addition or the presence of a readily utilizable nitrogen source. The only sequence that was similar to previously described RDX-degrading strains was a *Clostridium nitrophenolicum* strain 1DT, recovered from an enrichment with no added electron donor or RDX, but with NH_4. The majority of the sequences were not related to known degraders, and two sequences had no close matches in the public databases, which could indicate new, unknown organisms.

Results of screening enrichment samples for putative explosive-degrading genes is presented in Table 3.1-7. None of the genes for which screening was performed were detected in any samples.

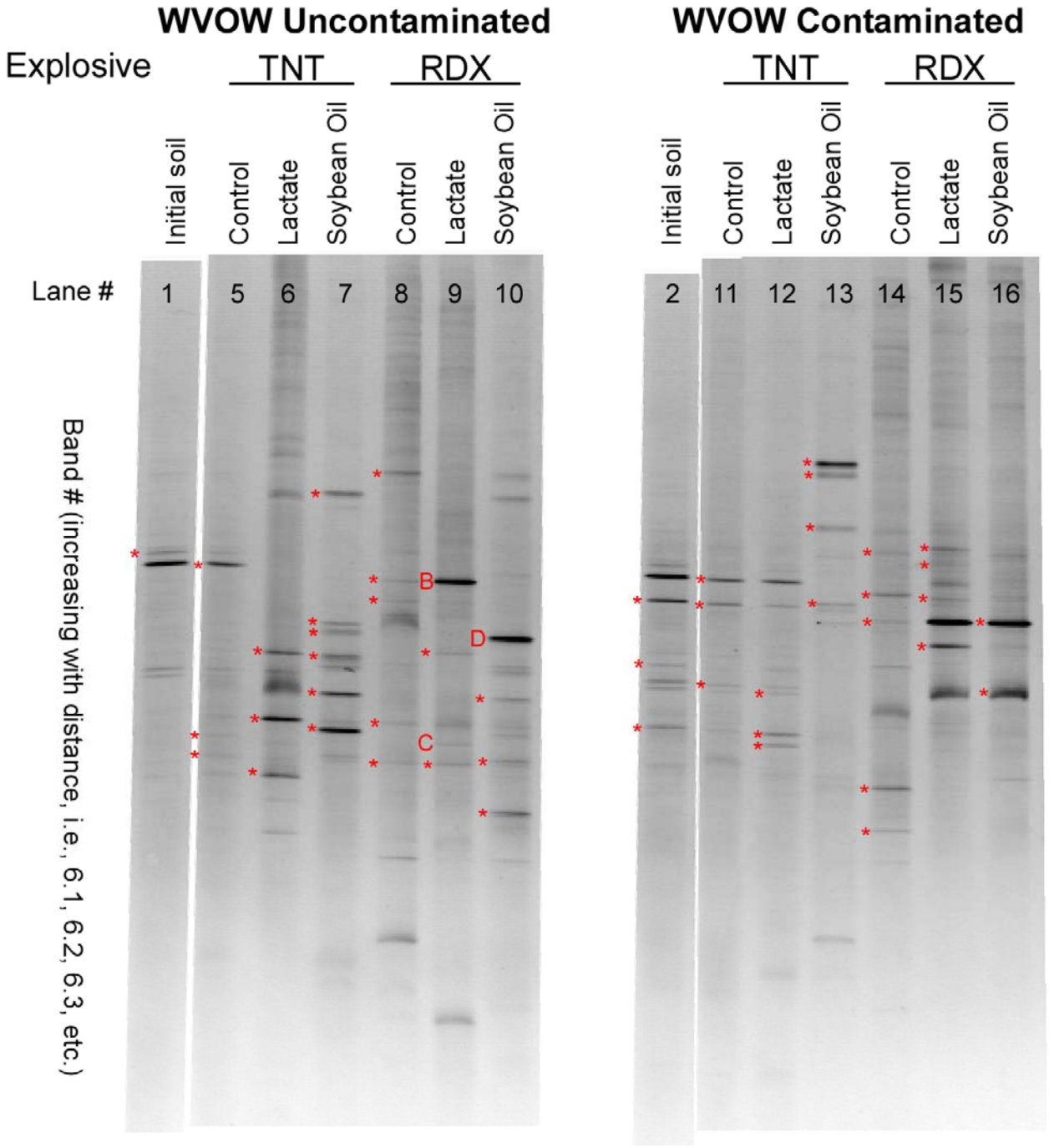


Figure 3.1-8. DGGE analysis of WVOW microcosm enrichments performed by Microbial Insights.

Table 3.1-4. Identification of bands from initial WVOW DGGE analysis. Samples are designated as source of WVOW sediment (clean or contaminated area), explosive added, and nutrient amendment.

Treatment	Band	Identification	Source
Clean soil/TNT/Control	5.2	Unidentified bacterium clone SD12.	Antarctic Research Station
Clean soil/TNT/Control	5.3	Uncultured bacterium	biofilm
Clean soil/TNT/Lactate	6.1	*Ralstonia* sp. Q3-8/14	soil
Contam soil/TNT/Lactate	12.1	Uncultured bacterium	biofilm
Clean soil/TNT/EOS	7.1	*Pseudomonas* sp. LCY16	oil contaminated soil (Antarctic)
Clean soil/TNT/EOS	7.6	*Burkholderia kirkii* strain 835429	leaf galls
Contam soil/TNT/EOS	13.1	Uncultured eubacterium clone LKB84.	anaerobic landfill leachate
Clean soil/RDX/Lactate	C	Uncultured bacterium clone 21BSF26	poplar trees - flooded rhizosphere
Clean soil/RDX/Lactate	B	Uncultured bacterium clone 015B-F04	uranium contaminated groundwater
Clean soil/RDX/Lactate	9.1	Uncultured bacterium clone 015B-F04	uranium contaminated groundwater
Clean soil/RDX/EOS	D	Uncultured *Aquabacterium* sp.clone C-23	bottled water
Clean soil/RDX/EOS	10.4	*Rhodoblastus sphagnicola*	peat bog
Contam soil/RDX/EOS	16.1	Uncultured bacterium clone 300I-F02	uranium contaminated groundwater

Table 3.1-5. Identification of bands from subsequent WVOW DGGE analysis.
All samples were from enrichments using WVOW contaminated sediment.

Treatment	Band	Identification	Source
CON+N, NO EXP	1	*Janthinobacterium* sp. blxAG	
	2	*Clostridium nitrophenolicum* strain 1DT	PNP degrader from subsurface soil
CS	1	*Deinococcus* sp. 4A6	
	2	uncultured bacterium clone W2-8	oil contaminated aquifer
EOS, NO EXP	1	uncultured gamma proteobacterium clone FH1-74	landfill cover soil
	2	uncultured *Geobacter* sp. clone X9Ba89	flooded soil
EOS+N	1a	uncultured gamma proteobacterium clone 1HP1-O20	coral disease agent
	1b	uncultured bacterium clone P3T_036	sulfate reducing mine drainage
	2a	uncultured bacterium clone 5S54	petroleum contaminated sediment
	2b	uncultured bacterium clone FW131	reject coal contaminated forest soil
SOY+NO EXP	1	uncultured *Pseudoxanthomonas* sp. clone LDC-8-c9	cellulose degrading culture
	2	uncultured *Sporichthyaceae* bacterium clone D25_13	tar oil contaminated aquifer
	3	uncultured alpha proteobacterium clone D10_12	tar oil contaminated aquifer
SOY+N	1a	uncultured bacterium clone FB47-26	uranium and nitrate bioremediation
	1b	unknown	
	2	uncultured bacterium clone 12C-A82	nitrate reducing methanol utilizers
LAC+N	1	unknown	
CS, Crude soybean oil; SOY, soybean oil; LAC, lactate; EOS, emulsified oil substrate			
+N, NH_4 added			
NO EXP, no RDX added			

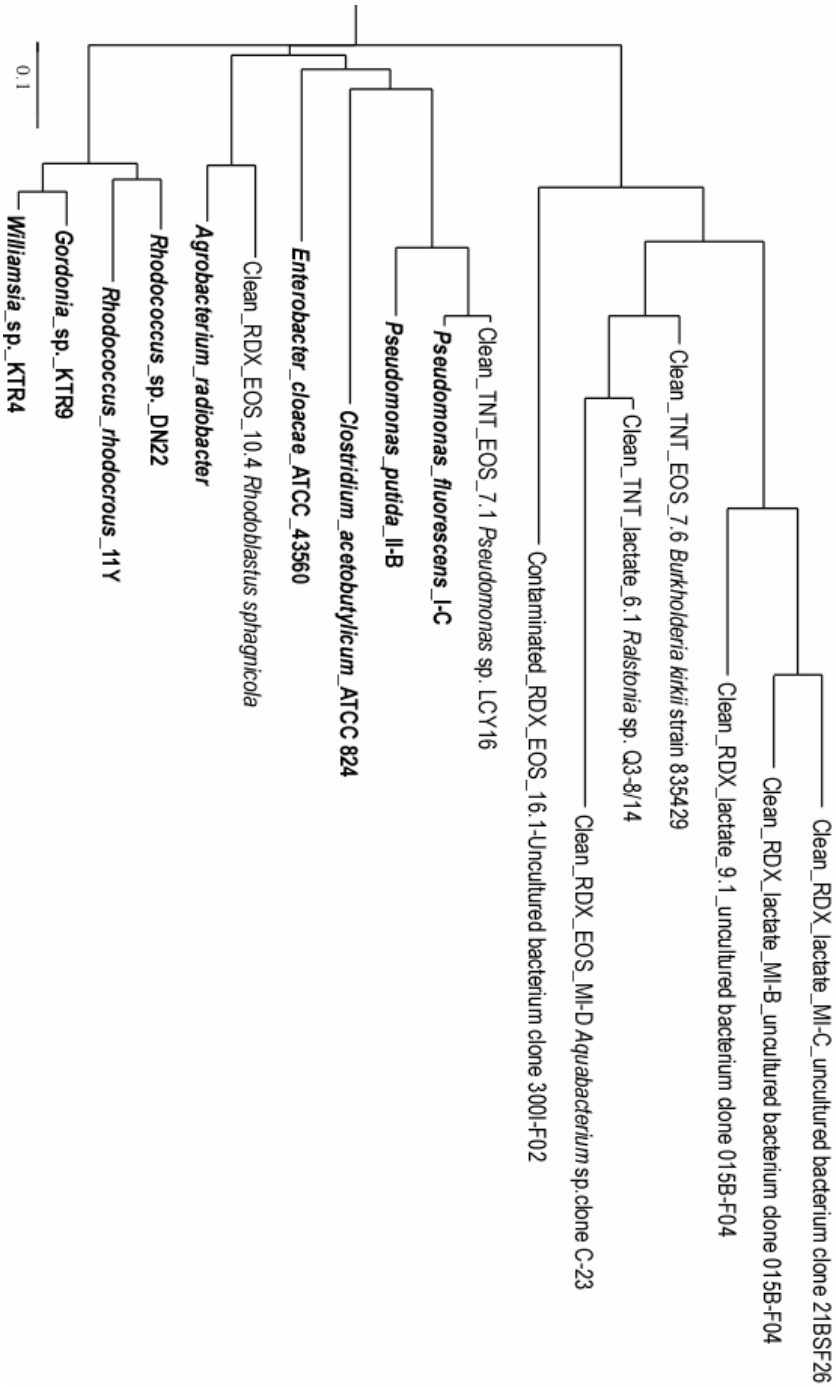


Figure 3.1-9. Initial phylogenetic analysis of sequences derived from in WVOW microcosm enrichments. Samples are designated as source of WVOW sediment (clean or contaminated area), explosive added, and nutrient amendment. The closest identities based on the sequences are given, and known explosive-degrading strains are included for reference (bold text). Bar = 10 nucleotide substitutions per 100 nucleotides in the 16S rRNA sequences.

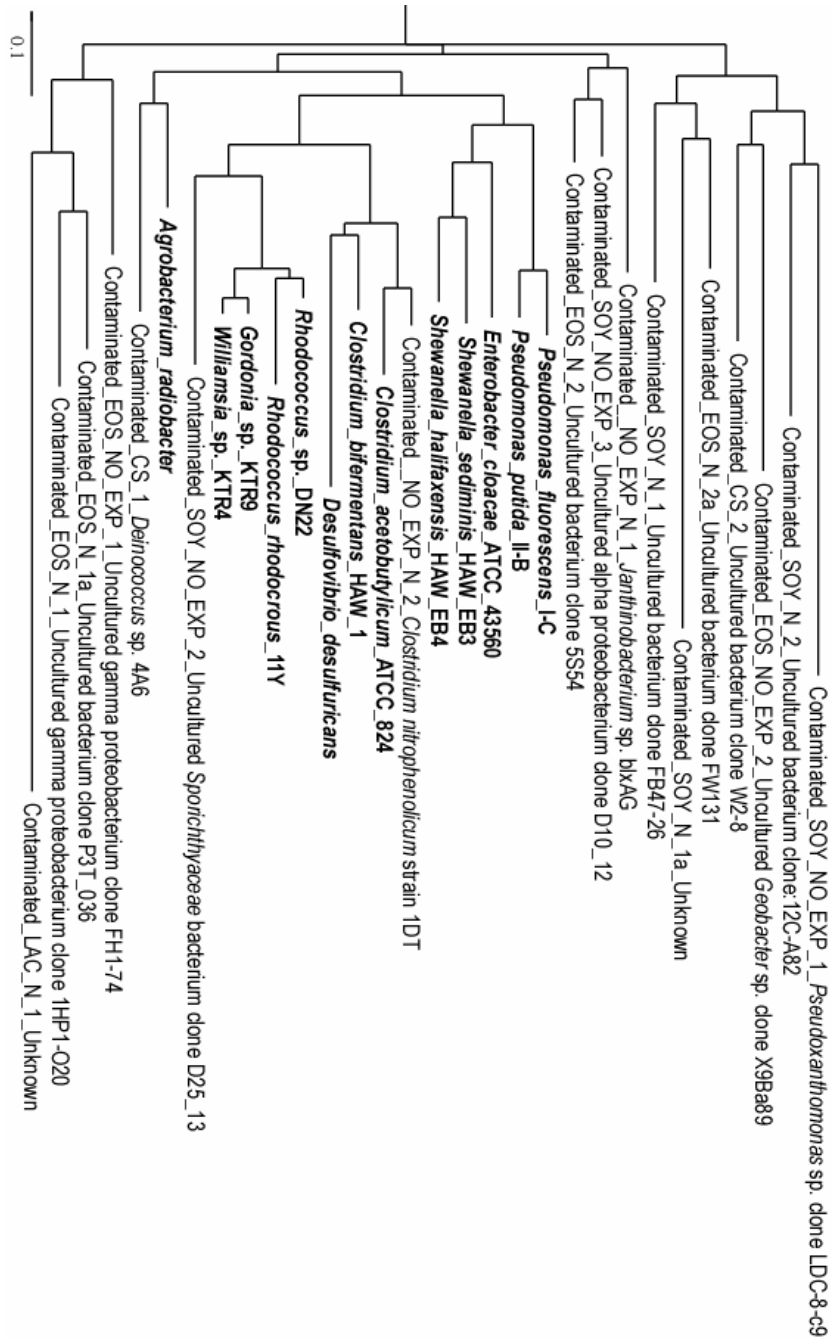


Figure 3.1-10. Refined phylogenetic analysis of sequences derived from in WVOW microcosm enrichments. All samples used WVOW contaminated sediment as the source and RDX as the explosive added except in those designated as NO_EXP. Nutrient amendments are as follows: SOY, soybean oil; CS, crude soybean oil; LAC, lactate; N, NH_4. The closest identities based on the recovered sequences are given, and known explosive-degrading strains are included for reference (bold text). Bar = 10 nucleotide substitutions per 100 nucleotides in the 16S rRNA sequences.

Table 3.1-6. Description of the genera detected in the WVOW enrichments.

Identification	Characteristics
Geobacter	iron-oxide reducing organoheterotroph, common in soil, river water, groundwater, and sewage
Clostridium	strictly anaerobic, fermentative bacteria, **some strains proven to degrade TNT, RDX**
Pseudomonas	widespread genera, extensive catabolic diversity, **some strain shown to degrade RDX**
Burkholderia	common environmental bacteria, **degrade many pollutants including nitro-compounds**
Ralstonia	common soil bacteria, diverse metabolic capabilities, **some strains degrade CL-20**
Aquabacterium	widespread aquatic bacteria in groundwater and drinking water systems
Rhodoblastus	purple non-sulfur bacteria, simple carbonotrophy, perform nonoxygenic photosynthesis
Janthinobacterium	aerobic soil and water bacteria
Deinococcus	dessication- and radiation-resistant environmental bacteria
Pseudoxanthomonas	aerobic and facultative anaerobic soil bacteria, **some strains degrade DNT**
Sporichthyaceae	actinobacteria

Table 3.1-7. Detection of putative RDX-degradative genes in the WVOW enrichments.

	PCR detection of:					
Treatment	**xenA**	**xenB**	**xplA**	**onr**	**hydA**	**nerA**
CON+N, NO EXP	-	-	-	ND	ND	ND
CS	-	-	-	ND	ND	ND
EOS, NO EXP	-	-	-	ND	ND	ND
EOS+N	-	-	-	ND	ND	ND
SOY+NO EXP	-	-	-	ND	ND	ND
SOY+N	-	-	-	ND	ND	ND
LAC+N	-	-	-	ND	ND	ND
CS, Crude soybean oil; SOY, soybean oil; LAC, lactate; EOS, emulsified oil substrate						
+N, NH_4 added						
NO EXP, no RDX added						
ND, Not determined						

3.2 PICATINNY ARSENAL

Multiple experiments were performed with groundwater and sediment from Picatinny Arsenal. This was done to leverage and complement the work being performed during ESTCP project ER-0425, "In Situ Bioremediation of Energetic Compounds in Groundwater", and in collaboration with the project Principal Investigator, Dr. Paul Hatzinger.

3.2.1 COLUMN EFFLUENT ANALYSES

3.2.1.1 METHODS

ESTCP project ER-0425, managed by Dr. Paul Hatzinger, established flow-through columns to evaluate explosives degradation in native Picatinny Arsenal soils prior to moving to the field demonstration phase of that project. Operation of these columns was maintained during this SERDP project to serve as a source of effluent enriched in explosive-degrading organisms and as a source for establishing batch microcosm enrichments. A full description of the columns can be obtained in the Technical Report for project ER-0425, but pertinent details are given here.

A schematic diagram and photographs of the columns are presented in Figure 3.2.1-1 and 3.2.1-2. Aquifer sediments were collected from Area 157 at Picatinny Arsenal (NJ) which has a history of soil and groundwater contamination. Five columns (approximately 15 cm x 2.5 cm ID) were prepared and operated similar to methods previously described (33). Groundwater from Picatinny Arsenal (157MW-4) was pumped through the columns in an upflow manner (~0.5 mL/h), and amended for each column as follows: column 1 (live control, CON), no amendment; column 2 (killed control, KIL), 0.09% (v:v) formaldehyde; column 3 (low cheese whey, CW1), 100 mg/L dissolved cheese whey; column 4 (high cheese whey, CW2), 1000 mg/L dissolved cheese whey; column 5 (yeast extract, YE), 100 mg/L yeast extract. The influent RDX concentrations in the groundwater were in the range of 30 to 50 µg/L, with similar concentrations of TNT and HMX, and lower concentrations of TNB, DNTs, and amino-DNTs. The groundwater contained concentrations of phosphate, nitrate, nitrite, and ammonia below the detection limit (<0.1 mg/L), a trace amount of TKN (0.52 mg/L), 15 to 20 mg/L sulfate, and 1 to 2 mg/L total/dissolved organic carbon.

The columns were operated for a total of 1034 days. The flow was reduced from 0.5 to 0.3 mL/h after 799 days. Due to changing field conditions, influent groundwater was changed from 157MW-4 to 157MW-8D after 887 days. Column effluent samples were collected periodically and analyzed for explosives concentrations by HPLC.

Effluent samples for initial molecular analyses were collected after 500 days as follows: Sterile 50 mL tubes were placed on ice and the column effluent lines from each were directed into the tubes. After the 24 hours of collection, the filled tubes were switched out with new tubes. The collected effluent was centrifuged at 9400 x *g* at 4°C for 45 minutes. The liquid was decanted with a pipette and the pellet was frozen at -70ºC. Each subsequent 24 h collection of effluent was transferred to the first tube with the frozen pellet, centrifuged, and the resulting pellet was refrozen. This process was repeated for a total of three days, and the final pellet was stored at -70ºC until DNA extraction.

The DNA was extracted from the pellets by thawing and resuspending the material in a minimal volume, which was then transferred to a bead beating tube. The DNA was extracted using bead beating, followed by QIAEX II extraction as previously described using 20 µL of the QIAEX suspension and two 20 µL elutions of sterile ultrapure TE, pH 8.0. The extracts were quantified using Quant-iT DNA Assay Kit (Molecular Probes), and amplified for DGGE analysis using touchdown PCR as previously described. Additionally, DNA was extracted by bead beating followed by purification using the ZR Soil Microbe DNA kit (Zymo Research Corporation, Orange, CA, USA).

Additional samples were collected after approximately 1000 days using Sterivex filters to concentrate the biomass in the effluent from the columns over several days. Excess water was pushed through the filters, which were then frozen and processed according to the standard procedures in Appendix 3.

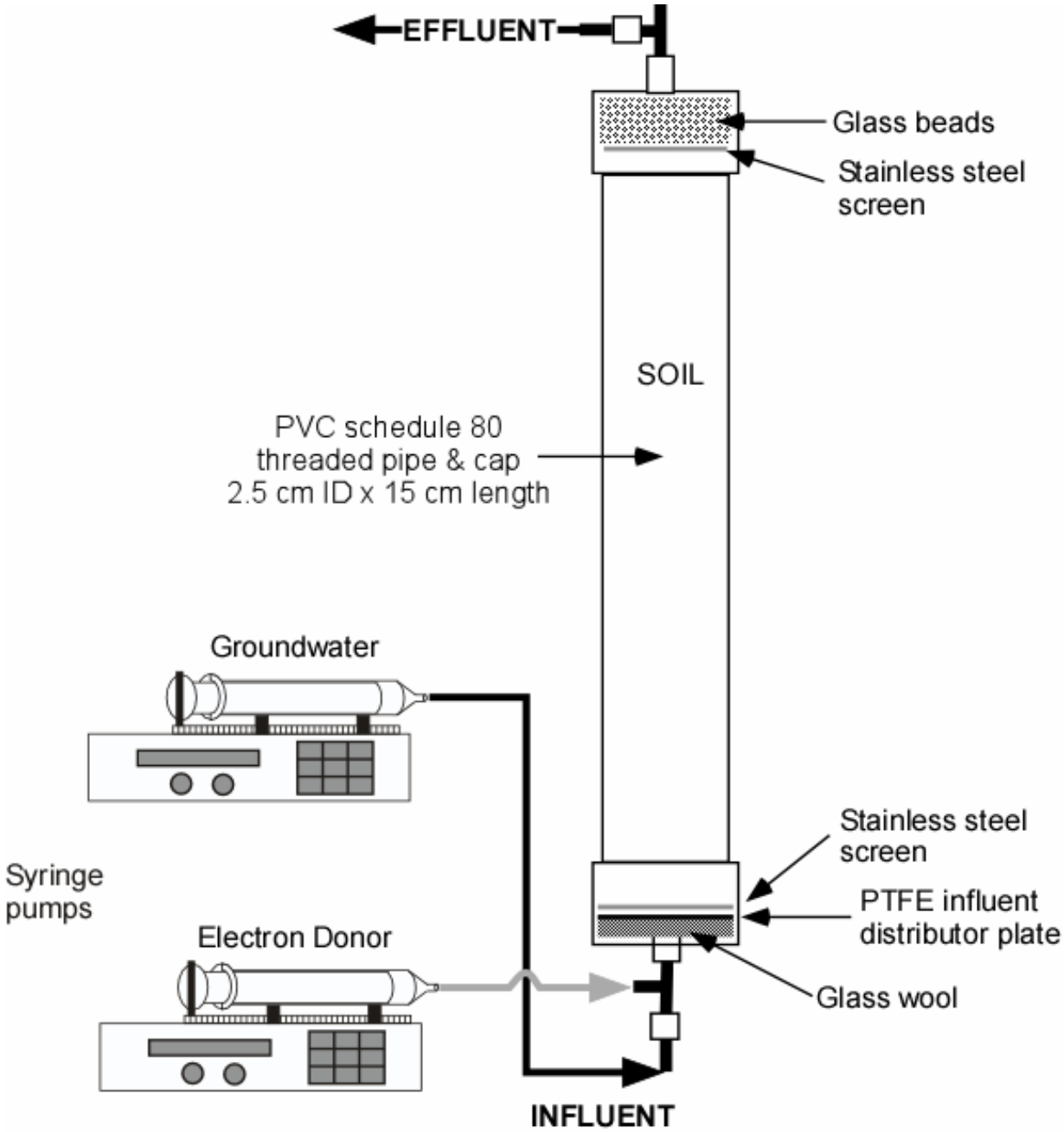


Figure 3.2.1-1. Schematic illustration of the columns used for this research.

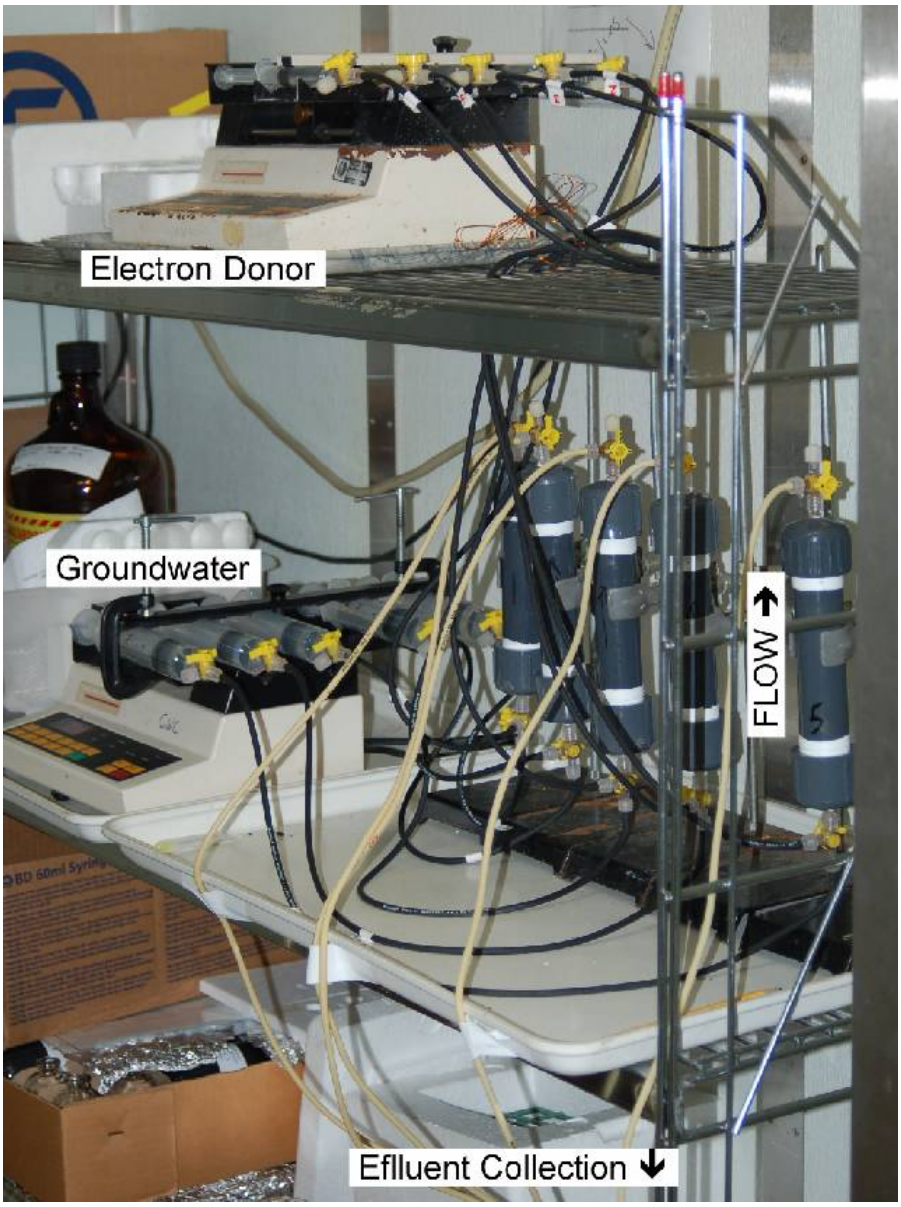


Figure 3.2.1-2. Photograph of the columns used for this research.

3.2.1.2 RESULTS

RDX degradation was achieved in the columns receiving electron donor amendments, with the greatest degradation in Column 4 (high cheese whey) and Column 5 (yeast extract). Minimal degradation was observed in the control and killed columns (Figure 3.2.1-3).

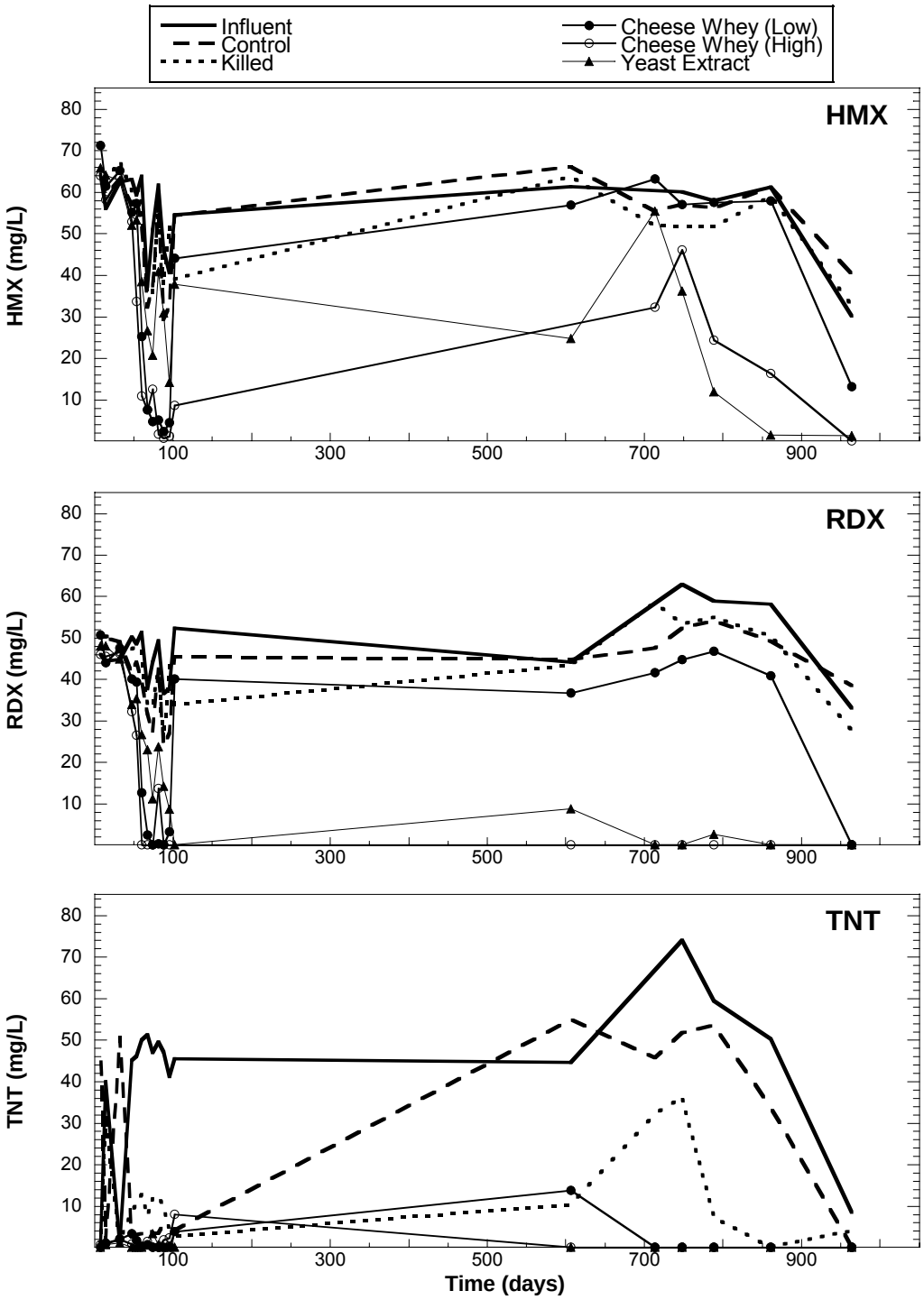


Figure 3.2.1-3. Concentrations of HMX, RDX, and TNT in Picatinny Arsenal columns. Initial bacterial isolations and molecular analyses occurred at around 500 days. Influent groundwater was switch from well 157MW-4 to 157MW-8D after 887 days, and samples for final molecular analysis were collected around 1000 days.

Samples for molecular analysis were collected after the degradative activity had stabilized. Initial attempts to isolate and analyze DNA directly from column effluent were only successful with samples from Column 4 (high cheese whey). The identities of the isolated sequences are presented in Table 3.2.1-1, and a phylogenetic tree relating these sequences to known degradative organism is shown in Figure 3.2.1-4. Several of the recovered sequences grouped near the known explosive-degrading strain *Clostridium acetobutylicum* ATCC 824, whereas many other sequences were similar to other characterized bacterial genera (i.e., *Desulfitobacterium* sp., *Sporolactobacillus* sp.) that have not been demonstrated to be explicitly associated with explosives degradation.

Supplemental analysis of effluent after collection of biomass using Sterivex filters after 1000 days yielded 16S rRNA gene sequences from all of the nutrient-amended columns (COL 3, 4, 5). A phylogenetic tree of the results is presented in Figure 3.2.1-5.

Table 3.2.1-1. Identification of bands from initial molecular analysis of Picatinny Arsenal column effluent.

Treatment	Band	Identification	Source
PA COL 4	1.1	*Sporolactobacillus* _sp.	study of lactic acid bacteria
	1.4	*Sporolactobacillus* _sp.	study of lactic acid bacteria
	1.5	*Desulfitobacterium* _sp.	study of TCE degrading strain
	2.2	*Sporolactobacillus* _sp.	study of lactic acid bacteria
	2.3	*Sporolactobacillus* _sp.	study of lactic acid bacteria
	2.4	*Sporolactobacillus* _sp.	study of lactic acid bacteria
	3.5	Uncultured_bacterium	bio-hydrogen producing culture
	4	*Sporolactobacillus_sp.*	
	5	*Desulfitobacterium* _sp.	
	6.1	*Desulfitobacterium* _sp.	study of TCE degrading strain
	6.2	Uncultured_bacterium	bio-hydrogen producing culture
	7.1	*Desulfitobacterium* _sp.	study of TCE degrading strain
	7.2	Uncultured_environmental_bacterium	human gut
	9	*Clostridium* _sp.	
	9.3	*Clostridium* _sp.	study of genus *Clostridium*
	10	Uncultured_clostridia_bacterium	
	14	*Sporolactobacillus* _sp.	study of lactic acid bacteria
	15	*Desulfitobacterium* _sp.	chlorophenol dechlorinating strain

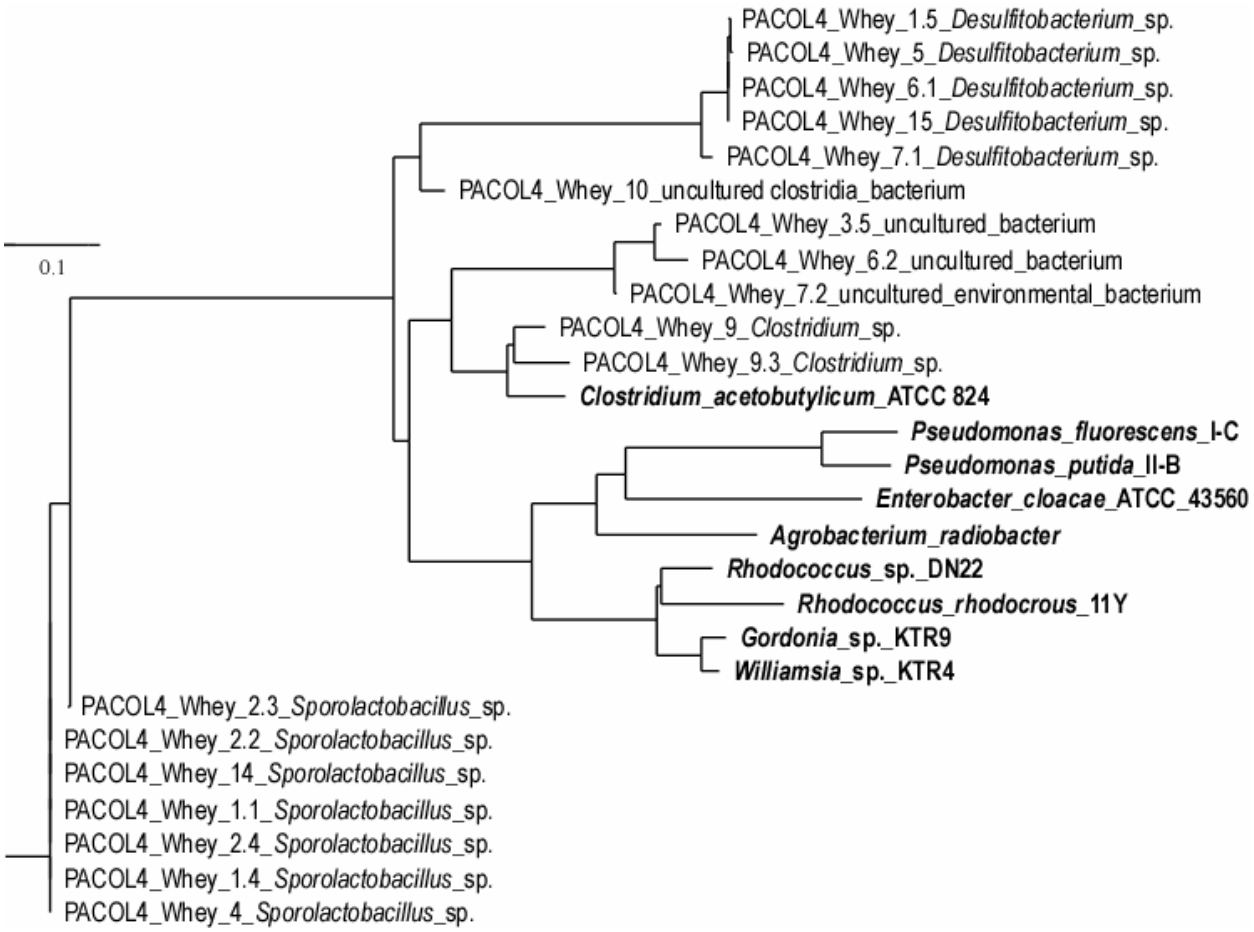


Figure 3.2.1-4. Initial phylogenetic analysis of sequences derived from Picatinny Arsenal column effluent samples. Only samples from Picatinny Arsenal Column 4 yielded sufficient DNA for molecular analyses. The closest identities based on the sequences are given, and known explosive-degrading strains are included for reference (bold text). Bar = 10 nucleotide substitutions per 100 nucleotides in the 16S rRNA sequences.

The samples collected used Sterivex filters attached directly to the effluent lines yielded somewhat different results with respect to the makeup of the microbial communities. The identities of the recovered sequences and a phylogenetic tree showing the sequences in relationship to previously described degradative strains are presented in Table 3.2.1-2 and Figure 3.2.1-5, respectively. Descriptions of the various genera detected are presented in Table 3.2.1-3. The differences between the initial and final molecular analyses could be due to the long duration between when samples were taken and/or the change in the influent groundwater source near the end of the experiment.

The results of screening the samples for putative explosive-degrading genes is presented in Table 3.2.1-4. The only gene detected was *xenA* in effluent from the column being fed a high

concentration of cheese whey (column 4). All other screens were negative. The detection in column 4 may have been due to the fact that the higher amount of cheese whey allowed a larger biomass to be sustained, and therefore the bacteria harboring the *xenA* gene increased to a level above the detection limit of the PCR screen.

Table 3.2.1-2. Identification of bands from molecular analysis of Picatinny Arsenal column effluent.

Treatment	Band	Identification	Source
PACOL3	1	Uncultured *Geobacteraceae* bacterium clone M21_9976	subsurface Fe-reducing sediment
	2	Uncultured bacterium clone GW10	nitrate contaminated aquifer
	3	Uncultured bacterium clone C3-8	acetate-utilizing lake bacteria (Isreal)
PACOL4	1a	*Azospira oryzae* strain N1/*Dechlorosoma* sp. KJ	selenate reducing strain
	1b	*Azospira oryzae* strain N1/*Dechlorosoma* sp. KJ	selenate reducing strain
	2a	Unknown organism	
	2b	*Streptomyces* sp. 10N31-4	deep-sea sediment
	5	Uncultured actinomycete clone CRE-FL43	Columbia river and estuary
PACOL5	1a	*Azospira oryzae* strain N1/*Dechlorosoma* sp. KJ	selenate reducing strain
	1b	Uncultured gamma proteobacterium clone 1HP1-O20	coral disease organism
	2	Uncultured bacterium clone 3M08	osmosis membrane biofilm

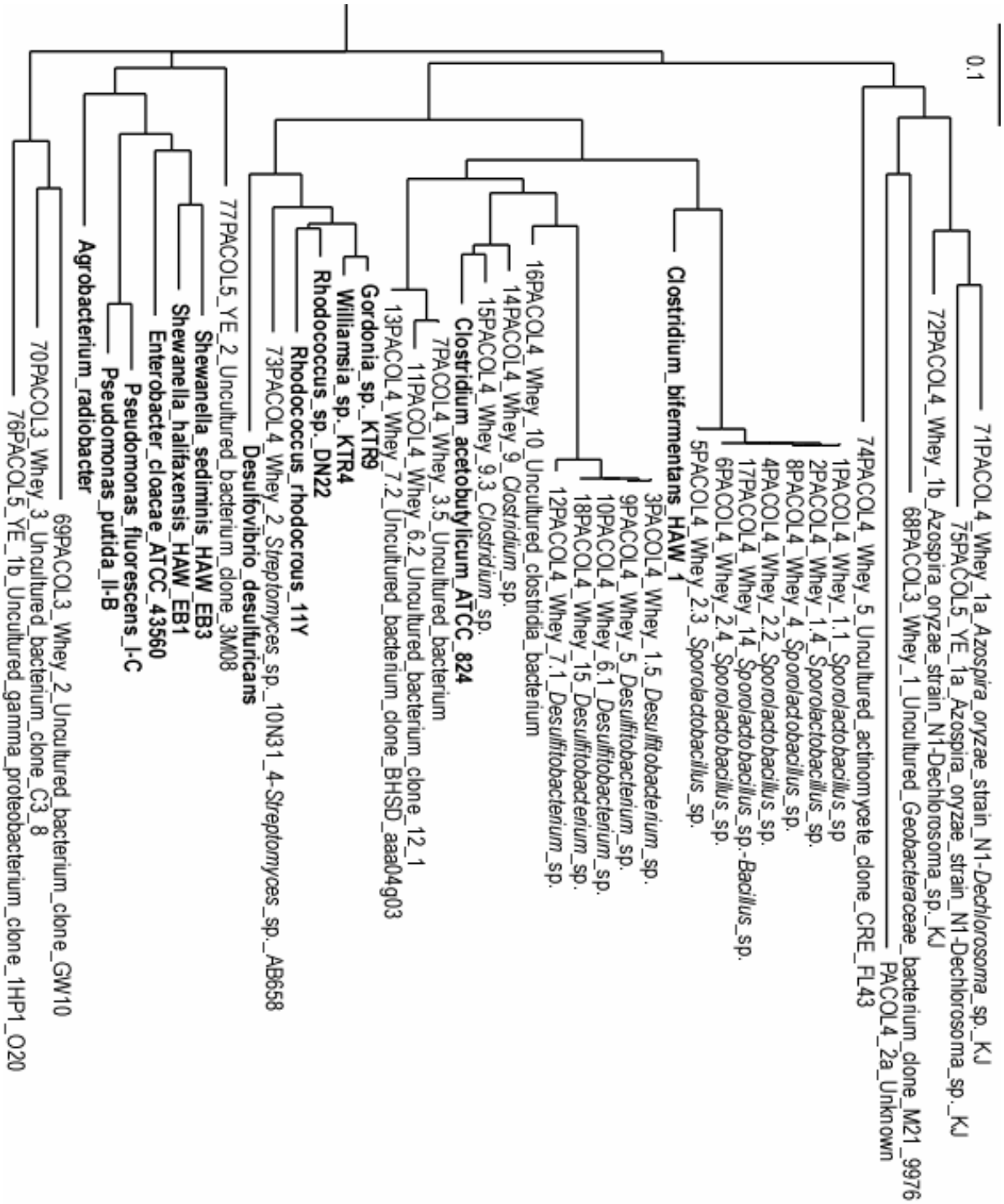


Figure 3.2.1-5. Phylogenetic analysis of sequences derived from Picatinny Arsenal column effluent samples (initial and after biomass collection using Sterivex filters). The closest identities based on the sequences are given, and known explosive-degrading strains are included for reference (bold text). Bar = 10 nucleotide substitutions per 100 nucleotides in the 16S rRNA sequences.

Table 3.2.1-3. Description of the genera detected in Picatinny Arsenal column effluent.

Identification	Characteristics
Geobacteraceae	iron-oxide reducing organoheterotroph, common in soil, river water, groundwater, and sewage
Streptomyces	soil and subsurface bacteria, extensive catabolic diversity, **some strains transform TNT**
Azospira	nitrogen-fixing bacteria found in groundwater and grass roots, some strains degrade perchlorate
Dechlorosoma	some strains reduce perchlorate, selanate, anaerobically oxidize iron

Table 3.2.1-4. Results of screening for putative RDX-degradative genes in Picatinny Arsenal column effluent.

	PCR detection of:					
Treatment	**xenA**	**xenB**	**xplA**	**onr**	**hydA**	**nerA**
PACOL 4 (initial)	-	-	-	ND	ND	ND
PACOL3	-	-	-	ND	ND	ND
PACOL4	+	-	-	ND	ND	ND
PACOL5	-	-	-	ND	ND	ND
ND, Not determined						

3.2.2 COLUMN EFFLUENT AND GROUNDWATER ISOLATES

3.2.2.1 METHODS

Agar plates were made using the following recipes: 1) Yeast extract (YE) plates: 1 gram yeast extract per L basal salts media (BSM) solidified with 20 grams of agar. 2) Cheese whey (CW) plates: 1 gram cheese whey per L of BSM, solidified with 20 grams of agar. These media were chosen because yeast extract and cheese whey were the only electron donors that supported the biodegradation of explosives in Picatinny Arsenal groundwater, as well as being the electron donors being used in the column experiments.

Samples of the influent groundwater (157MW-4) and effluent off the columns were collected in sterile tubes, plated undiluted (100 µL) and after serial dilution in PBS onto both types of plates and incubated at room temperature until colonies developed. Samples from columns 1 and 2 were plated on both types of plates, whereas samples from columns 3, 4 and 5 were plated on the plates with the same electron donor that the column was receiving (i.e., Column 3 and 4 on cheese whey, Column 5 on yeast extract). Picatinny groundwater was also plated diluted and undiluted as described on both types of plates.

Twenty two individual colonies with different morphology (different colors, margins, shapes, etc.) from each source were transferred directly into PCR tubes with sterile disposable loops. DNA in the cells was subjected to PCR amplification using universal primers followed by DGGE separation (180 volts / ~45 amps) for approximately three hours. The DGGE bands were excised and sequenced as previously described to allow species identification. In most cases, multiple bands were sequenced for each isolate. These sequences were entered into a 'BLAST-N' query in the National Institute of Health's National Center for Biotechnology Information website (http://www.ncbi.nih.gov/) for identification. A phylogenetic tree relating the isolate identifications to known explosive-degrading strains was constructed.

Twenty of the isolates were screened for RDX degradation in BSM with succinate or glucose and RDX as the sole nitrogen source. Briefly, glass 15 mL serum vials were combusted at 550º C overnight to burn off any trace carbon or nitrogen. Once cooled they were capped with Teflon®-lined butyl rubber septa and autoclaved. The liquid culture (5 mL) consisted of BSM, 1 g/L carbon (succinate, glucose, or a combination of the two) and 3 g/L RDX. Cells of each strain were scraped from BSM + succinate agar plates and diluted in 1 mL of sterile PBS. *Rhodococcus rhodocrous* 11Y cells were also obtained from BSM + glucose plates. The cells were vortexed for 30 seconds and 100 µL was used to inoculate the serum vials. Negative

controls with and without RDX, positive controls with ammonium as the nitrogen source, and RDX-only "carbon-negative" controls were prepared in the same manner. Over several weeks, the vials were scored for growth (based on turbidity) and samples were collected and analyzed for RDX and breakdown products via HPLC. During sampling, 1 mL of air was injected and 1 mL was withdrawn with a sterile syringe and needle.

The isolates closely related to the genus *Rhodococcus* were screened for the presence the known RDX degradative gene *xplA*. *Rhodococcus* sp. DN22 and *Rhodococcus rhodocrous* 11Y were used as positive controls. DNA was purified from pure cultures of these two strains using bead beating followed by QIAEX II purification, and yield was measured with the Quant-iT kit. Each yielded ~11 ng/µL and both 5 µL and 10 µL of template were tested in 100 µL reactions with 0.4 µL of each 50 µM primer. Cycling conditions were: 94ºC for 5 minutes to denature the DNA; 35 cycles of 95º C for 1 min - 56º C for 30 sec - 72ºC for 1 min, and a final extension at 72º C for 5 min.

3.2.2.2 RESULTS

Table 3.2.2-1 presents the identifications of the isolates. A phylogenetic tree illustrating how the isolates from the Picatinny Arsenal groundwater and from Picatinny Arsenal columns are related to the known explosive degrading strains is shown in Figure 3.2.2-1.

The isolates from the groundwater and columns were spread over multiple genera. Some of the isolates group with, and are hence related to, known degradative organisms, at least with respect to their identity. However, it should be noted that when this phylogenetic data is combined with the RDX degradation screening data, it is clear that not all strains of a given genera have the same degradative abilities. For instance, several of the column isolates were identified as *Rhodococcus* sp. and grouped with the known RDX degrader *Rhodococcus* sp. DN22.

Table 3.2.2-1. Identities of Picatinny Arsenal column and Picatinny Arsenal groundwater isolates based on sequencing of 16S rRNA genes.

Isolate	Source	Sequencing ID	% Identity
1	PA GW	*Pseudomonas* sp.	99
2	PA GW	*Kocuria* sp., *Arthrobacter* sp.	99
3	PA GW	*Pseudomonas* sp.	99
4	PA GW	*Burkholderiaceae* bacterium, *Ralstonia* sp.	99
5	PA GW	*Variovorax* sp.	100
6	PA GW	*Arthrobacter* sp.	98
7	PA GW	*Arthrobacter* sp.	99
8	PA GW	*Arthrobacter* sp.	99
9	PA GW	*Arthrobacter* sp.	99
10	PA Column CON	*Rhodococcus* sp.	100
11	PA Column CON	*Rhodococcus* sp., *Lechevalieria* sp., *Saccharothrix* sp., *Lentzea* sp.	100
12	PA Column CON	*Asticcacaulis benevestidus* , *Brevundimonas* sp.	100
13	PA Column CON	*Variovorax* sp., *Agrobacterium* sp.	99
14	PA Column CON	*Rhodococcus* sp., *Lechevalieria* sp., *Saccharothrix* sp., *Lentzea* sp.	100
15	PA Column 3 WHEY	*Sphingomonas* sp., *Sphingopyxis* sp.	99
16	PA Column 3 WHEY	*Asticcacaulis benevestidus* , *Brevundimonas* sp.	99
17	PA Column 3 WHEY	uncultured soil bacterium	97
18	PA Column 5 YE	*Pseudomonas* sp.	99
19	PA Column 5 YE	*Rhodococcus erythropolis* , *Lechevalieria / Lentzea / Saccharothrix* sp.	100
20	PA Column 5 YE	*uncultured soil bacterium*	99
21	PA Column 5 YE	*Microbacterium* sp.	95
22	PA Column 4 WHEY	*Bradyrhizobium* sp., *Nitrobacter* sp.	100

PA GW, Picatinny Arsenal groundwater; PA Column 1 CON (control), 3 WHEY (low cheese whey), 4 WHEY (high cheese whey), YE (yeast extract)

However, none of the 20 isolates screened degraded RDX under the screening conditions (Table 3.2.2-2). Additionally, none of the groundwater or column isolates yielded PCR products with the *xplA* gene primers (Figure 3.2.2-2). This correlates and corroborates with the RDX degradation screening results in which none of the isolates were observed to degrade RDX, and indicates that attempts to detect a specific genus of bacteria may not be the best approach for assessing the ability of the given microbial community to degrade RDX. The amount of product amplified by the *xplA* primers seemed to be greater from *Rhodococcus* sp. DN22 compared to *Rhodococcus rhodocrous* 11Y. This may reflect the fact that the primers were designed specifically for the *xplA* gene cloned from DN22, and that the *xplA*-like gene in 11Y is somewhat different. Alternatively, the difference in PCR product may reflect that *xplA* in DN22 has been determined to be plasmid-borne, and hence there may be multiple copies per cell, compared to only one or two copies of the *xplA*-like gene in 11Y (4, 34).

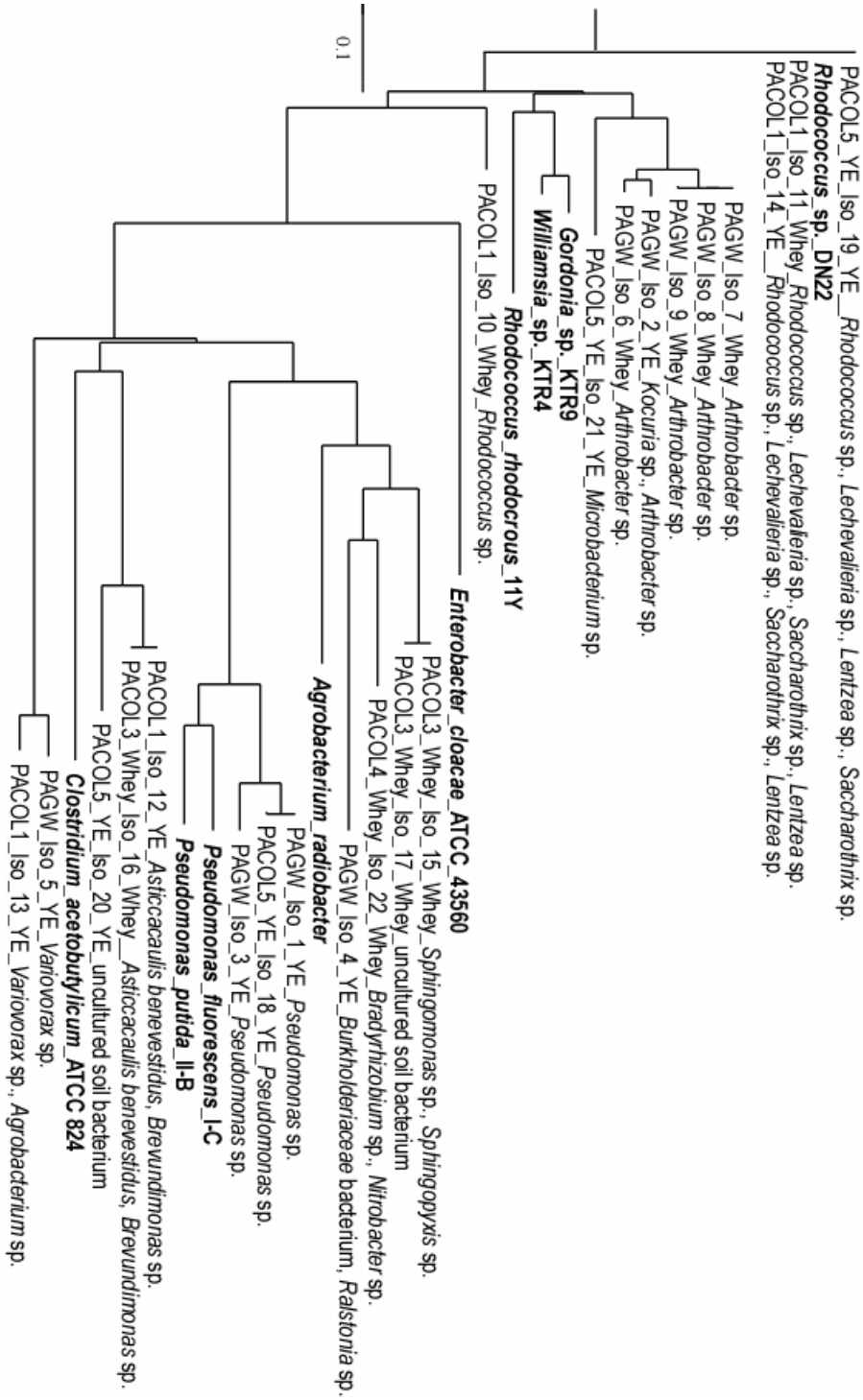


Figure 3.2.2-1. Phylogenetic analysis of Picatinny Arsenal column and Picatinny Arsenal groundwater isolates. Known explosive-degrading strains are included for reference (bold text). Bar = 10 nucleotide substitutions per 100 nucleotides in the 16S rRNA sequences.

Table 3.2.2-2. Degradation of RDX by isolates from Picatinny Arsenal columns and Picatinny Arsenal groundwater.

	RDX Degradation			RDX Degradation	
		N Source = RDX			N Source = NH4
Sample ID	**C Source**	**Aerobic/Anoxic**	**Anaerobic**	**C Source**	**Aerobic/Anoxic**
PA-GW Isolate 1	none	No	No		
	succinate	No	No	succinate	No
PA-GW Isolate 2	none	No	No		
	glucose	No	No	glucose	No
PA-GW Isolate 3	none	No	No		
	succinate	No	No	succinate	No
PA-GW Isolate 4	none	No	No		
	succinate	No	No	succinate	No
PA-GW Isolate 5	none	No	No		
	succinate	No	No	succinate	No
PA-GW Isolate 6	none	No	No		
	succinate	No	No	succinate	No
PA-GW Isolate 7	none	No	No		
	succinate	No	No	succinate	No
PA-GW Isolate 8	none	No	No		
	succinate	No	No	succinate	No
PA-GW Isolate 9	none	No	No		
	succinate	No	No	succinate	No
PA-COL 1 Isolate 10	none	No	No		
	succinate	No	No	succinate	No
PA-COL 1 Isolate 11	none	No	No		
	succinate	No	No	succinate	No
PA-COL 1 Isolate 12	none	No	No		
	glucose	No	No	glucose	No
PA-COL 1 Isolate 13	none	No	No		
	succinate	No	No	succinate	No
PA-COL 1 Isolate 14	none	No	No		
	succinate	No	No	succinate	No
PA-COL 3 Isolate 15	none	No	No		
	glucose	No	No	cheese whey	No
PA-COL 3 Isolate 16	none	No	No		
	glucose	No	No	cheese whey	No
PA-COL 3 Isolate 17	none	No	No		
	glucose	No	No	cheese whey	No
PA-COL 5 Isolate 18	none	No	No		
	glucose	No	No	yeast extract	No
PA-COL 5 Isolate 19	none	No	No		
	succinate	No	No	yeast extract	No
PA-COL 5 Isolate 20	none	No	No		
	glucose	No	No	yeast extract	No

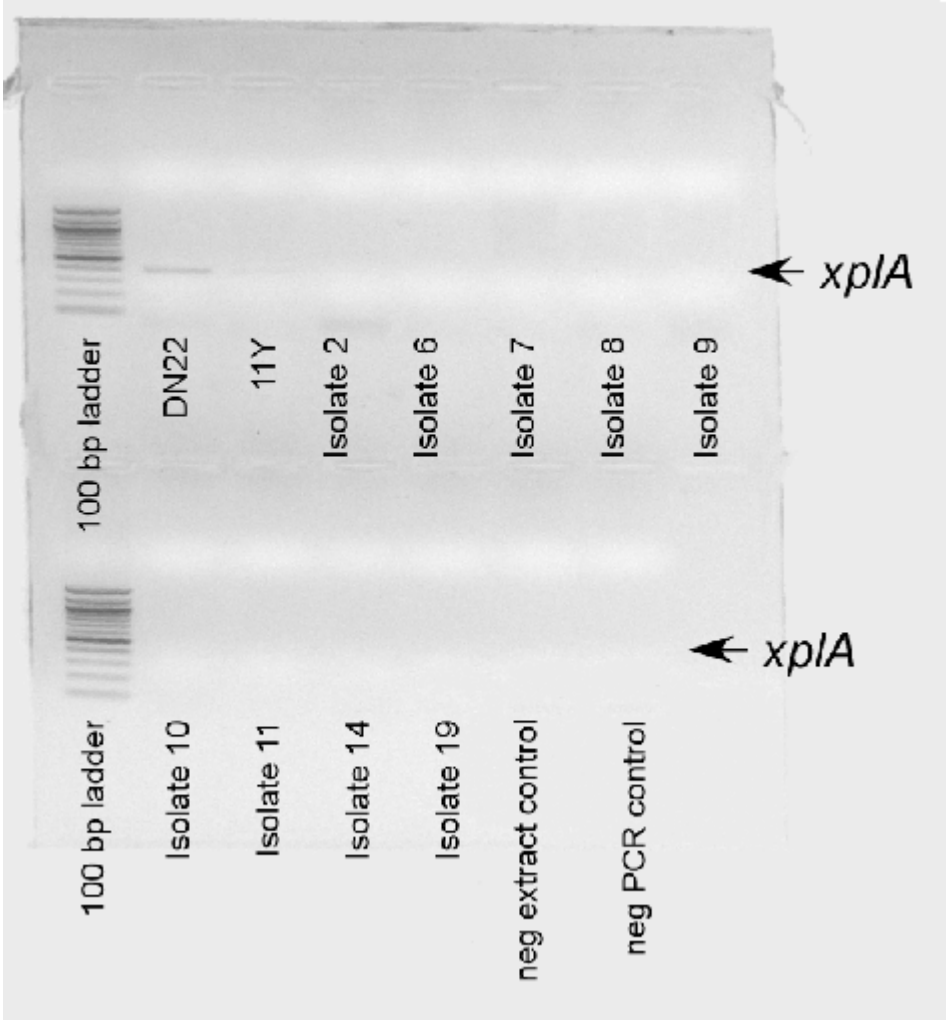


Figure 3.2.2-2. Screening of Picatinny Arsenal column and Picatinny Arsenal groundwater isolates for the RDX-degrading gene *xplA*. Known explosive-degrading strains are included for reference.

3.2.3 COLUMN EFFLUENT AND GROUNDWATER ENRICHMENTS

In order to facilitate identification of the range of potential RDX degraders, experiments with enrichment cultures derived from model aquifer samples with verified RDX degradation ability, as well as with contaminated groundwater, were established and examined with molecular techniques.

3.2.3.1 METHODS

Two screenings were performed with RDX (5 mg/L) as the sole nitrogen source (incubated under aerobic/anoxic and anaerobic conditions), and were amended with and without 1 g/L succinate or

glucose. Vials inoculated with column effluent (100 µl) or groundwater from well 157MW-5 (100 µl) were amended with both succinate and glucose (0.5 g/L each).

The third screening was performed with a full amendment of carbon and nitrogen (as NH_4) and RDX as a supplemental nitrogen source and/or as an alternate electron acceptor. Vials inoculated with Picatinny Arsenal column effluent were amended with the same carbon source as the respective column from which the effluent came (either cheese whey or yeast extract), with the exception of the vials inoculated with effluent from the live control column (receiving no carbon source) which were amended with a combination of succinate and glucose.

Selected enrichments were screened for their ability to degrade RDX in groundwater vs. the BSM to examine the effects of the full compliment of inorganic nutrients. Subsamples from established RDX-degrading enrichments were inoculated into either filtered (0.2 µm) Picatinny Arsenal groundwater or BSM, amended with RDX (5 mg/L) and succinate or glucose. Bottles were incubated at 15°C to more closely approximate groundwater temperatures, and shaken at 150 rpm. Samples were removed periodically and analyzed for RDX and breakdown products.

Established RDX-degrading enrichments were also screened for RDX degradation under aerobic vs. anoxic/anaerobic conditions. Glass serum bottles (10 mL) containing BSM amended with succinate and RDX were inoculated with 2 mL of the selected enrichments. The anaerobic bottles were set up and sampled in a glove bag. The aerobic bottles were set up in a glove bag, and sterile air was injected into headspace upon removal from the glove bag. Air was added to the headspace of the aerobic vials every few days. Samples were removed periodically and RDX concentrations were determined by HPLC. Once RDX degradation was observed, larger volume enrichments (100 mL liquid in 160 mL serum vials) were inoculated with 2 mL of the smaller enrichments in order to generate enough biomass for molecular analyses. The larger aerobic vials were equipped with air vents, and were purged with sterile air twice daily using an aquarium pump connected to a digital timer. RDX concentrations were monitored by HPLC. All vials were incubated at room temperature with shaking (150 rpm).

3.2.3.2 RESULTS

The results of these various enrichments are summarized in Tables 3.2.3-1 to 3.2.3-3.

Under aerobic/anoxic and anaerobic conditions with RDX as the sole nitrogen source and a defined carbon source, only the effluent from the column receiving high concentrations of cheese whey (Column 4) was observed to unequivocally degrade RDX. Some degradation may have been observed in the vials inoculated with effluent from the columns receiving low concentrations of cheese whey (Column 3) or yeast extract (Column 5). Under aerobic/anoxic conditions with RDX as a supplemental nitrogen source in addition to NH_4, effluent from the column receiving high cheese whey (Column 4) and yeast extract (Column 5) appeared to degrade RDX.

One replicate of the groundwater enrichments degraded RDX when it was supplied as the sole nitrogen source under anaerobic conditions, and one replicate degraded RDX under aerobic/anoxic conditions when RDX and NH_4 were both supplied. The fact that inoculation from groundwater resulted in RDX degradation in only some replicates indicates that RDX

degraders are likely sparse and heterogeneously distributed in the groundwater. It was interesting that degradation was observed in enrichments directly inoculated from groundwater, but that no RDX degradation was observed in enrichments inoculated with effluent from Column 1, which was essentially groundwater passing through sediment. This may have resulted from straining (filtration) or attachment of bacteria by the sediment, but may also just be a result of heterogeneous distribution of degraders.

Degradation under aerobic conditions was observed when air was added periodically, but scaled-up aerobic enrichments which were purged with air every day failed to degrade RDX. This likely indicates that the periodic air amendments did not maintain highly aerobic conditions, and the low oxygen concentrations resulting as the carbon source was consumed allowed RDX degradation to occur. Because the scaled-up aerobic enrichments failed to degrade RDX, no molecular analyses were performed.

Good RDX removal was observed at 15°C in complete culture medium (BSM) as well as groundwater under both aerobic/anoxic and anaerobic conditions. Degradation occurred in all enrichments except for aerobic/anoxic enrichments derived from Column 3 effluent that were amended with cheese whey and NH_4. Less robust degradation was observed in all the Column 5 effluent enrichments. Column 4 effluent and groundwater-derived enrichments exhibited fast and complete RDX degradation under all conditions tested.

Taken together, these results indicate that the microbial community in the Picatinny Arsenal aquifer and the communities developed during model aquifer experiments were able to degrade RDX under a range of conditions. RDX degradation was more favored under anaerobic (and possible anoxic) conditions compared to highly aerobic conditions (oxygen as an alternate electron acceptor). RDX was not degraded if a supplemental carbon source was not added. The presence of alternate exogenous nitrogen sources did not generally inhibit RDX degradation by the enrichments. The common breakdown products MNX, DNX, and TNX were detected in most of the enrichments.

Only the aerobic/anoxic enrichments with full nutrient amendments (carbon plus nitrogen source) produced enough biomass to get sufficient DNA for molecular analyses. A table summarizing the identities of the sequences retrieved is presented in Table 3.2.3-4, and descriptions of the various genera detected are presented in Table 3.2.3-5. A phylogenetic tree showing the relationship to the detected sequences to known explosive-degrading strains is shown in Figure 3.2.3-1. A few sequences grouped near the known degradative strain *Clostridium acetobutylicum* ATCC 824, and one actinobacterial sequence was detected (grouping near the known RDX-degrading actinobacteria). Two bands recovered from RDX-degrading groundwater amended with succinate, glucose, and ammonium had sequences which were related to a sequence detected in a TNT-degrading bioreactor (PA GW, bands 2.2 and 7).

In general, no single “biomarker” organism could be identified from these experiments. However, a large number of the sequences were closely related to *Pseudomonas* sp., which is a common environmental microbe. *Pseudomonas* sequences were detected in most samples across a wide range of conditions. In light of the other information above (Section 1.3.2), these results

indicate that this bacterial genera may be more involved with the degradation of explosives than previously reported.

Table 3.2.3-1. Degradation of RDX in enrichments derived from Picatinny Arsenal column effluent.

	RDX Degradation			RDX Degradation	
		N Source = RDX			N Source = NH_4
Sample ID	**C Source**	**Aerobic/Anoxic**	**Anaerobic**	**C Source**	**Aerobic/Anoxic**
PA Column 1 Effluent	none	No	No		
(live control)	succinate+glucose	No	No	succinate+glucose	No
PA Column 3 Effluent	none	No	No		
(low cheese whey)	succinate+glucose	**(Yes)**	No	cheese whey	No
PA Column 4 Effluent	none	No	No		
(high cheese whey)	succinate+glucose	**Yes**	**Yes**	cheese whey	**Yes**
PA Column 5 Effluent	none	No	No		
(yeast extract)	succinate+glucose	**(Yes)**	No	yeast extract	**Yes**

Table 3.2.3-2. Degradation of RDX in enrichments derived from Picatinny Arsenal groundwater.

	RDX Degradation			RDX Degradation	
		N Source = RDX			N Source = NH_4
Sample ID	**C Source**	**Aerobic/Anoxic**	**Anaerobic**	**C Source**	**Aerobic/Anoxic**
PA Groundwater	none	ND	No		
PA Groundwater-1	succinate+glucose	ND	No	succinate+glucose	**Yes**
PA Groundwater-2	succinate+glucose	ND	No	succinate+glucose	No
PA Groundwater-3	succinate+glucose	ND	**Yes**	succinate+glucose	No

Table 3.2.3-3. Degradation of RDX at 15°C in enrichments derived from Picatinny Arsenal column effluent and Picatinny Arsenal groundwater.

Sample ID	Base medium	RDX Degradation C Source	N Source = RDX Aerobic/Anoxic	Anaerobic
PA Column 3 Effluent	BSM	succinate+glucose	No / **Yes**	ND
	groundwater	succinate+glucose	No / (**Yes**)	ND
PA Column 4 Effluent	BSM	succinate+glucose	**Yes / Yes**	**Yes / Yes**
	groundwater	succinate+glucose	**Yes / Yes**	**Yes / Yes**
PA Column 5 Effluent	BSM	succinate+glucose	No / **Yes**	ND
	groundwater	succinate+glucose	No / **Yes**	ND
PA Groundwater-1	BSM	succinate+glucose	**Yes / Yes**	ND
	groundwater	succinate+glucose	**Yes / Yes**	ND
PA Groundwater-3	BSM	succinate+glucose	ND	**Yes / Yes**
	groundwater	succinate+glucose	ND	No / **Yes**

Sample ID	Base medium	RDX Degradation C Source	N Source = NH_4 Aerobic/Anoxic	Anaerobic
PA Column 3 Effluent	BSM	cheese whey	No / No	ND
	groundwater	cheese whey	No / No	ND
PA Column 4 Effluent	BSM	cheese whey	**Yes / Yes**	**Yes / Yes**
	groundwater	cheese whey	**Yes / Yes**	**Yes / Yes**
PA Column 5 Effluent	BSM	yeast extract	No / **Yes**	ND
	groundwater	yeast extract	No / **Yes**	ND
PA Groundwater-1	BSM	succinate+glucose	**Yes / Yes**	ND
	groundwater	succinate+glucose	**Yes / Yes**	ND
PA Groundwater-3	BSM	succinate+glucose	ND	**Yes / Yes**
	groundwater	succinate+glucose	ND	**Yes / Yes**

ND, Not determined

Results are presented for both 14 days and 120 days of incubation

Table 3.2.3-4. Identification of bacterial sequences recovered from Picatinny Arsenal groundwater and Picatinny Arsenal column effluent enrichments.

Inoculum	Carbon Source	Band	Identification	Source
PA COL 3	cheese whey	2	*Asticcacaulis* sp., *Brevundimonas* sp.	Antarctic hydrocarbon-degrading bacteria // dechlorinating cultures
		3	*Actinomycetales*	ginseng field
		4	uncultured *Methylobacteriaceae*	methylotroph community
		6	*Pseudomonas* sp.	Chinese gold mine tailings
		8	*Phaeospirillum* sp.	New Zealand thermal environments
		9	*Pseudomonas* sp.	
PA COL 4	cheese whey	1.2	*Pseudomonas* sp.	
		1.3	*Pseudomonas* sp.	
		1.4	*Pseudomonas* sp.	alpine grassland
		2	*Pseudomonas* sp.	
		3	*Pseudomonas* sp.	
		4	*Pseudomonas* sp.	
		6	*Pleomorphomonas oryzae*, *Kaistina koreensis*	
		9	*Clostridium* sp.	
		12	*Pseudomonas* sp.	
		13	*Pseudomonas* sp.	
		14	*Pseudomonas* sp.	alpine grassland
		18	uncultured *Clostridium* sp.	eutrophic soils of the Florida Everglades
PA COL 5	yeast extract	1	*Pseudomonas* sp.	
		2	*Pseudomonas* sp.	
		3	*Enterococcus* sp.	
		4	*Pseudomonas* sp.	swine manure-impacted environments
		5	*Pseudomonas* sp.	
		6	*Azospira* sp.	soil ultramicrocells
		8	*Pseudomonas* sp.	
		9	*Enterococcus* sp.	Peruvian traditional cheeses
		10	*Pseudomonas* sp.	
		12	*Azospira* sp., uncultured *Dechlorosoma* sp.	selenate reducers // denitrifying community
		13	*Pseudomonas* sp.	
PA GW	succinate-glucose	1	*Pseudomonas* sp.	
		1.7	*Pseudomonas* sp.	
		2.2	*Pelosinus fermentans*	**TNT-degrading bioreactor**
		2.3	*Pseudomonas* sp.	
		3.1	*Pelosinus fermentans*	
		3.3	*Pelosinus fermentans*	
		4	*Pseudomonas* sp.	
		5	*Pseudomonas* sp.	
		6	*Pseudomonas* sp.	
		6.1	*Pseudomonas* sp.	
		6.2	*Pseudomonas* sp.	
		6.3	*Pseudomonas* sp.	
		6.6	*Pseudomonas* sp.	
		7	*Pelosinus fermentans*	**TNT-degrading bioreactor**
		8	*Pseudomonas* sp.	
		12	*Pseudomonas* sp.	
		13	*Pelosinus fermentans*	acidic subsurface uranium(VI)-contaminated sediments
		14	*Pseudomonas* sp.	
		15	*Pseudomonas* sp.	

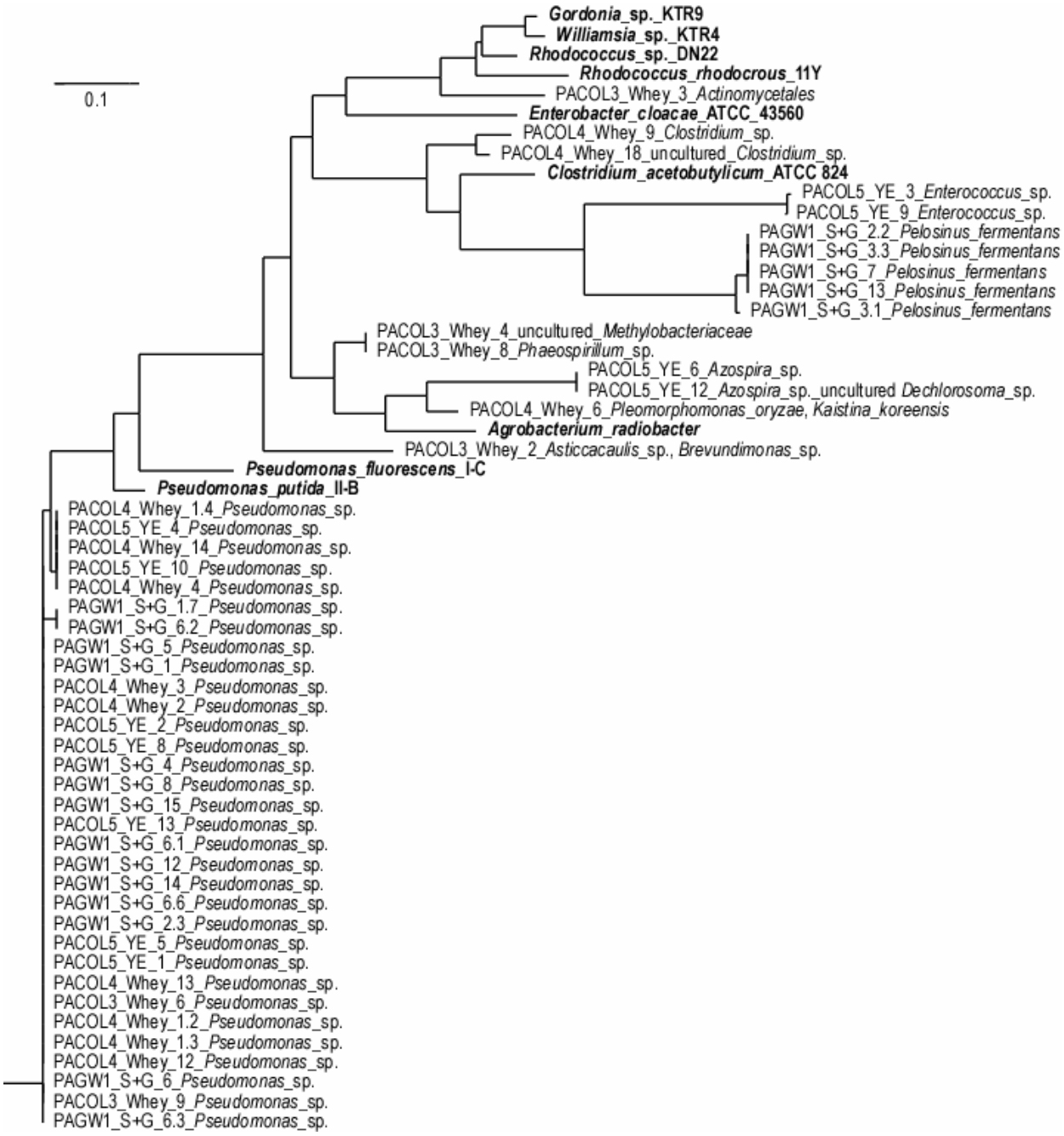


Figure 3.2.3-1. Phylogenetic analysis of Picatinny Arsenal column effluent and Picatinny Arsenal groundwater enrichments. Known explosive-degrading strains are included for reference (bold text). All enrichments had a carbon source (Whey=cheese whey, YE=yeast extract, S+G=succinate+glucose) and a nitrogen source (NH_4). Bar = 10 nucleotide substitutions per 100 nucleotides in the 16S rRNA sequences.

Table 3.2.3-5. Description of the genera detected in Picatinny Arsenal groundwater and Picatinny Arsenal column effluent enrichments.

Identification	Characteristics
Asticcacaulis	aerobic freshwater bacteria with prosthecae
Brevundimonas	prosthecate/nonprothecate bacteria found in fresh water and soil
Azospira	nitrogen-fixing bacteria found in groundwater and grass roots, some strains degrade perchlorate
Pleomorphomonas	nitrogen-fixing bacteria common in flooded soil
Pseudomonas	widespread genera, extensive catabolic diversity, **some strain shown to degrade RDX**
Pelosinus	iron-reducing soil and subsurface (kaolin clay) bacteria
Kaistina	nitrogen-fixing endophytic bacteria associated with rice
Enterococcus	fermentative bacteria common in animal guts
Dechlorosoma	some strains reduce perchlorate, selanate, anaerobically oxidize iron
Methylobacteriaceae	widespread environmental bacteria, methylotrophic
Actinomycetales	in the actinobacteria group, many genera of which are metabolically diverse
Phaeospirillum	obligately anaerobic phototrophic bacteria, detected in drinking water systems
Clostridium	strictly anaerobic, fermentative bacteria, **some strains proven to degrade TNT, RDX**

3.2.4 DIRECT MOLECULAR ANALYSIS OF GROUNDWATER SAMPLES

The native and biostimulated microbial community in the contaminated aquifer at Picatinny Arsenal was examined with molecular techniques after direct extraction of DNA from groundwater samples.

3.2.4.1 METHODS

Biomass in groundwater at Area 157 at Picatinny Arsenal was collected onto Sterivex filters. Wells inside and outside the zone of influence of the biostimulant (cheese whey) were sampled. Filters were returned to the laboratory and frozen at -80°C until processing.

The DNA in the biomass on the Sterivex filter membranes was extracted according to the standard procedure described in Appendix 3. The microbial community DNA was analyzed using DGGE/sequencing, as well as by PCR for specific RDX-degrading genes.

3.2.4.2 RESULTS

A range of organisms were detected both before and after biostimulation (Table 3.2.4-1). A few sequences were closely related to the previously described explosive-degrading *Clostridium acetobutylicum* ATCC 824, but no sequences were closely related to the other explosive-degrading strains (Figure 3.2.4-1). One sequence detected post-biostimulation was closely related to a sequence detected during RDX degradation under sulfate reducing conditions (PAGW_MW4_7.2). The groundwater in this well was quite anoxic at the time of sampling, and there was hydrogen sulfide being produced, indicating that sulfate-reducing conditions were likely present. Descriptions of the various genera detected are presented in Table 3.2.4-2.

Results of screening the samples for putative explosive-degrading genes are presented in Table 3.2.4-3. None of the genes for which screening was performed were detected in any samples.

Table 3.2.4-1. Identification of bacterial sequences recovered from Picatinny Arsenal groundwater before and after biostimulation.

Well ID	Native / Biostim	Band	Identification	Source
MW2	Native	1.1	uncultured_bacterium_clone_BHSML13	estuary, contaminated sediments
	Native	1.2	uncultured_*Geobacter*_sp._clone_06G	urban creek sediments, rice field, acidic fens
	Native	1.3	uncultured_bacterium_DGGE_band_12	gasoline-contaminated groundwater
	Native	1.4	uncultured_Clostridiales_bacterium_clone_wb1	microbial consortia degrading complex organic matter
	Native	6.1	*Paludibacter_propionicigenes*	estuary, contaminated sediments
	Native	6.2	uncultured_*Geobacter*_sp._clone_BEM21	Fe(III)-reducing subsurface environments
	Native	6.3	uncultured_*Caulobacter*_sp._clone_LiUU_1_5a	ocean crust, bacteria growing on natural organic carbon
MW4	Biostim	2.3	uncultured_*Thermoanaerobacteriaceae*_bacterium_clone_D15_17	tar oil contaminant groundwater
	Biostim	2.4	Uncultured_bacterium_clone_LaC20H43	perchlorate-respiring culture
	Biostim	7.1	uncultured_bacterium_clone_BHSML1	estuary // benzene-contaminated river sediments
	Biostim	7.2	uncultured_bacterium_clone_CH11	**RDX degradation under sulfate-reducing conditions**
	Biostim	7.3	uncultured_*Thermoanaerobacteriaceae*_bacterium_clone_D15_17	tar oil contaminant groundwater
	Biostim	7.4	unknown (no match in sequence database)	
MW5	Native	2.2	uncultured_environmental_bacterium	contaminated sediments (metals, nitrate, uranium, solvents)
	Native	3	uncultured_*Legionella*_sp.	drinking water
	Native	4.1	*Brevundimonas*_sp._*Caulobacter*_sp.	rocks in a gold mine, lake water
	Native	4.2	uncultured_environmental_soil_bacterium	Kansas agricultural soil
	Native	5	*Caulobacter*_sp._*Afipia*_sp.	lake water, linuron-mineralizing bacterial consortia
	Native	6	*Afipia*_genosp._14	anoxic wastewater treatment biofilm
	Biostim	3.1	uncultured_eubacterium_WD296	polychlorinated biphenyl-polluted soil
	Biostim	3.2	*Clostridium_gasigenes, Clostridium_carnis*	alkaline soil-water systems, vacuum-packed meat
	Biostim	8.1	uncultured_Sphingobacteriales_bacterium_clone_GASP_MA1W2_F06	former arable field, Michigan agricultural soil, Arctic tundra tussock
MW8S	Native	4.1	uncultured_gamma_proteobacterium_clone_HCM3MC90_1H_FF_RP3	sea sediment, corals, India lake
	Native	4.2	uncultured_*Hyphomicrobiaceae*_bacterium_clone_GASP_MB3S3_G02	Michigan agricultural soil
MW8D	Native	5.2	uncultured_*Hyphomicrobiaceae*_bacterium_clone_GASP_MB3S3_G02	Michigan agricultural soil

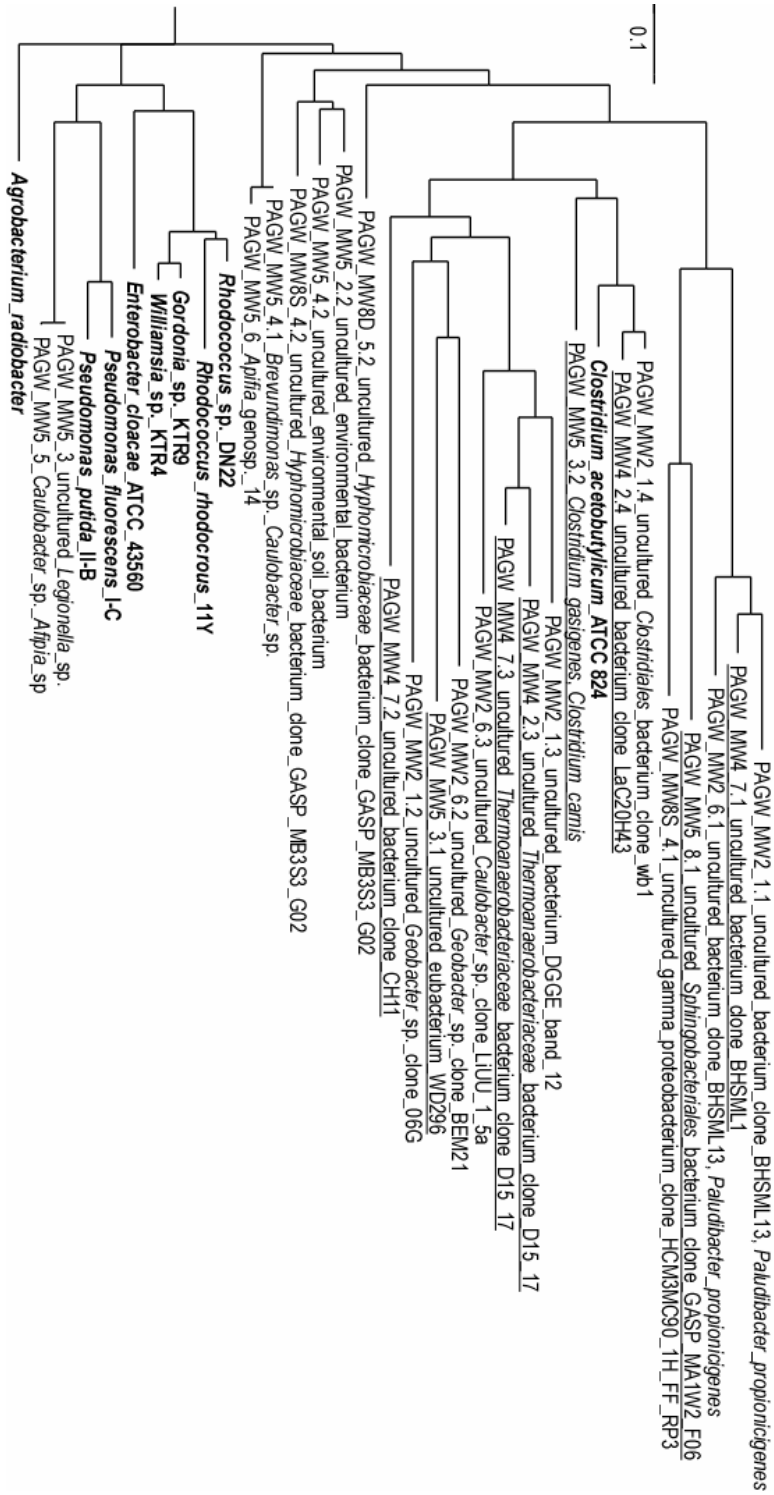


Figure 3.2.4-1. Phylogenetic analysis of Picatinny Arsenal groundwater before and after biostimulation. Underlined sequences denote post-biostimulation samples. Known explosive-degrading strains are included for reference (bold text). Bar = 10 nucleotide substitutions per 100 nucleotides in the 16S rRNA sequences.

Table 3.2.4-2. Description of the genera detected during *in situ* biostimulation of Picatinny Arsenal groundwater.

Identification	Characteristics
Caulobacter	common oligotrophic groundwater organism
Brevundimonas	prosthecate/nonprothecate bacteria found in fresh water and soil
Geobacter	iron-oxide reducing organoheterotroph, common in soil, river water, groundwater, and sewage
Legionella	aerobic surface water bacteria, found in cooling system and thermo-water sources
Clostridium	strictly anaerobic, fermentative bacteria, **some strains proven to degrade TNT, RDX**
Paludibacter	strictly anaerobic, propionate-producing bacteria in rice plant residue in anoxic rice-field soil
Hyphomicrobiaceae	hyphae- or prosthecae-forming soil and aquatic bacteria, oligomethylotrophic
Afipia	environmental bacteria, methylotrophic strains isolated, **detected in saturated RDX-contaminated soils**

Table 3.2.4-3. Results of screening for putative RDX-degradative genes following *in situ* biostimulation of Picatinny Arsenal groundwater.

		PCR detection of:					
Well	**Treatment**	**xenA**	**xenB**	**xplA**	**onr**	**hydA**	**nerA**
MW2	Native	-	-	-	-	-	-
MW4	Biostim	-	-	-	-	-	-
MW5	Biostim	-	-	-	-	-	-
MW8S	Native	-	-	-	-	-	-
MW8D	Native	-	-	-	-	-	-

3.3 MOLECULAR ANALYSIS OF GROUNDWATER FROM MULTIPLE SITES

3.3.1 METHODS

We identified sites where explosives-contaminated groundwater could be collected for molecular analysis by DGGE and PCR. Some sites also allowed collection of samples of groundwater undergoing *in situ* remediation. The sites from which groundwater was collected were:

-Pueblo Chemical Depot (PCD)
-West Virginia Ordnance Works (WVOW)
-Massachusetts Military Reservation (MMR)
-Nebraska Ordnance Plant (NOP)

An inventory of the samples collected is presented in Table 3.3-1. Concentrations of explosives in these samples are also shown, if known.

Groundwater from WVOW, MMR, and NOP was sent to the laboratory, where biomass was collected by filtration. Biomass in groundwater was collected by filtration in the field at the Pueblo Chemical Depot. All filters were stored at -80°C prior to DNA extraction. DNA extraction and analysis using DGGE and PCR was performed according to the standard protocols described in Appendix 3.

Table 3.3-1. Groundwater samples collected from various sites.

Well ID		Date Collected	Date Filtered	Volume Filtered (L)	µg/L RDX	TNT	pH	mg/L DO
Nebraska Ordnance Works (in-lab, 42 mm filters)								
	MW01	1/26/2005	2/2/2005	1.00	ND	ND	ND	ND
	MW03	1/26/2005	2/2/2005	1.00	ND	ND	ND	ND
	MW06	1/26/2005	2/2/2005	1.00	ND	ND	ND	ND
West Virginia Ordnance Works (in-lab, 42 mm filters)								
	TS-01	2/17/2005	2/23/2005	0.90	<0.5	340	5.20	4.63
	TS-02	2/17/2005	2/23/2005	0.90	<0.5	<0.5	5.10	5.52
	TS-03	2/17/2005	2/23/2005	0.90	<0.5	8.31	5.29	3.01
	TS-04	2/17/2005	2/23/2005	0.85	<0.5	4.75	5.22	7.22
	TS-06	2/17/2005	2/23/2005	0.90	<0.5	<0.5	5.53	7.64
	TS-07	2/17/2005	2/23/2005	0.85	0.64	<0.5	6.27	0.36
Peublo Chemical Depot (in-field, Sterivex)								
	R1A-1	7/16/2007	7/16/2007	1.00	3	ND	6.89	5.66
	R1B-1	7/16/2007	7/16/2007	1.00	ND	ND	6.66	0.14
	R3A-1	7/16/2007	7/16/2007	1.00	<0.2	ND	6.92	0.31
Massachussetts Military Reservation (in-lab, Sterivex)								
	MW80M1A	12/5/2006	12/22/2006	3.00	ND	ND	ND	ND
	MW80M2A	12/5/2006	12/22/2006	3.00	ND	ND	ND	ND
	58MW0016A	11/28/2006	12/22/2006	1.50	0.25-1.0*	ND	ND	ND
	58MW0016A	11/28/2006	12/22/2006	1.50	0.25-1.0*	ND	ND	ND
	58MW0015A	11/28/2006	12/22/2006	3.00	0.25-1.0*	ND	ND	ND
Picatinny Arsenal (Sterivex, both in-lab and in-field)								
	157MW2	2/28/2008	3/6/2008	2.00	4	<0.25	5.28	1.96
	157MW5	10/11/2006	various	various	55	75	ND	ND
	157MW8D	9/5/2007	6/16/2008	1.00	38	13	5.52	2.88
ND, Not determined								
*Historical data; more recent analyses indicated <0.25 µg/L								
WVOW data based on Ocotber 2004 sampling event								

3.3.2 RESULTS

About 50% of the groundwater samples yielded amplifiable DNA (Table 3.3-2). No DNA was recovered from the NOP groundwater, and only one out of six samples from the WVOW yielded amplifiable DNA. This may be due to differences in the way the biomass in this groundwater was collected. Flat membrane filters were used early in the project, whereas the Sterivex filters were used for later sampling. It is also possible that the longer storage time of the NOP and WVOW filters contributed to DNA degradation.

DGGE analysis of the recovered DNA yielded snapshots of the microbial communities at the time of sampling. For comparison, data from native (i.e., non-biostimulated) Picatinny Arsenal groundwater is included. Identities of individual bands from the DGGE gel are listed in Tables 3.3-3 and 3.3-4. Phylogenetic trees are presented in Figure 3.3-1 and 3.3-2.

Descriptions of the various genera detected are presented in Table 3.3-5. As with the laboratory samples, a variety of bacterial genera were detected, and there were multiple detections of sequences related to *Pseudomonas* spp. One sequence from non-stimulated PCD was related to an unidentified clone detected in RDX contaminated sediments (R1A-1, band 3a), and one PCD sequence downgradient of the mulch biowall that was stimulating *in situ* RDX degradation was related to a sequence recovered from a bioreactor degrading TNT (R3B-1, band 1b). In addition, one sequence recovered from a Picatinny Arsenal well undergoing *in situ* biostimulation was related to a sequence detected in a consortium degrading RDX under sulfate-reducing conditions (157MW-4, band 7.2).

Results of screening the samples for putative explosive-degrading genes is presented in Table 3.3-6. None of the genes for which screening was performed were detected in any samples.

Table 3.3-2. Recovery of amplifiable DNA from groundwater samples.

	Well ID	Amplifiable DNA
Nebraska Ordnance Works (in-lab, 42 mm filters)		
	MW01	No
	MW03	No
	MW06	No
West Virginia Ordnance Works (in-lab, 42 mm filters)		
	TS-01	No
	TS-02	No
	TS-03	No
	TS-04	No
	TS-06	Yes
	TS-07	No
Pueblo Chemical Depot (in-field, Sterivex)		
	R1A-1	Yes
	R1B-1	Yes
	R3A-1	Yes
Massachussetts Military Reservation (in-lab, Sterivex)		
	MW80M1A	Yes
	MW80M2A	Yes
	58MW0016A	Yes
	58MW0016A	Yes
	58MW0015A	Yes
Picatinny Arsenal (Sterivex, both in-lab and in-field)		
	157MW2	Yes
	157MW5	Yes
	157MW8D	Yes

Table 3.3-3. Identification of bands from DGGE analysis of non-stimulated groundwater from various sites.

Site	Well	Band	Identification	Source
WVOW	TS-06	1	uncultured_*Methylocystis*_sp.	methane oxidizers in soil
PCD	R1A-1	1	uncultured_bacterium_clone_BHSML13	estuary, contaminated sediments
		3	uncultured_bacterium_clone_5S2	petroleum contaminated sediments
		4	uncultured_bacterium_clone_BHSML13	estuary, contaminated sediments
		5	uncultured_Clostridiales_bacterium_clone_RsW01_064	termite gut
		1a	*Pseudomonas_fluorescens*_strain_SSR8_1	bacterial endophytes of plants
		2a	*Pseudomonas*_sp._MFY116	
		3a	uncultured_bacterium_clone_PTA_31	**RDX contaminated sediment**
MMR	MW80M1A	1	*Lysobacter_defluvii*_strain_IMMIB_APB_9T	municipal solid waste
	MW80M2A	1	*Pseudomonas*_sp._PD_6	Antarctic soil
		2	uncultured_bacterium_isolate_DGGE_gel_band_ASC12	rhizosphere
		3	uncultured_*Halomonas*_sp.	rodent natural flora
		4	uncultured_gamma_proteobacterium_clone_8_4	coal tar waste contaminated aquifer
	58MW0015A	1	uncultured_*Hyphomicrobiaceae*_bacterium_clone_D10_05	tar oil contaminated groundwater
	58MW0016A	1	*Pseudomonas*_sp._USTB_04	methyl-parathion degrading strain
		2	uncultured_*Pedomicrobium*_sp._clone_GASP_KB2W1_F09	Kansas agricultural soil
		3	*Pseudomonas*_sp._GOBB3_104_2	northern Baltic Sea
		4	uncultured_bacterium_isolate_DGGE_band_20	Alpine soil
		2a	uncultured_bacterium_clone_BHSML13	estuary, contaminated sediments
PA	157MW2	1.1	uncultured_bacterium_clone_BHSML13	estuary, contaminated sediments
		1.2	uncultured_*Geobacter*_sp._clone_06G	urban creek sediments, rice field, acidic fens
		1.3	uncultured_bacterium_DGGE_band_12	gasoline-contaminated groundwater
		1.4	uncultured_Clostridiales_bacterium_clone_wb1	microbial consortia degrading complex organic matter
		6.1	*Paludibacter_propionicigenes*	estuary, contaminated sediments
		6.2	uncultured_*Geobacter*_sp._clone_BEM21	Fe(III)-reducing subsurface environments
		6.3	uncultured_*Caulobacter*_sp._clone_LiUU_1_5a	ocean crust, bacteria growing on natural organic carbon
	157MW5	2.2	uncultured_environmental_bacterium	contaminated sediments (metals, nitrate, uranium, solvents)
		3	uncultured_*Legionella*_sp.	drinking water
		4.1	*Brevundimonas*_sp._*Caulobacter*_sp.	rocks in a gold mine, lake water
		4.2	uncultured_environmental_soil_bacterium	Kansas agricultural soil
		5	*Caulobacter*_sp._*Afipia*_sp.	lake water, linuron-mineralizing bacterial consortia
		6	*Afipia*_genosp._14	anoxic wastewater treatment biofilm
	157MW8S	4.1	uncultured_gamma_proteobacterium_clone_HCM3MC90_1H_FF_RP3	sea sediment, corals, India lake
		4.2	uncultured_*Hyphomicrobiaceae*_bacterium_clone_GASP_MB3S3_G02	Michigan agricultural soil
		5.2	uncultured_*Hyphomicrobiaceae*_bacterium_clone_GASP_MB3S3_G02	Michigan agricultural soil
	157MW8D	1	uncultured_*Comamonadaceae*_bacterium_clone_168E6	nitrate contaminated aquifer
		2	uncultured_bacterium_clone_104B408	iron- and sulfur-precipitating microbial mats at mud volcano

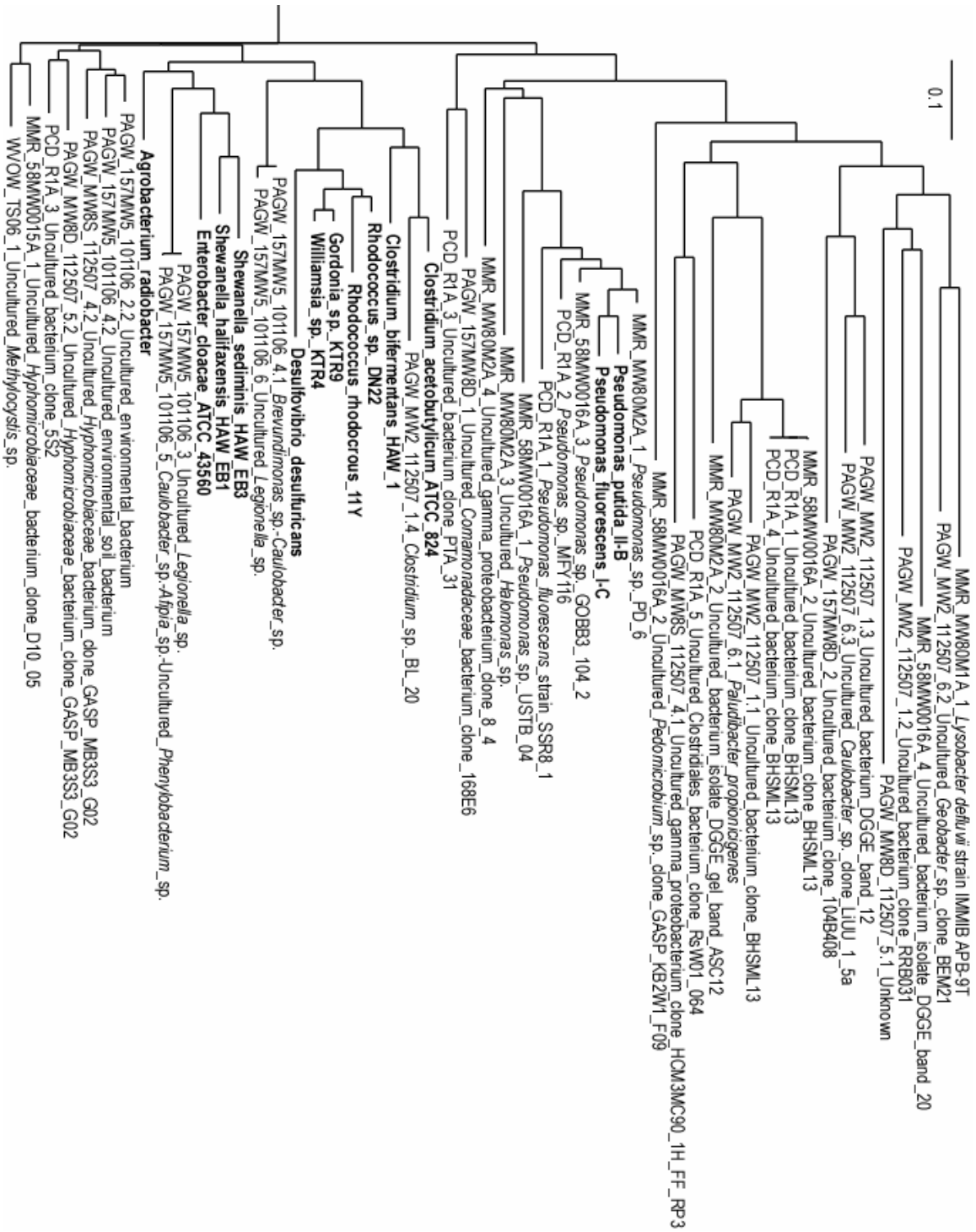


Figure 3.3-1. Phylogenetic analysis of non-stimulated groundwater from various explosive-contaminated sites. Known explosive-degrading strains are included for reference (bold text). Bar = 10 nucleotide substitutions per 100 nucleotides in the 16S rRNA sequences.

Table 3.3-4. Identification of bands from DGGE analysis of biostimulated groundwater from various sites.

Site	Well	Band	Identification	Source
PCD	R1B-1	1a	uncultured bacterium TA12	terephthalate degrading anaerobic sludge
		1b	soil bacterium Dicl_S1N_M1LLLSSL_3	bacteria growing on antibiotics
		2	uncultured bacterium clone SP3_a11	mammalian gut microbes
		3	uncultured alpha proteobacterium JG34_KF_349	uranium mine waste piles
	R3B-1	1a	uncultured bacterium isolate CH96H00073	
		1b	uncultured bacterium clone 24264	**TNT degrading bioreactor**
		2a	*Pseudomonas* sp. SSR5_2	bacterial endophytes of plants
		2b	soil bacterium Dicl_S1N_M1LLLSSL_3	bacteria growing on antibiotics
		3	uncultured Bacteroidetes bacterium clone DOK_CONFYM_clone242	soil
PA	157MW4	2.3	uncultured_*Thermoanaerobacteriaceae*_bacterium_clone_D15_17	tar oil contaminant groundwater
		2.4	Uncultured_bacterium_clone_LaC20H43	perchlorate-respiring culture
		7.1	uncultured_bacterium_clone_BHSML1	estuary // benzene-contaminated river sediments
		7.2	uncultured_bacterium_clone_CH11	**RDX degradation under sulfate-reducing conditions**
		7.3	uncultured_*Thermoanaerobacteriaceae*_bacterium_clone_D15_17	tar oil contaminant groundwater
		7.4	unknown (no match in sequence database)	
	157MW5	3.1	uncultured_eubacterium_WD296	polychlorinated biphenyl-polluted soil
		3.2	*Clostridium_gasigenes, Clostridium_carnis*	alkaline soil-water systems, vacuum-packed meat
		8.1	uncultured_Sphingobacteriales_bacterium_clone_GASP_MA1W2_F06	former arable field, Michigan agricultural soil, Arctic tundra tussock

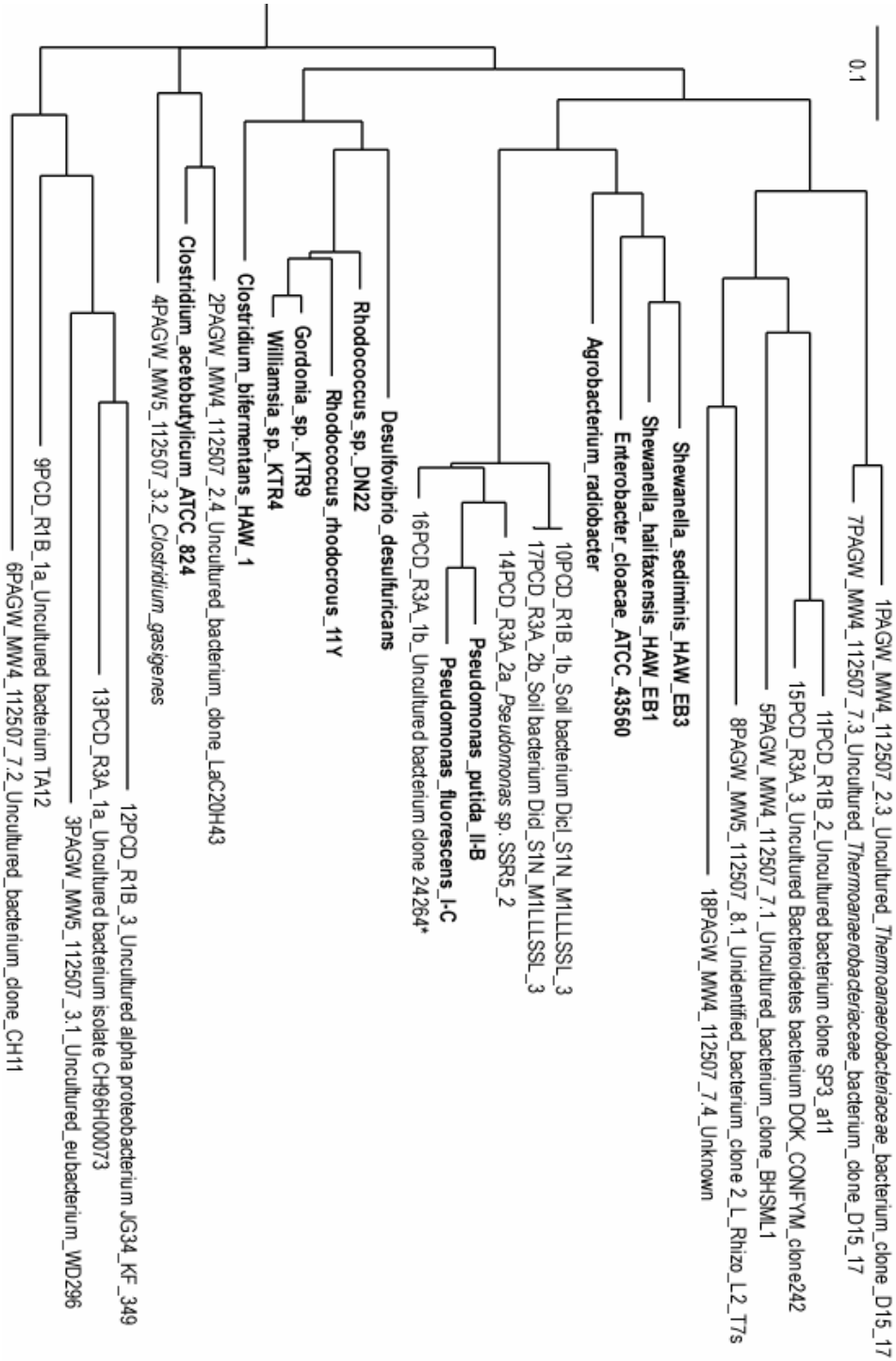


Figure 3.3-12. Phylogenetic analysis of biostimulated groundwater from various explosive-contaminated sites. Known explosive-degrading strains are included for reference (bold text). Bar = 10 nucleotide substitutions per 100 nucleotides in the 16S rRNA sequences.

Table 3.3-5. Description of the genera detected in native and biostimulated groundwater from various sites.

Identification	Characteristics
Caulobacter	common oligotrophic groundwater organism
Brevundimonas	prosthecate/nonprothecate bacteria found in fresh water and soil
Geobacter	iron-oxide reducing organoheterotroph, common in soil, river water, groundwater, and sewage
Legionella	aerobic surface water bacteria, found in cooling system and thermo-water sources
Clostridium	strictly anaerobic, fermentative bacteria, **some strains proven to degrade TNT, RDX**
Paludibacter	strictly anaerobic, propionate-producing bacteria in rice plant residue in anoxic rice-field soil
Hyphomicrobiaceae	hyphae- or prosthecae-forming soil and aquatic bacteria, oligomethylotrophic
Afipia	environmental bacteria, methylotrophic strains isolated, **detected in saturated RDX-contaminated soils**
Pseudomonas	widespread genera, extensive catabolic diversity, **some strain shown to degrade RDX**
Methylocystis	nitrogen-fixing, aerobic, obligate methanotrophic bacteria, common in flooded soils, sediments, sewage
Lysobacter	ubiquitous soil bacteria with gliding motility, degrade plant material with extracellular enzymes
Halomonas	common in saline environments, diverse metabolism
Pedomicrobium	budding hyphal bacteria in soil and aquatic environments, metal-oxidizing abilities

Table 3.3-6. Results of screening for putative RDX-degradative genes in native and biostimulated groundwater from various sites.

			PCR detection of:					
Site	**Well**	**Treatyment**	**xenA**	**xenB**	**xplA**	**onr**	**hydA**	**nerA**
WVOW	TS-06	Native	-	-	-	ND	ND	ND
PCD	R1A-1	Native	-	-	-	ND	ND	ND
	R1B-1	Biostim	-	-	-	ND	ND	ND
	R3B-1	Biostim	-	-	-	ND	ND	ND
MMR	MW80M1A	Native	-	-	-	ND	ND	ND
	MW80M2A	Native	-	-	-	ND	ND	ND
	58MW0015A	Native	-	-	-	ND	ND	ND
	58MW0016A	Native	-	-	-	ND	ND	ND
PA	157MW2	Native	-	-	-	-	-	-
	157MW4	Biostim	-	-	-	-	-	-
	157MW5	Biostim	-	-	-	-	-	-
	157MW8S	Native	-	-	-	-	-	-
	157MW8D	Native	-	-	-	-	-	-
ND, Not determined								

4. STABLE ISOTOPE PROBING

In response to SAB comments and in a desire to examine the use of new approaches for better understanding the degradation of explosives, we performed additional experiments using a new, powerful methodology called stable isotope probing (SIP). This methodology was developed to specifically examine microbial communities with respect to which strains are involved in carbon and nitrogen cycling (26, 44, 45). Briefly, a target compound that is enriched with ^{13}C or ^{15}N is added to a sample and allowed to incubate. The nucleic acids are then extracted from the sample, and the ^{13}C- or ^{15}N-enriched portion is separated using density centrifugation. The purified "heavy" stable isotope-enriched nucleic acids, which represent only those organisms that metabolized the target compound, are then manipulated using standard molecular methods (PCR, DGGE, etc.). A conceptual illustration of SIP is presented in Figure 4-1.

Explosive compound biodegradation is somewhat nonspecific, in that many organisms fortuitously degrade the compounds if excess electron donor is present and alternate electron acceptors are not present. However, it is not completely clear which, if any, of these organisms actually get carbon or nitrogen from the explosives they degrade. The exploration of the SIP methodology during this project was expected to yield results with a level of specificity, with respect to the identity of the organisms that can use the explosives as a carbon and nitrogen source, that would not have been possible using standard methods.

This project started the initial work on the methods to apply stable isotope probing to gain a better understanding of which organisms are most important and/or directly responsible for the biodegradation of RDX. This work was done in collaboration with Dr. Bella Chu at Texas A&M University.

NOTE: A draft manuscript detailing the ^{15}N-RDX SIP work is included in Appendix 1, but relevant results are presented here, along with results from the ^{13}C-RDX SIP work.

4.1 METHOD DEVELOPMENT

4.1.1 METHODS

Custom synthesis of $^{13}C_3$-RDX and $^{15}N_6$-RDX was performed by a private research corporation, and the material was delivered in July 2006. The 1 g of ^{13}C-labeled RDX was 99.6% chemically pure and fully labeled (i.e., all the carbons were ^{13}C). The 5 g of ^{15}N-labeled RDX was 99.2% chemically pure, but due to the synthesis route utilized, only the ring nitrogens were ^{15}N. This meant that SIP using the ^{15}N-RDX was most effective if the ring nitrogens were incorporated into the nucleic acids of the bacteria.

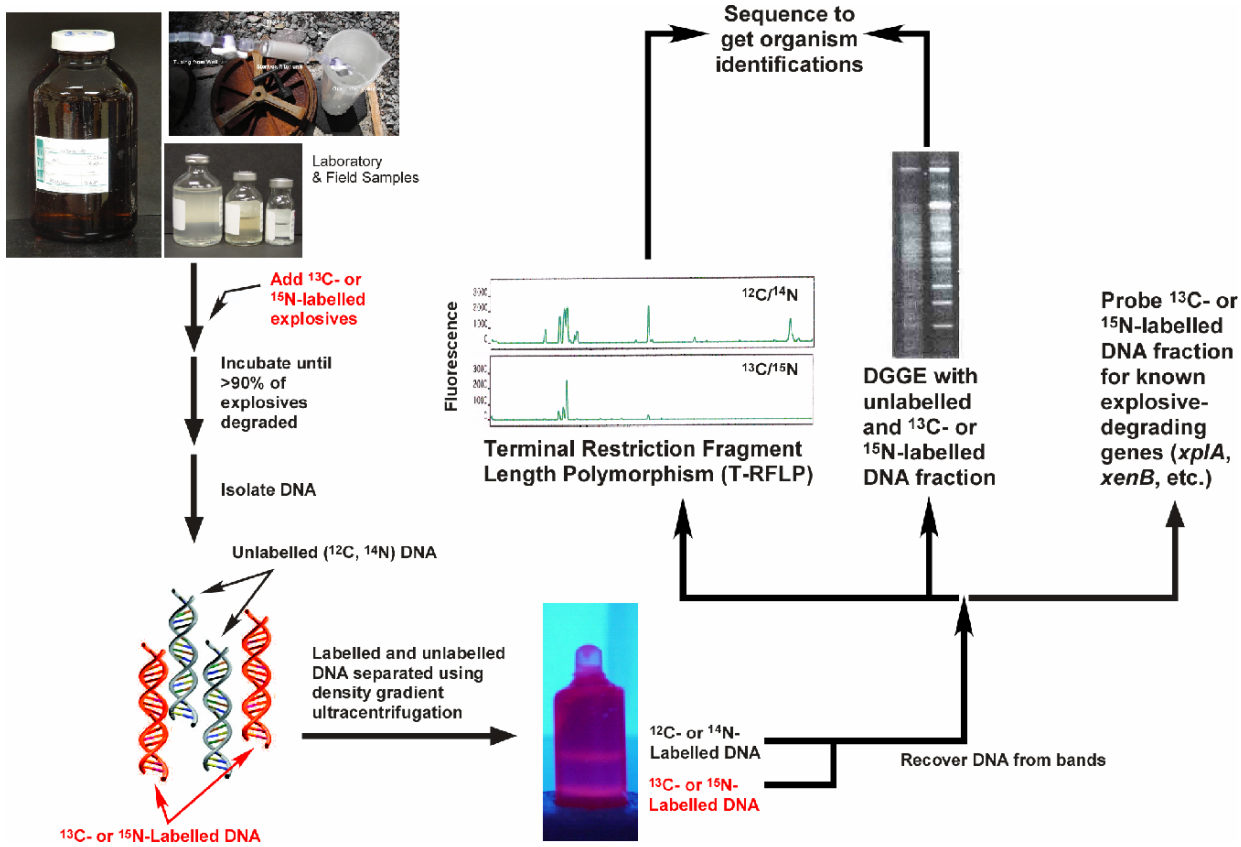


Figure 4-1. Conceptual illustration of SIP.

A procedure for separation of ^{15}N-enriched DNA from unenriched DNA using density gradient ultracentrifugation was developed and tested using *E. coli* cells grown on unlabeled and ^{15}N-labeled nitrate as the sole nitrogen source. Two densities of cesium chloride ($CsCl_2$) were tested.

Initial SIP evaluation was performed with the known RDX degrader strain *Rhodococcus* sp. strain DN22. An inoculum from a BSM-N-succinate plus RDX agar was grown up on succinate as the carbon source and RDX as the sole nitrogen source. This pure culture was then used to

inoculate three different media: BSM-N amended with succinate with ammonium as the nitrogen source, BSM-N amended with succinate with unlabeled RDX as the nitrogen source, and BSM-N amended with succinate and ^{15}N-RDX as the nitrogen source. The media were in 50 mL conical centrifuge tubes. The amount of total nitrogen was approximately the same in all the cultures. One 20-mL aliquot of BSM-N amended with succinate and ammonium and two 20-mL aliquots of the RDX media were inoculated. The cultures were incubated aerobically at 30°C with shaking. When growth appeared, additions of the carbon (succinate) and nitrogen (ammonium, RDX, or ^{15}N-RDX) sources were made. Once the cultures are fully grown, the cells were collected and shipped to Dr. Chu for DNA extraction and analysis. The extracted DNA from DN22 was loaded onto a $CsCl_2$ density gradient along with DNA from E. *coli* with unlabelled DNA, and *Pseudomonas putida* that had been grown in media with ^{15}N-nitrate as the sole nitrogen source.

4.1.2 RESULTS

A photograph of the separation of ^{15}N-enriched DNA from unenriched DNA from *E. coli* cells grown on unlabeled and ^{15}N-labeled nitrate as the sole nitrogen source is presented in Figure 4.1-1. The heavier ^{15}N-enriched DNA separated very well from the unenriched ^{14}N-DNA (lighter upper band).

Figure 4.1-2 presents images of the bands produced during ultracentrifugation of the differentially-labeled DNAs. Unlabeled and ^{15}N-labeled DNA from E. *coli* and *Pseudomonas putida*, respectively, were separated and resolved quite well. When ^{15}N-labeled *Rhodococcus* sp. DN22 DNA was added to the mix, the DN22 DNA migrated halfway between the unlabeled and ^{15}N-labeled "standard" DNAs.

These results yielded several conclusions. First, the SIP method was clearly amenable to the use of ^{15}N-labeled substrates, as opposed to only ^{13}C-labeled compounds. Second, the intermediate density of the ^{15}N-labeled DN22 DNA indicated that the *Rhodococcus* was able to assimilate both the nitro-group N and the ring N of RDX. Since it was initially assumed that DN22 only assimilated the nitro-group N, the current results indicate that larger amounts of ^{15}N can be incorporated into the nucleic acids of the organism.

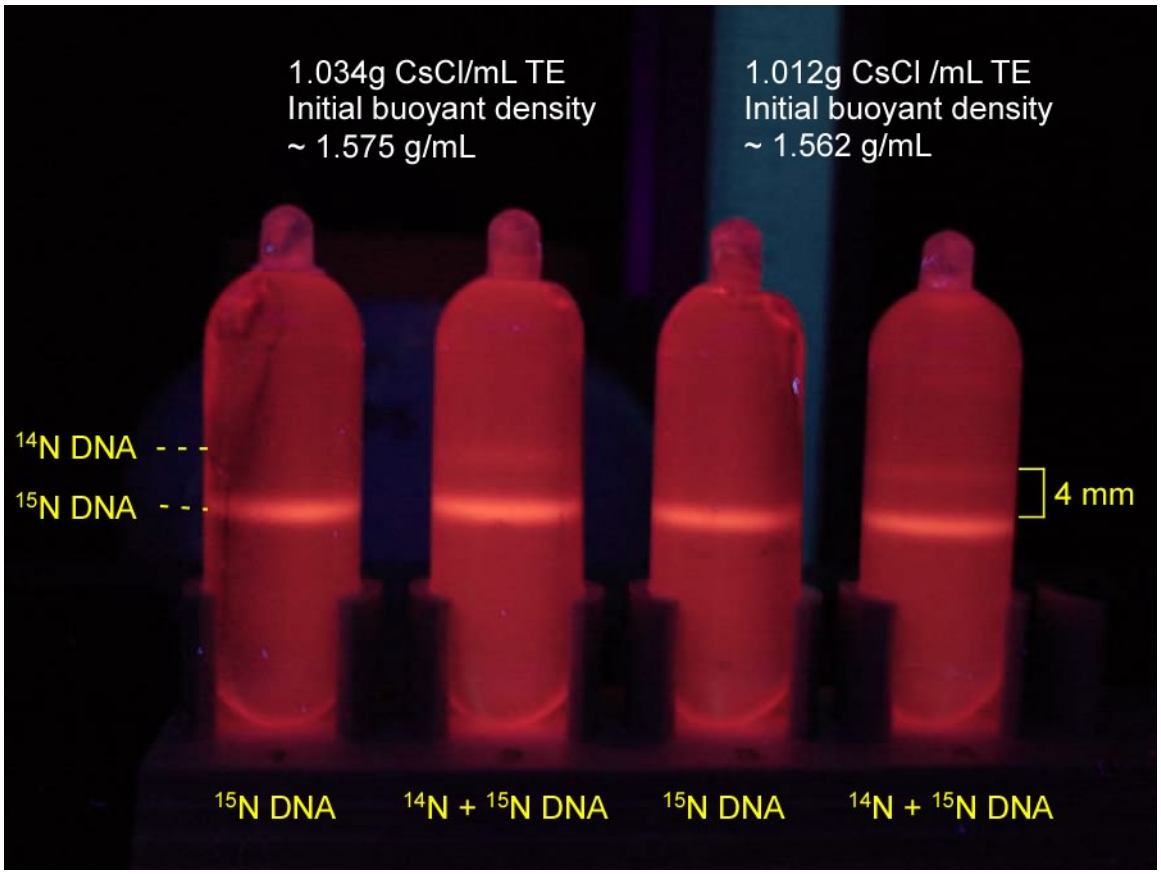


Figure 4.1-1. Photograph of separation of ^{15}N-enriched DNA from unenriched DNA from *E. coli* cells grown on unlabeled and ^{15}N-labeled nitrate as the sole nitrogen source. DNA bands in the tubes were visualized under long-wavelength (365 nm) UV light.

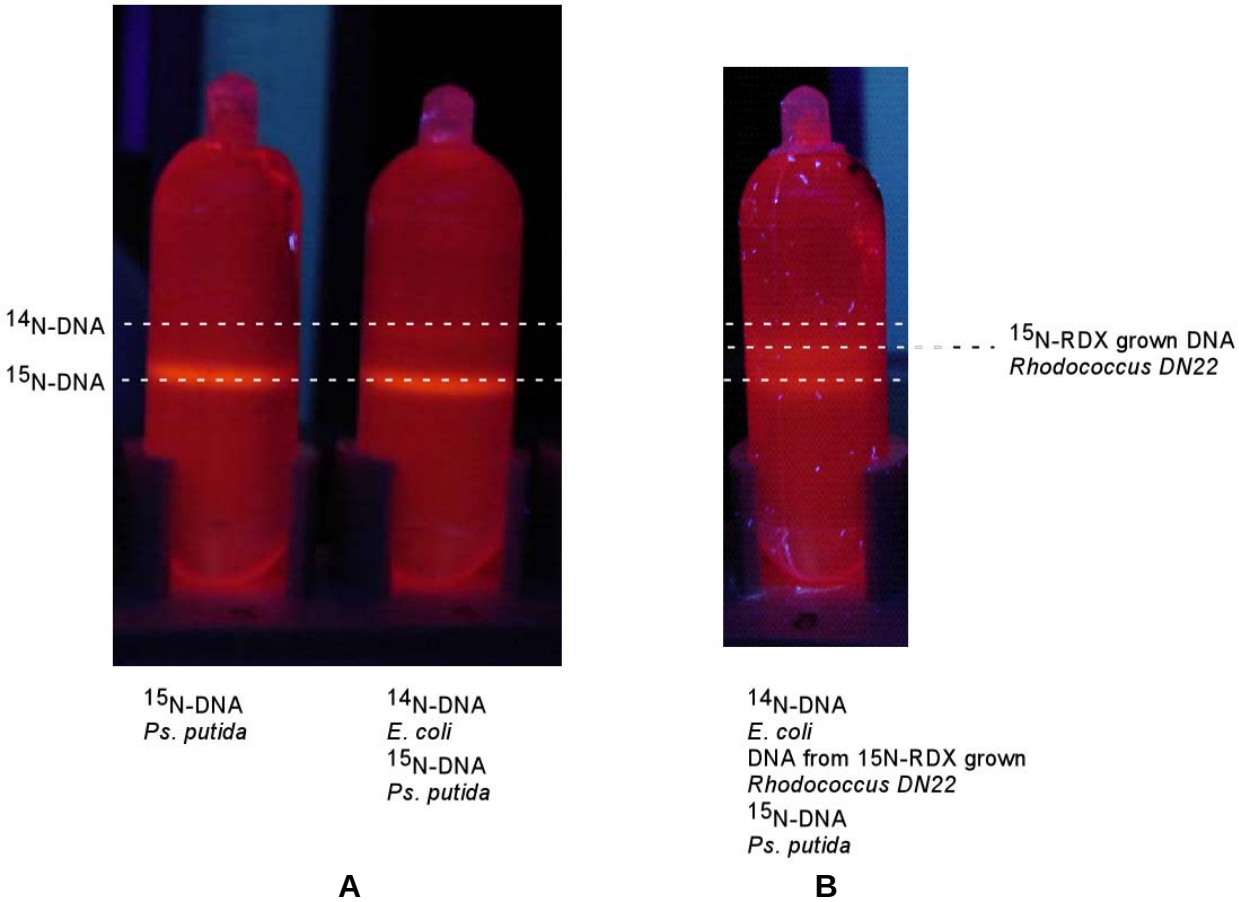


Figure 4.1-2. Separation of stable ^{15}N isotope-labelled DNA by ultracentrifugation. A) Separation of ^{14}N-DNA of *E. coli* from ^{15}N-DNA of *P. putida* after ultracentrifugation. The upper band is ^{14}N-DNA and lower band is 15N-DNA. (right)The band of 15N-DNA of *Rhodococcus* sp. DN22 (after degrading ring-^{15}N-labelled RDX) located between that of ^{14}N-DNA and ^{15}N-DNA controls.

4.2 SIP OF COLUMN EFFLUENT AND GROUNDWATER SAMPLES

Additionally, experiments were performed to apply both ^{15}N-RDX SIP and ^{13}C-RDX SIP to enrichments derived from the Picatinny Arsenal biostimulation project area in order to elucidate which organisms were involved with RDX biodegradation *in situ*.

4.2.1 METHODS

Effluent was collected for several days from Picatinny Arsenal columns 3, 4, and 5. The effluent (15 mL) was placed in glass serum bottles (160 mL), amended with either unlabeled or ^{15}N-labeled RDX (10 mg/L), cheese whey or yeast extract (1000 mg/L, filtered), and enough 0.22 µm

filter sterilized Picatinny Arsenal groundwater to give a final volume of 100 mL. The treatments were as follows:

Bottle #	Column	Nutrient source	RDX type
1	3	Cheese whey	RDX
2	4	Cheese whey	RDX
3	5	Yeast extract	RDX
4	3	Cheese whey	^{15}N-RDX
5	4	Cheese whey	^{15}N-RDX
6	5	Yeast extract	^{15}N-RDX

Bottles were incubated under anoxic/anaerobic conditions with shaking at 15°C, and RDX degradation was monitored by HPLC analysis. Bottles in which all the RDX was degraded were respiked with additional RDX or ^{15}N-RDX. Approximately half of the volume was removed when RDX degradation was complete or leveled off, and sent to Dr. Chu's laboratory. Dr. Chu isolated and purified the DNA, then performed density gradient centrifugation, followed by terminal restriction fragment length polymorphism (tRFLP) analysis/sequencing on the heavy and light DNA bands. PCR for the RDX-degrading gene *xplA* was also performed.

For groundwater SIP, several liters were collected from well 157MW-5 at the Picatinny Arsenal. The groundwater was homogenized in an anaerobic chamber and then divided among six 1-L glass bottles equipped with luer-lock ports in the bottle caps. The groundwater was amended as follows:

Bottle #	Nutrient source (0.3 g/L, dry powder)	RDX type (10 mg/L)
1	Cheese whey	RDX
2	Cheese whey	^{15}N-RDX
3	Cheese whey	^{13}C-RDX
4	Cheese whey	RDX + dicumarol (~4 g/L)

The final volume in each bottle was 0.8 L. Dicumarol (3,3'-methylene-bis(4-hydroxycoumarin)) was added to inhibit nitroreductases and allow comparison of the microbial community that developed with and without these enzymes being active. Bottles were incubated under anoxic/anaerobic conditions at 15°C with shaking, and samples were periodically removed and analyzed by HPLC for RDX. After degradation of the second RDX spike was complete, samples (120 mL) were removed, biomass was collected onto Sterivex filters, frozen, and sent to Dr. Chu for DNA extraction and separation and tRFLP analysis/sequencing.

4.2.2 RESULTS

See Appendix 1 for more details on the ^{15}N-RDX SIP experiments. Degradation was most robust in the bottles inoculated with Column 4 effluent (high cheese whey), whereas no RDX degradation was observed in bottles inoculated with Column 3 effluent (low cheese whey).

The application of ^{15}N-SIP was only successful with effluent from Picatinny Arsenal Column 4 effluent. A phylogenetic tree showing the relationship of the sequences recovered to known

explosive-degrading strains is shown in Figure 4.2-1, and the characteristics of the bacterial genera that incorporated ^{15}N from the ^{15}N-RDX are presented in Table 4.2-1.

Of the recovered sequences enriched in ^{15}N, several (i.e., RDX_1, RDX_4, RDX_5 RDX_6, RDX_12, and RDX_15) were related to strains that have been associated with the degradation of explosives, including *Enterobacter* and *Pseudomonas*. Again, several sequences related to methylotrophic (*Afipia*) and nitrogen-fixing (*Bradyrhizobium*, *Pleomorphomonans*, *Azospirillum*) organisms were recovered. It should be noted that these organisms were incorporating ^{15}N originating from the ring of RDX, which indicates that RDX degradation had reached at least the step of ring opening. It is possible that some or all of these ^{15}N-incorporating organisms were feeding off remnants of RDX produced by some other, unidentified organism. These results add additional evidence that the ability to take part in the overall RDX degradation process likely exists across a wider range of genera than is assumed based on pure culture studies, and that the genus *Pseudomonas* may be more involved than previously thought.

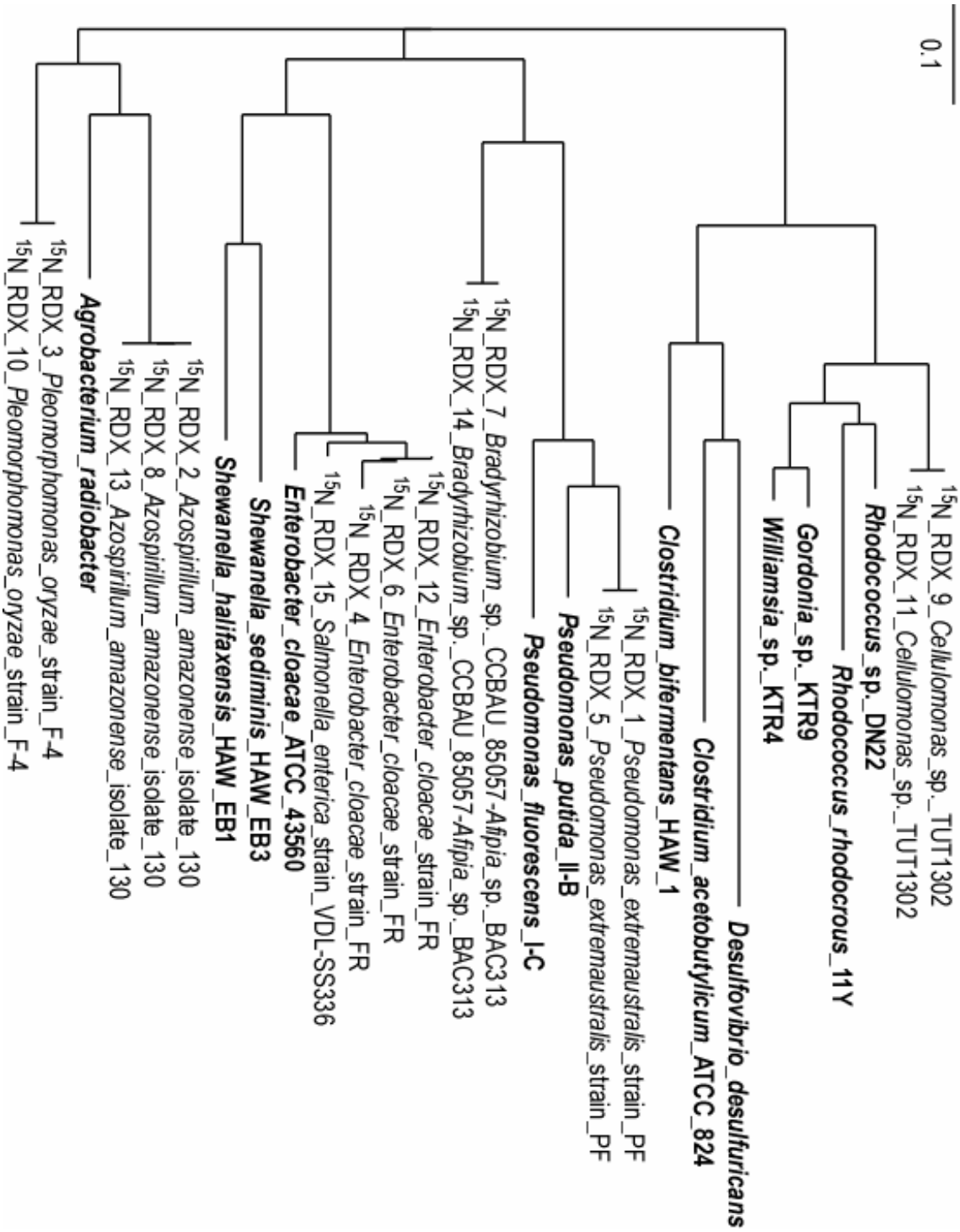


Figure 4.2-1. Phylogenetic analysis of recovered sequences after application of ^{15}N-RDX SIP to effluent from Picatinny Arsenal Column 4. Known explosive-degrading strains are included for reference (bold text). Bar = 10 nucleotide substitutions per 100 nucleotides in the 16S rRNA sequences.

Table 4.2-1. Description of the bacterial genera that incorporated ^{15}N from ^{15}N-RDX.

Identification	Characteristics
Caulobacter	common stalked bacteria found in oligotrophic groundwater
Cellulomonas	soil and subsurface bacteria, extensive catabolic diversity, some shown to degrade s-triazine compounds
Azospirillum	free-living, nitrogen-fixing soil bacteria capable of microaerophilic hydrocarbon degradation
Pleomorphomonas	nitrogen-fixing bacteria common in flooded soil
Pseudomonas	widespread genera, extensive catabolic diversity, **some strains shown to degrade RDX**
Bradyrhizobium	nitrogen-fixing plant symbiont, some shown to degrade 2,4-D herbicide
Afipia	environmental bacteria, methylotrophic strains isolated, **detected in saturated RDX-contaminated soils**
Enterobacter	facultative anaerobe, found in soil, water, animal intestinal tracts, **some strains shown to degrade RDX**
Salmonella	facultative anaerobe, common in amphibians and reptiles, **some strains shown to degrade TNT**

The degradation of RDX in Picatinny Arsenal groundwater during SIP is presented in Figure 4.2-2. The addition of the nitroreductase inhibitor dicumarol did not significantly alter RDX degradation.

The application of SIP only yielded results when ^{13}C-RDX was used as the labeling compound. Separation of the ^{15}N-enriched DNA from the unenriched DNA from this experiment was not successful. However, the ^{12}C/^{13}C DNA was readily separated and analyzed. A phylogenetic tree showing the relationship of the sequences recovered from the light (^{12}C) and heavy (^{13}C) bands observed after ultracentrifugation is presented in Figure 4.2-3. Descriptions of the organisms which incorporated ^{13}C from ^{13}C-RDX are given in Table 4.2-2. These organisms incorporated carbon from RDX even though a complete and labile source of carbon and nitrogen was supplied by the added cheese whey. The carbon in RDX is part of the ring, so RDX degradation at least through the ring-opening stage must have occurred.

The organisms incorporating ^{13}C included widespread genera, some of which are nitrogen-fixing (*Azospirillum*) or are known to have diverse catabolic capabilities (*Streptomyces*, *Dechloromonas*). As with the ^{15}N-RDX experiments, it is possible that an unidentified organism initiated degradation of the RDX but was not able to complete the process and incorporate the ^{13}C from the ring structure.

Taken together, these SIP experiments have provided additional and more specific insight into the degradation of RDX with respect to the responsible microbial community in groundwater. Additional studies are underway as part of another SERDP project to expand the application of SIP to RDX degradation under more varied conditions, as well as more studies with alternately-labeled ^{15}N-RDX.

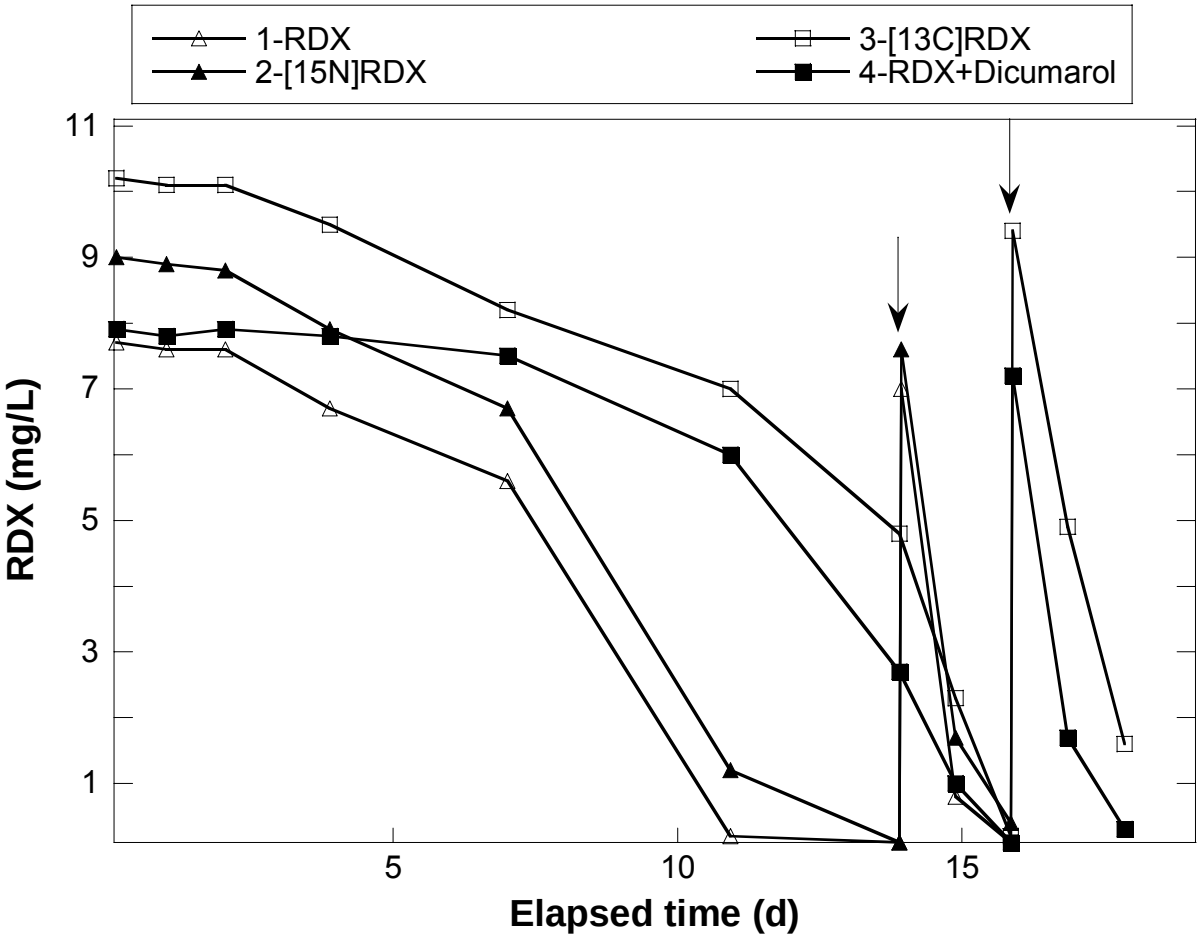


Figure 4.2-2. Degradation of RDX during application of SIP to Picatinny Arsenal groundwater. Arrows denote second additions of RDX.

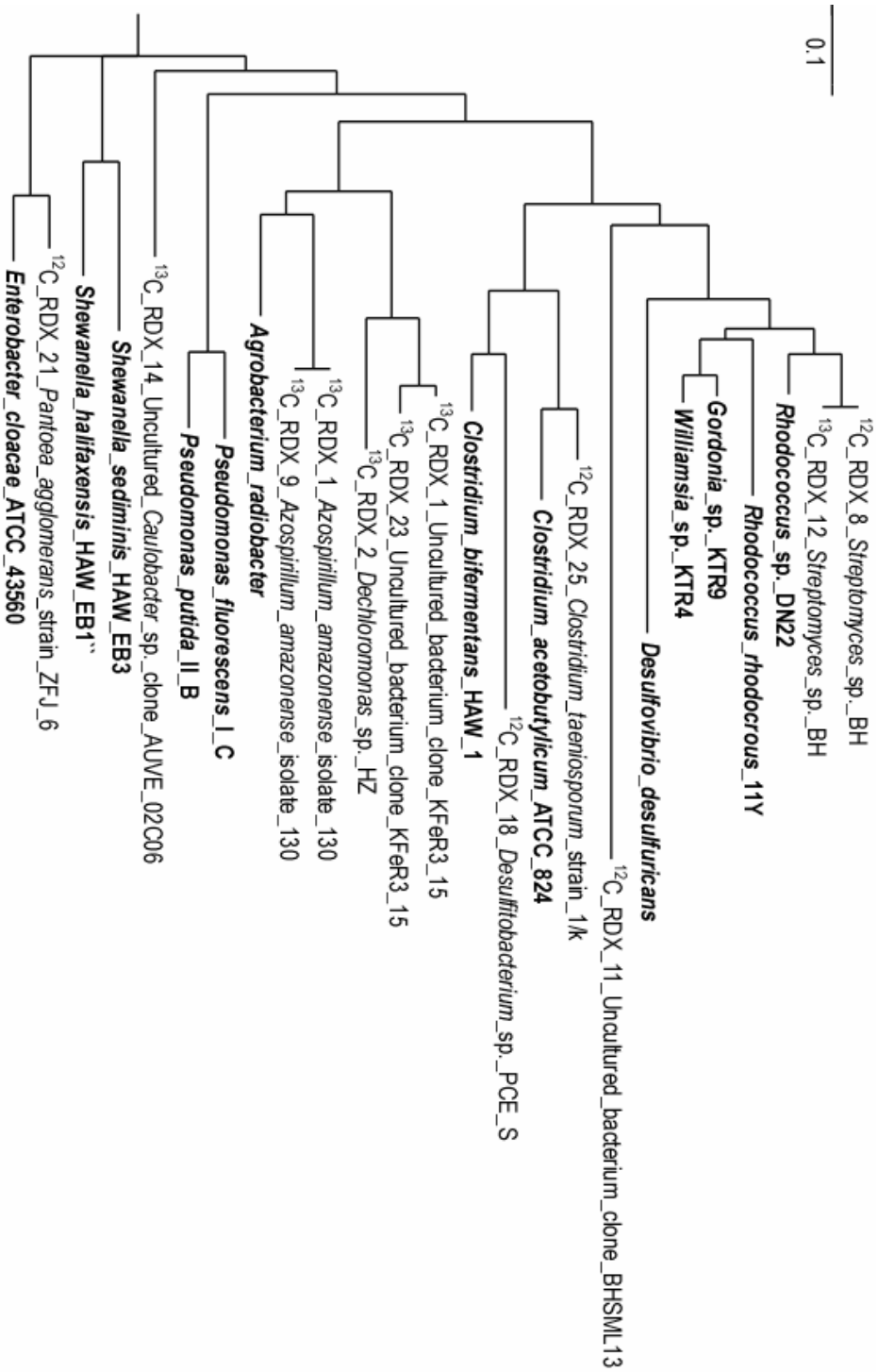


Figure 4.2-3. Phylogenetic analysis of recovered sequences after application of ^{13}C-RDX SIP to Picatinny Arsenal groundwater. Known explosive-degrading strains are included for reference (bold text). Bar = 10 nucleotide substitutions per 100 nucleotides in the 16S rRNA sequences.

Table 4.2-2. Identification and description of the bacterial genera that incorporated ^{13}C from ^{13}C-RDX.

Identification	Characteristics
Caulobacter	common oligotrophic groundwater organism
Streptomyces	soil and subsurface bacteria, extensive catabolic diversity, **some strains transform TNT**
Azospirillum	free-living, nitrogen-fixing soil bacteria capable of microaerophilic hydrocarbon degradation
Dechloromonas	facultative bacteria, wide range of electron acceptors and extensive catabolic diversity

V. CONCLUSIONS

This project demonstrated that RDX was amenable to biological degradation under a range of anoxic and/or anaerobic conditions. Both simple and complex nutrients stimulated degradation of RDX, and additional labile nitrogen (as NH4) appeared to enhance rather than inhibit RDX degradation under these conditions. The presence of RDX degraders in native groundwater appeared to be heterogeneously distributed.

Through the application of molecular techniques to a wide range of samples (microcosms, model aquifer columns, field samples) this project indicated that a wider range of organisms than were previously studied and described are likely involved in RDX degradation in groundwater under a range of anoxic/anaerobic conditions. However, the results did not lead to the identification of a specific "biomarker" organism for RDX biodegradation.

The development and application of the new SIP technique to examine RDX biodegradation employing both ^{13}C- and ^{15}N-labeled RDX, has indicated that, at a minimum, a range of different organisms are likely involved with the complete degradation of RDX in groundwater.

A summary analysis of all the molecular data yielded the following observations:

1) Several sequences detected during this project were related to sequences/strains observed during other nitro-organic-related research. These are listed in Table V-1. Given that only five of the 212 sequences recovered during this research were similar to previously described sequences likely indicates that the breadth of bacteria capable of explosive- and explosive-related compound transformation/degradation is much wider than would be inferred from the published literature.

2) Several bacterial genera were detected in multiple samples (microcosm enrichments, model aquifer columns, field samples, isolates). Of the total number of 212 sequences recovered, 149 were identified to the genus level. The number of detections of each genera identified are tabulated in Table V-2. Genera with strains previously identified with the ability to degrade RDX are indicated in bold face.

Pseudomonas sequences were the most abundant and widely detected, followed by *Clostridium* in second place. *Geobacter* and nitrogen-fixing *Azospira* and *Azospirillum* sequences were also repeatedly detected. The breakdown of recovered sequences by phyla is presented in Table V-3. Due to the high number of *Pseudomonas* sequences, the gammaproteobacterial phyla was the most frequently detected in terms of total sequences, followed by the alphaproteobacteria and firmicutes. However, based on the number of different genera in a given phylum that were detected, the alphaproteobacteria were most numerous, followed by about equal numbers of genera in the betaproteobacteria, gammaproteobacteria, actinobacteria, and firmicutes phyla.

3) The lack of detection of any of the putative RDX-degrading genes in the wide range of samples screened indicates that the current state of understanding about which enzymes/pathways may be involved in RDX degradation in complex groundwater microbial communities is incomplete. More work is needed to determine if there are other specific genes

involved in RDX degradation, or if more general, widespread metabolic processes (i.e., generic reductases) are the predominant players.

More research is warranted in this area to increase the microbial ecology knowledge base for explosives degradation in general, and RDX biodegradation in particular. Continued application of SIP to a wider variety of samples, and inclusion of differentially-labeled RDX, will result in a clearer understanding of which organisms are important to the overall degradation process.

Table V-1. Recovered sequences similar to those observed in other research.

Sample source	Condition	Sequence recovered similar to	Originally detected in
PA GW	succinate-glucose	*Pelosinus fermentans*	TNT-degrading bioreactor
PCD-R1A-1	native	uncultured_bacterium_clone_PTA_31	RDX contaminated sediment
PCD-R3B-1	mulch biostim	uncultured bacterium clone 24264	TNT-degrading bioreactor
PA-157MW4	cheese whey biostim	uncultured_bacterium_clone_CH11	RDX degradation under sulfate-reducing conditions
WYOW	native	*Clostridium nitrophenolicum* strain 1DT	PNP-degrading subsurface soil

Table V-2. Detection of sequences of specific genera across all samples analyzed.

Genera	Phyla	Total sequences recovered
Pseudomonas	Gammaproteobacteria	45
***Clostridium* (includes *Clostridiales*)**	Firmicutes	9
Sporolactobacillus	Firmicutes	7
Desulfitobacterium	Firmicutes	6
Geobacter (includes Geobacteraceae)	Deltaproteobacteria	6
Azospira	Alphaproteobacteria	5
Azospirillum	Alphaproteobacteria	5
Caulobacter	Alphaproteobacteria	5
Pelosinus	Firmicutes	5
Arthrobacter	Actinobacteria	4
Rhodococcus	Actinobacteria	4
Asticcacaulis	Alphaproteobacteria	3
Bradyrhizobium	Alphaproteobacteria	3
Brevundimonas	Alphaproteobacteria	3
Enterobacter	Gammaproteobacteria	3
Pleomorphomonas	Alphaproteobacteria	3
Streptomyces	Actinobacteria	3
Burkholderia (includes *Burkholderiaceae*)	Betaproteobacteria	2
Cellulomonas	Actinobacteria	2
Enterococcus	Firmicutes	2
Paludibacter	Bacteroidetes	2
Ralstonia	Betaproteobacteria	2
Variovorax	Betaproteobacteria	2
Afipia	Alphaproteobacteria	1
Aquabacterium	Betaproteobacteria	1
Dechloromonas	Betaproteobacteria	1
Deinococcus	Deinococcus-Thermus	1
Halomonas	Gammaproteobacteria	1
Janthinobacterium	Betaproteobacteria	1
Kocuria	Actinobacteria	1
Legionella	Gammaproteobacteria	1
Lysobacter	Betaproteobacteria	1
Methylocystis	Alphaproteobacteria	1
Microbacterium	Actinobacteria	1
Pantoea	Gammaproteobacteria	1
Pedomicrobium	Alphaproteobacteria	1
Phaeospirillum	Alphaproteobacteria	1
Pseudoxanthomonas	Gammaproteobacteria	1
Rhodoblastus sphagnicola	Alphaproteobacteria	1
Salmonella	Gammaproteobacteria	1
Sphingomonas (includes *Sphingobacteriales*)	Bacteroidetes	1

Table V-3. Summary of sequences by bacterial phyla across all samples analyzed.

Phyla	Number of genera detected	Sequences identified to genus level
Alphaproteobacteria	12	32
Betaproteobacteria	7	10
Gammaproteobacteria	7	53
Deltaproteobacteria	1	6
Actinobacteria	6	15
Firmicutes	5	29
Deinococcus-Thermus	1	1
Bacteroidetes	2	3

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APPENDIX 1

DRAFT MANUSCRIPTS WITH ADDITIONAL EXPERIMENTAL DETAILS

Degradation of RDX and other energetic compounds by xenobiotic reductases *xenA* and *xenB*.

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ABSTRACT

The degradation of the explosives, including hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), by xenobiotic reductases XenA and XenB (and the bacterial strains harboring these enzymes) under both aerobic and anaerobic conditions was examined was assessed. Under anaerobic conditions, *Pseudomonas fluorescens* I-C (*xenB*) degraded RDX faster than *Pseudomonas putida* II-B (*xenA*), and degradation occurred when the cells were supplied with sources of both carbon (succinate) and nitrogen (NH_4), but not when only carbon was supplied. Degradation was always faster under anaerobic conditions compared to aerobic conditions, with both enzymes exhibiting a O_2 concentration-dependent inhibition of RDX degradation. The primary degradation pathway for RDX was conversion to methylenedinitramine (MEDINA) and then to formaldehyde, but a minor pathway that produces 4-nitro-2,4-diazabutanal (NDAB) also appeared to be active during degradation by whole cells of *Pseudomonas putida* II-B and purified XenA. Both XenA and XenB also degraded the related nitramine explosives octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (CL-20). Additionally, purified XenB degraded more of the other explosive-related compounds screened than XenA. The results indicate that these two xenobiotic reductases may have the capacity to contribute to explosive compound biodegradation in natural and engineered environments under a range of redox conditions.

INTRODUCTION

Past and current activities at sites where munitions are manufactured and tested have resulted in the release of munition-related compounds. The environmental fate of these contaminants is an issue of significant concern to the United States Department of Defense (DoD), regulators, and the public because their mobility and persistence allows them to contaminate ground water supplies (33, 35). Recently, information describing the extent of soil and groundwater contamination at military training ranges has been published (15, 25).

Extensive research has examined the biological degradation of explosive compounds by pure cultures of bacteria and mixed consortia in soil and groundwater (see review (21)). Most research has focused on the dinitrotoluenes (DNT) and 2,4,6-trinitrotoluene (TNT), with interest in hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) increasing in recent years. RDX biodegradation has been observed under conditions ranging from fully aerobic (10, 16) to strictly anaerobic (2, 3, 6, 22, 27, 31), and at least three degradation pathways have been elucidated (Figure 1). Anaerobic processes involve either a direct attack on the ring structure or the successive reduction of the pendant nitro groups followed by ring cleavage (21, 28). Cytochrome P450 monooxygenases have been implicated in O_2-dependent denitration of RDX, leading to ring cleavage (18). Many bacterial strains can utilize RDX as a sole nitrogen source (13, 32, 38), but only recently has the use of RDX as a sole source of carbon, nitrogen, and energy been reported (32).

The degradation of nitroglycerin and TNT by the xenobiotic reductases (XenA and XenB) from the obligate aerobes *Pseudomonas putida* II-B and *Pseudomonas fluorescens* I-C, has been explored (11, 30). Though XenA and XenB are both members of the Old Yellow Enzyme family of flavoprotein oxidoreductases, and catalyze similar reactions, there are

significant differences in the catalytic rates and substrate specificities of the two. For example, purified XenB catalyzes the degradation of TNT ~5-fold faster than XenA, whereas the catalytic rates with nitroglycerin (NG) are approximately equal. However, XenA preferentially denitrates NG at the terminal positions (1 and 3 positions), whereas XenB preferentially denitrates NG at the interior position (2 position). Furthermore, the rate of TNT degradation by XenB was slightly enhanced under anaerobic conditions and the product distribution resulting from TNT degradation varied greatly under anaerobic conditions. Degradation of RDX by these enzymes was not characterized.

In the present study, the effect of decreasing O_2 tension on the catalytic characteristics of XenA and XenB expressed in their native bacterial hosts and as purified enzymes was explored. The results reveal that both enzymes are capable of degrading RDX, HMX, and a suite of related energetic compounds under reduced O_2 concentrations, but not necessarily under fully aerobic conditions. The observation that RDX can be degraded by aerobic organisms under reduced oxygen tensions could lead to enhanced bioremediation technologies and a better understanding of natural attenuation process.

MATERIALS AND METHODS

Chemicals. All chemicals were reagent grade or purer. The three nitroso-containing metabolites (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine; hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine; and hexahydro-1,3,5-trinitroso-1,3,5-triazine) of RDX were purchased from SRI International (Menlo Park, CA, USA). RDX (7% HMX as a manufacturing impurity) was a gift from James Phelan at Sandia National Laboratories (Albuquerque, NM, USA). HMX was a gift from Herb Fredrickson at the U.S. Army Engineer Research and

Development Center (Environmental Laboratory, Vicksburg, MS, USA). [^{14}C]-RDX (specific activity = 60.0 mCi/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA, USA). CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane) was obtained from ATK Launch Systems (Corinne, Utah, USA). Neat standards of nitroaromatic compounds were purchased from ChemService (West Chester, PA, USA).

Whole cell biodegradation assays. *Pseudomonas putida* II-B and *Pseudomonas fluorescens* I-C possessing the *xenA* and *xenB* genes, respectively, were screened for degradation of RDX, HMX, and CL-20. Two additional wild type organisms *Pseudomonas putida* F1 (39) and *Pseudomonas putida* KT2440 (a derivative of *P. putida* mt-2 cured of the TOL plasmid (34),) were included in this study because a BLAST search (4) using XenA and XenB as query sequences revealed that they both possessed multiple genes closely related to the xenobiotic reductases of *P. putida* II-B and *P. fluorescens* I-C. Thus *P. putida* F1 has two genes encoding peptides that are 96% and 45% identical to XenA, and one *xenB*-like gene that encodes a peptide that is 87% identical to XenB, while *P. putida* KT2440 has three genes encoding peptides that are 97%, 69%, and 45% identical to XenA, and one *xenB*-like gene that encodes a peptide with 87% identity.

A basal salts medium (BSM, (19)) was used for screening. The carbon source was succinate. Inocula were prepared by growing the strains in BSM plus succinate overnight, followed by concentration and washing of the cells twice with nitrogen-free BSM. The washed cells were used to inoculate vials of BSM medium amended with succinate (1 g/L) and RDX (~5 mg/L) or HMX (~1 mg/L). Vials were incubated at room temperature with shaking, and samples were removed periodically, passed through 0.45 µm glass microfiber filters into 2 mL glass sample vials, and analyzed for RDX, HMX, and breakdown products

by HPLC (see below). Experiments under anaerobic conditions were prepared and incubated in a glove bag.

Degradation of CL-20 (~1 mg/L initial concentration) by all four *Pseudomonas* strains was performed similarly, except that the screening was performed in polypropylene tubes instead of glass to prevent abiotic loss of CL-20. Anaerobic treatments were prepared and incubated in an anaerobic glove box. Samples were removed periodically, centrifuged in polypropylene microfuge tubes to remove biomass, and the supernatant was transferred to polypropylene high performance liquid chromatography (HPLC) vials for analysis (see below).

The effect that changes in the RDX concentration had on the rate and extent of degradation by *Pseudomonas putida* II-B and *Pseudomonas fluorescens* I-C was examined by adding washed cells to anaerobic BSM plus succinate amended with RDX at concentrations of 1, 3, 7, 14, and 28 mg/L. Samples were removed periodically and analyzed by HPLC. Direct toxicity of RDX (at 0, 19, and 34 mg/L) to these two strains was examined by monitoring cell density at 550 nm during aerobic growth in BSM plus succinate (a condition under which RDX was not degraded).

Production of nitrous oxide (N_2O) and nitrite (NO_2^-) from RDX were determined by incubating cultures of *Pseudomonas putida* II-B and *Pseudomonas fluorescens* I-C with RDX and periodically removing samples of the headspace and liquid for analysis (see analytical section below). To identify the RDX breakdown products, cultures were incubated with ~20 mg/L of RDX at room temperature with shaking, and frozen at -70°C after approximately 50% of the initial RDX had degraded. Frozen samples were shipped on dry ice to the Biotechnology Research Institute, National Research Council Canada for more

extensive analysis of RDX breakdown products according to previously described methods (23).

Cell free enzyme assays. Several experiments were performed to assess the catalytic properties of the xenobiotic reductases of *P. putida* II-B and *P. fluorescens* I-C, which were purified essentially as previously described (12, 30). The explosive degradation assays were performed with the test compounds dissolved in sodium phosphate buffer (100 mM, pH 7.4). A reductant in the form of NADPH was added to a final concentration of 1 to 2 mg/mL. Vials were purged with at least twenty volumes of O_2-free N_2 bubbled through the liquid, then transferred to an anaerobic chamber where 1 mL of the solutions were transferred to 2 mL glass screw cap auto-sampler vials (or polypropylene vials in the case of the explosive CL-20) and sealed with Teflon lined septa. To examine the effect of O_2 on the rate of RDX and HMX degradation, pure O_2 gas was added via a syringe inserted through the septum of the vial to bring the headspace O_2 concentration up to the desired percentage on a (v/v) basis with the headspace. An assay was initiated by injecting 1 µL of purified XenB (0.017 mg) or 1 to 5 µL of purified XenA (0.014 to 0.070 mg) through the septum. For kinetic assays, the vials were automatically and repeatedly analyzed via HPLC (see below). End-point experiments were incubated for no less than 24 h prior to analysis. Negative controls comprised of substrate, buffer, and NADPH were included in all experiments, and were used to detect and adjust for any non-enzymatic substrate losses.

To determine if RDX was converted to MNX during degradation by XenB, an experiment utilizing radiolabeled RDX was conducted. Briefly, the enzyme assay procedure described above was followed, except that the XenB and XenA enzymes were mixed with 16 mg/L of MNX and 12 mg/L [^{14}C]-RDX. Unlabeled MNX was included in the assay so that

if very small amounts of MNX were being formed and subsequently degraded by XenB during the degradation of RDX, the large pool of unlabelled MNX would slow down the degradation of the enzymatically formed [^{14}C]-MNX, which could then be detected using scintillation counting. The reaction vial was repeatedly sampled, and the degradation of the substrates was monitored via HPLC as described below, except that the HPLC eluant was collected at 20 s intervals into scintillation vials pre-filled with 3 mL of Optiphase HiSafe scintillation cocktail (Perkin-Elmer, Inc., Boston MA, USA). The time of elution of the radioactive peaks was compared with the elution time of the known explosive compounds and metabolites (RDX, MNX, DNX, and TNX) to determine if any of the [^{14}C]-RDX was being converted to [^{14}C]-MNX or other related compounds. Under the analytical conditions described below, there is more than a full minute separating the elution of MNX and RDX, which would be easily resolved with the described protocol.

Analytical. The concentrations of the explosives and their breakdown products were determined using HPLC according to a modified EPA Method 8330 using a Hewlett-Packard 1100 HPLC equipped with a Allure C18 column (Bellefonte, PA, USA) and a UV detector (230 nm). The mobile phase was 50:50 methanol:water at a flow rate of 0.9 mL/min. The column temperature was 25°C. The lower detection limit was approximately 25 µg/L for RDX and 50 µg/L for the RDX breakdown products. CL-20 was analyzed on the same system, except that the mobile phase was adjusted to 55:45 methanol:water, and detection was at performed at 228 nm.

Nitrous oxide was measured using GC-TCD. Nitrite was determined colorimetrically (Hach Company, Loveland, CO, USA). Ammonia was measured spectrofluorometrically (24). Hydrogen peroxide production was quantified using the Amplex Red Hydrogen

Peroxide/Peroxidase Assay Kit (Invitrogen Inc., Carlsbad, CA, USA) and a SpectraMax Gemini fluorescent plate reader (Molecular Devices Inc., Sunnyvale, CA, USA).

RESULTS

Degradation of RDX and other explosives by whole cells. During initial experiments all four *Pseudomonas* strains examined here were able to degrade TNT under aerobic conditions, but no aerobic degradation of RDX, HMX, or CL-20 was observed. Under anaerobic conditions, degradation of RDX was observed with *P. putida* II-B and *P. fluorescens* I-C, while HMX was degraded only by *P. fluorescens* I-C (Figure 2A, 2B). Neither RDX nor HMX was degraded by *P. putida* F1 nor KT2440. HMX was degraded to some extent by both *P. putida* II-B and *P. fluorescens* I-C when RDX was also initially present. The apparent first-order rate for RDX disappearance was about 14-fold higher for *P. fluorescens* I-C as compared to *P. putida* II-B (0.0084/h vs. 0.0006/h) at an initial RDX concentration of 6.8 mg/L. The RDX degradation rates of *P. putida* II-B and *P. fluorescens* I-C appeared to be concentration-dependent. The degradation rate decreased 3-fold and 10-fold for *P. putida* II-B and *P. fluorescens* I-C, respectively, as the initial RDX concentration increased from 0.7 to 28 mg/L. However, the aerobic growth of these two strains was not affected by the presence of RDX even at 34 mg/L. When incubated under conditions in which an initially aerobic medium was allowed to become O_2-depleted during the growth of the culture, both *P. putida* II-B and *P. fluorescens* I-C degraded RDX, but only *P. fluorescens* I-C degraded HMX. Degradation of CL-20 was observed by pure cultures of all four strains under anaerobic conditions, with *P. fluorescens* I-C degrading the compound

much faster than the other strains (Figure 2C). These findings suggested that O_2 either inhibited the expression or the activity of the catalytic enzymes in these strains.

RDX degradation by purified XenA and XenB enzymes. The fact that *P. putida* KT2440 and *P. putida* F1 both contain multiple genes similar to *xenA* and *xenB* raised the possibility that *P. putida* II-B and *P. fluorescens* I-C also carry multiple genes encoding xenobiotic reductases. In order to assure that results could be attributed to specific enzyme activities, experiments using purified XenA and XenB were conducted. Initial studies indicated that RDX was not degraded via a direct reduction of the nitro group (i.e., no nitroso-containing products were detected by HPLC), so a more detailed analysis of the degradation process was performed (Table 1). The product distribution resulting from RDX degradation differed not only between the XenA and XenB, but also between the purified enzymes and their source organisms. With both purified enzymes, the major products that accumulated indicated that RDX was degraded via the MEDINA pathway (Figure 1, Anaerobic II pathway), yet MEDINA did not accumulate in whole cell incubations. Formaldehyde was a major product of RDX metabolism by purified XenA and by XenB, whether degradation was performed with pure enzymes or in whole cells. The carbon mass balances for the degradation of RDX by the enzymes and whole cells ranged from 60% to 100% (mole C basis). With purified XenA, production of trace amounts of NDAB and MNX suggested that minor alternative reactions occurred with this enzyme that did not occur with XenB. However, detection of MNX was not reproducibly observed. Indeed, the [^{14}C]-RDX/MNX experiment gave no evidence that XenA or XenB produced MNX during the breakdown of RDX. All of the RDX derived radioactivity was contained in a broad peak that eluted before the known retention time of MNX, showing conclusively that MNX was not a

typical product of RDX breakdown by these enzymes. The identity of the compound (or compounds) in this early eluting peak were not identified, but based on other results reported here it is presumed that the peak is MEDINA.

Nitrogen mass balances ranged from 56% to 78%. As shown in Figure 1 (Anaerobic II pathway), RDX is converted to MEDINA and bis(hydroxymethyl)nitramine, and these compounds decay to form formaldehyde and nitramide, the latter of which may further break down to form nitrous oxide and nitrogen gas (20). Therefore, measurement of these inorganic nitrogenous products was performed, and percentages were calculated on the basis of the nitrogen present in the amount of RDX degraded during a given experiment. Nitrous oxide was not detected during RDX degradation with the purified enzymes, but small amounts of nitrous oxide (1 to 2 mol%) were detected during whole cell assays. Nitrite was detected during RDX degradation by purified XenB at a level of ~17 mol%. Nitrite was detected in whole cell assays with *P. putida* II-B and *P. fluorescens* I-C at levels ~2 mol% and ~12 mol%, respectively. Ammonia was detected during degradation of RDX by whole cells at levels equal to ~15 mol%, and during degradation of RDX by XenB (~23 mol%). However, the possibility that the assay was actually detecting one or more of the possible RDX breakdown products (eg. nitramide) rather than ammonia could not be ruled out. Inclusion of these inorganic nitrogenous products increased the nitrogen mass balances of the products produced during RDX degradation by *P. putida* II-B and *P. fluorescens* I-C to 75% and 102%, respectively (compared to 56% and 74% based on only the organic products with nitrogen are considered; Table 1). Similarly, the overall nitrogen mass balance for RDX degradation by XenB was increased to 118% when both organic and inorganic nitrogenous products are considered.

Co-degradation of RDX and HMX. Because HMX is a common contaminant of RDX preparations, and because the two compounds are often found together in the environment, we investigated whether HMX was degraded sequentially or consecutively with RDX. With purified XenB, degradation studies with high concentration RDX indicated that little to no HMX (present at approximately 10% the RDX concentration) was degraded in the presence of RDX. However, when RDX and HMX were present in equal concentrations, HMX and RDX were degraded simultaneously by XenB (Figure 3A). Similarly, when present as a mixture, the typical degradation products of RDX degradation (MNX, DNX, and TNX) all were degraded simultaneously with RDX (Figure 3B), with no obvious preference for any of the potential RDX metabolites.

Additional experiments were performed to determine the O_2 inhibition characteristics for RDX and HMX degradation. While XenB degraded RDX much faster than XenA (~30-fold), both enzyme systems had similar O_2 inhibition characteristics (Figure 4A, 4B). A similar effect was noted when HMX served as a substrate for XenB (Figure 4C). The percentage of saturation for O_2 that resulted in a 50% reduction in the initial linear degradation rates (derived from Figure 4) were 1.5 ± 0.3% and 1.6 ± 0.3% for RDX degradation by XenA and XenB, respectively, and 2.3 ± 0.4% for HMX degradation by XenB.

Aerobic and anaerobic degradation of various explosives by XenA and XenB. Purified XenA and XenB were examined for their ability to degrade a suite of explosive compounds under aerobic and anaerobic conditions (Table 2). Of the sixteen compounds tested, only six were degraded by XenA under aerobic conditions, whereas ten were degraded anaerobically. With XenB, a similar pattern was observed as nine of the

compounds degraded aerobically and fifteen were degraded anaerobically. In most cases where the substrates were degraded both aerobically and anaerobically, the reactions were faster and more extensive under anaerobic conditions. These results greatly expand the known substrate range of both XenA and XenB.

DISCUSSION

Only a single previous report has described the aerobic degradation of the nitramine explosive RDX by a *Pseudomonas* sp, though the enzymes involved and the degradation pathway were not discussed (14). In our study, RDX and HMX degradation by *Pseudomonas* spp. occurred under strictly anaerobic conditions, as well as under "anoxic" conditions created as cells consumed dissolved O_2 while growing on succinate. The biodegradation pathway described herein for RDX degradation by purified xenobiotic reductases and whole cells of *P. putida* II-B and *P. fluorescens* I-C leads to innocuous products (formaldehyde, nitrous oxide, etc), rather than more toxic nitrosolated compounds like those produced during other anaerobic processes (1, 36), or dead-end products like NDAB that is produced during aerobic degradation by some *Rhodococcus* spp (Figure 1, Aerobic pathway). These innocuous products are more desirable end point for bioremediation applications.

Unlike previously described *Rhodococcus* spp. (16, 29), RDX degradation by pure cultures in this study was not inhibited, but rather was facilitated, by the presence of utilizable nitrogen (NH_4). Degradation rates by whole cells decreased with increasing RDX concentrations, whereas the RDX degradation rate from purified XenB increased with

increasing RDX concentration. Additionally, the aerobic growth rates of *P. putida* II-B and *P. fluorescens* I-C were not inhibited with increasing RDX concentration. Taken together, these results suggest that although RDX itself is not toxic to either the cells or the degradative enzymes described here, the breakdown products may exert toxicity by an unknown mechanism. This finding is in general agreement with previous results showing toxicity in another pseudomonad during aerobic degradation of RDX (14).

Previous studies with xenobiotic reductases (and related enzymes) have shown that the presence of O_2 can impact the degradation of explosive compounds in more than one way. For example, Pak et al. (2000) noted that while TNT was degraded by XenB both aerobically and anaerobically, the presence of O_2 changed the product distribution. Most notably, certain TNT dimers accumulated, resulting in the release of nitrite only in the presence of O_2 (or other oxidants such as $NADP^+$) via an abiotic mechanism. In another study investigating degradation of RDX by three *Enterobacteriaceae* isolates, O_2 also played a key role in the final outcome, as RDX was degraded only under oxygen-depleted conditions (26). Similarly, it was reported that RDX degradation by *Klebsiella pneumoniae* strain SCZ-1 was completely quenched by the presence of O_2, though the concentrations of O_2 required to stop RDX degradation was not reported (37).

In the present study, O_2 had a large impact on the activity of XenA and XenB. When assayed under reduced O_2, these enzymes were capable of degrading a much broader suite of explosive compounds than previously recognized (Table 2). RDX and HMX were among these, which is a significant finding because these compounds are generally recognized as being more recalcitrant to biological degradation than TNT. It is also important to note that O_2 did not function as a binary on/off switch for the degradation of RDX (and HMX), but

rather it caused a gradual decrease in RDX degradation as a function of the initial O_2 concentration.

This pattern of O_2 dependence is consistent with auto-oxidation processes occurring concurrently with substrate reduction. Others have suggested that RDX degradation is initiated with the transfer of a single electron that creates an unstable RDX radical that undergoes spontaneous denitration and a series of rearrangements to yield MEDINA and bis(hydroxymethyl)nitramine as shown in Figure 1 (Anaerobic II pathway) (20). In this scenario, the presence of O_2 may oxidize the RDX radical, forming superoxide, and simultaneously return RDX to its original, stable form. If such a scenario is occurring during XenB-mediated RDX degradation, then superoxide would be expected to form during catalysis, with the concomitant production of hydrogen peroxide.

We attempted to determine if this was the case, but found that when supplied with NADPH, XenB generated hydrogen peroxide even in the absence of RDX, and surprisingly, NADPH was consumed more rapidly in the absence of RDX under aerobic conditions (data not presented). While these results cannot exclude the possibility that O_2 interferes with RDX degradation by quenching an unstable radical, the formation of hydrogen peroxide suggests that O_2 might simply be displacing RDX from the active site of XenB in a concentration dependent manner and be adventitiously reduced to hydrogen peroxide.

Furthermore, we observed that there was little difference between the degradation rates observed for the products of RDX nitro group reduction (MNX, DNX, and TNX) when these compounds were present in roughly equal proportions. While the products resulting from the degradation of these intermediate products has not been determined, the fact that TNX,

which contains no nitro groups, is transformed by XenB, clearly indicates that TNX is not transformed via an attack on a nitro group.

This work also adds to the information base for the new energetic compound CL-20. CL-20 was developed as a more environmentally friendly replacement for RDX in a wide variety of munitions. In general, CL-20 has been found to be more labile than RDX in soil (5, 17). Our studies have identified an anaerobic degradation mechanism for CL-20 by purified enzymes and whole cells that is different from the few previously published reports, which implicated monooxygenases (8), nitroreductases (7), and membrane-associated flavoenzymes (9). It is interesting to note that all four *Pseudomonas* spp. strains tested degraded CL-20, but only *P. putida* II-B and *P. fluorescens* I-C degraded RDX, likely reflecting the lower chemical stability of CL-20 compared to RDX cited above.

The addition of RDX, HMX, and CL-20 to the list of known substrates for the xenobiotic reductases, under reduced O_2 tension, has important implications for bio-remediation efforts. Basic research in environmental microbiology is often directed toward the isolation, and characterization of bacterial strains that use a target compound as a sole source nutrient (for carbon, nitrogen, or energy). However, actual field-scale bioremediation is dominated by biostimulation approaches (i.e., addition of nutrients to stimulate the indigenous microbial community) rather than the addition of specific strains which derive nutrients from a pollutant. The results reported here support the practice of general biostimulation approaches to effect remediation of explosives-contaminated sites as follows: 1) degradation occurs under a relatively broad range of O_2 concentrations (anoxic to anaerobic); 2) degradation is not inhibited by the presence of utilizable nitrogen, and; 3) degradation is performed by a class of enzyme that is widespread amongst bacterial genera. Several studies in our

laboratory examining the microbial ecology of RDX biodegradation have detected *Pseudomonas* spp. 16S rDNA sequences in RDX-degrading enrichments derived from groundwater from an explosives manufacturing site (unpublished data). Furthermore, given the widespread distribution of *Pseudomonas* spp. in the environment, it is likely that these organisms play a larger role in the degradation of nitramine explosives than previously thought, which could be further expanded when environmental conditions are manipulated to maximize their degradative potential.

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Table 1. Product distribution during degradation of RDX by purified XenA and XenB enzymes (average of duplicate assays), and by whole cells of *Ps. putida* II-B and *Ps. fluorescens* I-C (single replicates).

	RDX (μmoles)		Products (μmoles)				Mass Balance (%)	
Assay	Initial	Residual	MNX	MEDINA	NDAB	HCHO	C	N
Ps. fluorescens I-C	21.5	15.0	0.0	1.4	0.0	13.2	92	74
Ps. putida II-B	21.5	11.7	0.0	0.4	0.3	2.7	60	56
XenB	69.1	29.2	0.0	37.4	0.0	82.2	100	78
XenA	69.1	28.9	0.2	23.5	1.5	76.1	98	66

Table 2. Degradation of explosive-related compounds by xenobiotic reductases XenA and XenB under aerobic and anaerobic conditions. Minimum incubation time was 24 h. Negative results indicate that less than 10% of the initial concentration was degraded. A plus sign indicates that >10% of the compound was degraded in the timeframe of the experiment.

Compound		**Initial Concentration (µM)**	**Degradation**			
			XenA		**XenB**	
			Aerobic	**Anaerobic**	**Aerobic**	**Anaerobic**
Hexahydro-1,3,5-trinitroso-1,3,5-triazine	TNX	33	-	+	-	+
Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine	DNX	29	-	+	-	+
Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine	MNX	33	-	+	-	+
Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine	HMX	5	-	+	-	+
Hexahydro-1,3,5-trinitro-1,3,5-triazine	RDX	89	+	+	+	+
2,4,6,8,10,12-Hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane	CL-20	3	+	+	+	+
Nitrobenzene	NB	47	-	-	-	+
1,3-Dinitrobenzene	1,3-DNB	15	+	+	+	+
1,3,5-Trinitrobenzene	TNB	23	+	+	+	+
2-Nitrotoluene	2-NT	85	-	-	-	-
4-Nitrotoluene	4-NT	46	-	-	-	+
2,4-Dinitrotoluene	2,4-DNT	24	+	+	+	+
2,6-Dinitrotoluene	2,6-DNT	37	-	-	+	+
2-Amino-4,6-dinitrotoluene	2A-4,6-DNT	54	-	-	+	+
4-Amino-2,6-dinitrotoluene	4A-2,6-DNT	23	-	-	+	+
2,4,6-Trinitrotoluene	TNT	88	+	+	+	+

[a] ND, Not determined.

Figure 1. Known degradation pathways for RDX. Pathways derived/adapted from reference 18.

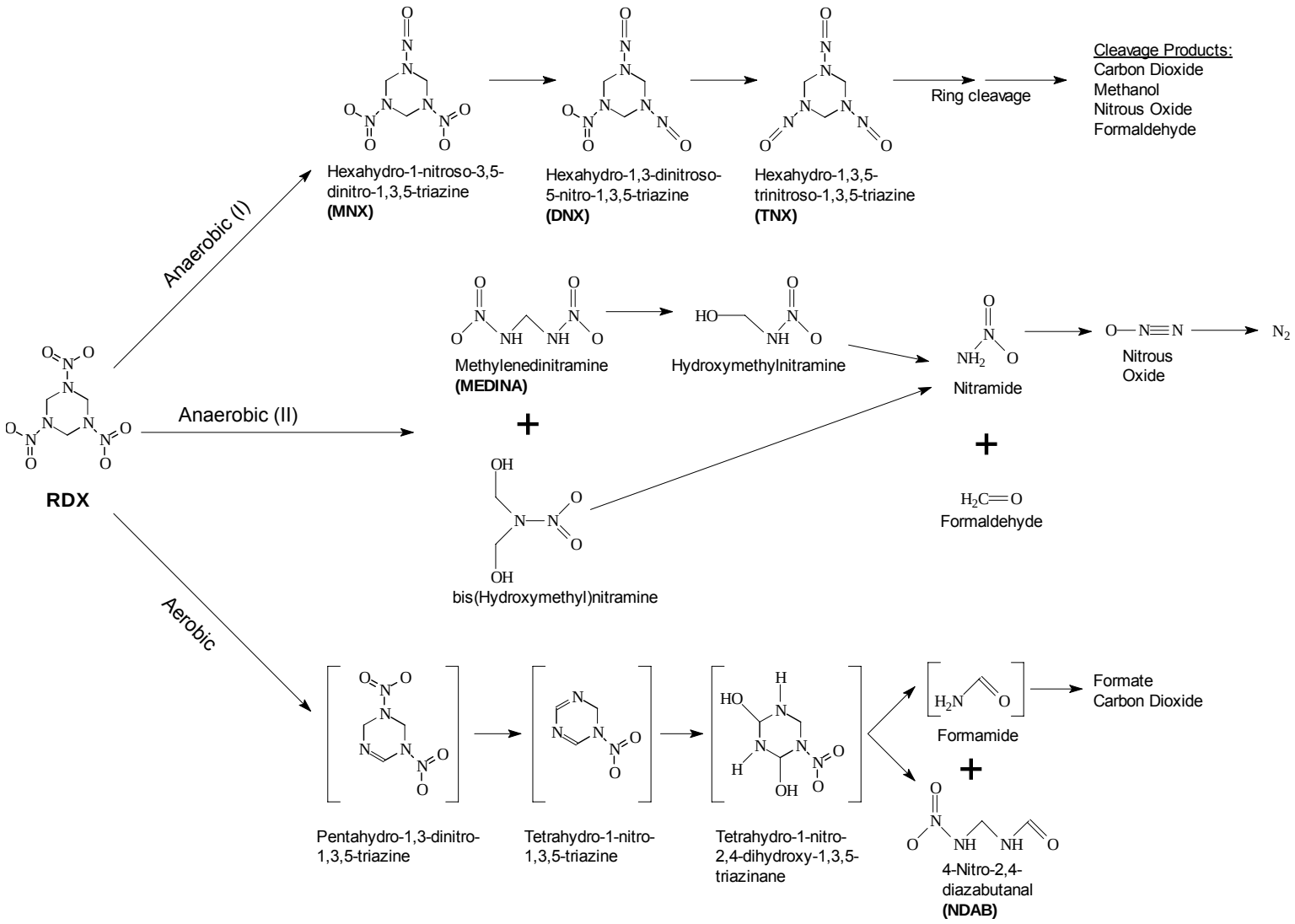


Figure 2. Degradation of A) RDX, B) HMX, and c) CL-20 by pure cultures of *Pseudomonas* spp. under anaerobic conditions. Sterile control (—); *P. putida* II-B (○); *P. fluorescens* I-C (□); *P putida* F1 (●); *P. putida* KT2440 (△). Datapoints represent average of two replicate cultures. Note difference in x-axis scales.

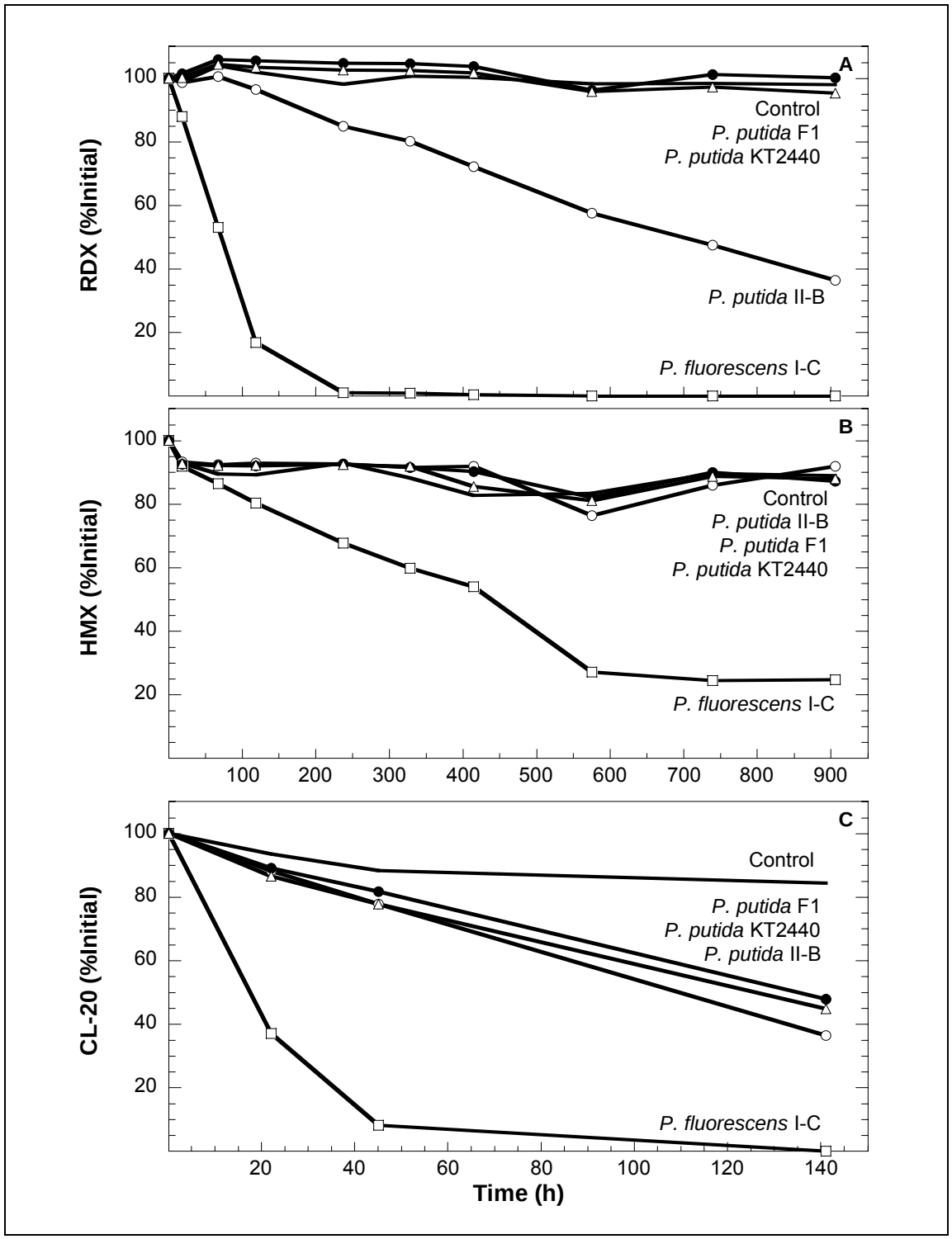


Figure 3. Simultaneous degradation of A) RDX/HMX and B) RDX/MNX/DNX/TNX by purified XenB enzyme. Data points represent average of two replicate cultures. Note difference in x-axis scales.

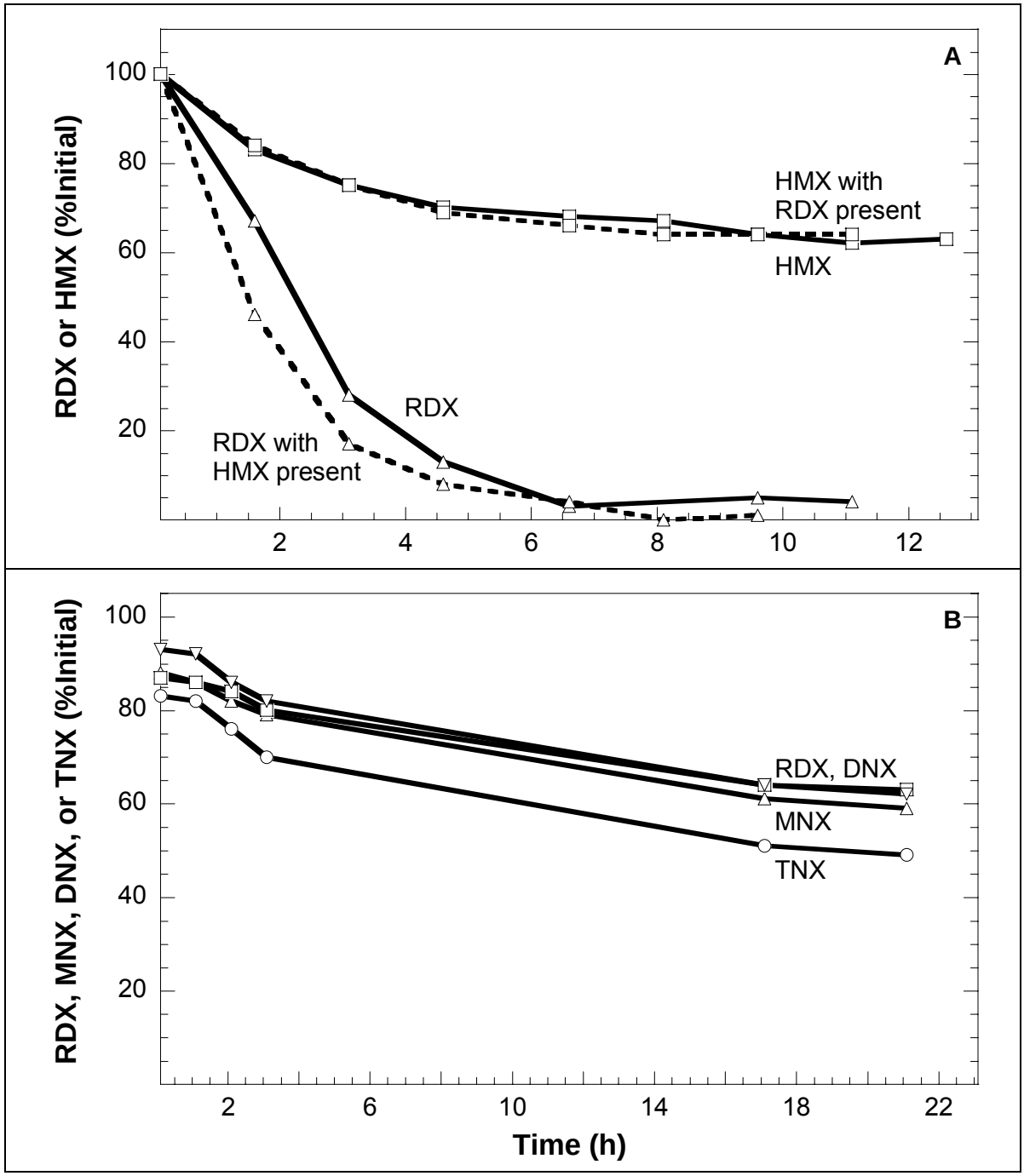
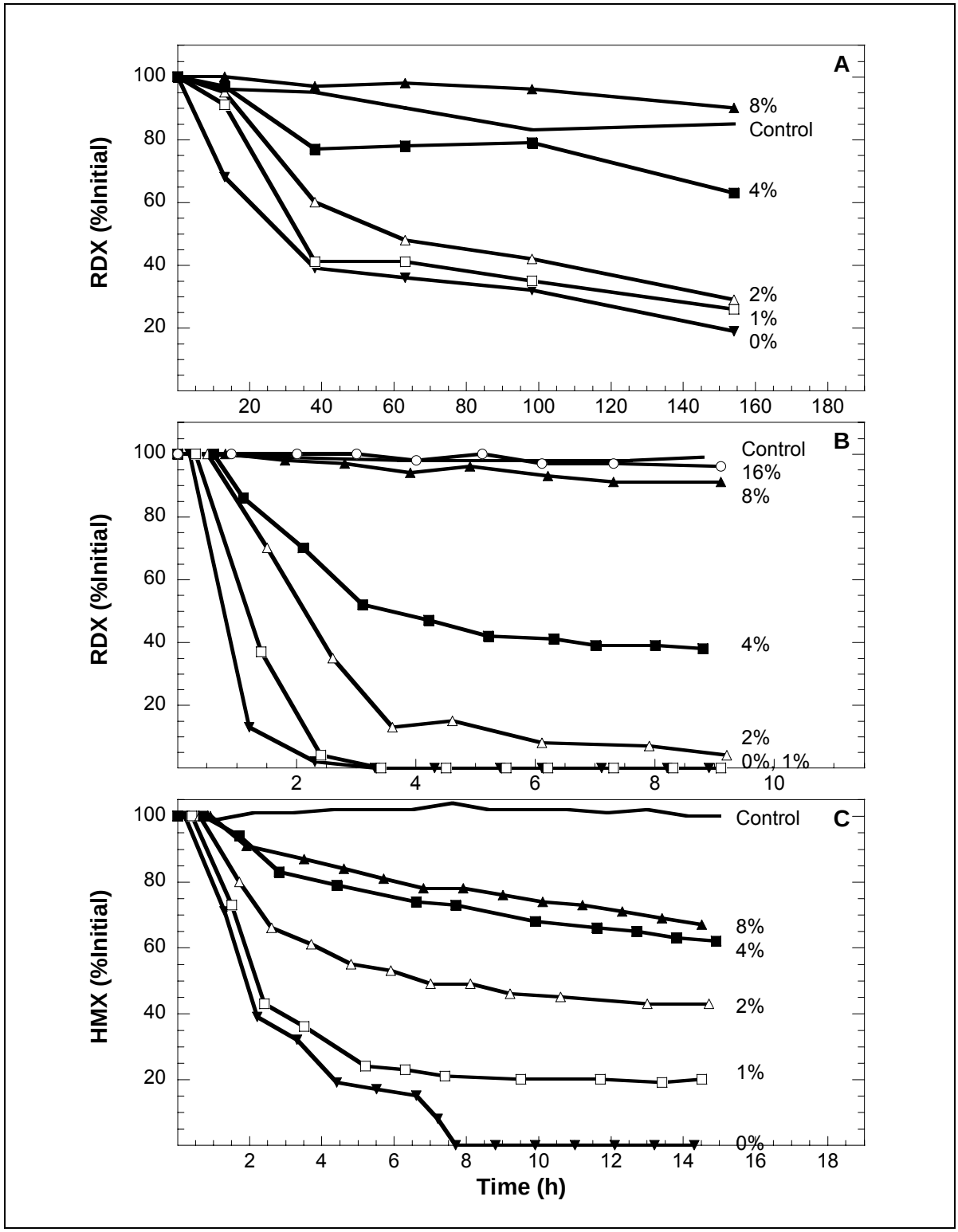


Figure 4. Degradation of RDX by purified A) XenA and B) XenB enzymes, and C) HMX by purified XenB enzyme under different initial oxygen concentrations. Each line represents data from two duplicates. Note difference in x-axis scales.



Anaerobic degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by three *Rhodococcus* strains.

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ABSTRACT

We report the anaerobic degradation of RDX by whole cells of three *Rhodococcus* strains. When succinate was supplied as the carbon source, RDX was used as the nitrogen source, and also produced the breakdown product 4-nitro-2,4-diazabutanal (NDAB), with lesser amounts of formaldehyde and methylenedinitramine (MEDINA) also observed.

Soil and groundwater contamination with explosive compounds has generated significant concern because of their mobility and persistence (26). The extent of contamination, which occurs during munition production and military training operations, is currently being assessed (7, 16).

One of the important energetic compounds of concern is hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). RDX biodegradation has been observed under conditions ranging from fully aerobic (5, 8) to strictly anaerobic (1, 2, 3, 12, 19, 22). Many bacterial strains can utilize RDX as a sole nitrogen source (6, 24, 28), but not until recently has the use of RDX as a sole source of carbon, nitrogen, and energy been reported (24). .

This work was undertaken to examine the ability of three *Rhodococcus* strains that have been previously shown to degrade and use RDX aerobically as a sole nitrogen source to anaerobically degrade RDX.

Chemicals and media. Research quantities of RDX (7% HMX as a production impurity) was a gift from James Phelan at Sandia National Laboratories (Albuquerque, NM, USA). All other chemicals were reagent grade or purer. Basal salts medium (BSM) was prepared

according to Hareland at al. (11). Nitrogen-free BSM (BSM-N) was prepared similarly, except that no ammonium chloride or nitriloacetic acid was added.

Bacterial strains. *Rhodococcus* strains capable of RDX biodegradation were acquired from the following sources (reference describing explosive degradative abilities in parentheses): *Rhodococcus rhodochrous* 11Y (NCIMB 40820), Dr. Neil C. Bruce, University of York, GB (via NCIMB Ltd., Aberdeen, UK) (23); *Rhodococcus* sp. DN22, Dr. Diane Fournier, National Research Council, Canada (8). Strains were maintained on R2A agar and minimal agar (BSM-N solidified with noble agar) supplemented with succinate (1000 mg/L) as the carbon source and RDX (5 mg/L) as the sole nitrogen source.

RDX and HMX degradation screening. *Rhodococcus* strains were grown overnight in BSM with succinate. Cells were pelleted by centrifugation (3400 rpm, 4°C), washed twice, and resuspended in anaerobic BSM-N. Duplicate vials (35 ml total volume in 40 ml glass vials) of BSM-N amended with RDX (~5 mg/L with 0.4 mg/L HMX), with and without succinate (1000 mg/L), were inoculated in a glove bag to achieve an optical density at 550 nm of 0.02. All solutions were purged with nitrogen for 10 minutes, then equilibrated in the anaerobic glove bag (Coy Laboratory Products, Grass Lake, MI, USA) overnight with stirring prior to use. Vials were incubated at room temperature with gentle shaking in the glove bag. Samples were removed periodically and analyzed for RDX, HMX, and metabolites. Degradation of HMX was examined similarly at an initial concentration of ~2 mg/L. Growth was assessed by measuring the optical density at 550 nm (OD_{550}) before and after each addition of RDX and/or succinate. Culture liquid streaked onto agar plates (BSM agar with succinate and RDX; R2A agar) and incubated in the glove bag indicated cultures remained pure.

To more precisely identify the RDX breakdown products, cultures were incubated similarly with 5 mg/L of RDX at room temperature with shaking, and frozen at -70°C after approximately 50% of the initial RDX had degraded. Frozen samples were shipped on dry ice to the Biotechnology Research Institute, National Research Council Canada for more extensive analysis for MEDINA, NDAB and formaldehyde according to previously described methods (13).

Analytical. The concentrations of the explosives and their breakdown products were determined using HPLC according to a modified EPA Method 8330 using a Hewlett-Packard 1100 HPLC equipped with a Allure C18 column (Bellefonte, PA, USA) and a UV detector (230 nm). The mobile phase was 50:50 methanol:water at a flow rate of 0.9 ml/min. The column temperature was 25°C. The lower detection limit was approximately 25 µg/L for RDX and 50 µg/L for the RDX breakdown products.

All three strains degraded RDX under anaerobic conditions when supplied with succinate (Figure 1A). Although the degradation rates were slow, strains 11Y and A degraded RDX with no apparent lag period, whereas a lag of several days was observed with strain DN22. Without succinate, the three strains also partially degraded RDX, but all three strains exhibited rapid RDX degradation upon succinate addition (Figure 1B). No degradation of HMX occurred.. Each addition of succinate and RDX resulted in cell growth based on the observed 2- to 4-fold increase in the OD_{550} of the cultures. Among the three strains, DN22 degraded RDX the least (both in terms of rate and extent) and grew only minimally. In contrast, strain 11Y exhibited the fastest RDX degradation rates compared to the other two strains.

The breakdown products from anaerobic RDX degradation by the three strains are presented in Table 1. NDAB was the major product, followed by a mixture of formaldehyde and MEDINA. NDAB has also been observed as main product of aerobic RDX degradation by DN22 (10), while MEDINA and formaldehyde have previously been detected during anaerobic RDX degradation processes (1, 3, 4, 13). These results do corroborate the findings of Jackson *et al.* (2007) (15) who found that the purified RDX-degrading enzymes from strain 11Y, XplA and XplB, produces NDAB under aerobic conditions but MEDINA under anaerobic conditions in cell-free assays. The reasons for the mix of products observed during this work is likely due to the use of whole cells as compared with purified enzymes.

The anaerobic degradation RDX by whole cells of these *Rhodococcus* strains is interesting, given that these organisms are usually considered to be aerobes (14). Rhodococci species have variable abilities to reduce nitrate, and some have been shown to grow and/or degrade specific compounds under denitrifying conditions (20, 25, 27). However, reports of activity and growth (albeit slow) under anaerobic conditions are sparse (9, 21). The most similar previous report detailed the transformation by two *Rhodococcus erythropolis* strains of the nitroaromatic compounds 2,4-dinitrophenol and picric acid (2,4,6-trinitrophenol) (17, 18). The authors indicated that transformation of both compounds by resting cells was much slower under anaerobic compared to aerobic conditions. Growth at the expense of these nitroaromatic compounds as a sole nitrogen source was not examined. We report here that under anaerobic conditions the three *Rhodococcus* strains tested were able to grow using succinate as the carbon source and RDX as the nitrogen source.

In the larger context of environmental remediation, this work indicates that RDX degradtion under anoxic and anaerobic conditions may be attributed to a wider range of bacterial species that would be normally assumed.

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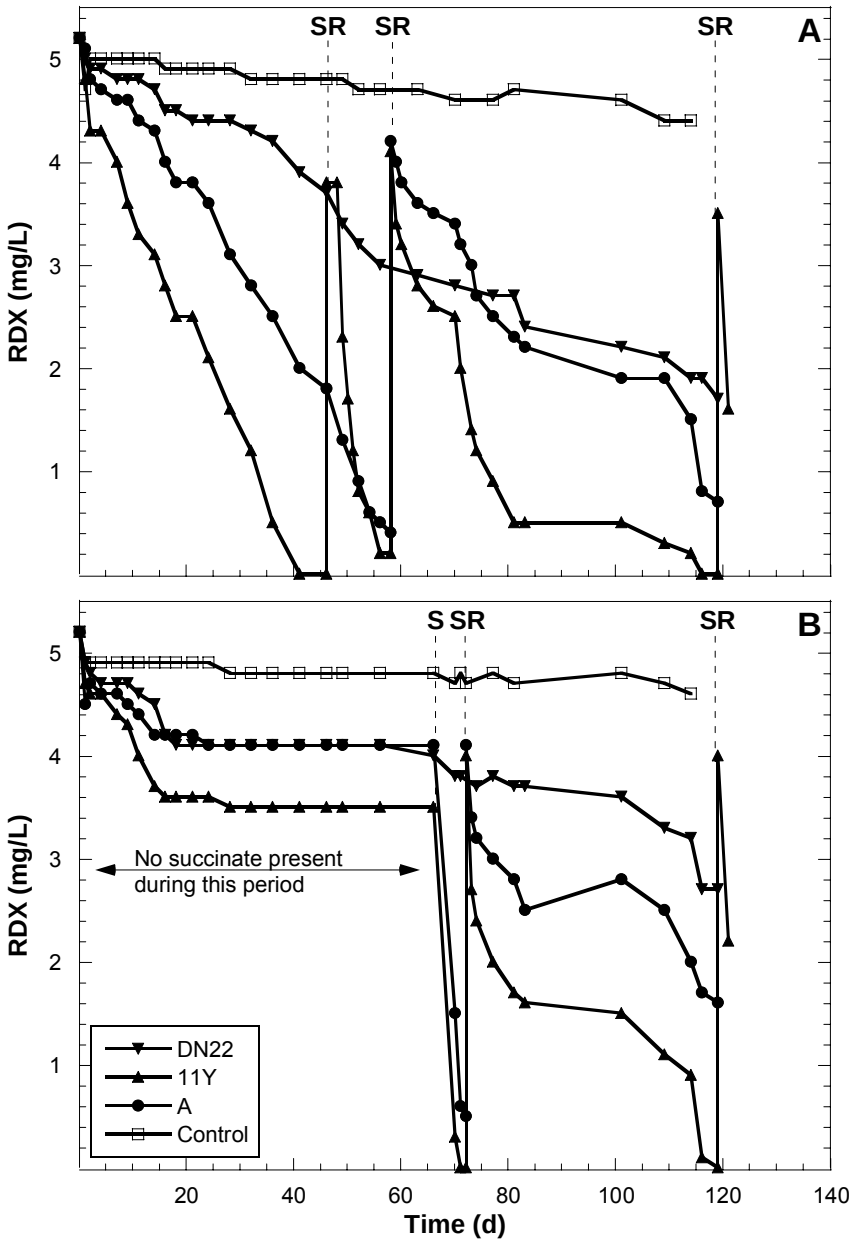
Table 1. Product distribution from anaerobic RDX degradation by three *Rhodococcus* strains.

	RDX (µmoles)		Products (µmoles)				Mass Balance (%)	
Assay	**Initial**	**Residual**	**MNX**	**MEDINA**	**NDAB**	**HCHO**	**C**	**N**
RDX only								
DN22	--[a]	--	--	--	--	--	--	--
11Y	21.5	11.3	0.0	0.2	7.4	1.9	80	70
Strain A	21.5	12.4	0.0	0.2	7.0	1.0	81	74
RDX+Succinate								
DN22	21.5	15.3	0.6	0.2	1.6	0.8	80	78
11Y	21.5	9.9	0.0	0.3	9.6	2.5	80	69
Strain A	21.5	8.8	0.0	0.5	8.9	5.9	78	63

[a] Strain DN22 did not degrade sufficient RDX without succinate present so product ananlysis was not performed

Figure 1. Degradation of RDX by three *Rhodococcus* strains in the presence (A) and initial absence (B) of succinate. When succinate was added to the initially succinate-free vials, RDX was quickly degraded. On the graphs, an S denotes when succinate was added, and an R denotes when RDX was added. Succinate was added to all vials including the controls. RDX was only added to vials in which most of the RDX had degraded. Mineral nutrients were added with the first supplemental succinate addition. Graph represents the average of two replicate vials, with the exception of the control in (B), where one control was lost during the experiment.



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Identification of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)-degrading microorganisms via ^{15}N-stable isotope probing

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Identification of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)-degrading microorganisms via ^{15}N-stable isotope probing

ABSTRACT

This study reported the application of ^{15}N-stable isotope probing (SIP) to identify active hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)-utilizing microorganisms in groundwater microcosms. Fifteen 16S rRNA gene sequences were derived from the ^{15}N-DNA fraction (contributed from active microorganisms capable of using RDX as a nitrogen source) of microcosms receiving cheese whey. The 16S rRNA gene sequences belonged to *Actinobacteria* (2 clones), α-*Proteobacteria* (7 clones) and γ-*Proteobacteria* (6 clones). Except for five sequences with high similarity to two known RDX-degraders (*Enterobacter cloacae* and *Pseudomonas fluorescens* I-C), our results suggested that phylogenetically diverse microorganisms were capable of using RDX as a nitrogen source. Six sequences of the *xpl*A gene (a known RDX-degrading catabolic gene) were also detected from the ^{15}N-DNA fraction. The *xpl*A gene sequences were 96 to 99% similar to the *xpl*A gene of *Rhodococcus* sp. DN22 (a known RDX-utilizer), suggesting that other RDX-utilizing bacteria might also contain *xpl*A-like genes. Twenty-five 16S rRNA gene sequences recovered from the unenriched, RDX-contaminated source groundwater clustered differently from those obtained from the ^{15}N-DNA fraction of the cheese whey amended microcosm. Our results suggested that active RDX-utilizing microorganisms can be stimulated by carbon source additions even if they are present at low densities, and that use of ^{15}N-SIP can help to identify these minority members of the microbial community.

INTRODUCTION

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a cyclic nitramine explosive that has been widely used in military and many civilian applications since World War II. The widespread use of RDX has resulted in contamination of many soils and groundwater in the United States and other countries [1-3]. Because RDX is soluble, non-volatile, and adsorbs poorly to soils [3], once it is released into soils it moves quickly into the groundwater, potentially impacting local drinking water supplies [4]. As RDX is a possible human carcinogen, a drinking water guideline of 2 μg RDX/L for lifetime exposure for adults is recommended by the U. S. Environmental Protection Agency (EPA) [5]. RDX is currently on EPA's Contaminant Candidate List 3.

Biodegradation of RDX has been reported under both aerobic and anaerobic conditions [6-22]. However, successful *in-situ* bioremediation of RDX has remained a challenge. This is in part due to our limited knowledge of the microbial ecology of RDX biodegradation under various geochemical conditions. Our understanding on RDX biodegradation is mainly derived from RDX-degrading isolates. Several microorganisms within a broad range of bacterial genera are known to degrade RDX through growth-linked reactions (RDX as a nitrogen source) or through non-growth-linked reactions. A wide range of anaerobic microorganisms, including two *Acetobacterium* species [15, 23], six *Clostridium* species [24-26], *Citrobacter freundii* NS2 [27], *Desulfovibrio desulfuricans* [26], *Enterobacter cloacae* [28], *Klebsiella pneumoniae* SCZ-1 [22], *Morganella morganii* B2 [27], *Providencia rettgeri* B1 [27], and *Serratia marcescens* [29], are known to degrade RDX.

Under aerobic conditions, several *Rhodococcus* isolates (including *Rhodococcus* sp. strain DN22 and *Rhodococcus rhodochrous* strain 11Y) are capable of utilizing RDX as a

nitrogen source [12, 30, 31]. A worldwide survey has shown that this ability is widespread in this genera and is mediated by the *xplA* and *xplB* genes [32]. Only recently did Thompson et al. [21] report the isolation of two bacteria (*Gordonia* sp. and *Williamsia* sp.) capable of using RDX as a sole carbon and nitrogen source, albeit at very slow rates. These results strongly indicated there are knowledge gaps regarding the ability of bacteria to use RDX as a sole C- and/or N-source.

Despite isolation of numerous RDX-degraders, the identities of the active microorganisms that are responsible for RDX biodegradation *in-situ* remains largely unknown. A powerful new technique called stable isotope probing (SIP) has allowed researchers to identify metabolically active microorganisms in complex engineered and natural systems [33-37]. This study explored the feasibility of using ^{15}N-SIP to identify active RDX-utilizing populations in microcosms amended with two different nutrient (carbon plus nitrogen) sources (cheese whey and yeast extract). The ^{15}N-SIP approach was first validated with two non-RDX-utilizers (*Escherichia coli* and *Pseudomonas fluorescens*) and a known RDX degrader (*Rhodococcus* sp. DN22), and then applied to RDX-degrading microcosms derived from RDX-contaminated groundwater. Active RDX-degrading microbial populations were identified after nucleic acid isolation and purification based on 16S rRNA gene sequences. This study also detected the presence of the known RDX-degrading gene *xpl*A in RDX-degrading microcosms. Results of this study demonstrate the effectiveness of ^{15}N-based SIP for identifying active compound transforming microorganisms, and enhance our understanding of the microbial ecology of RDX biodegradation.

MATERIALS AND METHODS

Chemicals. Ring-^{15}N-labeled-RDX (99.2% chemically pure, 50 mol% ^{15}N) was

synthesized by a private research corporation. Ammonium chloride ($^{15}NH_4^+$) and sodium nitrate ($^{15}NO_3^-$) were purchased from Isotec, Inc. (Miamisburg, OH). Cesium chloride (99.999% pure) was obtained from Fisher Scientific (Fair Lawn, NJ). Ethidium bromide (EtBr) was purchased from Promega Corp. (Madison, WI).

Bacterial cultures. Two non-RDX-utilizers (*Escherichia coli* and *Pseudomonas fluorescens*) and one RDX-utilizer (*Rhodococcus sp*. DN22, referred as DN22 hereafter) were used to validate the ^{15}N-SIP approach. *E. coli* (~ 51 % G+C content) and *P. fluorescens* (~ 62% G+C content) were used as reference bacteria in our previous studies [38, 39]. DN22 (~ 67% G+C content) is a known to use RDX as a sole nitrogen source (N-source) [9, 31] and was kindly provided by Dr. Nicholas V. Coleman, School of Molecular and Microbial Biosciences, University of Sydney. *E. coli* and *P. fluorescens* were supplied with glucose as a sole carbon source (C-source) and unlabeled NH_4^+ or $^{15}NO_3^-$ as a sole N-source. For DN22, succinate was supplied as a sole C-source and one of four N-sources (unlabeled NH_4^+, $^{15}NH_4^+$, RDX, and ring-^{15}N-labeled RDX) were supplied. All the three strains were grown at 30ºC overnight (optical density of cell suspension at A_{600} was about 1.0) before being harvested for DNA extraction. Extracted DNA was later used in SIP experiments.

Sample site, aquifer-enrichment columns, and microcosms. Aquifer sediments and groundwater were collected from Area 157 at the Picatinny Arsenal (New Jersey) which had a history of soil and groundwater contamination with explosives. The groundwater contained explosives including RDX, TNT (2,4,6-trinitrotoluene), HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine), TNB (1,3,5-trinitrobenzene), DNTs (2,4-dinitrotoluenes), and amino-DNTs) at concentrations of low to mid µg/L. Groundwater chemical analyses indicated low phosphate, TKN, and sulfate (15 to 20 mg/L), and total/dissolved organic carbon on the order of 1 to 2

92 mg/L. The biomass in the groundwater (well 157MW-5) was concentrated by filtering about
93 four liters through Sterivex filter cartridges (0.22 µm, Millipore Corp., Billerica, MA), which
94 were stored at -80ºC for later molecular analysis.

95 The groundwater and sediments were used for constructing five small columns (15 cm x
96 2.5 cm ID) similar to methods described elsewhere [40]. Briefly, the columns were operated
97 with an upward flow (0.5 ml/h) of groundwater containing RDX and other explosives (30 to 50
98 µg/L). Cheese whey (100 or 1,000 mg/L) or yeast extract (100 mg/L), were added to the influent
99 prior to entering the bottom of the soil columns. Effluent samples were collected periodically
100 and analyzed for explosive compounds by high performance liquid chromatography (HPLC, see
101 below). After RDX degradation in the columns was established, effluent samples from the
102 columns were used to setup the respective microcosms as described below.

103 Microcosms (160 ml serum bottles) were inoculated with column effluent (5 mL) and
104 amended to achieve final concentrations of RDX (5 mg/L), ammonium chloride (2 g/L), cheese
105 whey or yeast extract (1 g/L, corresponding to the carbon source of the column from which the
106 inoculum was taken), and nitrogen-free basal salts medium [41] to a final volume of 100 ml.
107 Either unlabeled or ring-^{15}N-labeled-RDX was added. Enrichment condition for each of the
108 microcosms is summarized in Table 1. Microcosms were prepared aerobically, and allowed to
109 become anoxic during growth after inoculation. Microcosms were incubated at 15°C with
110 shaking, and samples were removed periodically and analyzed for RDX and breakdown
111 products. When RDX was completely degraded, the bottles were respiked with additional RDX
112 (see Figure S1 in supporting material). All bottles were sacrificed after 25 days, and DNA was
113 extracted from liquid samples, and analyzed.

Chemical analysis. The concentrations of the explosives and their breakdown products were determined using HPLC according to a modified EPA Method 8330 using a Hewlett-Packard 1100 HPLC equipped with a Allure C18 column (Bellefonte, PA, USA) and a UV detector (230 nm) [42]. The mobile phase was 50:50 methanol:water at a flow rate of 0.9 ml/min. The column temperature was 25°C. The lower detection limit was approximately 50 μg/L for the RDX and breakdown products.

DNA extraction. Genomic DNA of each bacterium was extracted using FastDNA kit (Q-Biogene Bio 101, Carlsbad, CA) according to manufacturer's instructions. For microcosms and groundwater samples, the FastDNA SPIN kit for soil (MP Biomedical LLC, Solon, OH) was used as described by Yu and Chu [38] with a modified cell lysing process: the lysing matrix tube was processed 2 x 30s in FastPrep instrument. Concentrations of extracted DNA were measured by using a Hoefer DyNa Quant 200 fluorometer (Pharmacia Biotech, San Francisco, CA).

^{15}N-DNA and ^{14}N-DNA separation. The ^{15}N-DNA and ^{14}N-DNA fractions were separated by equilibrium centrifugation in CsCl-EtBr density gradients similar to that described by Yu and Chu [39]. Briefly, DNA solution was prepared in 3.9-mL Beckman centrifuge tubes containing 200 μL of EtBr (10mg/mL) and 1.034 g/mL CsCl solution in TE buffer. A tabletop Beckman TL-100 ultracentrifuge with a TLN-100 rotor was used for centrifugation at 77,000 rpm (265,000*g*) at 20 °C for 24 h. The ^{15}N-DNA and ^{14}N-DNA bands in the tubes were visualized under long-wavelength (365 nm) of UV light. The ^{15}N-DNA and ^{14}N-DNA bands (approximately 100-200 μL) were carefully withdrawn from the tube by using a disposable syringe (1 mL) with a sterile 21-gauge hypodermic needle. DNA was extracted from the CsCl-EtBr solution with water-saturated *n*-butanol, precipitated with ethanol and sodium acetate, and resuspended in HPLC water as described by Yu and Chu [39]..

PCR cloning and sequencing. Both ^{15}N-DNA and ^{14}N-DNA fractions of microcosm samples were used as templates for PCR amplification of 16S rRNA and *xpl*A genes. DNA extracted from the groundwater was used for 16S rRNA gene sequencing only. All PCR reactions were performed in a total volume of 25 µL, with *Taq* PCR Master Mix (QIAGEN Inc., Valencia, CA), 2-50 ng of DNA templates, and 400nM of primers. For 16S rRNA sequences, bacterial universal primers (8f (5'-AGAGTTTGATCMTG GCTCA G-3') and 1407r (5'-ACGGGCGGTGTGTACA-3')) and PCR thermal cycle were used as described by Yu and Chu [39], except that less number of PCR cycle (35 cycles) was used. Forward (5'-GGTGGGGATGGAGGACTTC-3') and reverse (5'-CATGATGGGCAGTTTCGC-3'), were newly designed for *xpl*A gene. The *xpl*A gene primers were designed by alignment of the *xpl*A gene sequences from 14 *Rhodococcus* species in GenBank (Accession number DQ487126-DQ487137, AF449421, DQ277709). The PCR thermal cycle for *xpl*A gene was 95°C for 15 min, followed by 50 cycles of 95°C for 30s, 57 °C for 45s, and 72°C for 30s, followed by a final elongation step of 72°C for 10 m. A series of diluted DNA concentrations were used as templates to examine any inhibition in PCR reactions. The fresh PCR product was cloned into the vector pCR4-TOPO (TA cloning; Invitrogen, Carlsbad, CA) as manufacturer's instruction. Clones with inserts were verified by PCR with M13 primers and *xpl*A gene primers. The amplified fragments were cleaned using a QIA quick PCR purification kit (Qiagen Inc., Valencia, CA), followed by digestion with enzymes, *Hae*III and *Hha*I (Promega Corp., Madison, WI). A total of 70 clones for 16S rRNA gene and 60 clones for *xpl*A gene were screened by analyzing the patterns of restriction fragment length polymorphism (RFLP) on 4% Metaphor agarose gels (Lonza, Rockland, ME). Clones with unique RFLP pattern were selected for sequencing as

159 described by Yu and Chu [39]. M13 primers and *xpl*A gene primers on the pCR4-TOPO
160 plasmid were used for sequencing of 16S rRNA genes and *xpl*A genes, respectively.

161 **Phylogenetic analysis.** A web-based Manipulate Sequences Program
162 (http://www.vivo.colostate.edu/molkit/manip/index.html) was used to assemble raw DNA
163 sequence data from both strands into full-length sequences. The assembled sequences were
164 checked for chimeras using the on-line computer tool, CHIMERA_CHECK version 2.7 of the
165 Ribosomal Database-II Project (http://rdp8.cme.msu.edu/docs/chimera_doc.html) and carefully
166 inspected manually. Three out of 43 sequences for 16S rRNA gene were found as suspects of
167 chimera and removed from phylogenetic analysis. Related sequences were identified by
168 comparing the partial 16S rRNA gene sequences or cytochrome P450 gene sequences in the
169 GenBank by using BLAST. The closest relatives identified from searches were aligned and
170 analyzed with bootstrap neighbor-joining method in CLUSTALX2 program [43]. The
171 phylogenetic tree was created by using Treeview 32 software. The sequences of 16S rRNA and
172 *xpl*A gene have been deposited in GenBank as accession numbers EU907865 to EU907904 for
173 16S rRNA genes and EU919740 to EU919745 for *xpl*A genes.

174 **RESULTS**

175 **Validation of ^{15}N-SIP approach with pure cultures.** The ^{15}N- and ^{14}N-DNA of RDX-
176 and non-RDX degrading cultures were used to validate the ^{15}N-SIP approach. Mixture of three
177 different DNAs (^{14}N-DNA of *E. coli* grown with unlabeled NH_4^+, ^{14}N-DNA of DN22 grown
178 with unlabeled NH_4^+, and ^{15}N-DNA of *P. fluorescens* grown with $^{15}NH_4^+$) were successfully
179 separated into three individual bands from the top to bottom of the gradient (Tube #1, Figure 1).
180 The distance between the ^{14}N-DNA of *E coli* (the top band) and ^{15}N-DNA of *P. fluorescens* (the
181 bottom band) was around 4 mm.

To determine the effects of the mol% of ^{15}N in labeled substrates on ^{15}N-SIP application, experiments were also conducted by using the DNA of DN22 grown with three different N-sources: NH_4^+, $^{15}NH_4^+$ (100% N is labeled), and ring-^{15}N-labeled RDX (i.e. 50% N is labeled). When $^{15}NH_4^+$ was the sole N-source, a single band of ^{15}N-DNA was observed and the band position (Tube #2 in Figure 1) was very close to that of ^{15}N-DNA of *P. fluorescens* (data not shown). The slight difference in band location was due to different G+C contents of DN22 (67% G+C) and *P. fluorescens* (63% G+C). However, no separation was observed between the DNA of DN22 grown with NH_4^+ and the DNA of DN22 grown with ring-^{15}N-labeled RDX (i.e. 50% N is labeled) (Tube #3, Figure 1). Furthermore, the distance between ^{14}N-DNA of DN22 (NH_4^+ as N-source) and ^{15}N-DNA of *P. fluorescens* ($^{15}NH_4^+$ as N-source) (bands a and c, in Tube #4) was only slightly bigger than the distance between ^{15}N-DNA of DN22 (Ring-^{15}N-labeled RDX) and ^{15}N-DNA of *P. fluorescens* ($^{15}NH_4^+$ as N-source) (bands b and c, in Tube #5).

Application of ^{15}N-SIP to RDX-degrading enrichment cultures. The genomic DNAs extracted from six microcosms (M#1- #6, Table 1) were ultracentifuged in CsCl-EtBr density gradients (Figure 2). ^{14}N-DNA of *E. coli* and ^{15}N-DNA of *P. fluorescens* were used as Control #1. ^{14}N- and ^{15}N-DNA of DN22 grown with NH_4^+ and $^{15}NH_4^+$ were used as Control #2. While there were two visible bands, one in M#3 and the other one in M#5 (Figure 2), these bands appeared to not be ^{15}N-DNA fractions when compared to the band positions of ^{15}N-DNA of two controls. Still, ^{14}N- and ^{15}N-DNA of all samples (M#1-#6) were extracted from the expected locations of those in controls. The extracted DNA fractions were examined for the presence of the *xpl*A gene and 16S rRNA gene sequences (Table 1). Genes of 16S rRNA were detected in ^{14}N- and ^{15}N-DNA fractions for all microcosms, except the ^{15}N-DNA fraction of microcosm #5.

As the *xpl*A gene was detected only in the ^{15}N-DNA fraction of microcosm #4, this fraction was used for cloning and sequencing of 16S rRNA and *xpl*A genes.

Identification of active organisms in RDX-degrading enrichments and native groundwater organisms in RDX-contaminated groundwater. The ^{15}N-DNA fraction of microcosm #4 yielded seventy 16S rRNA gene clones, fifteen of which showed unique RFLP patterns and were sequenced. The 15 unique sequences were compared to those deposited in GenBank, including reported RDX-degrading isolates [15, 17, 21, 23, 24, 31, 44, 45]. As shown in the phylogenetic tree in Figure 3, these fifteen sequences were found to cluster among *Acinobacteria* (2 clones), α-*Proteobacteria* (7 clones) and γ-*Proteobacteria* (6 clones). The sequences did not cluster near *Clostridia*, many strains of which are known RDX-degraders [26]. Three out of 15 sequences (RDX clone #2, #8, and #13) were closely related to nitrogen-fixing bacteria (*Azospirillum* sp., up to 98% similarity) and two sequences (RDX clones#1 and #5) were 99% similar to *Pseudomonas* sp. While five sequences (RDX clones # 1,4,5,6, and 12) showed high similarity to two known RDX-degraders (*Enterobacter cloacae* and *Pseudomonas fluorescens* I-C), the other 10 RDX clones were different from 16S rRNA gene sequences of known RDX-degraders that were deposited in GenBank (accessed on 05/21/2008).

Twenty five out of seventy clones recovered from unenriched, RDX-contaminated groundwater possessed unique RFLP patterns. These 25 gene sequences clustered among the phyla *Actinobacteria*, *Firmicutes*, and *Proteobacteria*. The sequences from the unenriched groundwater were dissimilar from both the 16S rRNA gene sequences recovered from the microcosms and any known RDX degraders (Figure 3).

***Xpl*A gene clones**. Six *xpl*A-like genes were derived from the ^{15}N-DNA fraction of microcosm #4. The sequences were compared to *xpl*A gene sequences deposited in the GenBank.

227 Clone#1, with one undetermined base out of 550 bp, was almost identical to *xpl*A gene of DN22.
228 The other five clones also showed high homology to the *xpl*A gene; 99% for clones #3- #5, 97%
229 for clone #2, and 96% for clone #6 (Figure 4).

230 **DISCUSSION**

231 Stable isotope probing has been recognized as a powerful, culture-independent tool to
232 study microbial ecology. Researchers can now glimpse active microbial populations within
233 complex matrices by following the carbon and/or nitrogen flows of labeled substrates [35, 36].
234 In this study, ^{15}N-SIP was validated using pure cultures, then applied to RDX-degrading
235 microcosms to identify which members of a complex microbial community were able to derive
236 nitrogen from RDX. To our knowledge, this is the first study to use ^{15}N-SIP to study active
237 microbial population using RDX as an N source.

238 Several limitations specific to the applications of the ^{15}N-SIP have been discussed
239 previously [46, 47], including resolution of ^{14}N/^{15}N bands, different GC contents of
240 microorganisms, and the effects of the percent of ^{15}N label in the substrates employed. The
241 distance between ^{14}N/^{15}N bands is much shorter than that between ^{12}C/^{13}C bands after
242 equilibrium centrifugation in CsCl-EtBr density gradients. In this study, ^{15}N-DNA of *P.*
243 *fluorescens* and ^{14}N-DNA of *E. coli* was successfully separated with a distance about 4 mm. As
244 expected, the distance was much smaller than that of ^{13}C-DNA and ^{12}C-DNA bands (~7 mm)
245 observed in our previous study [39]. Due to the variation of G+C contents in DNA, similar
246 buoyant densities are expected from 100% ^{15}N-labeled DNA with a low G+C content (like 51%
247 G+C content in *E. coli*) and from unlabeled DNA with a high G+C content (like 67% G+C
248 content in *P. aeruginosa*), making separation of these DNA difficult. The effects of the variation
249 of G+C contents are expected to be more profound on ^{15}N-SIP than ^{13}C-SIP. In this study, the

250 effects of G+C contents were observed in Tube #1 and short distances of separation were
251 visualized in Tubes #4 and #5 (Figure 1).

252 While the percent of ^{15}N label in substrates was considered as a limitation during ^{15}N-SIP
253 applications in previous studies [47], applications of substrates with various percentage of
254 labeling, and variations in the ^{15}N-labeled positions, can be used during SIP to validate known,
255 and/or to identify unknown, biodegradation pathways in pure cultures. This aspect of ^{15}N-SIP
256 was observed during the validation work with pure cultures. As Cadisch *et al*. [47] showed that
257 a clear separation of bands at 40% ^{15}N-DNA was possible, we were surprised that separated
258 bands in Tube #3, containing 50% ^{15}N-DNA, were not observed. This unexpected result can be
259 explained by the degradation pathway of RDX by DN22. Fournier *et al*. [48] reported that DN22
260 transformed two out of three Ns in the RDX ring into dead-end metabolites. Accordingly,
261 despite the fact that all three Ns in the ring of RDX were labeled, only one third of the labeled N
262 (~33%) was free to be integrated into the DNA of DN22. This low resolution problem might be
263 resolved by using a second ultracentrifugation with bisbenzimide as an intercalating agent to
264 alter buoyant density of DNA from high G+C organisms [46]. Nevertheless, by using RDX that
265 is systematically labeled at different locations in SIP, one can not only identify active RDX-
266 utilizers but also understand their degradation pathways.

267 The application of ^{15}N-SIP to RDX-degrading microcosms and molecular analysis of
268 groundwater microorganisms in RDX-contaminated groundwater were successful and yielded
269 interesting insights. Our results indicated that a phylogenetically diverse microbial population
270 was capable of using RDX as a nitrogen source in the presence of a complex nutrient source like
271 cheese whey (Figure 3). Some of identified RDX clones are similar to clones/strains previously
272 reported [28, 42 , 49], while, majority of the clones have not been linked to RDX biodegradation

273 [26, 49] nor closely related to the dominant clones (25 clones) from the unenriched groundwater.
274 For example, three RDX clones (#4, #6, and #12) were found to be very similar to an
275 *Enterobacter* sp. (clone AA4-23, 97- 98% similarity) [49] and to *Enterobacter cloacae* [28]
276 (98% similarity). Similarly, RDX clones #1 and #5 showed high homology to *Pseudomonas*
277 IC(92% homology) [42], *Pseudomonas putida* II-B (95% homology) [42] and a *Pseudomonas*
278 sp. (*clone* Z4-19 , 99% similarly) [49]. While previous studies detected *Clostridia* during RDX
279 degradation under anaerobic conditions [26, 49], none of the isolated RDX clones were related to
280 *Clostridia* and only one sequence from groundwater (GW clone #10) was identified at the genus
281 level as a *Clostridia* sp. Regardless of the difference in dominant species detected, previous
282 studies were unable to clearly link dominant species to their involvement in *in situ* explosive
283 biodegradation. Overall, our results suggested that these five RDX clones (#1, #4-#6, #12) might
284 not be dominant species in RDX contaminated sites, but they might be biostimulated and play an
285 important role during RDX biostimulation.
286 The catabolic gene *xpl*A encoding an RDX-degrading cytochrome P450 was first
287 identified from DN22 [9] and has been proposed as a biomarker for assessing potential and/or
288 progress of RDX biodegradation [32, 50]. In this study, six *xpl*A-like genes were derived from
289 the ^{15}N-DNA fraction of microcosm #4 (receiving cheese whey) and these *xpl*A-like genes are
290 highly similar (96 to 99%) to these *xlp*A gene sequences of DN22. However, the 16S rRNA
291 gene sequence of DN22 was not detected in GW nor in the ^{15}N-DNA fraction of microcosm #4,
292 indicating that other RDX-utilizing bacteria might also contain *xpl*A-like genes. Putative TNT-
293 degrading genes *xen*A and *xen*B, which code for xenobiotic reductases XenA and XenB, were
294 initially described as being involved in the biodegradation of TNT (2,4,6-trinitrotoluene) [51, 52].
295 These two enzymes have recently been shown to catalyze the degradation of a wide range of

energetic compounds including RDX [42]. However, the presence of these two genes (*xen*A and *xen*B) was not examined due to lack of ^{15}N-DNA fraction as a template. Future studies should examine the presence and diversity of a range of known catabolic genes that can degrade RDX.

The results of this study have several implications, including that (i) the development of biomarkers based on currently known RDX-degrading isolates might not be suitable, since the known RDX-degrading strains may not be present in field and the lack of these known strains does not imply the lack of RDX degradation potential; (ii) the genetic information from these RDX clones might be a better choice to be used for developing a suite of biomarkers for monitoring engineered RDX biodegradation potential and/or natural attenuation of RDX; (iii) some RDX-degraders show high similarity to *Azospirillum* sp. and *Pseudomonas* sp., suggesting that enhanced RDX biodegradation might be possible by creating *in situ* growth conditions similar to those strains; and (iv) by mimicking field conditions, new RDX-degrading cultures and/or mixed consortia might be able to be isolated from diverse field samples, and the new isolates might be used for detailed RDX degradation pathway studies and for RDX bioaugmentation.

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the authors and should not be construed as an official Department of the Army position or decision unless so designated by other official documentation.

Supporting Information Available

Degradation of RDX in RDX-degrading microcosms. This material is available free of charge via the Internet at http://pubs.acs.org.

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List of Figures

Figure 1. Separation of the ^{14}N- and ^{15}N-DNA of three reference strains: *E. coli*, *Rhodococcus sp*. DN22 and *P. fluorescens* by using equilibrium concentration in CsCl-EtBr density gradients at 77,000 rpm (265,000g) at 20˚C for 24 hr. Three clear bands, from top to bottom, ^{14}N-DNA of *E. coli* (NH_4^+ as N-source), ^{14}N-DNA of DN22 (NH_4^+ as N-source), ^{15}N-DNA of *P. fluorescens* ($^{15}NH_4^+$ as N-source), were observed in Tube #1. Separation of ^{14}N- and ^{15}N-DNA of DN22 grown with three different N-sources: NH_4^+ (Tube #2), $^{15}NH_4^+$, and Ring-^{15}N-labeled RDX (Tubes 2 and 3). The distance (a-c) between ^{14}N-DNA of DN22 (NH_4^+ as N-source), ^{15}N-DNA of *P. fluorescens* ($^{15}NH_4^+$ as N-source) was shown in Tube #4. Tube #5 showed the distance (b-c, in Tube #5) between ^{15}N-DNA of DN22 (Ring-^{15}N-labeled RDX), ^{15}N-DNA of *P. fluorescens* ($^{15}NH_4^+$ as N-source).

Figure 2. Application of ^{15}N-SIP to RDX-degrading microcosms that were amended with a nutrient source (cheese whey or yeast extract) and ^{15}N-RDX as an additional nitrogen source. Genomic DNA of six RDX-degrading microcosms (M#1-M#6) was ultracentrifuged in CsCl-EtBr density gradients at 77,000rpm (265,000g) at 20˚C for 24 hr. Two bands were observed in M #3 and M#5. However, these bands were not due to ^{15}N-DNA fractions, based on the ^{15}N-DNA band positions of two controls, C#1 and C#2. C#1 contained ^{14}N-DNA of *E.coli* and ^{15}N-DNA of *P. fluorescens*. C#2 contained ^{14}N- and ^{15}N-DNA of *Rhodococcus* sp. DN22.

Figure 3. Phylogenetic analysis of cloned bacterial 16S rRNA genes from ^{15}N-DNA fraction. Sequences found are contrasted with known RDX degraders and with sequences of cloned bacterial 16S rRNA genes from unenriched, RDX-contaminated groundwater. The tree was rooted with the 16S rRNA sequence of *Methanococcus thermolithotrophicus* (Bar = 10 nucleotide substitutions/100 nucleotides in 16S rRNA sequences). An asterisk (*) indicates a known RDX degrader. As the deposited sequences of three known RDX degraders (*Enterobacter cloacae* ATCC43560 [EF219421], *Pseudomonas fluorescens* I-C [EF219420], and *Pseudomonas putida* II-B [EF219419] [38]) were short (< 250bp), these sequences were not used included in the tree. In stead, *Enterobacter ludwigii* (**), *Pseudomonas veronii S1f-34(**)*, and *Pseudomonas* sp. J7(**) were used due to the availability of their 16S rRNA gene sequences (1,400bp) and their similarity to the three known RDX-degraders. BLAST analysis showed that *Enterobacter cloacae* is 96% similar to *Enterobacter ludwigii* (**). *Pseudomonas veronii S1f-34(**) and Pseudomonas* sp. J7 (**) showed 92-95% similarity to *Pseudomonas fluorescens* I-C and *Pseudomonas putida* II-B.

Figure 4. Phylogenetic analysis of *xpl*A genes cloned from ^{15}N-DNA fraction of microcosm #4 (amended with cheese whey). The RDX Clone #1, with one undetermined base out of 550bp, is almost identical to *xpl*A gene of DN22. Three *xpl*A gene sequences (Clone #3~ #5) showed 99% similarity to the *xpl*A gene of DN22. One sequence (clone #2) showed 97% homology, and the other (clone #6) showed 96% similarity.

Figure 1

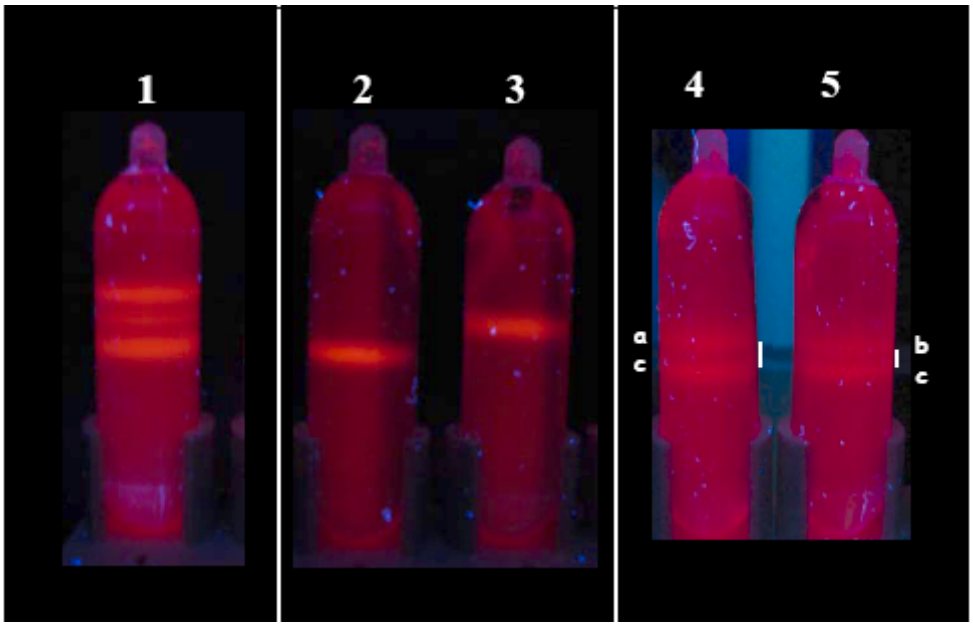

Figure 2. Application of stable isotope probing to ^{15}N-RDX enrichment cultures

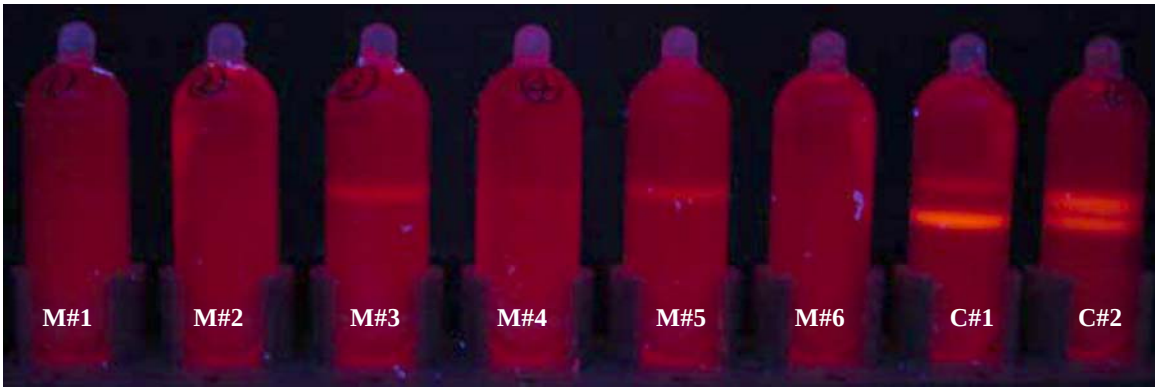

Figure 3. Phylogenetic analysis of cloned bacterial 16S rRNA genes from ^{15}N-DNA fraction.

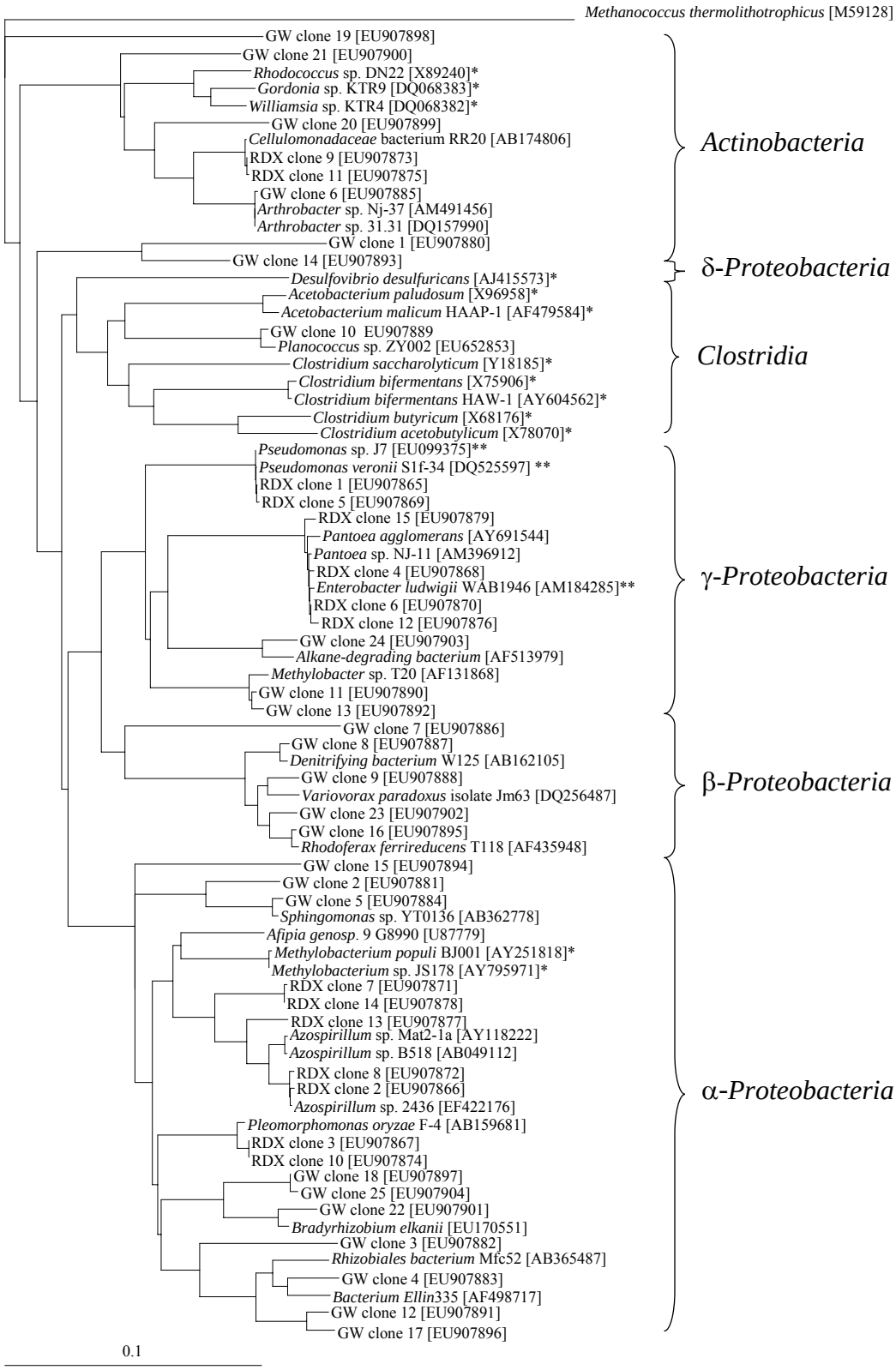

Figure 4. Phylogenetic analysis of cloned bacterial *xpl*A genes from ^{15}N-DNA fraction.

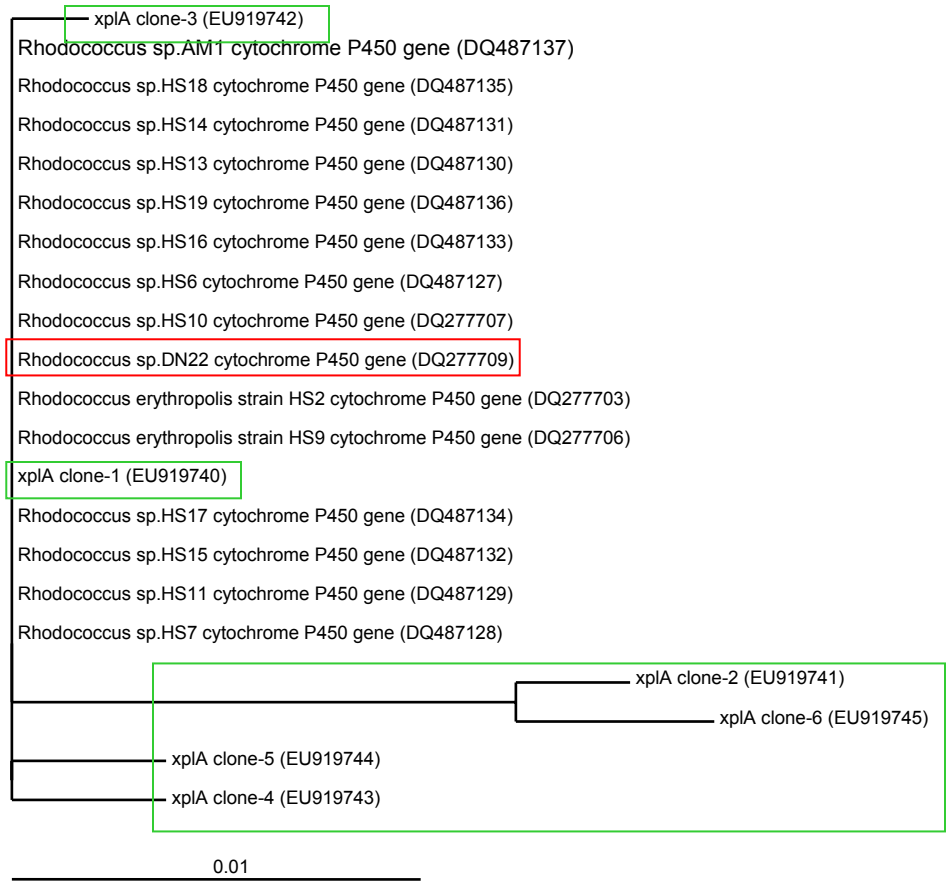

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Table 1. Examinations on the presence of 16S rRNA and *xpl*A gene of microcosms

Table 1. Summary of 16S rRNA and *xpl*A genes detected in microcosms

Microcosm	Treatment		Unfractionated genomic DNA		^{14}N-DNA fraction		^{15}N-DNA fraction	
	Nutrient Source (1 g/L)	Additional N-source RDX (5 mg/L)	16S rRNA	*xpl*A	16S rRNA	*xpl*A	16S rRNA	*xpl*A
M#1	Cheese whey	^{14}N-RDX	ND	+	+	–	+	–
M#2	Cheese whey	^{14}N-RDX	ND	+	+	–	+	–
M#3	Yeast extract	^{14}N-RDX	ND	+	+	–	+	–
M#4	Cheese whey	^{15}N-RDX	+	+	+	–	+	+
M#5	Cheese whey	^{15}N-RDX	ND	+	+	–	–	–
M#6	Yeast extract	^{15}N-RDX	ND	+	+	–	+	–

+,Detected; -, Not detected; ND, Not determined

Supporting Materials

for

Identification of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)-degrading microorganisms via ^{15}N-stable isotope probing

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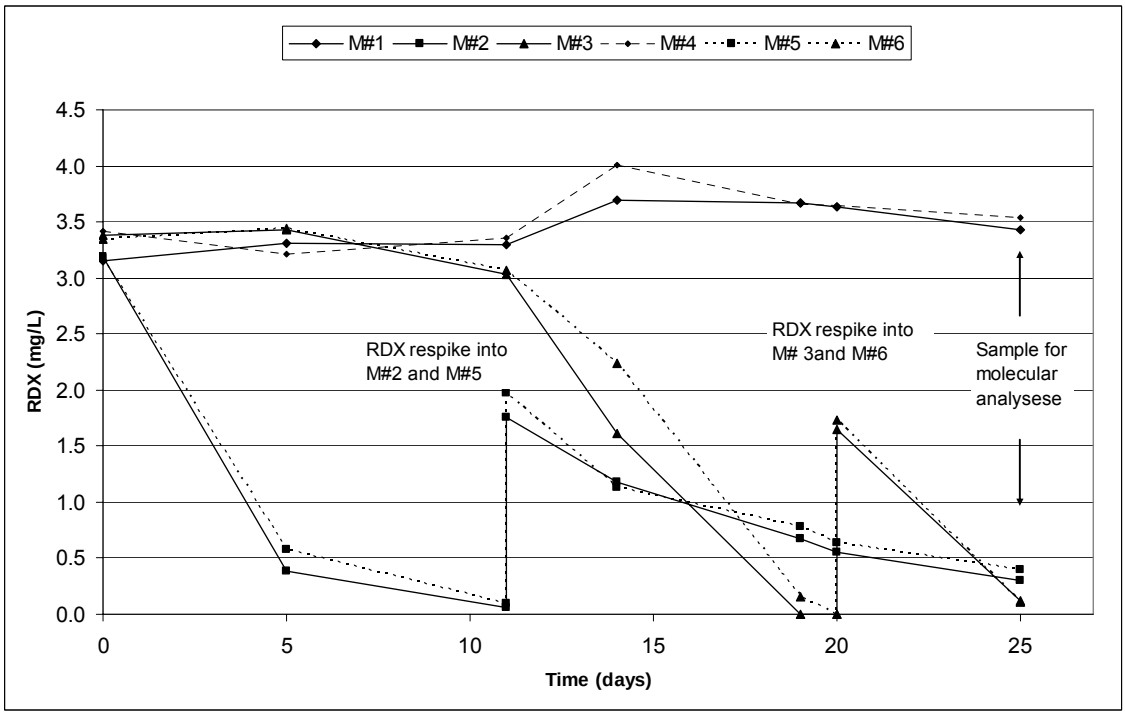
Figure S1 RDX degradation in microcosms M#1-M#6.



APPENDIX 2

TECHNICAL PRESENTATIONS

Fuller, M. E., R. J. Steffan, J. M. Lowey, and K. McClay. 2004. Microbial ecology of energetic compound biodegradation. The 2004 Partners in Environmental Technology Technical Symposium & Workshop. Washington, D.C., USA, November 30-December 2.

Groundwater at many DoD installations has become contaminated with various military-related chemicals. Our SERDP-funded research project (CU-1378) is examining the biodegradation of explosive compounds like TNT, RDX and HMX in the subsurface with respect to the microbial ecology and the effects of groundwater chemistry.

Initial research has focused on developing molecular tools and protocols to detect and quantify putative explosive-degrading genes, as well as identify the dominant microorganisms involved with explosive compound biodegradation. DNA primers for quantitative polymerase chain reaction analysis have been developed for the genes xenA, xenB, xplB, nbz, and onr. Probes to these genes have also been developed and are being used to confirm the identity of PCR products generated using the primers.

Sediments were collected from the Naval Surface Warfare Center - Indian Head Division (IHDIV) and the West Virginia Ordnance Works (WVOW). The IHDIV sediments were used to prepare soil slurries which were amended with different electron donors (lactate, emulsified vegetable oil, crude soybean oil) to stimulate the biodegradation of RDX and HMX by the indigenous microorganisms. This allowed the effect of biostimulant (i.e., addition of a complex or simple electron donor) on the microbial ecology of explosive-degrading bacteria to be assessed. The WVOW sediments were collected from areas inside and outside a known explosives plume, which allowed a comparison of the microbial ecology of explosive-degrading bacteria as it relates to exposure to low concentrations of explosive compounds over a long period of time.

The primers were used to analyze the microbial community in the IHDIV slurries and the WVOW unenriched sediments. The results indicated the presence of xenA in both the contaminated and uncontaminated sediments from WVOW, as well as in the unamended IHDIV slurry. The IHDIV slurry enrichments with various electron donors all evidenced degradation of RDX, and only xenA was detected in the enrichments. These results indicate that this gene (or similar genes detected using the primer set employed) may be the most widespread and possibly the dominant explosive degradative gene in the environment.

Development of these techniques will allow the microbial community at sites prior to and during passive (monitored natural attenuation) or active (biostimulation, bioaugmentation) remedial activities to be assessed. This will allow remedial approached to be specifically tailored to a given site, as well as allowing the progress of clean-up efforts to be monitored.

Fuller, M. E., R. J. Steffan, and M. Higham. 2005. Assessing the Microbial Ecology of Energetic Compound Biodegradation in Groundwater. The 2005 Partners in Environmental Technology Technical Symposium & Workshop. Washington, D.C., USA, November 28-30.

There is increasing concern about the presence of explosive compounds in the groundwater at many DoD installations. This SERDP-funded research project (CU-1378) is examining the biodegradation of explosive compounds like TNT, RDX and HMX in the subsurface with respect to the microbial ecology and the effects of groundwater chemistry.

Experiments using microcosms enriched under different conditions (i.e., electron donor, utilizable nitrogen, etc.), as well as analysis of groundwater from various explosive-contaminated sites, were conducted. Samples were analyzed for putative explosive-degrading genes using polymerase chain reaction (PCR). Microbial community analysis was performed using denaturing gradient gel electrophoresis (DGGE).

Results from enrichment cultures in which active explosive compound degradation was occurring indicated that very few of the dominant organisms were similar to microbial strains previously associated with explosives degradation. Analysis of groundwater samples yielded similar results. Additionally, putative explosive-degrading genes were not frequently detected.

The results indicate that the range of organisms (and genes) responsible for the biological degradation of explosives under actual field conditions may be broader than inferred from studies with single bacterial isolates. Development and use of these microbial community assessment methods will facilitate site-specific remediation for explosive-contaminated groundwater.

Fuller, M. E., R. J. Steffan, and M. Higham. 2007. Groundwater Microbial Ecology of RDX Biodegradation. The 2007 Partners in Environmental Technology Technical Symposium & Workshop. Washington, D.C., USA, December 4-6.

There is increasing concern about the presence of explosive compounds in the groundwater at many DoD installations. This SERDP-funded research project (CU-1378) is examining the biodegradation of energetic compounds, especially RDX, in the subsurface with respect to the microbial ecology and the effects of groundwater chemistry.

Samples from microcosms enriched under different conditions (i.e., electron donor, utilizable nitrogen, etc.), model aquifers, and groundwater from various explosive-contaminated sites, were collected. Microbial community analysis was performed using denaturing gradient gel

electrophoresis (DGGE), followed by DNA sequencing and determination of the dominant 16S rRNA sequences. Selected samples were analyzed for putative explosive-degrading genes using polymerase chain reaction (PCR). Methods to apply 13C/15N stable isotope probing (SIP) to more precisely identify the RDX-degraders in mixed microbial communities were also developed and evaluated.

Results to date from enrichment cultures indicate that only a few of the dominant 16S rRNA sequences detected were related to bacterial strains previously associated with explosives degradation (i.e., Rhodococcus, Clostridium). Analysis of groundwater samples have yielded similar results.

The putative explosive-degrading genes were not frequently detected in any of the samples. However, follow-on work has expanded the number of putative TNT-degrading genes that may also be involved in RDX biodegradation under certain environmental conditions.
These results indicate that the range of organisms (and genes) responsible for the biological degradation of RDX under actual field conditions may be broader than inferred from studies with single bacterial isolates. Further development and use of these microbial community assessment methods (especially SIP) will expand our understanding of the microbial ecology of explosive compound biodegradation, and facilitate site-specific remediation of explosive-contaminated groundwater.

Yu, C.-P., H. Roh, M. E. Fuller, and K.-H. Chu. 2007. Application of ^{15}N Stable Isotope Probing to Identify Microorganisms Utilizing Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) as a Sole Nitrogen Source. ASM 107th General Meeting. Toronto, Ontario, CANADA, May 21-25.

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a heterocyclic nitramine explosive commonly detected in soils and groundwater at army ammunition plants and other military sites. RDX is toxic and a possible human carcinogen. While RDX biodegradation has been reported under aerobic and anaerobic conditions and several RDX degrading isolates have been reported, microorganisms that are active for RDX biodegradation in-situ remain unidentified. With different 13C-labeled substrates, a newly developed technique, called stable isotope probing (SIP), has allowed researchers to identify metabolically active microorganisms in complex engineered and natural systems. However, 15N-based SIP has not been applied to identify microorganisms capable of degrading nitrogen-containing compounds, like RDX.

This study examines the feasibility of using 15N-DNA SIP to identify active RDX-utilizers in RDX-degrading microcosms. Two non-RDX-utilizing strains (Escherichia coli and Pseudomonas fluorescens) and one RDX-utilizing culture (Rhodococcus sp. DN22) were used to validate

15N-DNA SIP approach. Rhodococcus sp. DN22 can use RDX as a sole nitrogen source. All strains were grown with nitrogen-free mineral medium. For non-RDX-degraders, glucose was supplied as the carbon source, and unlabeled and 15N-labeled NO3- (as sodium nitrate) were used as the nitrogen source. The 15N-DNA and unlabeled-DNA from P. fluorescens and E. coli, respectively, were used as controls. Rhodococcus sp. DN22 was grown with unlabeled and ring-15N labeled-RDX (i.e.50% of N in RDX was labeled) as the sole nitrogen source, and succinate as the carbon source. After ultracentrifugation, the two non-RDX-degrading controls (unlabeled- and 15N-DNA) were successfully separated into two bands with an approximately distance of 4 mm. Interestingly, the band of the 15N-DNA of Rhodococcus sp.DN22 migrated halfway between these two control bands. The result suggested that Rhodococcus sp. DN22 can incorporate both ring- and nitro-group-nitrogen into its DNA. On-going research efforts will improve separation of 14/15N-DNA bands and to apply 15N-DNA SIP to RDX-degrading microcosms and groundwater microbial communities.

Fuller, M. E., M. Higham, K. McClay, H. Roh, K.-H. Chu, and R. J. Steffan. 2008. Groundwater Microbial Ecology of RDX Biodegradation. ASM 108th General Meeting. Boston, MA, USA, June 1-5.

There is increasing concern about the presence of explosive compounds in the groundwater at many DoD installations. This research project is examining the biodegradation of energetic compounds, especially RDX, in the subsurface with respect to the microbial ecology and the effects of groundwater chemistry.

Samples from microcosms enriched under different conditions (i.e., electron donor, utilizable nitrogen, etc.), model aquifers, and groundwater from various explosive-contaminated sites, were collected. Microbial community analysis was performed using denaturing gradient gel electrophoresis (DGGE), followed by DNA sequencing and determination of the dominant 16S rRNA sequences. Selected samples were analyzed for putative explosive-degrading genes using polymerase chain reaction (PCR). Methods to apply 13C/15N stable isotope probing (SIP) to more precisely identify the RDX-degraders in mixed microbial communities were also developed and evaluated.

Results to date from enrichment cultures indicate that only a few of the dominant 16S rRNA sequences detected were related to bacterial strains previously associated with explosives degradation (i.e., Rhodococcus, Clostridium). Analysis of groundwater samples have yielded similar results.

The putative explosive-degrading genes were not frequently detected by regular PCR, but 15N-SIP did reveal the presence of the RDX-degrading

gene xplA in enrichments from groundwater from a site undergoing biostimulation. Additionally, follow-on work has expanded the number of putative TNT-degrading genes that may also be involved in RDX biodegradation under certain environmental conditions.

These results indicate that the range of organisms (and genes) responsible for the biological degradation of RDX under actual field conditions may be broader than inferred from studies with single bacterial isolates. Further development and use of these microbial community assessment methods (especially SIP) will expand our understanding of the microbial ecology of explosive compound biodegradation, and facilitate site-specific remediation of explosive-contaminated groundwater.

Roh, H., D.-G. Lee, M. E. Fuller, R. J. Steffan, and K.-H. Chu. 2008. Deciphering Active Hexahydro-1,3,5,-trinitro-1,3,5-triazine (RDX) Utilizers and Their Associated Microbial Communities in RDX-Contaminated Groundwater. ASM 108th General Meeting. Boston, MA, USA, June 1-5.

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a heterocyclic nitramine explosive commonly detected in soils and groundwater. RDX is also a possible human carcinogen and is listed as a Drinking Water Contaminant Candidate by Environmental Protection Agency (EPA). A drinking water guideline of 2 µg RDX/L for lifetime exposure for adults is advised. While biodegradation of RDX is observed under aerobic and anaerobic conditions, successful engineered bioremediation and/or monitored naturally attenuation of RDX remains a great challenge due to our limited knowledge on active RDX-degraders and their microbial community.

The objective of this study is to better understand active RDX-utilizers and their microbial communities in RDX-contaminated groundwater. By using DNA-based stable isotope probing, we used 15N- and 13C- labeled RDX to track and identify microorganisms capable of using RDX as a sole nitrogen and/or carbon source. Real-time-t-RFLP will be used to quantitatively characterize their associated microbial community structure. To optimize separation of 15N- and 14N-DNA fractions, we add bisbenzimide, as an intercalating agent, to alter buoyant density of DNA from high G+C organisms during ultracentrifugation in CsCl-EtBr density gradients. Experiments were conducted in microcosms containing RDX-contaminated groundwater and spiked with unlabeled RDX, 13C-labled RDX, 15N-labeled RDX, and unlabeled RDX+ dicumarol (an inhibitor to Type I nitroreductase that is known to catalyze RDX degradation). The amended RDX in the microcosms were rapidly degraded within 2-4 weeks. After RDX was depleted, liquid samples were collected and used for DNA extraction. The extracted DNA is undergoing ultracentrifugation. Both lighter and heavier and DNA will be extracted and used for sequencing and cloning as well as for real-time-t-RFLP analysis.

The results of this study will not only offer technical considerations for applying 15N-DNA SIP to field samples but also potentially identify novel RDX-utilizers.

Fuller, M. E., K. McClay, H. Roh, K.-H. Chu, and R. J. Steffan. 2008. Microbial Ecology Assessment of RDX-Contaminated Groundwater and RDX-Degrading Enrichments. The 2008 Partners in Environmental Technology Technical Symposium & Workshop. Washington, D.C., USA, December 2-4.

There is increasing concern about the presence of explosive compounds in the groundwater at many DoD installations. This research project is examining the microbial ecology associated with RDX contamination and biodegradation in the subsurface.

Samples from microcosms enriched under different conditions (i.e., electron donor, utilizable nitrogen, etc.), model aquifers, and groundwater from various explosive-contaminated sites, were collected. Microbial community analysis was performed using denaturing gradient gel electrophoresis (DGGE), followed by DNA sequencing and determination of the dominant 16S rRNA sequences. Selected samples were analyzed for putative explosive-degrading genes using polymerase chain reaction (PCR). Methods to apply 13C/15N stable isotope probing (SIP) to more precisely identify the RDX-degraders in mixed microbial communities were also developed and evaluated.

Analysis of enrichments and native groundwater samples have yielded similar results using both standard DGGE and SIP. Few of the dominant 16S rRNA sequences recovered were related to, but not identical to, bacterial strains previously associated with explosives degradation (i.e., Rhodococcus, Clostridium). However, a very wide range of other bacterial genera were detected, most notably a large number from the genus Pseudomonas. Results from 13C and 15N SIP also revealed a range of bacterial strains able to derive carbon or nitrogen from RDX.

The putative explosive-degrading genes were not frequently detected by regular PCR. 15N-SIP did reveal the presence of the RDX-degrading gene xplA in enrichments from groundwater from a site undergoing biostimulation.

Follow-on work has confirmed RDX degradation by specific genera (and genes), indicating that RDX biodegradation may be carried out by a wider range of microorganism than previously reported under certain environmental conditions.

These results indicate that the range of organisms (and genes) responsible for the biological degradation of RDX under actual field conditions may

be broader than inferred from studies with single bacterial isolates. Our understanding of the microbial ecology of explosive compound biodegradation will expand with further development and use of these microbial community assessment methods, especially SIP.

Fuller, M. E., P. B. Hatzinger, K.-H. Chu, J. Hawari, N. C. Sturcio, and R. J. Steffan. 2008. Understanding the Biodegradation of RDX in Groundwater. SETAC North America 29th Annual Meeting. Tampa, FL, November 16-20.

There is increasing concern about the presence of explosive compounds in soil and groundwater at DoD installations. This research is exploring the biodegradation of the energetic compound RDX in the subsurface with respect to the microbial ecology and groundwater chemistry. This research will expand the range of organisms that are known to be associated with the biological degradation of RDX under actual field conditions.

RDX degradation in microcosms, model aquifers, and groundwater from various explosive-contaminated sites is being examined. Efforts are being directed towards identifying signature RDX breakdown products, and relating these products to geochemical and microbiological parameters. Microbial community analyses include use of denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphisms (tRFLP), coupled with 16S rRNA gene sequencing to identify the predominant microorganisms. Application of 13C/15N stable isotope probing (SIP) is being performed to improve identification of RDX-degraders in mixed microbial communities. Biological isotopic fractionation of RDX is also being examined in order to develop a diagnostic method to assess natural attenuation in groundwater.

Results to date indicate that some of the dominant 16S rRNA sequences detected were related to bacterial strains associated with explosives degradation (i.e., Rhodococcus, Clostridium). Results also have indicated that Pseudomonas spp., which are widespread environmental bacteria, are likely involved in RDX biodegradation under certain environmental conditions. Initial SIP experiments have more precisely identified some of the organisms (and degradative genes) directly metabolizing RDX and/or RDX breakdown products.

Further development and use of microbial community assessment methods (especially SIP), identification of signature products, and determination of biological fractionation factors for RDX will expand our understanding of explosive compound biodegradation in groundwater, and will facilitate site-specific assessment, monitoring, and remediation of explosive-contaminated groundwater.

APPENDIX 3

STANDARD OPERATING PROCEDURES

Sterivex Field- Filtering Procol

Revision 2.0
October 9, 2007

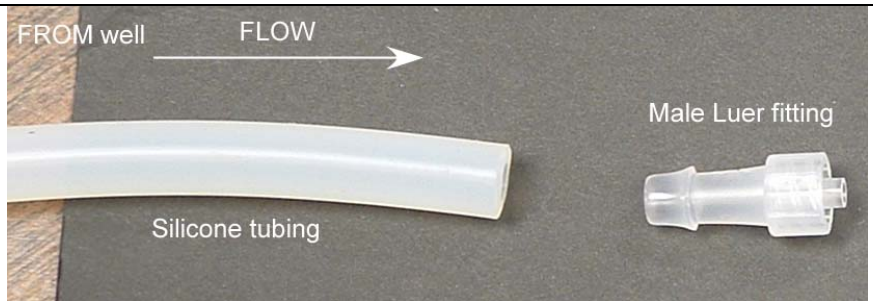
Summary
This Standard Operating Procedure (SOP) describes the techniques used to filter groundwater from installed wells.

Equipment need in field

- Graduated cylinder (1 L or similar)
- Zip lock bags and permanent marker
- Ice and cooler

A sampling kit with Sterivex filters and all small accessories, solutions, etc. needed for sample collection will be supplied for each well that will be sampled. Minimize touching the filters and other parts with bare hands, and avoid placing them on ground, etc. Change gloves between wells.

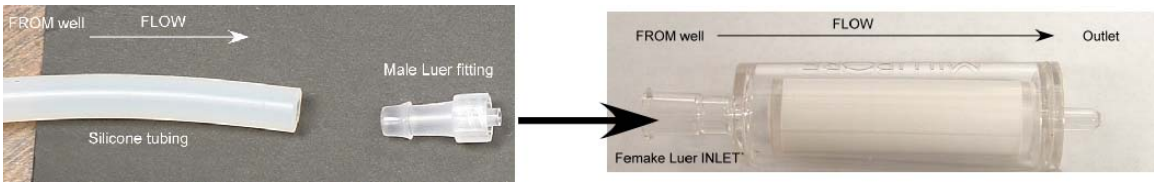
These items are in each ziplock bag:

Sterivex filter unit	
Well-to-Sterivex adaptor with silicone tubing	
60 ml syringe	

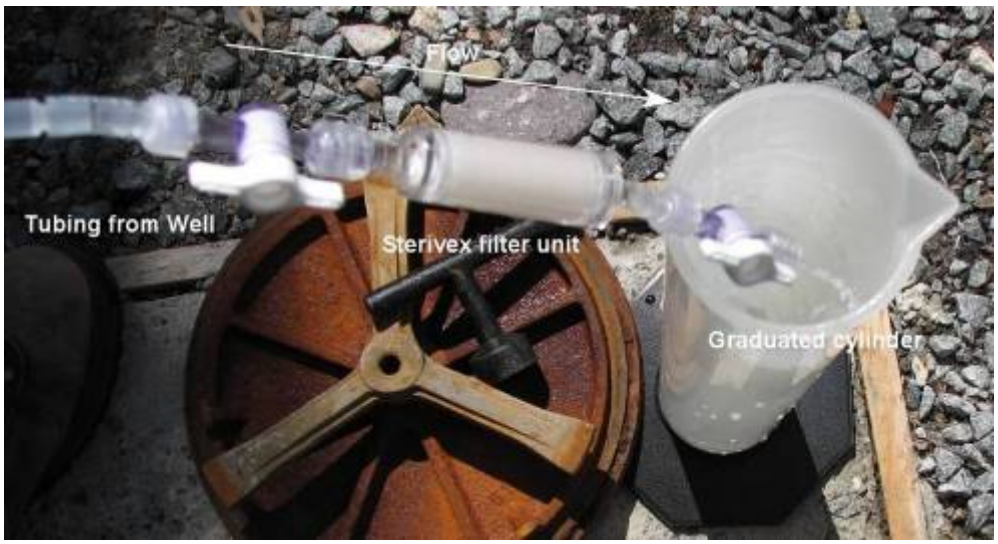
50 ml tube for used filter storage		

The following sampling procedure is performed for each well to be sampled:

1) **Filter 2 liters of groundwater (or as much as will pass through the filter unit) from well through the filter**
    a. Normalize pumping and flow as per SOP for sampling wells for other analyses and sample collection.
    b. Put on clean latex gloves.
    c. Remove one filter unit from the ziplock bag, and open the filter packaging.
    d. Label the first filter unit using a marker with the well ID. Label the 50 ml tube with the same information.
    e. Attach the male Luer x ¼" hose barb adaptor and tubing to the inlet end of the filter.



    f. Attach the silicone tubing to the well tubing.
    g. Begin pumping groundwater from the well into the filter unit. Collect the effluent from the filter into graduated cylinder.

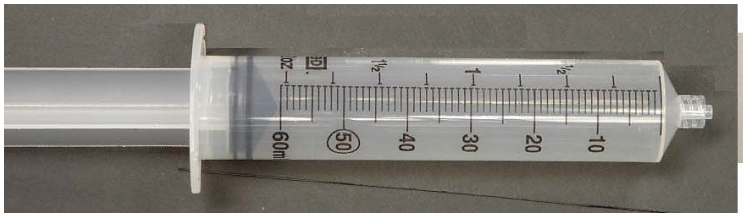


NOTE: The pressure and/or pump speed may need to be adjusted to account for back-pressure from the filter units in order to get good groundwater flow

h. A minim of 2 L of GW should be filtered, more if flow and time permit. Record the volume filtered for each filter on the CoC.
i. Disconnect the filter unit at the hose barb adaptor.

2) Remove all water from the filter

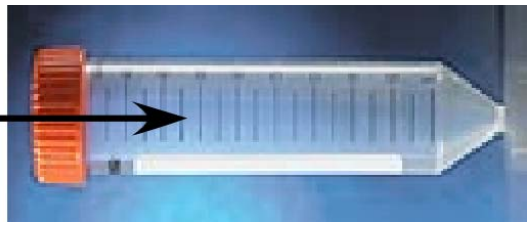
a. Filll the 60 mL syringe with air and attach it to the inlet of the Sterivex filter.
b. Force air into the filter to remove the groundwater that is still in the filter cartridge.



c. Detach the syringe, refill with 60 mL air and repeat step (b) to expel all residual water from the filter.

3) Store and ship the filter

a. Place the Sterivex filter with the outlet end down into the 50 ml tube.



b. Place the tubes upright in the styrofoam rack.
c. Place rack with tubes in cooler on ice. If need to store, place at 4°C.
d. Ship on plenty of ice.

STERIVEX FILTER SAMPLING
Chain of Custody

Location: _____

Collected by: _____

			Volume	GW Parameters (final reading during stabilization)				
Sample ID	**Date**	**Time**	**Filtered (L)**	**Temp (°C)**	**pH**	**DO (mg/L)**	**ORP (mV)**	**Notes**

Sterivex Filter Processing, DNA Purification and PCR Protocol

Preparing filters for DNA extraction.

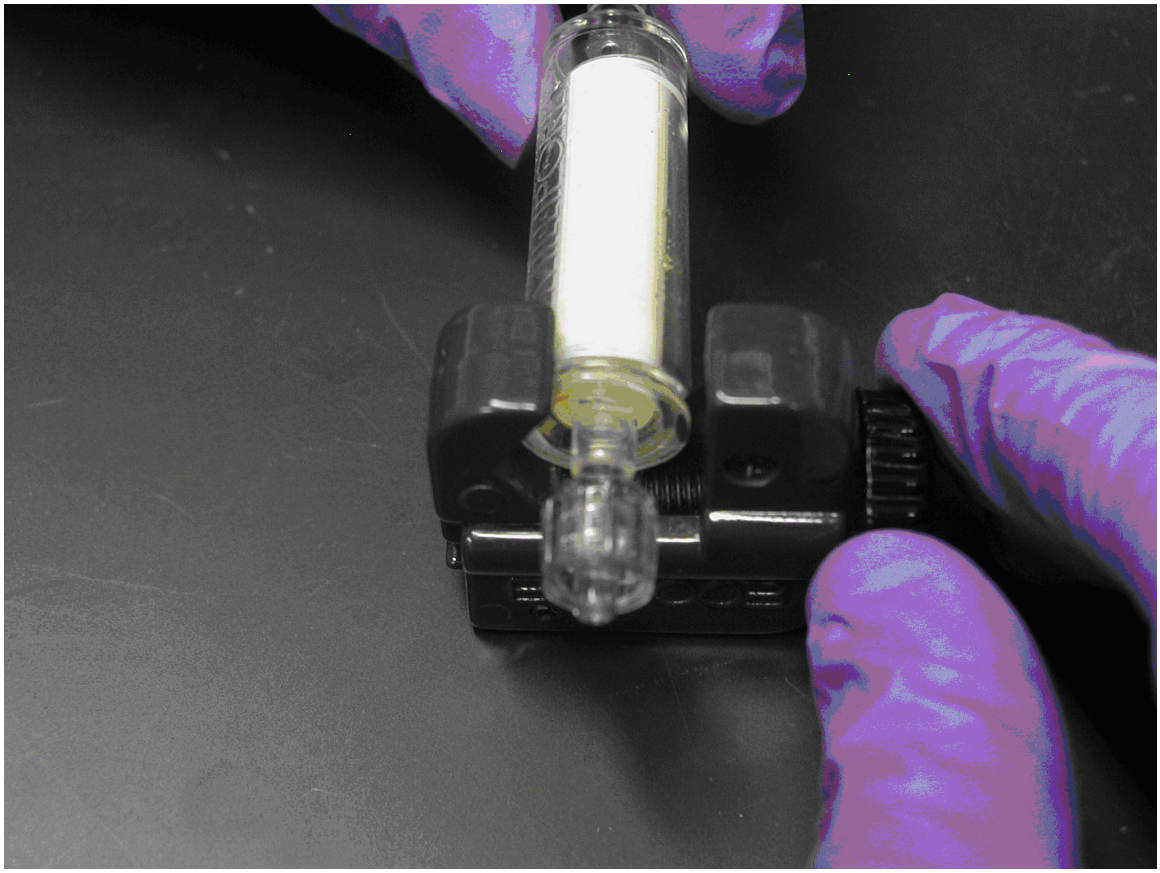
Filters are handled using the method developed in Dr. Frank Loffler's lab, reproduced here with slight modifications.

Materials

2 tweezers, tubing cutter, wide mouth ethanol reservoir, scalpel blades.

1. Clean tubing cutter by flaming with ethanol.

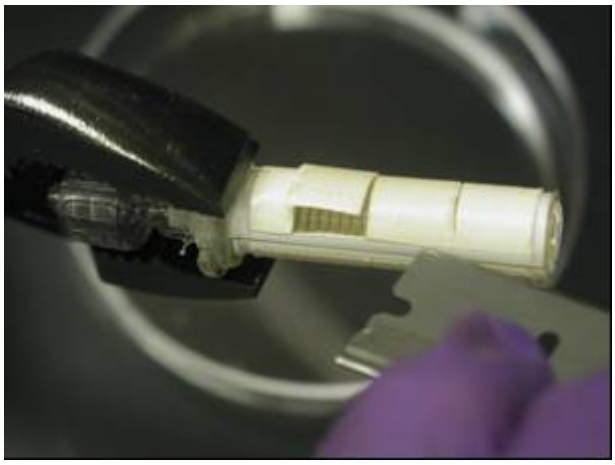
2. Place filter cartridge into tubing cutter. The blade of the tubing cutter should fit into the seam at the bottom of the Sterivex filter cartridge. Ideally the blade will easily work into the joint, making opening easier.



3. Tighten the cutting wheel, guiding it into the seam between the end cap and the outer wall of the filter cartridge. Spin the cutter around until the cartridge opens. While opening the cartridge, hold it vertically to prevent any liquid that may

remain in the cartridge from coming out. Once the filter cartridge is open, place the two halves of the filter in a sterile Petri dish.

4. If the 'bucket' part of the cartridge contains any liquid, collect it with a pipette and put it into the bead beater tube (from Zymo kit). If liquid volume is larger than 100 µl, concentrate it via centrifugation in a sterile micro-centrifuge tube before transferring. Discard the 'bucket' once any liquid has been recovered.



5. Grab the filter cartridge at the outlet end using a pair of pliers or your hand and use a sterile scalpel blade (straight razor is depicted below) to cut the filter along the seams at the terminal ends to liberate the filter from the support.

6. Remove the filter with flamed tweezers and place it in the Petri dish.

7. Discard the filter support.

8. Taking two flame sterilized tweezers, gently roll the filter into a tube shape with a small enough diameter to fit into the bead bashing tube. Use one of the tweezers to hold the filter in place and use the other tweezers to grasp the filter at one of its ends, such that the filter can be picked up and inserted into the bead beater tube.

9. Insert the filter into a bead bashing tube, and transfer any of the liquid material collected and/or concentrated into the tube.

10. Proceed with DNA purification as described below.

DNA isolation

Environmental sources such as soil or filtered groundwater are best extracted using the ZR Soil Microbe DNA Isolation Kit from Zymo Research.

Materials

Bead beater, kit solutions and tubes.

Before Starting: (environmental samples only) Zymo-Spin™ IV-HRC Spin Filters (green tops) need to be prepared prior to use by: 1) snapping off the base, 2), inserting into a Collection Tube, and 3), spinning in a microcentrifuge at exactly 8,000 x *g* for 3 minutes.

1. Add 750 µl of Lysis Solution to the tube containing the Sterivex filter. If DNA can not be extracted at this point, this is a good time to freeze them

2. Secure in a bead beater fitted with a 2 ml tube holder assembly and process at 4200 rpm for 1.5 minutes.

3. Centrifuge the ZR BashingBead™ Lysis Tube in a microcentrifuge at ≥10,000 x *g* for 1 minute.

4. Transfer up to 400 µl supernatant to a Zymo-Spin™ IV Spin Filter (orange top) in a Collection Tube and centrifuge at 7,000 rpm (~7,000 x *g*) for 1 minute.

5. Add 1,200 µl of Soil DNA Binding Buffer to the filtrate in the Collection Tube from Step 4.

6. Transfer 800 µl of the mixture from Step 5 to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute.

7. Discard the flow through from the Collection Tube and repeat Step 6.

8. Add 200 µl DNA Pre-Wash Buffer to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuge at 10,000 x *g* for 1 minute.

9. Add 500 µl Soil DNA Wash Buffer to the Zymo-Spin™ IIC Column and centrifuge at 10,000 x *g* for 1 minute.

10. Transfer the Zymo-Spin™ IIC Column to a clean 1.5 ml microcentrifuge tube and add 100 µl DNA Elution Buffer directly to the column matrix. Centrifuge at 10,000 x *g* for 30 seconds to elute the DNA. If fungi or bacterial cultures were sampled, the DNA is now suitable for PCR as well as other downstream applications.

11. Transfer the eluted DNA from Step 10 to a prepared Zymo-Spin™ IV-HRC Spin Filter (green top) (see above) in a clean 1.5 ml microcentrifuge tube and centrifuge at exactly 8,000 x *g* for 1 minute. The filtered DNA is now suitable for PCR and other downstream applications.

PCR set up

Materials

Sterile vapor barrier pipette tips for all pipettes used in set up, Go-Taq-Green Polymerase Master Mix, appropriate primers, RT-PCR grade water.

Using DNA from the final step in DNA purification protocol, set PCR reaction as follows (assuming 100 µl final volume is desired).

Component	**Volume**	**Final Conc.**
GoTaq® Green Master Mix, 2X	50µl	1X
upstream primer, 50 µM	1 µl	0.5µM
downstream primer, 50µM	1 µl	0.5 µM
DNA template	2-4 µl	~250 ng (source dependant)
PCR Water	to 100µl	N.A.

Amplification program

Melting	94°C for 30 seconds
Annealing	57°C for 30 seconds
Extension	72°C for 30 second
Repeat	X 40 cycles

Run PCR product on 1% agarose gel with Sybr Green or ethidium bromide stains and a 100 base pair DNA ladder to verify that DNA product was obtained and that it is of the appropriate size. If DNA is present, proceed with DGGE analysis of DNA product.

Denaturing Gradient Gel Electrophoresis (DGGE) Protocol

Making Denaturing Solutions:

Tips: Prepare in a fume hood

Place higher concentration denaturants into 32°C shaker to dissolve urea

Wrap top of all acrylamide containing bottles with parafilm during storage

Store large portions of 10% APS at -20°C - if it's not frozen it will start to breakdown

We run a 20%-70% denaturing gradient and 8% polyacrylamide gel

Water-saturated butanol is being stored in the stock room flammable cabinet on the left

Location of ingredients:

40% acrylamide/Bis (19:1)	Rm 165 – Red Bucket in Fridge
50x TAE	Kevin's Bench
Formamide	PP IV (flammable)
Urea	Rm 118 – stock shelves
10% APS (ammonium persulfate)	Rm 165 – 50 ml centrifuge tube in freezer

Note: 100% Denaturing Solution:

- **40 ml Formamide**
- **42g Urea**

20% Denaturant (100 ml)

40% acrylamide/Bis (19:1)	18.8 ml
50x TAE	2 ml
Formamide	8 ml
Urea	8.4 g
Water	62.8 ml

30% Denaturant (100 ml)

40% acrylamide/Bis (19:1)	18.8 ml
50x TAE	2 ml
Formamide	12 ml
Urea	12.6 g
Water	54.6 ml

55% Denaturant (100 ml)

40% acrylamide/Bis (19:1)	18.8 ml
50x TAE	2 ml
Formamide	22 ml
Urea	23.1 g
Water	34.1 ml

<u>70% Denaturant (100 ml)</u>

40% acrylamide/Bis (19:1)	18.8 ml
50x TAE	2 ml
Formamide	28 ml
Urea	29.4 g
Water	21.8 ml

Other Solutions to be made:

<u>Stacking gel Stock Soln for 8% Acrylamide (100 ml)</u>

40% acrylamide/Bis (19:1)	15 ml
50x TAE	2 ml
Water	83 ml

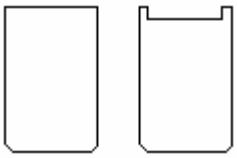
<u>Water-saturated butanol</u>

Combine water & butanol in an amber jar (2 ml water: 4.5 ml butanol)
Mix, and then allow to settle prior to use
Water-saturated butanol will be in top layer after the phases separate

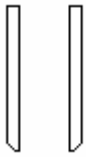
Preparing Gel Cast Assembly:

Tips: Clean plates before assembling; rinse 1x with EtOH, then 1x DI (use KimWipes to rinse and dry)
Make sure plates and all components are <u>completely</u> dry before assembling
Small spring clamps (clips) have the blue sticker on them and are used for pouring the gel; larger clips are used for attaching it to the running cassette

Parts:



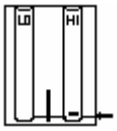
Glass Plates



Spacers (Thick – 1 mm)

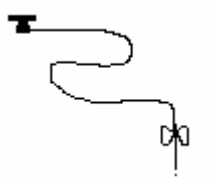
Blue Rubber Gasket

Comb

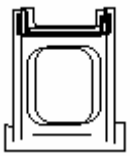


Gradient Mixer with Stir Bar in High Chamber

Allows flow of left chamber; up is closed



Tube and Butterfly Needle

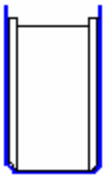


Dual Running Cassette

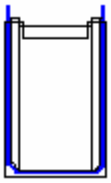
Spring clamps – small and big

- Put the blue gasket on the plate that is straight across the top (aim to get the slits in the corner) – larger overlap of gasket will be on outside of plate (not touching the gel)

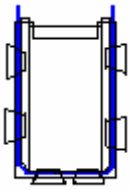
- Place the spacers on the plate with the gasket



- Place the second plate on top

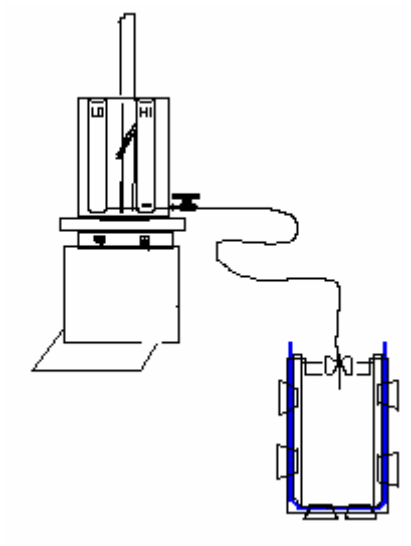


- Place small clips on each side – 2 per side



- Make sure that the comb fits between the plates (don't leave comb in)

- Assemble the stand



Place the gradient mixer on an elevated stir plate (use any box or contraption to raise the height as long as it is steady)

Attach the gradient mixer to the stand with clamps

Stand the plates on the bench top and fix the butterfly needle to deliver the denaturing solutions

Pouring the Gel:

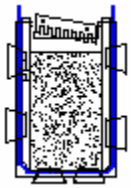
Tips: Melt 10% APS in water bath; cool prior to use
Make sure all components are chilled: polymerization is accelerated by warmer temperatures
Once TEMED and APS are added to working denaturing solutions, you have approximately 10-15 min before polymerization
Make sure the stir bar is in chamber B, and the stir plate is on

- Fill black bucket with ice
- Retrieve pre-made denaturing solutions from fridge, TEMED, and 10%APS
- Store all denaturing solutions, TEMED, and APS working solutions on ice while preparing gels
- Place two 50 ml tubes on ice and add 23 ml of low or high concentration denaturants
- Add 160 µl of 10% APS – mix gently
- Add 10 µl of TEMED
- Pour low concentration denaturant into chamber A of gradient mixer
- Pour high concentration denaturant into chamber B of gradient mixer
- Open the outlet to deliver to cast gel (stopcock)
- Immediately open middle valve (lower silver lever)
- Fill gel cast until top layer of gel reaches bottom of the butterfly needle
- Immediately redirect gradient mixer to empty 50 ml tubes, rinse <u>at least</u> one pour volume of each chamber with water
- Immediately place 1 ml of water-saturated butanol (in fume hood) on top of the gel using a bulb and Pasteur pipette. Allow to polymerize in hood: 1-2 hours minimum.

Pouring the Stacking Gel:

Tips: Make sure no bubbles get trapped in the combs
After the stacking gel solution has been filled to the top: wait 30-60s and fill up edges again (will continue to shrink). Repeat as necessary.

- Once the gel has polymerized, remove the layer of water-saturated butanol by dumping onto a napkin to evaporate in the fume hood
- Rinse the top of the gel several times with purified water
- Dry the gel cast assembly completely by inserting napkins or Whatman paper between the glass plates; drawing up the remaining purified water
- Insert the comb on an angle



- Prepare the Stacking Gel Working Solution as follows per gel
  In 50 ml cent tube: 5 ml of Stacking Gel Stock Solution
  5 µl TEMED
  50 µl 10% APS
- Mix gently
- Start filling the casting assembly with the Stacking Gel working solution using a Pasteur pipette and bulb.
- When the assembly is almost full, push the comb in all the way and finish filling to the top
- Allow the stacking gel to polymerize: 15-20 min

Preparing the Running Cassette:

Tips: Preheat tank minimum 1 hr (must reach 60°C to run samples)
Once the running cassette is assembled, pull the blue gasket away from the bottom of the glass plates or it will block the flow of electricity

- Remove small clips from polymerized gel
- Place the gel up against the side of the running cassette with the larger glass plate that is straight across the top facing the outside
- Using big clips, attach the glass plates (gel) to the running cassette by placing two on each side (left and right)
- Two gels can be placed on the running cassette; if only one gel is needed assemble a "false" gel by assembling a glass plate sandwich, spacers, etc and attaching to opposite side of running cassette. This is needed to create a 'buffer dam' in order to keep running buffer in the reservoir
- Place the running cassette into the tank
- Fill the reservoir with running buffer using one of the white tubes
- Attach white tube to the running cassette

Preparation of Sample:

Tips: PCR product is loaded onto the gel

- Amplify DNA with *Bacteria*-specific primers PRBA338F-GC and PRUN518R

Loading Samples and Running the Gel:

Tips: Make sure running buffer has reached 60°C to run samples
Rinse & load wells from L → R as you are facing the tank; re-circulation pump causes unpolymerized acrylamide to float out of well and to the right
Place 3 µl amounts of dye on parafilm, mix sample in, and then load each sample
Use long-nose tips for loading samples
Maximum of 70-75V has been achieved to date (even when set to 200V)
Wet gloves and surfaces that touch the gel, keeps it from breaking

- Rinse wells thoroughly with running buffer to remove unpolymerized acrylamide from wells
- Mix 25 µl DNA and 3 µl of 10X Loading Dye
- Load samples
- Attach one of the black electrical wires to the running cassette
- Start power at 200V for 5-8 hours (20 mA for one gel; 40 mA for two gels)
- Stop pump and power
- Remove running cassette from tank
- Remove gel from gel plate by removing clamps and one glass plate, leaving the gel on the second glass plate
- Incubate gel on glass plate in fresh 1X TAE/Ethidium Bromide: 30 min
- Visualize on UV illuminator
- Photo document

Other Notes:

- Chamber can hold up to 6 gallons of 1x TAE (usually fill with 20 L) and can be used 4-5 times
- Hose goes into the white opening
- The black wire goes into the gel cast that we put in

Technique Tips:

- Prior to casting gel, make sure the needle has no blockages – clean it
- Create a vacuum in the needle for the denaturing solutions before pouring gels
  To do so, squirt a little distilled water into the right channel (direct outlet line to waste) and let drain until its almost done – stop flow by turning the stopcock. If at any point a vacuum is lost and this cannot be done a blue needle (25g) attached to a 1 ml syringe will fit inside the butterfly needle and works well to draw a vacuum.
- Make sure that the valves are closed when putting denaturing solution into chambers
- Make sure that there are no leaks and the two glasses are tightly sealed
- Make sure the comb fits between the plates
- Gel shrinks a little when dried so let it fill up to the maximum height (with stacking gel)

- Load approximately 20-25 µl of DNA in each well
- Gel can be stored overnight at 4°C
- Stacking gels can be poured prior to overnight storage or the next morning immediately prior to running samples
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Acrylamide Spills

- Soak up with sorbent sheet
- Treat with 1.6% Potassium Persulfate
- Treat with 1.6% Sodium meta-Bisulfite

Clean-up

- Treat clean up as if it were a spill if it touched unpolymerized acrylamide
  i.e. all pipets, glassware, and workspace should be wiped down and treated with 1.6% Potassium Persulfate and 1.6% Sodium meta-Bisulfite
- All DGGE running apparatus should be cleaned with lab soap and warm water and allowed to air dry