New Approaches to Evaluate the Biological Degradation of RDX in Groundwater

Project Number (ER-1607)

Paul B. Hatzinger, Ph.D.
Shaw Environmental, Inc.

Brief To The Scientific Advisory Board

October 18, 2007

Performers

Dr. Kung-Hui (Bella) Chu
Texas A&M University
Molecular biology, stable isotope probing

Dr. Jalal Hawari
NRC, Biotechnology Research Institute
Analytical chemistry, metabolite analysis

Dr. Neil Sturchio
University of Illinois at Chicago
Geochemistry, stable isotope analysis

Dr. Steven Fallis
Naval Air Warfare Center, China Lake
Chemistry, organic synthesis

Drs. Paul Hatzinger & Mark Fuller
Shaw Environmental
Biodegradation, bioremediation
environmental microbiology

RDX-Problem Statement

Sources:
C4

Composition A, B, C, HBX, H-6, Semtex,
Manufacturing/melt-pour plant discharges
Firing & training ranges (partial detonations)

Fate:

Mobile in soils (K_d= 0.2-0.9 L/Kg; Log K_{ow}= 0.87)
Nonvolatile (1.2 x 10^{-5} atm-m^3/mol)
Recalcitrant

DoD Liability:

Army: 583 sites (82 installations)[1]
Explosives in groundwater

Treatment:

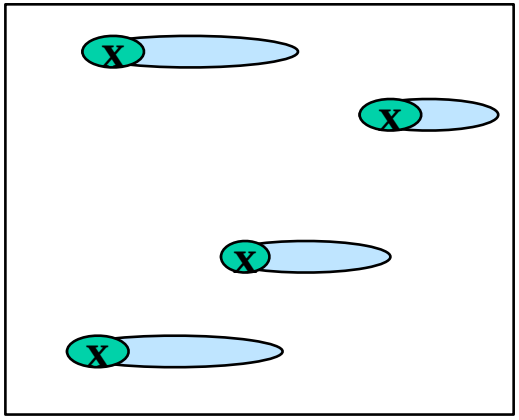
Soil: Composting, Slurry Reactors
Groundwater: Pump & treat with UV or GAC.

Issues:

Need for *in situ* remedial approaches.
No criteria for natural attenuation or enhanced bioremediation
- ***key organisms?***
- ***mode of biodegradation?***
- ***key metabolites?***

EPA Lifetime Health Advisory = 2 μg/L.

[1] Wani et al., 2003. Technical Report ERDC EL TR-03-11. USACE, Engineer Research and development Center, Vicksburg, MS



Technical Objectives

Develop Fundamental Understanding of RDX Biodegradation in Groundwater Under Differing Geochemical Conditions

1. ***Determine key metabolic intermediates.***

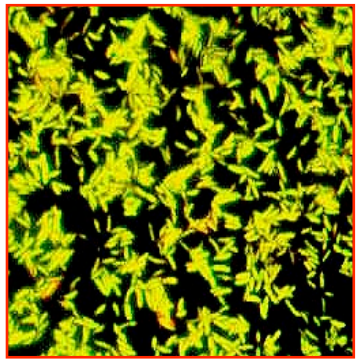
2. ***Identify important groundwater microorganisms & microbial communities via newly emerging molecular approaches.***

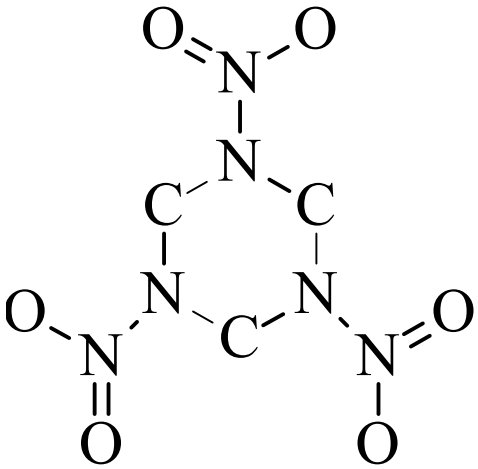
3. ***Develop stable isotope techniques to quantify in situ RDX biodegradation.***

RDX-Biodegradation Summary

Summary of Research

1. **Anaerobic RDX Biodegradation–sewage sludge & soils**
   - *** First degradation pathway proposed by McCormick et al., (1981)**
   - *** Additional pathway & detailed intermediates (Hawari, 2000-)**
   - *** Environmental studies & remediation (soils, compost, sludge)**
   - *** Pure cultures (*Clostridium, Klebsiella, Desulfovibrio*) from sludge**

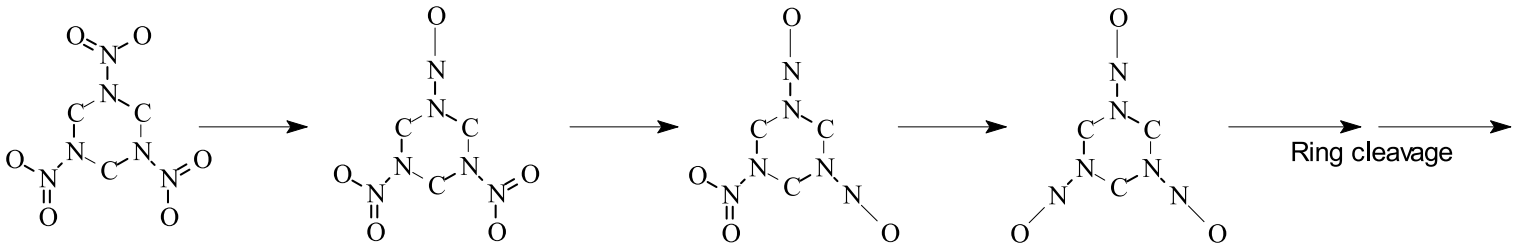




RDX

Technical Background

RDX Biodegradation: Anaerobic Pathway(s)



RDX

Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine **(MNX)**

Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine **(DNX)**

Hexahydro-1,3,5-trinitroso-1,3,5-triazine **(TNX)**

Cleavage Products:
Carbon Dioxide
Methanol
Nitrous Oxide
Formaldehyde

Hydrazine
Dimethylhydrazine

RDX-Biodegradation Summary

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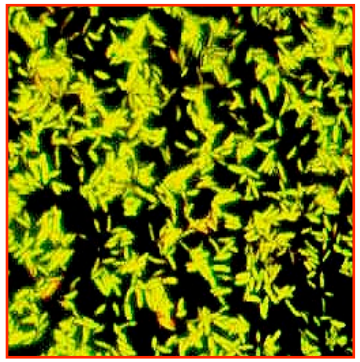
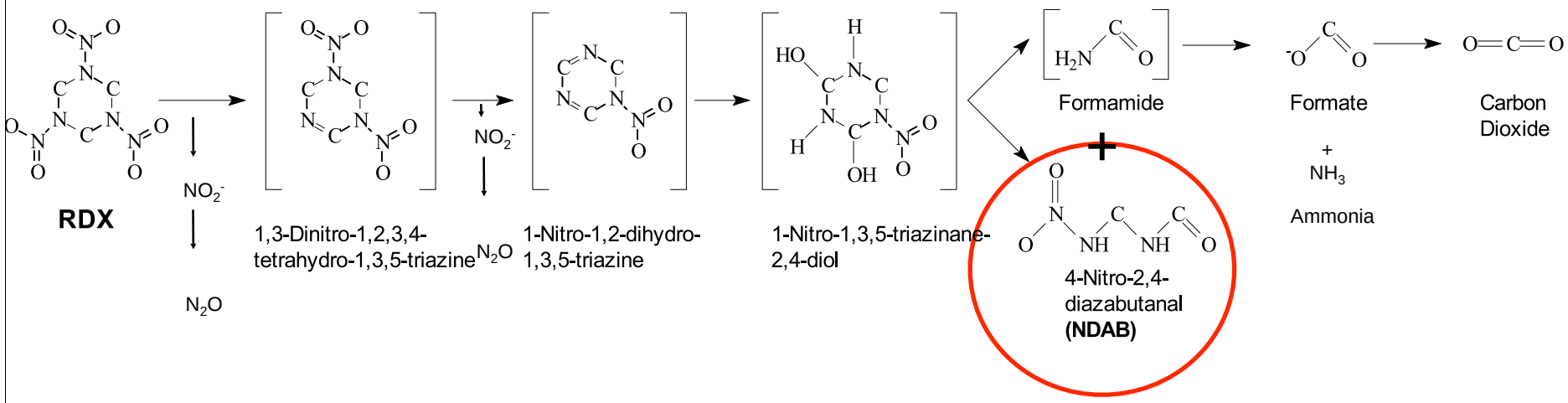


Technical Background

RDX Biodegradation: Aerobic Pathway



RDX-Biodegradation Summary

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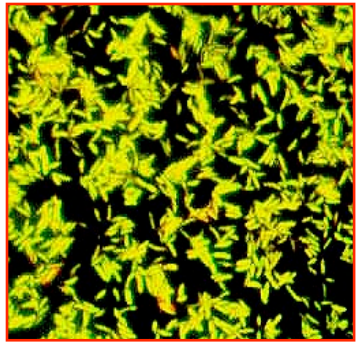
1. **Anaerobic RDX Biodegradation–sewage sludge & soils**
   - * First degradation pathway proposed by McCormick et al., (1981)
   - * Additional pathway & detailed intermediates (Hawari, 2000-)
   - * Environmental studies & remediation (soils, compost, sludge)
   - * Pure cultures (*Clostridium, Klebsiella, Desulfovibrio*) from sludge
   - * Type I nitroreductases; *hydA* from *Clostridium*

2. **Aerobic RDX Biodegradation –pure cultures**
   - * Cultures using RDX as a N source (*Stenotrophomonas* spp.; *Rhodococcus* spp. A1, DN22, 11Y) from soils
   - * Aerobic pathway described for DN22 (Fournier et al., 2002)
   - * Two recent soil cultures use RDX as a C, N and energy source (*Gordonia* & *Williamsia*, spp.) (Thompson et al., 2005)
   - * key enzyme/gene identified for *Rhodococcus* spp. (*xplA*)



Technical Background

SERDP Project ER-1378 " Microbial Ecology Effects on Explosives Biodegradation"

Picatinny Arsenal Groundwater

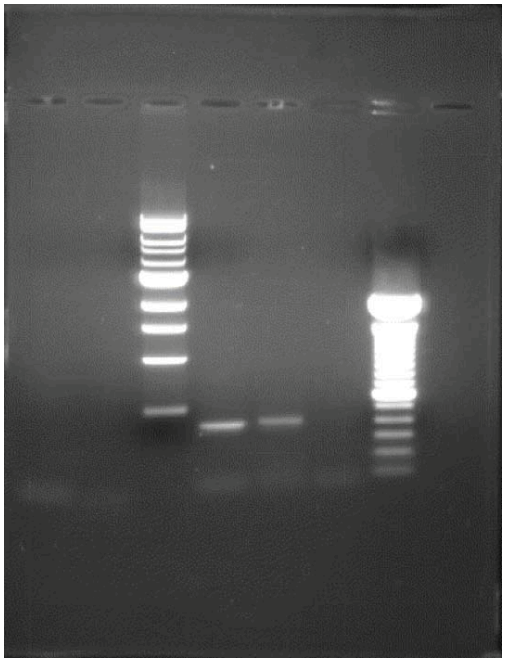
Sample		Vol filtered (L)	PCR Amplifiable**	PCR detection of: *xenA*	*xenB*	*xplA*	*onr*	*hydA*
40MW-4	Clean	1.95	Y	N	N	N	N	N
157MW-5	Contaminated	1.82	Y	N	N	N	N	N
40MW-7	Contaminated	1.95	Y	N	N	N	N	N

**DNA amplified using universal *Bacteria* DGGE primers.

Groundwater Data:

1. **Gene probes show absence of many suspected degradative genes?**

2. **Organisms identified via DGGE analysis of samples and enrichments very different from known degraders in soils?**



Key Questions

Is RDX degradation occurring in groundwater – under which geochemical conditions?

What are the key pathways and can we effectively measure "indicator" metabolites to differentiate these pathways?

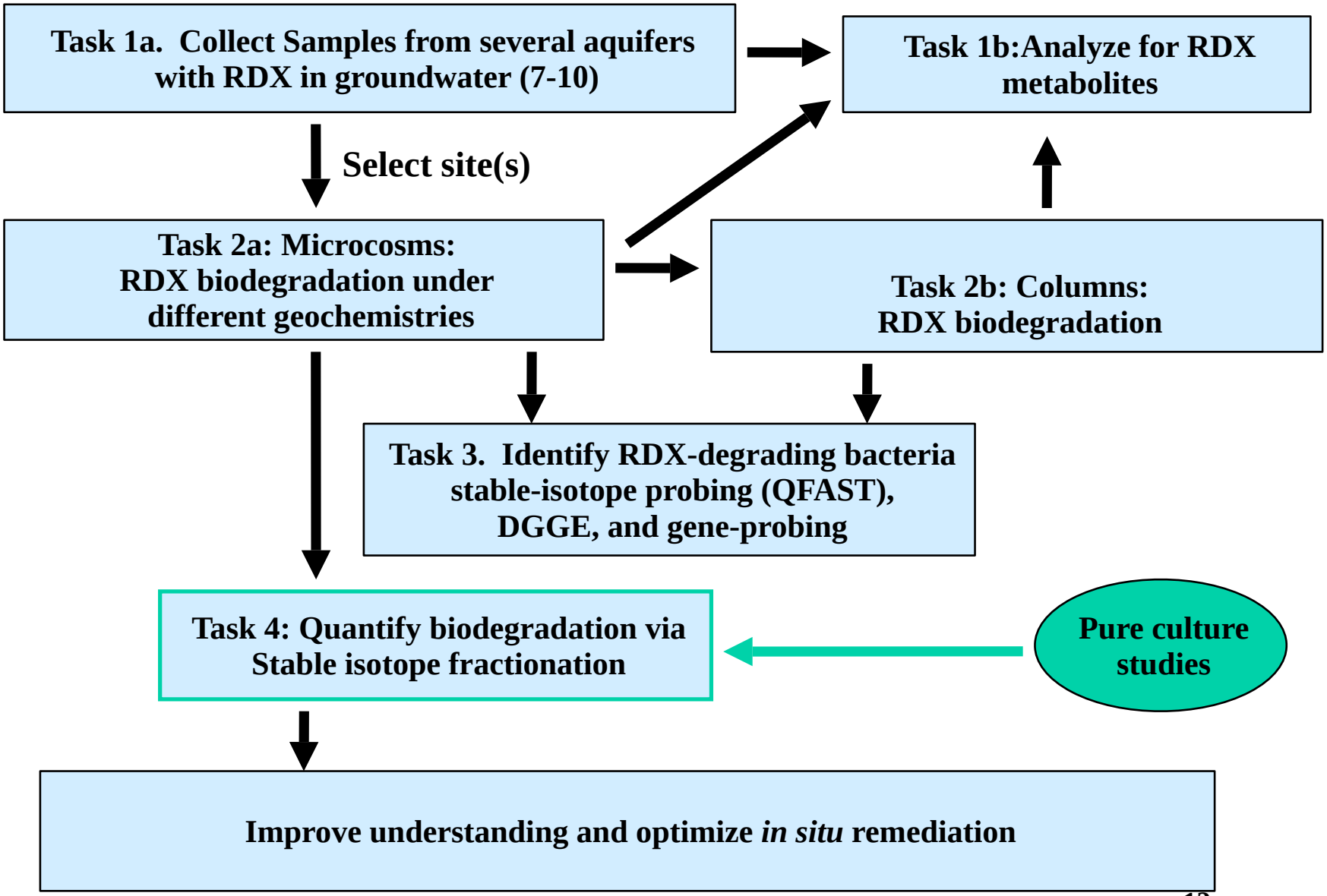
Which organisms are responsible?

How is RDX used by these organisms?

Can we effectively quantify and or verify the degradation process *in situ*?

How can we promote degradation and/or enhance rates?

Technical Approach



Technical Approach

Question: Is biodegradation occurring in aquifers and what are key pathways?

Task 1. Analyze Indicator Metabolites of RDX Degradation at Contaminated Sites.

Picatinny Arsenal (NJ)*
Mass Military Reservation
Iowa AAP
Umatilla Ordnance Depot (OR)
Pueblo Chemical Depot (CO)
Redstone Arsenal (AL)
Pantex Plant (TX)
Nebraska Ordnance Plant

Collect Groundwater
(7-10 Sites)
*** geochemical profile**

Conduct Metabolite analysis

NDAB
Formate
Formaldehyde
Nitrous Oxide

TNX, DNX, MNX
MEDINA
Formaldehyde
Hydrazine
Methanol
Nitramine
Nitrous Oxide

Biodegradation?

Technical Approach - Microcosms

Question: Under what geochemical conditions does degradation occur – key metabolites?

Task 2a. Evaluate RDX Biodegradation under Differing Geochemical Conditions

Collect Groundwater and Aquifer Solids (2+ sites)

Sites determined by Task 1 metabolite analysis

Prepare Microcosms

Aerobic
Denitrifying
Iron-reducing
Sulfate-reducing
Methanogenic
+/- nitrogen
+/- cosubstrate(s)

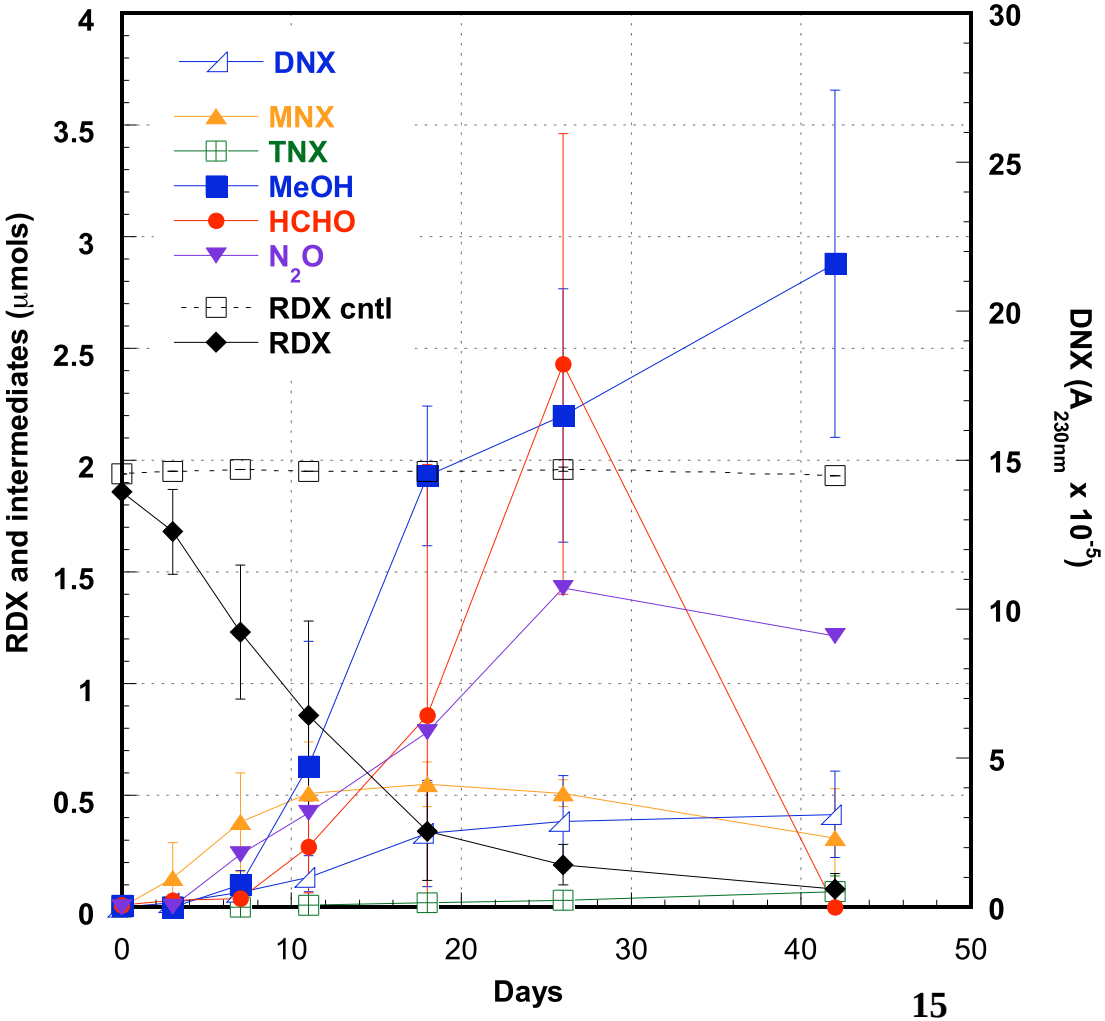
Site conditions

1. **RDX Loss**
2. **Metabolites**
3. **Geochemistry**
4. **Community Analysis**
5. **Stable Isotope Fractionation**

Quantify RDX Biodegradation, Metabolites, Microbial Communities

Technical Approach

RDX Degradation Intermediates Detected in Microcosms from Picatinny Arsenal Area 157: Anaerobic Incubation

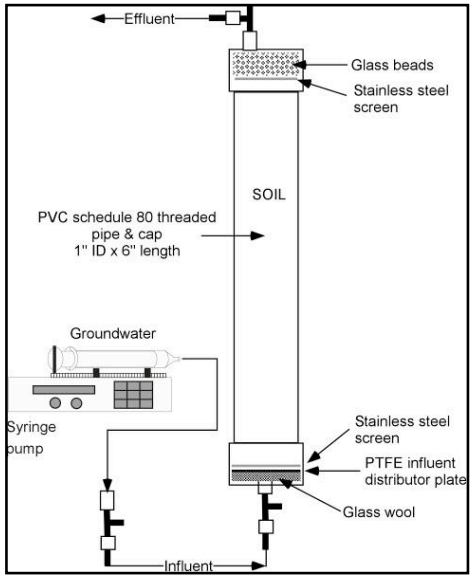


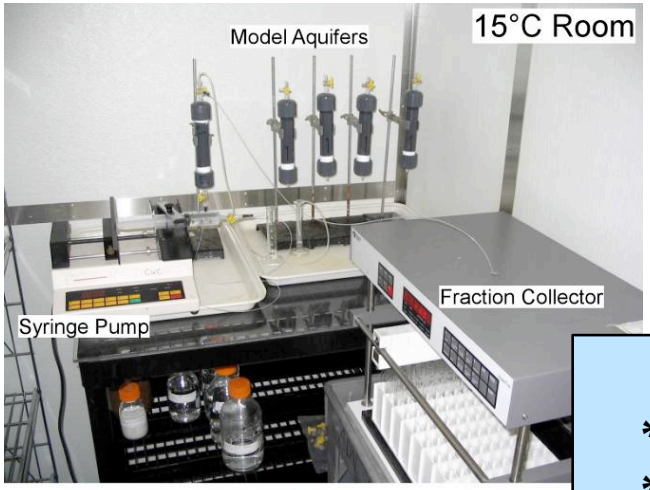
Technical Approach - Columns

Task 2b. Evaluate RDX Biodegradation: Long-Term Column Studies

Flow-Through Columns
Conditions based on Task 1 and Task 2a

<u>Example:</u>
Aerobic
Anoxic (denitrifying)
Anaerobic (methanogenic)





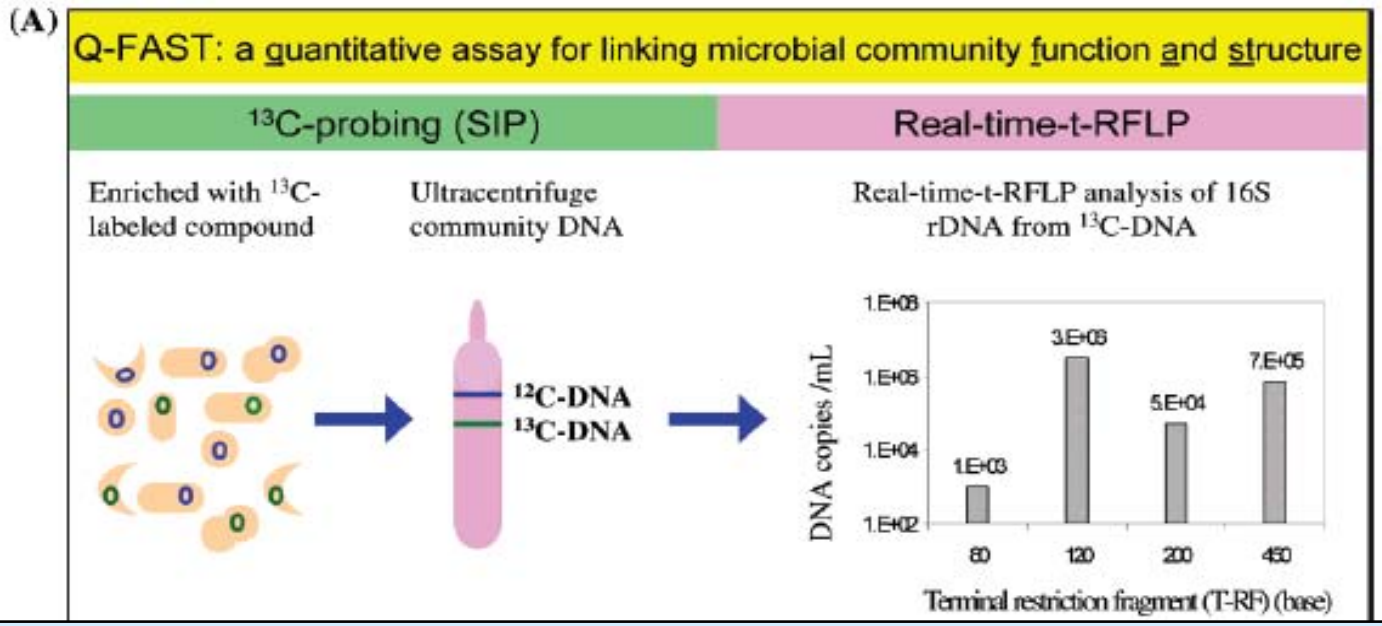
<u>Collect Effluent Samples</u>

- ***RDX Concentration***
- ***Geochemical Analyses***
- ***RDX Metabolites***
- ***Community Analysis (Task 3)***
- ***Isotope Fractionation (Task 4)***

Technical Approach

Question: Which microorganisms are responsible and how is RDX utilized?

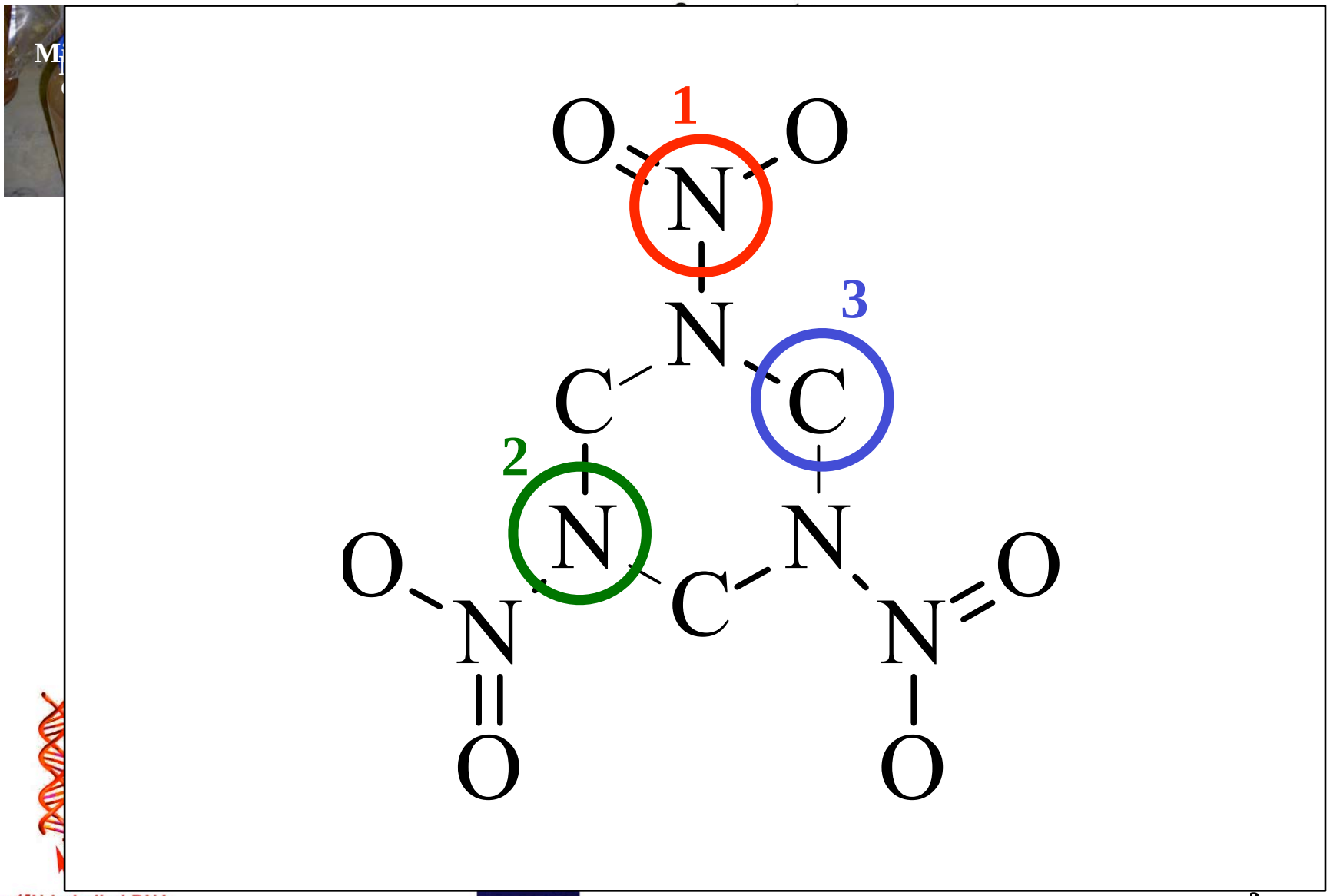
Task 3. Application of Stable Isotope Probing for Identification of RDX Degrading Bacteria



Benefits:

- *Directly link organisms with observed activity.*
- *No requirement for prior knowledge of strains/genes of interest.*
- *Quantify numbers of each active organism via rDNA copy number.*

Technical Approach
Task 3. Stable Isotope Probing



^{13}C- or ^{15}N-Labelled DNA

Technical Approach

Question: Can we effectively quantify and or verify the degradation process in situ?

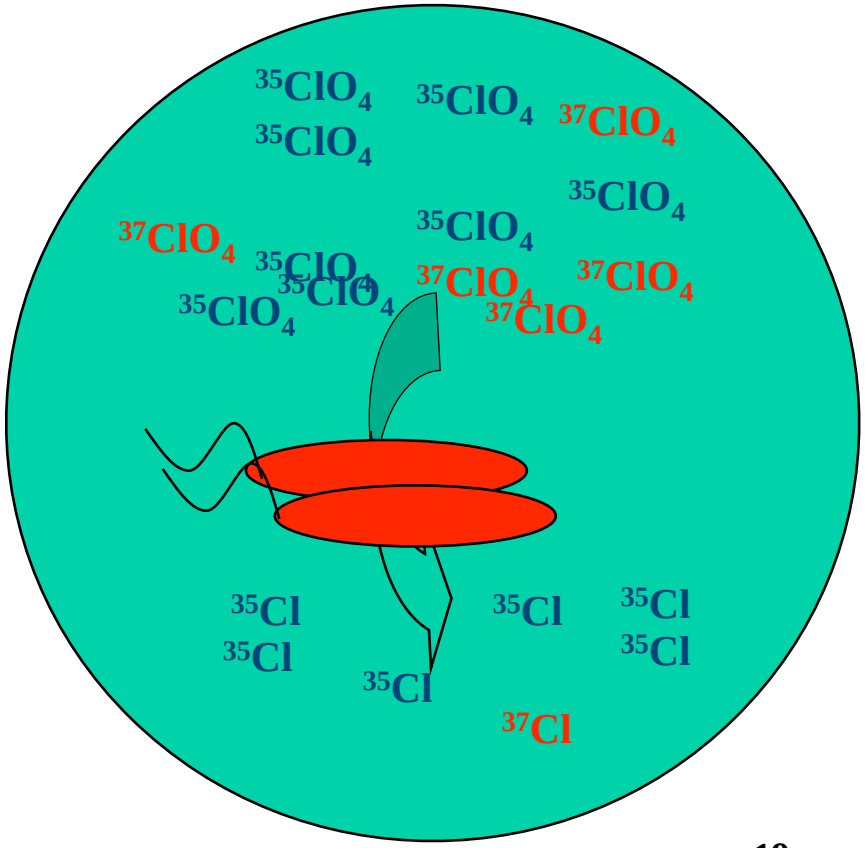
Task 4. Evaluate Biological Fractionation of N and C Isotopes in RDX during Biodegradation

Background:

Bacteria enrich heavy isotopes during biodegradation.
"Kinetic" isotope effect.

Enrichment can be used as a measure of biodegradation in flask and field.

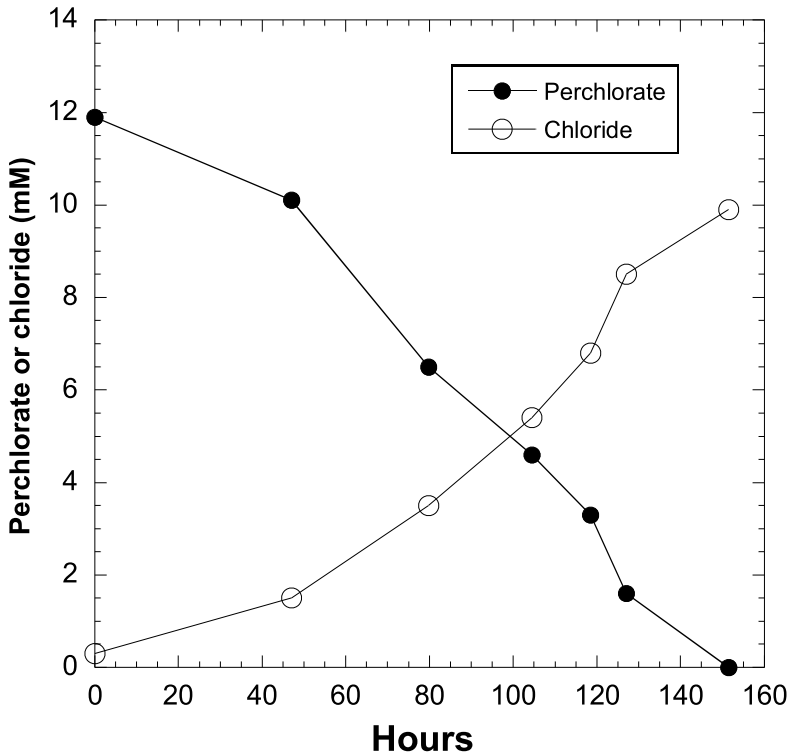
More powerful with multiple isotopes: fractionation factors and factor ratios.



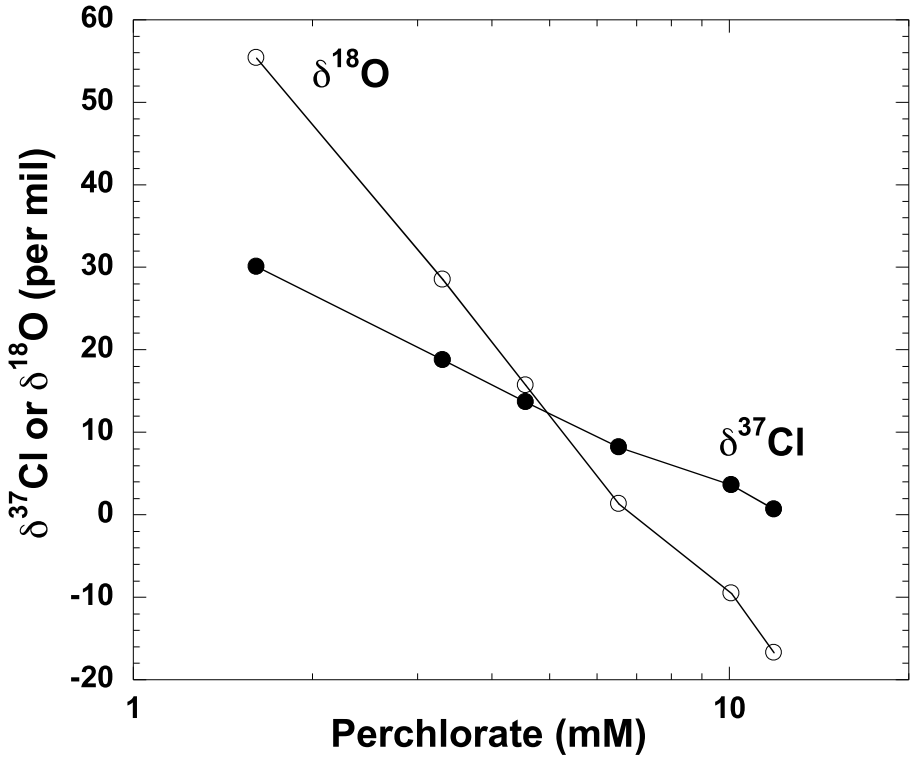
Technical Approach

Pure Culture Study: *Azospira suillum* JPLRND

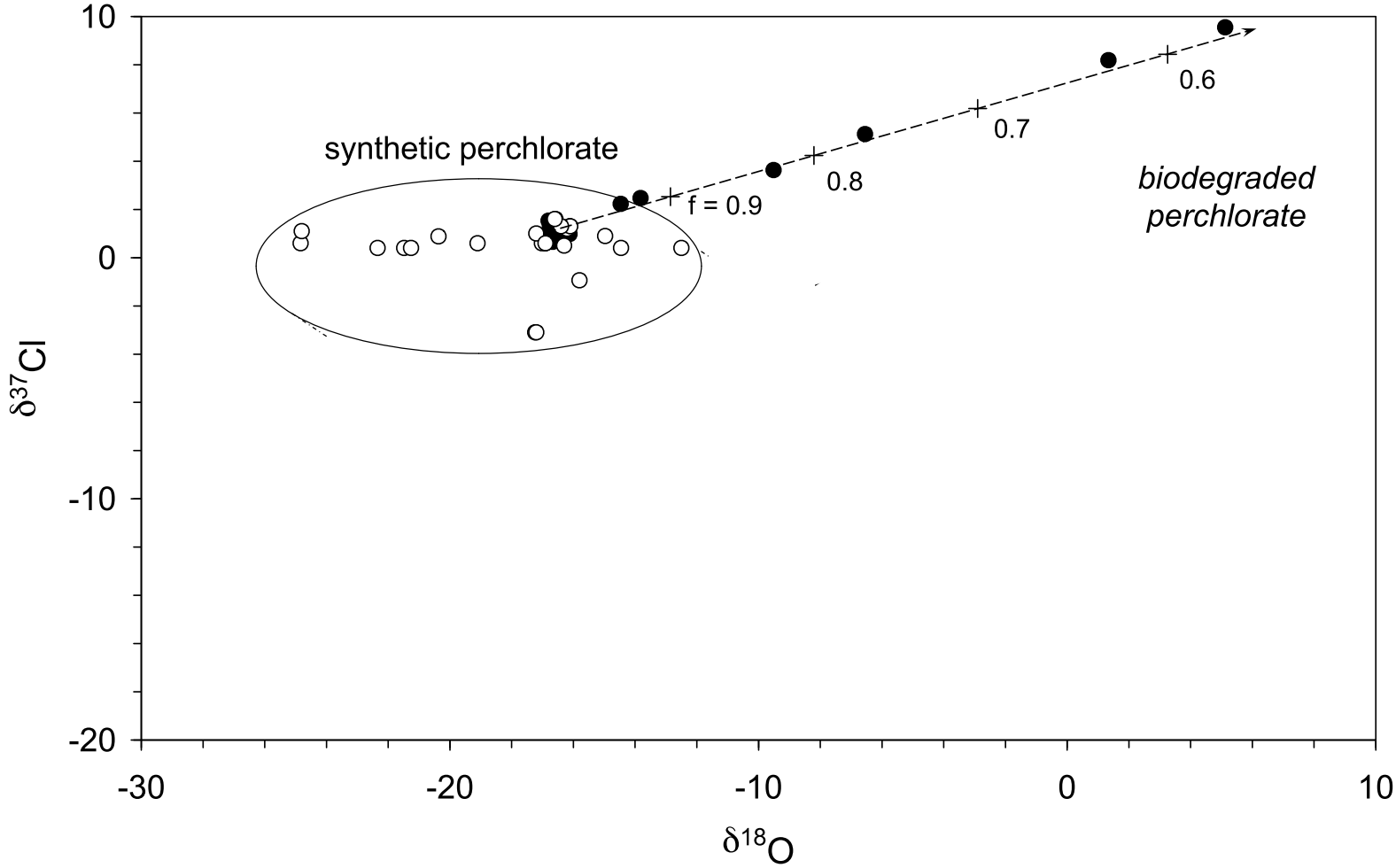
Perchlorate Biodegradation



Isotopic Fractionation



Technical Approach



Sturchio, N.C., J.K. Böhlke, A.D. Beloso, Jr., S.H. Streger, L. J. Heraty and P. B. Hatzinger. 2007. Oxygen and Chlorine Isotopic Fractionation During Perchlorate Biodegradation: Laboratory Results and Implications for Forensics and Natural Attenuation Studies. Environ. Sci. Technol. 41: 2796-2802.

Technical Approach

Question: Can we effectively quantify and or verify the degradation process in situ?

Task 4. Evaluate Biological Fractionation of N and C Isotopes in RDX during Biodegradation

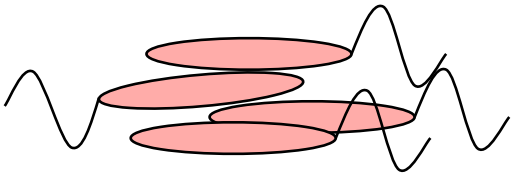
Method Development (GC-IRMS)

1. *Quantify $\delta^{15}N$ and $\delta^{13}C$ in RDX.*
2. *Purification of RDX from metabolites.*

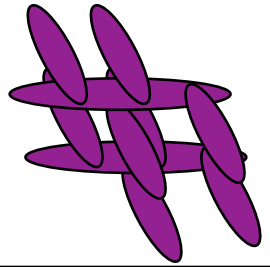
Pure Culture Studies

1. *Fractionation factors*
2. *Fractionation factor ratios*

Anaerobic pathway
e.g., Klebsiella sp.

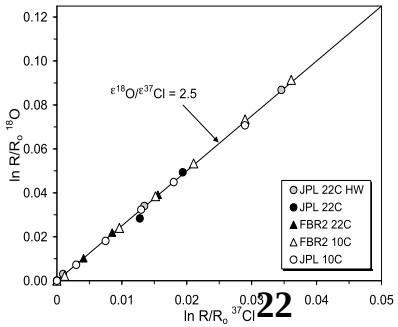


Aerobic pathway
e.g., Rhodococcus sp. DN22



Microcosms

1. *Fractionation factors*
2. *Fractionation factor ratios*



Year 1 Project Plan

Planned Year 1 Expenditures by Task **Cost (k)**

- **Task 1. Groundwater Sampling and Metabolite Analysis…… $158**
- **Task 2. Microcosm & Column Studies……$179**
- **Task 3. Stable Isotope Probing……$159**
  - ***Synthesis of labeled RDX*…… ….. $51**
- **Task 4. Stable Isotope Analysis……$110**

TOTAL $657

Overall Project Plan

The project will be performed over a 2.5 year period, with the task underway for the following durations:

	Year 1						Year 2						Year 3		
	1/2	3/4	5/6	7/8	9/10	11/12	1/2	3/4	5/6	7/8	9/10	11/12	1/2	3/4	5/6
Task 1 - Indicator Metabolites				*											
Task 2a - Microcosms						**									
Task 2b Column Studies															
Task 3 - Stable Isotope Probing															
Task 4 - Compound Specific Isotope Analysis															
Task 5 - Reports and Tech. Transfer															

•Decision point 1 – Choose sites to sample based on metabolite data

**** Go/No Go Point – If biodegradation does not occur in microcosms from site samples.**

Project Funding

YEAR	SERDP FUNDING (K)
FY08	$657
FY09	$482
TOTAL	$1139

Deliverables

PROJECT DELIVERABLES

- **New Methods to Document RDX Biodegradation and Bioremediation**
  - *QFAST Technique for Microbial Community Analysis*
  - *Biological Fractionation Factors for Both of N and C in RDX*
  - *Improved Technique for Natural Attenuation through Metabolite Analysis.*
  - *Discussion of Combined Use of All Methods*
- **Peer-Reviewed Publications (4-6 anticipated)**
- **Two Graduate Students and One Summer Intern Trained**

Backup Slides

Transition Plan

Presentations at National Meetings Attended by DoD Personnel

- Battelle In-Situ and On-Site Bioremediation Conference

- Joint Services Environmental management Conference and Exhibition

- SERDP Partners in Environmental Technology Symposium

Presentations to DoD Sites/DoD Contractors with RDX Contamination

- Picatinny Arsenal

- Massachusetts Military Reservation

Others Based on Task 1

Peer-Reviewed Journal Articles

4-6 Publications Anticipated

Reviewer Comments

Program Office Comments

1. Comment: There is some concern that the experiments described in Task 2 may be duplicative of past work.

1. Response: The microcosm and column experiments conducted in Task 2 are designed to serve as the experimental platform for detailed microbial community analysis via QFAST (Task 3), metabolite analysis, and isotopic fractionation studies (Task 4). While others have certainly performed such studies (i.e., degradation of RDX in microcosms with differing electron donors), the detailed community, metabolite, and isotope analyses listed above have not been performed. Moreover, the studies build upon initial results from ER-1378, utilizing new, more specific methods for community analysis.

2. Comment: Provide a plan to co-ordinate Dr. Jalal Hawari's efforts among similar projects.

2. Response: We have participated in an initial conference call with SERDP and all Project PIs to discuss Dr. Hawari's role in each project. We intend to cooperate with all other projects, and see only minimal overlap of activities.

Reviewer Comments

Reviewer 1 Comments

1. Comment: The molecular analysis in Task 1 lacks detail.

1. **Response:** The main purpose of Task 1 is to sample groundwater wells for analysis of RDX metabolites (indicative of *in situ* degradation). However, we have developed a technique to filter bacteria in the field and extract DNA for DGGE and gene probe analysis. Because we will be sampling wells, we intend to collect such samples. Initially, these will be archived, but based on geochemistry and metabolite results, we will have the samples in the future to perform community analysis via DGGE or to evaluate the presence of *alkB*, *xplA*, *xenA*, *xenB*, *hydA* and other genes suspected to be involved in RDX metabolism.

Reviewer Comments

Reviewer 2 Comments

1. **Comment:** The groundwater survey will depend on sampling aqueous phase - do the investigators consider this to be an accurate reflection of zone of degradation and/or process outcomes?

1. **Response:** The Task 1 groundwater survey is designed primarily to determine if soluble metabolites of RDX are present groundwater at various sites and under varying geochemical conditions. The collection of biomass is secondary – but may yield useful information as well. I believe questions concerning the relevance of attached vs. free-living bacteria in RDX degradation, however, are very relevant. We will be able to quantify both "communities" during microcosm studies, which is the advantage of this approach. In addition, we can perform an initial QFAST analysis on core samples (used for microcosm studies) from selected sites, particularly if these is evidence of natural attenuation at the site.

2. **Comment:** I am left with many components that should add up incrementally to new knowledge, however the overall picture of why all these components are included is not as clearly delineated.

2. **Response:** If successful, the research proposed will provide multiple lines of evidence for RDX biodegradation under differing geochemical conditions in groundwater. I believe any of the three main tasks (QFAST, metabolite analysis, stable isotope fractionation) could have been proposed individually. However, by combining these methods and completing all evaluations on an individual sample, I believe there is significant synergy. For example, if we observe RDX degradation under denitrifying conditions in microcosms, we should be able to quantify metabolites, characterize the responsible organisms, and determine the extent of biodegradation through isotopic fractionation. In sum, these data provide a much more thorough analysis of the RDX biodegradation and the community involved than any individual assay.

Reviewer Comments

Reviewer 3 Comments

1. **Comment:** The proposal lacks sufficient detail concerning… analytical methods, sites to be evaluated, molecular methods…

2. **Response:** It is difficult to provide complete details in the space of a 15-page proposal. The analytical methods employed have all been published by Dr. Hawari's group during the past several years. Additional details concerning the Molecular and stable isotope techniques are provided in this SAB briefing.

Reviewer Comments

Reviewer 4 Comments

1. **Comment:** Lots of work has already been devoted to metabolite analysis. Task 1 is not a wise investment…. There are better ways to confirm biodegradation, such as microcosms and push-pull tests.

2. **Response:** Many of the unusual metabolites that have been identified during pure culture and enrichment studies are not commonly analyzed for during site assessment studies of groundwaters contaminated with RDX. The nitroso-derivatives MNX, DNX, and TNX are not detected by routine EPA 8330 analysis at most commercial laboratories, and compounds such as MEDINA, NDAB, formaldehyde, and nitrous oxide are almost never included on an analyte list. Thus, I believe this task is worthwhile to evaluate whether degradation is occurring naturally at some sites, particularly with recent data showing the aerobic degradation potential of this nitramine. We intend to perform initial laboratory studies to assess whether samples can be preserved in the laboratory for detection of MEDINA, the most labile of the metabolites. Finally, we intend to perform microcosm and column studies using samples from at least two of the sites studied in Task 1 to confirm biodegradation under site conditions (if we see metabolites in the field) and/or to stimulate activity via geochemical modification (e.g., cosubstrate addition). A push-pull test, however, is also interesting idea if site data suggest that degradation is occurring. We completed a similar test recently for perchlorate (stable isotope studies primarily) with success.

Reviewer Comments

Reviewer 4 Comments *(continued)*

2. **Comment:** Task 2 & 3 lack originality and propose experimental approaches similar to the "rest of the crowd" and… the exploratory flavor makes it unclear that the proposed work will advance our fundamental understanding of nitroamine degradation…

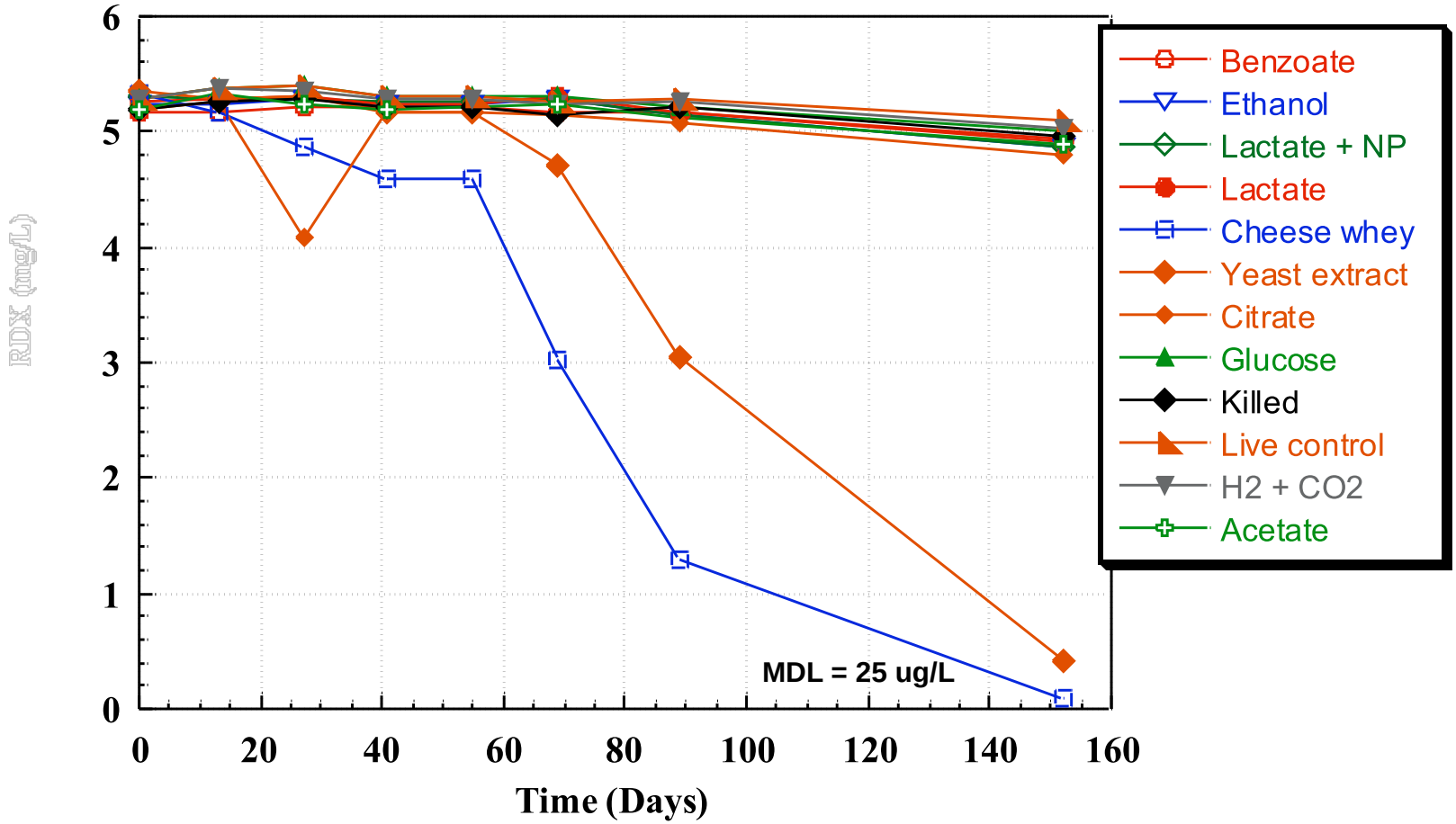
2. **Response:** The project is designed to provide multiple lines of evidence for RDX biodegradation in groundwater. The microcosm and column studies are not intended to be novel or to stand alone, but rather to provide controlled environments for the joint study of important metabolites, relevant degradative communities, and isotopic fractionation of RDX under differing geochemical conditions. Thus, analytical data verifying RDX pathways and degradation extent (metabolites and stable isotope fractionation) will be obtained in conjunction with molecular analysis of microbial communities via multiple techniques (QFAST, DGGE, gene probing). I believe a more thorough understanding of RDX degradation will result.

Supporting Information – BRI Role

Project	Analysis and Biological assays						
	Matrix	Strains	Assay	Conditions	Products	Kinetics, stoichiometry C & N mass balance	Pathways
ER1607 (Shaw, PH)	GW	Indigenous	Microcosms; Columns; Field	Field monitoring; Aerobic & anaerobic assays	Key products (Field & laboratory)	yes	yes
ER1608 (UW, SS; York, NB)	Soil (Can, UK, US)	11Y new strains	Metabolism	Whole cells Enzyme extracts P450 XplA enzyme Aerobic/anaerobic	yes	yes	yes
ER1609 (ERDC, FC)	Soil GW Soil GW	*Gordonia* KTR-9 and *Shewanella* MR-1, and	Metabolism KTR-9, MR-1, Physiology of RDX degradation Microcosm	Growing & Resting cells, Enzyme extracts Aerobic/anaerobic	yes	yes	yes

Supporting Information

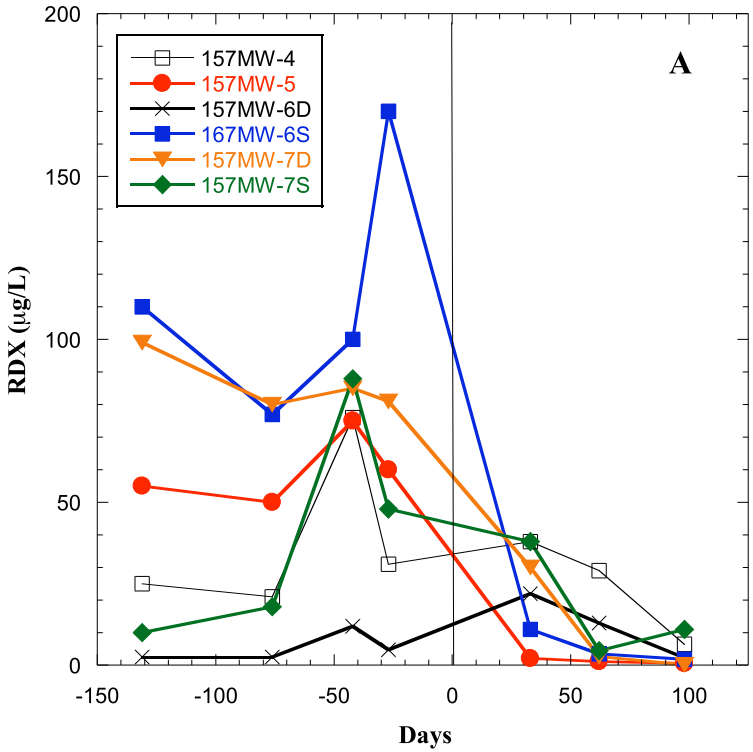
RDX Levels in Site 157 Microcosms Amended with Different Co-substrates



Supporting Information

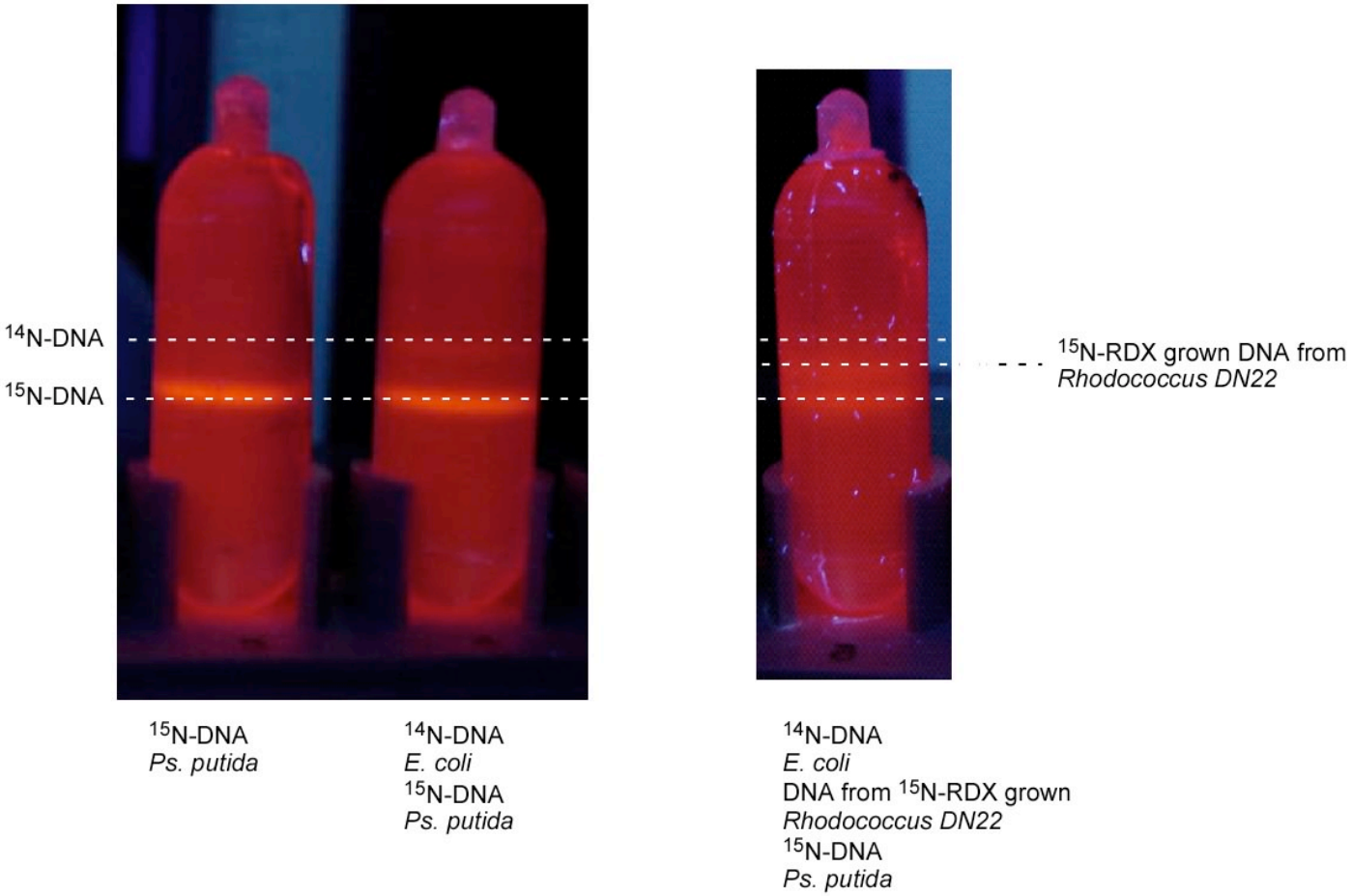
ESTCP-ER-0425 – In Situ Biodegradation of Explosives in Groundwater at Picatinny Arsenal: Recirculation System with Cheese Whey Addition

Treatment Wells



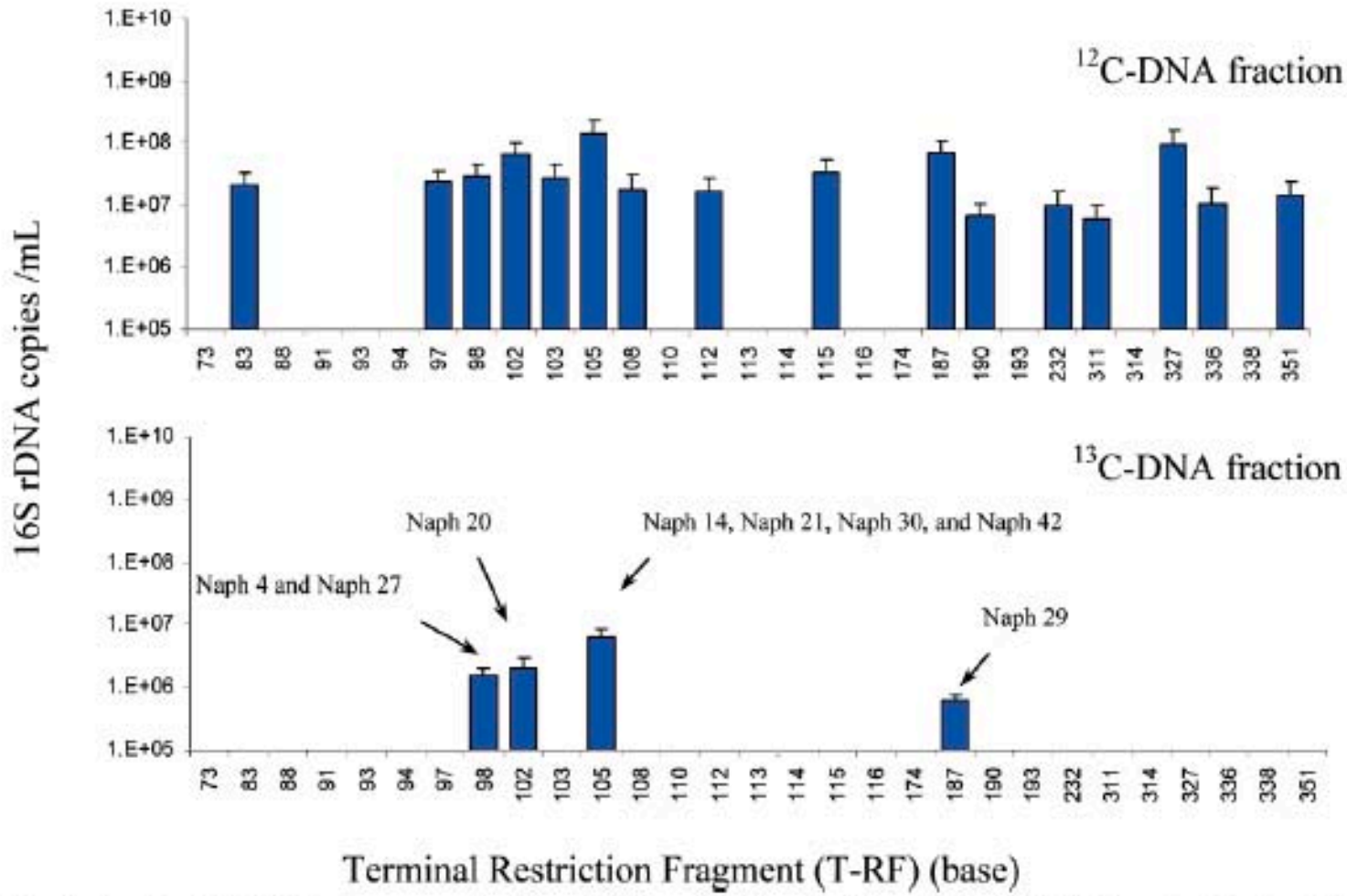
Supporting Information
Stable Isotope Probing Using ^{15}N-RDX

Separation of ^{15}N-labeled DNA from *Pseudomonas putida*, ^{14}N-labeled *DNA from Escherichia coli, and* ^{15}N-labeled DNA from RDX-grown *Rhodococcus* sp. DN22.



Supporting Information

Application of QFAST to quantification and identification of naphthalene-degrading bacteria from a contaminated sediment.



Supporting Information

Stable Isotope Terminology:

*Isotopic compositions of light elements are generally reported relative to a known standard as "delta" (δ) values and measured in parts-per-thousand (denoted "‰" = per mil).

Equation 1. δ (in ‰) = (Rx/Rs-1) * 1000

R = ratio heavy/light isotope (e.g., $^{37}Cl/^{35}Cl$)

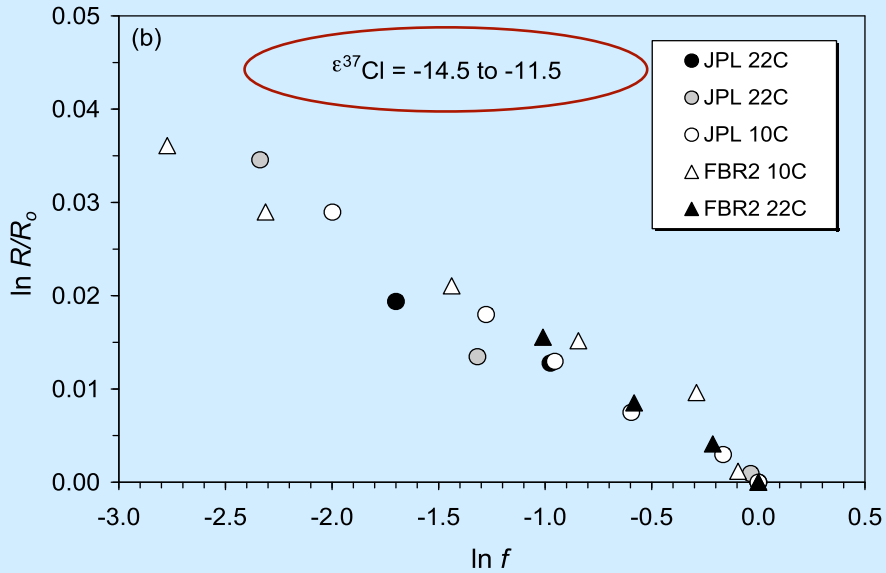
R_x = sample (e.g., $^{37}Cl/^{35}Cl$ in environmental sample)

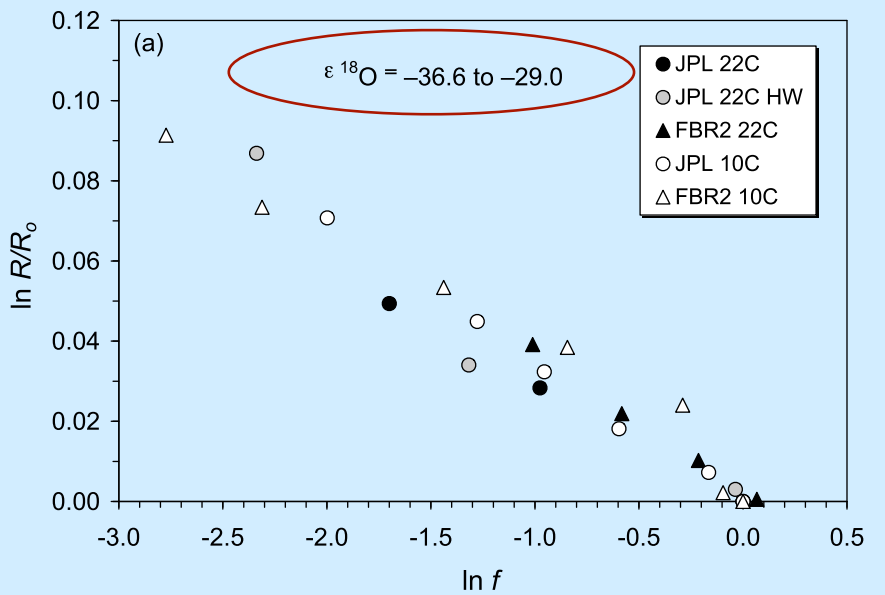
R_s = standard (e.g., $^{37}Cl/^{35}Cl$ in chlorine standard "SMOC")

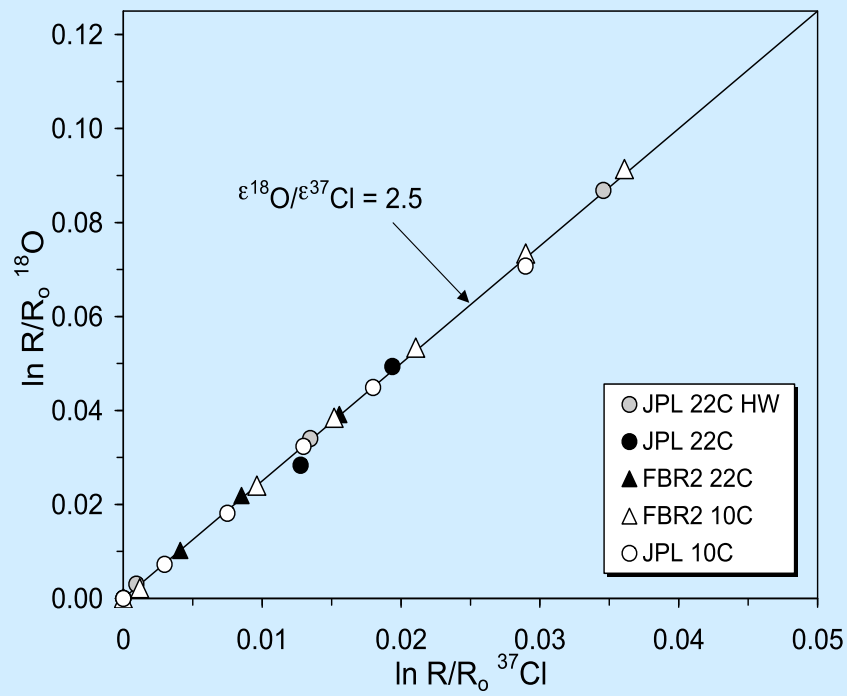
<u>* Example: $\delta^{37}Cl$ = + 30 ‰</u>

30 parts-per-thousand (3%) more ^{37}Cl in sample relative to a known standard (Standard Mean Ocean Chloride; SMOC; ^{18}O = SMOW; ^{13}C = VPDB)

Supporting Information





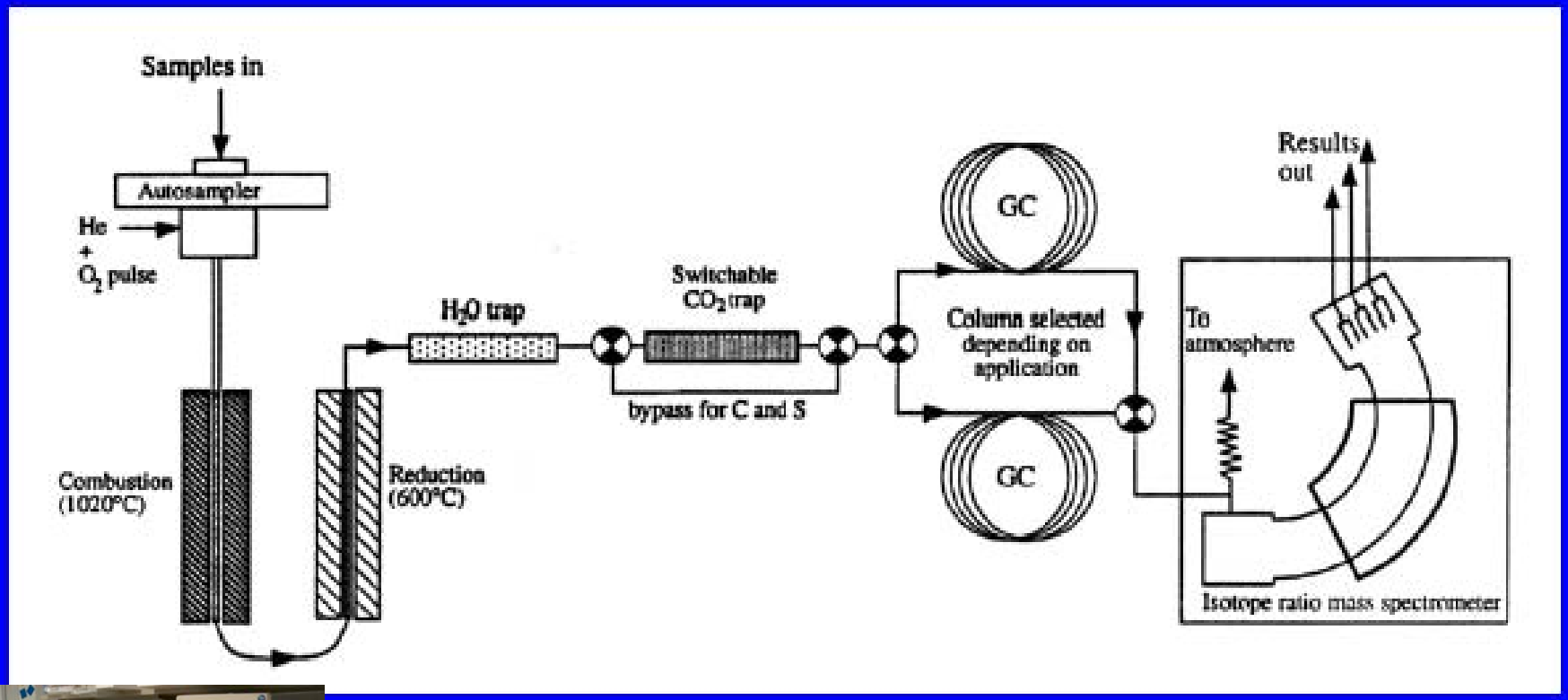


Fractionation Factors
&
Fractionation Factor Ratio

Strain independent
Temperature independent

Technical Approach

Continuous Flow IRMS System



Elemental analyzer – for automated combustion of RDX to produce CO_2 and N_2 for isotopic analysis of C and N; sends CO_2 and N_2 in continuous-flow He carrier gas stream to isotope ratio mass spectrometer

Supporting Information

Stable isotope analysis of TNT

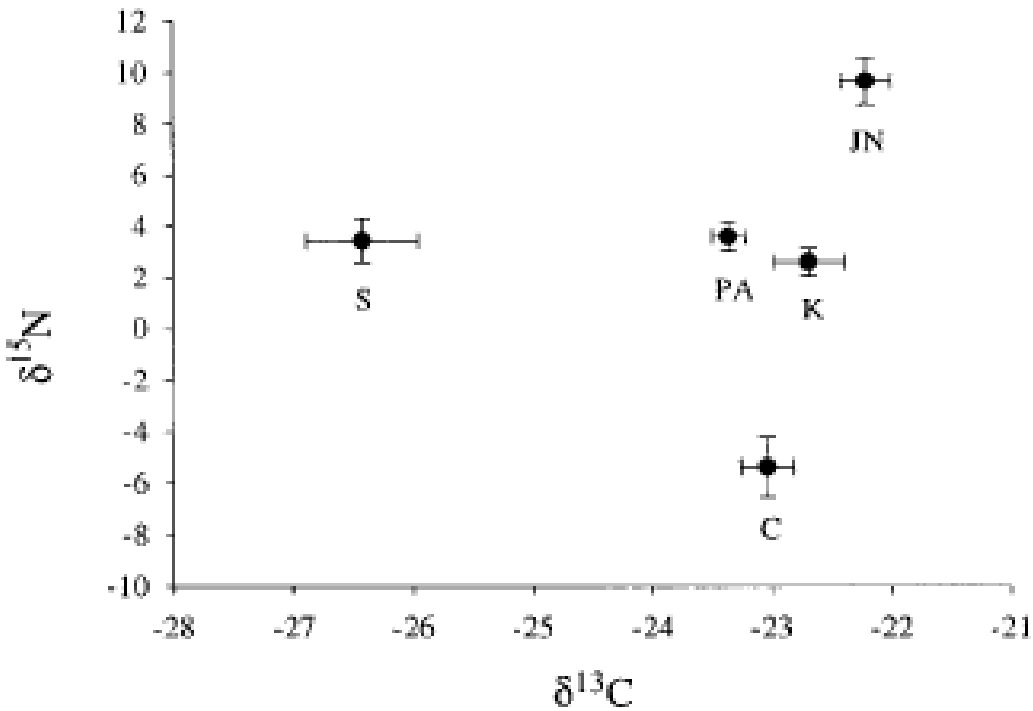


Fig. 2. Stable carbon and nitrogen isotope analysis of five sources of TNT. Sources include military grade from Croatia (K), U.S. military grade (C), Kodak technical grade (S), U.S. military grade from Sandia (JN), and U.S. military grade from the Picatinny Arsenal (PA). Ranges for each source are standard deviations of 10 to 12 runs on the gas chromatography/ion trap mass spectrometer/isotope ratio mass spectrometer.