New Approaches to Evaluate the Biological Degradation of RDX in Groundwater

ER-1607

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SERDP
DOD ▪ EPA ▪ DOE

Project Team

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environmental microbiology

* *Currently at McGill University*

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Background

2008 New Start SERDP Project

<u>Statement of Need:</u>

ERSON-08-02: "Improved Understanding of the Biological Degradation of Nitroamines in the Environment"

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Technical Objectives

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

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?

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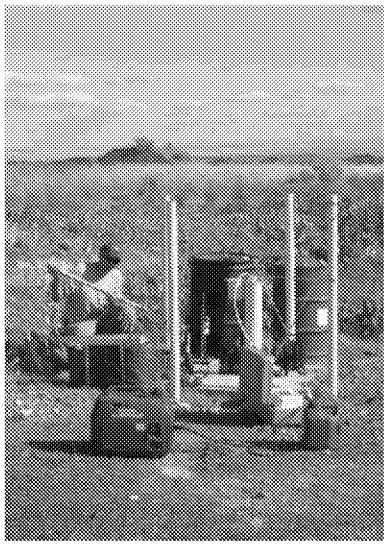
Technical Approach

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

Evaluate Preservation Methods



Collect Groundwater
(7-10 Sites)
* geochemical profile

NDAB
Formate
Formaldehyde
Nitrous Oxide

TNX, DNX, MNX
MEDINA
Formaldehyde
Methanol
Nitrous Oxide

Conduct Metabolite Analysis

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

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Technical Approach

Task 2. Evaluate RDX Biodegradation under Differing Geochemical Conditions

Collect Groundwater and Aquifer Solids (2 sites)

Prepare Microcosms, Mesocosms, Columns

Aerobic
Denitrifying
Iron-reducing
Sulfate-reducing
Methanogenic

Carbon sources

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

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

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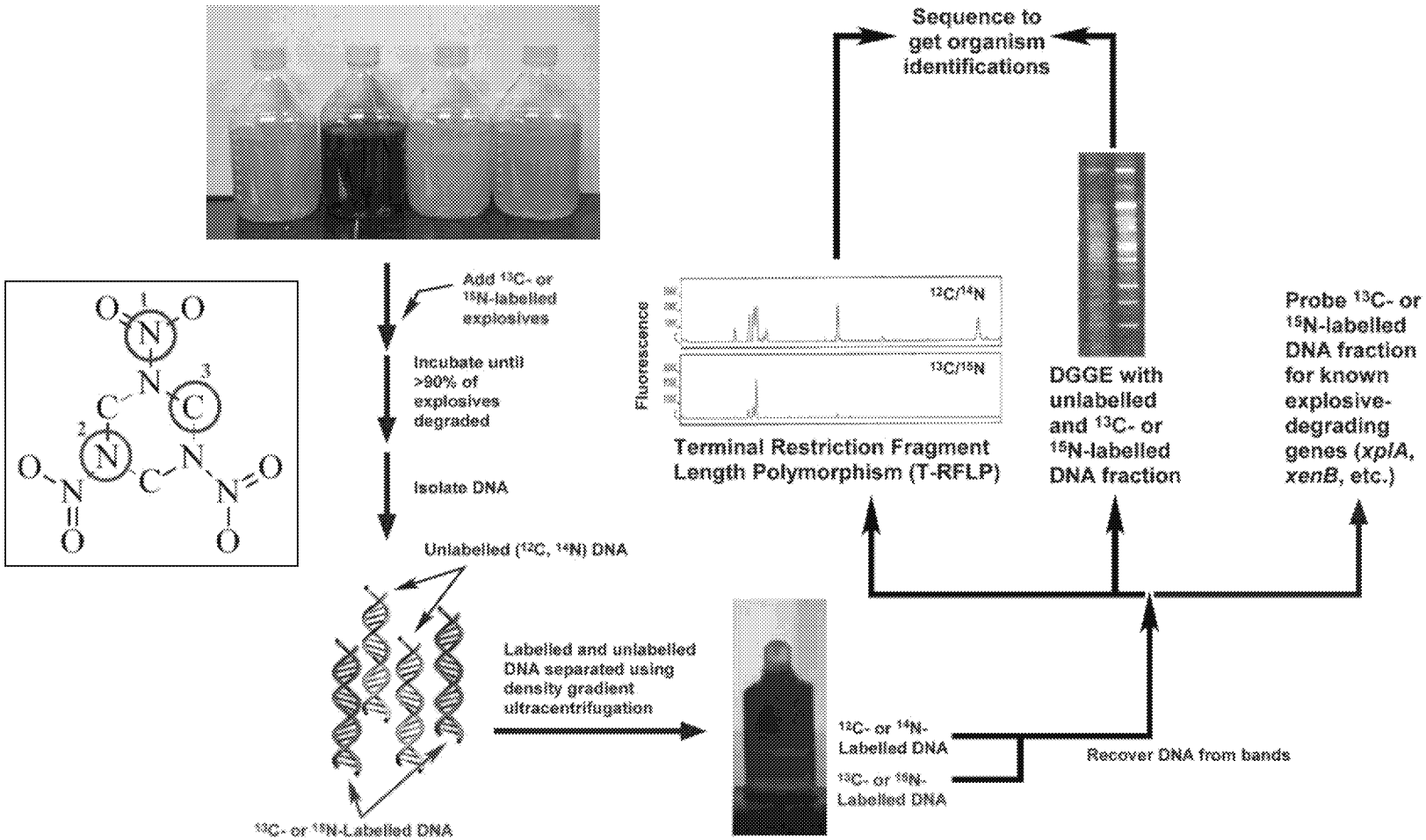
Technical Approach

Task 3. Apply Stable Isotope Probing (SIP) for Identification of RDX Degrading Bacteria



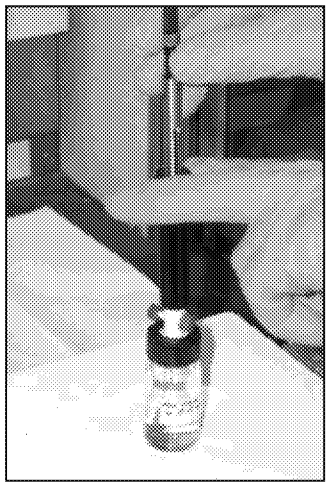
Question: Which microorganisms are responsible and how is RDX utilized?

Technical Approach

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

New Method (GC-IRMS)

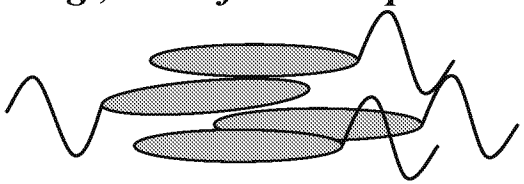
Method Development
Quantify $\delta^{15}N$ and $\delta^{13}C$ in RDX



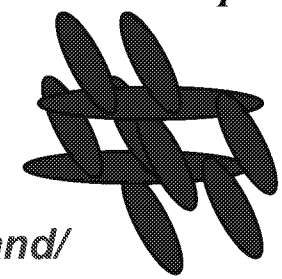
Pure Cultures

Quantify N and C Isotope Fractionation in RDX

Anaerobic pathway(s)
e.g., Desulfovibrio sp.

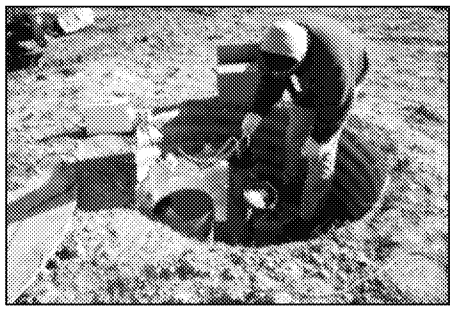


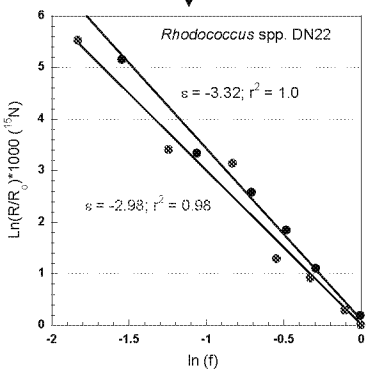
Aerobic pathway
e.g., Rhodococcus sp. DN22



Mesocosms

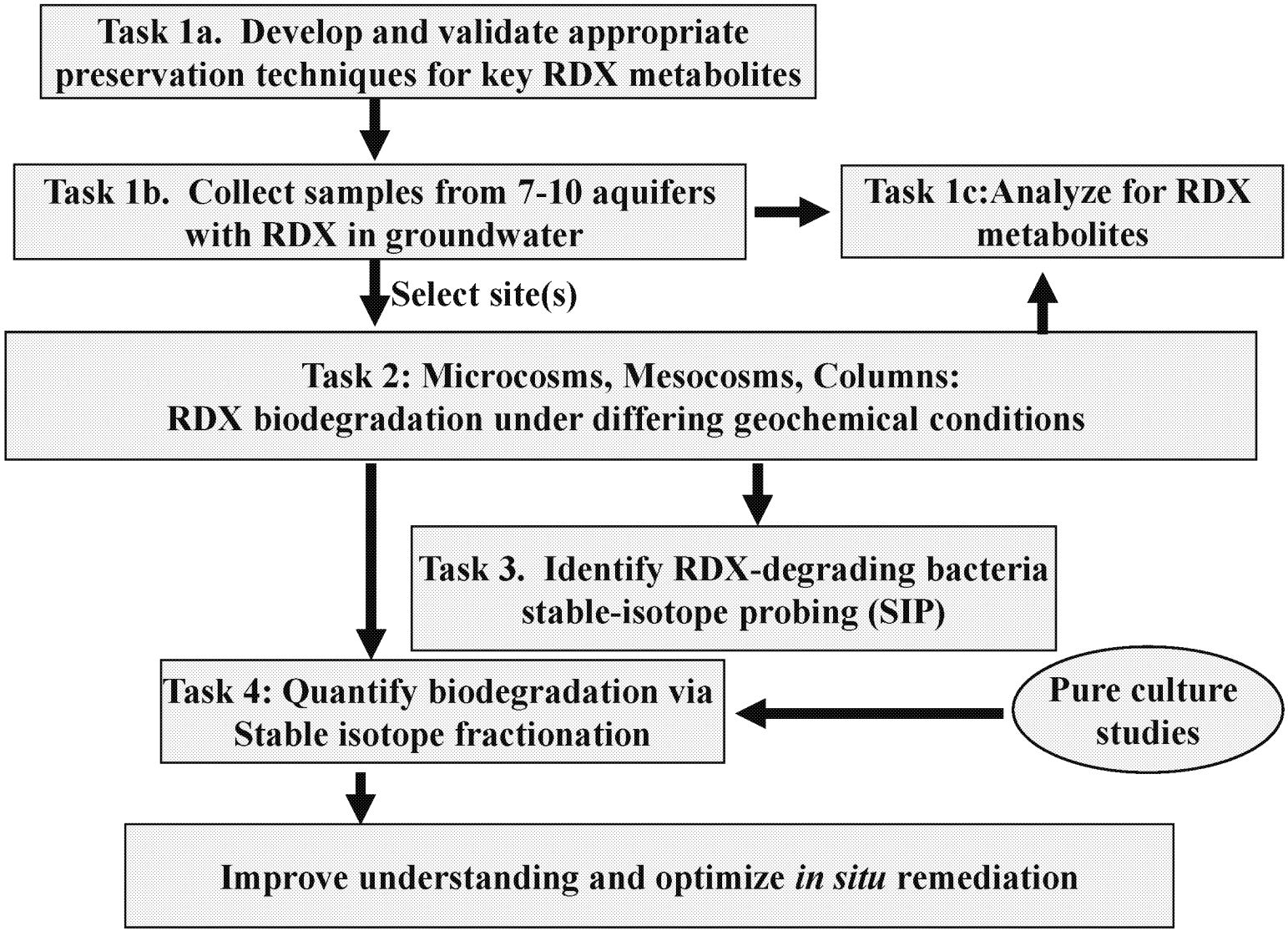
Quantify N and C Isotope Fractionation in RDX





Question? Can we effectively quantify and/ or verify RDX degradation using CSIA?

Technical Approach



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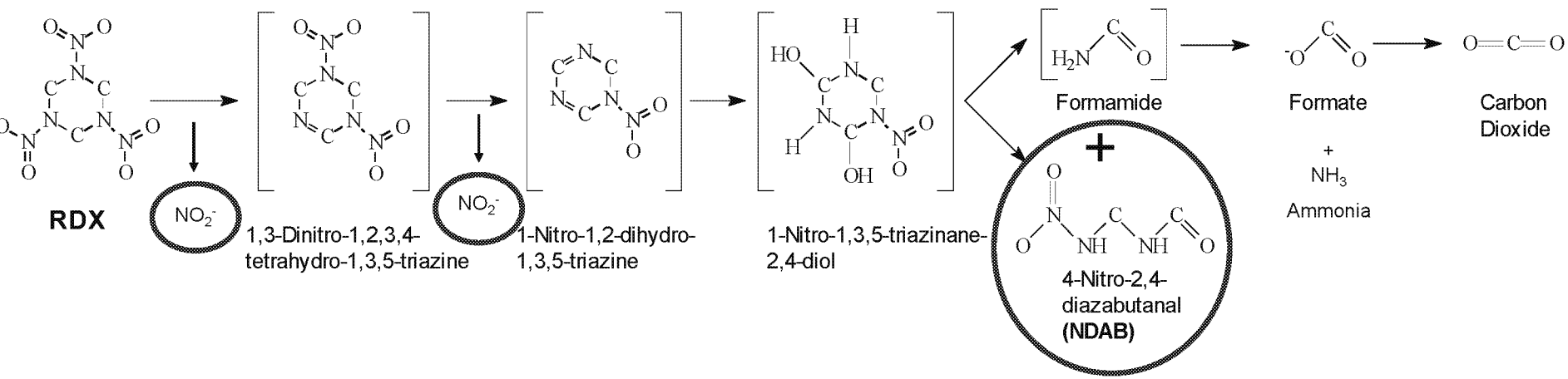
Background

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

RDX Biodegradation: Aerobic Pathway

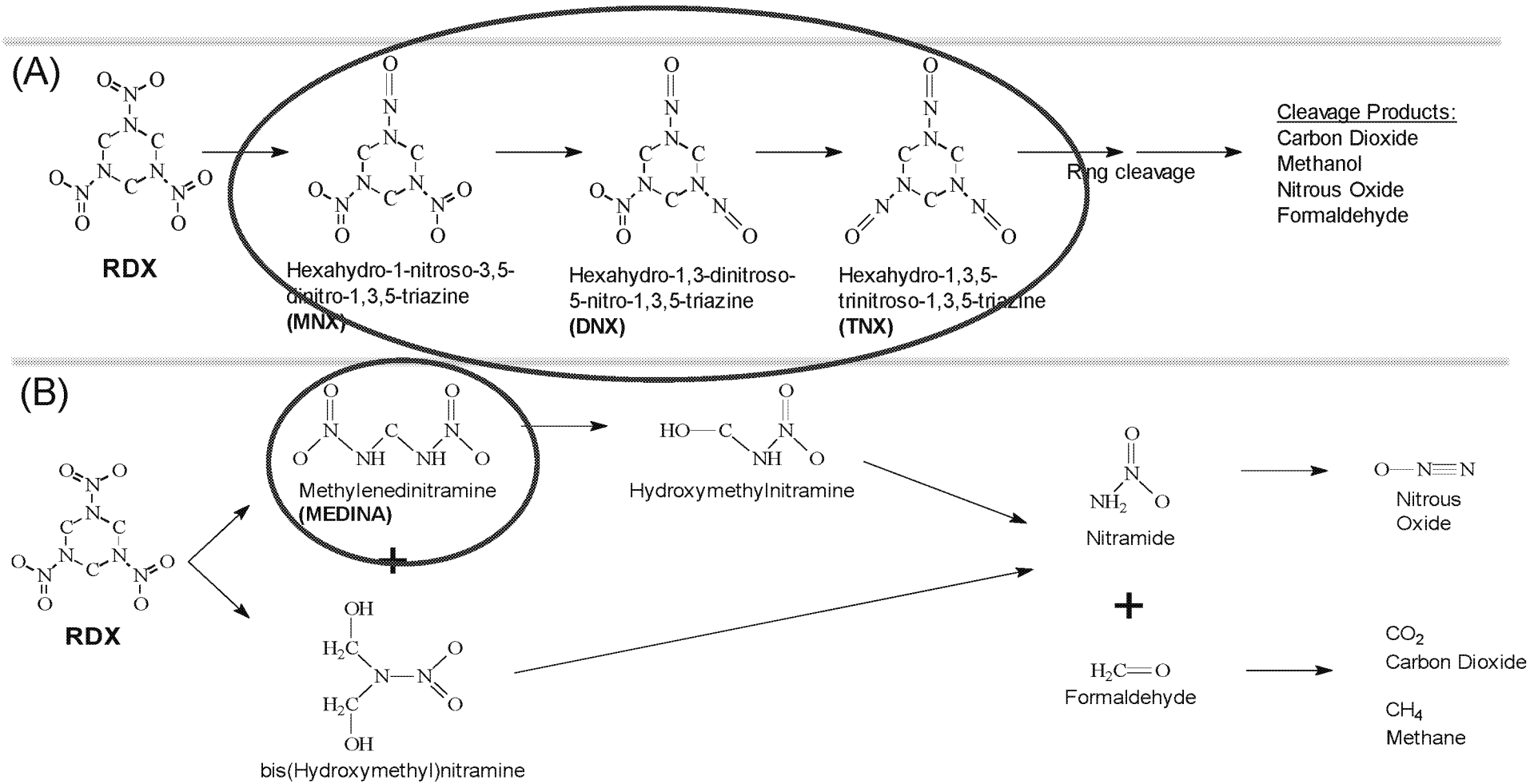


- **NDAB previously detected at Iowa Army Ammunition Plant & in soils at Canadian explosives manufacturer**

Background

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

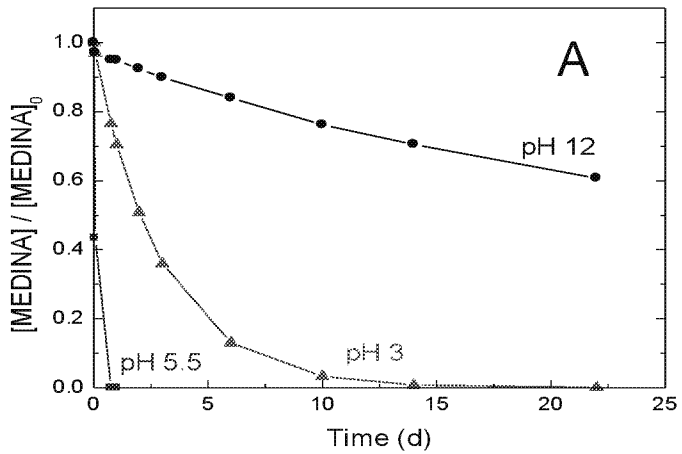
RDX Biodegradation: Anaerobic Pathways



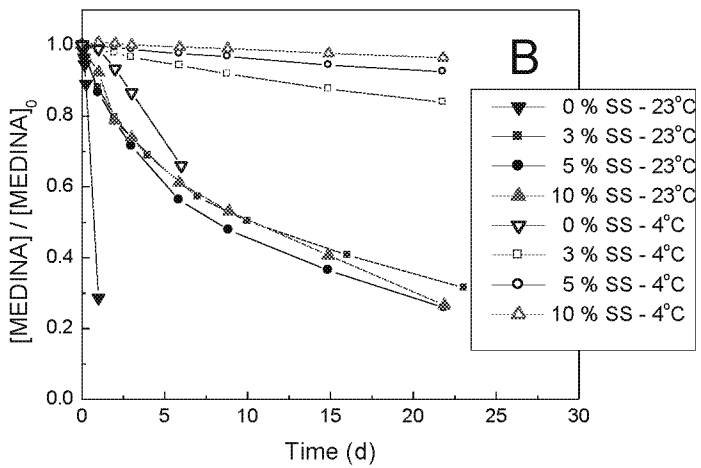
Results

Task 1a: Evaluation of Preservation Methods: MEDINA

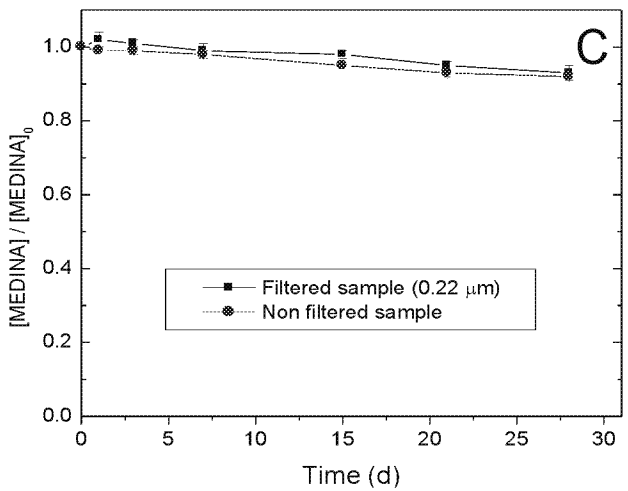
MEDINA in DI Water



MEDINA ± Sea Salt (4 or 23°C) in DI Water (pH 5.5)



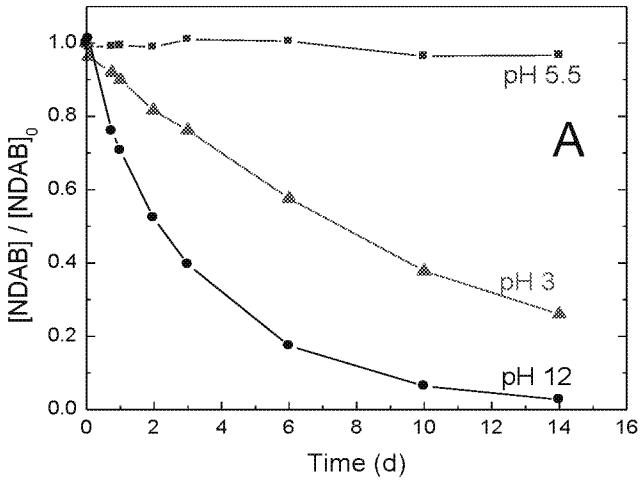
MEDINA + 10% Sea Salt (4°C) in Groundwater



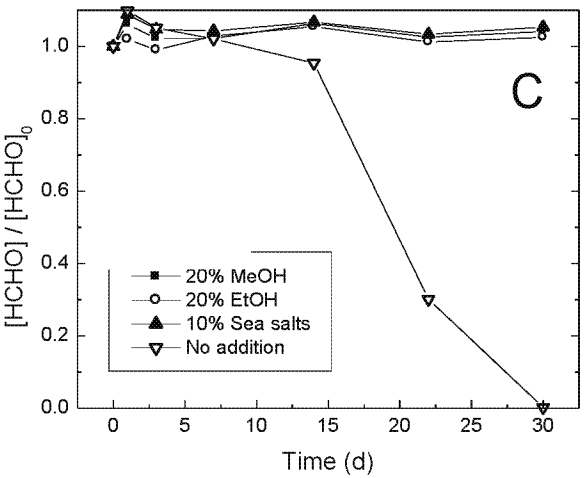
Results

Task 1a: Evaluation of Preservation Methods: NDAB, HCHO, NXs

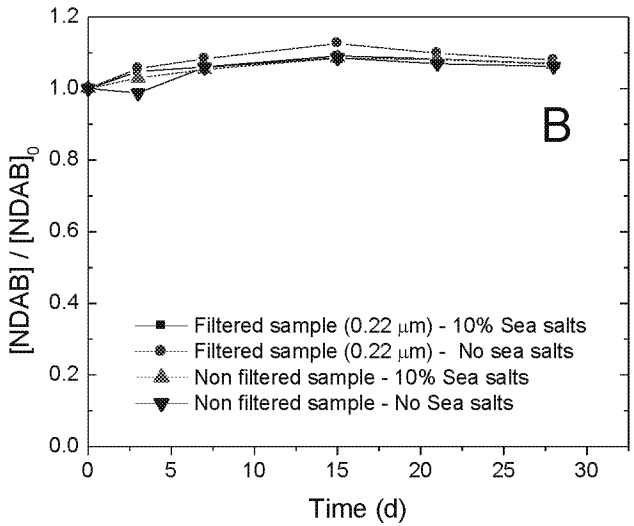
NDAB (40°C) in DI Water



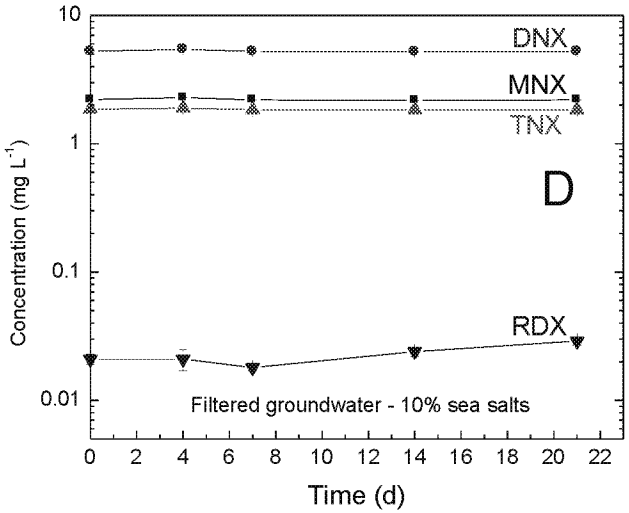
HCHO ± Sea Salts in Groundwater (4°C)



NDAB ± Sea Salt (4°C) in Groundwater

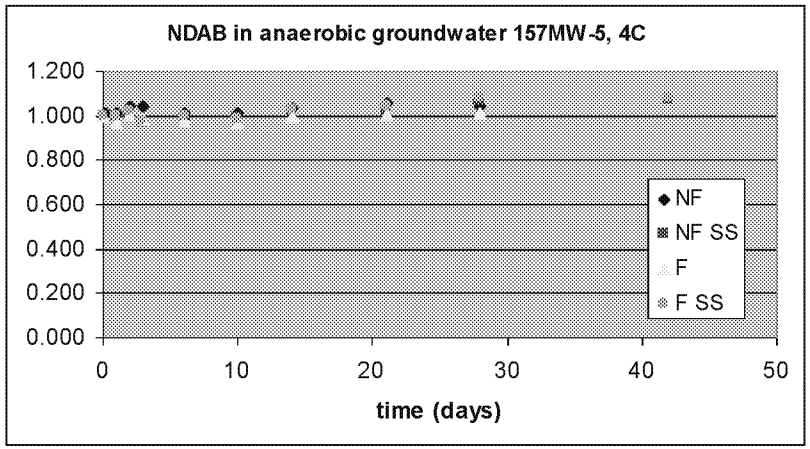


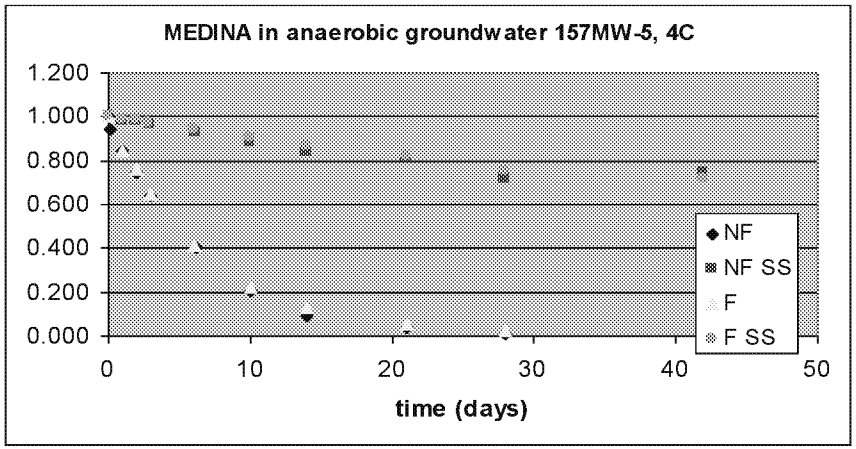
MNX, DNX, TNX ± Sea Salt (4°C) in Groundwater

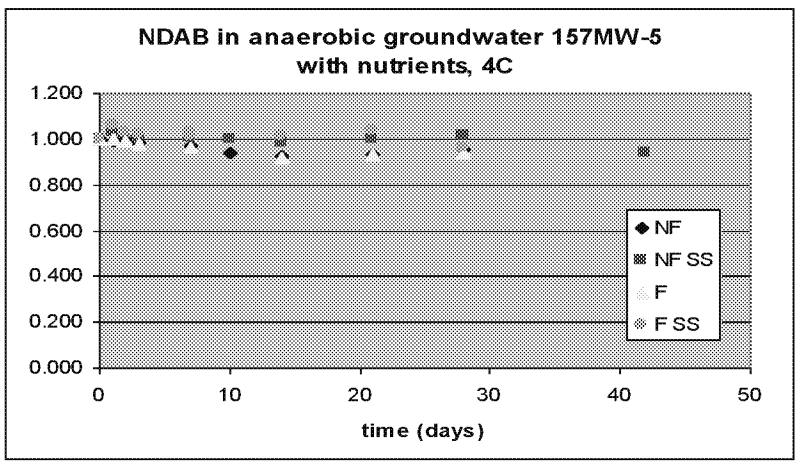


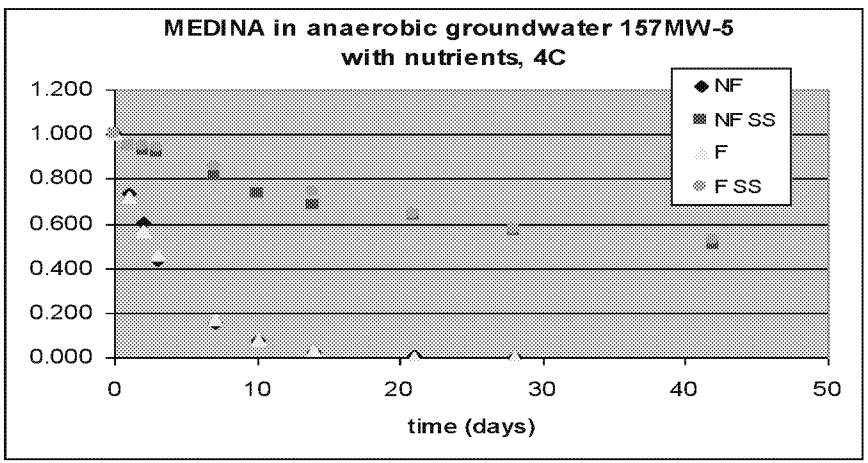
Results

Task 1a: Evaluation of Preservation Methods: Anaerobic Groundwater









Results

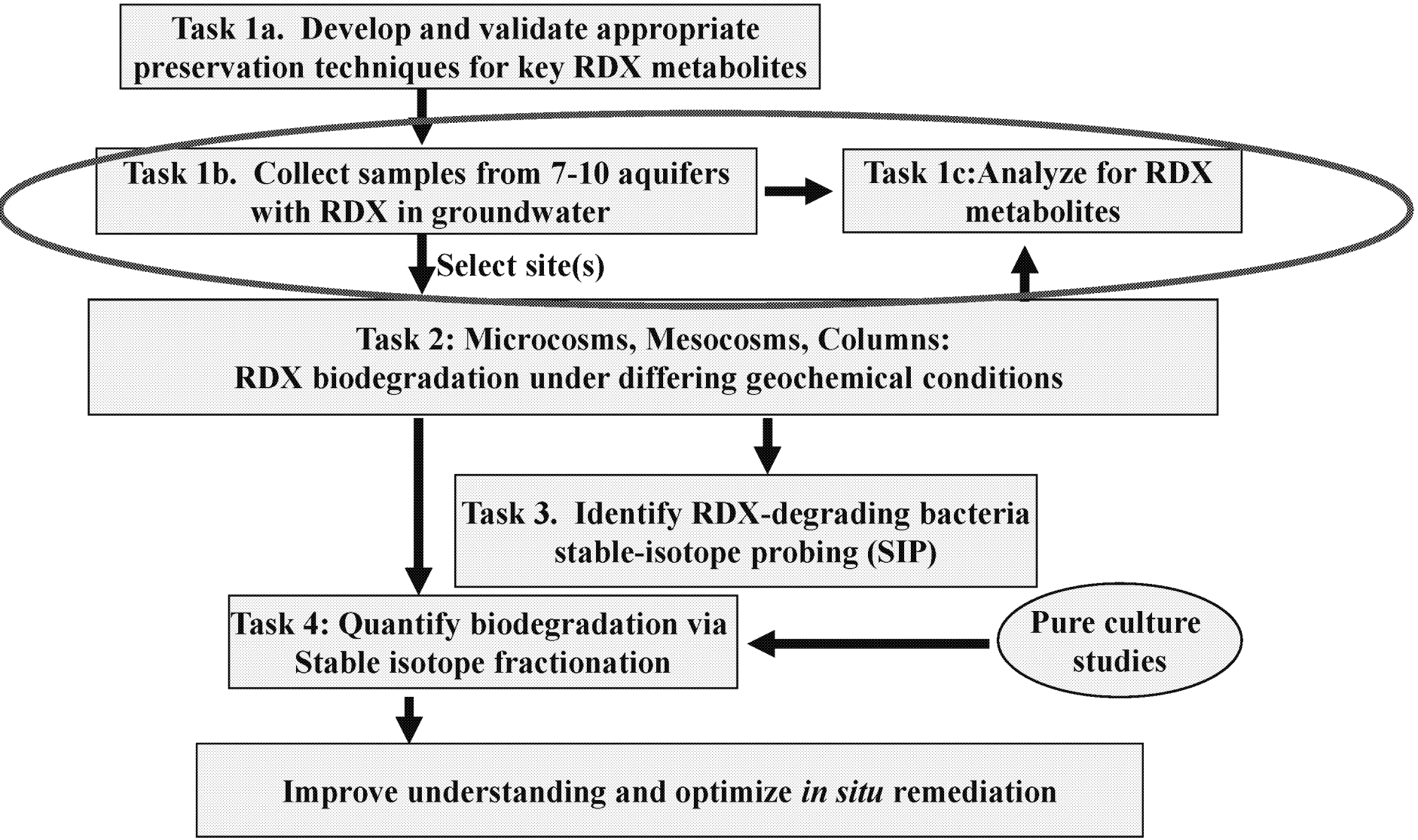
Key Conclusions: Metabolite Preservation

(1) NDAB is expected to be very stable in groundwater under most storage conditions

(2) MEDINA is extremely short-lived in groundwater

(3) 10% Sea Salts @ 4°C can be used to preserve MEDINA and HCHO and will not impact other, more stable intermediates (e.g., MNX, DNX, TNX, NDAB)

Technical Approach



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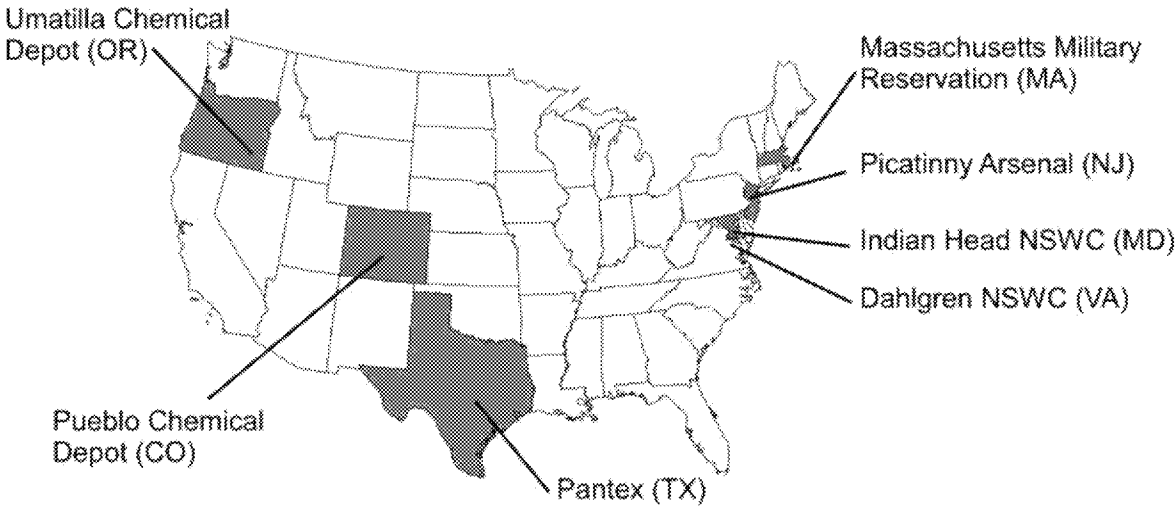
Results

Task 1b. Sites and Groundwater Sample Analytes and Methods

Analytes and Preservation Methods

Chemical	Preservation	Volume
Field parameters	NA	--
8330 Analysis	Ice	1 L
ICP-MS Metals	Filter (in line), HNO_3, ice	500 mL
Anions	Ice	250 mL
RDX, MNX, DNX, TNX	Ice	1 L
MEDINA	Sea salt/ice	10 mL *2
NDAB	Sea salt/ice	10 mL *2
MeOH + Formate	(1) 0.45 µm filter /ice and (2) Sea salt/ice	10 mL *2
HCHO	Sea salt/ice	10 mL*2
N_2O	Ice (serum bottle)	40 mL*2
NH_3	Ice/H_2SO_4	500 mL
RDX-SPME	Ice	1 L



44 Wells at 7 Sites (+ 7 background wells)

24 : +100 < ORP < +425

10 : 0 < ORP < +100

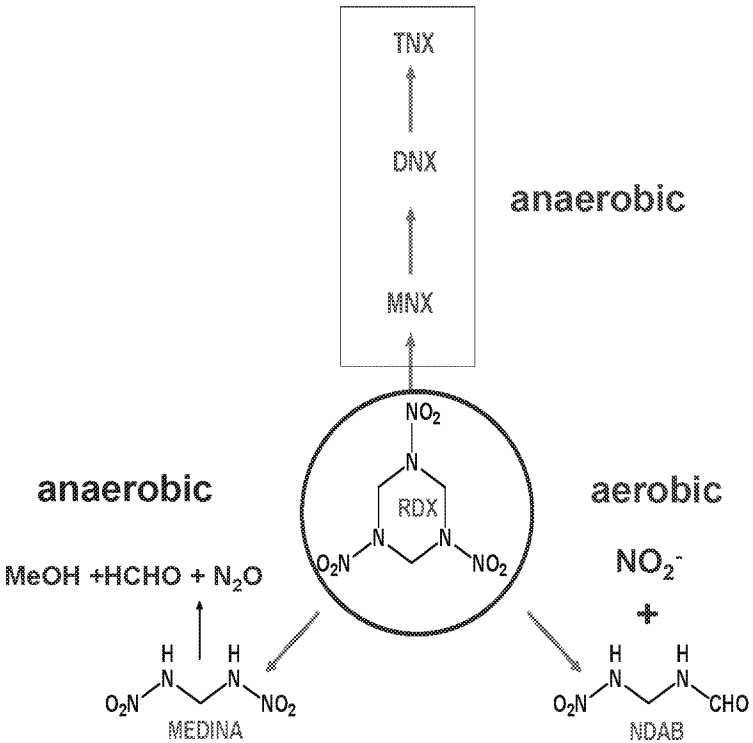
10 : -250 < ORP < 0

pH 7.1 ± 0.5 (n = 44)

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

Results

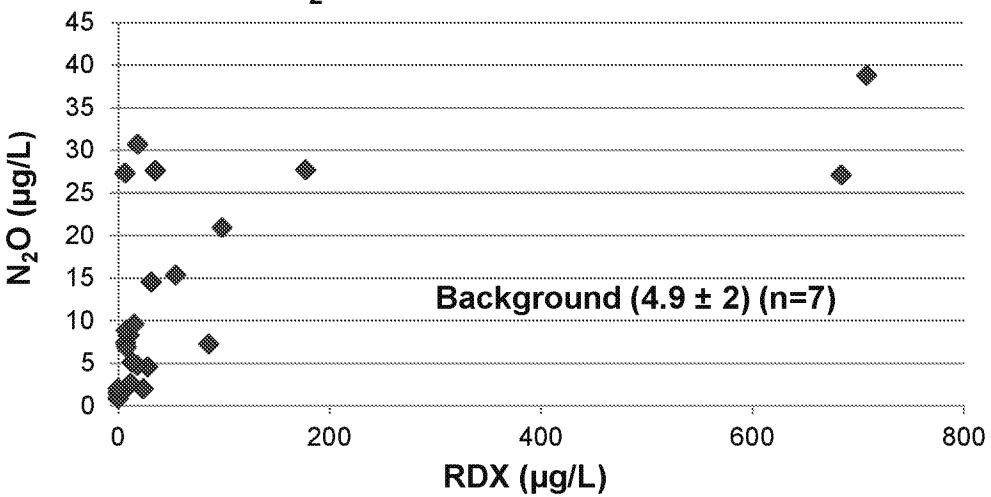
Task 1c. Analyze for Indicator Metabolites at RDX-contaminated Sites



Oxic Wells (ORP > 0; DO > 1.5 mg/L)

- No NDAB in any wells at 6/7 sites
  - NDAB at Pantex (2 wells)
- No significant formate or formaldehyde
- No NO_2^-
- No MEDINA
- Elevated N_2O in some wells – source?

Detection of N_2O in Aerobic RDX-contaminated wells



Results

Task 1c. Analyze for Indicator Metabolites at RDX-contaminated Sites

Geochemistry and Concentrations of Explosives in Pantex Plant Wells.

WELL ID	DO mg/L	ORP mV	NO_3 mg/L	NH_3 mg/L	HMX µg/L	MNX µg/L	DNX µg/L	TNX µg/L	RDX µg/L	MEDINA µg/L	NDAB µg/L	N_2O µg/L
PTX06-103	6.31	74	0.9	0.5	85.0	3.4	6.1	45.4	708	<10	**59**	39
PTX06-104	7.03	99	2.2	0.7	4.1	<0.1	<0.1	48.1	684	<10	**150**	27
PTX06-105	6.18	98	1.2	0.5	23.9	<0.1	2.1	10.6	178	<10	**<10**	28
PTX06-107	5.99	107	1.2	0.3	<0.1	<0.1	<0.1	<0.1	<0.2	<10	<10	4

- Only second environmental detection of NDAB in an aquifer (after IAAP)
- Some nitroso-compounds also present?

Groundwater (1039A and 1047A)

Bottles set up with different enrichment conditions
- + no additions (control)
- + mineral salts without nitrogen
- + mineral salts with nitrogen (as NH_4)
- + succinate (~1 mg/L)

AEROBIC

+/- trace minerals

4.5 months

+/- yeast extract

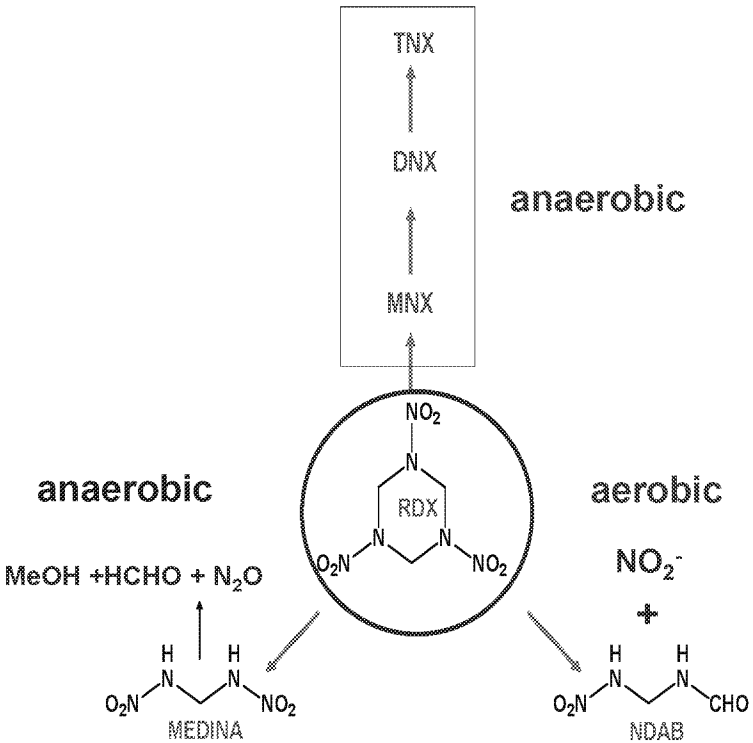
NO RDX DEGRADATION

Probed for *xplA*

NOT DETECTED

Results

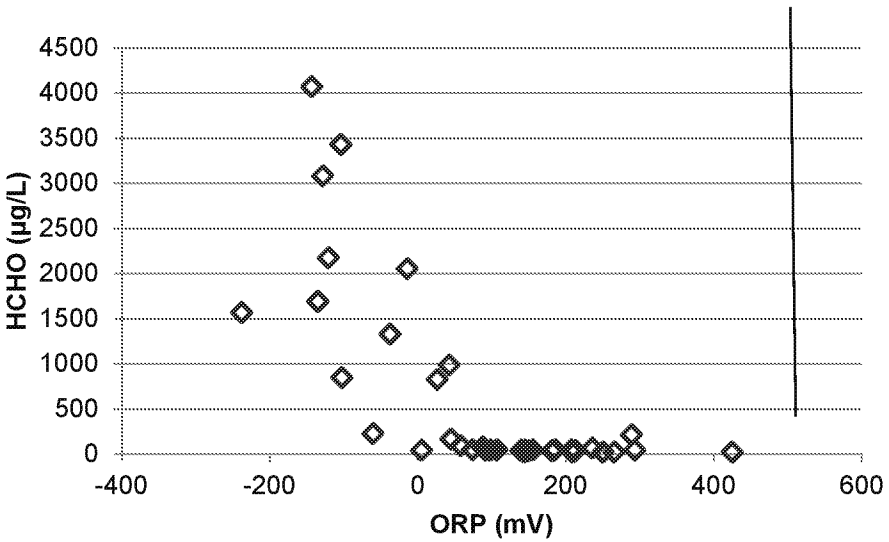
Task 1c. Analyze for Indicator Metabolites at RDX-contaminated Sites



Reducing Wells (ORP < 0)

- No MEDINA or NDAB
- Trace MNX (4 wells – Picatinny & Pantex)
- DNX, TNX (2 Wells - Pantex)
- No elevated N_2O
- Elevated Formaldehyde (HCHO)
- Elevated MeOH - few cases

Detection of HCHO in RDX-contaminated wells as a function of ORP



Results

Key Conclusions: Groundwater Sampling and Analysis

(1) NDAB is relatively stable in groundwater, but was only detected in a two aerobic wells.

(2) Where NDAB was detected (Pantex), NXs also detected – no *xpl*A – anoxic mechanism?

(3) Data suggest that aerobic RDX degradation may not be a prominent mechanism in the field

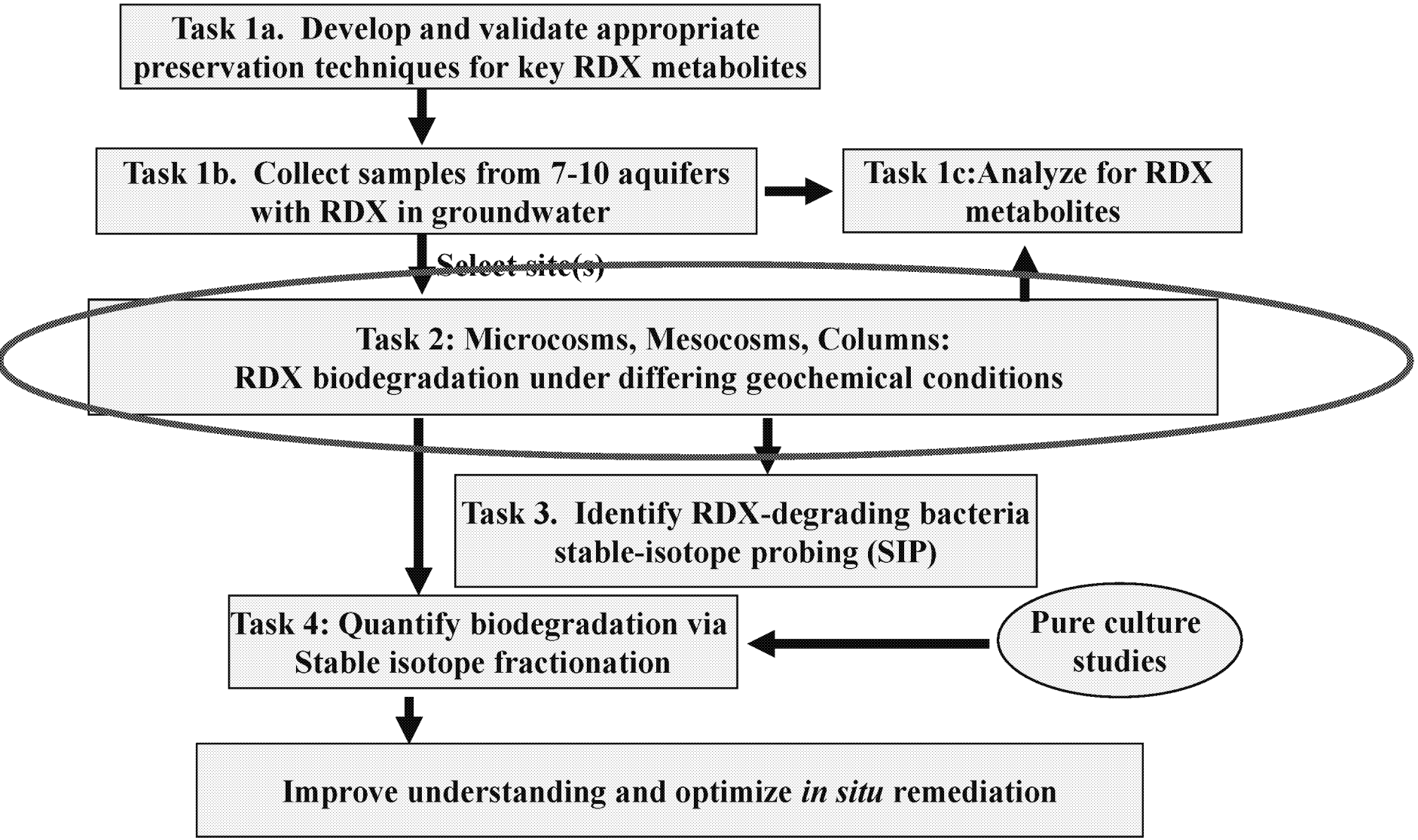
(4) MEDINA was not detected in any anaerobic wells – very labile compound

(5) HCHO and MEOH were detected in many anaerobic wells – multiple sources

(6) N_2O was detected in many aerobic wells and moderate correlation with RDX concentration – further investigation warranted.

Paquet, L, F. Monteil-Rivera, P.B. Hatzinger, M. Fuller, and J. Hawari. 2011. Analysis of the key intermediates of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) in ground water: Occurrence, stability and preservation. *Journal of Environmental Monitoring,* 13:2304-2311.

Technical Approach



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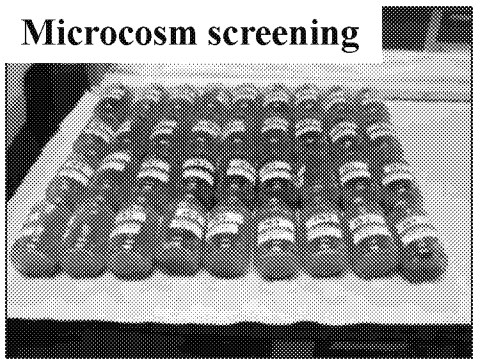
Results

Task 2a. Stimulate Aerobic and Anaerobic RDX Biodegradation

Collect Groundwater and Aquifer Solids

Microcosm screening

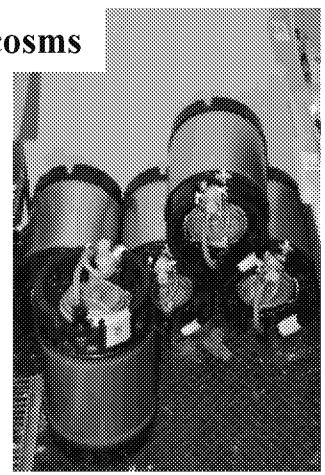


* *Aerobic vs. Anaerobic*
* *Multiple cosubstrates*
* *+/- Nitrogen*

RDX degradation

Task 2b. Evaluate Different Geochemical Conditions

Large-Scale Mesocosms



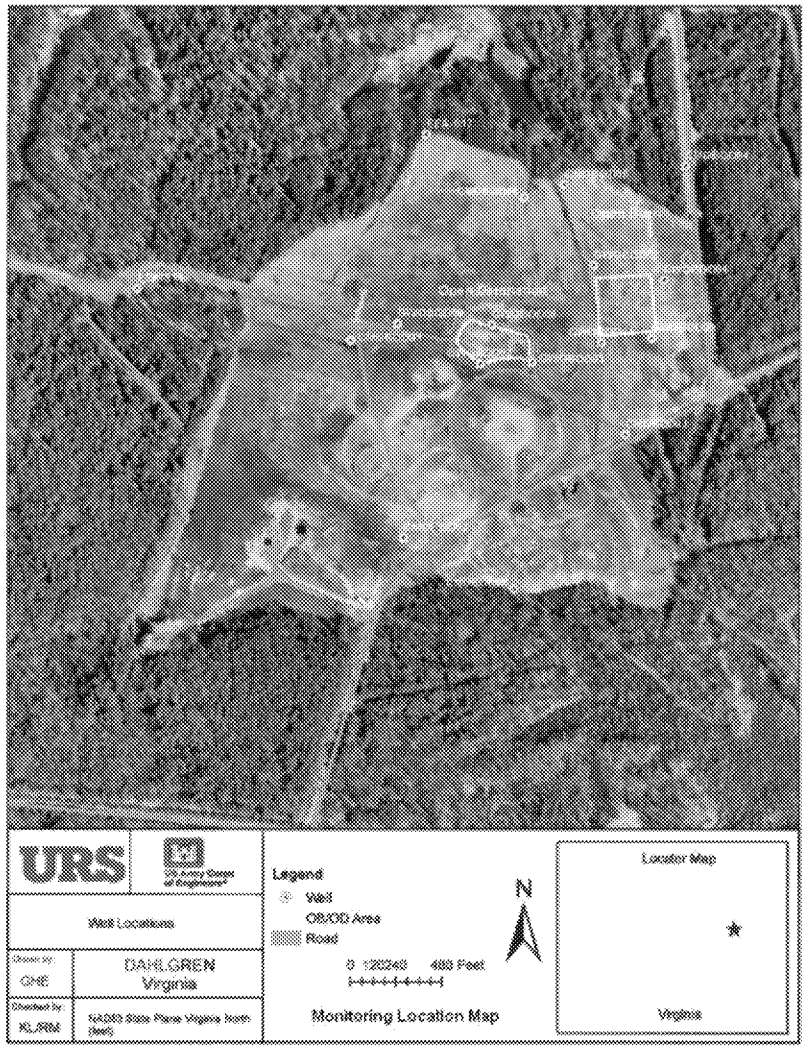
* *Succinate electron donor*
* *Nitrate reducing*
* *Fe reducing*
* *Mn reducing*
* *Sulfate reducing*
* *Methanogenic*

-RDX loss
-Metabolites
-Geochemistry
-Community analysis
-Stable isotope fractionation

Results

Field Site: Naval Surface Warfare Center, Dahlgren VA

US Navy Test Range



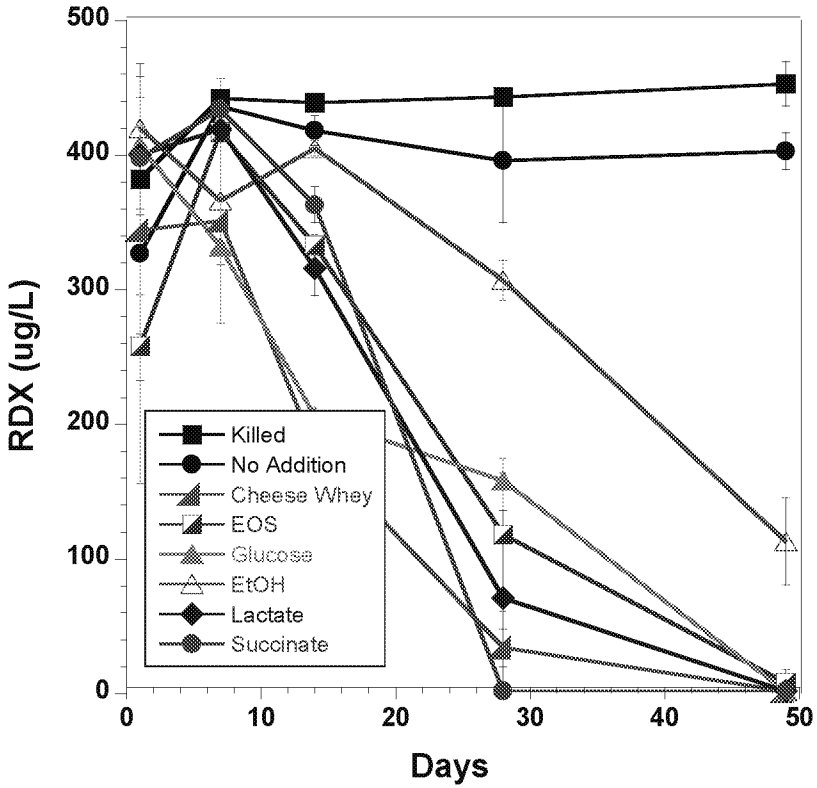
Groundwater	
RDX, HMX	**(10 – 100 µg/L)**
Perchlorate	**(5 – 600 µg/L)**
Aerobic	
pH ~5.0	

Results

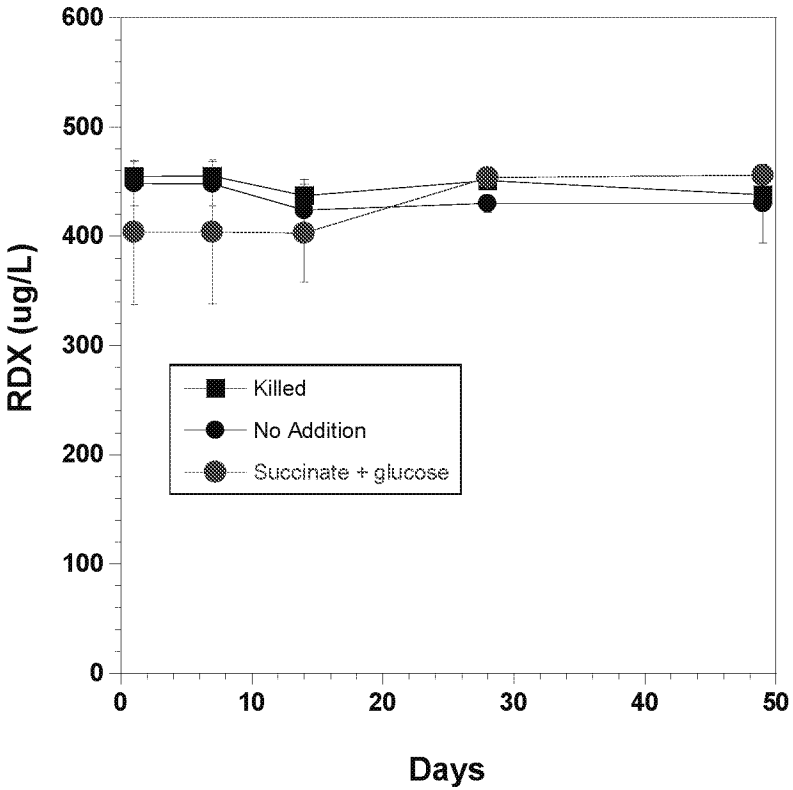
Task 2a. Stimulate RDX Biodegradation - Microcosms

Naval Surface Warfare Center, Dahlgren

Anaerobic Microcosms



Aerobic Microcosms

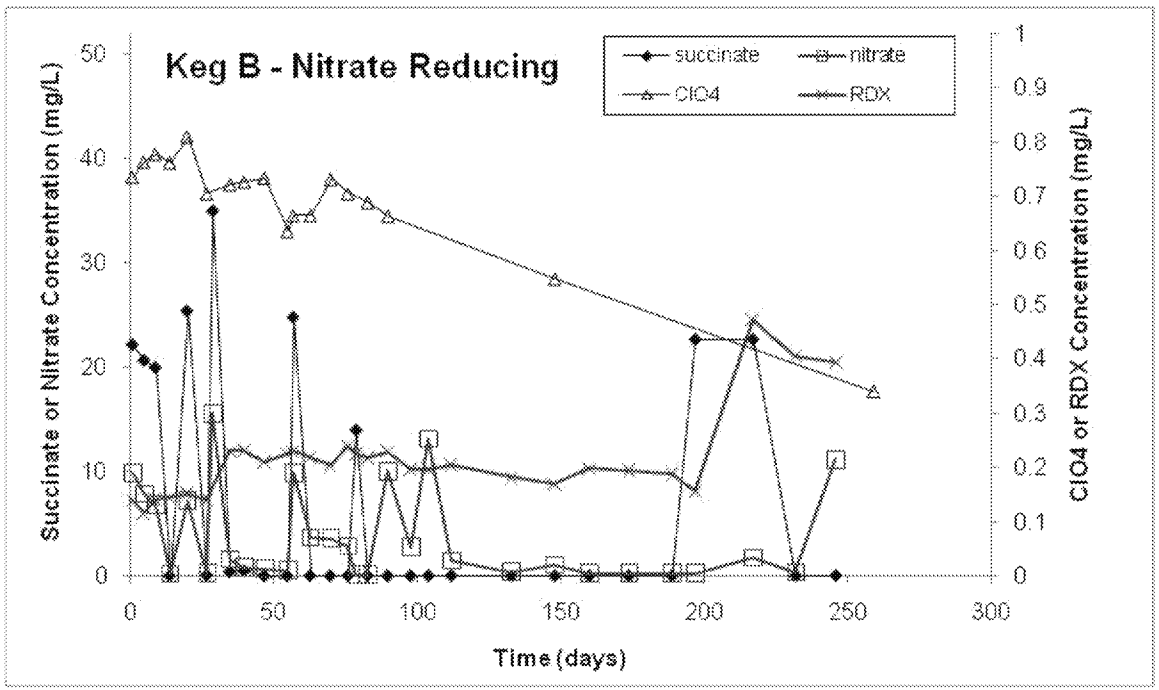


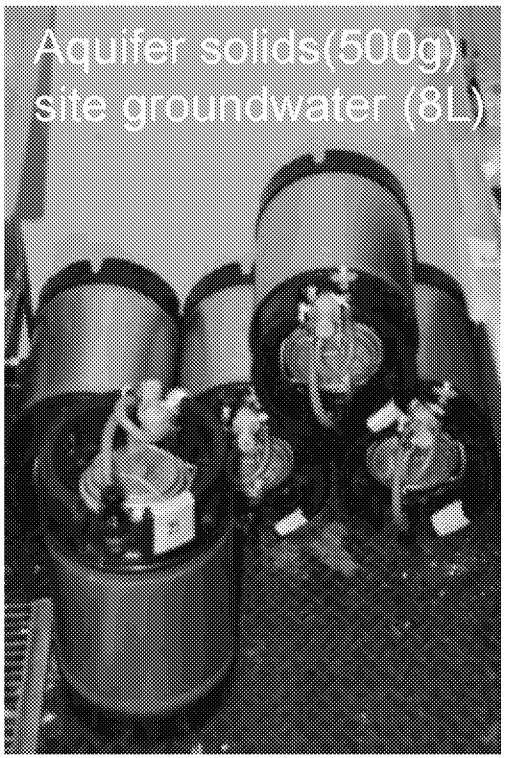
Results

Task 2b. Evaluate RDX Biodegradation under Differing Geochemical Conditions

Naval Surface Warfare Center, Dahlgren

Large-Scale Mesocosms: NO_3-Reducing





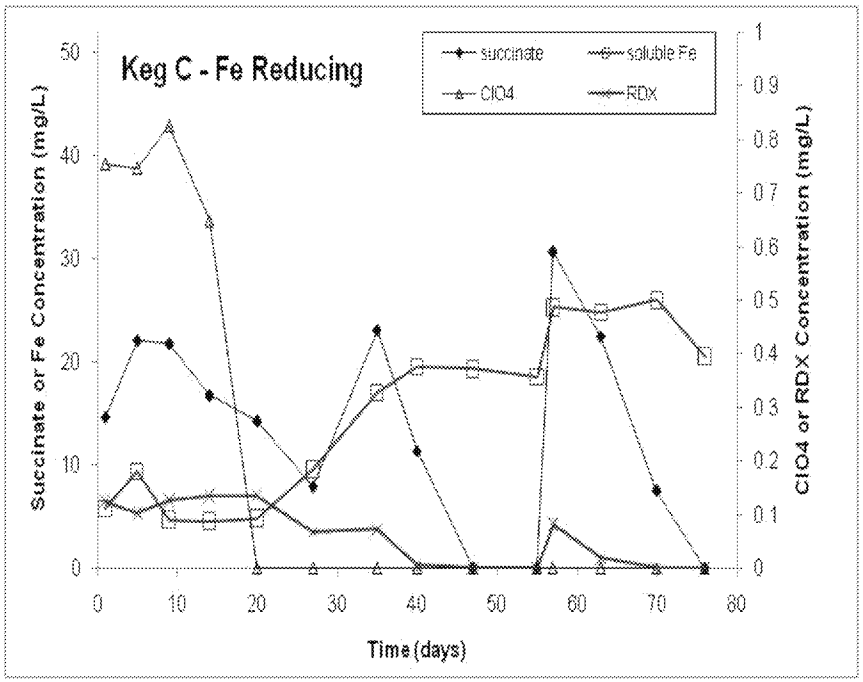
RDX degradation not observed under nitrate-reducing conditions

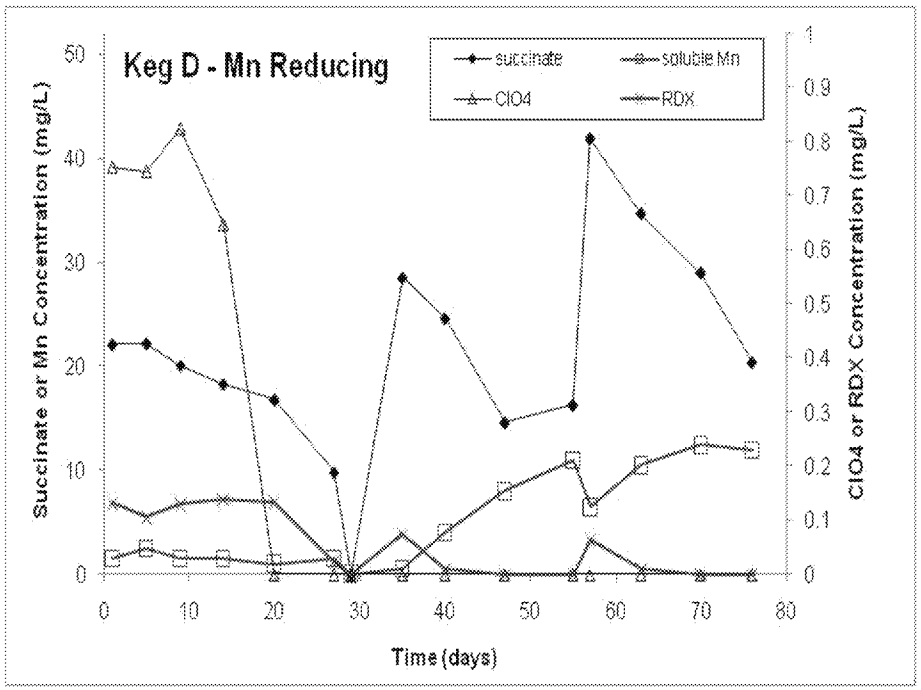
Results

Task 2b. Evaluate RDX Biodegradation under Differing Geochemical Conditions

Naval Surface Warfare Center, Dahlgren

Large-Scale Mesocosms: Fe- and Mn-Reducing





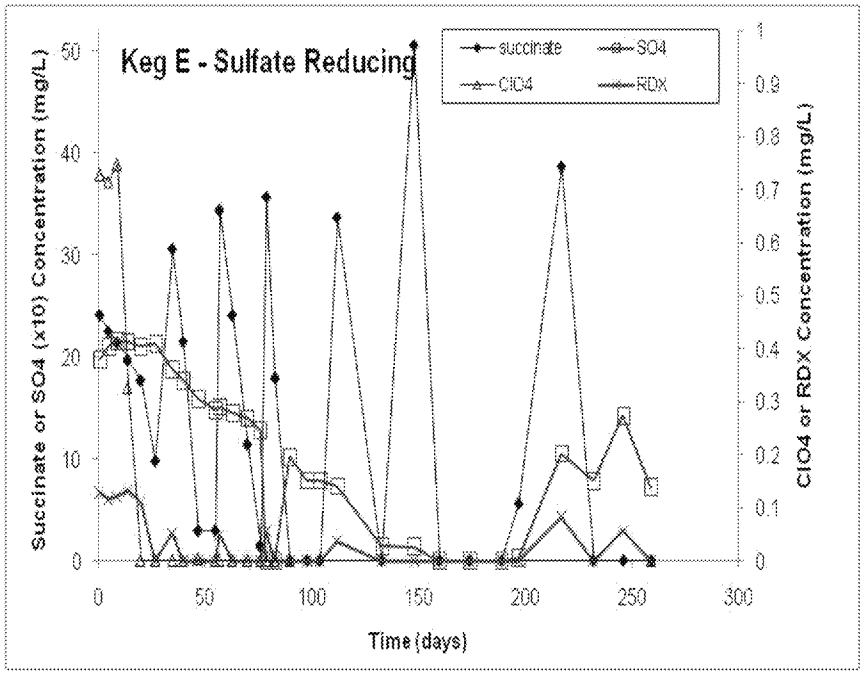
RDX degradation quickly established under both Fe- and Mn-reducing conditions

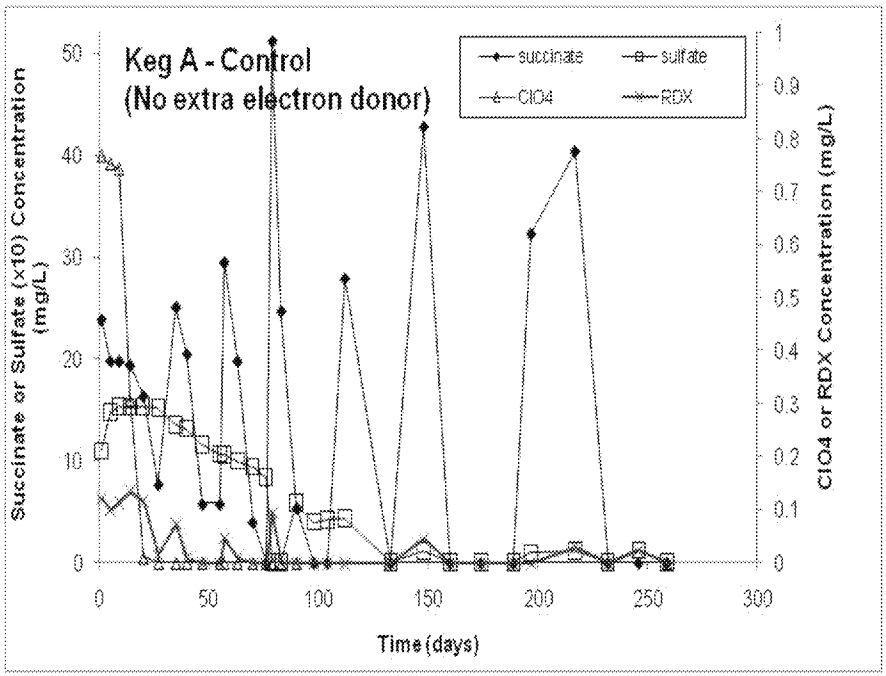
Results

Task 2b. Evaluate RDX Biodegradation under Differing Geochemical Conditions

Naval Surface Warfare Center, Dahlgren

Large-Scale Mesocosms: SO_4-Reducing and Methanogenic

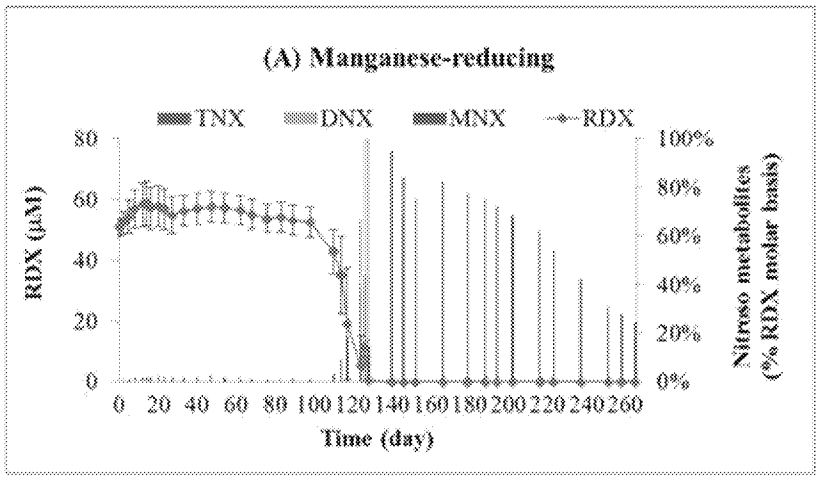


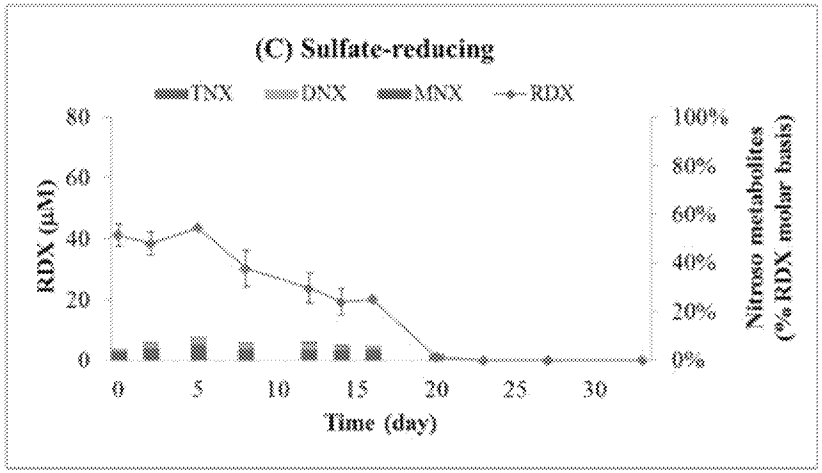


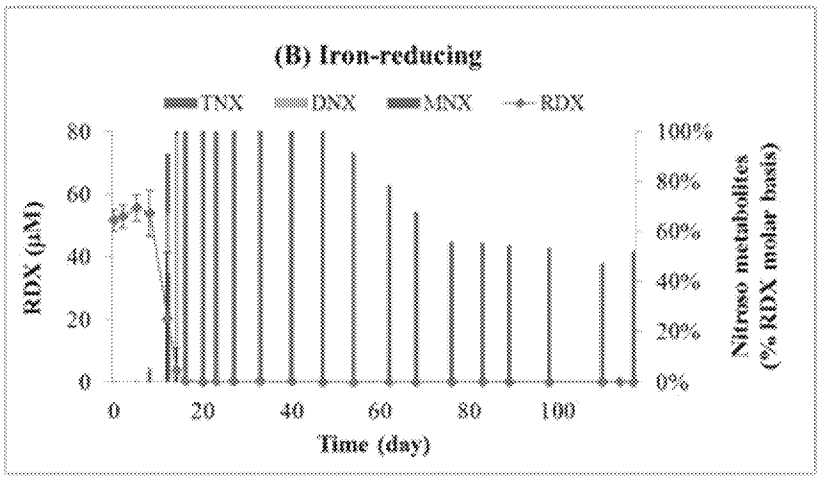
RDX degradation slowly established under SO_4^- reducing and methanogenic conditions

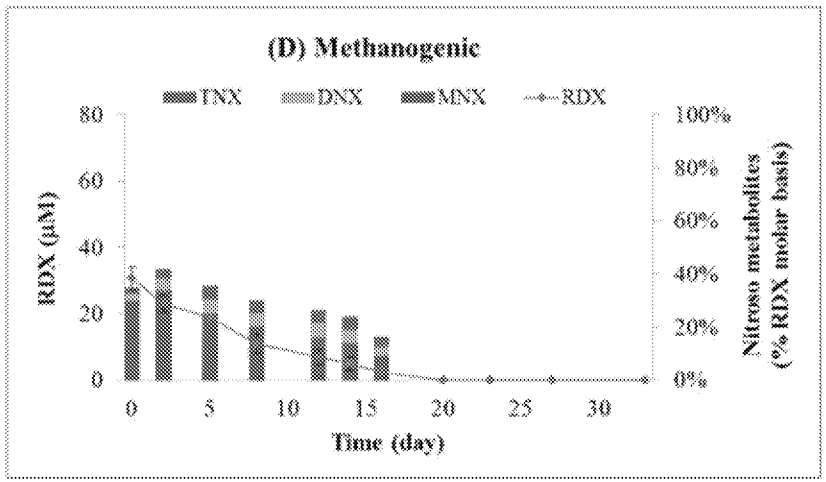
Results

Formation of MNX, DNX and TNX under different electron-accepting conditions.



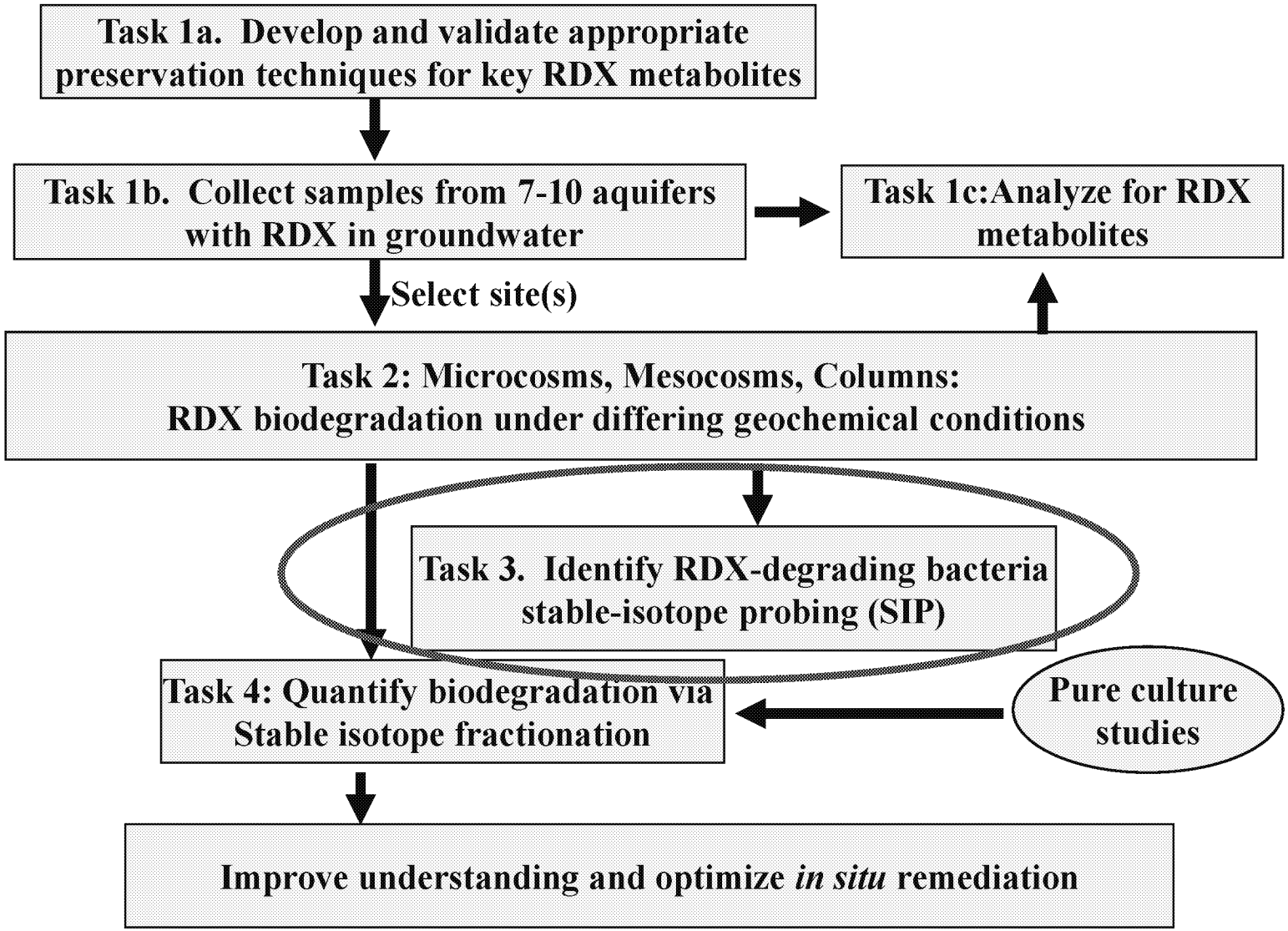






* Samples were transferred from mesocosms to 1L bottles for SIP analysis (next slide)

Technical Approach



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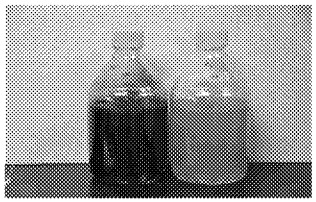
Results

Task 3. Stable Isotope Probing (SIP)

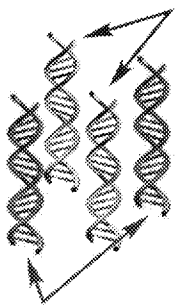


Add ^{13}C- or ^{15}N-labelled explosives

Incubate until >90% of explosives degraded

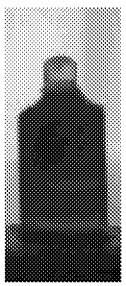
Isolate DNA

Unlabelled (^{12}C, ^{14}N) DNA



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

Labelled and unlabelled DNA separated using density gradient ultracentrifugation

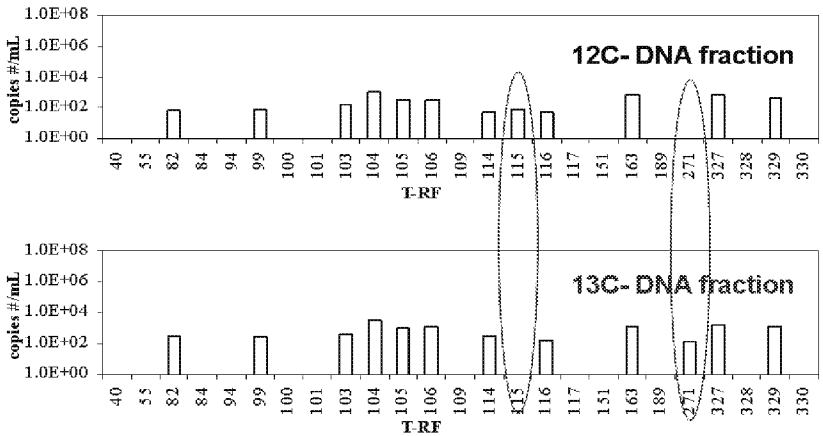


^{12}C- or ^{14}N-Labelled DNA

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

Recover DNA from bands

t-RFLP analysis of $^{12}C/^{13}C$-DNA
Abundance of 16s-rDNA fragments



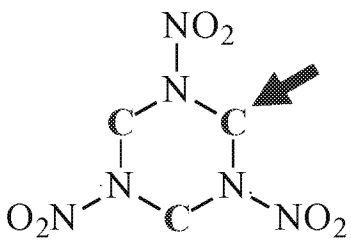
Benefits:

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

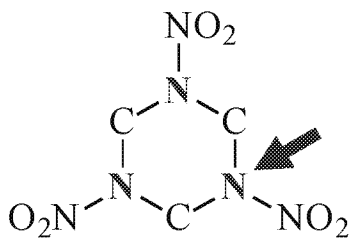
Results

Task 3. Stable Isotope Probing – Synthesis of ^{15}N- and ^{13}C-labeled RDX

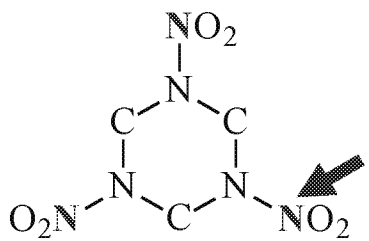
NAWC China Lake Synthesized 4 RDX Isotopomers



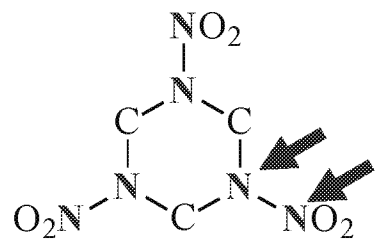
Ring ^{13}C



Ring ^{15}N



Nitro ^{15}N



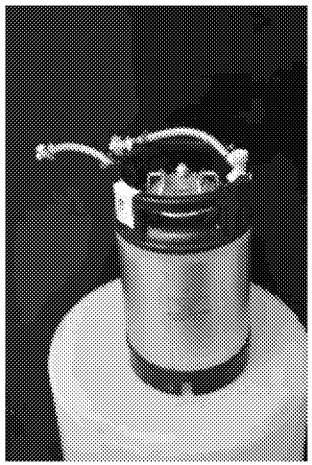
Full ^{15}N

Benefits:

- Allow distinction between organisms that use RDX for C vs. N source.
- Allow distinction between organisms that derive N from nitro-groups vs. those that can break the ring to use internal N.

Results

Task 3. Stable Isotope Probing – ^{13}C and ^{15}N SIP with Dahlgren GW and Sediment



Dahlgren mesocosms with different electron-accepting conditions (Task 2a)

Enrichment divided to five 2L bottles under each set of electron-accepting conditions with the following RDX additions:

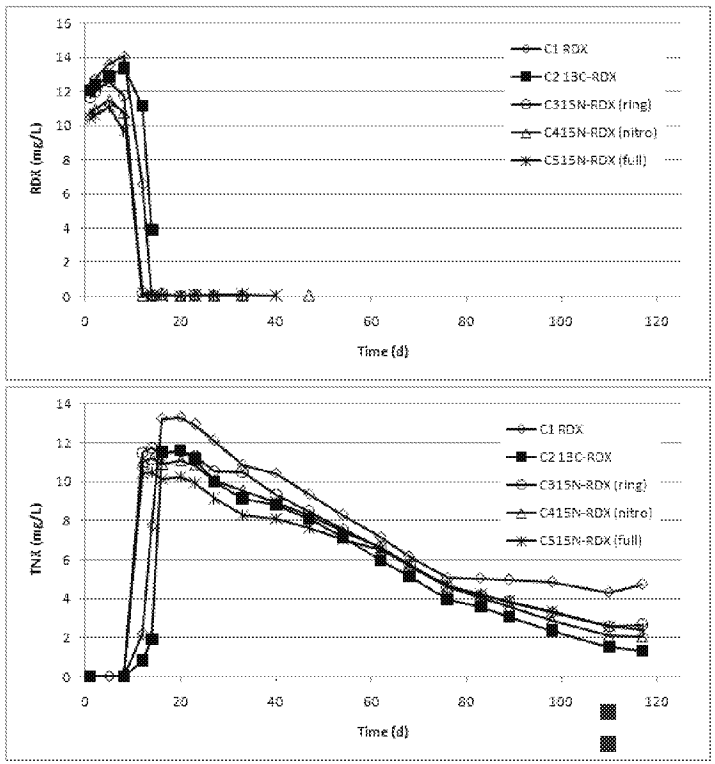
(1) RDX

(2) ^{13}C-RDX

(3) $^{15}N_{(ring)}$RDX

(4) $^{15}N_{(nitro)}$RDX

(5) $^{15}N_{(full)}$RDX

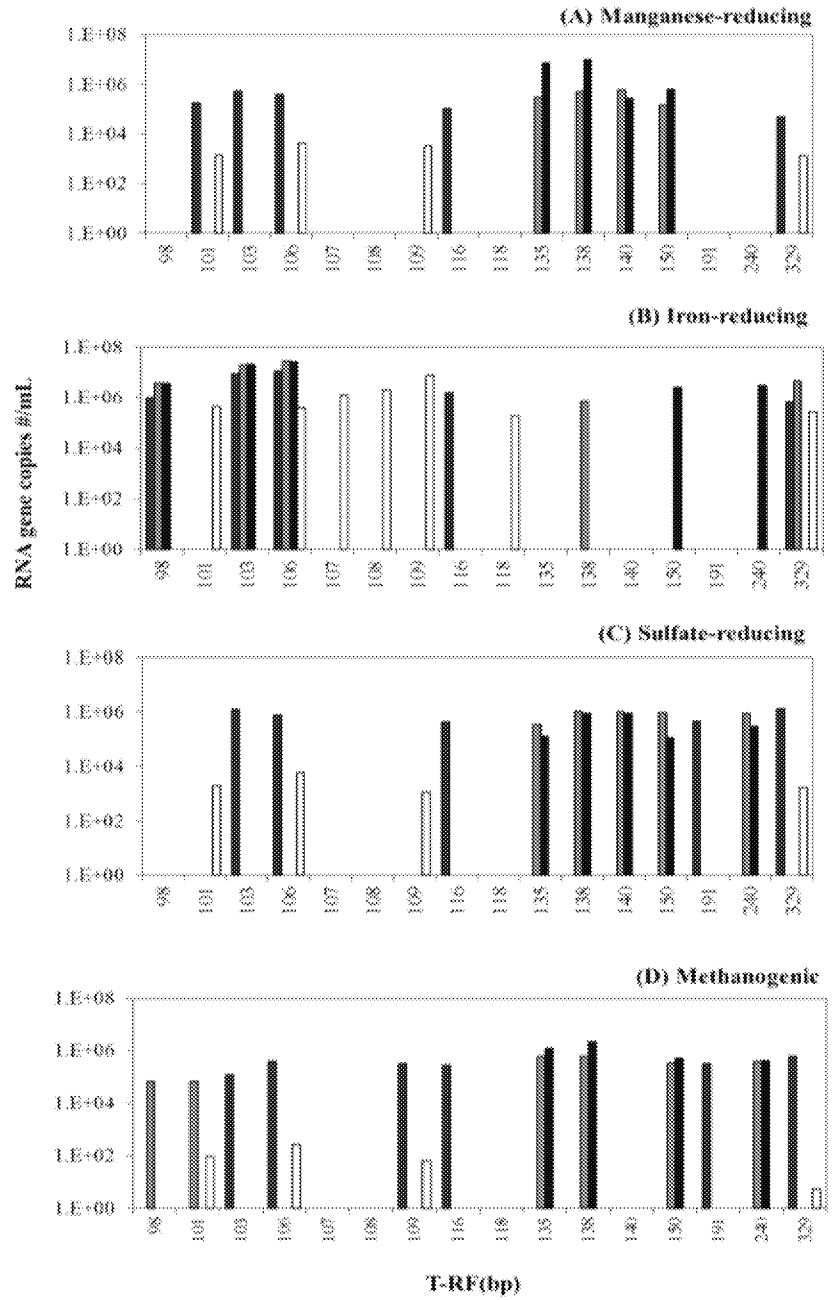


Biomass collected by filtration

Results

1. Numerous organisms involved in RDX metabolism for all electron-accepting conditions.
2. High numbers of some organisms.
3. Four ribotypes observed in all ^{13}C-RDX microcosms (101, 106, 109, 329).
4. Some unique ribotypes in Fe-reducing with ^{13}C-RDX (107, 108, 118).
5. More diverse communities with ^{15}N-labeled RDX.
6. Some clear differences between ring- and nitro-labeled RDX.

Microbial community structure within the ^{13}C-gradient fraction and the ^{15}N-gradient fractions of microcosms receiving labeled RDX.



Ring-15N-labeled RDX
Nitro-15N-labeled RDX
Fully-15N-labeled RDX
13C-labeled RDX

Results

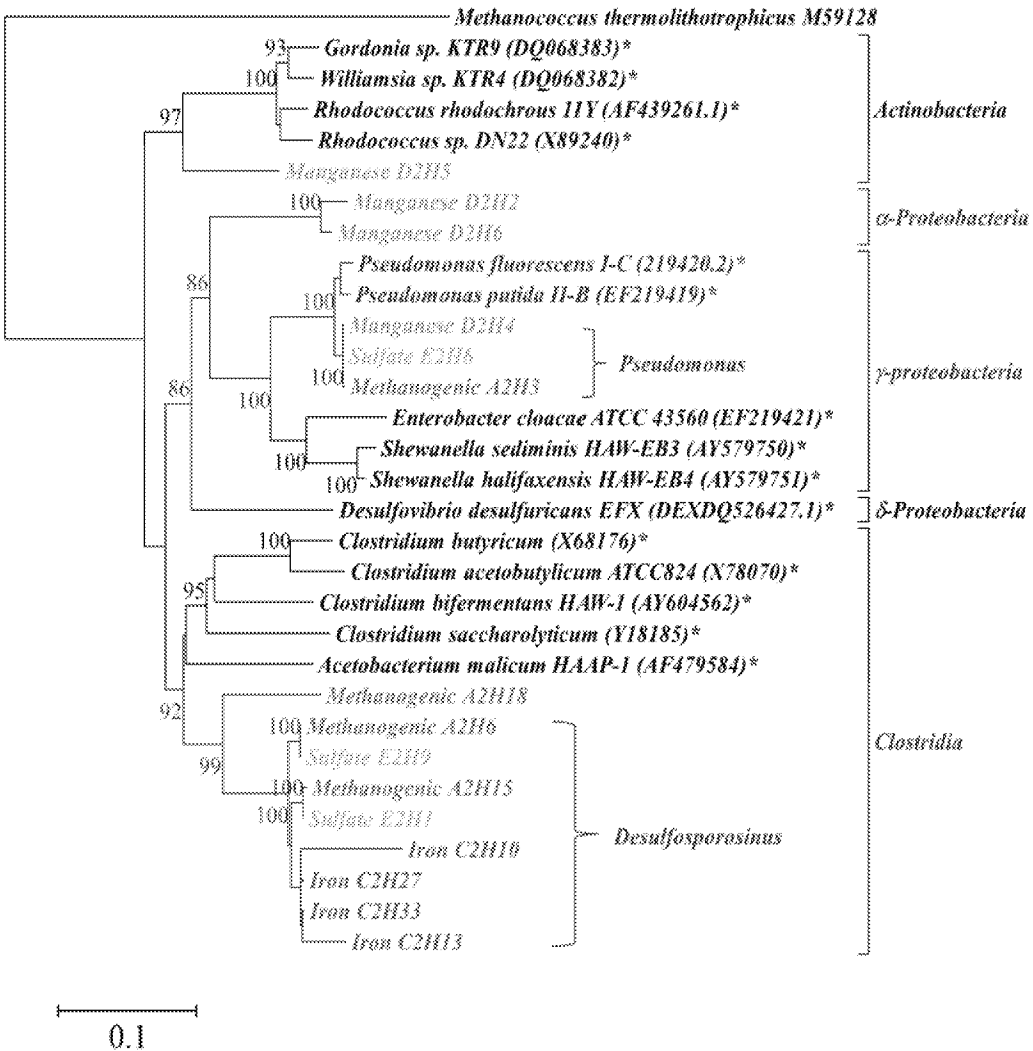
1. Predominance of *Desulfosporosinus* sequences labeled with ^{13}C and under iron reducing, sulfate reducing, and methanogenic conditions

2. *(2) Desulfosporosinus reduces sulfate, but has a diverse metabolism.* Not previously associated with RDX degradation.

3. Some *α*- and *γ-Proteobacteria* also labeled with ^{13}C under manganese and sulfate reducing conditions.

4. *Psuedomonas* spp. with Xen A/B genes close relatives

Phylogenetic Tree using SIP with ^{13}C-RDX

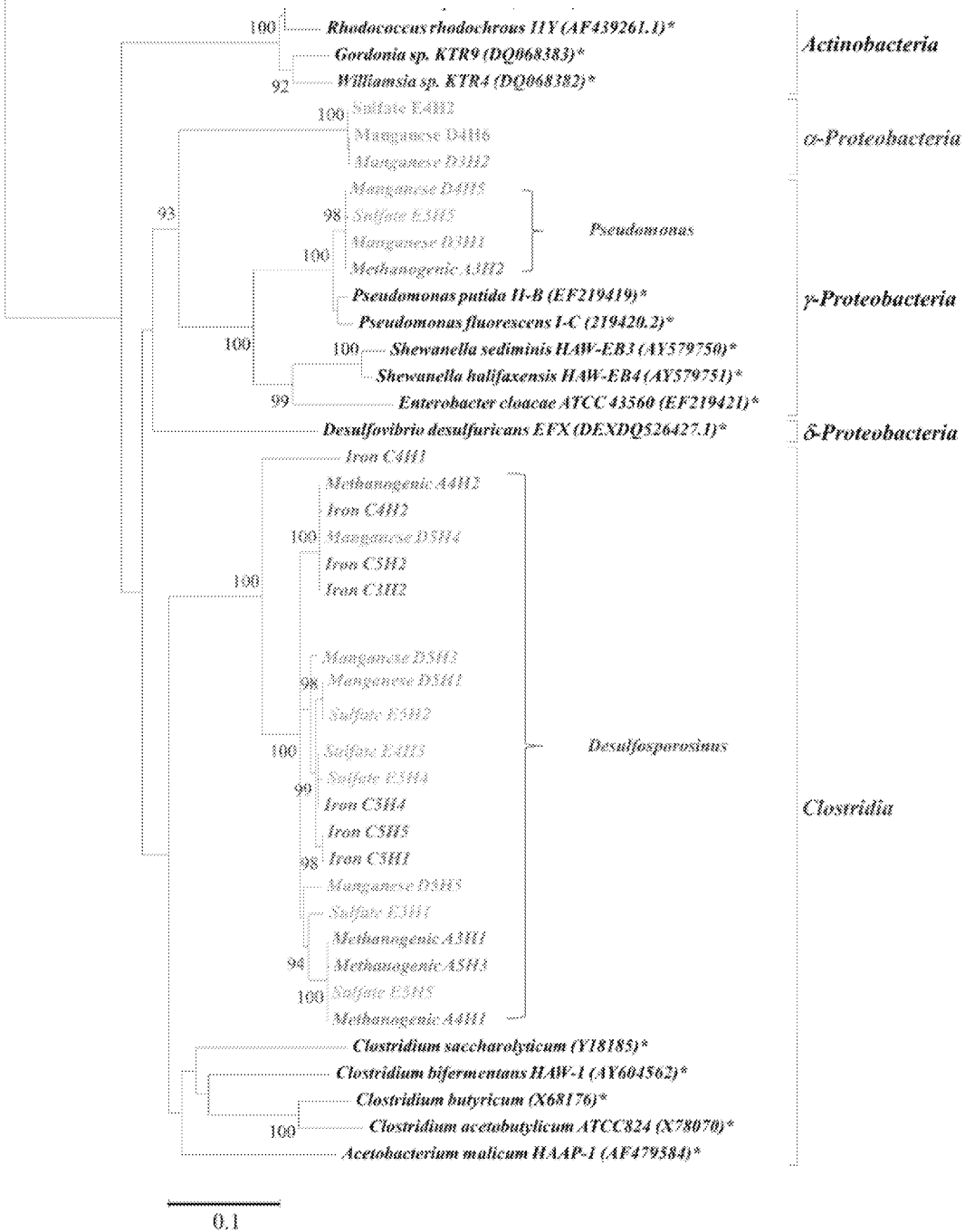


Results

1. Similar to ^{13}C-RDX data, *Desulfosporosinus* predominate under many different electron-accepting conditions

2. Some *α*- and *γ-Proteobacteria* also labeled with ^{13}C under manganese- and sulfate-reducing conditions.

Phylogenetic Tree using SIP with ^{15}N-RDX



Results

Key Conclusions: Geochemical Conditions and SIP Analysis

1. RDX degradation was inhibited in the presence of nitrate as a dominant electron acceptor.
2. Degradation pathways and metabolite accumulation may differ significantly based upon dominant electron-accepting conditions in an aquifer.
3. A variety of different organisms are involved in the degradation of RDX and its metabolites and can incorporate C and/or N from the molecule.
4. Organisms not previously shown to be involved in RDX degradation (e.g., *Desulfosporosinus)* were detected with this method.

Results

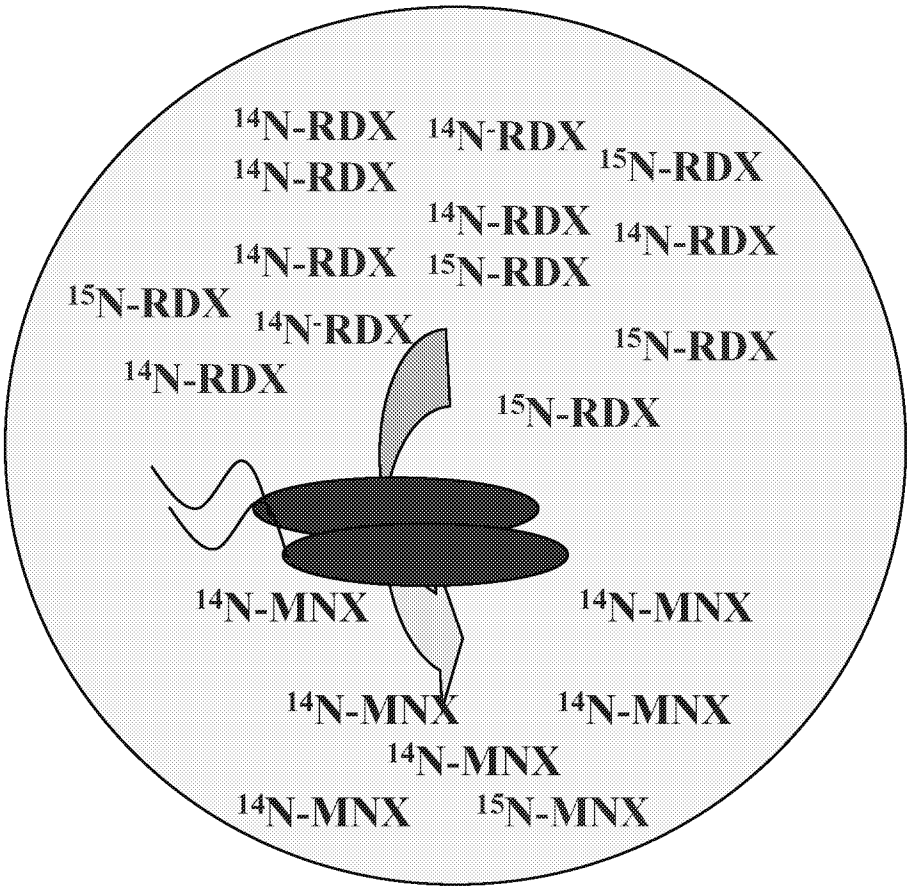
Task 4. Evaluate Biological Fractionation of N and C Isotopes in RDX during Biodegradation (CSIA)

Background:

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

Enrichment can be used as clear evidence of contaminant biodegradation

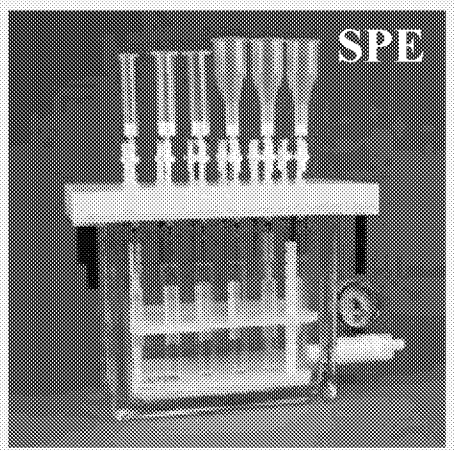
Even more powerful with isotope data from multiple elements



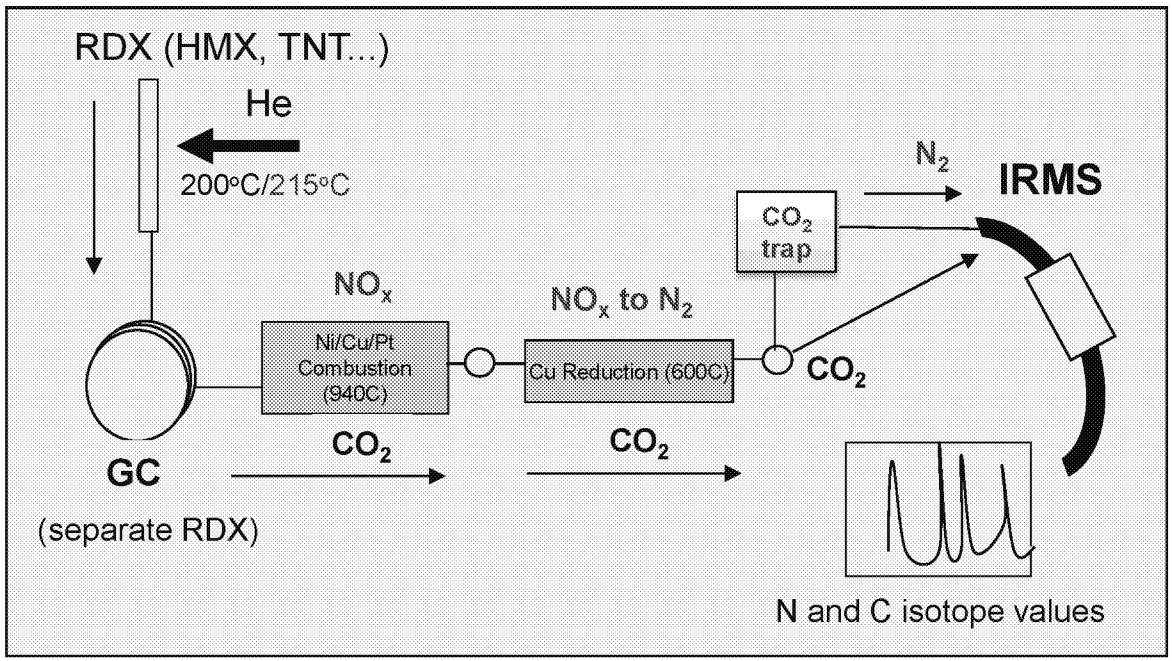
Results

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

1. Collect RDX from cultures/groundwater
2. Concentrate RDX via SPE - acetonitrile
3. Separate RDX (GC) and Quantify Stable Isotope Ratios (IRMS)



Continuous Flow GC-IRMS

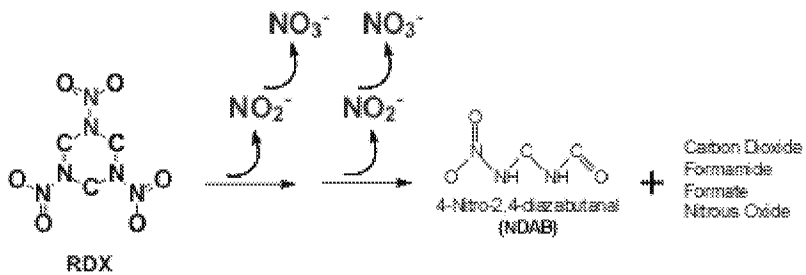


CO_2 and N_2 sent in continuous-flow gas stream to isotope ratio mass spectrometer (IRMS) - 2 different runs

* 10 μg RDX per analysis

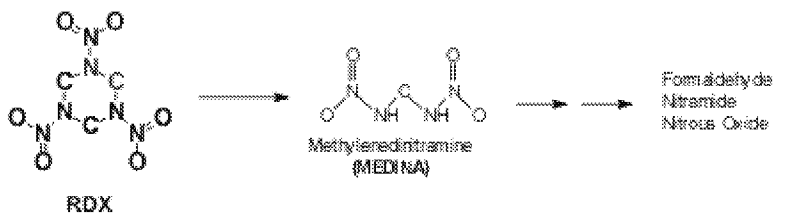
Results

Task 4. Pure Culture CSIA Studies: Different Pathways & Strains



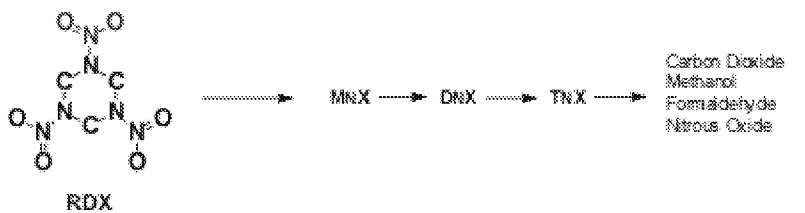
Pathway 1: Aerobic

Rhodococcus sp. DN22
Rhodococcus rhodocrous 11Y
Rhodococcus sp. Strain A
Gordonia sp. KTR9



Pathway 2: Anaerobic (Ring cleavage)

Pseudomonas putida II-B
Pseudomonas fluorescens I-C

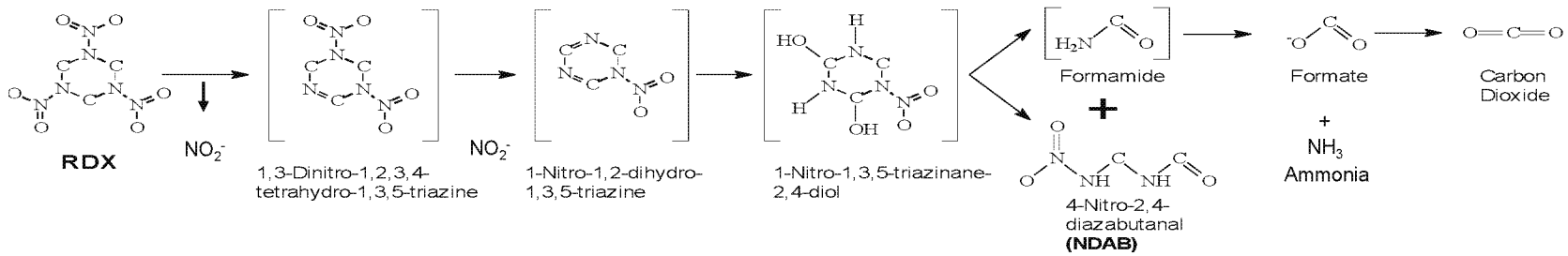


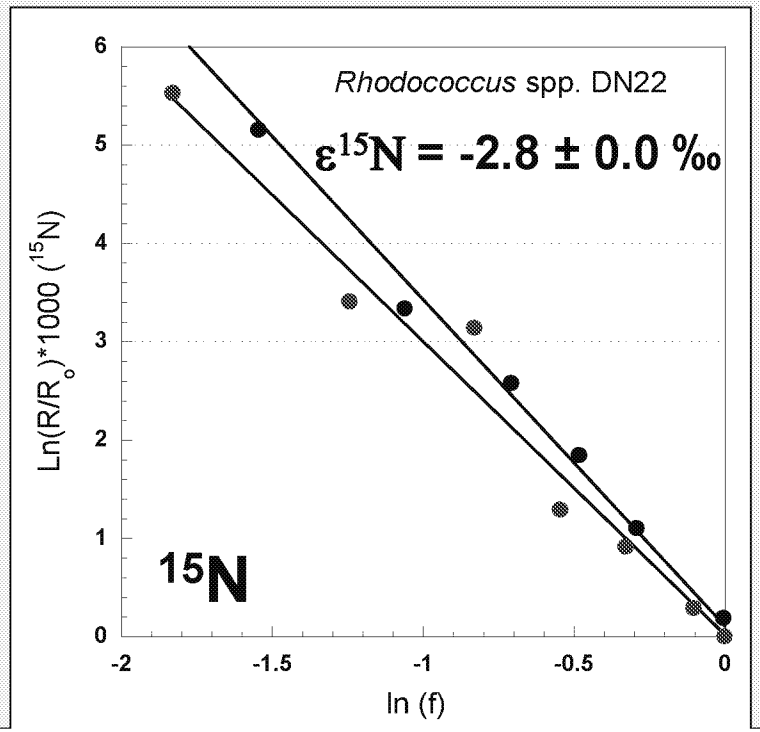
Pathway 3: Anaerobic (MNX)

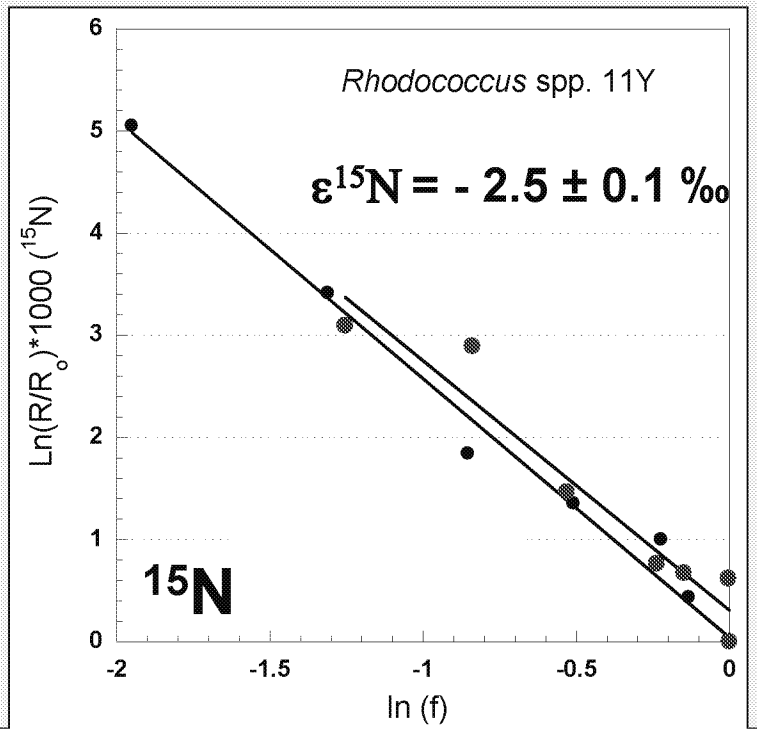
Shewanella sp. MR-1
Klebsiella sp. SCZ-1
Clostridium acetobutylicum ATCC824
Desulfovibrio spp.

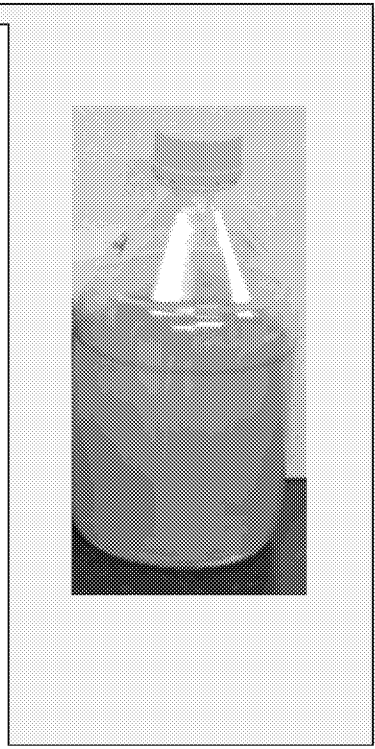
Results

Task 4. Fractionation of N during Aerobic Degradation of RDX - *xpl*A



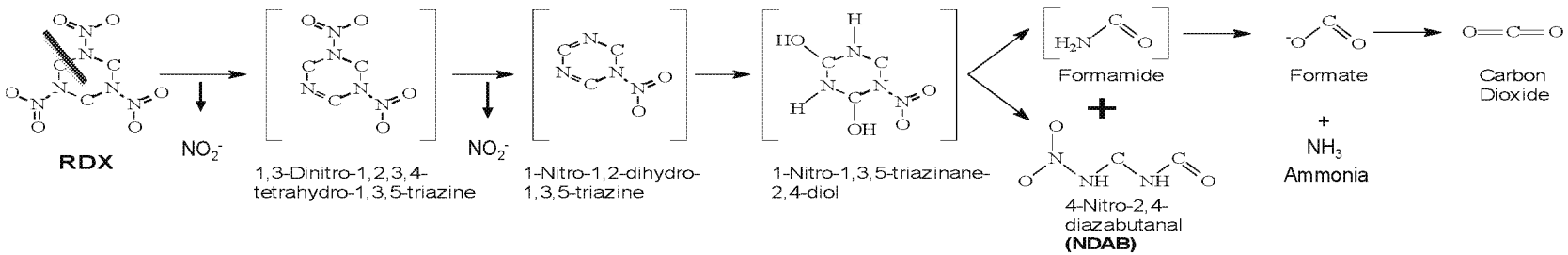




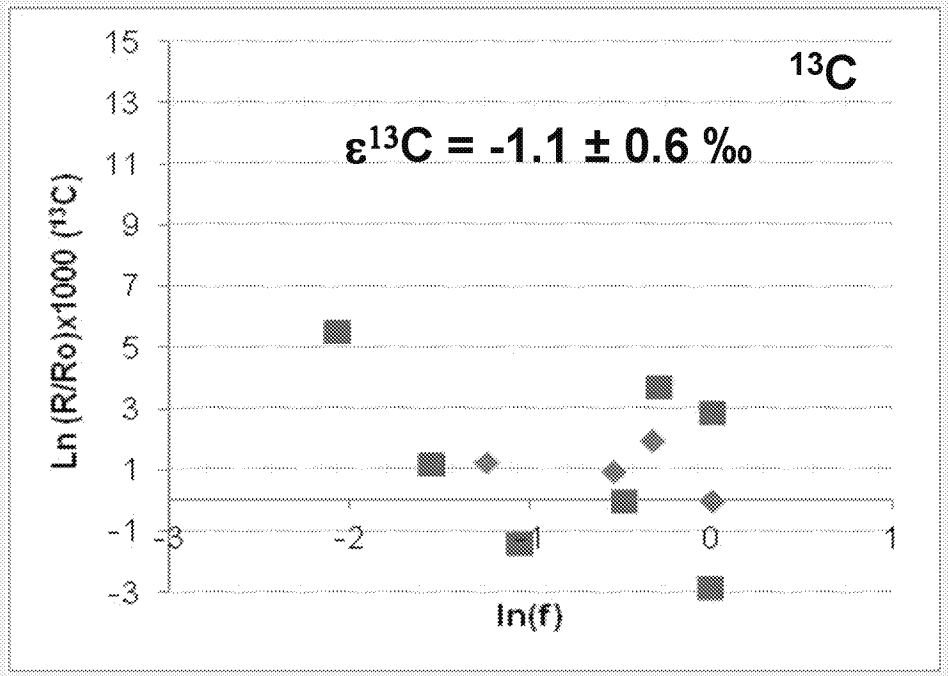


Results

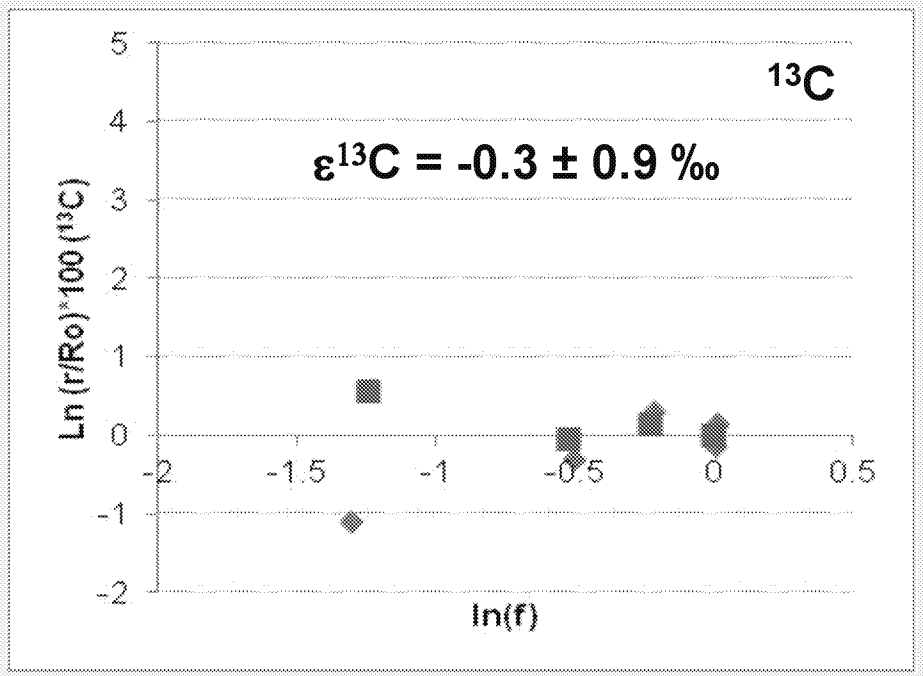
Task 4. Fractionation of C during Aerobic Degradation of RDX - *xpl*A



Rhodococcus spp. DN22

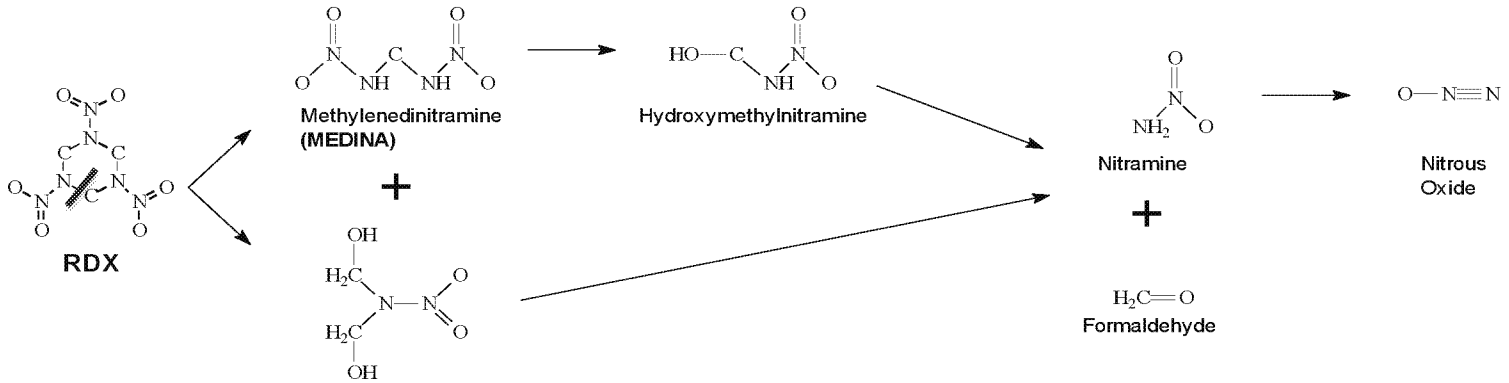


Rhodococcus spp. 11Y

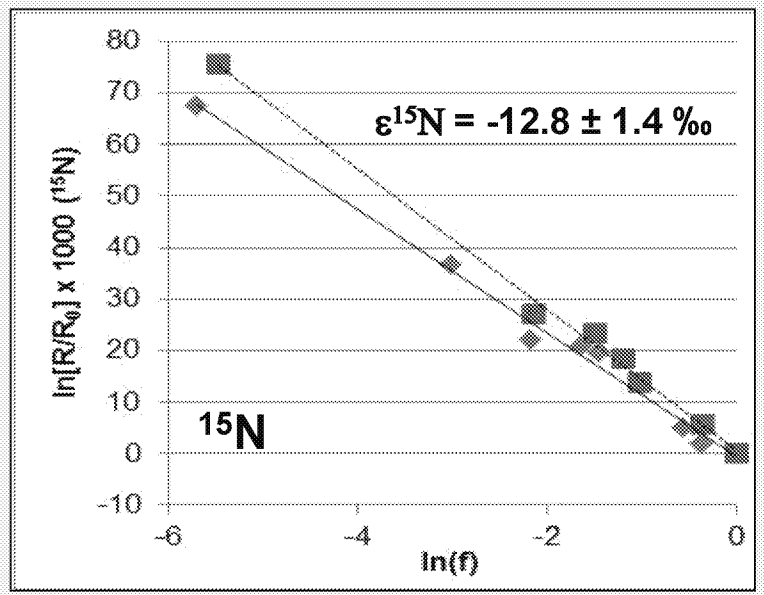


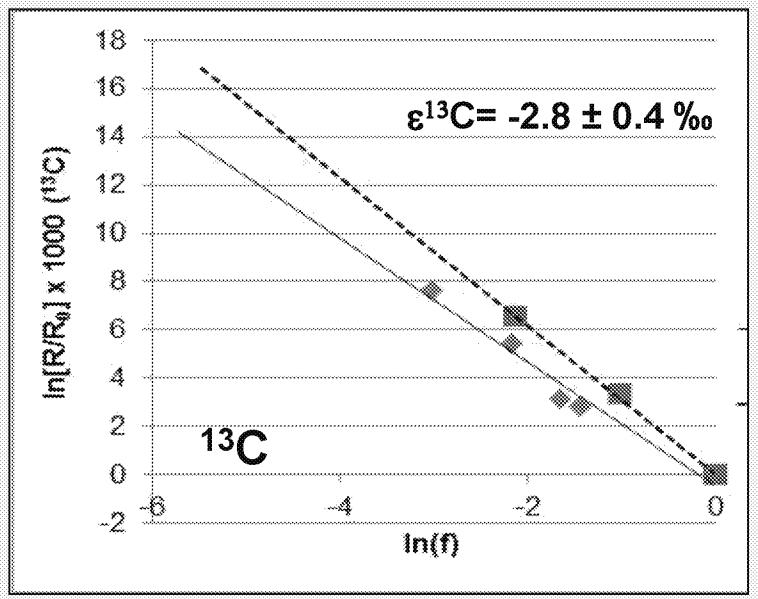
Results

Task 4. Fractionation of C during Anaerobic Degradation of RDX – *XenA/B*



Pseudomonas fluorescens I-C





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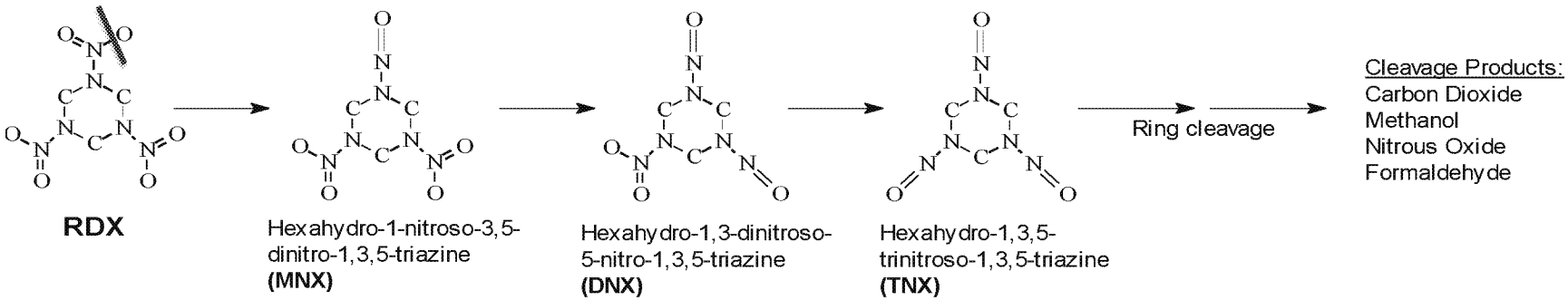
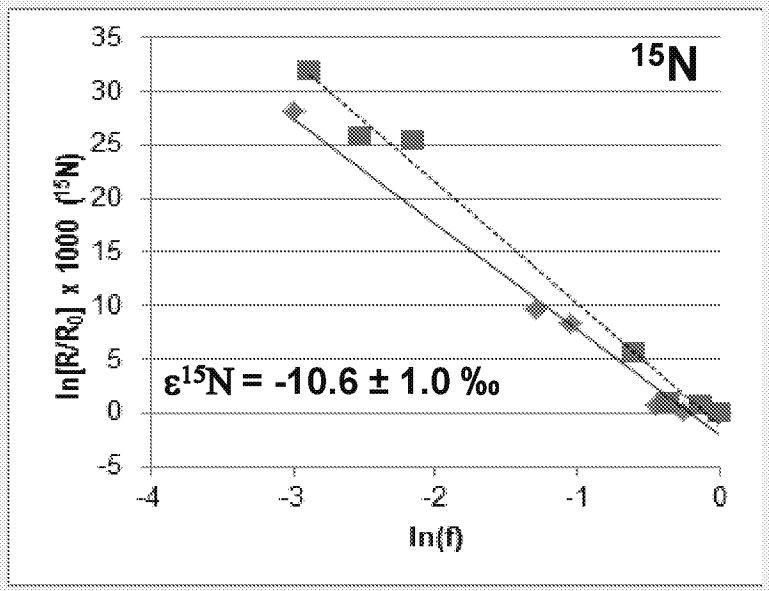
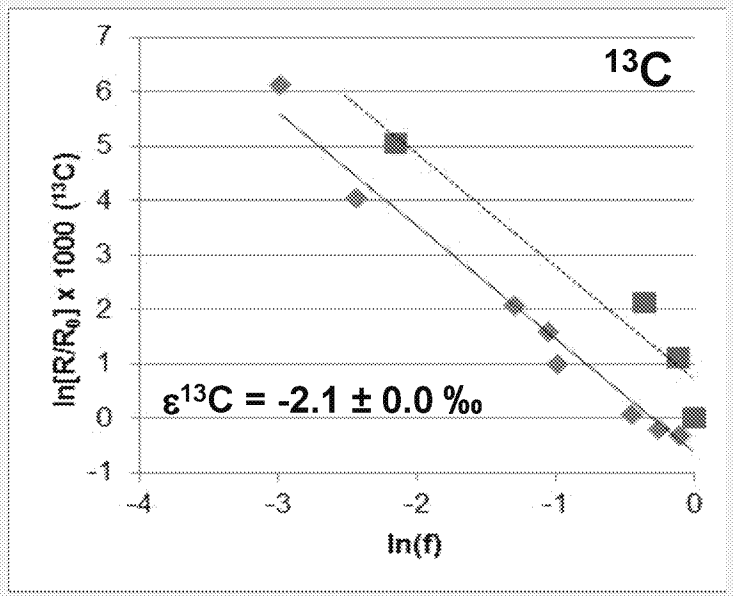
Results

Task 4. Fractionation of C during Anaerobic Degradation of RDX: Nitro-Reduction Pathway - MNX, DNX, TNX



Desulfovibrio spp.

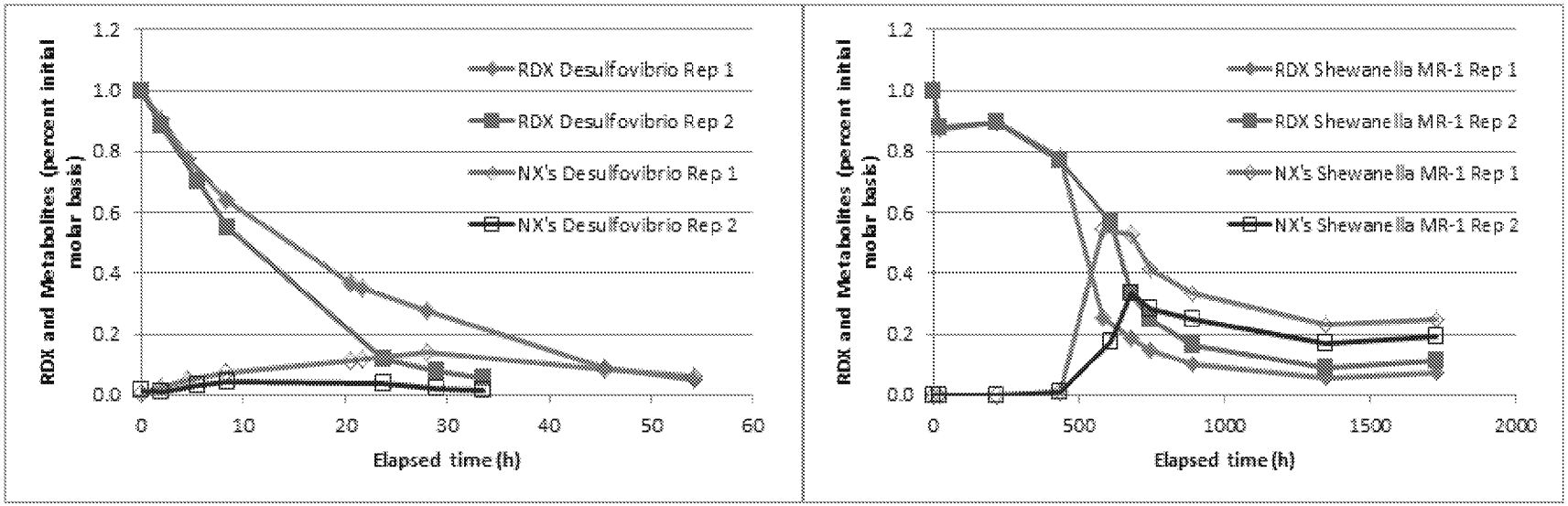




Results

Task 4. Fractionation of C during Anaerobic Degradation of RDX: Nitro-Reduction Pathway - MNX, DNX, TNX

Production of NXs by different strains considered to predominantly use the nitro-reduction pathway.



Data suggest that these strains may follow multiple degradation pathways for RDX

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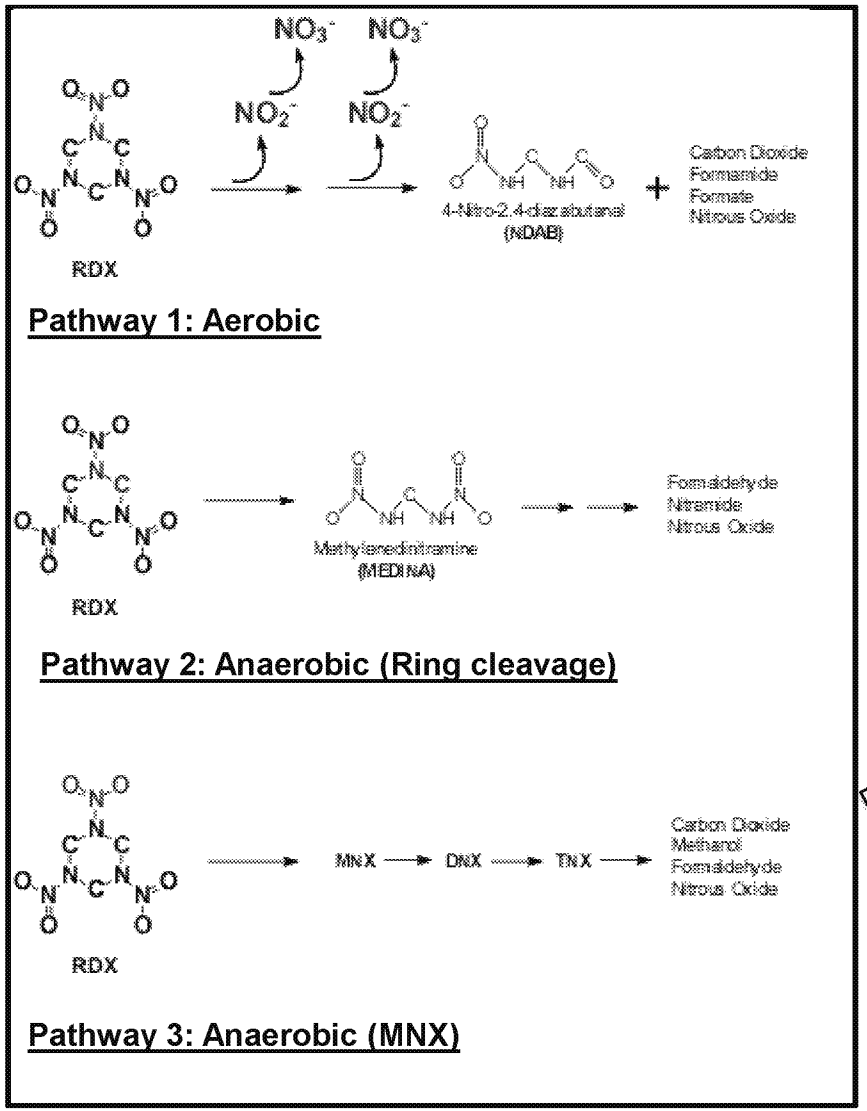
Results



Task 4. Summary of CSIA data for different strains

Rhodococcus sp. DN22
Rhodococcus rhodocrous 11Y
Rhodococcus sp. Strain A
Gordonia sp. KTR9

$\varepsilon^{15}N$	$\varepsilon^{13}C$
-2.4 ± 0.4	-0.4 ± 1
(-2.0 to -2.8)	(+0.4 to -1.1)

Pseudomonas putida II-B
Pseudomonas fluorescens I-C
Shewanella sp. MR-1
Klebsiella sp. SCZ-1
Clostridium acetobutylicum ATCC824
Desulfovibrio spp.

$\varepsilon^{15}N$	$\varepsilon^{13}C$
-10.8 ± 2.6	-3.8 ± 1.4
(-7.4 to -13.1)	(-2.1 to -6.4)

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Conclusions and Summary

Methods were developed and/or evaluated to better evaluate the degradation of RDX in the environment:

- RDX metabolite analysis
- Stable isotope probing
- Compound-specific isotope analysis

Application of these methods suggests the following:

- Pathways of RDX degradation may differ appreciably among different electron-accepting conditions
- Aerobic degradation of RDX may not be prevalent in groundwater aquifers.
- A variety of different organisms are involved in RDX metabolism in the environment, including organisms not previously associated with this activity
- CSIA may have broad application to document both aerobic and anaerobic degradation of RDX in the environment

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Transition Plan

- **Validate CSIA method for RDX in the field through ESTCP.**
- **Offer CSIA method for RDX on a per sample basis at the University of Delaware.**
- **Presentations at national and international conferences**
- **Peer-reviewed publications**
- **Participation as team member and trainer in ITRC *Environmental Molecular Diagnostics Team: CSIA and SIP included***

- **Future Research Needs**
  - Develop better understanding of fractionation of C and N in RDX at an enzymatic and mechanistic level
  - Evaluating stable isotope data for N (e.g., NO_2, NO_3) released from RDX
  - Further assessing RDX degradation pathways in the field – and relevant degradation products – as a function of geochemical conditions.
  - Application of CSIA method to assess RDX degradation at field sites
  - Continuing to define the role of key organisms involved in RDX degradation – *Pseudomonas* and *Desulfosporosinus* among them

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Publications

Paquet, L, F. Monteil-Rivera, P.B. Hatzinger, M. Fuller, and J. Hawari. 2011. Analysis of the key intermediates of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) in ground water: Occurrence, stability and preservation. *Journal of Environmental Monitoring,* 13:2304-2311.

Cho, K-C, P.G. Lee, H. Roh, M.E. Fuller, P.B. Hatzinger, and K-H Chu. 2013. Application of ^{13}C-stable Isotope probing to identify RDX-degrading microorganisms in groundwater. *Environmental Pollution* 178:350-360.

Cho, K.-C., D. G. Lee, M.E. Fuller, P.B. Hatzinger, C.W. Condee and K.-H. Chu. 2015. Application of ^{13}C and ^{15}N stable isotope probing to characterize RDX degrading microbial communities under different electron-accepting conditions using ^{13}C and ^{15}N stable isotope probing. *Journal of Hazardous Materials, 297: 42-51.*

Cho, K-C, P.G. Lee, M.E. Fuller, P.B. Hatzinger, and K-H Chu. 2015. Stable isotope probing to identify active microorganisms capable of using different nitrogen molecules in RDX structure. *Journal of Hazardous Materials* (in preparation).

Fuller, M.E., L. Heraty, C.W. Condee, S. Vainberg, N.C. Sturchio, J.K. Böhlke, and P.B. Hatzinger. 2015. Carbon and nitrogen isotopic fractionation during biological degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by various anaerobic and aerobic bacterial cultures. *Applied and Environmental Microbiology* (in preparation).

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