

GUIDANCE DOCUMENT

Guidance for Using Compound Specific Isotope
Analysis (CSIA) to Document the Biodegradation
and Natural Attenuation of RDX

ESTCP Project ER-201208

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14. ABSTRACT The objective of this project was to validate a stable isotope method that can be utilized by DoD to quantify aerobic and anaerobic biodegradation of RDX at field sites. Combined analysis of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in RDX was used to clearly document anaerobic RDX biodegradation in groundwater at a DOD range where a biobarrier was installed to promote this process. Documenting aerobic RDX biodegradation in the field proved to be more difficult because the expected fractionation of N is low (~2.4 ‰ based on pure culture studies) and no fractionation of C is expected. $\delta^{15}\text{N}$ measurements provided supporting data for aerobic RDX biodegradation in a push-pull test conducted at a DoD site, but results from other similar tests were inconclusive. In support of the field studies with RDX, a column method was developed to collect and concentrate RDX from wells with low concentrations for isotope analysis, and the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values in NO_2^- and NO_3^- generated from RDX during biodegradation were assessed as a potential second isotopic measure of RDX biodegradation.					
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List of Acronyms and Abbreviations

Acronym	Full text
amsl	above mean sea level
bgs	below ground surface
¹³ C	carbon-13
Cl ⁻	chloride
³⁷ Cl	chlorine-37
CO ₂	carbon dioxide
cm	centimeter
CSIA	Compound specific isotope analysis
d	day
1,2-DNT	1,2-dinitrotoluene
DNAN	2,4-dinitroanisole
2,4-DNT	2,4-dinitrotoluene
2,6-DNT	2,6-dinitrotoluene
DNX	hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine
DO	dissolved oxygen
DoD	Department of Defense
EOS	emulsified oil substrate
ESTCP	Environmental Security Technology Certification Program
ft	feet
GAC	granular activated carbon
GC	gas chromatograph
GC-IRMS	gas chromatography-isotope –ratio mass-spectrometry
GPS	Groundwater Protection Standard
h	hour
HMX	octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
HPLC	high performance liquid chromatography
ID	inner diameter
K	hydraulic conductivity
KIE	kinetic isotope effect
L	liter
MEDINA	methylenedinitramine
mL	milliliter
MNA	monitored natural attenuation
MNX	hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine
¹⁴ N	nitrogen-14
¹⁵ N	nitrogen-15
NDAB	4-nitro-2,4-diazabutanal
NO ₃ ⁻	nitrate
NO ₂ ⁻	nitrite
NSWC Dahlgren	Naval Surface Warfare Center, Dahlgren, VA
ORP	oxidation-reduction potential
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine
SERDP	Strategic Environmental Research & Development Program

SPE	solid phase extraction
TNT	2,4,6-trinitrotoluene
TOC	total organic carbon
SIP	stable isotope probing
SO ₄ ²⁻	sulfate
Sp. Cond.	specific conductance
TNX	hexahydro-1,3,5-trinitroso-1,3,5-triazine
μg	microgram
USACE	U.S. Army Corps of Engineers
USEPA	United States Environmental Protection Agency
VPDB	Vienna Pee Dee Belemnite
‰	per mil
δ	delta; per mil deviation of sample isotope ratios relative to those of reference materials
ε	epsilon; isotope fractionation factor

1.0 INTRODUCTION

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a common soil contaminant at current and former military facilities, including many training and testing ranges. Because RDX is readily transported through soils to the subsurface, this nitramine explosive now also impacts groundwater and drinking water at numerous locations across the country. A significant issue with RDX contamination on ranges and at other military installations is that it often occurs over expansive areas, where *in situ* or *ex situ* treatment technologies are difficult to implement. One potential alternative for military ranges and other facilities is monitored natural attenuation (MNA), in which contaminant degradation by natural processes, including biodegradation, are evaluated. However, one limitation of this approach for RDX is the inability to accurately evaluate whether the nitramine is degrading under field conditions, as rates may be slow. One potential technique to help address this limitation is the use of compound specific isotope analysis (CSIA), where contaminant destruction can be documented as changes in the ratio of stable isotopes of specific elements in a molecule; for RDX, ratios of $^{15}\text{N}/^{14}\text{N}$ and $^{13}\text{C}/^{12}\text{C}$ are relevant. The objective of this project was to develop and validate a CSIA method to confirm and constrain rates of aerobic and anaerobic biodegradation of RDX at field sites. This technique can be utilized to provide critical data to support monitored natural attenuation (MNA) as a remedy for treating this energetic in groundwater, and to confirm the effectiveness of *in situ* enhanced bioremediation remedies when implemented.

2.0 TECHNOLOGY

2.1 Compound specific isotope analysis (CSIA)

CSIA is increasingly being applied as an analytical tool to quantify the degradation and environmental fate of industrial and military pollutants, including chlorinated solvents, gasoline oxygenates, perchlorate, TNT, and most recently, RDX. Examining biodegradation with this technique relies on the fact that bacteria degrade heavier isotopocules (e.g., RDX with ^{15}N rather than ^{14}N in its structure) more slowly than lighter ones due to the greater bond stability of the heavier molecules. This leads to relative enrichment of heavier isotopes within the residual parent molecules as biodegradation proceeds (**Figure 2.1**). An analysis of stable isotope ratios of a contaminant along the flow path of a plume, in a single well over time, and/or in contaminated groundwater compared to the contaminant source material can be utilized to document degradation and natural attenuation *in situ* (as opposed to losses due to adsorption, dilution or other nondestructive mechanisms). In addition, CSIA can provide information on specific reaction mechanisms, particularly if isotopes of multiple elements are evaluated, since the breaking of specific bonds is typically associated with characteristic kinetic isotope effects (KIE), resulting in different isotopic enrichment factors for elements when different degradation pathways occur. Thus, CSIA is a powerful tool to detect, understand, and in some cases, quantify contaminant degradation in the environment. Further information on applications of CSIA for documenting pollutant degradation can be found in review articles (e.g., Meckenstock et al., 2004; Hatzinger et al., 2013; Elsner and Imfeld, 2016). In addition, a USEPA guidance document is available

describing the application of CSIA to evaluate the biodegradation and source identification of a variety of organic compounds (Hunkeler et al., 2008).

2.2 Method for analysis of C and N stable isotopes in RDX

A CSIA method has been developed that utilizes gas-chromatography coupled to isotope-ratio mass-spectrometry (GC-IRMS) to quantify C and N isotope ratios in RDX. Further details are provided later in this document and in recent publications (Hatzinger et al., 2018; Fuller et al., 2016). In summary, RDX is collected from groundwater and concentrated via solid-phase extraction (SPE) either in the field using a column developed during this project (primarily for wells with low RDX concentrations) or in the laboratory. The RDX is then eluted from the SPE columns into acetonitrile, concentrated, and analyzed for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in RDX using GC-IRMS.

2.3 Isotopic fractionation of N and C in RDX during biodegradation

Important to interpretation of field biodegradation data is an understanding of the extent to which isotopic enrichment (i.e., fractionation) occurs for each element of interest during the degradative process. This fractionation often varies based upon the mechanism (e.g., aerobic vs anaerobic biodegradation or different anaerobic degradation pathways) and is typically determined in controlled laboratory studies with pure cultures to allow interpretation of more complex field data. The key parameter calculated for most of the studies is the isotopic enrichment factor [ϵ]. The calculation of this factor is described later in this document (Section 5.3).

The biodegradation of RDX occurs under aerobic, microaerophilic, and anaerobic conditions and pathways for its catabolism by various bacteria have been characterized (**Figure 2.2**). Pure culture data indicate that significant N isotope fractionation occurs during RDX biodegradation (measured as a ^{15}N enrichment factor [ϵ]) but that ϵ varies based upon the degradative pathway. A summary of the three general pathways, and relevant isotope fractionation factors are provided in **Figure 2.3**, and compiled isotope data are provided in **Table 2.1**. For aerobic degradation of RDX via the XplA/B enzyme system (Pathway C in **Figure 2.2**), the $\epsilon^{15}\text{N}$ value averaged -2.4 ± 0.2 ‰ for four separate strains. The isotopic fractionation of N during degradation of RDX by strains with XenA/XenB enzymes under low oxygen/anaerobic conditions (Pathway B in **Figure 2.2**) was significantly higher than for aerobic degradation, with an average $\epsilon^{15}\text{N}$ value of -12.7 ± 0.8 ‰ observed for two strains. The most widely studied pathway of anaerobic RDX biodegradation, which proceeds through mono-, di-, and tri-nitroso derivatives of RDX (Pathway A in **Figure 2.2**) resulted in N fractionation ranging from -5.8 ‰ for a *Klebsiella* sp. to -10.9 ‰ for a *Desulfovibrio* sp. Some of this variability likely reflects the fact that some cultures follow mixed anaerobic pathways and/or that there are different initial reaction mechanisms that fractionate N differently. The composited $\epsilon^{15}\text{N}$ for all strains tested under anaerobic conditions was -11.9 ± 0.7 ‰.

Significant C isotope fractionation is not expected during aerobic RDX degradation via the XplA/B enzyme system because the initial enzymatic attack results in denitration of the molecule, and a C-

N bond is not broken (**Figure 2.2**; Pathway C). Isotope data for C during aerobic RDX degradation support this hypothesis, as the fractionation factor ($\epsilon^{13}\text{C}$) was $< 1\text{‰}$ ($-0.8 \pm 0.5 \text{‰}$) (**Table 2.1**). In contrast, for strains that initiate RDX degradation via direct ring cleavage to methylenedinitramine (MEDINA) under microaerophilic conditions (**Figure 2.2**; Pathway B), enrichment in ^{13}C can be reasonably anticipated because the initial step in this process is the cleavage of a C-N bond. For two *Pseudomonas* spp. strains degrading RDX anaerobically via XenA/XenB enzymes, a $\epsilon^{13}\text{C}$ value of $-2.7 \pm 0.7 \text{‰}$ was observed, with a corresponding $\epsilon^{15}\text{N}$ value -12.7‰ as previously noted. Fractionation of C under anaerobic Pathway A by four strains averaged $-4.7 \pm 1.1 \text{‰}$, with strain-to-strain variation ranging from -2.0 to -6.0‰ . Because the degradative intermediates in this reaction, such as MEDINA, are short-lived, stable isotope evidence ($\epsilon^{13}\text{C}$ and $\epsilon^{15}\text{N}$) for biodegradation via this route may be the only evidence obtainable that this process is occurring *in situ*.

2.4 Potential field application of CSIA for documenting RDX biodegradation

Significant and consistent fractionation of N isotopes occurred for all of the different cultures that degraded RDX under anaerobic conditions, representing multiple mechanisms of RDX degradation ($\epsilon^{15}\text{N} = -11.9 \text{‰}$), and lesser but consistent enrichment in ^{15}N occurred during aerobic degradation ($\epsilon^{15}\text{N} = -2.4 \text{‰}$). These data are in general concurrence with the few previous reports using different microbial cultures or mixed cultures, although our $\epsilon^{15}\text{N}$ values for anaerobic degradation are somewhat more negative (Bernstein et al., 2008, 2010, 2013). In contrast, there was no measurable fractionation of C isotopes in RDX during aerobic degradation, which is consistent with the proposed mechanism of degradation by the XplA/B cytochrome P450 system. Interestingly, there was significant C isotopic fractionation during anaerobic RDX degradation for all of the cultures for which data were available, although the extent of fractionation was highly variable. This variability most likely reflects the fact that (1) cultures appear to degrade RDX by multiple pathways in some instances, and (2) the initial degradative step for RDX as it breaks down to MEDINA (which may be a multi-step process) may vary by culture and reflect attack by different enzymes.

Based on pure culture studies and initial field evaluation (detailed later), the CSIA technology described herein is useful for documenting the biological degradation of RDX in groundwater by both aerobic and anaerobic mechanisms. When RDX is degraded aerobically via the typical denitration pathway, the extent of N fractionation is expected to be low ($\epsilon = \sim -2.4 \text{‰}$) and C is not expected to fractionate measurably based on pure culture studies. Thus, for the method to be useful for field samples, losses of RDX in groundwater either over distance (e.g., along a groundwater flowpath) or time (e.g., in an individual well) must be substantial, on the order of 80% or higher from initial concentrations. It is recommended that additional lines of evidence of RDX biodegradation under these conditions be considered along with N isotope analysis of RDX, as described further in Section 8.0.

When RDX is biodegraded via anaerobic mechanisms, C and N stable isotopes are both applicable to document this process due to relatively large fractionation factors based on culture studies ($\epsilon = \sim -4.7\text{ ‰}$ for C and $\epsilon = \sim -11.9\text{ ‰}$ for N on average). Dual isotope measurements with C and N can be utilized to confirm biodegradation. Many of the general lines of evidence suggested for evaluating aerobic RDX biodegradation are also applicable for anaerobic biodegradation as documented in Section 8.0.

Figure 2.1 Illustration of the isotopic enrichment process of nitrogen in RDX during biodegradation.

Lighter RDX, containing more ^{14}N , is preferred over ^{15}N -containing RDX by the enzymes degrading the RDX. As the degradation process progresses, the percentage of ^{15}N -RDX increases, thereby changing the measured $^{15}\text{N}/^{14}\text{N}$ isotope ratio.

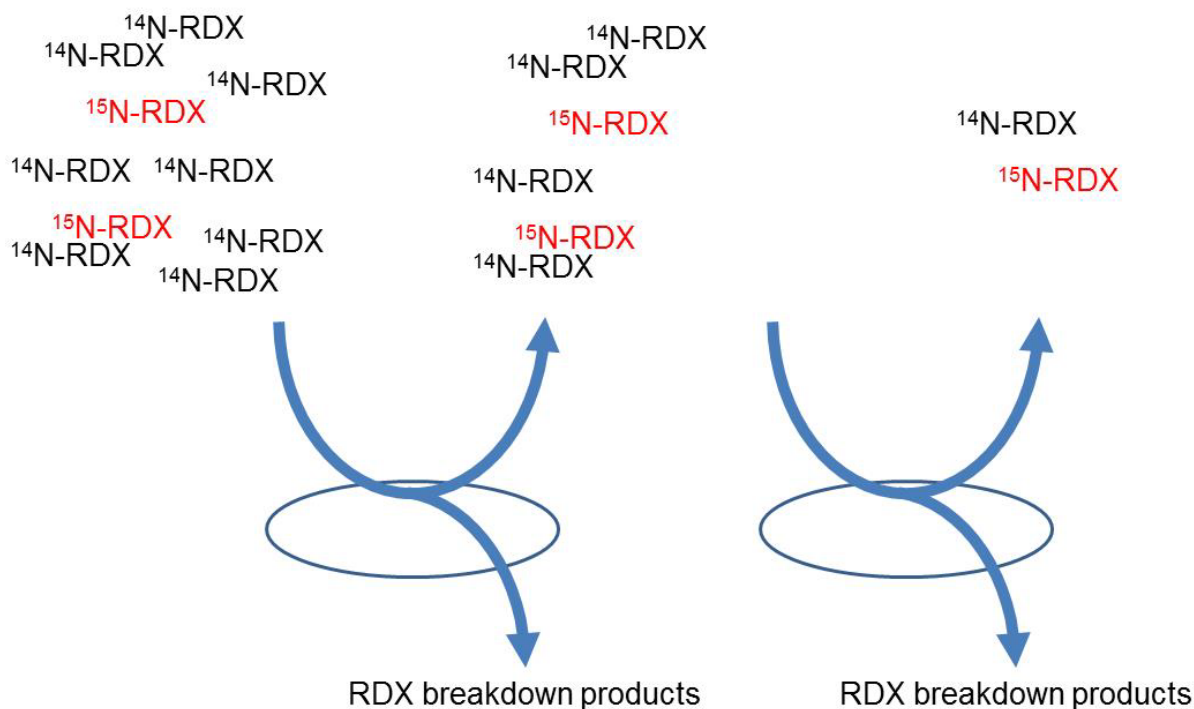
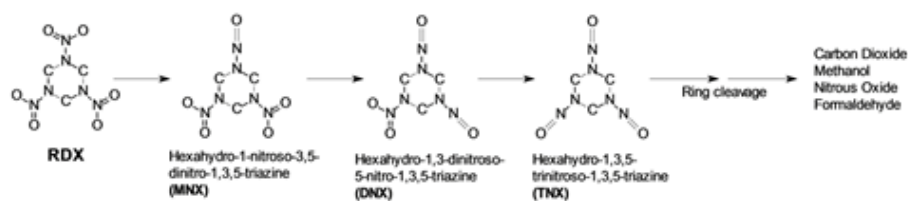


Figure 2.2 Biodegradation pathways for RDX.

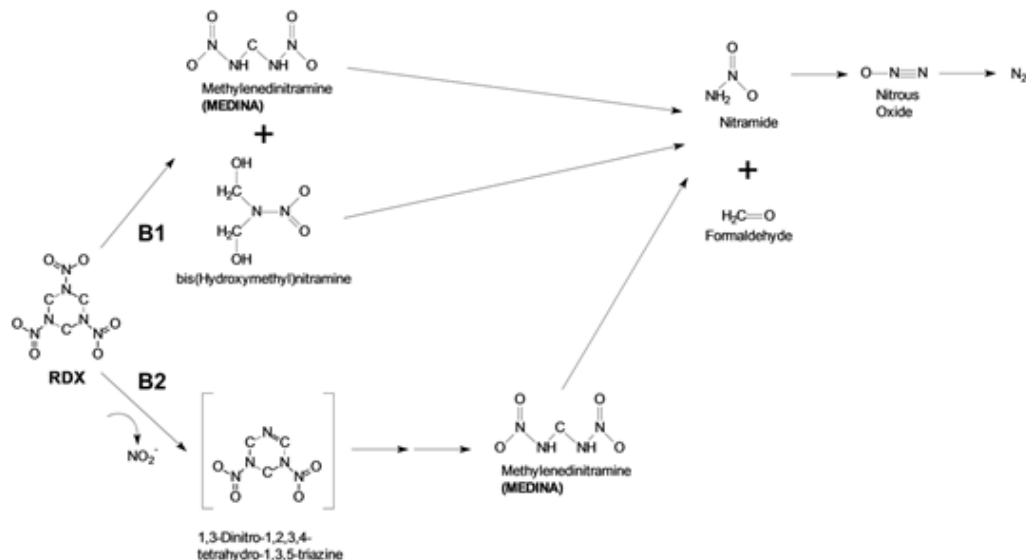
Aerobic conditions via the anaerobic conditions via nitroreductases or other enzymes (Pathway A); anaerobic conditions via XenA and XenB enzymes (Pathway B); via the XplA/B enzyme system (Pathway C). Figure reproduced from Fuller et al., 2016.

Anaerobic Pathways

A. Nitro-reduction



B. Ring cleavage



Aerobic Pathway

C. Denitration

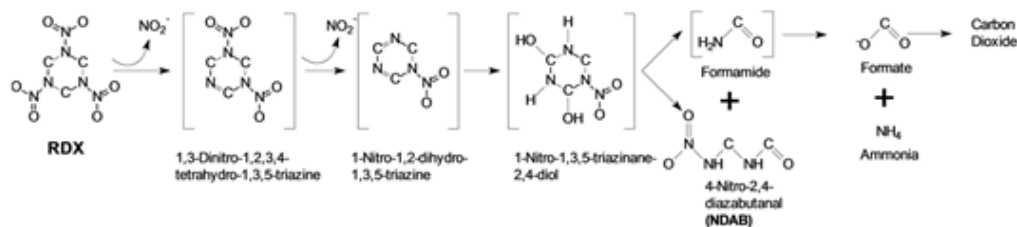


Table 2.1 Summary of bulk ^{13}C and ^{15}N fractionation factors (ϵ) by different bacteria and via different RDX degradation pathways.

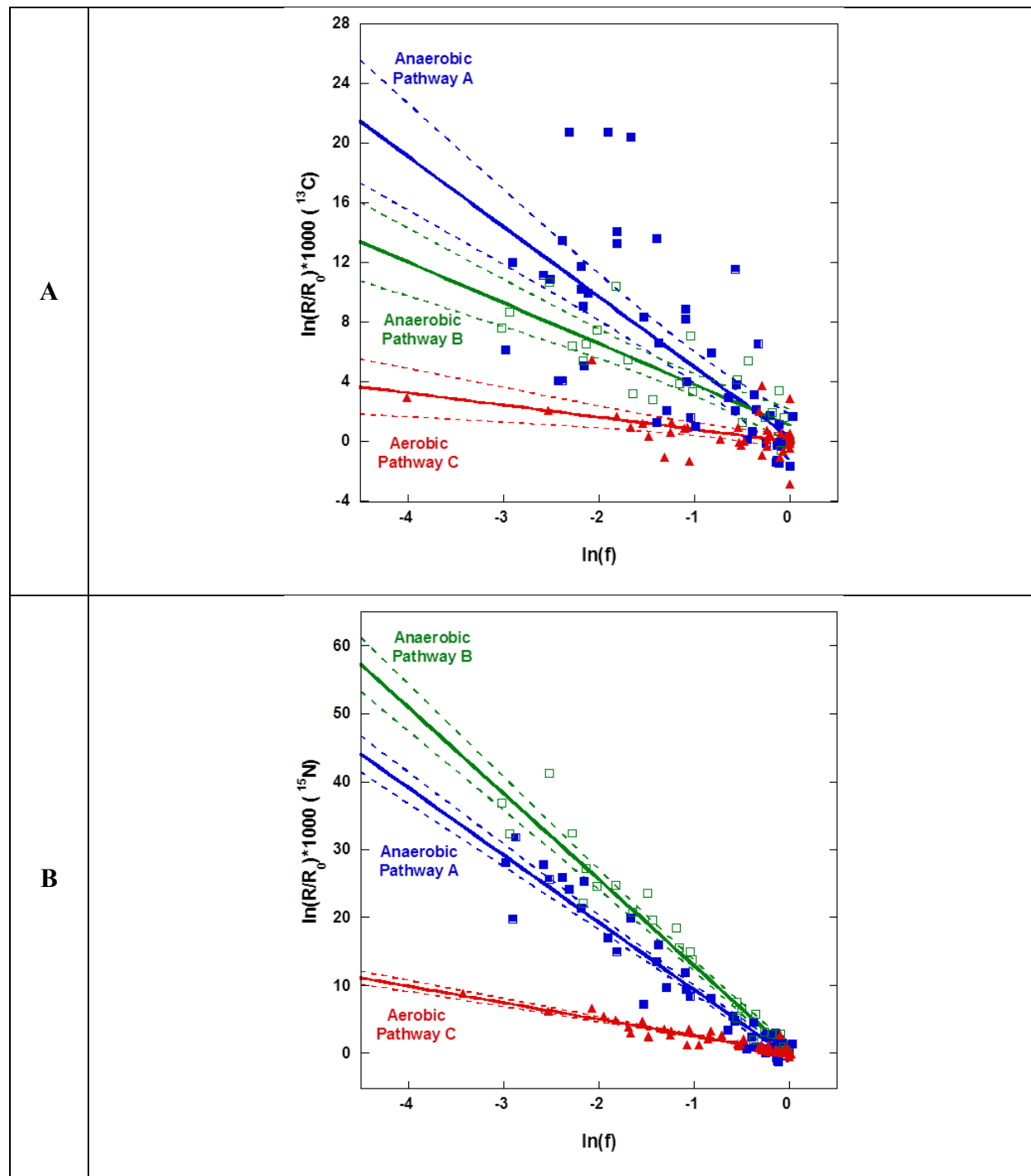
Values represent the slope of the linear regression of the combined data for duplicate fractionation experiments of each culture $\pm$ the 95% confidence interval of the slope. P-values represent the probability that the observed slope is different from zero. Data from Fuller et al., 2016.

Strain	$\epsilon^{13}\text{C}$			$\epsilon^{15}\text{N}$		
	‰	$\pm$	P-value	‰	$\pm$	P-value
Anaerobic Pathway A						
<i>Shewanella oneidensis</i> MR-1	-6.0	2.6	2.0E-04	-9.6	1.3	5.6E-12
<i>Klebsiella pneumoniae</i> SCZ-1	-4.1	1.6	4.7E-05	-5.8	2.6	6.3E-04
<i>Desulfovibrio</i> sp.	-2.0	0.5	2.8E-06	-10.9	1.2	5.2E-10
<i>Clostridium acetobutylicum</i> ATCC 824	-2.8	1.6	2.7E-03	-8.0	5.5	1.9E-02
Composited Pathway A data ^a	-4.7	1.1	1.8E-11	-9.9	0.7	1.8E-32
Anaerobic Pathway B						
<i>Pseudomonas putida</i> II-B	-3.1	1.0	1.3E-05	-13.3	1.5	6.0E-12
<i>Pseudomonas fluorescens</i> I-C	-2.1	1.3	6.2E-03	-12.7	1.1	1.7E-12
Composited Pathway B data ^a	-2.7	0.7	8.7E-08	-12.7	0.8	9.0E-26
Composited Pathway A and B data ^a	-4.0	0.8	3.4E-15	-11.9	0.7	7.5E-52
Aerobic Pathway C						
<i>Rhodococcus</i> sp. DN22	-1.3	2.3	2.2E-01	-2.8	0.5	7.1E-09
<i>Rhodococcus rhodocrous</i> 11Y	0.3	0.8	4.0E-01	-2.6	0.3	2.3E-10
<i>Rhodococcus</i> sp. Strain A	-0.7	0.3	2.1E-04	-2.3	0.4	2.6E-06
<i>Gordonia</i> sp. KTR9	-0.9	0.6	7.2E-03	-2.3	0.5	2.0E-06
Composited Pathway C data ^a	-0.8	0.5	1.2E-03	-2.4	0.2	3.3E-25

^a All data from the strains associated with designated pathway included in a single regression analysis.

Figure 2.3 Isotopic fractionation of $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ during biodegradation of RDX by different pathways.

Dashed lines represent 95% confidence intervals around the best fit solid lines with slopes as reported in Table 2.1. Refer to Figure 2.1 for pathways. Data from Fuller et al., 2016.



3.0 COLLECTION, PRESERVATION, STORAGE AND PROCESSING OF SAMPLES

3.1 Selection of sampling locations

There are multiple ways to document degradation of RDX in an aquifer using CSIA. Groundwater samples can be collected along a transect with the direction of groundwater flow, (e.g., from an expected source area downgradient) and/or from the same wells over a period of months to years (or shorter if active bioremediation approaches are ongoing). The overall objective is to look for isotopic enrichment in N (and C for anaerobic sites) over distance (transect approach) or time (repeated sampling in individual wells).

3.2 Sample collection, preservation, and storage

While a single stable isotope analysis for either C or N in RDX requires only 5 to 10 µg of RDX, sufficient groundwater should be collected and processed (in-field or in the laboratory) to obtain enough RDX mass for multiple analyses, typically 50 to 100 µg in the final extract. Side-by-side testing at a contaminated site has indicated that in-field and laboratory processing provide comparable CSIA data for $\delta^{15}\text{N}$ in RDX (**Table 3.1**). The same results are expected for $\delta^{13}\text{C}$ although this was not tested. In general, for wells with RDX concentrations greater than ~ 25 µg/L, collection of samples in bottles with laboratory SPE is recommended, whereas for sites with lower concentrations, in-field SPE is recommended to reduce the number of bottles requiring shipping and processing.

Table 3.1 Comparison of RDX mass recovered and $\delta^{15}\text{N}$ for in-field vs. laboratory SPE.

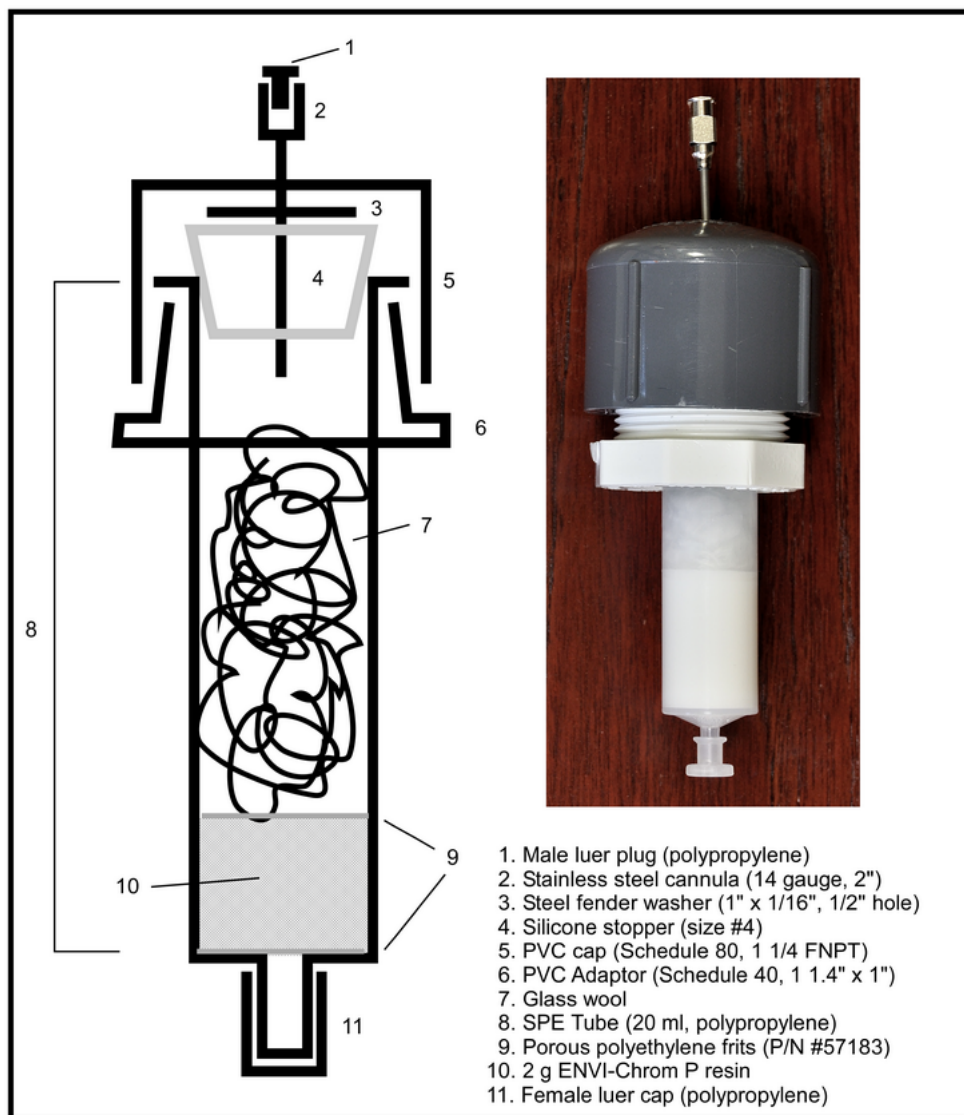
Well ID	RDX mass collected (µg)			$\delta^{15}\text{N}$	
	Lab	Field	(%)	Lab	Field
1	262	74	NA*	2.8	2.8 ± 1.6
2	264	253	104	1.5 ± 0.3	2.5 ± 1.0
3	185	159	116	1.5 ± 0.4	1.3 ± 1.3
4	225	246	91	2.1 ± 0.1	3.1 ± 1.7
5	364	106	NA*	0.1 ± 0.5	-1.0 ± 0.7
6	44	110	NA*	1.5 ± 0.3	1.0 ± 1.9
7	164	152	108	0.0 ± 0.2	-1.0 ± 2.5

* Different groundwater volumes processed preclude direct comparison of lab vs. field for these samples.

Appropriate low-flow sampling procedures (e.g., Puls et al., 2003) should be used in order to assure collection of representative groundwater samples irrespective of whether samples are concentrated via SPE in the field or in the laboratory. For laboratory processing, which is likely to be the most common technique, samples should be collected into pre-cleaned amber glass, HDPE, or polypropylene containers. Protection from direct sunlight is advised to minimize RDX photodegradation. Sufficient groundwater should be collected to allow concentration of the required mass for isotopic analysis of RDX. Samples should be preserved with hydrochloric acid sufficient to reduce the pH to below 2 SU and stored on ice in the field and at 4 °C before processing.

For in-field SPE, columns similar to that shown in **Figure 3.1** can be constructed. All necessary materials are provided in the figure legend and modifications are acceptable. Most importantly, ENVI-Chrom P SPE resin should be used as the sorptive matrix. Each column tested in our laboratory was prepared with 2 g of dry resin. Groundwater should be pumped directly from the well (after stabilization via low-flow sampling) through the SPE column at a rate of 50 to 100 mL/min. Flow is continued until the expected mass of RDX sorbed to the resin is sufficient for CSIA analysis (e.g., 50 to 100 µg). If there is enough sediment in the groundwater to cause clogging, multiple columns can be used per well. The column packing should then be dried using a portable vacuum pump and/or air compressor, and the columns stored at room temperature until extraction. If long-term storage is required, samples can also be preserved with dilute HCl.

Figure 3.1 Design of in-field large capacity SPE apparatus for concentration of RDX from groundwater.



3.3 Sample processing

The general procedure for groundwater collected in the field in bottles and shipped back the laboratory for isotopic analysis is as follows:

- 1) If the groundwater sample contains excessive solids, prefilter the water through a disposable plastic filter funnel (Nalgene 145-2020) holding a glass microfiber filter (Whatman 1823-047, 2.7 μm nominal pore size). Use multiple filters as needed.
- 2) Clean (or prefiltered) samples should be passed over a pre-conditioned Supelclean[™] ENVI-Chrom P solid phase extraction (SPE) column (250 mg packing; Sigma-Aldrich, St. Louis, MO, USA) to concentrate residual RDX and organic degradation products. The internal recovery standards 1,2-dinitrotoluene (1,2-DNT) and 2,4-dinitroanisole (DNAN), which are typically added to samples concentrated by SPE for concentration analysis by EPA Method 8330, should not be added to samples used for CSIA prior to passage through the SPE column due to potential for interference with analysis of RDX.
- 3) The analytes on the SPE column should then be eluted with 12 mL acetonitrile (or as per SPE column manufacturer's recommendations), then the extract should be evaporated to a final volume of 1 mL. A small aliquot (e.g., 5 - 50 μL based upon expected RDX) can be used for measuring RDX concentration in the extract (Section 4.1), and the remainder for C and N isotopic analysis of RDX. If multiple SPE columns were used for a given sample, then the extracts can be combined and evaporated to 1 mL. The final extract should remain in 100 % acetonitrile.

The general procedure for SPE performed in-field is as follows:

- 4) The analytes on the SPE column should be eluted with 100 mL acetonitrile, then the extract evaporated to a volume of 1 mL. A small aliquot (e.g., 5 - 50 μL based upon expected RDX) can then be removed for determination of RDX concentration, and the remainder used for C and N isotopic analysis of RDX. If multiple SPE columns were used for a given sample, then the extracts can be combined and evaporated to 1 mL. The final extract should remain in 100 % acetonitrile.

4.0 SAMPLE ANALYSIS

4.1 RDX analysis of extracts

Extracts described in Section 3.3 should initially be analyzed to determine RDX concentrations prior to further processing for CSIA as described in Section 4.2. The concentrations of the RDX in the final acetonitrile extract can be determined via HPLC using a Dionex 3000 Ultimate HPLC or equivalent with a Agilent Zorbax Bonus-RP column (4.6 x 75 mm, 3.5 μm particle diameter), variable wavelength detector (254 nm), and a photodiode array detector collecting peak spectral data. The mobile phase is 50:50 methanol:0.2 % (v:v) trifluoroacetic acid in water, at a flow rate of 1 ml/min. The column temperature should be set at 33 °C. The method detection limit is typically $\sim 10 \mu\text{g/L}$ for RDX.

4.2 Compound specific isotope analysis of C and N in RDX

4.2.1 Analysis of C isotope ratios in RDX

For analysis of $\delta^{13}\text{C}$ in RDX, carbon is converted to CO_2 gas after chromatographic separation and prior to IRMS analysis. Isotopic measurements are typically done with 5 μg aliquots of RDX, which is at the low end of the feasible range for isotopic analysis of RDX according to Gelman et al. (2011) who used 50 μg to obtain ± 0.3 to 0.4 ‰ precision on pure RDX. However, injecting smaller amounts of RDX yielded improved GC resolution for C isotopic analysis and provided a good balance between better chromatographic separation and slightly lower analytical precision. Gas chromatographic interferences near RDX, as observed in C isotopic analyses, were much less problematic in N isotopic analyses. If the RDX peak cannot be fully resolved for a given sample, isotopic data for that sample should be rejected.

An RTX-5MS GC column (30 m length x 0.25 mm inner diameter x 0.25 μm film thickness; Restek, Inc.), with a 1.5 mL/min flow of He can be used for analysis. The inlet temperature should be 190 °C, which will avoid thermal decomposition of the injected RDX. A PTV inlet with ramped temperature capabilities is preferred. The following GC program can be used for C separation: 75 °C x 1.5 min, ramp at 10 °C/min to 200 °C, then ramp 20 °C/min to 300 °C, which is maintained for 5 min. From the GC column the sample passes through a pre-oxidized Ni/Cu/Pt combustion furnace at 940 °C, and the combustion products are then reduced in a separate furnace of Cu at 600 °C. The resulting CO_2 is introduced to the isotope-ratio mass spectrometer (IRMS; Thermo Finnigan Delta Plus XP) by an open split interface (Thermo Finnigan GCCIII) for quantification of C isotope amounts. Newer instrumentation from Thermo Scientific combines combustion and reduction in a single furnace reactor at 1000°C in its ISOLINK GC interface.

4.2.2 Analysis of N isotope ratios in RDX

The N in RDX is converted to N_2 gas prior to IRMS analysis. The method for N isotopic analysis of RDX is similar to that described for $\delta^{13}\text{C}$ with modifications as follows: 1) The GC oven temperature program starts at 75 °C x 1.5 min, ramps at 10°C/min to 220 °C, then ramps at 20 °C/min to 300 °C, which is maintained for 5 min. 2) From the GC column the sample passes through a Ni/Cu/Pt combustion furnace which was not pre-oxidized (unlike that for CO_2) in order to minimize formation of NO, then passes through a Cu reduction furnace at 600 °C (Single reactor combined combustion and reduction at 1000°C is an equivalent treatment). CO_2 must be trapped

from the continuous flow He stream using liquid N₂ before the analyte N₂ is introduced to the mass spectrometer as described for CO₂. 3) Following each N₂ isotopic measurement, the open-split interface is isolated and the trapped CO₂ is flushed away by removing the liquid nitrogen trap from the He stream prior to the next analysis. Multiple GC-IRMS injections (two to seven) should be completed per sample.

5.0 CALCULATIONS AND DATA ANALYSIS

5.1 Calculation of $\delta^{13}\text{C}$

The C isotope ratio in RDX is reported as $\delta^{13}\text{C}$:

$$\delta^{13}\text{C}_{\text{sample}} = (^{13}\text{C}/^{12}\text{C}_{\text{sample}}) / (^{13}\text{C}/^{12}\text{C}_{\text{VPDB}}) - 1 \quad (5-1)$$

where VPDB is the Vienna Pee Dee Belemnite standard. The $\delta^{13}\text{C}$ data are reported in per mil (‰) as obtained by multiplying the right side of equation 5-1 by 1,000 and are calibrated by analyzing an RDX standard reference material for which a $\delta^{13}\text{C}$ value is determined independently by comparison with international L-glutamic acid isotopic reference materials USGS40 and USGS41 (Qi et al., 2003).

5.2 Calculation of $\delta^{15}\text{N}$

The N isotope ratio in RDX is reported as $\delta^{15}\text{N}$:

$$\delta^{15}\text{N}_{\text{sample}} = (^{15}\text{N}/^{14}\text{N}_{\text{sample}}) / (^{15}\text{N}/^{14}\text{N}_{\text{AIR}}) - 1 \quad (5-2)$$

where AIR is N₂ in air. The $\delta^{15}\text{N}$ data are reported in per mil (‰) as obtained by multiplying the right side of equation 5-2 by 1,000, and were calibrated by analyzing an RDX standard reference material for which a $\delta^{15}\text{N}$ value is determined by comparison with international L-glutamic acid isotopic reference materials USGS40 and USGS41 (Qi et al., 2003)

5.3 Calculation of fractionation factors (α) and enrichment factors (ϵ)

The isotopic fractionation factor, α , is defined as

$$\alpha = R_A / R_B \quad (5-3)$$

where R is an isotope-amount ratio, and A and B represent the instantaneous product and reactant for kinetic reactions. For C and N in RDX, R represents the isotope ratios $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$, respectively. Values of α are often observed to be reasonably constant with time and can be determined from experimental data by assuming the Raleigh-type function

$$R / R_0 = f^{\alpha-1} \quad (5-4)$$

where R and R_0 are the C or N isotope ratios of the residual RDX and the initial (unreacted) RDX, respectively, and f is the fraction of RDX remaining at the sample time point (C/C_0). In terms of the δ values, Equation 5-4 can be rewritten as:

$$(\delta + 1) / (\delta_0 + 1) = f^{\alpha-1} \quad (5-5)$$

where δ is the isotopic composition of RDX at any value f , and δ_0 is the isotopic composition at $f = 1$. The value of α can be obtained from the natural log of Equation 5-4:

$$\alpha = [\ln (R/R_0) / \ln f] + 1 \quad (5-6)$$

This describes the Rayleigh-type isotopic fractionation that accompanies biodegradation and other kinetic processes. Isotopic enrichment factors are commonly expressed as ε , where

$$\varepsilon = \alpha - 1 \quad (5-7)$$

Values of ε are given in per mil (‰), and negative ε values ($\alpha < 1$) indicate “normal” kinetic isotope effects with relative enrichment of light isotopes in products and heavy isotopes in residual reactants.

Linear regression can be used to generate an ε value from RDX samples undergoing biodegradation in the field, such as those taken along a plume transect, or taken from a single well over time. The value of ε is equivalent to the slope of a linear regression of $\ln (R/R_0)$ vs. $\ln f$. The independent variable (x-axis) is $\ln f$, i.e. the natural logarithm of the RDX concentration in each sample divided by the starting concentration (e.g., source sample or first sample in a single well). The dependent variable (y-axis) is $\ln (R/R_0)$, that is calculated from the measured δ values

$$\ln (R/R_0) = \ln [(\delta + 1) / (\delta_0 + 1)] \quad (5-8)$$

substituting the RDX isotopic data ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$) for each of the samples into the equation. An example spreadsheet layout is presented in **Figure 5.1**.

It may also be appropriate to calculate or plot confidence intervals for the isotope data. An example of this type of analysis is presented in **Figure 5.2** and is derived from <http://people.stfx.ca/bliengme/exceltips/regressionanalysisconfidence2.htm>.

Figure 5.1 Example spreadsheet calculations for determining enrichment factors (ϵ).

The following example shows the how the isotope enrichment factor ($\epsilon^{15}\text{N}$) observed during biodegradation of RDX by a pure bacterial culture can be calculated. The same approach can be applied to C fractionation.

Sample	RDX (ug/L)	Fraction RDX		$\delta^{15}\text{N}$ (Air)	1000* ln (R/R ₀)
		remining (f)	ln (f)		
1	15.35	1.00	0.00	7.26	0.00
2	13.89	0.91	-0.10	8.84	1.57
3	10.78	0.70	-0.35	12.71	5.40
4	5.90	0.38	-0.96	19.42	12.00
5	3.63	0.24	-1.44	26.68	19.10
6	1.78	0.12	-2.15	33.20	25.43

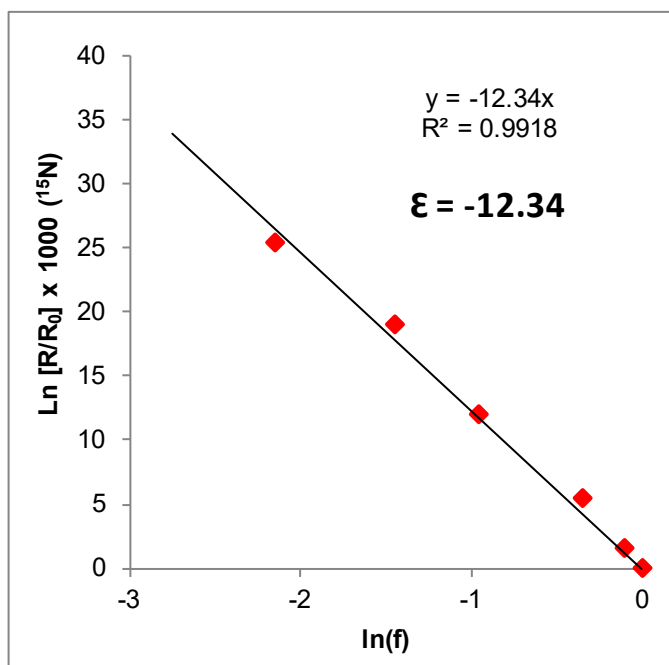
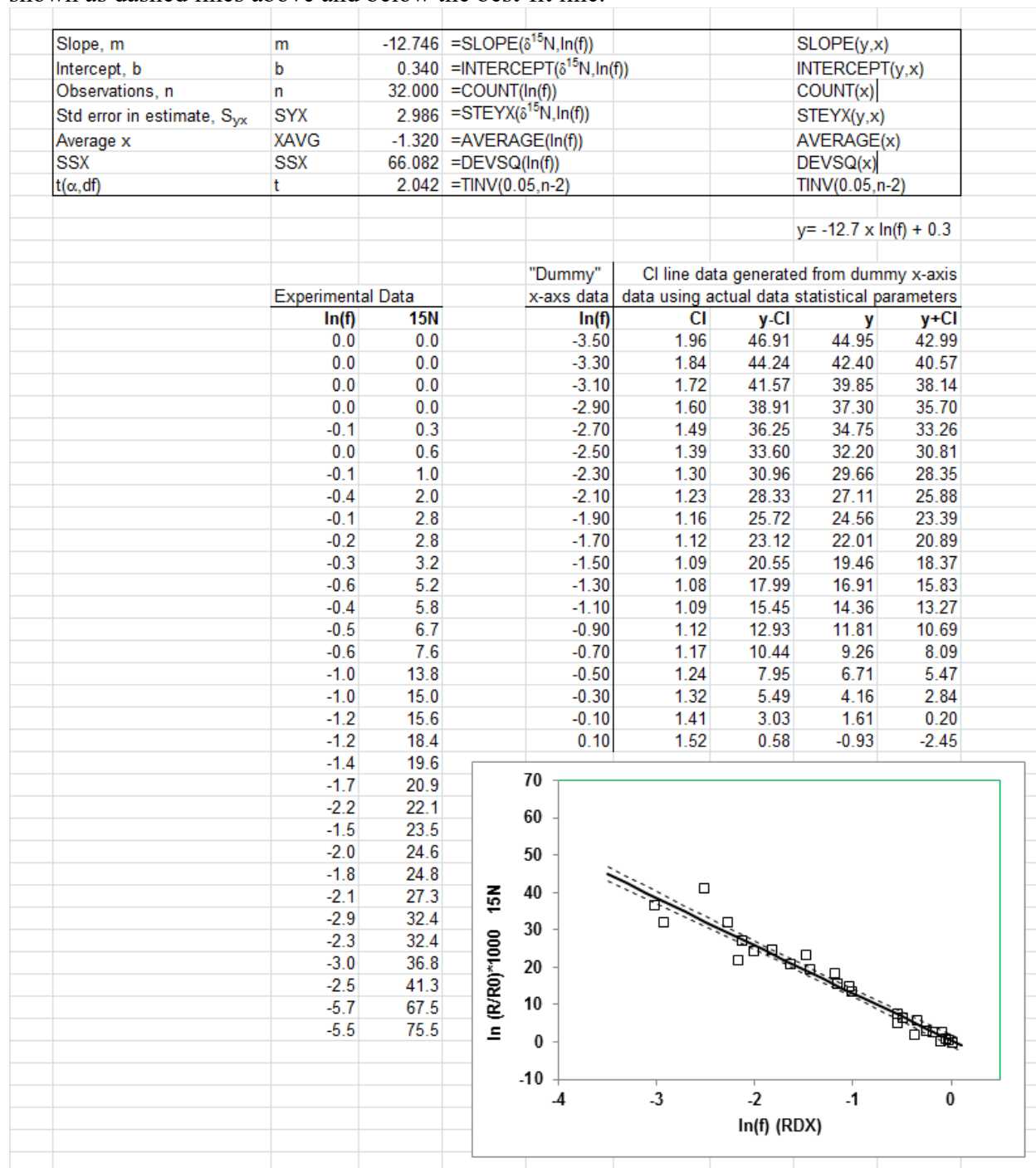


Figure 5.2 Example spreadsheet calculations for calculating and plotting the 95% confidence interval of the isotope fractionation data.

The following example shows the 95% confidence interval for the $\ln(f)$ vs. $\delta^{15}\text{N}$ data observed during biodegradation of RDX by a pure bacterial culture. The same approach can be applied to C fractionation. Experimental data are represented by the open symbols. Confidence intervals are shown as dashed lines above and below the best-fit line.



6.0 APPLICATION OF CSIA DATA FOR FIELD INVESTIGATIONS OF RDX BIODEGRADATION

6.1 Relevant equations

CSIA data can be used for a number of purposes during field investigations of RDX fate. The degree of transformation/degradation can be estimated between two points along the flow path, which can be used to provide estimates of degradation rates.

The relevant equations are presented below, and an example is given at the end of this section. These equations are presented using N isotope data but can also be applied using C isotope data for anaerobic biodegradation of RDX.

To quantify the fraction of RDX remaining (f) based on the isotope ratio data, the following equation can be used:

$$f = (\delta^{15}\text{N}_D + 1 / \delta^{15}\text{N}_U + 1)^{1/(\alpha-1)} \quad (6-1)$$

where

$\delta^{15}\text{N}_D$ and $\delta^{15}\text{N}_U$ describe N isotope composition of RDX in the downgradient and upgradient locations, respectively, and,

$\alpha-1$ is the ^{15}N isotope enrichment factor (i.e., ϵ value) for RDX degradation by a given process.

The change in the RDX isotope ratio from one location to another in the plume can be used for two purposes. The CSIA data can be used to test the hypothesis that the concentration decrease is predominantly due to degradation (e.g., as a line of evidence for degradation). The data can also be used to extrapolate the removal that would be expected along the flow path. It is important to note however, that an appropriate ϵ value from pure culture data or other sources is required for this calculation. If several values have been reported, which is common, it is recommended that such calculations are conducted over a range of representative ϵ values to bracket the data. Higher assumed ϵ values will provide more conservative estimates of contaminant degradation (i.e., reflecting less contaminant transformation).

The expected concentration at a downgradient location can be estimated as

$$C_D = C_U \times f \quad (6-2)$$

where

C_D is the expected RDX concentration at the downgradient location,

C_U is the RDX concentration at the upgradient location, and,

f is given by Equation 6-1 as an estimated fraction of RDX remaining (i.e., not degraded)

If the reductions in RDX concentrations are primarily due to a process that has a characteristic $\varepsilon^{15}\text{N}$ value, then the value of f obtained from Equation 6-1 should substitute into Equation 6-2 to predict a value for C_D that is in reasonable agreement with the measured RDX concentration at the downgradient location. If the RDX concentration values are not in good agreement, it is possible that other processes such as dilution or dispersion along the flow path are significant factors impacting attenuation. It is also possible that there is an error in the site conceptual model, that other degradative processes are active, or that the isotope fractionation factor (ε) is a poor estimate for the actual *in situ* reaction occurring.

The CSIA data can also be used to extrapolate RDX concentrations further down the flow path. The first order rate constant for RDX removal can be estimated from:

$$f = C_D / C_U = \exp(\lambda_t \times t) \quad (6-3)$$

where

t is the average travel time of RDX between the upgradient and downgradient locations along the flow path, and,

λ_t is a first order degradation rate constant for RDX due to biotic or abiotic processes.

Solving Equation 6-3 for λ_t and then substituting Equation 6-1 for f yields:

$$\lambda_t = - [(1/\varepsilon) \times \ln(\delta^{15}\text{N}_D + 1 / \delta^{15}\text{N}_U + 1)] / t \quad (6-4)$$

The calculated rate constant represents the rate due to RDX degradation. The rate of removal is distinct from the bulk attenuation rate constant, k , that is calculated by plotting the natural logarithm of the RDX concentration against the time of travel along the flow path (Newell et al., 2002). The bulk attenuation rate also includes the effects of dilution/dispersion on the concentration in addition to RDX removal.

Equation 6-3 can be solved for the travel time required along the flow path (t_{req}) to attain any desired RDX concentration (C_{req}) at the field-scale rate of removal, λ_t , as follows:

$$t_{req} = \ln (C_{req} / C_U) / \lambda_t \quad (6-5)$$

Multiplication of t_{req} by the RDX velocity ($V_{seepage}$) yields an equation for the distance along the flow path from the upgradient location that is required to reduce the RDX to the desired concentration (L_{req}):

$$L_{req} = [\ln (C_{req} / C_U) \times V_{seepage}] / \lambda_t \quad (6-6)$$

where

$V_{seepage}$ is the actual seepage velocity of RDX in the plume along the flow path.

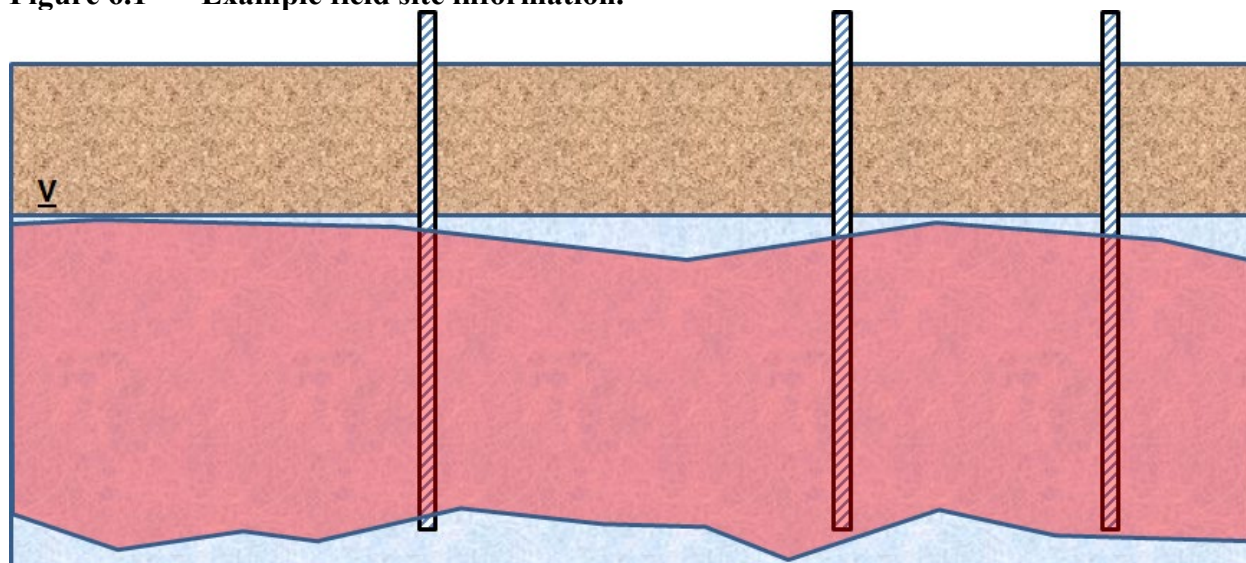
The seepage velocity is usually estimated by dividing the Darcy velocity by the effective porosity, and then dividing by the RDX retardation factor, if relevant.

6.2 Example application

The following presents an example of how RDX isotope data can be used to calculate various parameters relevant to field investigations.

Figure 6.1 illustrates a fictitious RDX contaminated site. RDX concentration and N isotope data are given below the illustration, as well as some relevant groundwater parameters. For this example, assume that anaerobic biostimulation has resulted in sulfate reducing conditions in the aquifer, yielding a value from Table 7.2 for $\epsilon^{15}\text{N-RDX}$ of -6.4 ‰.

Figure 6.1 Example field site information.



Well ID	Well A	Well B	Compliance Well C
Distance (m)	0	45	100
RDX ($\mu\text{g/L}$)	200	20	?
$\delta^{15}\text{N-RDX}$ (‰)	-4.7	-0.8	?
$\epsilon^{15}\text{N-RDX}$ Biodeg (‰)	-6.4 (sulfate reducing conditions)		

RDX travel time (yr)	0	0.9	2
Darcy velocity (m yr⁻¹)	15		
Porosity	0.3		

One question that may arise is whether concentrations and N isotope data at Well B are consistent with anaerobic degradation under sulfate reducing conditions. Using previous equations,

$$C_B = C_A * f = C_A * \left(\frac{\delta_B + 1}{\delta_A + 1} \right)^{1/\varepsilon} = 200 \mu g/L * \left(\frac{-0.0008 + 1}{-0.0047 + 1} \right)^{1/-0.0064} = 200 \mu g/L * 0.54$$

$$= 108 \mu g/L$$

The measured concentration at Well B was 20 $\mu g/L$, approximately five times lower than expected by anaerobic biodegradation under sulfate reducing conditions. This discrepancy is likely the result of dilution, a larger ε value than expected for the conditions, or some other alternative process.

One can also calculate a biodegradation rate constant, λ_t , based on N isotope data and a bulk attenuation rate constant, k , based on concentrations.

$$\lambda_t = \frac{-\ln(f)}{t} = \frac{-\ln(f)}{x/V_{seepage}} = \frac{-\ln(0.54)}{45m / \frac{15m \text{ yr}^{-1}}{0.3}} = \frac{0.616}{45/50 \text{ yr}^{-1}} = 0.684 \text{ yr}^{-1}$$

$$k = \frac{-\ln\left(\frac{C_B}{C_A}\right)}{t} = \frac{-\ln\left(\frac{20}{200}\right)}{x/V_{seepage}} = \frac{-\ln(0.1)}{45m / \frac{15m \text{ yr}^{-1}}{0.3}} = \frac{2.3}{45/50 \text{ yr}^{-1}} = 2.56 \text{ yr}^{-1}$$

As above when examining expected concentrations at Well B, the bulk natural attenuation indicates faster attenuation than is attributable to biodegradation alone using isotope data.

Given these data, RDX concentrations at the compliance point C can be estimated. Based on k and concentrations at Wells A and B,

$$C_{C,AC} = C_A * \exp(-k * t) = 200 \mu g/L * \exp\left(-2.56 \text{ yr}^{-1} * \frac{100 \text{ m}}{50 \text{ m yr}^{-1}}\right) = 1.2 \mu g/L$$

$$C_{C,BC} = C_B * \exp(-k * t) = 20 \mu g/L * \exp\left(-2.56 \text{ yr}^{-1} * \frac{55 \text{ m}}{50 \text{ m yr}^{-1}}\right) = 1.2 \mu g/L$$

Based on λ_t and N isotopes, and concentrations at Well A and B,

$$C_{C,AI} = C_A * \exp(-k * t) = 200 \mu g/L * \exp\left(-0.684 \text{ yr}^{-1} * \frac{100 \text{ m}}{50 \text{ m yr}^{-1}}\right) = 50.9 \mu g/L$$

$$C_{C,BI} = C_B * \exp(-k * t) = 20 \mu g/L * \exp\left(-0.684 \text{ yr}^{-1} * \frac{55 \text{ m}}{50 \text{ m yr}^{-1}}\right) = 9.42 \mu g/L$$

If only RDX concentration data were available, one might conclude that RDX concentrations could be below the health advisory level of 2 $\mu g/L$ at the compliance point C. Examination of RDX isotopes, however, suggests that concentrations at a compliance point might exceed the health advisory by up to 25x. While these isotope-based estimates are conservative, they may be more appropriate. For the case of isotope-based rates, we can also estimate distances beyond compliance point C at which RDX remains in excess of the health advisory level.

$$x + 100 \text{ m} = \frac{-\ln\left(\frac{2}{200}\right)}{\lambda_t / V_{seepage}} = \frac{-\ln(0.01)}{0.684 \text{ yr}^{-1} / 50 \text{ m yr}^{-1}} = \frac{4.6}{0.684 \text{ yr}^{-1} / 50 \text{ m yr}^{-1}} = 336 \text{ m}$$

$$x = 336 \text{ m} - 100 \text{ m} = 236 \text{ m}$$

$$x + 55 \text{ m} = \frac{-\ln\left(\frac{2}{20}\right)}{\lambda_t / V_{seepage}} = \frac{-\ln(0.1)}{0.684 \text{ yr}^{-1} / 50 \text{ m yr}^{-1}} = \frac{2.3}{0.684 \text{ yr}^{-1} / 50 \text{ m yr}^{-1}} = 168 \text{ m}$$

$$x = 168 \text{ m} - 55 \text{ m} = 113 \text{ m}$$

7.0 CASE STUDY: DOCUMENTING ANAEROBIC RDX BIODEGRADATION

An emulsified biobarrier was installed on a US Navy Test Range at the Dahlgren Naval Surface Warfare Center in Dahlgren VA (Fuller et al., 2019). The biobarrier consisted of a pH buffer and an emulsified oil substrate to promote anaerobic RDX biodegradation. Initial RDX concentrations upgradient of the barrier were in the vicinity of 100 µg/L. Samples for C and N isotopic analyses of RDX were collected from several different wells in the field test plot upgradient and downgradient from the biobarrier (**Figure 7.1**). RDX concentrations along the centerline of wells before (October 2012) and after (June 2013) the biobarrier was installed are shown in **Figure 7.2** as a function of distance upgradient and downgradient of the biobarrier. The complete dataset of all wells sampled is provided in **Table 7.1**. Groundwater samples for CSIA were collected in 950-ml amber bottles and RDX was processed and analyzed as described previously in Sections 3 and 4.

The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of RDX along the centerline of wells at the two timepoints (before and after amendment injection) are presented in **Figure 7.3**. Enrichment of both ^{13}C and ^{15}N are apparent in many of the downgradient test plot samples in June 2013 (post-biobarrier) compared to October 2012 (pre-biobarrier), with the highest fractionation occurring over the initial 20 ft downgradient of the plot. This is consistent with the zone of influence of the initial injection and an estimated groundwater flow in the range of up to 20 ft/yr based upon pump test data and groundwater elevation measurements. However, the geology at this shallow aquifer was extremely complex, consisting of small sand seams interspersed within thick clay layers, so a groundwater flow rate through the plot was difficult to determine as some of the sand seams appeared to be terminal, possibly leading to slower transport of groundwater within the plot.

Field C and N enrichment factors (ϵ) are shown graphically in **Figure 7.4**, and the best-fit linear regressions are presented in **Table 7.2**, along with ϵ values for anaerobic RDX degradation using pure cultures and RDX degradation under various electron acceptor conditions in enrichments derived from Dahlgren NSWC sediments. Because groundwater flow through the plot could not be readily determined (as previously noted), and likely was exceedingly slow, the calculation of R/R_0 from the plot was based on a “batch” system, with values for R and R_0 compared from individual wells assuming the parcel of water in the plot was largely static.

Using the batch calculation, values of $\epsilon^{13}\text{C}$ and $\epsilon^{15}\text{N}$ in the plot were -2.2 ‰ and -6.8 ‰, respectively. Smaller $\epsilon^{13}\text{C}$ and $\epsilon^{15}\text{N}$ values in the field compared to laboratory studies have been observed during the degradation of other compounds, and is often attributed abiotic effects (e.g., dispersion, dilution, incomplete mixing) occurring in the aquifer matrix compared to a well-mixed system (e.g., Mariotti et al., 1988; Abe and Hunkeler, 2006; Hatzinger et al., 2009). The ratio of isotope fractionation factors ($\epsilon^{15}\text{N} / \epsilon^{13}\text{C}$) for the samples was ~ 3.1 which falls within the relatively

wide range determined from anaerobic pure cultures (1.5-5.5), and most closely matches that determined for *Clostridium acetobutylicum* ATCC 824, at ~2.9. The relatively broad range of this parameter most likely reflects the differing or mixed anaerobic pathways of RDX degradation. More studies are required to determine if such dual isotope ratios can be linked to specific mechanisms of anaerobic degradation because many of the cultures tested appeared to degrade RDX via multiple mechanisms (Fuller et al., 2016). However, this study clearly documents anaerobic RDX biodegradation in an aquifer using C and N stable isotope analysis.

Figure 7.1 Map (top) and layout (bottom) of the Dahlgren NSWC passive *in situ* emulsified oil biobarrier.

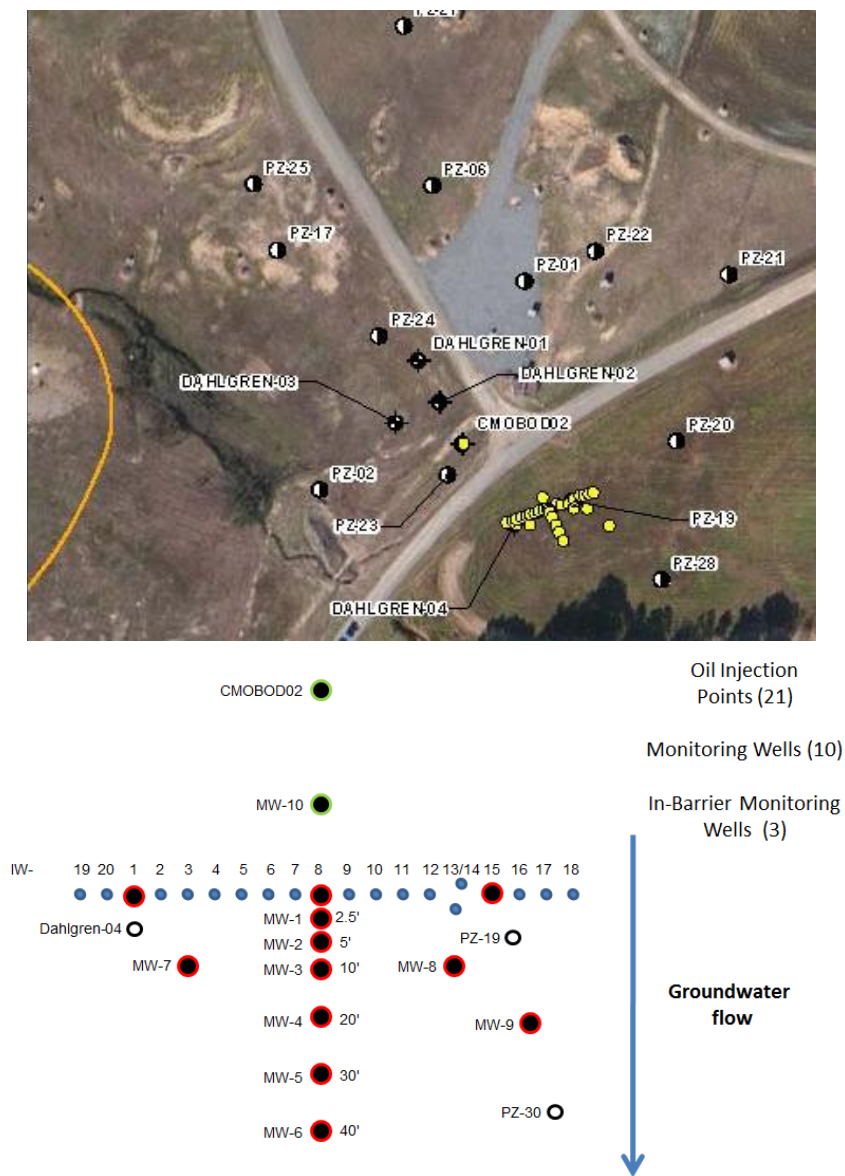


Figure 7.2 RDX concentrations along the centerline of the biobarrier before (October 2012) and after (June 2013) oil injection.

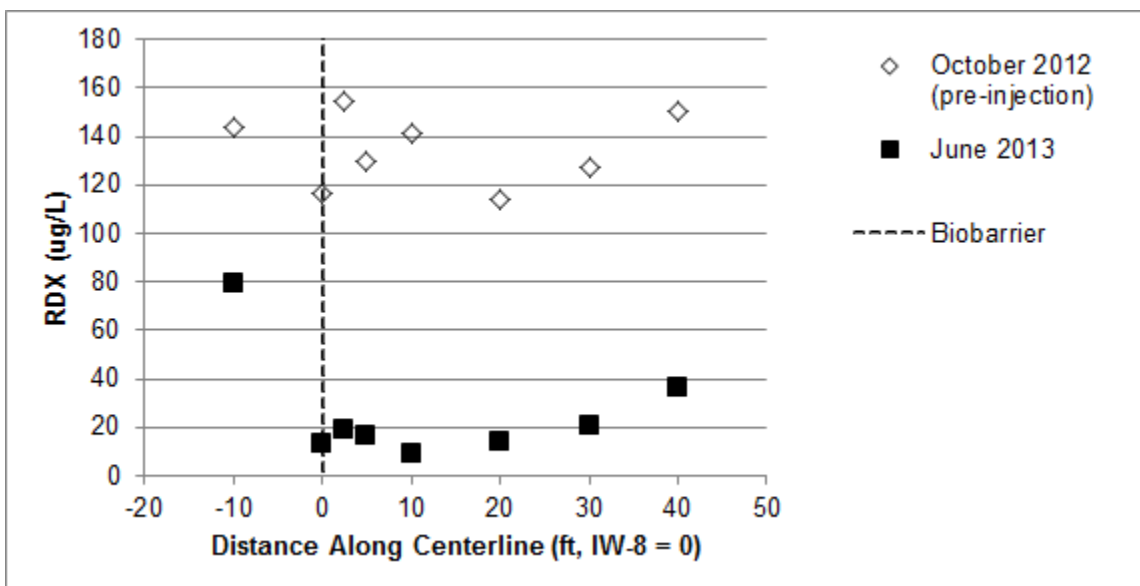


Table 7.1 $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ from RDX in biobarrier wells at Dahlgren NSWC.

Samples were collected prior to barrier installation (October, 2012), and approximately 130 days after installation (June, 2013).

Well ID	October, 2012 (pre injection)			June, 2013 (post injection)		
	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	$\mu\text{g/L}$	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	$\mu\text{g/L}$
CMOBOD02	-35.66	3.32	102.85	-34.89	2.19	120.39
MW-1	-35.36	2.80	130.70	-29.88	13.16	18.71
MW-2	-35.91	3.50	67.52	NA	NA	16.77
MW-3	-35.59	2.30	118.29	-28.01	18.32	9.01
MW-4	-35.52	2.50	108.07	-31.28	12.68	14.00
MW-5	-35.91	2.95	107.11	NA	NA	20.60
MW-6	-35.74	1.91	109.53	-34.58	3.26	36.60
MW-8	NA	NA	97.23	-35.04	2.34	60.07
MW-9	-34.94	1.40	64.34	-34.67	5.15	30.76
MW-10	-35.50	NA	104.08	-35.95	1.84	79.51
PZ-19	-35.08	2.80	57.37	NA	NA	20.98
IW-1	-36.22	2.68	125.18	NA	NA	16.19
IW-8	-35.50	2.63	105.62	NA	NA	13.44
PZ-30	NA	NA	72.57	-34.89	2.04	84.35

NA - data not available

Figure 7.3. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of RDX collected from sampled along the centerline of the biobarrier before (October 2012) and after (June 2013) oil injection.

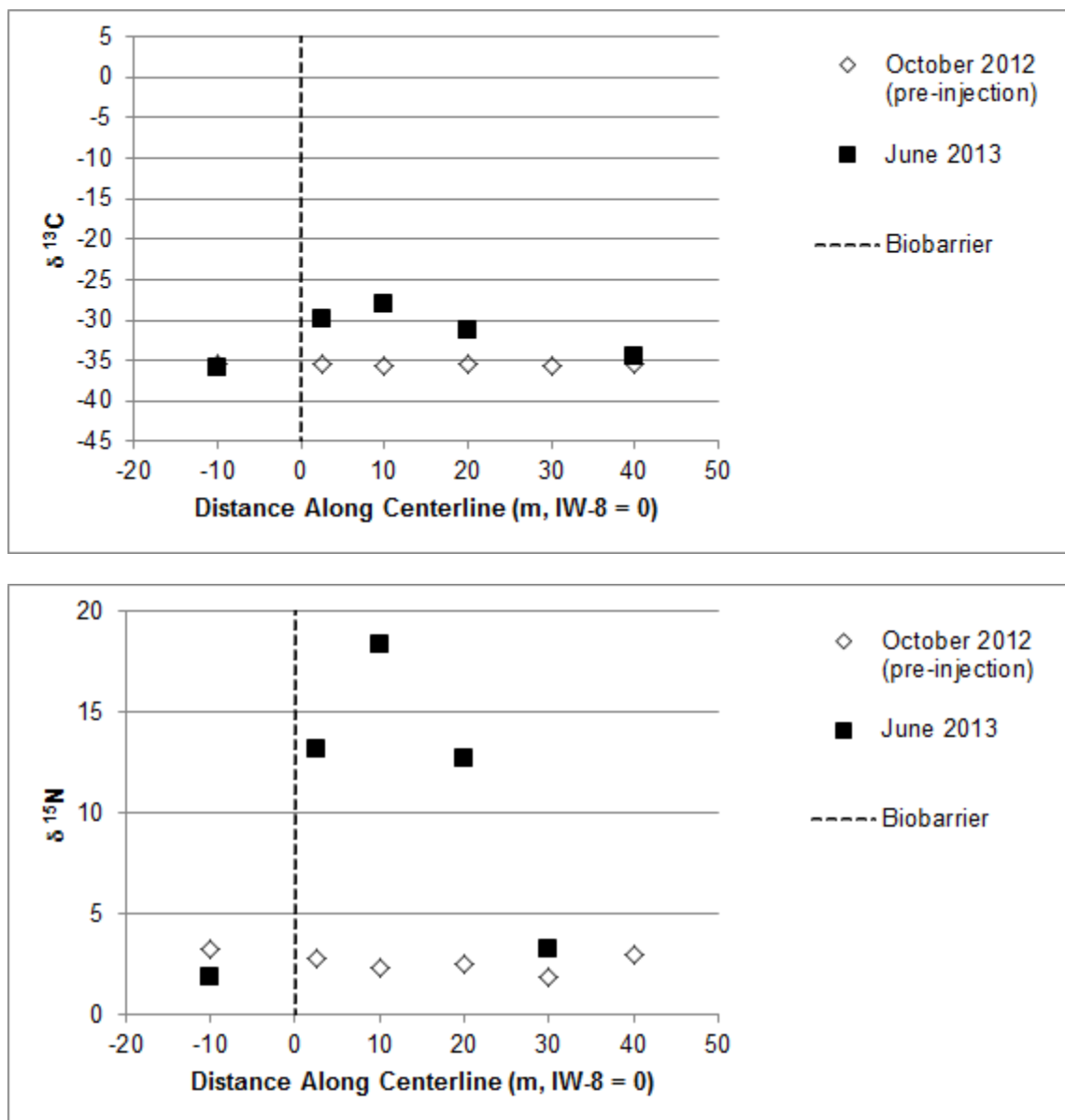


Figure 7.4 Plot of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ and versus RDX concentration expressed as $\ln(f)$ for samples collected along the centerline of the Dahlgren biobarrier in June 2013. Fractionation factors (ϵ) are shown based on the slopes of the fitted lines.

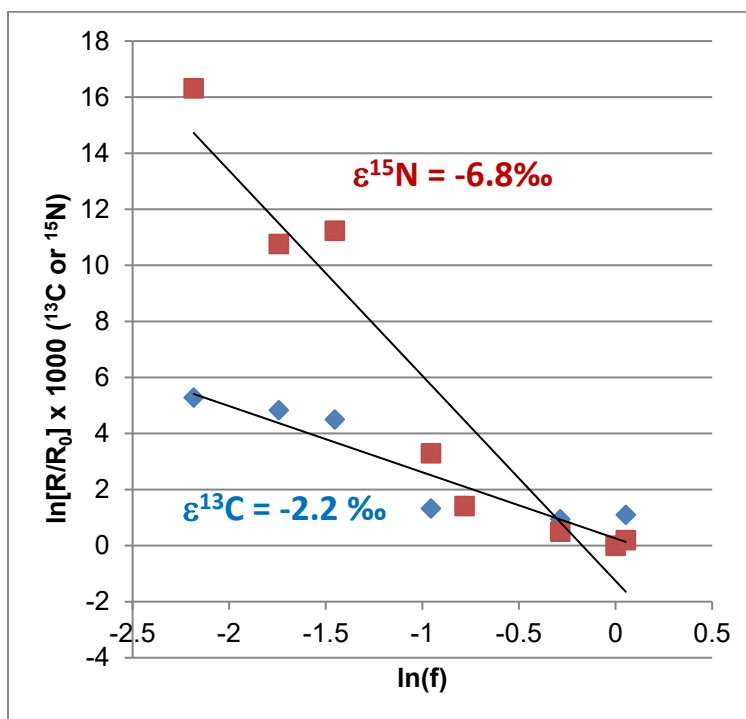


Table 7.2 Compiled ^{13}C and ^{15}N RDX fractionation factors from Dahlgren NSWC biobarrier sampling.

	$\epsilon^{13}\text{C}$			$\epsilon^{15}\text{N}$		
	‰	±	P-value	‰	±	P-value
Dahlgren NSWC Anaerobic Biobarrier^a	-2.2	1.1	4.7E-03	-6.8	3.1	3.7E-03
Anaerobic Pure Cultures						
<i>Shewanella oneidensis</i> MR-1	-6.0	2.6	2.0E-04	-9.6	1.3	5.6E-12
<i>Klebsiella pneumoniae</i> SCZ-1	-4.1	1.6	4.7E-05	-5.8	2.6	6.3E-04
<i>Desulfovibrio</i> sp.	-2.0	0.5	2.8E-06	-10.9	1.2	5.2E-10
<i>Clostridium acetobutylicum</i> ATCC 824	-2.8	1.6	2.7E-03	-8.0	5.5	1.9E-02
Composited Anaerobic Pathway^a	-4.7	1.1	1.8E-11	-9.9	0.7	1.8E-32
Dahlgren NSWC Anaerobic Enrichment Cultures						
Mn Reducing	4.0	0.0	9.1E-01	-4.0	0.0	4.0E-07
Fe Reducing	8.9	7.7	3.4E-02	-5.8	3.3	1.2E-02
Sulfate Reducing	-0.4	0.7	1.6E-01	-6.4	0.7	2.5E-06
Methanogenic	-1.2	4.4	5.0E-01	-4.2	2.7	1.2E-02

^a Values represent the slope of the linear regression of the field data $\pm$ the 95% confidence interval of the slope. P-values represent the probability that the observed slope is different from zero.

8.0 TECHNOLOGY IMPLEMENTATION AND LIMITATIONS

The primary end-users of this technology are expected to be DoD site managers and their contractors, consultants and engineers. The general concerns of these end users are likely to include: (1) technology availability and cost; (2) appropriate application of the technology at DoD sites; and (3) interpretation of CSIA data. The C and N stable isotope analyses described herein are not currently available in commercial laboratories to our knowledge. However, the analyses are currently being conducted at the University of Delaware, Environmental Isotope Geochemistry Laboratory (EIGL) under the supervision of Dr. Neil Sturchio on a per sample basis.

The CSIA technology described herein is applicable for documenting the biological degradation of RDX in groundwater by both aerobic and anaerobic mechanisms. However, when RDX is degraded aerobically via the typical denitration pathway, the extent of N fractionation is expected to be low ($\epsilon = \sim -2.4\text{ ‰}$) and C is not expected to fractionate measurably based on pure culture studies. Thus, for the method to be useful for field samples, losses of RDX in groundwater either over distance (e.g., along a groundwater flowpath) or time (e.g., in an individual well) must be substantial, on the order of 80% or higher from initial concentrations. In many instances, and given the observed variability in this measurement, it is unlikely that aerobic biodegradation of RDX in the field will be definitively proven by N isotope fractionation. It is recommended that additional lines of evidence of RDX biodegradation under aerobic conditions be assessed along with N isotope analysis of RDX, including (1) measurements of NDAB as a possible degradation intermediate; (2) molecular analysis of aquifer samples for the presence of *xplA/xplB* genes, which encode key enzymes involved in aerobic RDX biodegradation; (3) laboratory microcosms or columns incubated aerobically to document RDX biodegradation under controlled conditions; and (4) application of stable isotope probing (SIP) in laboratory microcosms or mesocosms to identify organisms that aerobically degrade RDX. The combination of one or more of these techniques in conjunction with N stable isotope analysis of RDX at a field site is recommended to clearly document aerobic RDX biodegradation or confirm the absence of this process.

When RDX is biodegraded via anaerobic mechanisms, C and N stable isotopes are both applicable to document this process, due to the relatively large fractionation factors measured in culture studies ($\epsilon = \sim -4.7\text{ ‰}$ for C and $\epsilon = \sim -9.9\text{ ‰}$ for N). Dual isotope plots can be used to confirm biodegradation, as was done for Dahlgren NSWC field samples downgradient of an emulsified oil biobarrier. Many of the general lines of evidence previously suggested for evaluating aerobic RDX biodegradation are also applicable for anaerobic biodegradation, including (1) evaluation of degradation intermediates, but in this case MNX, DNX and TNX rather than NDAB; (2) laboratory microcosms or columns incubated anaerobically; and (3) application of SIP in laboratory microcosms or mesocosms to identify anaerobic RDX degraders. As previously noted for evaluating aerobic biodegradation, a combination of one or more of these techniques in conjunction with C and N stable isotope analyses of RDX is recommended to document anaerobic RDX biodegradation.

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