EXECUTIVE SUMMARY

Validation of Stable Isotope Ratio Analysis to Document the Biodegradation and Natural Attenuation of RDX

ESTCP Project ER-201208

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1.0 INTRODUCTION

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a common soil contaminant at current and former military facilities, which impacts groundwater and drinking water at numerous locations. RDX contamination often occurs over expansive areas, making in situ or ex situ treatment technologies difficult to implement. One potential alternative for managing RDX sites is monitored natural attenuation (MNA), in which contaminants are controlled by natural processes, including biodegradation. However, one limitation of this approach for RDX is that biodegradation rates can be relatively slow under field conditions, making accurate rate measurements difficult. Compound-specific stable isotope analysis (CSIA) may overcome this limitation, because it allows measurements of slow degradation rates by measuring changes in the ratios of the stable isotopes of present in RDX, specifically the ratios of $^{15}\text{N}/^{14}\text{N}$ and $^{13}\text{C}/^{12}\text{C}$.

CSIA relies on the fact that bacteria biodegrade heavier isotopes (e.g., RDX with $^{15}\text{N}$ rather than $^{14}\text{N}$ in its structure) more slowly than lighter ones, due to the greater bond stability of the heavier molecules. As a result, the residual parent molecules become increasingly enriched in the heavier isotopes as biodegradation proceeds. Analyzing stable isotope ratios of RDX 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 therefore document degradation and natural attenuation in situ (as opposed to losses due to dilution, volatilization 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.
2.0 OBJECTIVES

The objective of this project was to validate a CSIA method to confirm and constrain rates of aerobic and anaerobic biodegradation of RDX at field sites. If successful, this method can be used by DoD to provide critical data to support MNA as a remedy for RDX in groundwater, and also to confirm the effectiveness of \textit{in situ} enhanced aerobic or anaerobic bioremediation. The stable isotopic composition of NO$_3^-$ and NO$_2^-$ were also measured when these anions co-occurred with RDX, to evaluate whether these potential degradation products from RDX could be used to further demonstrate MNA in the field.
3.0 TECHNOLOGY DESCRIPTION

A CSIA method was developed that utilizes gas-chromatography coupled to isotope-ratio mass-spectrometry (GC-IRMS) to quantify C and N isotope ratios in RDX. In summary, RDX collected from groundwater is 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 $\delta^{15}N$ and $\delta^{13}C$ in RDX using GC-IRMS. To evaluate the use of CSIA to document anaerobic biodegradation of RDX, $\delta^{15}N$ and $\delta^{13}C$ in RDX were measured in a series of wells along a groundwater flow path at Dahlgren Naval Surface Warfare Center, MD (Dahlgren NSWC) before and after injection of emulsified oil into a biobarrier to promote RDX biodegradation. To evaluate aerobic biodegradation of RDX via CSIA, $\delta^{15}N$ in RDX was measured in groundwater samples collected both (1) during a series of push-pull tests in which a culture capable of aerobically degrading RDX was bioaugmented into an aquifer at Umatilla Chemical Depot, OR (UCMD) along with a low dose of carbon substrate, and (2) from the bulk aerobic aquifer at Dahlgren NSWC along the flowpath of two RDX plumes. $\delta^{13}C$ values were not measured for RDX during these field tests because previous studies with pure cultures indicated no measurable fractionation of C during aerobic RDX biodegradation.
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4.0 PERFORMANCE ASSESSMENT

At Dahlgren NSWC, the calculated $\varepsilon^{13}$C and $\varepsilon^{15}$N values downgradient of the biobarrier were -2.2‰ and -6.8‰, respectively. These results compare favorably to pure culture data for strains degrading RDX under anaerobic conditions (which averaged -4.7‰ and -9.9‰ for C and N respectively, and included individual strains whose $\varepsilon$ values were as low as -2.0‰ and -5.8‰, respectively). The marginal depression in the $\varepsilon^{13}$C and $\varepsilon^{15}$N values in the field compared to laboratory studies has been observed during the degradation of other compounds and is commonly attributed to abiotic effects (e.g., dispersion, dilution, incomplete mixing). The isotope fractionation factor ratio ($\varepsilon^{15}$N / $\varepsilon^{13}$C) for the samples was ~ 3.0, which falls well within the range determined from anaerobic cultures (1.5 to 5.5), and most closely matches that determined for a *Clostridium* sp. at ~ 2.9. The range most likely reflects the differing or mixed anaerobic pathways of RDX degradation. To our knowledge this project represents the first field study clearly showing dual-element CSIA can be used to document RDX biodegradation under anaerobic conditions.

At UMCD, under bulk aerobic conditions, the fractionation of $^{15}$N during one of the push-pull tests provided a clear indication of aerobic RDX biodegradation, with an $\varepsilon^{15}$N value of -1.5 to -1.6‰, irrespective of the modeled mixing between the added and background groundwater. Results from the additional tests at the site, however, were less conclusive and varied with the interpretation of the mixing scenario. The relatively small $\varepsilon^{15}$N value for aerobic RDX biodegradation (averaging -2.4‰ for four different pure cultures) made documenting this process more difficult than for anaerobic biodegradation, where $\varepsilon^{15}$N is much larger and $\varepsilon^{13}$C can also be evaluated. While documenting aerobic RDX biodegradation in the field is certainly possible at this $\varepsilon$ value, the precision of N isotope measurements in RDX as well as the effects of field heterogeneity will make it more difficult.

$\delta^{15}$N values were also measured for RDX from 11 different wells at Dahlgren NSWC. The field-collected $\delta^{15}$N data along assumed transects through two RDX plumes did not provide an indication of natural attenuation via aerobic RDX biodegradation. While laboratory tests suggested the potential for natural biodegradation of RDX at this site, field data revealed no detectable NDAB (an aerobic degradation product of RDX) or MNX, DNX, or TNX (common anaerobic degradation products) in groundwater from the site. Taken together ($\delta^{15}$N in RDX and absence of intermediates), the data indicate a lack of measurable biological attenuation of RDX plume at this location.

$\delta^{15}$N and $\delta^{18}$O in nitrate (NO$_3^-$) were also measured at the Dahlgren NSWC site as a potential indication of RDX biodegradation. With the exception of one sample, the combined $\delta^{15}$N and $\delta^{18}$O data fell within the range of biogenic NO$_3^-$ on a dual isotope plot, typically a result of NO$_3^-$ produced via nitrification in soils. The $\delta^{18}$O values for most of the Dahlgren NSWC samples were in the general range of those of nitrite (NO$_2^-$) produced during aerobic degradation of RDX, but the $\delta^{15}$N values were appreciably higher. The one NO$_3^-$ sample that did not fall within this range had a very high value of $\delta^{15}$N (> +30‰) and a somewhat elevated value of $\delta^{18}$O (nearly +20‰), which could be consistent with N isotope fractionation during biological degradation of biogenic NO$_3^-$. Thus, it is likely that most of the NO$_3^-$ that was analyzed from Dahlgren NSWC did not originate from RDX, a conclusion that is consistent with the absence of evidence for MNA of RDX at this site (no measurable metabolites or $^{15}$N enrichment, as previously described).
This study demonstrated that $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ in NO$_2^-$ and/or NO$_3^-$ can be a useful marker of aerobic or anaerobic RDX biodegradation, provided that the amounts of these anions generated from RDX are not overwhelmed by those generated from other sources. Further, the combination of RDX and NO$_2^-$/NO$_3^-$ stable isotope analyses can be used to confirm natural degradation processes, particularly under anaerobic conditions.
5.0 COST ASSESSMENT

Stable isotope analysis of N and O in nitrate is currently estimated at $149 per sample (for 6 to 20 samples) from the USGS Reston, VA Stable Isotope Laboratory and C and N isotope analysis in RDX at $500 per sample by the University of Delaware EIGL Laboratory of Dr. Neil Sturchio, which is currently the only laboratory performing this method on a per sample basis. The estimated total cost for sampling and analysis of 20 wells to support a natural attenuation evaluation of RDX was just over $21,000.
6.0 IMPLEMENTATION ISSUES

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. Analyses of N and O stable isotopes in NO$_3^-$ are available on a routine per sample basis from various laboratories including the USGS in Reston, VA. Isotopic analyses of NO$_2^-$ may be available by special arrangement at some laboratories.

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 denitrification pathway, the extent of N fractionation is expected to be low ($\varepsilon = \sim -2.4$ ‰) 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) analysis of N and O stable isotopes in NO$_2^-$ and/or NO$_3^-$ that co-occur with RDX (particularly if initial RDX concentrations are in the mg/L range or higher); (4) laboratory microcosms or columns incubated aerobically to document RDX biodegradation under controlled conditions; and (5) 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 ($\varepsilon = \sim -4.7$ ‰ for C and $\varepsilon = \sim -9.9$ ‰ 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) analysis of N and O stable isotopes in NO$_2^-$ and/or NO$_3^-$ that co-occur with RDX; (3) laboratory microcosms or columns incubated anaerobically; and (4) 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.