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**Cover Page Footnote**

The use of trade, product, or firm names in this report is for descriptive purposes only and does not imply endorsement by the U.S. Government. The tests described and the resulting data presented herein, unless otherwise noted, were obtained from research conducted under the Environmental Technology (EQT) Program of the United States Army Corps of Engineers by the USAERDC. The findings of this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

# BIO-GEOCHEMICAL FACTORS THAT AFFECT RDX DEGRADATION

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## ABSTRACT

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a secondary high explosive that has been identified as a contaminant of concern in groundwater and soil as a result of military training activities (Wani et al., 2002). Remediation technologies that have been proposed for use on these training ranges include *in situ* techniques. An obstacle to using *in situ* approaches for the treatment of RDX contaminated soils and groundwater is the lack of information concerning the biogeochemical factors that influence transformation. This research compares paired (biotic and poised abiotic systems) RDX degradation experiments in which Eh-pH conditions conducive to RDX degradation were established.

Degradation of RDX under iron-reducing conditions was studied in biological and chemical systems. The redox conditions created by the biological systems were simulated by poised chemical systems in order to compare RDX transformation. The poised chemical systems used an iron-ligand complex to achieve the necessary Eh values for RDX degradation. RDX degraded in both biological and chemical systems and final reaction solutions from both systems were analyzed to determine which degradation pathway was followed. The results from this effort will expand the basic knowledge of energetic transformation over a range of biologically-induced conditions, by isolation of enzymatic pathways from abiotic redox mechanisms.

Keywords: energetics, RDX degradation, redox potentials, terminal electron accepting processes, bio-geochemical factors.

## 1. INTRODUCTION

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a secondary high explosive that is widely used by the military in shells, bombs, and demolition charges (Gorontzy et al., 1994). The molecular structure of RDX is shown in Figure 1. RDX has been identified as a contaminant of concern in groundwater and soil as a result of training activities on hand grenade and open burn open detonation (OBOD) ranges (Wani et al., 2002). The Department of Defense has identified more than 1,200 sites contaminated with RDX that

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require cleanup (Schmelling et al., 1997). The toxicity of RDX necessitates its removal from the environment (USEPA, 2006; ATSDR, 1995; USDHHS, 1995). The treatment of these explosive contaminated sites is therefore a major environmental concern for the Army, particularly those sites used for training purposes. Remediation technologies that have been proposed for use on these training ranges include *in situ* technologies, which remediate the contaminant in place, without the need to remove soil or groundwater from the field site (Wani et al. 2002, Boparai et al. 2008, Beller 2002, Waisner et al. 2000). A fundamental obstacle to the use of an *in situ* approach for the treatment of RDX-contaminated soils and groundwater is the lack of information concerning the biogeochemical factors that influence RDX transformation. This research compares paired biotic and abiotic experiments in which Eh-pH conditions conducive to RDX degradation were established to help provide some basic knowledge concerning the transformation of energetic materials under environmental conditions.

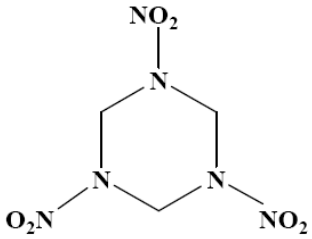


*Figure 1*. Molecular structure of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX).

RDX is relatively resistant to degradation under aerobic conditions; however, it is readily degraded under anaerobic conditions (Hawari, 2000). Ubiquitous soil bacteria from the genera *Desulfovibrio* (Arnett and Adrian, 2009), *Pseudomonas* (unpublished data) and *Geobacter* (Kwon and Finneran, 2006) possess the unique ability to degrade RDX utilizing one of three proposed pathways under anaerobic conditions (Bhushan et al. 2002). One pathway involves direct ring-cleavage, a second involves the sequential reduction of the nitro functional groups, and the third involves the hydrolytic substitution of the nitro functional groups and subsequent ring destabilization and cleavage. The dynamics which influence degradation pathways that follow environmentally relevant terminal electron accepting processes (TEAP) are not well understood and the role redox potentials play in the process are unknown. RDX degradation could be enhanced and/or stimulated if the bio-geochemical factors that regulate this process could be determined and controlled.

The anaerobic biodegradation of RDX has been well studied (Hawari, 2000; Kitts et al., 1994; McCormick et al., 1981), in contrast much less is known about the abiotic processes. Recent reports have shown that RDX can be degraded chemically under anaerobic conditions using iron, as either zero valent iron (ZVI) ( Parks et al., 2004; Oh et al., 2001; Singh et al. 1998) or ferrous ions (Fe II) (Seok-Young et al., 2005; Gregory et al., 2004). These studies clearly demonstrate the potential for abiotic degradation of RDX, however the role microorganisms play in these processes are uncertain. Suggested

pathways include extracellular electron transfer reactions (Kwon and Finneran 2006, 2008).

The final reaction products following both biotic and abiotic RDX degradation are similar, inferring that the reaction pathways may be similar. Nitroso derivatives have been noted after biological degradation (Wani and Davis, 2003; Freedman and Sutherland, 1998; Bhushan et al. 2002) and after abiotic transformation (Gregory et al., 2004; Oh et al., 2001). Methylenedinitramine (MDNA) has also been produced following RDX degradation using both biotic and abiotic systems (Halasz et al., 2002).

Current research on other contaminants has shown that redox potentials play a key role in degradation processes. Shrout and Parkin (2006) studied perchlorate biodegradation at fixed redox potentials. While the greatest rate of biodegradation was noted at -200 mV, significant degradation was achieved at redox potentials as high as +180 mV. We believe similar geochemical processes may influence RDX degradation and have a role in microbial processes.

## 2. MATERIALS AND METHODS

### 2.1 Biological Experiments

Isolates of *Pseudomonas aeruginosa*, and *Geobacter metallireducens* GS-15 were obtained from American Type Culture Collection (ATTC, Rockville, MD) and *Desulfovibrio desulfuricans* G20 was graciously donated by Dr. Judy Wall, University of Missouri at Columbia. The three species were combined to create a mixed culture, which represented members capable of nitrate, iron, and sulfate-reduction respectively. The media prior to inoculation had an Eh of approximately 300 mV (no reducing agent added). A mixed culture capable of growth under broad redox conditions was chosen to allow the system to achieve a static redox state biologically. Studies were carried out under facultative conditions in 160 mL serum bottles containing 100 mL Robert S. Tanner minimal media (Tanner, 1989) amended with 10 mM ferric citrate as the primary electron acceptor and 35 µM RDX. Yeast extract (0.1% w/v) was added to serve as a source of carbon and energy in each system. Headspaces were pressurized to 10 psi with nitrogen to facilitate a moderately anaerobic system and redox potentials were allowed to stabilize by biological means. Experimental conditions tested were: heat killed sterile controls (120°C for 20 min), no cells (uninoculated control), viable cultures without RDX amendment, and active (RDX-amended) cultures. All experiments were performed in triplicate and incubated in the dark, shaking, at 30ºC.

Liquid and gas samples were taken periodically and analyzed for RDX, nitroso-RDX intermediates, ferrous iron, and nitrous oxide. Explosives and nitrous oxide analysis were performed as previously described by Adrian and Arnett (2004). Ferrous iron was quantified by the Ferrozine assay as previously described (Lovley and Phillips, 1987). Additionally, redox potentials and pH were measured in a Plas Labs Anaerobic Chamber ($N_2$:$H_2$, 95:5) using a portable Horiba D-53 pH/ORP meter (Horiba, Ltd., Irvine, CA)

equipped with a platinum electrode and a silver/silver chloride reference electrode for measuring ORP.

## 2.2 Abiotic Experiments

Chemical redox systems were established using solutions of ferrous sulfate, ferric sulfate, and a substituted catechol, Tiron, in varying proportions, in a 0.1 M Tris buffer. Trion was used as a model compound for humic acids. All systems used deionized water that was purged with nitrogen prior to use and all solutions were prepared in an anaerobic nitrogen-filled glove box. The structure of Tiron (1,2-dihydroxyl-benzene- 3,5-disulfonic acid) is shown in Figure 2. Solutions of various Eh values (-610 to -410), obtained by varying proportions of ferric and ferrous ions, were tested in order to determine the optimal range for RDX degradation in the abiotic system. Redox potential (Eh) was measured using a platinum electrode and a silver:silver chloride reference. The accuracy of the platinum probe was verified using Light's solution, which yields a value of 476 mV; the Light's Solution check standard readings were within 10 mV of this value, which is deemed acceptable (Light, 1972).

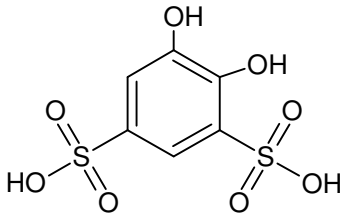


*Figure 2.* Molecular structure of 1,2-dihydroxyl-benzene-3,5-disulfonic acid (Trion).

The solution pH was adjusted to between 7.5 and 8 using 5% sodium hydroxide or 10% hydrochloric acid added drop-wise as needed. The iron-organic ligand complex that was produced in this system was previously reported by Kim and Strathmann (2007). This pH range mimicked the conditions achieved in the biotic systems. The resulting reagent was amended with RDX for a final solution concentration of 2 mg/L and allowed to react for 24 hours. Eh and pH values for these abiotic systems were also monitored during the experiments.

Explosives analyses of the samples from the abiotic systems were performed using the same equipment used for the biological samples. This allowed direct comparison of the results from both systems for RDX, intermediate, and final products.

## 2.3 Methods Used for Organic Analysis

RDX and any potential degradation products for both biotic and abiotic experiments were analyzed following modifications of EPA methods 8330B and 8321 using high pressure liquid chromatography (HPLC). The instrumentation used was an Agilent 1200 HPLC equipped with a photodiode array detector and interfaced to a Bruker 6000

Electrospray ionization ion trap mass spectrometer. Briefly, the HPLC achieved separation of analytes of interest using a 50:45:5 (aqueous 20 mg/L NaCl: methanol: acetonitrile) mobile phase pumped at 1.0 mL/min through an Agilent Eclipse XDB-C18 column. UV absorbance traces were collected at 254 nm and the mass spectrometer monitored masses in the range 50-750 m/z. Methanol was detected by GC-FID following a modified EPA method 8015D. Other analytes were determined using a Varian 3800 GC equipped with a Supelco 80/20 Porapak Q column and an ECD. Analytical conditions included a flow rate of 50 mL/min, a run time of 6 m, and injection volume of 1 mL.

## 3. RESULTS AND DISCUSSION

### 3.1 Biological Experiments

Initial redox readings were taken immediately after inoculation with each culture. Cultures containing viable cells (RDX-unamended and active treatments) showed a nearly instantaneous decreased redox potentials compared to the heat killed and uninoculated controls (Figure 3). Over the course of the study the RDX-unamended and active treatments averaged approximately -63 mV. Redox potentials remained near 0 mV through out the experiment for the heat killed and uninoculated controls and the pH remained neutral under all incubation conditions throughout the study. The variations in redox potentials between viable cultures and controls preclude assessing degradation exclusively to biotic process. However, these data do demonstrate the mixed culture readily decreased the redox potential well within the range of iron reduction, suggesting the *Geobacter* was likely responsible for RDX degradation.

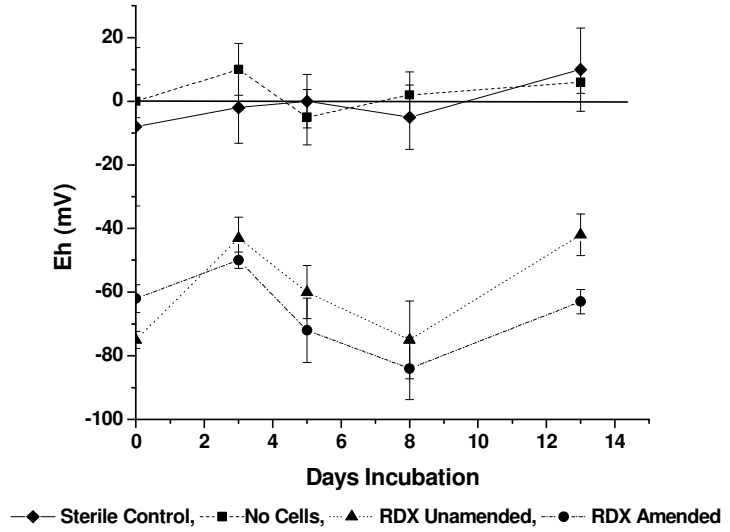


*Figure 3*. Redox potentials determined during biological experiment under iron-reducing conditions

RDX degradation in the active treatment was complete in 3 days (Figure 4) with no RDX transformation evident in the control cultures. Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-nitro-1,3,5-triazine (DNX), or hexahydro-

1,3,5-trinitroso-1,3,5-triazine (TNX) were not detected; however, sequential reduction could not be ruled out due to sampling frequency. Nitrous oxide (1.32 µmoles) was detected in the active treatment (data not shown). This was approximately ten times the amount produced in the control bottles. The production of nitrous oxide suggests RDX mineralization, which will be confirmed by radiological studies in the future. Near stoichiometric amounts of ferrous ions were only produced in the RDX-unamended and active treatments, indicating iron-reducing conditions were created by viable cells (Figure 5). Interestingly, concentrations of ferrous iron differ significantly between the RDX amended and unamended controls for the first five days of incubation. The lower concentrations in the RDX amended cultures suggested possible electron shuttling on to RDX (Kwon and Finneran, 2006). Due to the lack of observed MNX and reduced ferrous iron production, it was likely RDX was degraded initially by denitration followed by ring-cleavage (Crocker et al., 2006).

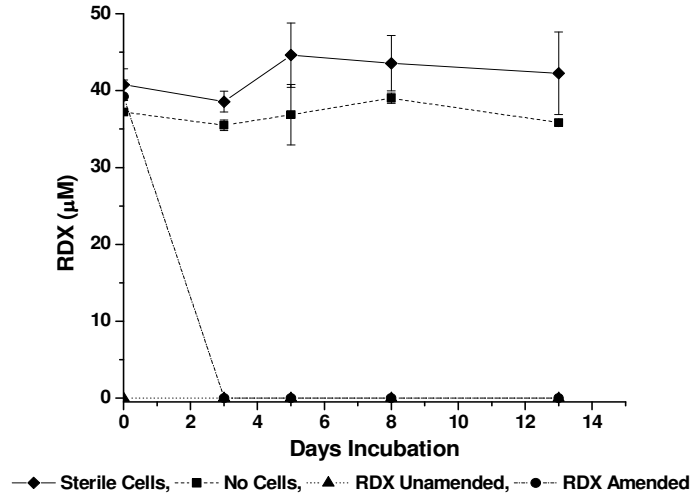


*Figure 4.* RDX concentrations determined during biological experiments under iron-reducing conditions. RDX was added to a target concentration of 40 □M.

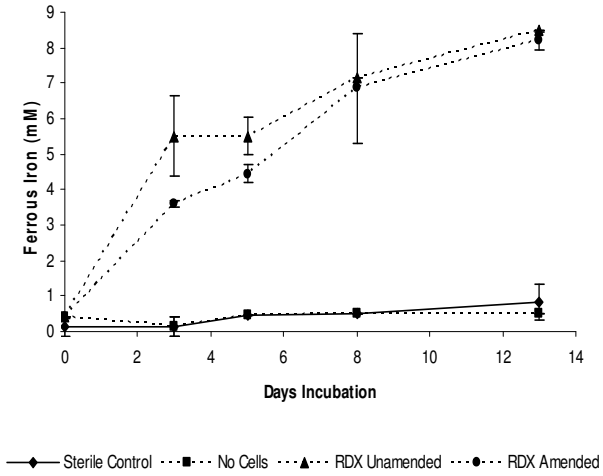


*Figure 5.* Ferrous iron production during biological experiments. Ferric iron was added to a final concentration of 10 mM.

## 3.2 Abiotic Experiments

Figure 6 illustrates RDX degradation that occurred within 24 h reaction time over a range of Eh values from -410 to -610 and pH values from 5.1 to 8.1. The squares indicate degradation and dots indicate that no degradation occurred in those samples within 24 hours. RDX degradation occurred below an average Eh value of about -500 mV in the abiotic systems. RDX was observed to degrade over time in the abiotic system, with no degradation products identified, suggesting complete degradation of the ring system to very small organic moieties that cannot be detected by ESI-IT-MS.

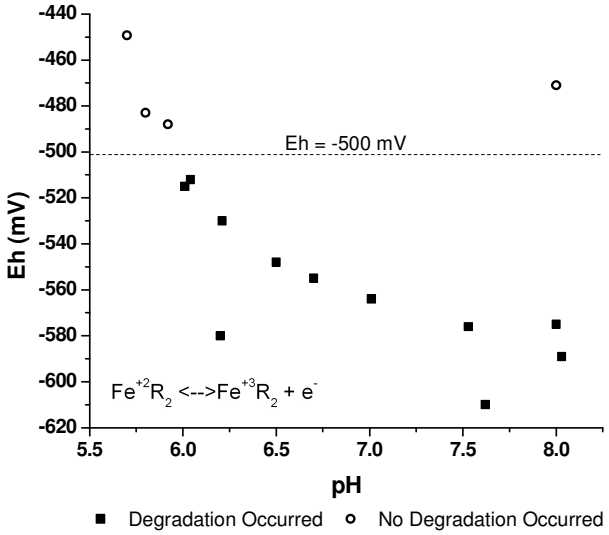


*Figure 6.* Influence of Eh and pH on RDX degradation.

## 3.3 Comparison of biotic and abiotic systems

It appears that RDX degradation proceeds by electron shuttling and subsequent ring-cleavage under biotic conditions and direct ring-cleavage in abiotic systems. Kim and Strathmann (2007) reported that RDX degradation is believed to involve direct ring cleavage using 2 electrons in the Fe-tiron system. RDX degradation occurred in the biotic system by direct ring cleavage mediated by enzymatic processes, since no sequential reduction products were detected. There was a significant difference in the Eh values of the two systems. Abiotic degradation occurred at approximately ≤-500 mV compared to -63 mV in the biotic system. The much higher Eh values for the biotic system suggest a biologically driven pathway was involved rather than purely chemical reduction. It is possible that microenvironments created by the microbes created suitable conditions (lower Eh values) for RDX degradation that are different from the bulk media being measured. Nitrous oxide concentrations were not determined for the abiotic systems so no direct comparison is possible at this time.

### 3.4 Other Chemical and Biological Systems

Nitrogen and sulfur systems are also being investigated using nitrate and sulfate reducing bacteria and mimicking these systems abiotically with a variety of nitrogen and sulfur species to achieve the required redox potential for RDX abiotic degradation. Additionally, hydroquinone systems will be investigated, as similar ligand moieties were needed in the abiotic iron system described above to achieve RDX degradation.

## 4. CONCLUSION

Biotic and abiotic degradation of RDX was studied under anaerobic conditions using iron-reducing bacteria and a ferrous iron-organic ligand system. Both systems were able to degrade RDX, with the abiotic system achieving complete degradation within 24 hours. The biotic system had complete degradation within 72 hours. Additionally, the biotic systems did not require as low a reducing environment as did the abiotic system, suggesting that a biological pathway was involved rather than purely chemical reduction. However, it is possible that microenvironments created by the microbes created suitable conditions for RDX degradation that are different from the bulk media being measured. Future studies will investigate this possibility.

The results from this effort will expand the basic knowledge of energetic transformation and could ultimately be used to select optimal remedial treatments that promote transformation reactions of energetic materials under environmental conditions in surface and sub-surface environments.

## ACKNOWLEDGEMENTS

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