RDX Loss in a Surface Soil under Saturated and Well Drained Conditions

D. B. Ringelberg,* C. M. Reynolds, M. E. Walsh, and T. F. Jenkins

ABSTRACT

RDX attenuation is typically favored under reduced conditions where the formation of nitroso intermediates precedes ring cleavage and the formation of hydrazines (McCormick et al., 1981). However, on military training ranges, low-order, incomplete detonations deposit RDX into surface soils, where well-drained and unsaturated (oxidative) conditions often predominate. Hawari et al. (2000) have suggested that aerobic enzymatic attacks on the H–NO2 or C–H bonds in RDX may lead to the formation of unstable intermediates that then undergo spontaneous decomposition. As a result, aerobic biodegradation of RDX could serve as a basis for a viable bioremediation technology. However, in surface soils, soil water potential, especially matric potential, can have a significant effect on plant and microbial growth and the subsequent production of exogenous enzymes. The effect of different soil water potentials on soil microbial activity and subsequent biotransformation or biodegradation of RDX has not been well characterized.

Augmenting soils with contaminants to determine the fate and biodegradability of a pollutant is a common practice. Unfortunately, the influence of an organic solvent on the extant microbiota and how this in turn can affect contaminant fate is rarely taken into consideration. Brinch et al. (2002) recently showed that the use of dichloromethane, often used to spike soils with PAHs, had a marked effect on indigenous soil microorganisms. Dichloromethane applied to the entire soil sample had an initial inhibitory effect on all microorganisms, which was then followed by an increase in Pseudomonas sp. to a cell density approximately 1000× greater than that in the control soil. Such an artificial enrichment could have an overall effect on contaminant biodegradation, albeit an unrealistic effect in terms of natural soil attenuation.

In this study, we examined RDX attenuation in a military training range soil incubated with equal concentrations of RDX, but at three different acetonitrile concentrations and two different soil water potentials. Our objective was to compare the effects of soil water potential and acetonitrile concentrations on RDX attenuation. In a companion study, we examined the response of the extant microbiota to the differences in soil treatment and how community structure and function were related to RDX biodegradation.

MATERIALS AND METHODS

The surface soil used in this study was obtained from Ft. Greely, AK, which is located approximately 160 km (100 miles) southeast of Fairbanks, AK, and 560 km (350 miles) northeast of Anchorage, AK. The physical-chemical parameters of the soil in this study were: 58% sand, 35% silt, 7% clay (sandy loam), pH 6.7, 10.4% organic matter (on a dry

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RDX was added at a concentration of 0, 0.1, and 1% (v/v). Saturated microcosms also received 100 μL of a 0.1% resazurin solution as a redox indicator. Control microcosms were autoclaved at 120°C for 20 min three consecutive times to attenuate the native microorganisms with an 8-h resting or growth period between autoclaving.

After RDX was added, microcosms were incubated for a total of 840 h in the dark without agitation at room temperature (21 ± 2°C). Three replicate vials were sacrificed at 0, 48, 168, 504, and 840 h to obtain contaminant concentrations. Contaminant concentrations were determined for both the aqueous and solid phases of the saturated microcosms. Following centrifugation at 2000 rpm for 20 min, the aqueous phase was decanted and 1 mL was diluted in 1 mL of ACN and 3 mL of Milli-Q H2O. The solution was then filtered through a Millipore Millex-FH hydrophobic PTFE 0.45-μm syringe filter (Bedford, MA) and analyzed by HPLC. The solid phase of the saturated and unsaturated microcosms were extracted with 10 mL of ACN for 18 h at 20°C with sonication; 2 mL of the supernatant were then filtered and diluted with 8 mL of Milli-Q H2O before HPLC analysis.

Concentrations of RDX, MNX (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine), DNx (hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine), and TNX (hexahydro-1,3,5-trinitroso-1,3,5-triazine) were determined by HPLC. For the primary separation, a 15-cm by 3.9-mm (4-μm) Nova Pak C8 (Waters Millipore) column was eluted with 1.4 mL min⁻¹ 15:85 isopropanol–water. For the confirmation separation, a 25-cm by 4.6-mm (5-μm) cyanopropyl column was eluted with 1.2 mL min⁻¹ 12:23:62 methanol–acetone–water. The injection volume for each separation was 100 μL. Following these HPLC separations, we recorded absorbance at 254 nm on a Spectra Physics Spectra 100 variable wavelength UV detector.

Rates of RDX loss were calculated as described in Miyares and Jenkins (2000). The observed concentrations for RDX were plotted as \( \ln(C/C_0) \) vs. time \( t \), where \( C \) is the RDX concentration at time \( t \) and \( C_0 \) is the RDX concentration at time 0. Lines were then fitted to the data to determine that a first-order rate process was apparent. Using the following rate equation, \( \ln(C/C_0) = kt \) (where \( k \) is the rate constant equal to the slope of the line), we calculated the half-life for RDX under the varying scenarios by dividing the natural log of 0.5 (−0.693) by the rate constant \( k \).

Total microbial biomass was estimated from the concentration of ester-linked polar lipid fatty acids (PLFAs) recovered from the soils. The PLFAs were quantitatively recovered as described in White and Ringelberg (1998) and PLFA concentrations were converted to cell numbers as described in Balkwill et al. (1988).

Statistical evaluations were made using the Statistica software package, version 6.0 (StatSoft, Inc. Tulsa, OK). Significant differences between treatments were determined by an analysis of variance using the Scheffe test in a post hoc analysis at an α level of 0.05.

### RESULTS AND DISCUSSION

The unamended soil, as collected, did not contain RDX but did show trace amounts of explosive residues, including 1.0 mg kg⁻¹ nitroglycerine (NG), 0.01 mg kg⁻¹ 2-amino-4-nitrotoluene (2am-4NT), and 0.01 mg kg⁻¹ 2,4-dinitrotoluene (2,4-DNT). The nature of the training activities and RDX being detected in adjacent soils suggests that past exposure to RDX was likely. The soil supported a substantial viable microbial population under all of the conditions of this study. Initial cell biomass was measured at 6 × 10¹¹ cells kg⁻¹ following 72 h of pre-incubation under the saturated condition and 4 × 10¹¹ cells kg⁻¹ under the unsaturated condition. The presence of acetonitrile (0.1 or 1%) had no significant effect on the microbial biomass at the 48-h sampling point (Table 1).

The saturated microcosms produced the greatest extents and rates (first order assumption) of RDX loss (Fig. 1). Loss of RDX from the saturated microcosms was approximately 7.5 times greater than that observed under the unsaturated condition (Fig. 2). The mean first order rate coefficient for RDX loss in the active microcosms was 0.0073 ± 0.0004 (Table 2). Despite the fact that RDX was not detected in the aqueous phase following 504 h of incubation, \( r^2 \) values for the three equations were highly significant (Table 2).

Following centrifugation, the saturated microcosms showed residual RDX in the remaining soil pellet. This RDX accounted for 32% of the added RDX at the 48 h time point and 1% or less of the added RDX at all subsequent time points (data not shown). Because RDX

### Table 1. Microbial biomass estimates in Fort Greely soils under saturated and unsaturated soil moisture potentials.

<table>
<thead>
<tr>
<th>Moisture potential</th>
<th>CH,CN</th>
<th>48 h</th>
<th>168 h</th>
<th>504 h</th>
<th>840 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPa (bar)</td>
<td>%</td>
<td>cells kg⁻¹ (CV)†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.015 (0.15)</td>
<td>0</td>
<td>5.0 × 10¹⁰ (10)</td>
<td>3.4 × 10¹¹ (17)</td>
<td>4.4 × 10¹¹ (39)</td>
<td>9.7 × 10¹⁰ (20)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>4.0 × 10¹⁰ (11)</td>
<td>4.1 × 10¹¹ (19)</td>
<td>2.3 × 10¹⁰ (15)</td>
<td>9.0 × 10¹⁰ (6)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>4.2 × 10¹⁰ (12)</td>
<td>5.0 × 10¹⁰ (19)</td>
<td>2.9 × 10¹⁰ (46)</td>
<td>2.5 × 10¹⁰ (46)</td>
</tr>
<tr>
<td>Saturated</td>
<td>0</td>
<td>6.2 × 10¹⁰ (33)</td>
<td>3.9 × 10¹⁰ (31)</td>
<td>1.1 × 10¹⁰ (36)</td>
<td>3.3 × 10¹⁰ (3)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>8.6 × 10¹⁰ (55)</td>
<td>5.1 × 10¹⁰ (12)</td>
<td>1.0 × 10¹⁰ (72)</td>
<td>3.2 × 10¹⁰ (9)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>6.1 × 10¹⁰ (21)</td>
<td>3.3 × 10¹⁰ (32)</td>
<td>1.9 × 10¹⁰ (32)</td>
<td>3.0 × 10¹⁰ (17)</td>
</tr>
</tbody>
</table>

† Coefficient of variation.
has a low binding affinity for soil and that approximately 24% of the starting aqueous volume remained in the soil following centrifugation, it is likely that the RDX detected was associated with the interstitial water and not bound or sorbed to the soils. It is also assumed that the RDX added to the unsaturated soils was recovered as solution RDX as well.

In the saturated treatments, there was no significant difference in RDX loss as a result of ACN concentration. Although the first-order rate coefficient for the 1% exposure was slightly lower than that of the 0 and 0.1% treatments, the significance of the decrease was minor (Table 2). RDX loss (as well as mean RDX concentrations) between the active and control microcosms were significantly different (Fig. 2). First-order rate coefficients for the control microcosms averaged 0.001 ± 0.0002 across the two acetonitrile treatments, six times less than that observed in the three active microcosms (Table 2). The differences in rates and extents of RDX loss between the active and control microcosms indicated that RDX degradation was, at least partially, attributable to biotic processes. Conversely, the observed loss of RDX in the control microcosms could not be entirely attributed to abiotic processes, as viable bacteria were observed in the control microcosms at study termination (data not shown). It is acknowledged that autoclaving does not completely sterilize a soil, but does attenuate the bacterial signal. In this study, the attenuation was significant. It is also acknowledged that abiotic degradation of RDX by thermolysis, photolysis, or reduction by zero valent metals can occur. In each of these abiotic processes as well as in certain biotic degradation pathways, an initial attack of the cyclic nitramine results in ring cleavage and the formation of the intermediate methylenenitramine that is then further degraded to N₂O and HCHO. Based on a comparison to the autoclaved controls, biotic process were typically two to three times greater than abiotic processes at the 168, 504, and 840 h sampling periods. In addition, samples were incubated in the dark, at room temperature with no supplemental metals.

Singh et al. (1998) have shown that Fe⁰ can increase the rate and extent of RDX degradation. However, their experiments involved amendments with Fe⁰ so that final Fe⁰ concentrations were 10 to 50 g kg⁻¹ soil. Excevable Fe present in the Ft. Greely soils used in this study were 0.25 mg kg⁻¹ and Fe was not added. In addition, Singh et al. found that RDX activity remained unchanged at aqueous Fe⁰ concentrations of <2 g L⁻¹.

The high organic matter content of the soil (approximately 10%) suggests that available C other than RDX was abundant for microbial metabolism. Given the availability of organic C, it seems unlikely that RDX
Table 2. First-order rate of loss equations for RDX in Fort Greely soil.

<table>
<thead>
<tr>
<th>Moisture potential</th>
<th>CH$_3$CN</th>
<th>Rate equation†</th>
<th>$t^2$</th>
<th>Half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPa (bar)</td>
<td>%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsat.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.015 (0.15)</td>
<td>0</td>
<td>$\ln \frac{C}{C_0} = -0.0010t$</td>
<td>0.9355</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>$\ln \frac{C}{C_0} = -0.0010t$</td>
<td>0.9612</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>$\ln \frac{C}{C_0} = -0.0006t$</td>
<td>0.9420</td>
<td>48</td>
</tr>
<tr>
<td>Ctrl. 0</td>
<td></td>
<td>$\ln \frac{C}{C_0} = -0.0004t$</td>
<td>0.9454</td>
<td>72</td>
</tr>
<tr>
<td>Ctrl. 1.0</td>
<td></td>
<td>$\ln \frac{C}{C_0} = -0.0004t$</td>
<td>0.8605</td>
<td>72</td>
</tr>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00 (0.00)</td>
<td>0</td>
<td>$\ln \frac{C}{C_0} = -0.0073t$</td>
<td>0.9624</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>$\ln \frac{C}{C_0} = -0.0073t$</td>
<td>0.9325</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>$\ln \frac{C}{C_0} = -0.0066t$</td>
<td>0.8384</td>
<td>4</td>
</tr>
<tr>
<td>Ctrl. 0</td>
<td></td>
<td>$\ln \frac{C}{C_0} = -0.0012t$</td>
<td>0.8472</td>
<td>24</td>
</tr>
<tr>
<td>Ctrl. 1.0</td>
<td></td>
<td>$\ln \frac{C}{C_0} = -0.0009t$</td>
<td>0.8208</td>
<td>32</td>
</tr>
</tbody>
</table>

† $C$ is the concentration of RDX at time ($t$), and $C_0$ is the concentration at time zero.

would be a desirable substrate. However, in other studies, RDX serves as a source of N and not C. In fact, *Rhodococcus rhodochrous* strain 11Y is capable of using RDX as a sole source of N but not as a sole source of C (Seth-Smith et al., 2002). In addition, high concentrations of soil organic matter promote the formation of reduced conditions due to high levels of microbial activity and RDX biodegradation is favored under a reduced environment.

Although abiotic processes of RDX degradation were undoubtedly present in the saturated and unsaturated microcosms of this study, the extent of abiotic degradation appeared to be far less than that attributable to biotic processes. Precise descriptions of the nature of any abiotic processes present were outside the scope of this research effort.

Loss of RDX in the saturated microcosms coincided with a gradual increase in the anaerobicity of the system, which was indicated by a color loss in the added resazurin dye. The increase in anaerobicity also coincided with the occurrence of nitroso intermediates in both the active and control microcosms (Fig. 3). The MNX and DNX concentrations reached a maximum 168 h into the time course and were transient in nature in the active microcosms, falling to below detection limits by study termination. The concentration of TNX reached a maximum at 840 h into the time course (Fig. 3). No other known intermediates (of RDX biodegradation) were targeted for detection. The results described here for RDX loss, nitroso intermediate formation, and subsequent nitroso intermediate disappearance in conditions that are microaerophilic to anaerobic are consistent with the findings of Light et al. (1997), Shen et al. (1997), Young et al. (1997), Boopathy et al. (1998), and Guiot et al. (1999). No nitroso intermediates were detected in the soil extract following centrifugation (i.e., sorbed to the soil), which is consistent with the finding of Shermata et al. (2001).

RDX concentrations in the unsaturated soil never decreased to an undetectable level, remaining at 42% of the initial addition at study termination (Fig. 2). Significant differences in mean RDX concentrations and rates of RDX loss were measured between the active and control treatments. First-order rate coefficients for RDX loss in the unsaturated soil were $-0.0010$ in the 0 and 0.1% ACN additions, and $-0.0006$ in the 1% addition (Table 2). These rate coefficients were 7 to 11 times less than those observed in the saturated microcosms. Half-lives for RDX in the unsaturated soil were calculated to be 29, 29, and 48 d for the 0, 0.1, and 1.0% ACN additions, respectively (Table 2). These values are similar to those reported by Speitel et al. (2001), who found half-lives for RDX in microaerobic soils to range from 60 to 43 d, depending on nutrient concentrations.
The core material examined by Speitel et al. (2001) was at 8% gravimetric moisture level but the water potential was not given. Our treatment was at 40% gravimetric moisture and was equivalent to −0.015 MPa for this soil. The results of this study, of Speitel et al. (2001), and of Grant et al. (1993) indicate that RDX biodegradation in unsaturated soils is either directly or indirectly dependent on soil moisture potential.

No nitroso-intermediates were detected in the unsaturated microcosms. Although Sheremata and Hawari (2000) reported the presence of MNX as an RDX intermediate during the aerobic biodegradation of RDX by the white rot fungi, Phanerochaete chrysosporium, this organism was not present in this study.

The ACN had a significant effect on RDX biodegradation in the unsaturated soil only. The 1% ACN addition (10,000 ppm) inhibited RDX degradation by approximately 25% (Fig. 2). Because microorganisms were present, approximately 4 × 10^11 cells kg^−1 at 48 h, the inhibition of RDX catabolism suggests either that: (i) the acetonitrile acted as a selective toxicant, or (ii) that the acetonitrile served as an alternative C source. Acetonitrile has a low toxicity to bacteria, cyanobacteria, green algae, and protozoans, with thresholds above 500 μL L^−1 (Hashimoto et al., 1993; USEPA, 1985). In this study acetonitrile final concentrations ranged from 1000 (0.1%) to 10,000 μL L^−1 (1.0%), which are well above the presumed threshold. However, there was no significant loss in viable cell numbers over the time course of the experiment as a result of the 1% ACN addition (Table 1). If it is assumed that toxicity would result in a decrease in viable cell numbers, then these data suggest that acetonitrile was not toxic at the levels administered.

Acetonitrile has been shown to be readily degraded by the aerobic heterotroph, Pseudomonas putida (Nawaz et al., 1989). In addition, available N, such as NH₃, has been shown to interfere with RDX biodegradation under aerobic conditions (Binks et al., 1995; Coleman et al., 1998; Yang et al., 1983). Acetonitrile may function in a similar manner to NH₃ by serving as an alternative source of N. Although not presented here, results show that a shift in microbial community structure occurred as a result of the 1% acetonitrile addition. The shift in community structure is hypothesized to be an enrichment of organisms capable of utilizing N or C from acetonitrile for metabolism.

CONCLUSIONS

When the sandy loam examined in this study was spiked with RDX in water and maintained at a moist but unsaturated condition, there was a significant increase in the half-life for RDX compared to the same soil under a saturated condition (Table 2). Miyares and Jenkins (2000) found the same to be true for TNT over a wide range of soil types and temperatures. Half lives for RDX in the unsaturated soil were further affected by the presence of acetonitrile, which may have served as an alternative carbon source for microbial respiration. Acetonitrile, at 1% (10,000 μL/L), significantly reduced the rate at which RDX was lost in the unsaturated soil by a factor of 7.5, but had no significant effect in saturated soil.

The results of this study demonstrated that RDX and its nitroso-derivatives can be biodegraded by an indigenous microbiota in a cold region soil with relatively high organic content. No distinction is made between biotransformation or biomineralization. For this soil, RDX loss rates were significantly greater when the soil was saturated and was rapidly becoming reduced. Although it is acknowledged that autoclaving does not completely sterilize soils, it does attenuate the in situ microbiota. Therefore, the significantly greater loss of RDX in the active microcosms, as compared with the autoclaved controls, suggested that biodegradation was a significant component to the observed RDX loss. The transient nature of nitroso-intermediates was viewed as a further indication of probable microbial activity (Walker and Kaplan 1992).

The fact that organic solvents can influence microbial degradation activity is well established, but nevertheless, these solvents are often used to introduce organic substrates into soils. The manner in which these solvents interfere with the degradation process is not well understood. In this study, the C or N in acetonitrile appears to have interfered with the aerobic biodegradation of RDX when present at 1% of the total aqueous volume, but had no detectable effect on the anaerobic catabolic process. The differential response of the microbiota to the presence of the solvent highlights the dynamic and intricate nature of an indigenous microbial community. These results emphasize that caution may be needed when extrapolating laboratory-based results in the making of far reaching land management decisions.

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