Hexahydro-1,3,5-trinitro-1,3,5-triazine Mineralization by Zerovalent Iron and Mixed Anaerobic Cultures

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Hexahydro-1,3,5-trinitro-1,3,5-triazine Mineralization by Zerovalent Iron and Mixed Anaerobic Cultures

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Soil microcosms were used to evaluate the potential benefits of an integrated microbial−Fe^0 system to treat groundwater contamination by RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine). Microcosms amended with both Fe^0 filings and municipal anaerobic sludge mineralized RDX faster and to a greater extent than separate treatments, with up to 51% $^{14}CO_2$ recovery after 77 d. The nitroso byproducts 1,3-dinitro-5-nitroso-1,3,5-triazacyclohexane (MNX), 1,3-dinitroso-5-nitro-1,3,5-triazacyclohexane (DNX), and 1,3,5-trinitroso-1,3,5-triazacyclohexane (TNX) were detected in all microcosms, although these compounds never accumulated above 5% of the added RDX on a molar basis. A soluble intermediate that was tentatively identified as methylenedinitramine [$(O_2NNH)_2CH_2$] was relatively persistent, although it accumulated to a much lower extent in combined-treatment reactors than in sets with Fe^0 or sludge alone. Some of the radiolabel was bound to soil and Fe^0 and could not be extracted with CH_3CN. This fraction, which was recovered by combustion with a biological oxidizer, was also found at lower concentrations in combined-treatment reactors. This work suggests that permeable reactive Fe^0 barriers might be an effective approach to intercept and degrade RDX plumes and that treatment efficiency might be enhanced by biogeochemical interactions through bioaugmentation.

Introduction

RDX (Hexahydro-1,3,5-trinitro-1,3,5-triazine) is the British code name for Research Department Explosive (*1*). RDX is a suspected carcinogen that represents a major remediation challenge at numerous munitions facilities due to its recalcitrance to biodegradation, low volatility, and high mobility in aquifers. While several ex situ physical−chemical and biological processes have been proposed to manage RDX contamination (*2*, *3*), many of these approaches are relatively expensive for groundwater treatment or are limited by the accumulation of transformation products of equal or even greater toxicity (*4*). The need exists for an in situ remediation strategy that is easy, cost-effective, less prone to accumulate toxic byproducts, and addresses both chemical and microbiological advantages and constraints.

Encouraging results in laboratory and field experiments have recently stimulated a rapid increase in the use of zerovalent iron (Fe^0) as a reactive material to remove redox-sensitive contaminants from groundwater (*5*). Semipermeable Fe^0 barriers are particularly attractive for in situ remediation in that they conserve energy and water and, through long-term low operating costs, have the potential to be considerably less costly than conventional cleanup methods (*6*). This approach has been mainly used to remove waste chlorinated solvents (*6*−*8*) and redox-sensitive metals such as chromium and uranium (*9*, *10*), and recent studies have reported that Fe^0 can also chemically reduce RDX in contaminated water and soil (*11*, *12*).

Research on Fe^0 systems has focused primarily on abiotic processes. Nevertheless, we found that hydrogen gas produced from the reduction of water-derived protons during Fe^0 corrosion (eq 1) can serve as an electron donor for the biotransformation of reducible contaminants:

$$Fe^0 + 2H_2O \rightarrow Fe^{2+} + 2OH^- + H_2 \qquad (1)$$

In fact, combining Fe^0 with an active methanogenic consortium significantly enhanced both the rate and the extent of transformation of chlorinated methanes (*13*). Further experiments were conducted with pure cultures of methanogens, including hydrogenotrophic species that could grow on H_2 as well as aceticlastic species that could not (*14*). These experiments demonstrated that cathodic H_2 could stimulate anaerobic bioremediation of chlorinated solvents, even when H_2 does not serve as growth substrate. In addition, Fe^0 stimulated *Methanosarcina thermophila* to excrete an extracellular factor with protein-like characteristics that degraded both carbon tetrachloride and chloroform (*15*). We also showed that a similar approach, combining Fe^0 and autotrophic denitrifiers, significantly enhanced the treatment of nitrate-contaminated water by increasing removal rates and improving the end product distribution, favoring N_2 over NH_4^+ (*16*). Thus, previous experiments suggest that some biogeochemical interactions can significantly enhance the efficacy of Fe^0 barriers.

This study investigated the potential benefits of bioaugmenting Fe^0 barriers to enhance the removal of RDX from contaminated groundwater. Emphasis was placed on determining whether combining anaerobic municipal sludge with Fe^0 filings is synergistic in terms of the rates and the extent of RDX mineralization. The fate of RDX in combined versus separate treatment systems was also compared.

Materials and Methods

Chemical Reagents. ^{14}C-Ring-labeled RDX was synthesized according to Ampleman et al. (*17*), resulting in a purity of 98%. The specific activity of the radioactive compound was 0.32 μCi/mmol. An analytical standard of RDX was purchased from Chem Service Inc. (West Chester, PA). Mono-, di-, and trinitroso transformation products of RDX [1,3-dinitro-5-nitroso-1,3,5-triazacyclohexane (MNX); 1,3-dinitroso-5-nitro-1,3,5-triazacyclo-hexane (DNX); and 1,3,5-trinitroso-1,3,5-triazacyclohexane (TNX)] were obtained from SRI International (Menlo Park, CA). All other chemicals used were reagent grade. Zerovalent iron (Fe^0) filings (medium coarse, 1.06 m^2/g; *18*) were obtained from Master Builders Inc. (Cleveland, OH).

Soil. Uncontaminated soil was obtained from the Iowa Army Ammunition Plant in Middletown, IA. Prior to experimentation and analysis, the soil was air-dried and ground to pass through a 1-mm sieve. Physicochemical properties of the soil were determined by Western Laboratories Inc. (Parma, ID) (Table 1).

Anaerobic Sludge. The source of microorganisms for bioaugmented treatments was anaerobic (methanogenic) sludge from Iowa City's wastewater treatment plant. The

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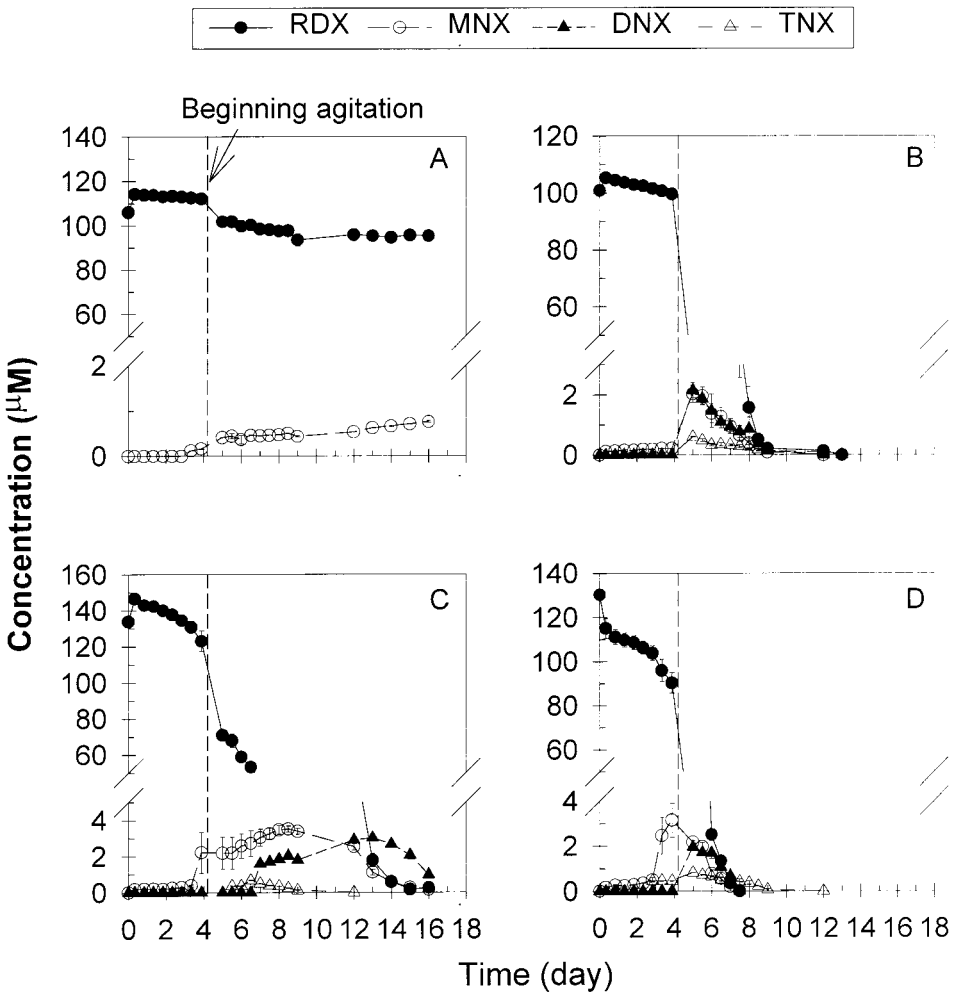

FIGURE 1. Changes in RDX, MNX, DNX, and TNX concentrations in unamended (no-treatment) controls (A) and in reactors amended with Fe^0 filings (10% of soil weight, w/w) (B), anaerobic sludge (10%, v/v) (C), and both (D). Bars denote standard deviations from the mean of triplicate reactors.

TABLE 1. Characteristics of the Uncontaminated Soil Obtained from the Iowa Army Ammunition Plant in Middletown, IA

soil property	uncontaminated soil
soil pH with H_2O	7.7
organic matter (%)	5.6
cation exchange capacity (mequiv)	28
sand (%)	22
silt (%)	74
clay (%)	4

sludge concentration was 6.6 g/L of volatile suspended solid (VSS). The sludge was always used fresh.

Microcosms Preparation. The anaerobic medium for all microcosms contained (in mg (L of distilled water)$^{-1}$): NH_4-Cl (400), KCl (400), $MgCl_2 \cdot 6H2O$ (400), $Na_2S \cdot 9H_2O$ (300), $(NH_4) \cdot 2HPO_4$ (80), $FeCl_2 \cdot 4H_2O$ (40), $CaCl_2 \cdot 2H_2O$ (25), $(NaPO_3)_6$ (10), KI (2.5), $CoCl_2 \cdot 6H_2O$ (2.5), $MnCl_2 \cdot 4H_2O$ (0.5), NH_4VO_3 (0.5), $ZnCl_2$ (0.5), $NaMoO_4 \cdot 2H_2O$ (0.5), H_3BO_3 (0.5), $NiCl_2 \cdot 6H_2O$ (0.5), and cysteine (10). The medium was buffered with $NaHCO_3$ (1000). Microcosms were prepared by adding 150 mL of mineral medium, 100 g of soil, and 25 mg of RDX L^{-1} to 250-mL serum bottles capped with screw-cap Mininert valves. Contents were purged for 2 h with N_2/CO_2 (80/20, v/v) to remove dissolved oxygen. Four sets were prepared in triplicate: no-treatment control (without Fe^0 filings and bacteria addition), Fe^0 filings alone (poisoned with 350 mg of $HgCl_2$ L^{-1} plus 10 g of Fe^0 filings), anaerobic sludge alone (15 mL, 10%, v/v), and combined treatment (10 g of Fe^0 filings plus 15 mL of sludge). Emphasis was placed on comparing the removal efficiency and the fate of RDX in different treatments.

All microcosms were incubated quiescently at 30 ± 2 °C in the dark in a Coy anaerobic chamber. After 4.2 d, all microcosms were agitated once per day. At preselected times, aqueous samples were collected with disposable syringes and filtered using a 0.2-μm syringe filter. Changes in the concentration of RDX and its degradation products were monitored. The initial pH of the microcosms was 7.4, and it increased up to 9.0 by the end of the experiment due to Fe^0 corrosion (eq 1).

RDX Mineralization. Experiments were also conducted to determine the potential benefits of an integrated microbial−Fe^0 system for RDX mineralization using ^{14}C-labeled RDX. Reactors were prepared in triplicate using wide-mouth jars under anaerobic conditions, as described above. ^{14}C-Labeled and unlabeled RDX were added to reactors to achieve an initial concentration of about 1 μCi of total radioactivity and 25 mg L^{-1} (113 μM). A 50-mL test tube containing 5 mL of 1 M NaOH was placed inside each jar to trap $^{14}CO_2$ from RDX mineralization. Test tubes were removed and replaced every 7 days. RDX mineralization was determined from trapped $^{14}CO_2$ at each sampling by liquid scintillation counting (LSC). All reactors were incubated quiescently at 30 ± 2 °C in the dark in a Coy anaerobic chamber and shaken manually every sampling event.

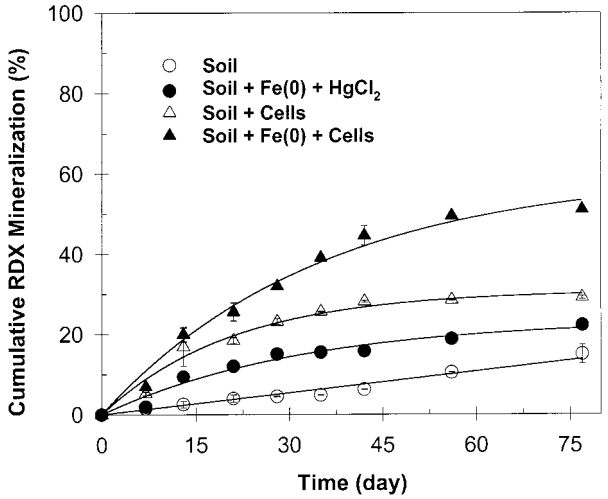

FIGURE 2. Cumulative mineralization of [^{14}C]RDX to $^{14}CO_2$ in unamended (no-treatment) controls and in reactors amended with Fe^0 filings (10% of soil weight, w/w), anaerobic sludge (10%, v/v), and both. Bars denote standard deviations from the mean of triplicate reactors.

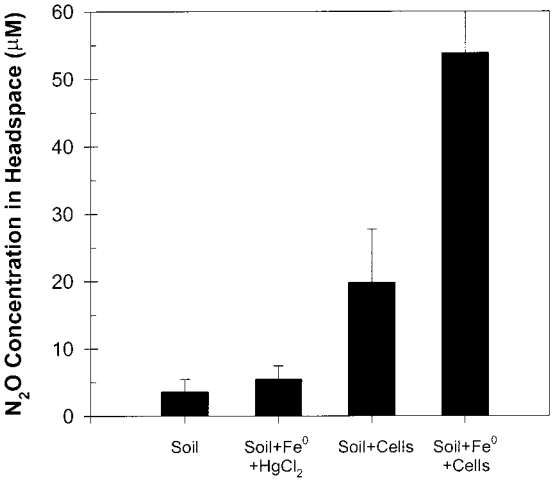

FIGURE 3. Nitrous oxide concentration in no-treatment controls and in reactors amended with Fe^0 filings (10% of soil weight, w/w), anaerobic sludge (10%, v/v), and both after 28-d incubation. Bars denote standard deviations from the mean of triplicate reactors. The initial RDX concentration was 25 mg L^{-1} (113 μM).

TABLE 2. Mineralization Rate Coefficients for Different Treatments

treatment	first-order mineralization rate coefficient (k) (d^{-1})
no treatment	0.002 ± 0.0001
Fe^0 alone	0.003 ± 0.0004
cells alone	0.004 ± 0.0009
Fe^0 + cells	0.010 ± 0.0012

Total ^{14}C Recovery. At the end of the mineralization measurements, the microcosms were sacrificed in order to determine a radioactive carbon balance. After the microcosms were centrifuged for 20 min at 10 000 × g, the aqueous phase was collected, and the soils were sequentially extracted with 300 mL of 3 mM $CaCl_2$ (shaking for 24 h at 25 °C) and 300 mL of CH_3CN (sonication for 24 h at 20 °C) to determine readily available and potentially available ^{14}C-labeled residue, respectively. After the extractions, the soil was dried (for 24 h at 95 °C), and the Fe^0 was separated using a magnetic stirrer. The soil and Fe^0 were combusted in a biological oxidizer (R. J. Harvey Instrument Co., Hillsdale, NJ) to determine remaining bound (unextractable) ^{14}C-labeled residue. The recovery efficiency of the oxidizer ranged from 94% to 99% as determined by combustion of known amounts of [^{14}C]mannitol. Total ^{14}C in each pool was determined by LSC.

Analytical Methods. High-performance liquid chromatography (HPLC) analysis of RDX and its nitroso derivatives MNX, DNX, and TNX was conducted using a Hewlett-Packard 1100 Series HPLC equipped with a 250 × 4.6 mm Supelcosil LC-18 column (Supelco, Bellefonte, PA). The mobile phase was isocratic, consisting of deionized water and methanol (4:6, v/v) at a flow rate of 1.0 mL/min. Detection was spectrophotometric at 240 nm. [^{14}C]RDX and its ^{14}C-labeled metabolites were quantified by HPLC using a radioactivity detector (Radiomatic, Series A-500, Packard Instrument Co., Downers Grove, IL). RDX mineralization was determined from trapped $^{14}CO_2$ by mixing 1 mL of sample with 10 mL of LSC cocktail (Fisher Scientific, Fair Lawn, NJ) and counting on a Beckman LS 6000IC liquid scintillation counter (Beckman Instr. Inc., Fullerton, CA). For nitrous oxide (N_2O) measurements, headspace samples were taken through the Mininert valves using a 100-μL gastight syringe (Dynatec Precision Sampling Corp.). N_2O was measured by injecting headspace (100 μL) into a HP 5890 series II GC. The GC was equipped with a thermal conductivity detector (TCD). Separation was achieved using an Alltech (Deerefield, IL) packed Haysep Q molecular sieve column.

A QuattroLC (Micromass Corporation) LC/MS/MS equipped with a photodiode array detector was used to characterize a soluble RDX metabolite detected via radiochemical HPLC analysis. The mass spectrometer was operated in negative-ion electrospray (ES−) mode with desolvation and source temperatures of 150 °C. Nitrogen flows were 75 L/h for the nebulizer and 700 L/h for desolvation. The capillary voltage was −3 kV, the cone was at −25 V, and the extraction voltage was −3 V. The mass resolution was 15, and the multiplier was at 650 V.

Initially, the LC/MS elution conditions followed that of Casetta and Garofolo (*19*) utilizing a C18 column (Supelco, 15 cm × 2.1 mm, 5 μm) and acetonitrile/2 mM ammonium acetate (50/50) eluent pumped at 200 μL/min. For confirmation purposes, a CN column (Alltech, 25 cm × 4.6 mm, 5 μm) with methanol/water (50/50) eluent pumped at 1 mL/min (with postcolumn flow splitting) was employed as described by Hawari et al. (*20*, *21*).

Results and Discussion

RDX Removal. Microcosm experiments suggest that an integrated microbial−Fe^0 treatment approach can effectively degrade RDX. Very little RDX removal was observed in no-treatment controls (Figure 1A), corroborating the notion that RDX is a relatively recalcitrant compound. Traces of MNX were detected in nonsterile controls, suggesting a low level of indigenous microbial transformation. RDX was removed faster in the combined-treatment microcosms (Figure 1D) than in treatments with Fe^0 (Figure 1B) or municipal anaerobic sludge alone (Figure 1C). RDX degradation rates increased significantly during agitation (up to 5 mg L^{-1} d^{-1}), which suggests that degradation kinetics under quiescent conditions were limited by inadequate contact between RDX and Fe^0 and/or cells. MNX, DNX, and TNX were detected as transient intermediates in both bioaugmented and abiotic microcosms, although these compounds never accumulated above 5% of the added RDX on a molar basis. The detection of these heterocyclic nitroso compounds has been reported by several other researchers studying RDX degradation by anaerobic sludge (*4*, *21*), Fe^0 (*11*), thermolysis (*22*), and photolysis (*23*).

TABLE 3. Distribution of ^{14}C in Microcosms Amended with Fe^0 Filings (10% of Soil Weight, w/w), Anaerobic Sludge (10%, v/v), None, or Both after 77-d Incubation[a]

amendments	released $^{14}CO_2$ (%)[b]	aqueous phase (%)	solid phase (%)				total (%)
			3 mM $CaCl_2$	CH_3CN	unextractable soil	unextractable Fe^0	
soil	15.0 (2.3)[c]	49.6 (7.2)	8.8 (0.4)	3.7 (0.1)	13.6 (3.1)		90.7
soil + Fe^0 + $HgCl_2$	22.3 (0.2)	22.0 (3.9)	9.3 (0.5)	3.6 (0.2)	28.0 (1.3)	13.8 (1.3)	99.0
soil + cells	29.1 (0.4)	28.7 (1.2)	8.7 (1.2)	3.0 (0.1)	14.5 (2.9)		84.0
soil + Fe^0 + cells	51.0 (0.2)	4.6 (0.9)	3.5 (0.2)	2.6 (0.1)	14.3 (0.3)	11.9 (0.5)	87.9

[a] Initial RDX concentration was 25 mg/L (spiked with 1 μCi [^{14}C]RDX). [b] Recovery of ^{14}C is percent of total ^{14}C added. [c] Parenthetic values indicate sample standard deviations.

RDX Mineralization. Previous studies have reported that Fe^0 can enhance RDX mineralization in soil microcosms (*11*), although the role of microorganisms was not investigated. In this paper, we studied chemical and biological degradation processes separately and interactively. Combined-treatment microcosms were more effective in mineralizing RDX, as shown by $^{14}CO_2$ recovery over 77-d incubation (Figure 2). In unamended (no-treatment) controls, 15% of the RDX was mineralized during the 77-d incubation. When Fe^0 was added under sterile conditions, cumulative mineralization reached up to 22% for 77 d. Abiotic RDX mineralization was verified in additional incubations with Fe^0 and without soil, although cumulative mineralization was less than 10% (data not shown). When anaerobic sludge was added to the microcosms, mineralization increased to 29%. In the combined treatment with Fe^0 and anaerobic sludge, cumulative mineralization increased to 51% for 77 d. This degree of RDX mineralization compares favorably to that reported by Singh et al. (*11*) for soil microcosms treated with Fe^0 and by Sheremata and Hawari (*24*) for the white-rot fungus *Phanerochaete chrysosporium*, which mineralized 52% of [^{14}C]RDX to $^{14}CO_2$ in 60 d under aerobic conditions.

RDX mineralization followed first-order kinetics (i.e., $C = C^*[1 - e^{-kt}]$, where $C = {}^{14}CO_2$ concentration at time t, and C^* is the asymptotic value). This model is depicted as solid lines in Figure 2. Rate coefficients were estimated by fitting the data to this model (Table 2). Interestingly, the first-order rate coefficient (k) for the combined Fe^0 and cells treatment (0.010 d^{-1}) was 30% greater than the sum of the k values for the separate Fe^0 and cells treatments (i.e., 0.003 + 0.004 = 0.007 d^{-1}), suggesting that the combination may have been synergistic with respect to the rate of RDX mineralization. This putative synergism could be due to several factors. Fe^0 corrosion rapidly induces anaerobic conditions that favor RDX degradation. The production of cathodic (water-derived) hydrogen by Fe^0 corrosion (eq 1) would increase the availability of an excellent electron donor to support microbial reduction of RDX (*12*) and the further degradation of some dead-end products that could accumulate during abiotic reduction by Fe^0. In addition, some iron-reducing bacteria commonly present in anaerobic consortia could enhance iron reactivity by reductive dissolution and activation of some oxides that passivate the iron surface (*25*, *26*).

Nitrous oxide (N_2O) was found in the headspace of all microcosms, which corroborates the work of Hawari et al. (*21*), who found that N_2O can be an end product of RDX degradation. More N_2O was detected after 28 d in the headspace of the combined-treatment microcosms (55 μM) than in the headspace of microcosms with anaerobic sludge alone (20 μM) or Fe^0 alone (5 μM) (Figure 3). This reinforces the notion that the combined treatment results in greater transformation of RDX to innocuous products.

Fate of Carbon-14. Radiolabel recovery ranged from 84 to 99% (Table 3). In these experiments, little ^{14}C was recovered by sequential extraction. Some of the radiolabel was bound to soil and Fe^0 and could not be extracted with CH_3CN. This bound residue, which was recovered by combustion with a biological oxidizer, was greater for treatments with Fe^0 alone (28% in soil and 14% in Fe^0) than with sludge alone (14% in soil) or with both Fe^0 plus sludge (14% in soil and 12% in Fe^0). Apparently, RDX byproducts can be irreversibly bound with some surface material as an alternative pathway to mineralization (*27*). On the basis of previous RDX degradation pathway analysis (*28*), we hypothesize that amine-containing RDX metabolites can bind to the carbonyl functional groups of humics and other organic residues, forming amide linkages as shown in Figure 4. Whether this "sequestration" pathway leads to an acceptable treatment end point (due to lack of bioavailability) remains to be determined.

A soluble ^{14}C-labeled metabolite was detected in all microcosms by HPLC with radiochromatographic detection (data not shown). This intermediate was common to both biological and abiotic degradation pathways. At the end of the 77-d incubation period, it accounted for about 50% of the radiolabel in treatments with soil alone, 29% with sludge alone, 22% with Fe^0 alone, and 5% with sludge plus Fe^0 (Table 3). The accumulation of this metabolite suggests that its degradation may be a rate-limiting step in RDX mineralization. This metabolite was persistent for two months in microcosms amended with Fe^0 plus anaerobic sludge, with the amount decreasing gradually with time.

Soluble Metabolite. We hypothesized that methylenedinitramine (MDNA) [$(O_2NNH)_2CH_2$] would be present in our system based on previous reports for RDX decomposition under a variety of conditions (*21*, *23*). Accumulation of this metabolite could be a potential problem since its human toxicity is unknown. Using the C18 column setup with acetate buffer and a scan range of m/z 61−300, the total ion chromatogram and mass spectrum (Figure 5) was collected. The signal at m/z 195 indicates the presence of an MDNA−acetate adduct ion, $[M + 59]^-$. A signal at m/z 61 would be anticipated from the reported $[O_2NNH]^-$ fragment from MDNA, but a signal at m/z 62 was measured instead. This could have been due to poor low-mass calibration or the presence of a compound other than MDNA. The large signal at m/z 97 was thought to be from phosphate $[H_2PO_4]^-$ present in the eluent. The significance of the peaks at m/z 217 and 80 is unclear at this time.

The analysis was repeated with the CN column and no acetate buffer to confirm the presence of MDNA since a standard was not available for comparative purposes. Without buffer, an ion at m/z 135 $[M - H]^-$ for MDNA was expected, so single ion monitoring at m/z 135 was used to enhance sensitivity. Two compounds with m/z 135 were detected (Figure 6), and UV absorbance (240 nm) was measured only for the compound eluting at 3.4 min. MDNA is thought to be UV active because of its nitro groups. Therefore, two sets of spectral data and UV absorbance analysis support the notion that this peak corresponded to MDNA.

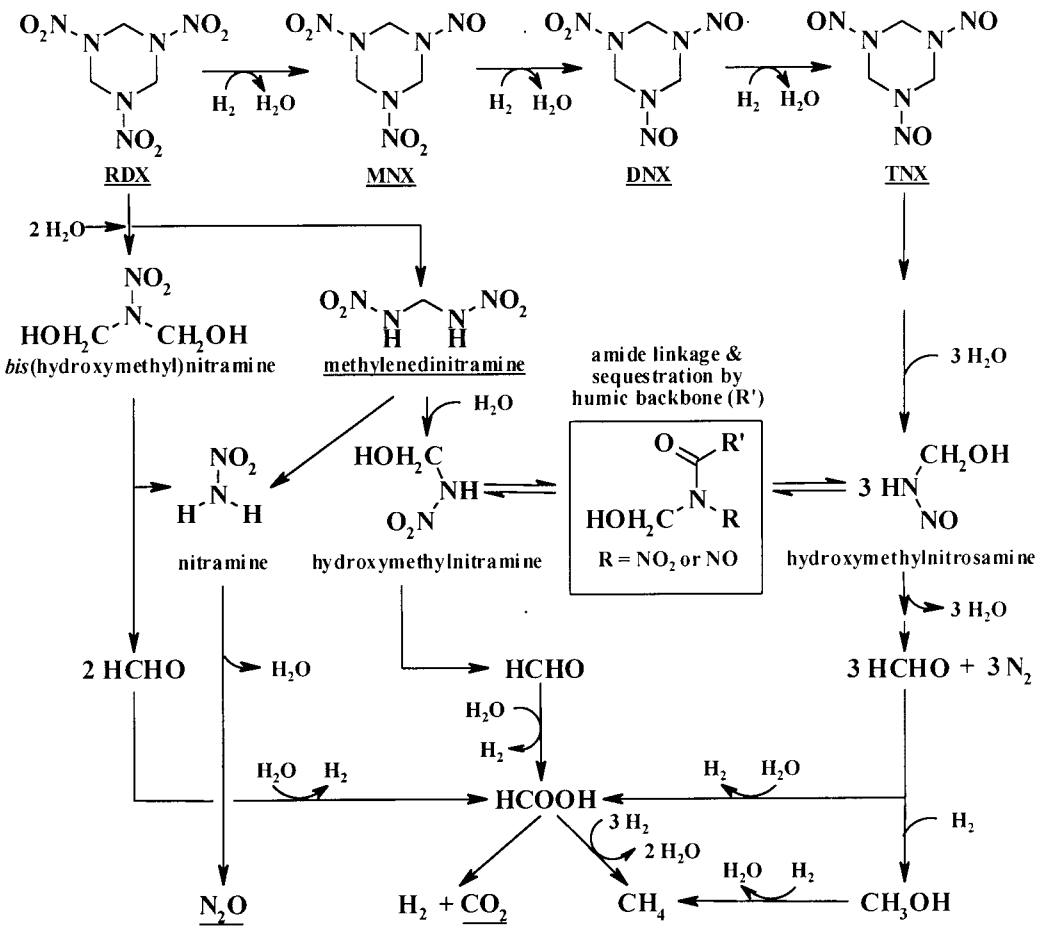

FIGURE 4. Proposed RDX degradation pathway, based on work by Hawari (*28*). Species detected in this research are underlined.

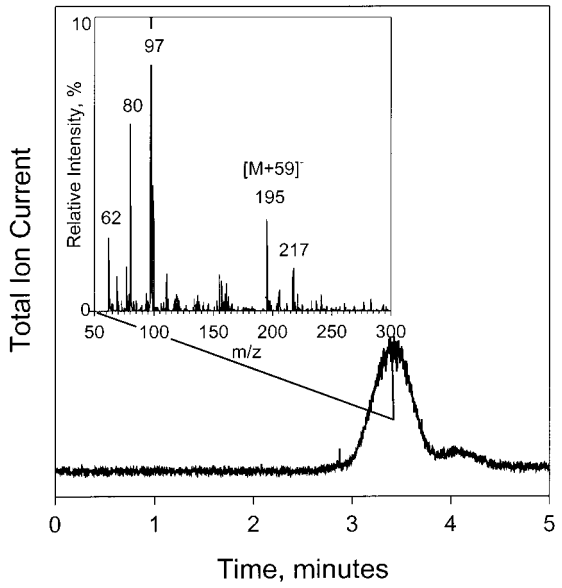

FIGURE 5. Total ion chromatogram and mass spectrum for the RDX metabolite using a C18 column and acetate-buffered eluent. The signal at *m/z* 195 suggests the presence of the MDNA−acetate adduct ion [M + 59]⁻.

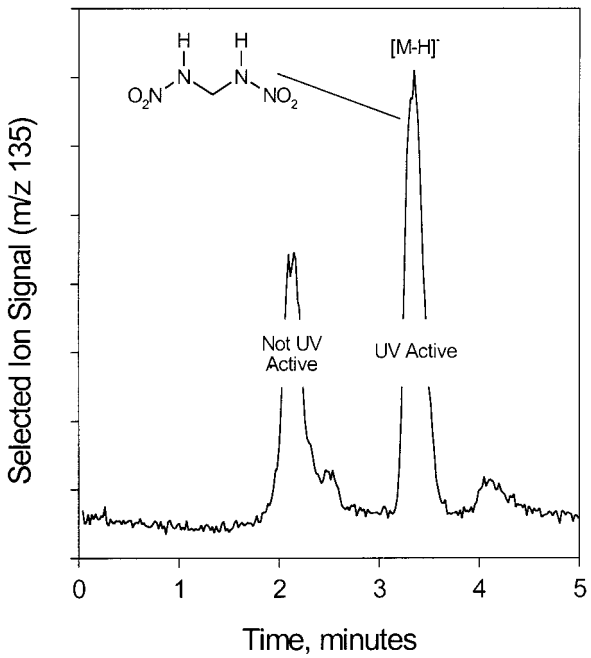

FIGURE 6. Selected ion chromatogram at *m/z* 135 for the RDX metabolite using a CN column without acetate-buffered eluent. Two compounds were detected via mass spectrometry, but UV absorbance (which is expected for MDNA) was measured only for the compound that eluted at 3.4 min.

Conclusion

Laboratory experiments suggest that permeable reactive Fe^0 barriers might be a viable alternative to intercept and degrade RDX plumes and that system performance can be enhanced by some biogeochemical interactions through bioaugmentation. This integrated approach resulted in enhanced RDX mineralization, presumably related to the cumulative effect of chemical and biological processes and the exploitation of cathodic depolarization and bioremediation as metabolic

niches. This approach should probably be also considered for the treatment of other redox-sensitive pollutants, such as chlorinated solvents, nitroaromatic compounds, nitrate, hexavalent chromium, hexavalent uranium, and some pesticides. Nevertheless, further studies are needed to delineate better the applicability and limitations of iron-based bioremediation.

Acknowledgments

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