THE EFFECT OF ELECTRON ACCEPTORS ON RDX BIODEGRADATION AND METABOLITE FORMATION

By

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INTRODUCTION

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a commonly used high explosive for military and demolition applications. RDX, short for royal demolition explosive, is used alone in detonators or combined with TNT and wax for use in bombs. RDX can enter water through the disposal of wastewater from munitions factories, and it can enter both soil and water through spills, leaks, or improper disposal at munitions plants and other hazardous waste sites. RDX particles can also enter the air if it is disposed of through open burning. Left unchecked, RDX contamination can migrate from soil into ground water.

Currently, RDX is produced in the United States only at the Holston Army Ammunition Plant in Kingsport, Tennessee. RDX is handled, however, at 11 facilities throughout the country where weapons are assembled. Past disposal practices, such as open burning and burial, have left a number of current and former munitions facilities with considerable soil contamination. These facilities include the former Pantex Ordnance Plant near Amarillo, Texas and the Nebraska Ordnance Plant in eastern Nebraska. Both sites have been placed on the National Priorities List.

The purpose of this study was to determine the electron accepting conditions through which RDX could be biologically degraded in soil slurry reactors. Previous experiments by Light et al. (1998) using large amounts of soil in slurry reactors suggested that RDX biodegradation might be most effective under nitrate-reducing conditions. However, the large amount of RDX-contaminated soil used in these reactors and the low solubility of RDX prevented the observation of any reduction in aqueous phase

concentrations during the one year period of study. Furthermore, the results of RDX reactor experiments without soil by Light et al. (1998) and results from other nitrate-reducing RDX reactor experiments (Freedman and Sutherland 1998) conflict with the previously mentioned soil slurry results, suggesting that nitrate-reducing reactors may not be most effective in degrading RDX.

In order to avoid the problems encountered by Light et al. (1998), bench-scale soil slurry reactors were designed with much smaller amounts of soil. These reactors were maintained under nitrate-reducing, sulfate-reducing, or fermentative conditions similar to confirm the previous experiments of Light et al. (1998). Metabolite formation was also examined in order to characterize the pathway through which RDX degradation would occur under each experimental condition. The goal was to determine under which conditions a soil slurry bioreactor could most effectively treat RDX-contaminated soils.

LITERATURE REVIEW

Properties of RDX

RDX is a hexacyclic ring with three nitrogen atoms and three carbon atoms in the ring and a nitro group attached to each of the ring-associated nitrogen atoms (Fig. 1). Its solubility in water is low, ranging from 21.8 mg/L at 10°C to 67 mg/L at 30°C (Smith-Simon and Goldhaber 1995, McLellan et al. 1992). RDX does not adsorb strongly onto soils. Singh et al. (1998a) found that only 34 percent of RDX added to soil was sorbed within a half-hour, and that sorption increased to only 37 percent after 168 days. The unsorbed RDX has log K_{OC} of 1.76-2.00, making it fairly mobile in soils (Rosenblatt et

al. 1991, Singh et al. 1998a). The low sorption and high mobility of RDX in soils make ground water contamination a major concern for sites where soil is contaminated with the explosive.

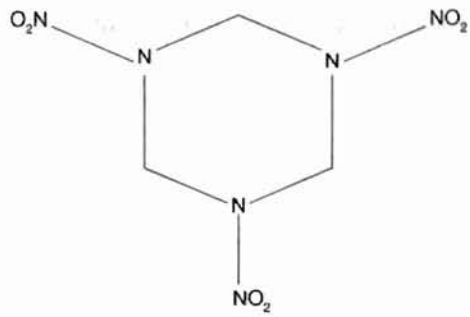


FIGURE 1. Chemical structure of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX).

Toxicology

The EPA has classified RDX as a Class C possible human carcinogen. One study has demonstrated that RDX may cause cancer in mice, but no carcinogenic effects have been observed in rats (Testud et al. 1996). No long-term data have been collected regarding possible carcinogenic or other chronic complications in humans. The possible carcinogenic effects, however, have led the EPA to publish a water quality criterion of 0.3 µg/L for RDX. Toxicology of the nitroso forms of RDX (MNX, DNX, and TNX) is not known at this time.

Acute RDX exposure has been linked to seizures and possible liver, kidney, and heart damage in humans (Testud et al. 1996). Nausea and vomiting have also been reported. In rats, the LD50 for RDX is 70-80 mg/kg, while 12.5 mg/kg is enough to cause seizures (Testud et al. 1996). The Agency for Toxic Substances and Disease Registry set a minimum risk level of 0.06 mg/kg•day for acute exposure and 0.03

mg/kg•day for intermediate (15-364 days) exposure. Most reported cases of human RDX poisoning have occurred as a result of inhalation of RDX powder by munitions factory workers (Testud et al. 1996). The most common symptoms found in these individuals were seizures and disorientation. In each case the seizures and other symptoms cleared up without any residual effects. No human deaths have been reported as a result of RDX exposure.

A number of studies have tested both acute and chronic effects of RDX exposure in aquatic animals. Peters et al. (1991) found that RDX was not acutely toxic to three different aquatic invertebrate species at the solubility limit in water. Chronic exposure did, however, reduce the reproduction rate of the daphnid *Ceriodaphnia dubia* by 48.9 percent at a concentration of 16.4 mg/L (Peters et al. 1991). The lowest observed effects concentration (LOEC) in *C. dubia* was 6.01 mg/L. RDX has been found to be both acutely and chronically toxic to a variety of fish, including rainbow trout, catfish, and bluegill (Burton et al. 1994). The acute 96-hour LC50 for 15-17 day old fathead minnows (*Pimephales promelas*) in the Burton study was 12.7 mg/L. Chronic exposure to RDX at 2.4 mg/L caused weight reduction, while a concentration of 4.9 mg/L decreased juvenile survival rates.

Physical and Chemical Treatment of RDX-Contaminated Soils

Until recently, physical and chemical methods have been the most common means of disposing of soils contaminated with RDX. Such soils were often incinerated or transported to approved hazardous waste landfills. Dumping RDX in landfills is now prohibited. While incineration is often an effective treatment option for RDX disposal,

its use poses many problems that may make it unfeasible, especially for large clean-up sites. The high monetary cost involved is the major factor making incineration difficult to implement. The cost of incinerating contaminated soils averages $120-$410/ton (U.S. EPA 1998). Incineration facilities must charge large amounts of money because of the multitude of laws and regulations that must be followed and the large amount of energy that must be expended in the process. Transportation of RDX-contaminated wastes is expensive. Often, the EPA does not approve shipping hazardous wastes off-site, preventing both off-site incineration and dumping in landfills.

Portable incineration units can be used to dispose of RDX-contaminated soil on-site, eliminating the need for transporting soils off-site. These incineration units, however, must be transported to the clean-up site, limiting their size. The largest portable incineration unit observed in EPA studies was 14 feet in outside diameter (U.S. EPA 1998). Community acceptance of incinerators may also be an issue if residents perceive problems such as noise, emissions, and the possibility of outside wastes being transported to the incinerator to be a risk.

Recently, zero-valent iron has been examined as a method for chemically treating RDX-contaminated soils. Singh et al. (1998b) found that 32 mg RDX/L was completely removed in liquid reactors within 72 hours when 10 g Fe^0/L was added. Nitroso derivatives of RDX accounted for 26 percent of the RDX lost within the first 24 hours. These intermediates disappeared within 96 hours. RDX degradation in contaminated soils was also tested using this method (Singh et al. 1998b). When soils containing 3600 mg RDX/kg were amended once with 50 g Fe^0/kg, and a moisture content of 0.35-0.40 kg water/kg soil was maintained, 52 percent of the RDX disappeared within 12 months. The

researchers proposed that addition of zero-valent iron could be used as a method for in situ remediation of RDX-contaminated soils.

Biological Treatment of RDX

Biological treatment has been studied in depth recently as a more feasible method of RDX removal from contaminated soil and munitions factory waste streams. A number of researchers have reported the biodegradation of RDX. McCormick et al. (1981) found that anaerobic sewage sludge microorganisms reduced the nitro groups on the RDX in a stepwise fashion forming the mono-, di-, and trinitroso intermediates MNX, DNX, and TNX. The ring cleavage products hydrazine, dimethylhydrazine, formaldehyde, and methanol were also produced. McCormick et al. (1981) postulated that the nitroso groups were further reduced to hydroxylamino groups, which would destabilize the ring structure and leave it open to hydrolytic attack. Other researchers have also reported the formation of MNX, DNX, and TNX (Kitts et al. 1994; Freedman and Sutherland 1998).

TNX may not need to be produced for ring cleavage to occur. Young et al. (1997) observed ring cleavage of MNX without the production of DNX or TNX when a consortium of horse manure microorganisms was used. The reduction of RDX to MNX reportedly occurred only during stationary growth phase and was described as following first order kinetics. RDX was transformed at a rate of 0.022 L/g cells•hr.

The degradation of RDX appears to be inhibited by the presence of nitrogen compounds. Ammonium has been reported to inhibit RDX degradation (Yang et al. 1983). Freedman and Sutherland (1998) found that the presence of nitrate also inhibited RDX degradation. Once the nitrate was depleted, however, RDX was transformed. The

presence of RDX had no effect on the reduction of nitrate. RDX degradation also appears to be inhibited by the presence of the nitroaromatic TNT (Light et al. 1998; Shen et al. 1998). TNT contamination often coincides with RDX contamination in soils at munitions facilities.

Most studies have demonstrated RDX degradation under anaerobic conditions but not in the presence of oxygen (Kitts et al. 1994; McCormick et al. 1981). Binks et al. (1995) reported, however, that a strain of *Stenotrophomonas maltophilia* was able to transform RDX aerobically under nitrogen-limiting conditions. This microorganism was reportedly able to use the RDX as a sole source of nitrogen. The only intermediate identified in the study was methylene-N-(hydroxymethyl)-hydroxylamine-N'-(hydroxymethyl)nitroamine. Jones et al. (1995) also reported the aerobic degradation of RDX under nitrogen-limiting conditions.

Composting has also been studied as a method for biologically treating RDX contaminated soils. Despite being a largely aerobic method of bioremediation, a few studies have found that RDX concentrations were significantly reduced through composting (Isbister et al. 1984; Griest et al. 1991, 1995). Isbister et al. (1984) reported a 78.4 percent reduction in the detectable RDX concentration after six weeks of composting. No intermediates were identified, but 55 percent of ^{14}C-labeled RDX was recovered as CO_2. Griest et al. (1991) reported that 97.2 percent of RDX could no longer be detected after composting. The degree of RDX removal, however, was dependent on the soil concentration in the compost. Lower concentrations of contaminated soil had much higher RDX removal efficiencies. It was also found that a non-aerated windrow

compost was slightly more efficient than an aerated windrow compost (Griest et al. 1995).

So far, composting experiments have failed to completely characterize the fate of RDX as it is degraded. Under the thermophilic conditions common to composting, it is possible for either RDX or its metabolic breakdown products to become more tightly bound to the soil. Another explanation for the apparent contradiction between composting studies and other aerobic reactor studies is that anaerobic conditions may develop over time in compost piles, especially if aeration is incomplete.

MATERIALS AND METHODS

Bacterial Culture and Media

Seed reactors were established using municipal anaerobic digester sludge or secondary clarifier underflow as initial inoculum. In a 120-ml serum bottle, 5 mL of anaerobic sludge or clarifier underflow was added to 95 ml of liquid nutrient medium and 0.5 g of soil. One seed reactor was set up with anaerobic sludge and 20 mM additional sodium sulfate, one with clarifier underflow and 20 mM sodium nitrate added, and one with anaerobic sludge and no added electron acceptor. After flushing the reactor contents and filling the head-space with nitrogen, the serum bottles were sealed with a rubber stopper and metal serum bottle seal. Molasses (0.3%) was used as the carbon source based on experiments by Boopathy et al. (1994) showing that TNT degradation occurred most quickly when molasses was supplied as a substrate for growth. Table 1 outlines the contents of the nutrient medium used in the reactors.

TABLE 1. Contents of the nutrient medium used in the soil slurry reactors.

Compound	Concentration (g/L)	Concentration (mM)
K_2HPO_4	3.50	40
KH_2PO_4	1.50	22
NH_4Cl	0.40	25
NaCl	0.05	0.85
$CaCl_2$	0.04	0.36
MgCl	0.01	0.10
$NaHCO_3$	0.75	8.93
yeast extract	0.10	
$FeSO_4 \cdot 7H_2O$	0.20	
$ZnSO_4 \cdot 7H_2O$	0.01	
$MnCl_2 \cdot 4H_2O$	0.003	
$CoCl_2 \cdot 6H_2O$	0.02	
$CuCl_2 \cdot 2H_2O$	0.001	
$NiCl_2 \cdot 6H_2O$	0.002	
$Na_2MoO_4 \cdot 2H_2O$	0.003	

Experimental reactors were set up similarly to the seed reactors, except 2 mL of inoculum from the seed reactors was added to 98 mL of nutrient medium and varying amounts of soil unless otherwise specified. The seed reactor with added sodium sulfate was used for sulfate experiments, the no-added-electron-acceptor seed was added to no-added-electron-acceptor experimental reactors, and sodium nitrate supplemented seed was used for the experimental nitrate reactors unless otherwise specified. The experimental reactors were flushed with either nitrogen gas or argon prior to sealing. All experimental reactors were maintained in the dark on an Eberbach reciprocating shaker table set at a rate of 135/min. A pH of 6.5 was maintained in the experimental reactors. For most of the experiments ambient temperature was monitored, but not controlled. The temperature range was between 25°C and 30°C until around day 150 of the varied electron acceptor experiments when the temperature dropped to a range of 17°C to 22°C. After day 218 in the varied electron acceptor experiments, and for the duration of the

TNX/nitrate and no inoculum experiments, a small 1000 W desktop heater was placed near the shaker table and a temperature of approximately 32°C was maintained.

Description of Experimental Reactors

Abiotic control reactors

Duplicate abiotic control reactors were established with 0.23 g of soil, 100 mL of nutrient medium, and 0.5 g of sodium azide. No seed inoculum was added to these reactors. Both reactors were flushed with argon gas and the headspace filled with argon. Fully solubilized, the RDX concentration in the reactor liquid phase from the contaminated soil would have been approximately 40 mg/L, determined by prior methanol extraction of samples of the same soil. Procedures for the methanol extraction are detailed later in this section.

RDX degradation under different electron accepting conditions

Duplicate reactors were set up with three different soil concentrations and three different electron acceptor applications. Either sodium sulfate (20 mM), sodium nitrate (20 mM), or no extra electron acceptor was added to duplicate reactors containing 0.33 g, 0.83 g, or 2.5 g of soil. Sulfate reactors were inoculated with seed from the sulfate seed reactor. Nitrate reactors were inoculated with seed from nitrate seed reactors, and no-extra-electron-acceptor reactors were inoculated with seed from no-extra-electron-acceptor seed reactors. A total of 18 separate reactors (9 different conditions being tested) were established.

Effect of TNT presence on RDX degradation

The concentration of TNT was varied, while soil volume was held constant. Duplicate reactors were set up with 0.22-0.23 g of soil from three separate soil subsamples (labeled soil A, soil B, and soil D) with similar RDX concentrations, but different TNT concentrations. This soil mass contained an amount of RDX equivalent to approximately 40 mg/L if all of the RDX was solubilized into the liquid phase of the reactors. The TNT concentration would be 10.7 mg/L fully solubilized in soil A reactors, 4.9 mg/L in soil B reactors, and 0.4 mg/L in soil D reactors. Sulfate was added to each reactor at a concentration of 20 mM. Each reactor was inoculated with sulfate-amended seed. The reactors were flushed and the headspace filled with nitrogen prior to sealing.

Additional nitrate reactors

In addition to the nitrate reactors established in the electron acceptor experiment, two additional reactors were set up using 0.23 g of soil D, 20 mM sodium nitrate, and seed that was not originally amended with added electron acceptor. These reactors were flushed with nitrogen gas prior to sealing. Sodium nitrate was again added to the reactors after the first nitrate reduction cycle, but no additional nitrate was added after subsequent depletion. To determine if the nitrogen headspace was responsible for stalling of the nitrate reduction activity in these reactors, two more duplicate reactors were established using the aqueous phase of the RDX-degrading reactor in the previous pair as seed inoculum. Argon was used as a headspace gas rather than nitrogen for these two reactors.

Determination of Soil TNT, RDX, and HMX Concentrations

To determine the concentration of contaminants in soil, 150 mL of contaminated soil was placed in 4 separate 500-mL glass jars. Each jar was hand-shaken for about 30 seconds to ensure even distribution of the contaminants. Next, 0.50 g of soil from each jar was placed in a separate 120-mL serum bottle, and 100 ml methanol was added to each bottle. The bottles were sealed and placed on a shaker table for five days. After 5 days, the methanol was analyzed by HPLC for RDX, TNT, and HMX concentrations.

Sample Preparation and Analysis

Aqueous phase samples were withdrawn from the reactors via syringe at a volume of 1 mL for HPLC and IC analyses. These samples were then centrifuged at full speed for 6 minutes using a 1 Amp International Clinical Centrifuge. Aqueous phase RDX, TNT, HMX, and RDX metabolite concentrations were analyzed using a Beckman HPLC with a 20 µl manual injection loop, a model 127 solvent pump, and a model 166 absorbance detector set at a wavelength of 254 nm. A Beckman reverse-phase C-18 column was used. The solvent used was 55% methanol and 45% water at a flow rate of 1.5 mL/min. Verification of RDX metabolite identities was performed using a Waters HPLC with automated injection, a C-18 reverse phase column, and a photodiode array detector capable of producing UV absorption spectra. A solvent of 35% methanol and 65% water and an injection volume of 20 µl was used with the Waters HPLC. RDX, TNT, and HMX standards were obtained from Chem Service (West Chester, PA), while Dr. Spanggord of SRI International (Menlo Park, CA) provided the RDX metabolites MNX, DNX, and TNX.

Prior to ion analysis the centrifuged samples were diluted 1:10. Analysis was then performed using a Dionex Series 2000i ion chromatograph with a 25 µL sample loop and a Dionex AS4A-SC 4-mm column. The eluent consisted of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ at a flow rate of approximately 2.0 mL/min. The regenerant was 25 mN sulfuric acid. Centrifuged samples from the reactor were diluted 1:10 with deionized distilled water prior to injection.

The headspace was periodically analyzed for the presence of methane using a Gow-Mac Series 350 gas chromatograph with a thermal conductivity detector. Gas samples of 1 ml were injected onto a Gow-Mac Porapak column. The carrier gas was helium set at a rate of 60 ml/min. Column temperature was 70°C, detector temperature was 170°C, and the injector temperature was 105°C. Bridge current was set to 70 mA.

Presence/Absence Test for Sulfate-Reducing Bacteria

A presence/absence test for sulfate-reducing bacteria was performed on one reactor that had been given 20 mM sulfate, one reactor that had not been given additional sulfate, one that had been given additional nitrate in cycles, and one reactor that had not been inoculated with anaerobic digester sludge. Small 6-mL vials were filled with 0.5 ml of supernatant from the reactor to be tested and enough sulfate reducer medium to completely fill the vial, leaving no headspace. The sulfate reducer medium was adapted from Dianou et al. (1998) and modified to be a liquid medium by omitting the addition of agar. The medium contained lactate as a carbon source, sulfate as an electron acceptor, thioglycolate as a reducing agent, and resazurin as a redox indicator. The presence of sulfide precipitate is a positive result for the presence of sulfate-reducing bacteria.

RESULTS

Concentration of Explosives in the Pantex Soil

Soil from the Pantex site was divided into four subsamples and labeled as soil A, soil B, soil C, and soil D. Soil A had 18,600 mg RDX/kg soil, 4960 mg TNT/kg, and 1600 mg HMX/kg. Soil B had concentrations of 18,200 mg RDX/kg, 2220 mg TNT/kg, and 1080 mg HMX/kg. Soil C contained 15,500 mg RDX/kg, 266 mg TNT/kg, and 1820 mg HMX/kg. Soil D had concentrations of 17,000 mg RDX/kg, 173 mg TNT/kg, and 2130 mg HMX/kg. The mean concentration of each contaminant in the soil from the Pantex site was 17,300 mg RDX/kg, 1910 mg TNT/kg, and 1660 mg HMX/kg.

Desorption Kinetics of RDX in Experimental Reactors

A pair of duplicate abiotic soil reactors served as test reactors for determining the rate of RDX desorption from soil and solubilization into the reactor liquid phase (Fig. 2). The chemically sterilized reactors contained 0.23 g of soil and 100 ml of nutrient medium. The initial transport of RDX from soil to liquid was quick in response to the initial agitation from adding the nutrient media to the reactors. Also, some of the RDX may have been only loosely bound to the soil. After a day, however, the transport of RDX into the liquid phase slowed dramatically and appeared to follow first order kinetics. No degradation of RDX ever occurred in the abiotic reactors. RDX dissolved into the liquid phase at a rate of 0.85 mg/L•day.

Effect of Soil TNT on RDX Degradation

Soil slurry reactors were set up to determine if the TNT content in the Pantex soil would affect the rate of RDX degradation when low concentrations of soil were used. Previous experiments using 20 g soil/100 ml reactor aqueous phase had demonstrated a noticeable inhibition of RDX degradation in the presence of TNT (Light et al. 1998). Soil from subsamples A, B, and D (described above) were used because they offered a range of TNT concentrations that could be found in the entire soil sample from the Pantex site. These reactors contained 0.22-0.23 g of soil and 20 mM sulfate. The fully desorbed and solubilized RDX concentration would be approximately 40 mg/L, just below the solubility limit of RDX at 20°C. The initial concentration of TNT if fully solubilized would be approximately 10.7 mg/L in the soil A reactors, 4.9 mg/L in the soil B reactors, and 0.4 mg/L in the soil D reactors.

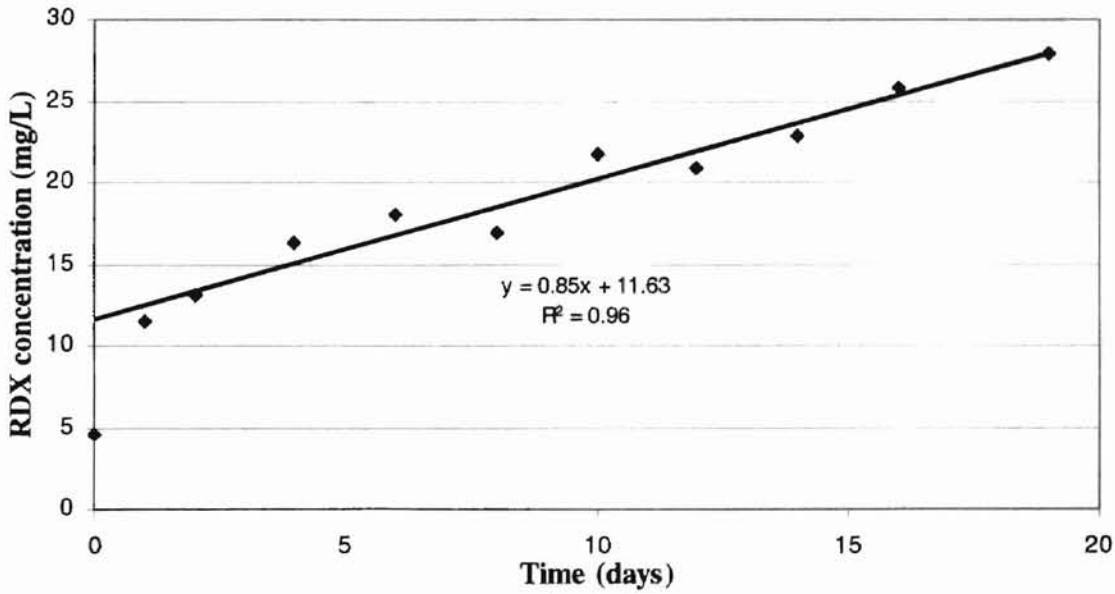


FIGURE 2. Desorption of RDX from soil into the liquid phase of abiotic soil slurry reactors. Data are the means of the duplicate reactors. Trendline ignores day 0 data.

Table 2 summarizes the time required for the degradation of RDX and its intermediate reduction products (MNX, DNX, and TNX) in each reactor set. No correlation existed between TNT concentration and the residence time of RDX and its intermediates. Moreover, TNT was never detected in the liquid phase of any of the reactors. TNT was also not detected in the soil after completion of the experiment. TNT is known to sorb strongly onto soils; therefore its degradation in the reactors likely occurred as rapidly as desorption. With the small amount of soil that was used in these reactors, the concentration of TNT in the Pantex soil was not high enough to noticeably affect the rate of RDX degradation.

TABLE 2. Amount of time RDX and RDX metabolites remained in reactors with varied TNT concentrations. RDX and TNT in the reactors were initially sorbed onto the soil. All reactors had 0.23 g soil and 20 mM sodium sulfate added.

TNT Concentration	Days Until No RDX Remained	Days Until Only TNX Remained	Days Until No Intermediate Remained
10.7 mg/l	13	13-15	31-36
4.9 mg/l	8	8	54-83
0.4 mg/l	10	10-13	54-57

RDX Degradation Under Different Electron Accepting Conditions

Different electron-accepting conditions were established in another set of duplicate reactors containing various amounts of RDX-contaminated soil. These reactors were either given 20 mM sodium nitrate, 20 mM sodium sulfate or no additional electron acceptor. Although no additional electron acceptor was given to some of the reactors, the nutrient medium used in the soil slurries still had about 1 mM sulfate without the addition of extra sodium sulfate. Table 3 summarizes the amount of time that RDX and its

intermediate degradation products persisted in reactors containing 0.33 g, 0.83 g, and 2.5 g of soil and the different amounts of electron acceptor.

TABLE 3. Amount of time RDX and RDX metabolites remained in reactors with varied soil volume and added electron acceptor.

Mass of Soil Added	Electron Acceptor Added	Days Until < 2mg/L RDX Remained	Days Until Only TNX Remained	Days Until No Intermediates Left
0.33 g	sulfate	12-16	44	75-111
	no electron acceptor	5-40	54	97-160
	nitrate		No significant activity	
0.83 g	sulfate	26-33	60-68	153-167
	no electron acceptor	19-37	75	243-250
	nitrate		No significant activity	
2.5 g	sulfate	35-44	68-111	271-295
	no electron acceptor	33-37	60	264-271
	nitrate		No significant activity	

Sulfate reactors

RDX was completely transformed into products that could not be detected by the HPLC methods used in all reactors given 20 mM sodium sulfate. The intermediates MNX, DNX, and TNX were all formed and subsequently degraded. TNX was degraded much more slowly than RDX, MNX, and DNX in all reactors. The lack of detectable intermediates after TNX suggests that ring cleavage occurred. HMX degradation occurred at a much slower rate than RDX degradation; however, an estimate of the degradation rate and the fate of metabolites could not be determined because of the low solubility of HMX. Visibly, sulfide production could not be detected until TNX was the only remaining RDX metabolite in the reactors. A black precipitate formed in each of the reactors after RDX, MNX, and DNX were depleted. Methane was not produced in any of the sulfate reactors, but acetate was detected through ion chromatography, indicating fermentation. A presence/absence test was used to attempt to isolate sulfate-reducing

bacteria from these reactors after the experiments were completed. The test was positive for sulfate reducers. Examination of the presence/absence test microorganisms under a microscope revealed motile vibrio-shaped organisms, which were likely to be members of the genus *Desulfovibrio*.

The initial concentration of RDX added to the duplicate 0.33-g soil reactors in the form of contaminated soil was approximately 59 mg/L (total dissolved and undissolved RDX). RDX concentration in these reactors dropped from above the solubility limit to less than 2 mg/L within 12 days in one reactor and 16 days in the other reactor. After this point a small concentration of RDX remained until day 44 in both reactors, suggesting that the rate of RDX degradation was limited by the slow rate of desorption described previously (Fig. 2). TNX was the only remaining RDX metabolite detected after 44 days in both reactors. TNX could no longer be detected in the liquid phase after 75 days and 111 days respectively in the duplicate reactors.

The initial total RDX concentration (dissolved and undissolved) from the contaminated soil in the duplicate 0.83-g soil reactors was approximately 144 mg/L. RDX concentration in these reactors fell below 2 mg/L after 26 days and 33 days in duplicate reactors. TNX was the only remaining metabolite after 60 days and 68 days, and TNX was completely degraded after 153 days and 167 days in these two reactors.

The initial total RDX concentration (dissolved and undissolved) in the duplicate 2.5-g soil reactors was approximately 450 mg/L. RDX concentration in these reactors fell below 2 mg/L after 35 days and 44 days in duplicate reactors. TNX was the only remaining detectable intermediate after 68 days and 111 days. TNX was completely degraded within 271 days and 295 days in each of the two reactors.

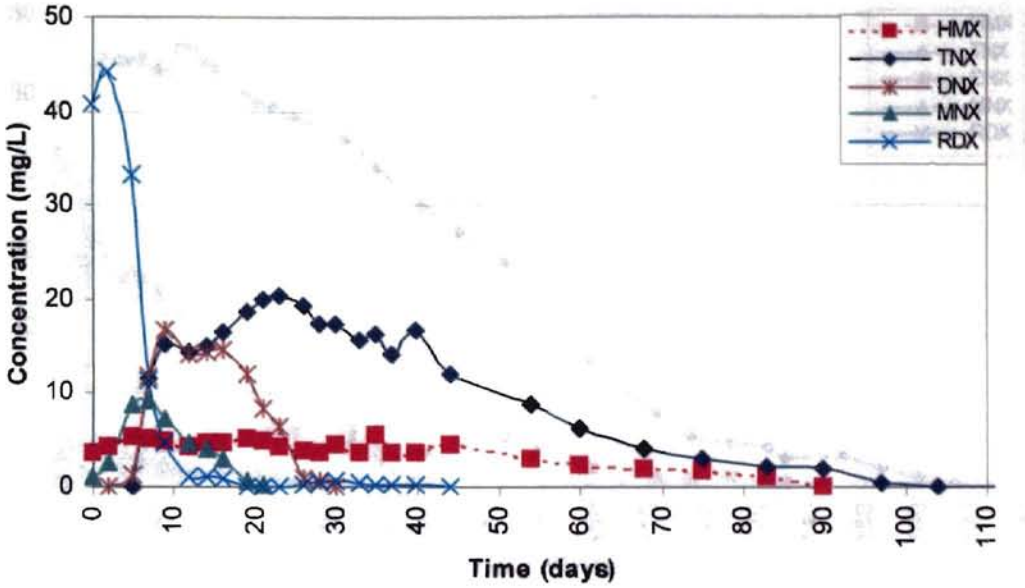
No extra electron acceptor

Reactors not given sodium sulfate acted similarly to the reactors dosed with extra sulfate, as shown in Figures 3-5. Methane production did not occur in any of these reactors. Acetate formation could be detected by ion chromatography, however, which is indicative of fermentative metabolism. The intermediates MNX, DNX, and TNX were produced and subsequently degraded. No intermediates after TNX could be detected by HPLC. The rate of TNX degradation was slightly slower in the reactors not given extra sulfate. Again, no intermediates after TNX could be detected by HPLC, suggesting that ring cleavage had occurred. A presence/absence test for sulfate reducers performed after the experiments were completed was positive for sulfate reducers in these reactors. Motile vibrio-shaped organisms, most likely members of the genus *Desulfovibrio*, were isolated from this test.

RDX concentration in the duplicate 0.33-g soil reactors (59 mg RDX/L total dissolved and undissolved from the soil) dropped below 2 mg/l after 5 days in one reactor and after 40 days in the other reactor. Trace detectable concentrations of RDX continued to persist until day 54 in both reactors. TNX was the only remaining RDX metabolite after 54 days in both reactors. TNX could no longer be detected in the liquid phase after 97 days and 160 days respectively in the duplicate reactors.

RDX concentration in the duplicate 0.83-g soil reactors (144 mg RDX/L total dissolved and undissolved from the soil) fell below 2 mg/l after 19 days and 37 days, respectively. TNX was the only remaining RDX metabolite after 75 days in both reactors. TNX was completely degraded after 243 days and 250 days in these reactors.

(a)



(b)

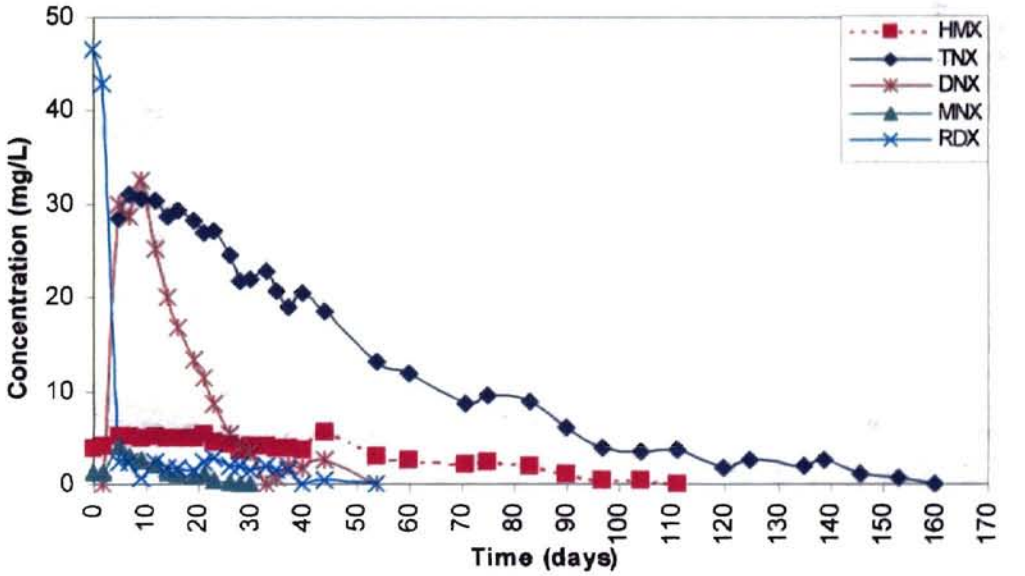
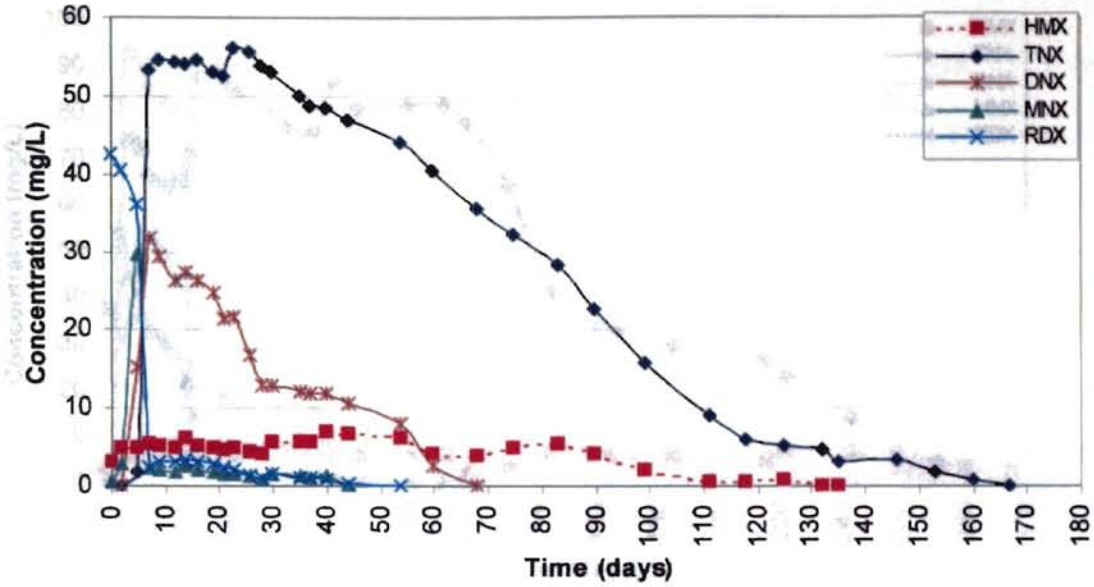


FIGURE 3. Composite plot of duplicate reactors containing 0.33 g soil and (a) 20 mM sodium sulfate added; (b) no extra electron acceptor added.

(a)



(b)

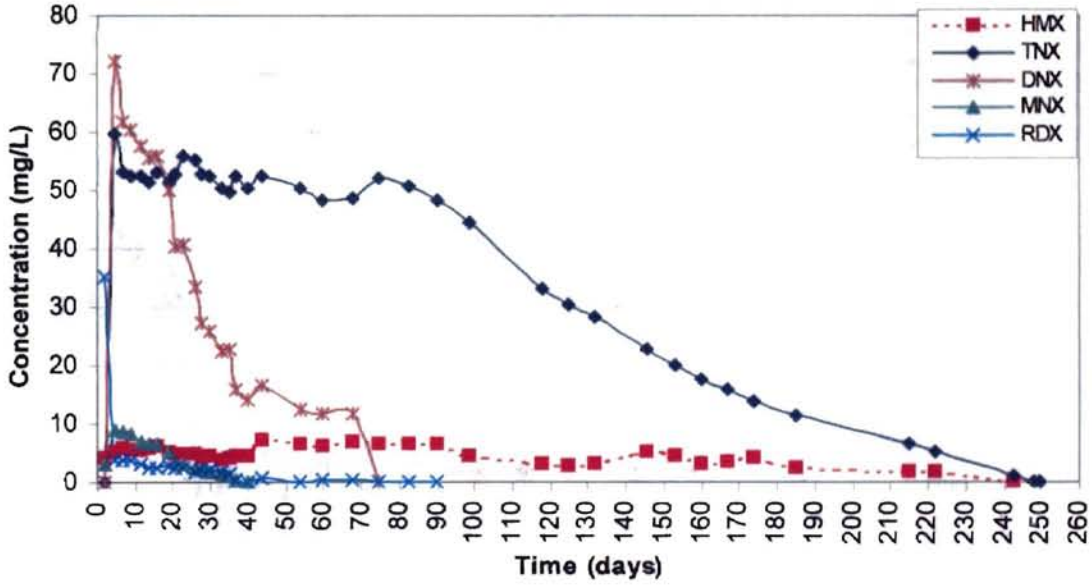


FIGURE 4. Composite plot of duplicate reactors containing 0.83 g soil and (a) 20 mM sodium sulfate added; (b) no extra electron acceptor added.

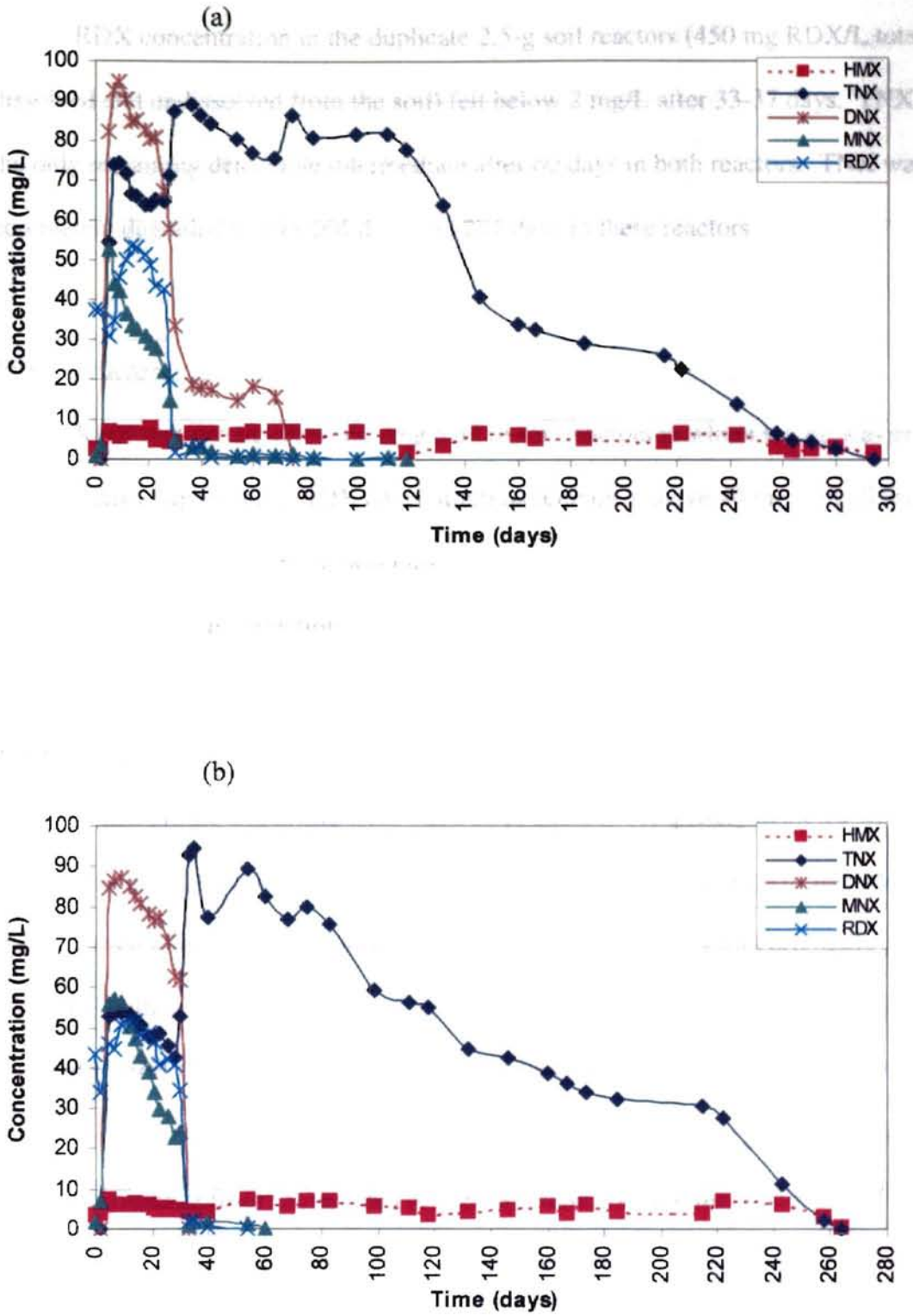


FIGURE 5. Plot of RDX and RDX intermediates versus time in reactors containing 2.5 g soil and (a) 20 mM sodium sulfate added; (b) no extra electron acceptor added.

RDX concentration in the duplicate 2.5-g soil reactors (450 mg RDX/L total dissolved and undissolved from the soil) fell below 2 mg/L after 33-37 days. TNX was the only remaining detectable intermediate after 60 days in both reactors. TNX was completely degraded within 264 days and 271 days in these reactors.

Nitrate reactors

RDX was not significantly degraded in soil reactors in which nitrate was present. After 38 days, liquid phase RDX concentrations remained above 40 mg/L in all reactors containing nitrate regardless of soil mass (Fig. 6). Turbidity increased, gas was produced, and nitrate reduction did occur after inoculation, suggesting that active nitrate-reducing microorganisms were present. Nitrate was replaced in these reactors when it was reduced.

When nitrate and nitrite were allowed to become completely depleted without replacing it, RDX was degraded (Fig. 7). No significant accumulation of intermediates ever occurred after nitrate depletion, however. Unlike in the reactors originally given additional sulfate or no extra electron acceptor, MNX was the only RDX metabolite detected by HPLC after nitrate depletion. MNX concentration never exceeded 4 mg/L. Ring cleavage of MNX apparently occurred before additional nitro groups were reduced. Another difference from the reactors not given nitrate was that the production of sulfide precipitate occurred at the same time as RDX degradation even while RDX was still present.

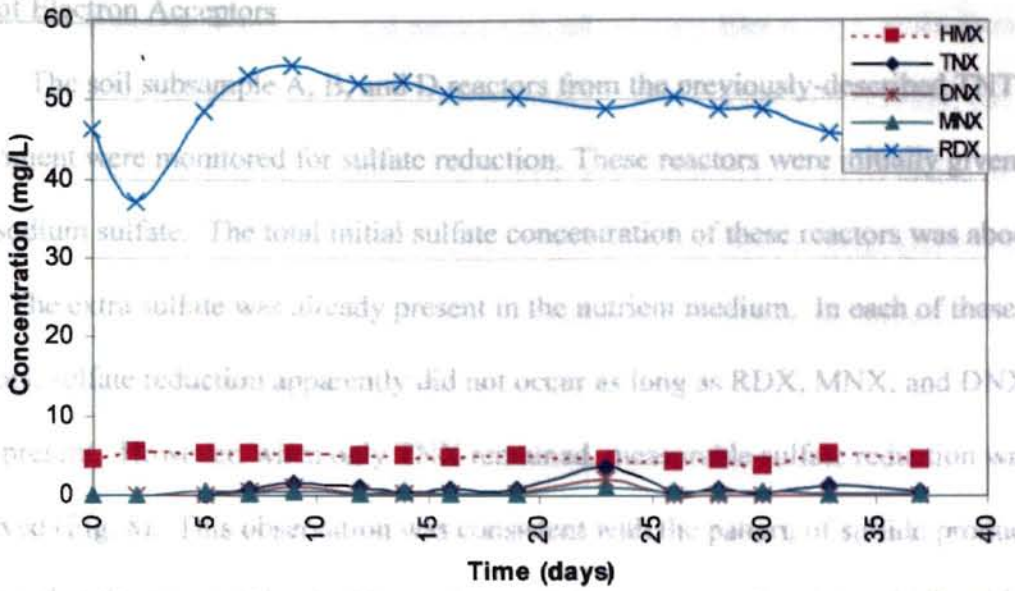


FIGURE 6. Composite plot of RDX and RDX metabolites versus time in duplicate reactors containing 0.33 g soil and 20 mM sodium nitrate added.

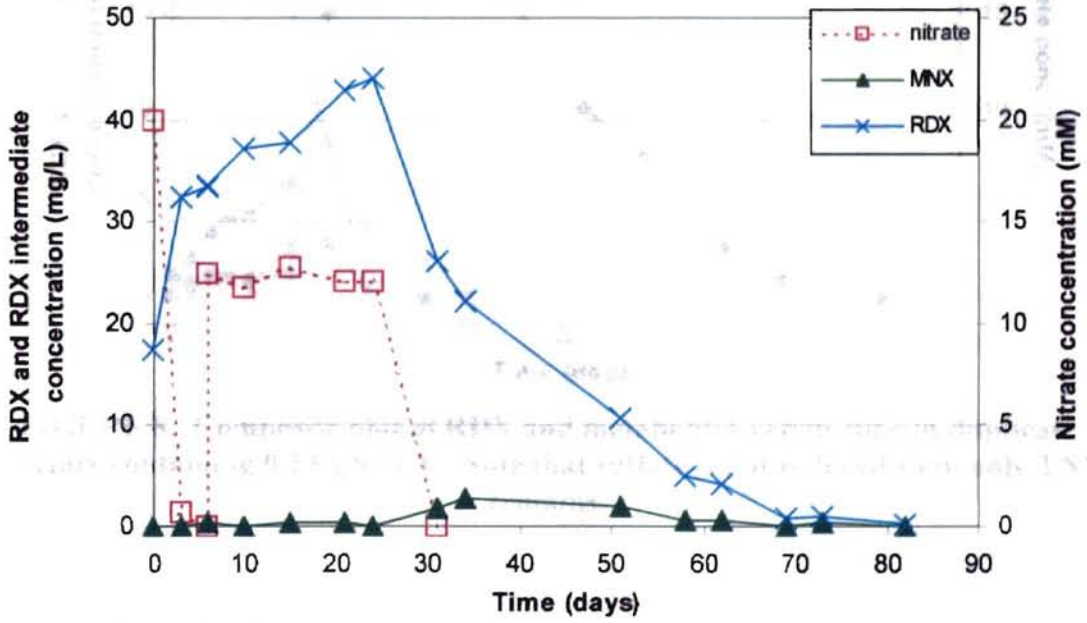


FIGURE 7. Plot of RDX and RDX metabolites versus time in one reactor containing 0.23 g soil and given nitrate as an added electron acceptor.

Fate of Electron Acceptors

The soil subsample A, B, and D reactors from the previously-described TNT experiment were monitored for sulfate reduction. These reactors were initially given 20 mM sodium sulfate. The total initial sulfate concentration of these reactors was about 21 mM. The extra sulfate was already present in the nutrient medium. In each of these reactors, sulfate reduction apparently did not occur as long as RDX, MNX, and DNX were present. However, when only TNX remained, measurable sulfate reduction was observed (Fig. 8). This observation was consistent with the pattern of sulfide production witnessed in the reactors that had been given varying amounts of soil, in which sulfide was only produced when TNX was the only RDX metabolite present.

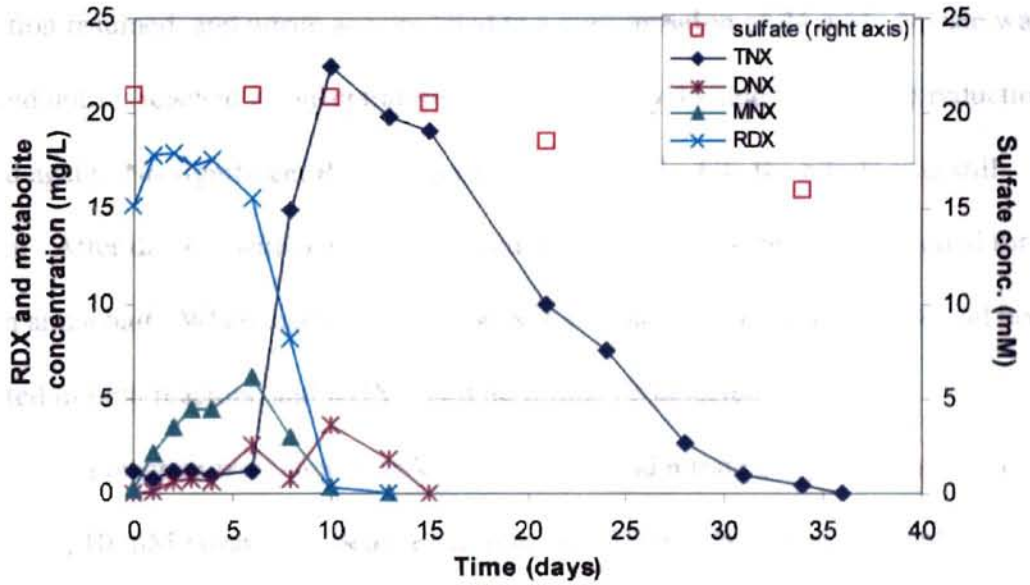


FIGURE 8. Composite plot of RDX and metabolites versus time in duplicate reactors containing 0.23 g Soil A. Note that sulfate is not reduced until only TNX remains.

Unlike with sulfate reduction, nitrate reduction occurred immediately. Nitrite was formed and accumulated until nitrate was completely depleted. In the reactor

characterized in Figure 6, additional nitrate was added once after 6 days, when nitrate had been completely depleted. Further nitrate reduction did not occur immediately, but within 25 days of adding more nitrate, the nitrate was completely reduced in one of two duplicate reactors. Nitrate reduction remained stalled in the other reactor, and RDX degradation did not occur. A drop in RDX concentration followed the complete reduction of nitrate and nitrite in the active reactor.

Two more nitrate-reducing reactors were set up to confirm these results. After one day, the initial nitrate concentration of 20 mM had been reduced. Nitrite was still present at a concentration of approximately 13 mM. After an additional 20 mM nitrate was added, both nitrate and nitrite reduction stalled until day 35. At this time nitrate reduction resumed, and nitrite accumulated to a concentration of 24 mM. Nitrate was reduced until it reached a concentration of 1 mM on day 45, and then nitrate reduction stalled again. No significant RDX degradation occurred while the nitrate was still present. After day 67, with activity still stalled, the reactors were left unattended for a month and a half. When analyzed again on day 115, all the nitrate and nitrite had been depleted in both reactors, and RDX could no longer be detected.

Both reactors were then respiked with RDX and nitrate in cycles (Fig. 9). In the first cycle, 10 mM nitrate was depleted in less than 5 days. Nitrite was completely reduced immediately following nitrate reduction, apparently at too quick a rate to quantify. Once both electron acceptors were depleted, RDX was quickly degraded without accumulation of metabolites. When nitrate was added in a second cycle, RDX degradation again stalled. Nitrate at a concentration of 14 mM was depleted in six days and nitrite accumulated until the nitrate was gone, but was quickly depleted after nitrate

was completely reduced. RDX was again subsequently degraded without significant metabolite formation at a rate of more than 3.5 mg/L•day. After a third cycle of nitrate addition, however, nitrate reduction slowed, and nitrite did not quickly disappear after nitrate depletion. Additional molasses was added, but this had no effect on nitrate or nitrite reduction. Unlike when nitrate was present, RDX degradation did occur in the presence of nitrite only, although at a slower rate than in the two previous cycles when nitrite was fully reduced. This trend is illustrated in Figure 9 after day 27.

A presence/absence test for sulfate-reducing bacteria was performed at the end of the nitrate cycling experiment. This test confirmed that sulfate reducers were still present after all of the nitrate addition cycles had been completed. A small, motile, vibrio-shaped microorganism and a large, fat, motile rod were both present in the presence/absence test.

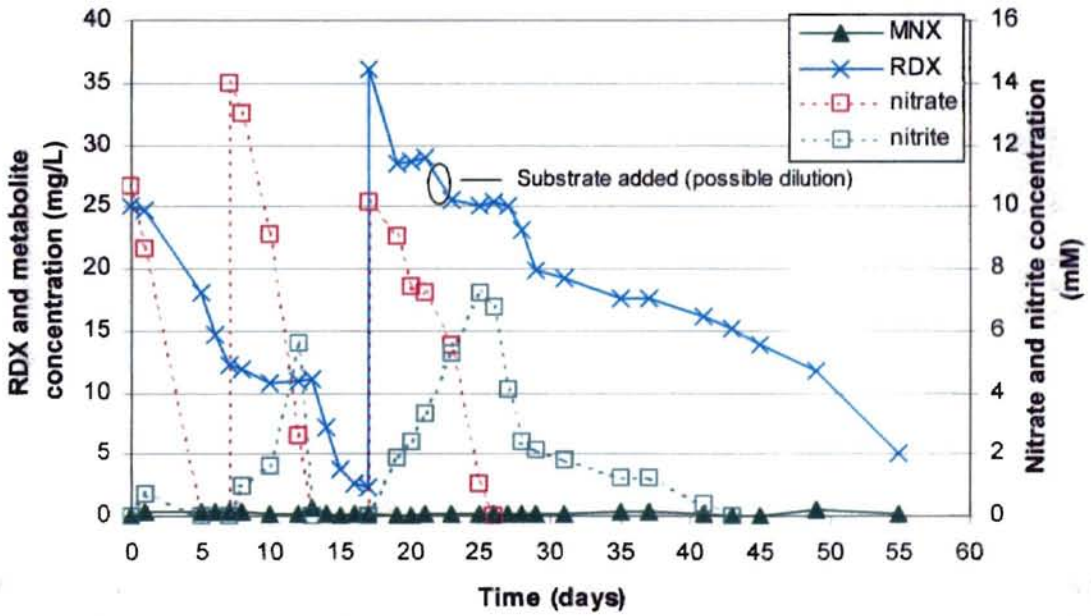


FIGURE 9. Composite plot of duplicate reactors respiked with RDX and nitrate. Note that nitrate and nitrite reduction activity significantly slows during the third cycle of nitrate addition. Substrate addition was in the form of molasses in water.

Potential use of a two-stage anaerobic system for TNX degradation

As demonstrated above, RDX was not transformed in the presence of nitrate. Reduction of nitrate occurred before any nitro groups were reduced on the RDX molecule. This experiment was set up to determine whether the reduced trinitroso metabolite could be oxidized under nitrate-reducing conditions. Two reactors that had previously completely degraded TNX under sulfate-reducing conditions were respiked with TNX, and sodium nitrate was added. While nitrate was reduced in both reactors to nitrite, the nitrite was persistent, and TNX was not significantly degraded in the presence of either potential electron acceptor (Fig. 10).

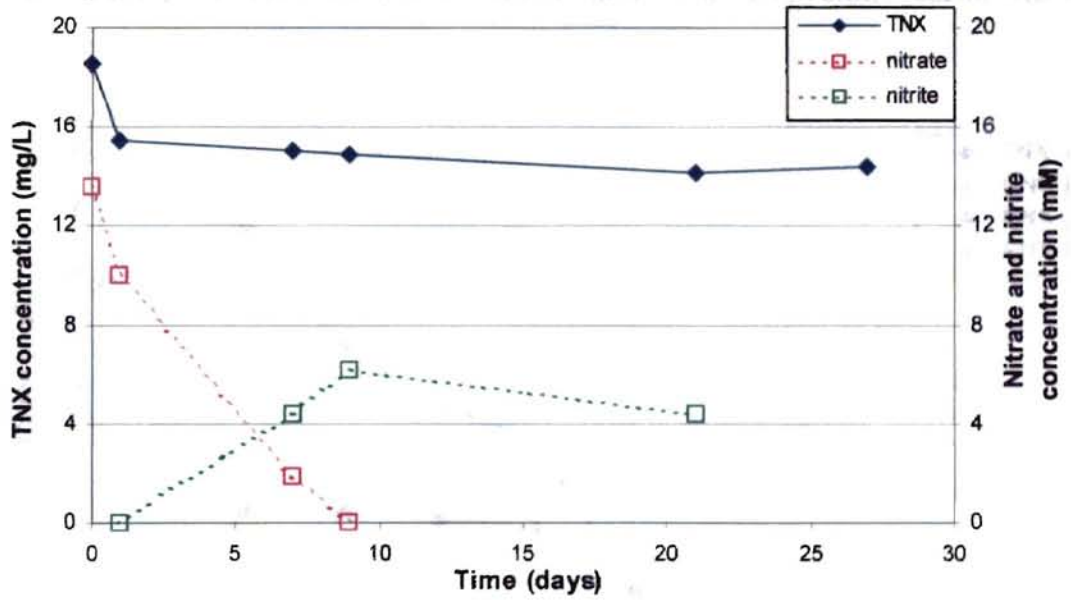


Figure 10. Composite plot of duplicate active TNX-degrading reactors with TNX respiked and sodium nitrate added. Reactors had previously completely degraded TNX under sulfate-reducing conditions.

RDX Biodegradation Potential Using Only Native Soil Bacteria

Each of the reactors discussed previously was inoculated with anaerobic sewage sludge to supply a population of active anaerobic fermenting and sulfate-reducing microorganisms. In this experiment a pair of duplicate reactors was set up without inocula to examine the necessity of this addition of outside organisms. The native soil bacteria were able to degrade RDX in a similar fashion to the anaerobic sewage sludge bacteria, except that the metabolites MNX, DNX, and TNX accumulated at lower concentrations when using the native soil bacteria (Fig. 11). TNX was only detected in trace concentrations. When 0.23 g of soil were used (40 mg RDX/L sorbed onto soil) without inocula, RDX and its metabolites were undetectable in the liquid phase within 13-19 days. Similar reactors with inocula had TNX present until at least day 31 (see Table 2 and Fig. 8). The highest RDX concentration observed in either reactor was 16.4 mg/L.

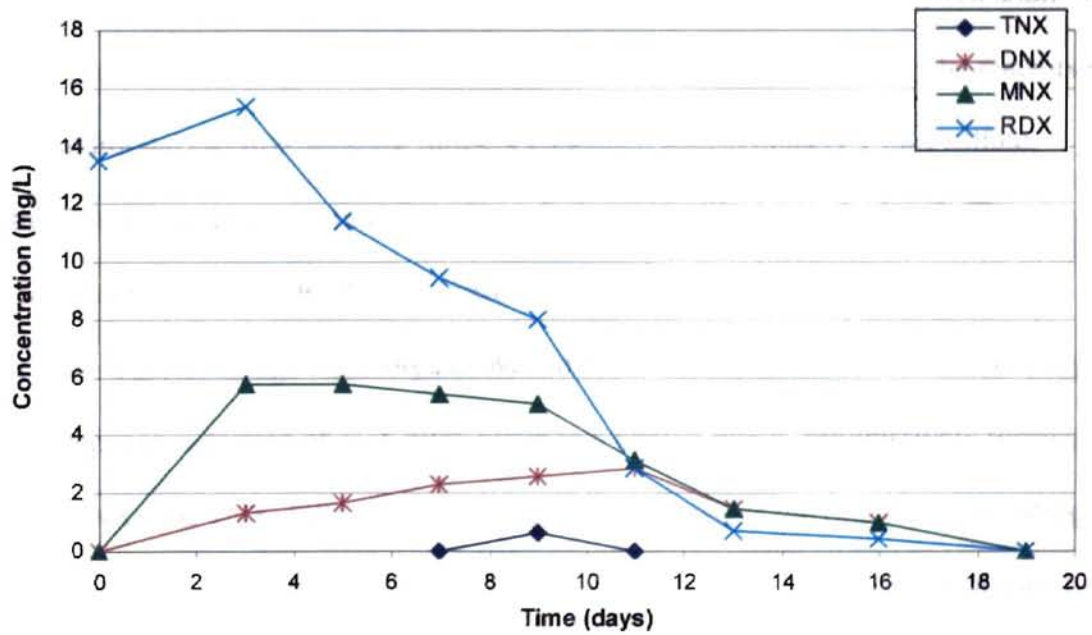


Figure 11. Composite plot of duplicate reactors showing RDX and its metabolites when only native soil bacteria were used.

A presence/absence test for sulfate-reducing bacteria showed that no sulfate reducers were present in these reactors. Microbiological examination of the bacteria from the presence/absence test revealed that the major organisms present were gram-negative motile rods capable of fermenting lactose to acetate. Members of the family Enterobacteriaceae were likely the dominant bacteria in the uninoculated reactors.

DISCUSSION

When comparing reactor performance when nitrate, sulfate, or no additional electron acceptor was added, reactors given either sulfate or no electron acceptor degraded RDX much more quickly than reactors given nitrate. The presence of nitrate in reactors appeared to almost completely inhibit the transformation of RDX. No significant difference in RDX transformation was seen between reactors given additional sulfate and reactors not given any additional electron acceptor. Although the reactors not given additional electron acceptor initially contained approximately 1 mM sulfate, metabolite degradation continued unaffected after this sulfate was depleted. The predominant pathway of RDX degradation in reactors inoculated with municipal anaerobic digester sludge microorganisms and either sulfate or no extra electron acceptor was a stepwise reduction of each nitro group to a nitroso group, progressively forming MNX, DNX, and TNX, followed by the apparent cleavage of the ring structure. Ring cleavage was the slowest step in the degradation process in these reactors, indicated by the significant accumulation of TNX before eventual degradation.

Since the concentration of sulfate remained unchanged until TNX was the only remaining RDX metabolite, an outside electron acceptor is apparently unnecessary for the transformation of RDX, MNX, and DNX. Sulfate reduction did occur while TNX was present, but no stoichiometric relationship existed. Degradation of TNX proceeded regardless of sulfate availability. The nitro groups were reduced before sulfate, possibly as the preferred electron acceptor. The preferential use of reducible nitrogen compounds (ie. nitrate, nitrite) over sulfate is consistent with at least one species of the sulfate-reducing bacteria *Desulfovibrio* (Krekeler and Cypionka 1995). Since high substrate concentration tends to select for this genus over other sulfate-reducers (Widdel 1986), it is likely that *Desulfovibrio* was present in the experimental reactors, which all received molasses as primary substrate. The morphology of sulfate reducers isolated from these reactors further corroborates this assumption. Since sulfate was used in the presence of TNX but only in the absence of any nitro groups, the sulfate reducers apparently could not further reduce the nitroso groups.

Further evidence for the use of the nitro groups as an electron acceptor for metabolic processes comes from the reactors fed extra sodium nitrate. In the presence of nitrate, nitro group reduction did not occur and RDX was not degraded. A nitro group is simply a nitrite attached to the ring structure. The genus *Desulfovibrio* reduces nitrate first, then nitrite, and then sulfate (Krekeler and Cypionka 1995). Consequently, when nitrate is readily available, nitro constituents remain in the oxidized form. When only nitrite was present in the experimental reactors, nitrite was reduced and RDX was degraded simultaneously, albeit at a slow rate. Nitro groups were reduced most quickly

when only a potential electron acceptor with an energy availability lower than the nitro groups was present.

Even when all of the nitro groups were reduced to nitroso constituents, the ring structure still could not be cleaved in the presence of nitrate. TNX was not degraded when nitrate was added to the reactors. The fact that oxidation of TNX did not occur while nitrate was being reduced suggests that the breakdown of TNX required a further reduction of the nitroso groups. This suggestion is in agreement with the postulation by McCormick et al. (1981) that nitroso groups are further reduced to unstable hydroxylamino intermediates, leading to ring cleavage. The only detectable RDX metabolites that accumulated in experimental reactors were the nitroso compounds MNX, DNX, and TNX. If any further reduction of the nitroso constituents occurred, it resulted in the destabilization and rapid cleavage of the ring structure.

The sulfate-reducing bacteria in the reactors apparently could not reduce the nitro groups any further than to the nitroso group, as seen by the immediate switch to sulfate reduction when only TNX remained. Furthermore, TNX degradation occurred independently of the presence of sulfate. Therefore, it is likely that the sulfate reducers were not responsible for the ring cleavage that occurred. Instead, it is more likely that fermentative bacteria both further reduced one of the nitroso constituents and oxidized the ring structure.

Data from reactors in which nitrate was added and then depleted and reactors not inoculated with anaerobic sludge microorganisms raise questions regarding the degradation mechanism described above. When no inoculum was added, the soil flora was able to degrade the RDX ring structure, while only producing a trace of TNX. DNX

was the last metabolite to accumulate. The divergence from the described process was even more pronounced in the reactors in which nitrate was added and then depleted. The only metabolite detected as RDX was disappearing from these reactors was a trace amount of MNX. Both sets of reactors show that it is not necessary to reduce each nitro group to the nitroso form in order for the ring structure to be cleaved.

However, results from these reactors do not completely contradict the proposed mechanism leading to ring cleavage. In both cases it is still conceivable that a nitro group is reduced to a nitroso group and then further reduced, allowing ring cleavage to occur. In the reactors containing only soil bacteria, TNX did not accumulate before a nitroso group was reduced. In the nitrate-depleted reactors, nitroso group reduction apparently occurred as quickly as MNX was formed.

The native microbial population in the soil is different from the bacteria found in anaerobic sewage sludge. The anaerobic digester sludge microorganisms existed in a long-term anoxic environment, where anaerobic respiratory bacteria would be present in higher concentrations. Significant populations of sulfate-reducing bacteria were found in reactors inoculated with anaerobic digester sludge, but not in uninoculated reactors. The soil, on the other hand, was dry and in constant contact with oxygen. The most likely soil microorganisms to survive in the anaerobic reactors would be facultative species. In fact, facultative fermenters were isolated from the reactors that had not been inoculated with the anaerobic digester sludge. It follows, then, that the reactors not inoculated with anaerobic digester microorganisms (containing only contaminated soil) likely had a higher percentage population of fermentative bacteria. As noted above, fermenters were the most likely organisms responsible for nitroso group reduction and ring cleavage. A

higher percentage of this type of organism could increase the rate at which nitroso group reduction occurs in reactors, limiting the accumulation of the later-occurring metabolites.

The pattern of RDX degradation in the nitrate reactors is the most difficult to explain. Nitrate reduction displayed a pattern of stalling in these reactors. The sulfate-reducer *Desulfovibrio* reduces nitrate and nitrite to ammonium, but not to nitrogen gas (Barton et al. 1983; Krekeler and Cypionka 1995; Mitchell et al. 1986). Facultative soil bacteria also commonly display dissimilatory nitrate reduction to ammonium (Tiedje 1988). Furthermore, high carbon/nitrate ratios and continuously anoxic environments select for dissimilatory nitrate reduction to ammonium over denitrification. Consequently, if ammonium is produced more quickly than the bacterial cells can use it, the ammonium may accumulate in a closed system and inhibit additional nitrate reduction. As long as nitrate is present, conditions are not favorable to the reduction of other less energetic compounds. When nitrate reduction was inhibited in the experimental reactors, most apparent activity stalled and then returned after a prolonged time period. The return of measurable activity may have occurred after enough ammonium was consumed or otherwise disappeared.

Activity as measured by nitrate or RDX disappearance stalled for about a month in some reactors. This stalling was not caused by a lack of electron donor, since the addition of more molasses did not have an effect. During this time the bacterial cells may have become starved for energy as electron acceptor usage was inhibited. Once the ammonium concentration was low enough to once again allow for nitrate reduction, the cells may have been in a state of higher than normal energy use. The increased energy consumption would require a more rapid rate of electron acceptor reduction. The initial

rate of RDX degradation immediately following stalling and nitrate depletion was almost three times more rapid than in the reactors initially given sulfate or no added electron acceptor. The observation of sulfate reduction concurrent with RDX degradation in only the nitrate-depleted reactors also supports the possibility of unusually rapid electron acceptor reduction under these conditions.

CONCLUSIONS

The most successful reactors in terms of RDX degradation rate in these experiments were reactors given either added sulfate or no added electron acceptor. Since no significant difference in performance was observed under either of these conditions, it is recommended that no electron acceptor be added to soil slurry bioreactors implemented to treat RDX-contaminated soils. No experiments were run to compare directly the rate of RDX degradation in reactors inoculated with anaerobic digester sludge and those only containing soil microorganisms. However, it appeared that uninoculated reactors, which were run at a later time, were able to degrade RDX more quickly than inoculated reactors tested previously under similar conditions. These soil-only reactors also had less accumulation of metabolite products than reactors inoculated with anaerobic digester sludge. Bench-scale studies would need to be performed using soil from the specific site being remediated, but it appears that the introduction of outside microorganisms would not be needed or recommended for the start-up of a soil slurry bioreactor.

The presence of nitrate completely inhibited the degradation of RDX in the experimental reactors. Even though high RDX degradation rates were observed after added nitrate had been reduced in these reactors, results were sporadic at best. These reactors exhibited extended periods of inactivity and were highly unpredictable. Further study would be needed to support the hypothesis of sequenced electron acceptor availability proposed to explain the lack of metabolite accumulation in these nitrate-depleted reactors. If this hypothesis held up, future study might be warranted regarding the feasibility and effectiveness of using anaerobic microorganisms that have developed this response as inocula for soil slurry reactors used to treat RDX.

Further study is also needed to verify the species of microorganisms responsible for the observed RDX degradation activity. This paper proposes that sulfate-reducing bacteria may have been responsible for the accumulation of the MNX, DNX, and especially TNX in experimental reactors, while fermenting microorganisms may have initiated ring cleavage. Pure culture experiments using sulfate-reducing and fermenting bacteria will be needed to determine if the role of each type of microorganism has been properly described based on the foregoing experimental results.

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VITA

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Master of Science

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