4-2006

Anaerobic Biodegradation of RDX and TCE: Single- and Dual-Contaminant Batch Tests

Travis S. M. Young
Environmental Engineer, Sacramento, CA

Matthew C. Morley
University of Nebraska - Lincoln

Daniel D. Snow
University of Nebraska at Lincoln, dsnow1@unl.edu

Published in *Practice Periodical of Hazardous, Toxic, and Radioactive Waste Management* 10:2 (April 1, 2006), pp. 94–101;
doi: 10.1061/(ASCE)1090-025X(2006)10:2(94)

Submitted July 6, 2005; approved July 6, 2005.

Anaerobic Biodegradation of RDX and TCE: Single- and Dual-Contaminant Batch Tests

Travis S. M. Young,[1] Matthew C. Morley, P.E., M.ASCE,[2] and Daniel D. Snow [3]

1 Environmental Engineer, CH2M-Hill, 2485 Natomas Park Dr., Suite 600, Sacramento, CA 95833.

2 Assistant Professor, Dept. of Civil Engineering, University of Nebraska–Lincoln, Lincoln, NE 68588-0531. E-mail: mmorley2@unl.edu

3 Research Assistant Professor, Water Sciences Laboratory, University of Nebraska–Lincoln, Lincoln, NE 68588-0844.

Abstract
Several sites in the United States have groundwater contaminated with mixtures of high explosives and chlorinated solvents. This research examined the ability of two microbial cultures (anaerobic sludge and a facultative enrichment culture) to biodegrade single- and dual-contaminant mixtures of trichloroethene (TCE) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) under anaerobic conditions. In single component batch tests, both cultures degraded 0.6–1 mg RDX/L and its nitroso metabolites to below detection limits in <7 days. During initial 9-day TCE biodegradation tests, the anaerobic sludge did not transform TCE, whereas the facultative culture transformed approximately 10% of the initial 1.4 mg TCE/L. Prior to dual-contaminant batch tests, both cultures were grown in the presence of TCE. Subsequently, both acclimated cultures rapidly biodegraded mixtures of RDX and TCE. Both cultures degraded RDX and RDX-nitroso compounds to below detection limits in <4 days. In the same tests, TCE-acclimated anaerobic sludge converted TCE primarily to *cis*-dichloroethene (*cis*-DCE), while the acclimated facultative culture produced *cis*-DCE and other chlorinated metabolites. These preliminary results demonstrate that anaerobic bioremediation may be part of a feasible groundwater remediation alternative for mixtures of TCE and RDX.

Keywords: biodegradation, anaerobic treatment, TCE, ground-water pollution, water treatment

Background and Objectives

Chlorinated solvents and high explosives are two common classes of environmental contaminants at defense-related facilities. At least five current and former defense-related sites in the United States, including the former Nebraska Ordnance Plant (NOP) in Mead, Nebraska (USEPA 1997), have groundwater contaminated with mixtures of these contaminants. One of the most common chlorinated solvents is trichloroethene (TCE), a volatile chlorinated ethene and degreasing agent that has been found in groundwater at about 60% of the sites on the National Priorities List (ATSDR 1997). Hexahydro-1,3,5-trinitro-1,3,5-triazine, also known as RDX (royal demolition explosive), is a semivolatile cyclic nitramine that is one of the most widely used high explosives in the world (Spain et al. 2000). RDX is a common soil and groundwater contaminant at current and former munitions production facilities and military training ranges (Pennington and Brannon 2002; Clausen et al. 2004).

TCE and RDX pose significant risks to human health, and both are possible human carcinogens (ATSDR 1995, 1997). The USEPA has established a maximum contaminant level of 0.005 mg/L for TCE and a lifetime health advisory (HA) for RDX of 0.002 mg/L in drinking water (USEPA 2004). Chronic exposure to TCE in drinking water may have detrimental health effects including neurological damage, immunological abnormalities, and birth defects in unborn children (ATSDR 1997). The lifetime HA for RDX is based on potential noncarcinogenic effects.

Groundwater contaminated with mixtures of TCE and RDX is typically remediated using extraction and treatment by adsorption to granular activated carbon (GAC) (BWXT Pantex, 2004; Hyun 2004). Although GAC effectively eliminates TCE and RDX from the groundwater (e.g., Carter et al. 1992; Morley et al. 2005), this treatment method requires long term pumping of contaminated groundwater, resulting in excessive operating, maintenance, and disposal costs. Because of the limitations of pump and treat groundwater remediation, methods for *in situ* groundwater remediation are being explored to reduce the time and costs for remediation.

In situ bioremediation may be a potential alternative for treating TCE- and RDX-contaminated groundwater. The biodegradation of TCE and RDX has been extensively characterized as single contaminants, but not as a mixture. Under reducing conditions, both RDX (e.g., McCormick et al. 1981; Hawari et al. 2000) and TCE (Freedman and Gossett 1989; He et al. 2002) can be readily biodegraded. Both contaminants can be biodegraded by anaerobic, methanogenic, or facultative cultures (Smatlak et al. 1995; Sharma and McCarty 1996; Young et al. 1997a,b; Hawari et al. 2000). Using microcosm or column studies with media from contaminated sites, enhanced anaerobic biodegradation processes have been explored for both chlorinated solvents (e.g., Fennell et al. 2001) and high explosives (e.g., Speitel et al. 2001; Wani and Davis 2003). Numerous electron donors have been effectively utilized for biodegrading both RDX (e.g., Davis et al. 2004) and TCE (Fennell et

al. 1997; Schollhorn et al. 1997; Lee et al. 1998). Hydrogen has been noted as an important electron donor for biodegradation of chlorinated contaminants (Smatlak et al. 1995; Fennell et al. 1997) and high explosives (Beller 2002; Adrian et al. 2003).

Anaerobic biodegradation of TCE typically proceeds by reductive dehalogenation, in which TCE serves as an electron acceptor (Vogel et al. 1987). Reduction of TCE initially produces one of the dichloroethene (DCE) isomers (*cis*-DCE or *trans*-DCE), which may be further reduced to vinyl chloride (VC) (Mohn and Tiedje 1992; Bradley 2000). VC itself is a known human carcinogen and has a drinking water MCL of 2 µg/L (USEPA 2004). Under strongly reducing conditions, VC can be further reduced to ethene, which poses negligible risk and can be readily mineralized.

RDX biodegradation under anaerobic conditions was first reported by McCormick et al. (1981), who proposed RDX biodegradation by reduction of the RDX nitro (–NO_2) groups to nitroso (–NO) groups to produce hexahydro-1-nitroso-3,5-dinitro- 1,3,5-triazine (MNX); hexahydro-1,3-dinitrso-5-nitro-1,3,5- triazine (DNX); and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). Further reduction of the three nitroso-RDX derivatives produces unstable hydroxylamino compounds that may be rapidly reduced to methanol, 1,1-dimethylhydrazine, 1,2-dimethylhydrazine, and formaldehyde. Hawari et al. (2000) proposed a significantly different pathway, asserting that RDX biodegradation is due to a combination of biological and chemical processes. Enzymatic attack on the inner C–N bond of RDX produces two unstable metabolites, methylenedinitramine and bis-(hydroxymethyl) nitramine. These two metabolites hydrolyze rapidly to produce nitramine, formic acid, and formaldehyde, which are further degraded biotically and abiotically to carbon dioxide, nitrous oxide, methane, and water.

Bioremediation has been proposed as a remediation alternative individually for TCE or RDX in groundwater, but little is known about the feasibility of biodegrading mixtures of these dissimilar contaminants. Although these contaminants have different physical and chemical properties, each can be biodegraded under anaerobic conditions. Mixtures of TCE and RDX could potentially be biodegraded if an appropriate microbial consortium is present and can be stimulated. The intent of this research was to make an initial assessment of the feasibility of biodegrading aqueous mixtures of RDX and TCE under anaerobic conditions in laboratory microcosms. The major objectives of this research were to assess the ability of two different microbial consortia to biodegrade TCE and RDX as single contaminants and as mixtures under anaerobic conditions, and to determine the metabolites produced by each culture.

Experimental Methods

Contaminants

Stock solutions used for spiking microcosms were prepared in methanol using high purity TCE, *cis*-DCE, *trans*-DCE, and VC (Acros Organics or Ultra Scientific). Preparative liquid chromatography was used to produce purified RDX from technical grade RDX which contained about 10% octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) by mass (donated by the U.S. Biomedical Research and Development Laboratory, Frederick, Md.). The three nitroso-RDX compounds (MNX, DNX, and TNX) were synthesized according to Brockman et al. (1949) or purchased from SRI International (Menlo Park, Calif.).

Cultures

Previous studies have shown that anaerobic sludge can be used as a source of microbes capable of biodegrading RDX (Hawari et al. 2000) and TCE (Freedman and Gossett 1989; Gu et al. 2004). Anaerobic sludge was obtained from the anaerobic digesters at the Theresa Street Wastewater Treatment Plant (Lincoln, Neb.). The sludge consists of organic solids from both the primary and secondary clarifiers, and the digesters have mean cell residence times of 15-20 d. Between 1 and 2 L of fresh anaerobic sludge were collected and used as the seed culture for microcosms.

The second culture was a facultative culture enriched from GAC backwash solids collected from the sedimentation tank at the U.S. Army Corps of Engineer's groundwater treatment plant near Mead, Neb. This treatment plant has a capacity of 2,000 gpm and treats RDX- and TCE-contaminated groundwater using 8 GAC contactors operated in four lead-lag pairs. Each contactor is periodically backwashed to remove accumulated fines and to reduce head losses. Bacteria associated with settled backwash solids were grown aerobically by adding 100 mL of the backwash solids slurry to 900 mL of Oxoid Nutrient Broth No. 2 (Difco, Detroit) in stirred 1-L flasks. Bacteria were cultured by wasting half of the suspension every 4 days, and centrifuging and resuspending the remaining mixture in fresh nutrient solution. After 20 days of culturing, sediment particles were settled for 30 min, and 50 mL of the supernatant was transferred to 950 mL of fresh Oxoid Nutrient Broth. Total suspended solids (TSS) and volatile suspended solids (VSS) levels after additional culturing were similar, indicating very low levels of inorganic solids. Loss assays with poisoned cultures confirmed that reductions in contaminant concentrations were due to biological degradation, rather than adsorption to solids. The culture was grown aerobically, with periodic exchange of the nutrient solution, until sufficient biomass was available for microcosm experiments.

Microcosms

Biodegradation experiments were conducted in 250-mL amber glass bottles. Each reactor had a 125-mL headspace and 125 mL of an aqueous solution consisting of biomass, substrate, contaminant, and mineral solution. Sodium acetate (2000 mg/L), which has been used to support microbial consortia capable of transforming chlorinated ethenes (Schollhorn et al. 1997; Chang et al. 1998; He et al. 2002; Sung et al. 2003) and RDX as single contaminants (Brenner et al. 2000; Davis et al. 2004), was used as the main carbon source. The sterile mineral solution (Binks et al. 1995; Morley et al. 2002) used for all biodegradation experiments was prepared aseptically and consisted of the following (all concentrations as mg/L): 226 $NaHCO_3$; 37.0 $MgSO_4 \cdot 7H_2O$; 27.7 $CaCl_2$; 4.36 Na_2EDTA; 1.0 H_3BO_3; and 0.316 $FeCl_3 \cdot 6H_2O$; 0.1 thiamine; 0.023 $ZnSO_4 \cdot 7H_2O$; 0.0066 $Na_2MoO_4 \cdot 2H_2O$; 0.0064 $CuSO_4$; 0.0119 $CoCl_2 \cdot 6H_2O$; 0.0005 d-biotin; and 0.0005 vitamin B_{12}. Additionally, the culture medium was buffered at near-neutral pH with 1.96 g K_2HPO_4 / L and 1.92 g KH_2PO_4/L; relatively high phosphate concentrations were used to prevent drastic pH fluctuations. This mineral solution stabilized the pH between 6.9 and 7.7 during each microcosm experiment.

Microcosms were filled aerobically, placed in a nitrogen filled glove box (Kewaunee Scientific; Adrian, Mich.), degassed by bubbling N_2 through the solution, and sealed with Mininert

valves. For RDX degradation experiments, an RDX stock solution (~40 mg RDX/L) was prepared by dissolving solid RDX in HPLC-grade water, filtering, and diluting in microcosms to the desired initial RDX concentration. For TCE degradation experiments, 166 µL of a TCE stock solution in methanol was spiked into the reactor through the Mininert valve after degassing and sealing. This method of TCE addition resulted in an initial methanol concentration of about 32.8 mM (1,050 mg/L); therefore methanol may have been an important electron donor in microcosms with TCE added. Reactors were continuously mixed with magnetic stirrers inside the N_2-filled glove box for the duration of the experiment. The range of oxygen concentrations in the glove box, monitored using a Gas Alert meter (BW Technologies; Arlington, Tex.), was 0.8–3.0% for all assays. Control reactors were prepared identically to experimental microcosms without biomass and contained 100 mg/L of $HgCl_2$ to prevent microbial growth. All experiments were conducted at room temperature (21±2°C).

For each sampling event, one control microcosm and two experimental microcosms were sacrificially sampled. Reactors were removed from the glove box, and the headspace immediately sampled for chlorinated ethenes through the Mininert valves. Dissolved oxygen and pH were measured after opening each reactor, and the liquid phase was sampled for analysis of RDX and RDX degradates. TSS and VSS measurements were used to surrogate estimates of biomass concentrations.

Analytical Methods

Aqueous samples (2 mL) were centrifuged (15 min at 13,000 rpm) to remove biomass and analyzed by high performance liquid chromatography (HPLC) on a Waters 2695 Separations Module. RDX, MNX, DNX, and TNX were separated with a C18 column (Phenomenex Luna 5 µm; 250 length × 4.6 mm inside diameter; Torrance, Calif.) and detected at 254 nm with a Waters 2996 photodiode array detector. The eluent consisted of 25% HPLC grade methanol (Fisher Optima) and 75% HPLC grade water (Burdick and Jackson) at a flow rate of 1.5 mL/min, yielding a run time of 16 min per sample. External standards were prepared from RDX standards obtained from Ultra Scientific (North Kingstown, R.I.) or purified nitroso-RDX compounds. The practical quantitation limits (PQL) were estimated to be 4.0, 9.0, 8.0, and 10.7 µg/L for RDX, MNX, DNX, and TNX, respectively.

Chlorinated ethenes were quantified using gas chromatography (GC) with solid phase microextraction (SPME) and flame ionization detection (FID) (Xu et al. 1996). Analytes were extracted from microcosm headspace on preconditioned 100 µm polydimethylsiloxane coated fibers (Supelco; Bellefonte, Pa.) at room temperature. Microcosms were magnetically stirred throughout the 5-min SPME adsorption time and the fiber was immediately placed into the GC injection port (3 min desorption time). Nonchlorinated volatile compounds from microbial metabolic processes and from the anaerobic sludge produced interfering peaks and required a shorter adsorption time and longer desorption time than those reported by Xu et al. (1996).

Chlorinated ethenes were quantified with a Varian (Walnut Creek, Calif.) Star 3600 CX GC, equipped with a FID, and a Star 6.2 Chromatography Workstation. A 60-m RTX-VRX (Restek; Bellefonte, Pa.) capillary column (0.53-mm internal diameter; 0.5-µm film thickness) was used to separate the analytes. The temperature program held the initial oven temperature of 40°C for 3.75 min, increased to 140°C at 20°C/min, and then held at 140°C for 2.5 min. Detector and injection port temperatures were held constant at 230 and 210°C, respectively. The carrier gas was high purity helium (80 psi; 1.5 mL/min), and the make-up gas was high purity nitrogen (80 psi; 30 mL/min). The hydrogen and dry air flow rates to the FID were 30 and 300 mL/min, respectively. Chlorinated solvent standards were prepared for each sampling event in 250 mL amber glass bottles with 125 mL of the nutrient solution used in the microcosms. Because the aqueous solubility of chlorinated solvents is affected by other dissolved constituents, NaCl was added to the standard solutions to yield a similar conductivity to the experimental solutions. Standards prepared in methanol were injected into the bottles, and after 30 min of stirring, measured identically to experimental microcosms. Method detection limits ranged from 2.5 to 8.0 µg/L. Dimensionless Henry's Law constants used to calculate liquid phase concentrations were interpolated for room temperature (21°C) from values reported by Gossett (1987) and were as follows: TCE 0.3276; *cis*-DCE 0.1386; *trans*-DCE 0.3297; and VC 0.9718.

Dissolved oxygen, pH, conductivity, TSS, and VSS were measured for all reactors. A YSI 5100 dissolved oxygen (YSI Incorporated; Yellow Springs, Ohio) meter equipped with a YSI 5010 BOD probe was used to measure the DO concentration of the aqueous microcosm solutions. Conductivity was measured using a VWR Scientific Products (West Chester, Pa.) Model 2052 conductivity meter equipped with a 525 Dip Cell Pt probe. An Accumet Basic AB15 (Fisher Scientific) pH meter was used to measure the pH after daily calibration pH 4, 7, and 10 buffers. TSS and VSS were determined following Clescceri et al. (1998).

Kinetic Modeling

To provide a consistent means of comparison between the two cultures and different experimental conditions, all batch biodegradation experiments were modeled according to a pseudo-first-order rate equation (Young et al. 1997b; Morley et al. 2002)

$$dC/dt = - k_1 XC \quad (1)$$

where C = contaminant concentration (mg contaminant/L); X = biomass concentration (mg VSS/L); t = time (days); and k_1 = pseudo-first-order degradation rate constant (L/mg VSS day). Equation (1) governs the biotransformation of a contaminant to its first metabolite, rather than the overall mineralization of a contaminant (Young et al. 1997b). This type of pseudo-first-order analysis has been used to analyze a diverse range of biodegradation experiments, including reductive dechlorination of TCE (Tandoi et al. 1994) and anaerobic biodegradation of RDX (Young et al. 1997a,b; Morley et al. 2002). Because of the simplicity of this kinetic model, the effects of inhibition and competition were not explicitly included in the kinetic analyses.

All contaminant concentrations used for kinetic analyses were averages from sacrificial sampling of two batch reactors. In cases in which RDX or TCE was undetectable by the end of the experiment, only the first such data point (set equal to the contaminant detection limit) was used when calculating the rate constant. Time-weighted average VSS concentrations (summarized in Tables 1 and 2) were used as the biomass concentration for each experiment. With a constant biomass concentration, the integration of Equation (1) yields

$$\ln(C/C_0) = -k_1 Xt \quad (2)$$

where C_0 = initial contaminant concentration (mg contaminant/L). Rate constants for degradation of RDX and TCE were determined by linear regression of Equation (2) using a spreadsheet; a straight line with the slope of $-k_1$ was found by plotting $\ln(C/C_0)$ as a function of Xt. The spreadsheet data analysis also provided the standard error for each estimated rate constant and the coefficient of determination (R^2) for each linear regression fit.

Results and Discussion

Single Component RDX Degradation

In a 9-day single-component RDX biodegradation test, anaerobic sludge that had not been previously exposed to RDX rapidly biodegraded RDX (initial average concentration of 0.61 mg/L) with minimal accumulation of nitroso-RDX metabolites (Figure 1a). By day seven, the concentration of RDX had fallen below the detection level in both of the experimental microcosms. Of the three nitroso-RDX metabolites, only TNX was detected in the experimental reactors on days 5 and 9 at average concentrations of 79 and 73 µg/L, respectively. The pseudo-first-order RDX degradation rate constant was 1.4×10^{-3} L/mg VSS day (Table 1).

The facultative culture degraded RDX slightly faster than the anaerobic sludge (Figure 1b). Starting with 1.04 mg/L, RDX could no longer be detected by day seven. The pseudo-first-order degradation rate constant for RDX was 2.1×10^{-3} L/mg VSS day (Table 1). TNX was detected at concentrations up to 50 µg/L.

In both of the single-component RDX biodegradation tests, TNX was detected in the control reactors between days 7 and 9 (data not shown), coinciding with decreases in RDX concentrations in the control reactors. The appearance of TNX and loss of RDX was attributed to significant biomass growth in the control reactors. Control reactors in subsequent experiments were poisoned with 100 mg/L $HgCl_2$.

Table 1. Pseudo-First-Order Rate Constants for Biodegradation of RDX

Experiment	Rate constant[a] (L/mg VSS day)	R^2	Data points	VSS (mg/L)
Anaerobic sludge–single component	$1.40\times10^{-3}\pm3.2\times10^{-4}$	0.82	6	470
Facultative culture–single component	$2.12\times10^{-3}\pm1.3\times10^{-4}$	0.99	6	390
Acclimated anaerobic sludge–dual component	$3.31\times10^{-3}\pm7.2\times10^{-4}$	0.91	4	580
Acclimated facultative culture–dual component	$4.73\times10^{-3}\pm1.0\times10^{-3}$	0.91	4	360

[a] Rate constants shown are $k_{1,RDX}$ calculated by linear regression of Equation (2); ± value is the standard error for the $k_{1,RDX}$ estimate.

Table 2. Pseudo-First-Order Rate Constants for Biodegradation of TCE

Experiment	Rate constant[a] (L/mg VSS day)	R^2	Data points	VSS (mg/L)
Facultative culture–single component	$8.32\times10^{-5}\pm2.5\times10^{-5}$	0.79	5	560
Acclimated anaerobic sludge–dual component	$2.93\times10^{-3}\pm8.2\times10^{-4}$	0.87	4	580
Acclimated facultative culture–dual component	$1.26\times10^{-3}\pm5.1\times10^{-4}$	0.56	4	360

[a] Rate constants shown are $k_{1,TCE}$ calculated by linear regression of Equation (2); ± value is the standard error for the $k_{1,TCE}$ estimate.

Single Component TCE Degradation

Biotransformation of TCE was not observed in microcosms prepared from anaerobic sludge (9 days) during the single component experiment (data not shown). None of the TCE degradation products were detected, and TCE decreases in experimental reactors were similar to controls. In contrast, the facultative culture degraded about 30% of the initial 1.42 mg TCE/L relative to the control, and both *cis*- and *trans*-DCE were detected (data not shown). *trans*-DCE and *cis*-DCE were detected at concentrations up to 140 and 75 µg/L, respectively. Vinyl chloride was not detected. The pseudo-first-order TCE degradation rate constant was 8.3×10^{-5} L/mg VSS day (Table 2).

Acclimation to TCE

Because the anaerobic sludge did not immediately degrade TCE, both cultures were exposed to TCE in an attempt to acclimate them to the chlorinated solvent. Various lag periods have been observed prior to the onset of dechlorination by anaerobic

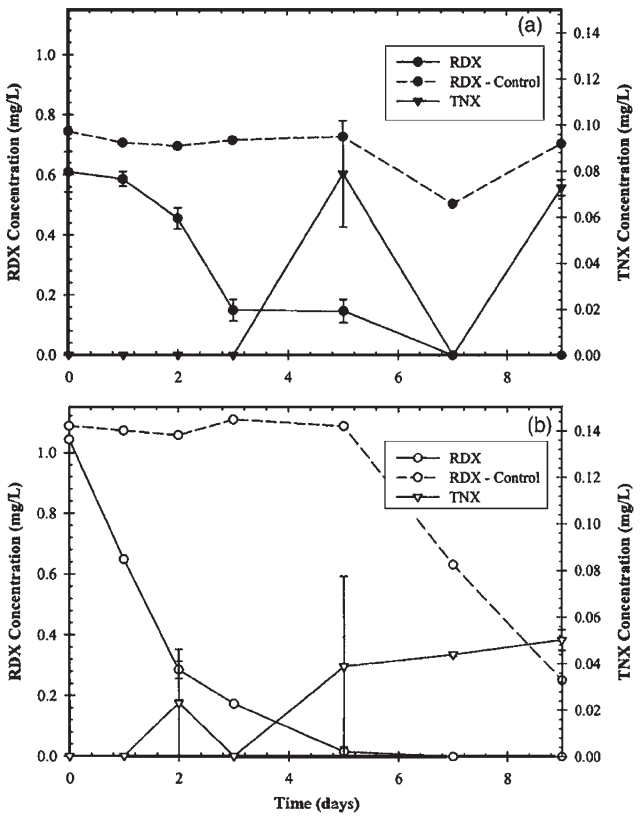

Figure 1. Single component biodegradation of RDX by (a) anaerobic sludge and (b) the facultative culture. For experimental reactors, symbols are averages of duplicate reactors, and error bars show the range of detected concentrations. Where error bars are absent, they fall within the symbol.

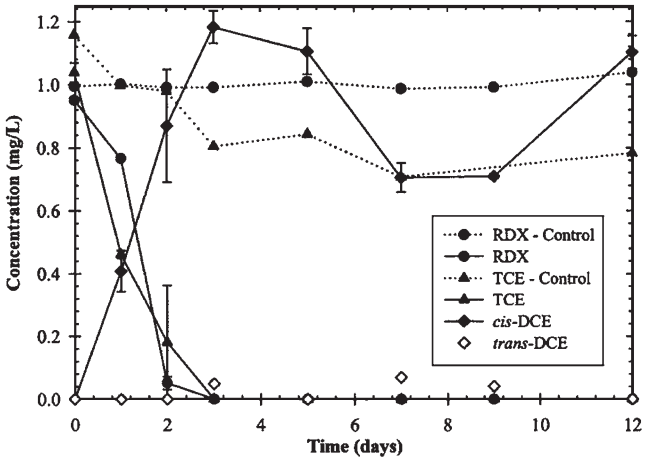

Figure 2. Biodegradation of RDX and TCE by the acclimated anaerobic sludge. For experimental reactors, symbols are averages of duplicate reactors, and error bars show the range of detected concentrations. Where error bars are absent, they fall within the symbol.

cultures (Schollhorn et al. 1997; Gu et al. 2004). Thus both cultures were grown in the presence of TCE for 3–5 weeks. Cultures were acclimated separately under anaerobic conditions in sterilized 1,300-mL glass airtight bottles with the same mineral solution and a higher sodium acetate concentration (4,000 mg/L) due to a higher VSS concentration (~800 mg VSS/L). Every 7 days, chlorinated ethenes were measured in each culture prior to replenishing TCE (1 mg/L) and acetate; this procedure provided each culture with both acetate and methanol as possible carbon sources.

After 21 days of acclimation, the anaerobic sludge completely transformed TCE to *cis*-DCE with trace amounts of *trans*-DCE. In contrast, the facultative culture transformed only a limited amount of TCE during acclimation. After 4 weeks, TCE was still present with low concentrations of *cis*- and *trans*-DCE detected. Acclimation of the facultative culture was terminated after 35 days.

Dual Component Assay – Acclimated Anaerobic Sludge

Anaerobic sludge grown in the presence of TCE rapidly degraded TCE, RDX, and the nitroso-RDX compounds during a 12-day experiment (Figure 2). RDX concentrations decreased from 0.95 mg/L to below detection by day 3. The only RDX metabolite detected was MNX, which was transiently detected prior to day 5 at concentrations <20 µg/L. The pseudo-first-order degradation rate constant for RDX was 3.3×10^{-3} L/mg VSS day.

TCE (initially 1.04 mg/L) was rapidly dechlorinated by the acclimated anaerobic sludge and was not detectable by day 3 (Figure 2), yielding $k_{1,TCE} = 2.9 \times 10^{-3}$ L/mg VSS day. The first TCE metabolite detected was *cis*-DCE, which was detected on day 1 at 0.408 mg/L. The concentration of *cis*-DCE continued to increase up to day 3 as TCE was transformed, and *trans*-DCE was detected later in the experiment at concentrations up to 0.07 mg/L. VC was detected in one reactor on day 9 at a concentration of 0.076 mg/L. During this experiment, TCE concentrations in the controls decreased by about 30%. The decreasing concentration of TCE from some control reactors was likely due to leakage rather than biodegradation because no TCE metabolites were detected in the controls.

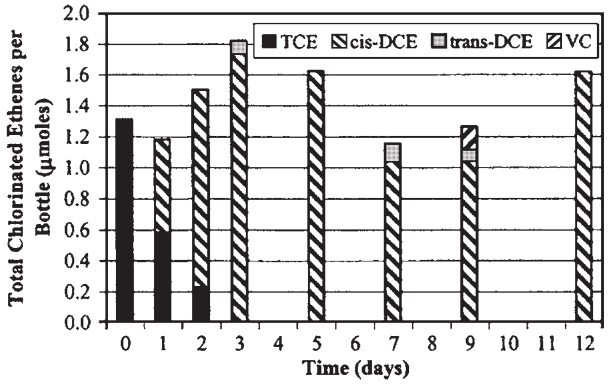

Figure 3. Mass balance analysis of chlorinated ethenes during two component biodegradation by the acclimated anaerobic sludge.

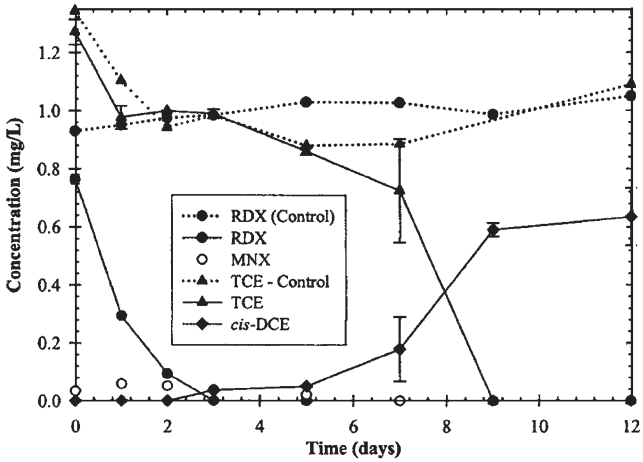

Figure 4. Biodegradation of RDX and TCE by the acclimated facultative culture. For experimental reactors, symbols are averages of duplicate reactors, and error bars show the range of detected concentrations. Where error bars are absent, they fall within the symbol.

The anaerobic sludge partially dechlorinated TCE, but had a very limited capability to further dechlorinate the primary TCE metabolite (*cis*-DCE). Although we were unable to directly measure production of ethene, a mass balance calculation (Figure 3) suggests that ethene production was unlikely. Over the course of an experiment, a decrease in the total molar quantity of chlorinated ethenes could indicate conversion to ethene. Although the distribution of chlorinated ethenes changed throughout the experiment, the average (moles of chlorinated ethenes in the experimental reactors did not decrease, suggesting that chlorinated ethenes were not converted to ethene.

Dual Component Assay – Facultative Culture

RDX was rapidly degraded by the acclimated facultative culture in the presence of TCE (Figure 4). The spiked RDX concentration (1.0 mg/L) had decreased to 0.77 mg/L approximately 6 h after microcosm setup and was no longer detectable by day 3. The degradation rate constant for RDX in this two component study was 4.7×10^{-3} L/mg VSS day (Table 1). The only nitroso-RDX metabolite detected was MNX, which was detected on day 1 at 60 µg/L and decreased to below detectable levels by day 5.

Despite very little TCE transformation during acclimation, the facultative culture transformed TCE during this experiment

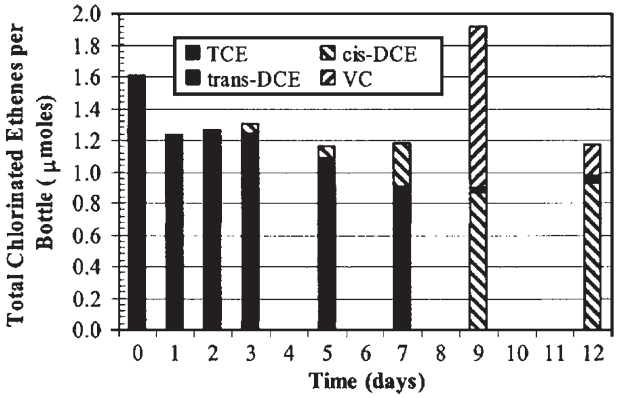

Figure 5. Mass balance analysis of chlorinated ethenes during two component biodegradation by the acclimated facultative culture.

(Figure 4). The initial TCE concentration was 1.27 mg/L. By day 9, TCE was no longer detectable in the experimental reactors, yielding $k_{1,TCE}$=1.3 × 10^{-3} L/mg VSS day. *cis*-DCE was first detected on day 3 and continued to accumulate through the end of the experiment. *trans*-DCE was detected in one microcosm on day 9 (23 µg/L) and one microcosm on day 12 (24 µg/L). VC was detected on day 9 (average concentration of 260 µg/L) and in one microcosm on day 12 (100 µg/L).

Mass balance calculations (Figure 5) show that the average total µmoles of chlorinated ethenes in the experimental reactors decreased by about 25% over the entire 12-day experiment. This decrease occurred during the first day of the experiment, when no TCE metabolites were detected. Similar TCE losses were observed in the control microcosms (Figure 4), suggesting leakage from the microcosms. The lack of any significant decrease in the total molar quantity of chlorinated solvents beyond day 2 in the experimental reactors (Figure 5) suggests that the facultative culture did not completely dechlorinate TCE. Reports of dechlorination by facultative cultures are rare. Sharma and McCarty (1996) found that two strains of facultative bacteria dechlorinated tetrachloroethene (PCE) to *cis*-DCE via TCE in the absence of oxygen and nitrate. These organisms could utilize acetate as an electron donor, and strongly reducing conditions, as required for sulfate reduction or methanogenesis, were not required for dechlorination of PCE.

Discussion

Pseudo-first-order rate constants for RDX ($k_{1,RDX}$) and TCE ($k_{1,TCE}$) are summarized in Tables 1 and 2, respectively, along with other details of how these rates were calculated. RDX at initial concentrations of 0.61–1.04 mg/L was readily degraded by both the anaerobic sludge and the facultative culture, both in the presence and absence of TCE (Figures 1, 2, & 4). The calculated pseudo-first-order RDX degradation rates ranged from 1.4 × 10^{-3} to 4.7 × 10^{-3} L/mg VSS d. Based on these results, it appears the facultative culture degraded RDX slightly faster than the anaerobic sludge.

Low concentrations of nitroso-RDX metabolites were detected in all RDX degradation assays, but none of these compounds accumulated. In the absence of TCE, the only nitroso-RDX compound detected was TNX, which was detected towards the end of each experiment. In contrast, when TCE was present, MNX was the only nitroso-RDX metabolite detected above its detection limit, generally early in each dual component experiment. The presence of nitroso-RDX compounds suggest that sequential reduction of one or more RDX nitro groups (McCormick et al. 1981; Hawari et al. 2000) was a first step in the biodegradation of RDX by these two cultures.

Interestingly, estimated RDX degradation rates for each culture increased by more than 100% from the single component to the dual component experiments. This indicates that the presence of TCE did not adversely affect RDX biodegradation. During acclimation to TCE, neither culture was supplied with a readily available nitrogen source, although some nitrogen would have been available due to endogenous decay. When these nitrogen-deprived cultures were used for the dual component tests, a nitrogen source (RDX) was available for both RDX and TCE degraders. RDX can be biodegraded under nitrogen-deficient conditions when present as the sole nitrogen source (Binks et al. 1995; Brenner et al. 2000; Morley et al. 2002).

Prior to acclimation, TCE was not transformed by the anaerobic sludge and was minimally transformed by the facultative culture, which produced small quantities of both DCE isomers. Both cultures benefited from the relatively short TCE acclimation periods, particularly the anaerobic culture. A similar observation has been reported by Gu et al. (2004), who found that lactate-fed methanogens developed an ability to dechlorinate TCE and *cis*-DCE after 3 to 4 weeks of exposure to these compounds. In our experiments, improved TCE degradation rates after acclimation (Table 2) may be due to acclimation as well as the presence of RDX as a potential nitrogen source in the dual contaminant experiments.

TCE was not completely dechlorinated despite more rapid degradation in the dual contaminant mixtures. The major byproduct for both cultures was *cis*-DCE, although small quantities of *trans*-DCE and VC were also detected (Figures 2–5). Accumulation of *cis*-DCE or VC has been observed in numerous studies (e.g., Vogel and McCarty 1985; Sharma and McCarty 1996; Schollhorn et al. 1997; Chang et al. 1998). Several factors may explain the inability of these cultures to completely dechlorinate TCE, including the short experimental durations, insufficiently reducing conditions, or the absence of microorganisms that cannot completely dechlorinate TCE. Additionally, the absence of sufficient hydrogen as an electron donor may have limited dechlorination by our cultures. Acetate can be used as an electron donor without supplemental hydrogen during reductive dechlorination (He et al. 2002; Sung et al. 2003), but other more complex organic compounds, including methanol, can be utilized as carbon sources, electron donors, and H_2 sources, typically resulting in rapid dechlorination (e.g., Fennell et al. 1997; Gu et al. 2004). Further research should examine the ability of these cultures to utilize other electron donors and carbon sources during reductive dechlorination.

When TCE is only partially dechlorinated under reducing conditions, anaerobic biotransformation may only be effective as part of a remedial response to groundwater with mixtures of TCE and RDX, or similar contaminant mixtures. Use of electron donors other than acetate that supply hydrogen may allow more complete reduction of chlorinated compounds, or bioaugmentation may be needed to enhance populations of dechlorinating organisms (Ellis et al. 2000; Lendvay et al. 2003). Alternatively, anaerobic biodegradation of RDX and TCE could be coupled with aerobic or anaerobic oxidation processes, which can effectively eliminate DCE and VC (Lee et al. 1998; Bradley 2000).

Conclusions

Mixtures of TCE and RDX can be rapidly biotransformed by acclimated microbial cultures under anaerobic conditions. RDX was readily degraded by a facultative culture and by microbes from anaerobic sludge, with very little accumulation of nitroso-RDX compounds. MNX and TNX were occasionally detected at very low concentrations, but were not persistent in any of the microcosms. These cultures also partially dechlorinated TCE, but only after 3–5 weeks acclimation to TCE. The major chlorinated ethene produced by both cultures was *cis*-DCE, with trace quantities of *trans*-DCE and VC produced. Mass balance analyses suggest that complete dechlorination was not achieved, possibly because the experimental duration was too short or the cultures had insignificant populations of organisms that can dechlorinate *cis*-DCE to VC under the conditions of these experiments. These preliminary results suggest that *in situ* bioremediation may be an effective approach for remediating groundwater with mixtures of TCE and RDX, with at least partial biotransformation occurring under reducing conditions. Further research is needed to optimize conditions for bioremediating mixtures of these compounds and to determine techniques for eliminating the persistent chlorinated ethenes.

Acknowledgments

This research was funded by a grant from the U.S. Geological Survey. The writers thank Mostafa Fatemi for assistance with purification, preparation of analytical standards, and determination of detection limits for RDX and the nitroso-RDX compounds. This paper is a contribution of the University of Nebraska Agricultural Research Division, Lincoln, Nebraska, Journal series No. 14906.

References

Adrian, N. R., Arnett, C. M., and Hickey, R. F. (2003). Stimulating the anaerobic biodegradation of explosives by the addition of hydrogen or electron donors that produce hydrogen. *Water Res.*, 37, 3499–3507.

Agency for Toxic Substances and Disease Registry (ATSDR). (1995). *Toxicological profile for RDX, Rep. TP-78*, U.S. Dept. of Health and Human Services, Public Health Service, Atlanta.

Agency for Toxic Substances and Disease Registry (ATSDR). (1997). *Toxicological profile for trichloroethylene, Rep. TP-19*, U.S. Dept. of Health and Human Services, Public Health Service, Atlanta.

Beller, H. R. (2002). Anaerobic biotransformation of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) by aquifer bacteria using hydrogen as the sole electron donor. *Water Res.*, 36, 2533–2540.

Binks, P. R., Nicklin, S., and Bruce, N. C. (1995). Degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by *Stenotrophomonas maltophilia* PB1. *Appl. Environ. Microbiol.*, 61(4), 1318.

Bradley, P. M. (2000). Microbial degradation of chloroethenes in groundwater systems. *Hydrogeol. J.*, 8, 104–111.

Brenner, A., Ronen, Z., Harel, Y., and Abellovich, A. (2000). Use of hexahydro-1,3,5-trinitro 1,3,5-triazine as a nitrogen source in biological treatment of munitions wastes. *Water Environ. Res.*, 72(4), 469–475.

Brockman, F. J., Downing, D. C., and Wright, G. F. (1949). Nitrolysis of hexamine tetramine III, preparation from pure cyclonite. *Can. J. Phys.*, 27B, 469–474.

BWXT Pantex. (2004). 2003 Site Environmental Report for Pantex Plant, Environmental Protection/Restoration Dept., Environment, Safety, Health and Quality Division–Pantex Plant for the U.S. Dept. of Energy, Albuquerque Operations Office, Amarillo Area Office, Amarillo, Tex. Online (May 19, 2005) at http://www.pantex.com/aser/2003/index2003.html

Carter, M. C., Weber, W. J., Jr., and Olmstead, K. P. (1992). Effects of background dissolved organic matter on TCE adsorption by GAC. *J. Am. Water Works Assoc.*, 84(4), 81–91.

Chang, Y. C., Hatsu, M., Jung, K., Yoo, Y. S., and Takamizawa, K. (1998). Degradation of a variety of halogenated aliphatic compounds by an anaerobic mixed culture. *J. Ferment. Bioeng.*, 86(4), 410–412.

Clausen, J., Robb, J., Curry, D., and Korte, N. (2004). A case study of contaminants on military ranges: Camp Edwards, Massachusetts, USA. *Environ. Pollut.*, 129, 13–21.

Clesceri, L. S., Greenberg, A. E., and Eaton, A. D. (1998). *Standard methods for the examination of water and wastewater*, 20th Ed., American Public Health Association, Washington, D.C.

Davis, J. L., Wani, A. H., O'Neal, B. R., and Hansen, L. D. (2004). RDX biodegradation column study: Comparison of electron donors for biologically induced reductive transformation in groundwater. *J. Hazard. Mater.*, 112(1-2), 45–54.

Ellis, D. E., et al. (2000). Bioaugmentation for accelerated in situ anaerobic bioremediation. *Environ. Sci. Technol.*, 34, 2254–2260.

Fennell, D. E., Carroll, A. B., Gossett, J. M., and Zinder, S. H. (2001). Assessment of indigenous reductive dechlorination potential at a TCE-contaminated site using microcosms, polymerase chain reaction analysis, and site data. *Environ. Sci. Technol.*, 35, 1830–1839.

Fennell, D. E., Gossett, J. M., and Zinder, S. H. (1997). Comparison of butyric acid, ethanol, lactic acid, and propionic acid as hydrogen donors for the reductive dechlorination of tetrachloroethene. *Environ. Sci. Technol.*, 31, 918–926.

Freedman, D. L., and Gossett, J. M. (1989). Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. *Appl. Environ. Microbiol.*, 55, 2144–2151.

Gossett, J. M. (1987). Measurements of Henry's Law constants for C1 and C2 chlorinated hydrocarbons. *Environ. Sci. Technol.*, 21, 202–208.

Gu, A. Z., Hedlund, B. P., Staley, J. T., Strand, S. E., and Stensel, H. D. (2004). Analysis and comparison of the microbial community structures of two enrichment cultures capable of reductively dechlorinating TCE and cis-DCE. *Environ. Microbiol.*, 6(1), 45–54.

Hawari, J., et al. (2000). Characterization of metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) with municipal anaerobic sludge. *Appl. Environ. Microbiol.*, 66(6), 2652–2657.

He, J. Z., et al. (2002). Acetate versus hydrogen as direct electron donors to stimulate the microbial reductive dechlorination process at chloroethene-contaminated sites. *Environ. Sci. Technol.*, 36 (18), 3945–3952.

Hyun, Y. (2004). Optimization study of an existing GAC treatment system, former Nebraska Ordnance Plant, Mead, NE. Master of Science thesis, Environmental Engineering, The University of Nebraska–Lincoln, Lincoln, Neb.

Lee, M. D., Odom, J. M., and Buchanan, R. J., Jr. (1998). New perspectives on microbial dehalogenation of chlorinated solvents: Insights from the field. *Annu. Rev. Microbiol.*, 52, 423–452.

Lendvay, J. M., et al. (2003). Bioreactive barriers: A comparison of bioaugmentation and biostimulation for chlorinated solvent remediation. *Environ. Sci. Technol.*, 37, 1422–1431.

McCormick, N. G., Cornell, J. H., and Kaplan, A. M. (1981). Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine. *Appl. Environ. Microbiol.*, 42(5), 817–823.

Mohn, W. W., and Tiedje, J. M. (1992). Microbial reductive dehalogenation. *Microbiol. Rev.*, 56, 482–507.

Morley, M. C., Henke, J. L., and Speitel, G. E., Jr. (2005). Adsorption of RDX and HMX in rapid small-scale column tests: Implications for full-scale adsorber applications. *J. Environ. Eng.*, 131(1), 29–37.

Morley, M. C., Shammas, S. N., and Speitel, G. E., Jr. (2002). Biodegradation of RDX and HMX mixtures: Batch screening experiments and sequencing batch reactors. *Environ. Eng. Sci.*, 19(4), 237–250.

Pennington, J. C., and Brannon, J. M. (2002). Environmental fate of explosives. *Thermochim. Acta*, 384, 163–172.

Schollhorn, A., Savary, C., Stucki, G., and Hanselmann, K. (1997). Comparison of different substrates for the fast reductive dechlorination of trichloroethene under groundwater conditions. *Water Res.*, 31(6), 1275–1282.

Sharma, P. K., and McCarty, P. L. (1996). Isolation and characterization of a facultatively aerobic bacterium that reductively dehalogenates tetrachloroethene to cis-1,2-dichloroethene. *Appl. Environ. Microbiol.*, 62 3), 761–765.

Smatlak, C. R., Gossett, J. M., and Zinder, S. H. (1995). Comparative kinetics of hydrogen utilization for reductive dechlorination of tetrachloroethene and methanogenesis in an anaerobic enrichment culture. *Environ. Sci. Technol.*, 30(9), 2850–2858.

Spain, J. C., Hughes, J. B., and Knachmuss, H. J. (2000). *Biodegradation of nitroaromatic compounds and explosives*, Lewis Publishers, New York.

Speitel, G. E., Jr., Engels, T. L., and McKinney, D. C. (2001). Biodegradation of RDX in unsaturated soil. *Biorem. J.*, 5, 1–11.

Sung, Y., et al. (2003). Characterization of two tetrachloroethene-reducing, acetate-oxidizing anaerobic bacteria as *Desulfuromonas michiganensis* sp. nov. *Appl. Environ. Microbiol.*, 69(5), 2964–2974.

Tandoi, V., DiStefano, T. D., Bowser, P. A., Gossett, J. M., and Zinder, S. H. (1994). Reductive dehalogenation of chlorinated ethenes and halogenated ethanes by a high-rate anaerobic enrichment culture. *Environ. Sci. Technol.*, 28(5), 973–979.

U.S. Environmental Protection Agency (USEPA). (1997). EPA Superfund Record of Decision: Nebraska Ordnance Plant (Former), Mead, NE Operable Unit 2. *EPA ID NE6211890011, EPA/ROD/R07-97/143*.

U.S. Environmental Protection Agency (USEPA). (2004). Drinking water standards and health advisories. *EPA 822-R-04-005*, Office of Water, Washington, D.C.

Vogel, T. M., Criddle, C. S., and McCarty, P. L. (1987). Transformations of halogenated aliphatic compounds. *Environ. Sci. Technol.*, 21, 722–736.

Vogel, T. M., and McCarty, P. L. (1985). Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. *Appl. Environ. Microbiol.*, 49(5), 1080–1083.

Wani, A. H., and Davis, J. L. (2003). RDX biodegradation column study: Influence of ubiquitous electron acceptors on anaerobic biotransformation of RDX. *J. Chem. Technol. Biotechnol.*, 78(10), 1082–1092.

Xu, N., Vandegrift, S., and Sewell, G. W. (1996). Determination of chloroethenes in environmental biological samples using gas chromatography coupled with solid phase micro extraction. *Chromatographia*, 42(5 /6), 313–317.

Young, D. M., Kitts, C. L., Unkefer, P. J., and Ogden, K. L. (1997a). Biological breakdown of RDX in slurry reactors proceeds with multiple kinetically distinguishable paths. *Biotechnol. Bioeng.*, 56(3), 258–267.

Young, D. M., Unkefer, P. J., and Ogden, K. L. (1997b). Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a prospective consortium and its most effective isolate *Serratia marcescens*. *Biotechnol. Bioeng.*, 53(5), 515–522.