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Anaerobic biotransformation of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) by aquifer bacteria using hydrogen as the sole electron donor

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Abstract

RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) is a nitramine explosive that has contaminated soil and groundwater at military installations throughout the US. Although anaerobic RDX metabolism has been reported, the process is not well understood, as past studies have typically involved complex, undefined media with multiple potential electron donors and acceptors. In this study, bacteria enriched from RDX-contaminated aquifer sediments consumed RDX in a defined, bicarbonate-buffered, anaerobic medium containing hydrogen as the sole electron donor and RDX as a potential electron acceptor and sole nitrogen source. RDX was not consumed in live controls that did not contain hydrogen. Transient formation of mononitroso- and dinitroso-RDX metabolites (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine and hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine, respectively) was documented by liquid chromatography-mass spectrometry. However, studies with ^{14}C-labeled RDX suggested that mineralization to carbon dioxide was negligible (<2%), which is consistent with cometabolic transformation. Several lines of evidence suggest that the RDX-transforming bacteria under study were homoacetogens, including correlations between RDX consumption and acetate production. Methanogens were unlikely to be responsible for RDX metabolism, as the presence of 2-bromoethanesulfonate, an inhibitor of methanogenesis, did not appear to affect RDX metabolism. The presence of nitrate reversibly halted RDX metabolism, whereas ammonium had no discernible effect, which implies that: (i) nitrate, which commonly occurs in RDX-contaminated groundwater, may inhibit in situ RDX metabolism, and (ii) although RDX may act as both a nitrogen source and cometabolic electron sink, the latter role predominates, as RDX reduction will proceed regardless of whether or not a more favorable nitrogen source is present. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: RDX; Anaerobic; Hydrogen; Acetogen; Groundwater

1. Introduction

RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine; Fig. 1) is a cyclic nitramine explosive that has contaminated groundwater, soil, and surface water at military installations throughout the US, prompting concerns about potential toxic effects [1–3]. Although aerobic bacterial RDX metabolism has been observed [4,5], anaerobic metabolism has been reported more frequently [1,6–13] and has proven to be more favorable when aerobic and anaerobic incubation were directly compared [11–14]. Our understanding of the potential for in situ bioremediation of RDX in aquifers is very limited, as experimental conditions in existing studies were not designed to be directly relevant to that environment. For example, studies of anaerobic RDX metabolism have typically involved complex, undefined media (e.g., containing nutrient broth or yeast extract) with multiple potential electron donors and acceptors,

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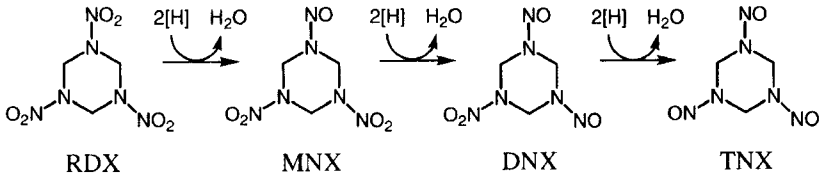


Fig. 1. Structures of RDX and the anaerobic metabolites MNX, DNX, and TNX.

or in some cases, single organic electron donors at relatively high concentrations (e.g., ⩾10 mM glucose or ethanol). This article addresses the fate of RDX (i.e., metabolite formation and degree of mineralization) in aquifer-derived, anaerobic cultures using H_2 as the sole electron donor. Although RDX biotransformation may involve cometabolic reduction reactions and abiotic hydrolysis reactions that are of little overall benefit to the bacteria involved, it is noteworthy that RDX mineralization coupled with hydrogen oxidation could potentially yield considerable energy and nitrogen, as illustrated by the equation below (thermodynamic data were taken from [15–17]).

$$C_3H_6N_6O_6(\mathrm{RDX}) + 6H_2 + 3H^+ + 3H_2O \rightarrow 3HCO_3^- + 6NH_4^+$$
$$\Delta G^{0\prime} = -1498\ \mathrm{kJ/reaction}.$$

2. Materials and methods

Chemicals used in this study were of the highest purity available and were used as received. Although a stock solution of RDX was available for use as a quantitative standard (Radian International, Austin, TX), standards of the nitroso-substituted RDX metabolites under study (Fig. 1) were not commercially available. MNX and TNX were synthesized at Lawrence Livermore National Laboratory (LLNL) by P. Pagoria using procedures described by Brockman et al. [18], and DNX (98%) was custom-synthesized by Radian. The [*ring*-U-^{15}N]RDX used as an internal standard for liquid chromatography-mass spectrometry (LC-MS) analysis was synthesized at LLNL from acetamide-^{15}N (99 atom%; Isotec, Inc., Miamisburg, OH) based on techniques described elsewhere [19,20]. The purity of standards synthesized at LLNL is estimated to be ⩾95% based on nuclear magnetic resonance, LC-MS, and melting point analyses. The DNX and [*ring*-U-^{15}N]RDX standards became available after most studies described in this article were completed, and should be assumed to not have been used unless noted otherwise. The RDX used for amendment of microcosms and enrichment cultures was derived from a high-purity stock at LLNL because the concentration and solvent (acetonitrile) in the analytical RDX standard were not suitable for this purpose. For radiolabeled assays, cultures were amended with uniformly labeled [U-^{14}C]RDX (⩾99% purity, 7.75 mCi/mmol, Chemsyn Science Laboratories, Lenexa, KN). Reagent water (18 MΩ resistance) was obtained from a Milli-Q UV Plus system (Millipore, Bedford, MA). Ultra high-purity (99.999%) nitrogen was used as a nebulizer and desolvation gas for LC-MS.

The inoculum used for this study was derived from an LLNL (Site 300) groundwater monitoring well with historical RDX contamination. After collection, 18 L of groundwater were stored in autoclaved, 2-L medium bottles and transported to an anaerobic glove box within 1 h. The suspended bacteria and sediment in the groundwater were harvested anaerobically by centrifugation (12,800 × *g*, 15°C, 20 min) in sealed polycarbonate bottles. The final pellet was resuspended in 200 mL of growth medium and the resulting suspension was used to inoculate microcosms that served as the source of enrichment cultures for this study. The experiments described in this article were performed with enrichment cultures that had undergone at least two transfers from the original microcosms. Prior to these experiments, cultures were incubated with hydrogen as the sole electron donor and were constantly supplied with RDX.

The anaerobic growth medium used in this study contained RDX as the sole nitrogen source, hydrogen as the sole electron donor, and RDX and bicarbonate as the only potential carbon sources and electron acceptors. The medium composition was similar to that described by Beller et al. [21] with the following major differences: (i) the $NaHCO_3$ concentration used in this study was 60 mM rather than 30 mM, (ii) NH_4Cl, $FeSO_4$, and toluene were omitted in the present study, and (iii) a lower concentration of sodium sulfide (75–100 μM) was used for pre-reduction of the medium in the present study. RDX was added to the medium by injecting a small volume (typically 5–30 μL) of acetone stock solution (ca. 30 μg RDX/μL acetone) into an empty glass bottle using a microliter syringe, allowing the acetone to evaporate completely, adding medium to the bottle, and allowing the RDX to dissolve overnight before inoculation. Initial RDX concentrations in various experiments were typically between 10 and 40 μM. All preparation and incubation of enrichment cultures was performed at 30°C under strictly anaerobic

conditions in an anaerobic glove box (Coy Laboratory Products, Inc., Grass Lake, MI) with a gas composition of approximately 90% N_2–7.5% CO_2–2.5% H_2. Sterile controls were used in all experiments and were prepared in a manner identical to active cultures except that the inoculum was first autoclaved under strictly anaerobic conditions (in a sealed serum bottle) and sodium azide (2 g/L) was added to the medium. RDX, which is photochemically reactive, was protected from light by using amber glass bottles or clear glass bottles covered with aluminum foil. Bottle sizes used in different experiments were either 20 mL (with 5 or 10 mL of liquid culture), 120 mL (with 50 mL of liquid culture), or 250 mL (with 100 mL of liquid culture). Unless noted otherwise, the bottles were sealed with Mininert Teflon, screw-cap valves (Alltech Associates, Inc., Deerfield, IL).

2.1. Analytical methods

RDX, MNX, DNX, and TNX were analyzed by LC-MS with a Quattro LC (Z-spray) triple quadrupole MS (Micromass, Inc., Manchester, UK) equipped with a Waters 2690 Separations Module (Waters, Milford, MA) and operated in the electrospray, negative ionization mode. The mobile phase was a 50:50 (vol/vol) mixture of methanol and reagent water flowing isocratically at 200 μL/min through a Nova-Pak C_{18} column (4-μm particle size, 60 Å, 2.1-mm inner diameter by 150-mm length; Waters, Milford, MA) and a 20-mm guard column containing the same stationary phase. The sample injection volume was 25 μL. Data acquisition was in the selected ion monitoring (single quadrupole) mode with a 0.4-s dwell time for each analyte. The capillary voltage was 2.75 kV and sample cone voltage varied between 9 and 15 V in correspondence with the optimal response determined for each analyte. RDX and all metabolites were detected as $[M+75]^-$ adducts; the adduct composition is postulated as $[M+NO_2+CH_3O-2H]^-$, which implies the involvement of ion–molecule reactions. As [*ring*-U-^{15}N]RDX was unavailable for most of the experiments described in this article, it should be assumed that external standard quantification was applicable unless noted otherwise. In the absence of an authentic DNX standard, the DNX response factor was estimated as the average of the MNX and TNX response factors.

Acetate and nitrate were determined with an ion chromatograph (model DX 500, Dionex Corporation, Sunnyvale, CA) equipped with an AG14 (4 mm) guard column, an AS14 (4 mm) separation column, a micromembrane suppressor, and an electrochemical conductivity detector. Analyses of 100 μL samples were isocratic, with a 3.5 mM Na_2CO_3–1 mM $NaHCO_3$ eluent flowing at a rate of 1.2 mL/min.

^{14}C-labeled RDX was used to investigate the extent of RDX mineralization in these aquifer-derived enrichment cultures. Analysis of ^{14}C activity in culture liquid and headspace was performed with a Wallac model 1409 liquid scintillation counter (PerkinElmer–Wallac, Inc., Gaithersburg, MD), which carried out chemical and color quench correction as well as chemiluminescence correction on a sample-specific basis. Sample processing methods were very similar to those described previously [21,22]. By this method, three fractions of ^{14}C activity were determined in culture samples: $^{14}CO_2$, nonvolatile carbon (potentially including RDX, biomass, and nonvolatile metabolites), and volatile carbon (volatile metabolites).

3. Results

3.1. RDX metabolism, nitroso-metabolite formation, and acetate production with H_2 as the sole electron donor

The aquifer-derived enrichment cultures metabolized RDX with hydrogen as the sole electron donor under strictly anaerobic conditions (Fig. 2). In the experiment represented in Fig. 2, the cultures were incubated in butyl rubber-stoppered, 120-mL serum bottles under the following conditions: (i) with a headspace of 80% N_2–10% CO_2–10% H_2 (duplicates; square symbols in Fig. 2); (ii) with a headspace of 80% N_2–10% CO_2–10% H_2 and 2-bromoethanesulfonate (BESA; 1 mM) in solution (duplicates; triangular symbols in Fig. 2A); (iii) with a headspace of 90% N_2–10% CO_2 until Day 59, at which time the headspace was replaced with 80% N_2–10% CO_2–10% H_2 (duplicates; circular symbols in Fig. 2); and (iv) for sterile controls, with a headspace of either 80% N_2–10% CO_2–10% H_2 (one replicate) or 90% N_2–10% CO_2 (one replicate). In all bottles, the headspace was established by three repetitions of vacuum–gas cycling (i.e., evacuation of the headspace under vacuum followed by addition of the desired gas under pressure).

After a lag period of 30–40 days, RDX consumption began in the active cultures that contained hydrogen (Fig. 2A). The presence of BESA, an inhibitor of methanogenesis [23,24], did not appreciably affect RDX consumption (Fig. 2A). Until Day 59, RDX concentrations in the sterile controls and active controls without hydrogen remained relatively constant (relative standard deviation = 6.9%; $n = 32$ data points for 4 cultures). After the addition of hydrogen to the active controls on Day 59, RDX consumption commenced within 2 weeks (Fig. 2A). In all the active cultures, the metabolite MNX was formed as RDX was consumed (Fig. 2B), and subsequently, DNX was formed as MNX was consumed (Fig. 2C). TNX was also observed, but at relatively low concentrations (typically not exceeding

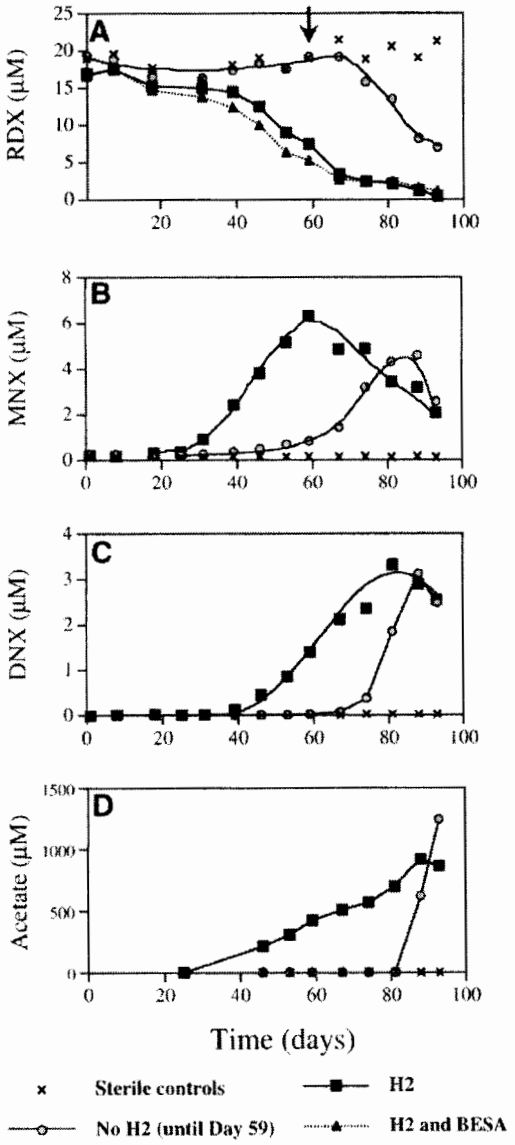


Fig. 2. Concentrations of RDX (A), MNX (B), DNX (C), and acetate (D) in aquifer-derived enrichment cultures in which hydrogen (when present) was the sole electron donor and RDX was the sole nitrogen source. Data points represent the averages of results of duplicate cultures. The arrow at Day 59 represents the time that hydrogen was added to the headspace of live controls (circular symbols). DNX concentrations should be considered as estimates because an authentic standard was not available during this experiment (see text).

0.5 μM; data not shown). Acetate was formed in the cultures that contained hydrogen (Fig. 2D). Although observations are limited by the sampling frequency and detection limit for acetate (ca. 100 μM), there appears to be a correspondence between the onset of RDX metabolism and the onset of acetate formation (Fig. 2A and D). Concentration trends for the hydrogen-plus-BESA-amended cultures were similar to those for the hydrogen-amended cultures; for the sake of clarity, only the RDX data for the hydrogen-plus-BESA-amended cultures are shown in Fig. 2.

The relationship between RDX metabolism and acetate formation was addressed in another experiment with enrichment cultures. In this experiment, RDX consumption and acetate formation were monitored in four active replicates and two sterile controls. As a result of variability that cannot be accounted for by different experimental conditions, two different levels of activity were observed, with two faster cultures and two slower cultures (Fig. 3). Although the cause of the variable activity is unknown, it is apparent that the cultures that metabolized RDX faster also produced acetate more rapidly (Fig. 3).

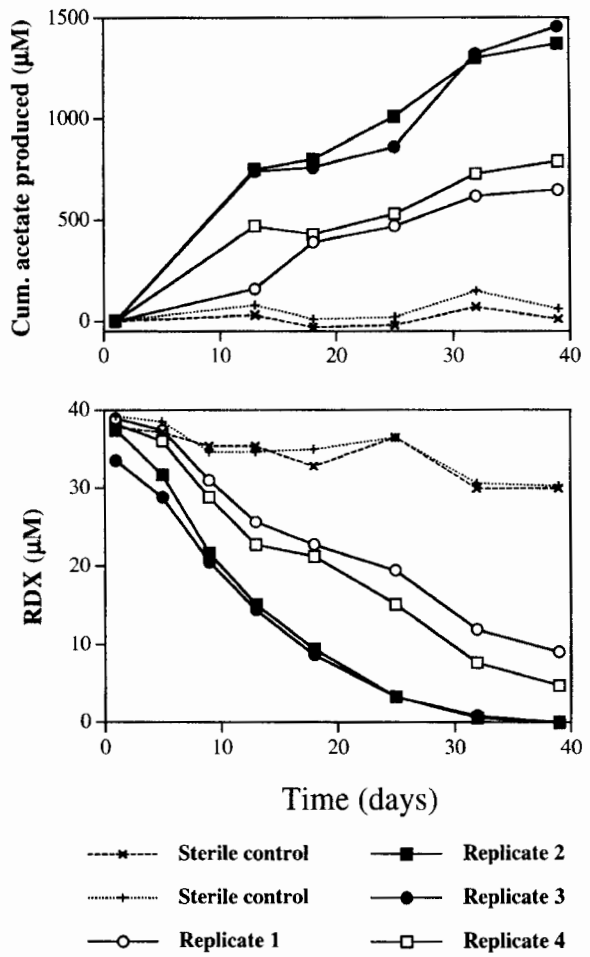
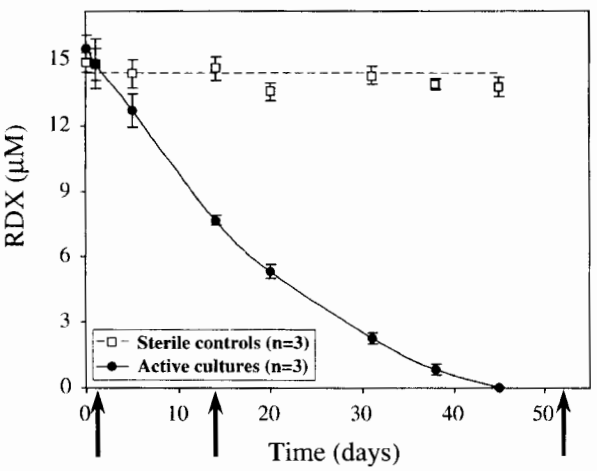


Fig. 3. Cumulative acetate production and RDX concentration in aquifer-derived enrichment cultures in which hydrogen was the sole electron donor.

3.2. Extent of RDX mineralization to CO_2 with H_2 as the sole electron donor

RDX mineralization was investigated by amending enrichment cultures with [U-^{14}C]RDX. Active cultures and sterile controls (all run in triplicate) were amended with ca. 10 μM RDX (0.39 μCi) and were analyzed sacrificially at four time points. A parallel experiment was performed with unlabeled RDX (ca. 15 μM) to determine strategic sampling times for ^{14}C analysis; the cultures with unlabeled RDX were sampled repeatedly rather than sacrificially. LC-MS analyses for the unlabeled RDX, MNX, DNX, and TNX relied on internal standard quantification with [*ring*-U-^{15}N]RDX; in this study, an authentic standard was available for DNX quantification.

The results of a mineralization experiment are presented in Fig. 4. Unlabeled RDX was depleted by Day 45 (Fig. 4) and LC-MS analysis of selected cultures confirmed that radiolabeled RDX was depleted by Day 52 (data not shown). The ^{14}C data indicate that RDX mineralization by these enrichment cultures was negligible (Fig. 4). By Day 69, only 1.3±0.2% (mean±SD) of the carbon had been converted to $^{14}CO_2$ and nonvolatile carbon accounted for virtually all (97.9±0.2%) of the ^{14}C. Volatile carbon (other than CO_2) never accounted for >0.8% of the ^{14}C on any sampling day. LC-MS analysis of the unlabeled cultures on Day 45 indicated that the nonvolatile metabolites MNX, DNX, and TNX constituted ≤0.5% of the total RDX carbon (data not shown). Thus, these enrichment cultures ultimately converted RDX almost completely to nonvolatile metabolites other than MNX, DNX, and TNX. Metabolites that have previously been identified in anaerobic RDX-metabolizing cultures and may have been operationally included with nonvolatile carbon in this experiment include hydrazine, 1,1-dimethyl- and 1,2-dimethylhydrazine, methylenedinitramine [$(O_2NNH)_2CH_2$], bis(hydroxymethyl)nitramine [$(HOCH_2)_2NNO_2$], formaldehyde, and methanol [1,9,12]; in the present study, specific analyses were not performed to identify these compounds, some of which are known to be labile in aqueous solution. Although ^{14}C in biomass was not directly measured in this study, it is likely that the negligible observed mineralization to CO_2 indicates negligible assimilation of RDX carbon into biomass (e.g., [11,12]).



RADIOCARBON DISTRIBUTION

Time (day)	Control		Active	
	Nonvolatile	CO_2	Nonvolatile	CO_2
1	97.7±2.1%	1.8±1.9%	96.5±0.7%	3.2±0.5%
14	98.9±0.6%	0.7±0.2%	98.9±0.1%	0.9±0.2%
52	98.7±1.0%	0.6±0.6%	98.4±0.5%	1.4±0.2%
69	99.2±0.4%	0.5±0.2%	97.9±0.2%	1.3±0.2%

Fig. 4. RDX metabolism and radiocarbon distribution in aquifer-derived enrichment cultures in which hydrogen was the sole electron donor. The data are from parallel experiments (see text) with unlabeled RDX (graphical data) and radiolabeled RDX (tabular data). Data points represent the averages of results of triplicate cultures; error bars represent 1 SD. Radiocarbon distribution data (nonvolatile ^{14}C and $^{14}CO_2$) are presented as mean±SD (triplicate cultures). Arrows indicate the times of the first three radiocarbon samplings. Mass balances of ^{14}C were 95.7–100.8% (relative to the 0.39 μCi added initially) for all individual replicates, including controls and active cultures.

3.3. The effect of nitrate on RDX metabolism with H_2 as the sole electron donor

The effect of nitrate on anaerobic RDX metabolism was investigated, as nitrate is a potential electron acceptor and nitrogen source that is present in RDX-contaminated groundwater at LLNL Site 300 (~55 mg/L in the well that served as the inoculum source; unpublished data) and in munition wastewater in general [8,12]. In one experiment, two identically prepared sets of duplicate enrichment cultures were allowed to metabolize RDX until Day 13, at which time 0.5 mM nitrate was added to one set of duplicates (Fig. 5A). The nitrate was completely depleted by Day 25 (Fig. 5C). Between Days 13 and 25, RDX metabolism ceased in the nitrate-amended cultures, whereas an additional 18% of the RDX was metabolized in the cultures without nitrate (Fig. 5A). After Day 25, when nitrate had been depleted, RDX consumption resumed in the nitrate-amended cultures and proceeded at a rate similar to that of the unamended cultures. On Day 39, 0.5 mM ammonium, which is a potential nitrogen source but not electron acceptor, was added to the cultures that were previously nitrate-amended. There was no clear effect of ammonium addition on the rate of RDX metabolism (Fig. 5A). Thus, nitrate (a favorable elec-

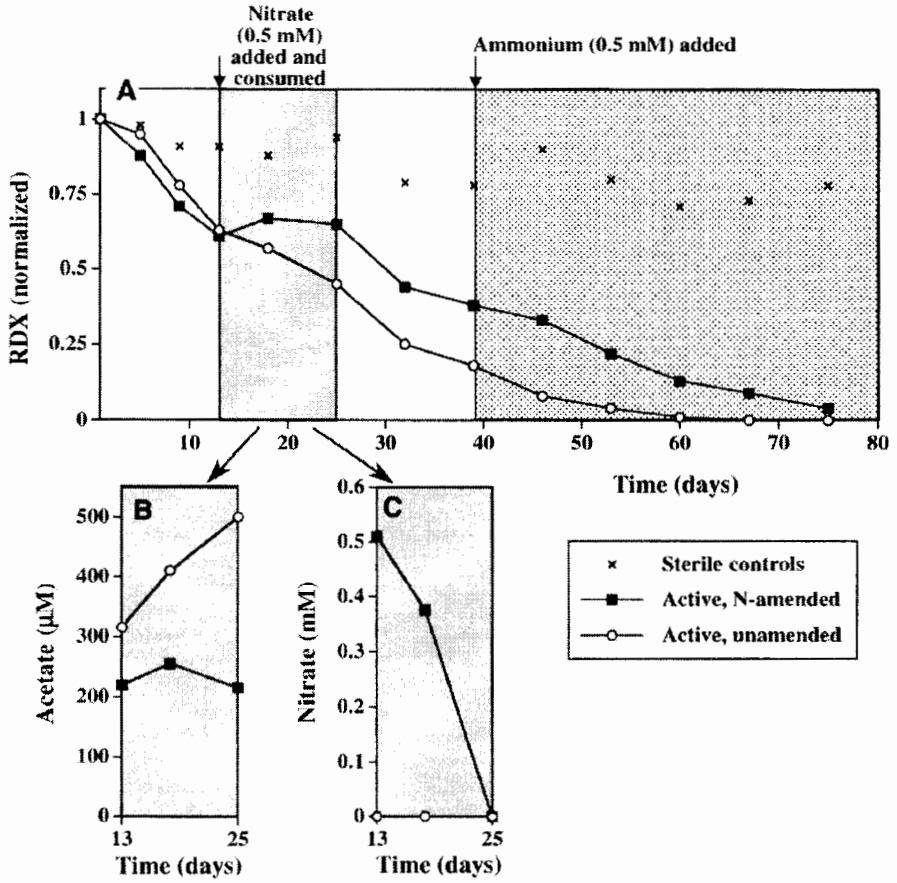


Fig. 5. (A) The effect on anaerobic RDX metabolism of adding nitrate and ammonium to aquifer-derived enrichment cultures in which hydrogen was the sole electron donor. The shaded region between Days 13 and 25 represents the interval during which 0.5 mM nitrate was added to duplicate cultures (square symbols) and was subsequently consumed. The stipled region beginning at Day 39 represents the interval during which 0.5 mM ammonium was present in duplicate cultures (square symbols). Data points represent the averages of results of duplicate cultures. RDX concentrations are normalized to their initial values, which ranged from ca. 39 to 44 μM. (B) and (C) show the concentrations of acetate and nitrate, respectively, between Days 13 and 25.

tron acceptor or sink) halted RDX consumption, whereas ammonium (a favorable nitrogen source) had no apparent effect. These results have several implications: (i) nitrate, which commonly occurs in RDX-contaminated groundwater, may inhibit in situ RDX metabolism, and (ii) although RDX may act as both a nitrogen source and cometabolic electron sink, the latter role predominates, as RDX reduction will proceed regardless of whether or not a favorable nitrogen source is present. Notably, inhibition of anaerobic RDX metabolism by nitrate was also observed in a study of ethanol-amended cultures inoculated with a biofilm from a wastewater treatment plant [8]. In addition to its effect on RDX metabolism in the present study, nitrate also appeared to halt acetate production (Fig. 5B).

4. Discussion

This study, carried out under defined acetogenic conditions, provides a new information on anaerobic RDX metabolism with H_2 as the sole electron donor and confirms trends observed in more complex systems. Experiments including live and sterile controls (Fig. 2) demonstrated that H_2 can serve as the sole electron donor for anaerobic RDX transformation by aquifer bacteria. There has been another report of RDX metabolism with H_2 as the electron donor [6]; however, the fate of RDX in that methanogenic enrichment culture was not addressed and the inoculum was not aquifer-derived. In this study, nitroso-substituted metabolites (particularly MNX and DNX; Fig. 2) were transiently formed during RDX metabolism; this

phenomenon has been reported for various other RDX-metabolizing cultures that used organic electron donors such as nutrient broth or yeast extract [8,9,11–13]. The ultimate fate of RDX in the present study appears to be nonvolatile metabolites other than MNX, DNX, and TNX; mineralization of radiolabeled RDX to $^{14}CO_2$ was negligible (<2%) and 98% of the ^{14}C was nonvolatile after RDX was depleted (Fig. 4). Similarly, McCormick et al. [12], in their seminal study of anaerobic RDX metabolism in the presence of nutrient broth, reported that only 1.5% of the total carbon from [^{14}C]RDX was converted to volatile carbon (including $^{14}CO_2$) and 2% was associated with solids (a maximum estimate of biomass created from RDX carbon). Low yields of $^{14}CO_2$ (5–9%) and biomass ($\leqslant 1\%$) were also observed in a study of anaerobic RDX metabolism by three *Enterobacteriaceae* species in the presence of yeast extract [11]. In contrast, a study of RDX metabolism by cultures inoculated with municipal anaerobic sludge and amended with 11.7 mM glucose resulted in ca. 50% $^{14}CO_2$ yields and other evidence of ring cleavage [9].

Several lines of indirect evidence suggest that the RDX-metabolizing bacteria in the present study may have been homoacetogens, such as the concurrent onset of RDX consumption and acetate formation (Fig. 2) and the correspondence of rates of RDX consumption and acetate formation (Fig. 3). Considering the medium composition used in this study, the enrichment of homoacetogenic bacteria would not be surprising. Furthermore, methanogens were unlikely to be responsible for RDX metabolism, as the presence of BESA, an inhibitor of methanogenesis, did not appear to affect RDX metabolism. Adrian and Lowder [6] proposed acetogens as the RDX-metabolizing members of a methanogenic enrichment culture amended with ethanol.

The results of the present study are consistent with the metabolism (or cometabolism) of RDX as an electron acceptor (or electron sink) by homoacetogens. In this light, the apparent inhibition of RDX metabolism by nitrate (Fig. 5) is noteworthy. Biochemical studies with acetogenic bacteria (in particular, *Clostridium thermoaceticum*, renamed *Moorella thermoacetica*) have shown that nitrate can alter electron transport and inhibit CO_2 reduction via the acetyl coenzyme A pathway [25,26], and can repress the expression of carbon monoxide dehydrogenase (CODH), hydrogenase(s), and certain electron transport proteins [27]. Notably, some of these proteins could play a crucial role in the use of RDX as an electron sink. For example, in vitro studies have demonstrated that CODH and hydrogenase can catalyze the reduction of nitro groups on TNT (2,4,6-trinitrotoluene) or 2,4-diamino-6-nitrotoluene [28,29]; nitroso intermediates were proposed for CODH-mediated TNT reduction [28]. The cometabolism of RDX by CODH (or other enzymes that participate in CO_2 reduction) and the inhibition of such enzymes by nitrate would explain why both RDX consumption and acetate production ceased in the presence of nitrate (Fig. 5A and B).

In conclusion, this study documents the anaerobic transformation, but not mineralization, of RDX by an aquifer-derived consortium under defined acetogenic conditions with hydrogen as the sole electron donor. Transient formation of nitroso intermediates (MNX, DNX, and TNX) was observed. Several lines of evidence suggest that the RDX-transforming bacteria were homoacetogens, and it is possible that the initial reduction of RDX nitro groups was cometabolically mediated by enzymes or electron transport proteins associated with acetogenesis. In contrast, the anaerobic, RDX-transforming bacteria isolated to date are not known homoacetogens, may use enzymes unrelated to acetogenesis for the reduction of RDX nitro groups (e.g., type I nitroreductase [30]), and their RDX metabolism was studied in the presence of complex, undefined medium [11,13]. A realistic assessment of the potential for in situ RDX bioremediation will, at a minimum, require more study of the metabolic diversity of anaerobic, RDX-transforming bacteria cultured under defined conditions and a fundamental understanding of the role(s) of RDX in this process (i.e., as an electron acceptor, electron sink, nitrogen source, and/or carbon source).

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References

[1] Hawari J, Beaudet S, Halasz A, Thiboutot S, Ampleman G. Microbial degradation of explosives: biotransformation versus mineralization. Appl Microbiol Biotechnol 2000;54:605–18.

[2] Palmer WG, Small MJ, Dacre JC, Eaton JC. Toxicology and environmental hazards. In: Marinkas PL, editor. Organic energetic compounds. Commack, NY: Nova Science Publishers, Inc., 1996. p. 289–372.

[3] US Environmental Protection Agency. Drinking water health advisory for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Washington, DC: Office of Drinking Water, 1988.

[4] Binks PR, Nicklin S, Bruce NC. Degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by *Stenotrophomonas maltophilia* PB1. Appl Environ Microbiol 1995;61:1318–22.

[5] Coleman NV, Nelson DR, Duxbury T. Aerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) as a nitrogen source by a *Rhodococcus* sp., strain DN22. Soil Biol Biochem 1998;30:1159–67.

[6] Adrian NR, Lowder A. Biodegradation of RDX and HMX by a methanogenic enrichment culture. In: Alleman BC, Leeson A, editors. Bioremediation of nitroaromatic and haloaromatic compounds. Proceedings from the Fifth International In Situ and On-Site Bioremediation Symposium. Columbus, OH: Battelle Press, 1999. p. 1–6.

[7] Boopathy R, Gurgas M, Ullian J, Manning JF. Metabolism of explosive compounds by sulfate-reducing bacteria. Current Microbiol 1998;37:127–31.

[8] Freedman DL, Sutherland KW. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) under nitrate-reducing conditions. Water Sci Tech 1998;38:33–40.

[9] Hawari J, Halasz A, Sheremata T, Beaudet S, Groom C, Paquet L, Rhofir C, Ampleman G, Thiboutot S. Characterization of metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) with municipal anaerobic sludge. Appl Environ Microbiol 2000;66:2652–7.

[10] Kaplan DL. Biotechnology and bioremediation for organic energetic compounds. In: Marinkas PL, editor. Organic energetic compounds. Commack, NY: Nova Science Publishers, Inc., 1996. p. 373–416.

[11] Kitts CL, Cunningham DP, Unkefer PJ. Isolation of three hexahydro-1,3,5-trinitro-1,3,5-triazine-degrading species of the family *Enterobacteriaceae* from nitramine explosive-contaminated soil. Appl Environ Microbiol 1994;60:4608–11.

[12] McCormick NG, Cornell JH, Kaplan AM. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine. Appl Environ Microbiol 1981;42:817–23.

[13] Young DM, Unkefer PJ, Ogden KL. 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 1997;53:515–22.

[14] Spanggord RJ, Mill T, Chou T-W, Mabey WR, Smith JH, Lee S. Environmental fate studies on certain munition wastewater constituents. Final Report, Phase II—Laboratory studies. Menlo Park, CA: SRI International, 1980.

[15] Madigan MT, Martinko JM, Parker J. Brock biology of microorganisms, 8th ed. Upper Saddle River, NJ: Prentice-Hall, 1997.

[16] Meyer R. Explosives, 2nd ed. Deerfield Beach, FL: Verlag Chemie, 1981.

[17] Thauer RK, Jungermann K, Decker K. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 1977;41:100–80.

[18] Brockman FJ, Downing DC, Wright GF. Preparation of pure cyclonite. Can J Res-B 1949;27:469–74.

[19] Coon CL, Hill ME, Ross DL. Nitramine cyclization and nitrolysis reactions. Final Report AD 905 582L. Menlo Park, CA: SRI International, 1972.

[20] Gilbert EE, Leccacorvi JR, Warman M. Preparation of RDX from 1,3,5-triacylhexahydro-s-triazines. ACS Symposium Series 1976;22:327–40.

[21] Beller HR, Spormann AM, Sharma PK, Cole JR, Reinhard M. Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. Appl Environ Microbiol 1996;62:1188–96.

[22] Beller HR, Grbic-Galic D, Reinhard M. Microbial degradation of toluene under sulfate-reducing conditions and the influence of iron on the process. Appl Environ Microbiol 1992;58:786–93.

[23] Oremland RS. Biogeochemistry of methanogenic bacteria. In: Zehnder AJB, editor. Biology of anaerobic microorganisms. New York, NY: Wiley, 1988. p. 641–705.

[24] Schink B. Diversity, ecology, and isolation of acetogenic bacteria. In: Drake HL, editor. Acetogenesis. New York, NY: Chapman & Hall, 1994. p. 197–235.

[25] Fröstl JM, Seifritz C, Drake HL. Effect of nitrate on the autotrophic metabolism of the acetogens *Clostridium thermoautotrophicum* and *Clostridium thermoaceticum*. J Bacteriol 1996;178:4597–603.

[26] Seifritz C, Daniel SL, Gössner A, Drake HL. Nitrate as a preferred electron sink for the acetogen *Clostridium thermoaceticum*. J Bacteriol 1993;175:8008–13.

[27] Arendsen AF, Soliman MQ, Ragsdale SW. Nitrate-dependent regulation of acetate biosynthesis and nitrate respiration by *Clostridium thermoaceticum*. J Bacteriol 1999;181:1489–95.

[28] Huang S, Lindahl PA, Wang C, Bennett GN, Rudolph FB, Hughes JB. 2,4,6-trinitrotoluene reduction by carbon monoxide dehydrogenase from *Clostridium thermoaceticum*. Appl Environ Microbiol 2000;66:1474–8.

[29] Preuss A, Fimpel J, Diekert G. Anaerobic transformation of 2,4,6-trinitrotoluene (TNT). Arch Microbiol 1993;159:345–53.

[30] Kitts CL, Green CE, Otley RA, Alvarez MA, Unkefer PJ. Type I nitroreductases in soil enterobacteria reduce TNT (2,4,6-trinitrotoluene) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine). Can J Microbiol 2000;46:278–82.