Biodegradation (2005) 16: 539–547

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degradation by *Acetobacterium paludosum*

Leslie A. Sherburne, Joshua D. Shrout & Pedro J.J. Alvarez*
*Department of Civil and Environmental Engineering, The University of Iowa, Seamans Center, Iowa City, IA 52242, USA (*author for correspondence: current address: Rice University, Department of Civil and Environmental Engineering-MS 317, Houston, TX 77251-1892, USA; e-mail: alvarez@rice.edu)*

Accepted 23 November 2004

Key words: ammonium, autotrophic, homoacetogens, hydrogen, nitrous oxide, RDX

Abstract

Substrates and nutrients are often added to contaminated soil or groundwater to enhance bioremediation. Nevertheless, this practice may be counterproductive in some cases where nutrient addition might relieve selective pressure for pollutant biodegradation. Batch experiments with a homoacetogenic pure culture of *Acetobacterium paludosum* showed that anaerobic RDX degradation is the fastest when auxiliary growth substrates (yeast extract plus fructose) and nitrogen sources (ammonium) are not added. This bacterium degraded RDX faster under autotrophic (H_2-fed) than under heterotrophic conditions, even though heterotrophic growth was faster. The inhibitory effect of ammonium is postulated to be due to the repression of enzymes that initiate RDX degradation by reducing its nitro groups, based on the known fact that ammonia represses nitrate and nitrite reductases. This observation suggests that the absence of easily assimilated nitrogen sources, such as ammonium, enhances RDX degradation. Although specific end products of RDX degradation were not determined, the production of nitrous oxide (N_2O) suggests that *A. paludosum* cleaved the triazine ring.

Abbreviations: RDX – hexahydro-l,3,5-trinitro-l,3,5-triazine

Introduction

The explosive RDX (hexahydro-l,3,5-trinitro-l,3,5-triazine) is a toxic and persistent groundwater contaminant found at many military installations (Held et al. 1997; Schmelling et al. 1997). The U.S. EPA has classified RDX as possible human carcinogen, and RDX is also toxic to the neurosystem and to other mammals, algae, invertebrates, and fish (McLellan et al. 1992; Testud et al. 1996). Possibly more toxic than RDX are some potential degradation metabolites, such as the nitroso heterocyclic compounds MNX (1,3-dinitro-5-nitroso-l,3,5-triacyclohexane), DNX (1,3-dinitroso-5-nitro-l,3,5-triazacyclohexane), and TNX (1,3,5-trinitroso-1,3,5-triazacyclohexane), as well as potential ring fission products 1,1- and 1,2-dimethylnitrosamine, azoxymethane, and hydrazine, which are known to be mutagens, carcinogens, or both (Fiala 1977; Greenhouse 1976; McCormick et al. 1981; Skopek et al. 1978). The toxicity of RDX and its potential metabolites is a major driving force for the remediation of contaminated sites.

One emerging strategy that holds great potential for treating RDX-contaminated groundwater is the use of zero-valent iron (Fe^0) in permeable reactive barriers (PRBs) (Hundal et al. 1997). Previous studies showed that indigenous aquifer microorganisms or mixed cultures from anaerobic digesters can enhance both the rate and extent of RDX transformation in Fe^0 systems (Oh et al.

2001; Wildman & Alvarez 2001). This enhancement was postulated to be due, in part, to cathodic hydrogen production during anaerobic Fe^0 corrosion:

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

Apparently, hydrogen has a biostimulatory effect and is used as an electron donor to drive the bacterial reduction of RDX (Adrian et al. 2003; Beller 2002). Hydrogen is also a common electron donor in anaerobic systems, which adds relevance to the study of RDX degradation by hydrogenotrophs.

Among the potential hydrogen-utilizers that could enhance RDX removal are homoacetogenic bacteria. Homoacetogens are strict anaerobes that can use H_2 and CO_2 for growth and have been found to colonize the Fe^0 layer in flow-through columns treating RDX (Oh & Alvarez 2002). In theory, homoacetogens could also commensally support heterotrophic activity in anaerobic systems by producing acetate. Increased heterotrophic activity due to higher availability of such a C source might be beneficial for RDX removal, especially if RDX is utilized as an N source by heterotrophs. Homoacetogens have also been implicated in RDX degradation by methanogenic sludge (Adrian & Lowder 1999) and other mixed cultures (Oh & Alvarez 2002), and recently, a pure homoacetogenic culture that degrades RDX was isolated (Adrian & Arnett 2004). However, the ability of homoacetogens to degrade RDX under autotrophic (H_2-fed) and nitrogen-deficient conditions that are likely to be encountered in groundwater as well as in and around Fe^0 barriers has not been previously reported.

Adding nutrients such as ammonia to contaminated sites or bioreactors is a common biostimulation practice. However, nutrient addition can have a detrimental effect if it inhibits bacteria adapted to oligotrophic environments (Morgan & Watkinson 1992) or if it stimulates the degradation of carbon compounds other than the target pollutants. Whether ammonium enhances or hinders RDX degradation by homoacetogens has not been previously investigated.

This paper is the first to report RDX degradation by the homoacetogenic species *Acetobacterium paludosum*. Emphasis was placed on (1) comparing RDX degradation under heterotrophic *versus* autotrophic conditions; (2) characterizing RDX degradation rates and products (including the potential for RDX mineralization); and (3) determining the effect of an easily assimilated nitrogen source, such as ammonium, on RDX degradation. This information contributes to our understanding of microbial niches in RDX contaminated environments.

Materials and methods

Culture conditions

Acetobacterium paludosum (ATCC # 51793), isolated by Kotsyurbenko et al. (1995) from sediment of a marsh 100 km north of Moscow, Russia, was utilized because of its ability to grow at environmentally relevant temperatures (≤20 °C) as well as its ability to be cultured more easily than other homoacetogenic bacteria (Sherburne 2003). Bacteria were routinely cultured in closed 25 ml Balch anaerobic culture tubes (18 × 150 mm, Bellco Glass Co., Vineland, NJ) capped with 20 mm butyl rubber septum stoppers (Bel1co Glass, Co., Vineland, NJ) under anaerobic conditions in liquid ATCC 1019 *Acetobacterium* medium with a headspace consisting of N_2/CO_2 (95/5, v/v) (Balch et al. 1977). In experiments conducted under autotrophic conditions, fructose and yeast extract were omitted from the medium and a headspace consisting of H_2/CO_2 (80/20, v/v) was used. All experiments were conducted at room temperature (i.e., 20 °C), which is the optimal growth temperature for *A. paludosum* (Kotsyurbenko et al. 1995).

Comparison of RDX degradation under autotrophic versus *heterotrophic conditions*

RDX biodegradation was compared under autotrophic and heterotrophic conditions to evaluate the effect of alternative carbon sources on bacterial performance. Degradation assays were conducted in 25 ml Balch anaerobic culture tubes amended with 6 ml of autoclaved ATCC 1019 medium (containing or omitting organic carbon), 1.5 ml of liquid cell culture (washed twice and resuspended in HEPES buffer), and RDX (approximately 3 mg l^{-1}) The liquid cell culture was taken from stock *A. paludosum* grown at 20 °C in ATCC 1019 medium (containing fructose as carbon source) with a headspace consisting of 20 ml of H_2/CO_2

(80/20, v/v) gas mixture. The headspace for the degradation assays also consisted of 20 ml of H_2/CO_2 (80/20, v/v) gas mixture. A third treatment set was used to investigate the growth of *A. paludosum* using RDX as the sole source of carbon. The headspace consisted of H_2/N_2 (5/95 v/v). Two to four replicates were studied for each set. Controls without bacteria were also monitored to obtain a baseline for comparing RDX degradation and acetate production.

The tubes were capped and crimped with 20 mm butyl rubber stoppers, covered in aluminum foil to prevent RDX photolysis, and rotated continuously on a Roto-Torque Heavy Duty Rotator (Cole-Parmer Instrument Co., Vernon Hills, IL) at 20 °C. Liquid samples (0.7 ml were collected with sterile disposable syringes, filtered using 0.2 μm syringe filters, and analyzed by high pressure liquid chromatography (HPLC). The optical density of each tube was recorded throughout the experiment to determine bacterial growth. Each time the reactors were spiked with RDX, an additional 20 ml of H_2/CO_2 headspace (80/20, v/v) was added to prevent electron donor and carbon source depletion.

Evaluation of ^{14}C-RDX mineralization under autotrophic conditions

Serum bottles (120 ml) were prepared with 54 ml of ATCC 1019 medium omitting yeast extract and fructose. $NaHCO_3$ (3 g l^{-1}) was added to provide a source of inorganic carbon. Six milliliters of pure culture were washed twice and resuspended in HEPES buffer (pH 7) before transfer to each treatment to obtain a 10% (v/v) bacteria/medium concentration. Two sets were prepared in triplicate: (1) *Acetobacterium paludosum* in HEPES buffer and (2) a no-bacteria control consisting of 5.8 ml of HEPES buffer with 0.1 ml l^{-1} Kathou® CG/ICP biocide (5-Chloro-2-methyl-3(2H)-isothiazolone and 2-Methyl-3(2H)-isothiazolone solution; Sigma–Aldrich, St. Louis, MO). ^{14}C-ring-labeled RDX (PerkinElmer Life Sciences, Boston, MA) and unlabeled RDX were added to obtain the initial conditions of 1 μCi total radioactivity and 3 mg l^{-1} The activity of the radioactive stock solution was 0.084 μCi μl^{-1}. Each bottle held a small test tube with 2 ml of 0.5 N NaOH to trap $^{14}CO_2$. Contents were sparged for 10 min with H_2/CO_2 (80/20, v/v) to add hydrogen to the system. All serum bottles were capped and crimped with 20 mm butyl rubber stoppers, covered in aluminum foil to prevent possible photodegradation of RDX, and incubated quiescently at 20 ± 2 °C in a Coy anaerobic chamber.

Samples were collected within the anaerobic chamber using sterile syringes, and filtered using 0.2 μm syringe filters. The headspace of each bottle was subsequently purged for 10 min with H_2/CO_2 mixture (80/20, v/v). RDX degradation and metabolite formation were tracked by analyzing the samples by HPLC and with a liquid scintillation counter.

Effect of ammonium on RDX degradation

Similar assays were conducted in 25 ml Balch anaerobic culture tubes with washed cells to determine if ammonium (an easily assimilated nitrogen source) inhibits RDX degradation. The autotrophic medium consisted of one of four substrate combinations: (1) RDX (3 mg l^{-1}) but no ammonium; (2) ammonium (1.0 g l^{-1}) but no RDX, (3) RDX and ammonium; and (4) neither ammonium nor RDX. The headspace consisted of 20 ml of an H_2/CO_2 (80/20, v/v) gas mixture.

Production of N_2O from RDX degradation

The production of nitrous oxide (N_2O) during RDX degradation (Figure 1) was investigated to determine if RDX ring cleavage occurred. *A. paludosum* incubations were prepared with RDX (approximately 2.5 ml l^{-1}, and N_2O concentrations in headspace samples (100 μl) were determined by gas chromatography. These incubations were prepared in duplicate 100 ml sealed glass serum bottles containing ATCC medium 1019, which included yeast extract, fructose (1.3 ml of 20% solution), and ammonium chloride, and were sparged with H_2/CO_2 headspace (80/20, v/v). Controls without RDX were also prepared to determine baseline N_2O production levels.

Analytical methods

Analysis of RDX and its nitroso derivatives MNX, DNX, and TNX was performed using a Hewlett Packard 1100 Series HPLC equipped with a 250 × 4.6 mm Supelcosil™ LC-18 column, herein referred to as the HPLC-1 method. The mobile

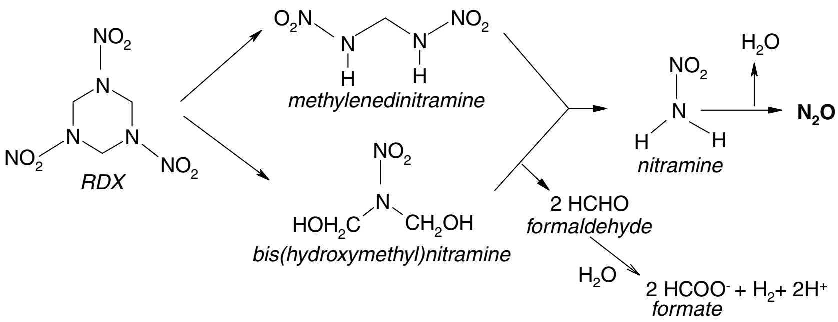

Figure 1. Production of nitrous oxide (N_2O) and formaldehyde from RDX and hypothetical transformation to formate (Hawari et al. 2000; Oh et al. 2001; Zhao et al. 2002, 2003b).

phase consisted of deionized water and methanol (4:6, v/v) at a flow rate of 1 ml min^{-1} (UV detection was at 240 nm. ^{14}C-RDX and ^{14}C-metabolites (e.g., methanol and formate) were analyzed by HPLC using a radioactivity detector (Radiomatic, Series A-500, Packard Instrument Co., Downers Grove, IL), herein referred to as the HPLC-RAD method. Analysis for ^{14}C-formaldehyde was performed using the HPLC-RAD method after derivatization using EPA method # 8315A (omitting the extraction by methylene chloride, due to the small volume of sample used). RDX mineralization was determined from trapped ^{14}C-CO_2 in the small tubes containing 0.5 N NaOH. Half a milliliter of sample from each of the small tubes was mixed with 9.5 ml of LSC cocktail (Ultima Gold) and was counted on a Beckmann LS 6000IC liquid scintillation counter (Beckman Instr. Inc., Fullerton, CA).

Nitrous oxide analysis was performed using a Hewlett Packard 5890 Series II gas chromatograph instrument with an electron capture detector and a HayeSep Q capillary column (Valco Instruments Co. Inc., Houston, Texas).

Acetate was measured using a Hewlett Packard 1100 Series HPLC equipped with a 150 × 6.5 mm Alltech IOA-2000 Organic Acids column (Deerfield, IL), herein referred to as the HPLC-2 method. The isocratic mobile phase consisted of 0.001 N sulfuric acid in distilled water at a flow rate of 1.0 ml min^{-1}. Detection was spectrophotometric at 210 nm, which resulted in a level of detection of less than 2.5 mM.

Bacterial growth was determined by measuring optical density at 660 nm (OD_{660}) using a Milton-Roy Spectronic 401 (Milton-Roy Co., Rochester, New York). The limit of detection was approximately 0.001 absorbance units.

Results and discussion

Comparison of RDX degradation under autotrophic versus *heterotrophic conditions*

Homoacetogens such as *Acetobacterium paludosum* are strict anaerobic mixotrophs that can use H_2 and CO_2 for growth and the production of acetate (Kotsyurbenko et al. 1995). While these bacteria have received considerable attention for their participation in municipal wastewater treatment, our understanding of their role of in aquifer bioremediation is very limited. Thus, experiments were conducted to determine if *A. paludosum* could degrade RDX under environmentally relevant conditions; i.e., when easily assimilated organic carbon sources are absent and H_2 (commonly present in anaerobic systems and Fe^0 barriers) might be the prevalent electron donor.

Experiments were performed to compare RDX degradation by *A. paludosum* under autotrophic *versus* heterotrophic conditions. No significant RDX removal was observed in abiotic controls, indicating that RDX disappearance was due to biodegradation. Treatments containing *A. paludosum* and RDX as the sole carbon source (i.e., no CO_2, yeast extract, nor fructose present) degraded approximately 70% of the initial amount of RDX (approximately 3 mg l^{-1}) after 9 days incubation (data not shown). Faster degradation was observed in treatments incubated under autotrophic conditions (containing CO_2) or heterotrophic conditions (containing fructose), where all the RDX was removed within three days. Apparently, the presence of alternative (inorganic or organic) carbon sources enhanced bacterial growth and RDX degradation.

In theory, *A. paludosum* could metabolize RDX by transforming it to formate (Figure 1), which is a known growth substrate (Kotsyurbenko et al. 1995). However, the observed RDX degradation in the absence of alternative carbon sources does not necessarily imply that this bacterium metabolized RDX, because H_2 that was present in the headspace could have served as an electron donor in the initial (reductive) transformation of RDX. Furthermore, an internal storage of carbon present in the (heterotrophically grown, then washed) bacteria could have also served as the electron donor for RDX transformation. No detectable growth of *A. paludosum* was observed when RDX was provided as the sole C source. This suggests that this bacterium did not metabolize RDX-derived carbon, which does not necessarily rule out RDX utilization as an N source.

The autotrophic and heterotrophic treatments were respiked with RDX, and the concentration *versus* time data were fit by an exponential decay model (i.e., $C = C_o\ e^{-kt}$) using SigmaPlot 8.0 software (Figure 2). RDX degradation was faster under autotrophic (H_2 and CO_2-fed) conditions (96% removal within 10 days) than under heterotrophic (yeast extract plus fructose-fed) conditions (73% removal), even though the latter contained a higher biomass concentration due to higher cell yield and faster growth under heterotrophic conditions (0.510 ± 0.039 *versus* 0.07 ± 0.004 OD_{660}). The faster removal for the autotrophic treatment is accentuated when normalized first-order decay coefficients (k) are considered. The specific k value was six times higher for the autotrophic than the heterotrophic treatment (1.67 ± 0.04 *versus* 0.28 ± 0.05 $(\text{day}^*OD_{660})^{-1}$).

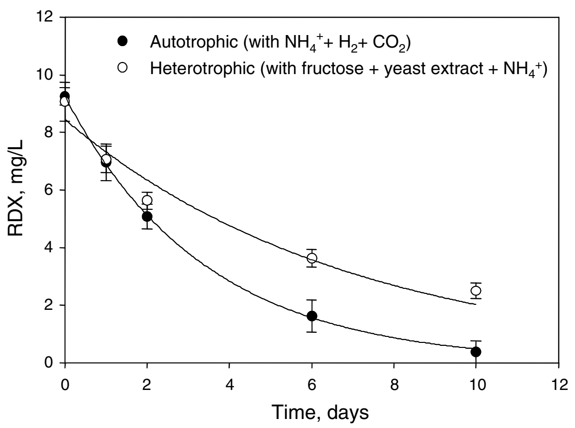

Figure 2. Effect of carbon source on RDX removal by *A. paludosum*. Initial OD_{660} was 0.077 ± 0.004 for the autotrophic treatment and 0.510 ± 0.039 for the heterotrophic treatment. Error bars represent 95% confidence intervals.

These experiments suggest that hydrogen is a better electron donor than fructose and yeast extract for promoting RDX degradation by *A. paludosum,* even though the latter are the recommended carbon sources for the growth medium (Balch et al. 1977). The lower RDX removal efficiency for the heterotrophic treatments is counterintuitive because heterotrophic conditions resulted in faster growth and higher acetate production after three days (i.e., 30.4 mM acetate (heterotrophic) and 5.9 mM acetate (autotrophic), corresponding to normalized values of 153 mM*$(OD_{660})^{-1}$ and 31 mM *$(OD_{660})^{-1}$, respectively). Further research will be needed to determine if this observation reflects differences in catabolic activities inherent to autotrophic *versus* heterotrophic metabolism. For example, autotrophic metabolism generates more reducing power (for CO_2 fixation) leaving the potential for more electrons to be diverted towards RDX reduction. It may also be possible that the availability of easily assimilated organic carbon sources hinder RDX degradation due to metabolic flux dilution (Lovanh & Alvarez 2004).

Degradation of ^{14}C-RDX under autotrophic conditions

A. paludosum degraded RDX (3 mg l^{-1}) within 9 days, converting it to soluble radio-labeled metabolite(s) (Figure 3). Less than 1% of the radiolabeled RDX was recovered as $^{14}CO_2$ after 20 days, indicating that mineralization did not occur. However, separate experiments indicated that production of N_2O occurred only in treatments containing RDX (Table 1), which is evidence of ring fission (Figure 1). Recent studies with another homoacetogen, *Acetobacterium malicum,* also reported RDX ring cleavage but no mineralization (Adrian & Arnett 2004).

Anaerobic production of N_2O from RDX has also been shown for *Clostridium bifermentans*

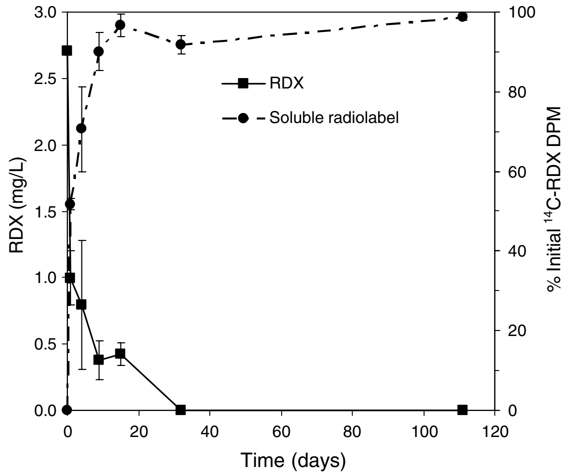

Figure 3. RDX degradation (2.7 mg l^{-1}, 1 μCi/bottle) and soluble ^{14}C-metabolite(s) formation by H_2-fed *A. paludosum* (autotrophic conditions, Inoculum $OD_{660} = 1.27$). Error bars represent 95% confidence intervals from the mean of triplicate reactors.

HAW-1 (Zhao et al. 2003a, b). Recovered N_2O accounted for 64% of N-RDX in these experiments compared to 29.5% reported for C. *bifermentans* HAW-1 (Zhao et al. 2003a), which suggests a different end-products distribution by these two anaerobic organisms. RDX transformation by *A. paludosum* was less rapid than that reported for C. *bifermentans* HAW-l (Zhao et al. 2003a).

Attempts to identify the radiolabeled byproduct(s) after 111 days of incubation were unsuccessful. Several potential RDX metabolites, that have been reported by others (e.g., Adrian & Chow 2001; Hawari et al. 2000, 2001; McCormick et al. 1981; Oh et al. 2001; Zhao et al. 2003a, b) were not detected using the HPLC-1 or HPLC-RAD analysis methods used for this research. For example, Zhao et al. (2003a) showed *C. bifermentans* HAW-1 transformed RDX transiently to MNX, DNX, and TNX, which were further transformed to methanol, formaldehyde, carbon dioxide, and nitrous oxide. However, no MNX, DNX, TNX, formaldehyde, formic acid, or methanol were detected in our analyses. Whereas the radiolabel eluted as one peak (2.8 min) using the RDX (HPLC-RAD) method, it eluted as two peaks (1.8 and 3.0 min) using the acetate (HPLC-2) method, suggesting the presence of two compounds that were not acetate (3.5 min). Retention times for ^{14}C-labeled formaldehyde (7.0 min), formate (2.7 min), and methanol (3.1 min) were also determined with the HPLC-RAD method. These elution times suggest that formate might have been one of the unidentified RDX metabolites (Figure 1). The HPLC-2 method was not run with formate to verify this notion. However, other previously reported RDX degradation products such as hydroxylamino metabolites (Adrian & Chow 2001), methylenedinitramine and bis(hydroxymethyl)nitramine (Figure 1) (Hawari et al. 2000; Oh et al. 2001), are relatively short lived (Adrian & Chow 2001; Bhushan et al. 2002) and are unlikely to persist as long as the unidentified metabolites did in this experiment (Figure 3). Similarly, no metabolites were identified using an Agilent 1100 series liquid chromatograph/mass spectrometer, presumably due to lack of sensitivity in full scan mode.

The volatility and reactivity of the radiolabeled metabolites towards oxygen was also investigated. Two 20 ml LSC vials were prepared with 5 ml of the medium remaining from the autotrophic experiment in which *A. paludosum* transformed ^{14}C-RDX. Both vials were covered with aluminum foil and lightly capped with aluminum foil to prevent photo-interactions but still allow for volatilization. One vial was placed in the anaerobic chamber and the other was exposed to air outside

Table 1. Production of N_2O by *A. paludosum* incubated with RDX

Treatment	RDX degraded (μM)	Maximum theoretical N_2O produced (μM)[a]	Measured N_2O produced (mM)	Percentage of Theoretical Maximum N_2O produced (μM)[b]
With RDX	1.50	4.50	2.9	64%
With RDX, duplicate	1.53	4.59	0.9	18%

[a]Theoretical calculation assumes that 3 M of N_2O gas could be produced from 1 M RDX.
[b]Corrected for background N_2O detected under conditions without RDX.

on the lab bench. After one week, the samples were analyzed by LSC and HPLC. No loss of radioactivity and no changes in HPLC peak elution times had occurred, indicating that the metabolites were not volatile and did not spontaneously react with oxygen.

Effect of ammonium on RDX degradation

Figure 4 shows the degradation of three subsequent spikes of RDX by *A. paludosum* under autotrophic conditions in the presence and absence of ammonium (1.0 g l^{-1} NH_4Cl, which is the recommended concentration for the *A. paludosum* growth medium). RDX degradation rates decreased for both treatments upon subsequent RDX spikes, possibly due to toxicity associated with RDX biotransformation or to the accumulation of inhibitory metabolites. Ammonium had no significant effect on the degradation of the first spike of RDX. Estimated *k* values (normalized to the initial optical density) were 4.43 ± 0.7l and 4.57 ± 0.30 $(day*OD_{660})^{-1}$ with and without ammonium, respectively. However, ammonium had an inhibitory effect on the degradation of the second spike of RDX, decreasing the *k* value by about one-half, from 4.52 ± 0.57 to 2.41 ± 0.18 $(day*OD_{660})^{-1}$ (Figure 4). The inhibitory effect of ammonium was reproducible during the degradation of the third RDX spike, where the *k* value for the treatment without ammonia, (1.66 ± 0.12 $(day*OD_{660})^{-1}$) was significantly higher ($p < 0.05$) than the value for the treatment with ammonia (0.74 ± 0.13 $(day*OD_{660})^{-1}$).

The inhibition of RDX degradation by ammonium might be due to its preferential utilization over RDX as a nitrogen source. Whereas we did not demonstrate *A. paludosum* assimilation of RDX-derived nitrogen (which would have required the use of ^{15}N-Iabeled RDX), numerous studies have shown that RDX can serve as a nitrogen source to bacteria (Beller 2002; Binks et al. 1995; Coleman et al. 1998; Sheremata & Hawari 2000) Thus, further research is recommended to test this hypothesis and to evaluate whether ammonium represses enzymes that initiate RDX degradation by reducing its nitro groups (Bhushan et al. 2002), as is the case for ammonia repression of assimilatory nitrate and nitrite reductases (Madigan et al. 2000).

Summary and conclusions

Little is known about the role of homoacetogenic bacteria in bioremediation. This study is the first report of RDX biodegradation by *A. paludosum,* which degraded RDX under both heterotrophic and autotrophic conditions that might prevail, respectively, in bioreactors and in the vicinity of iron barriers. Although RDX was not mineralized to CO_2, evidence of ring fission (per N_2O accumulation) with possible conversion to innocuous formate was obtained, and no objectionable heterocyclic nitroso derivatives (i.e., MNX, DNX, and TNX) were detected. However, not all degradation products were identified, which precludes our full endorsement of this pathway for bioremediation purposes.

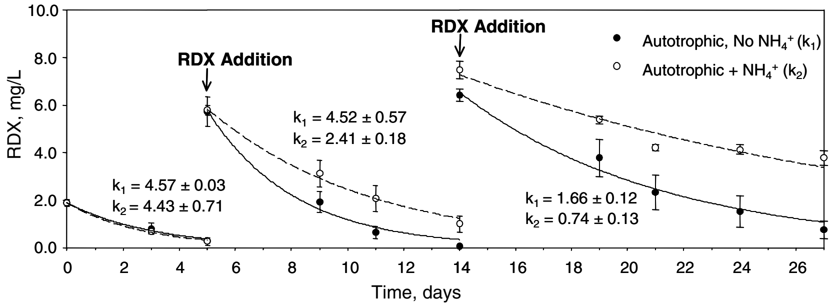

Figure 4. Degradation of three spikes of RDX by *A. paludosum* in the presence or absence of ammonium. The experiment was conducted under autotrophic, anaerobic conditions with an average initial optical density (at 660 nm) of 0.080 ± 0.009 for reactors with ammonium and 0.073 ± 0.003 for reactors without ammonium. The depicted first-order rate coefficients, *k*, have units of $(days*OD_{660})^{-1}$. Error bars represent 95% confidence intervals from the mean of triplicate reactors.

RDX was inhibitory to A. *paludosum* growth, and its degradation was more efficient under autotrophic (H_2-fed), nitrogen deficient conditions, even though faster growth occurred under heterotrophic (yeast extract plus fructose-fed) conditions. The addition of ammonia had an inhibitory effect on RDX degradation, possibly by relieving selective pressure for the utilization of RDX as a nitrogen source. Demonstration of ^{15}N-RDX incorporation into biomass is recommended for future studies to confirm assimilation of RDX nitrogen by *A. paludosum*. Nevertheless, these results suggest that the common practice of biostimulation through the addition of auxiliary substrates and nutrients should be carefully evaluated on a case by case basis to prevent a counterproductive effect on RDX bioremediation.

Acknowledgements

Funding for this research was provided by SERDP (Grant # DACA72-00-P-0057). We are very grateful for the skillful assistance with analytical measurements and analyses provided by Craig Just and Phil Larese-Casanova.

References

Adrian NR & Arnett CM (2004) Anaerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5- triazine (RDX) by *Acetobacterium malicum* strain haap-1 isolated from a methanogenic mixed culture. Curr. Microbiol. 48: 332–340

Adrian NR, Arnett CM & Hickey RF (2003) Stimulating the anaerobic biodegradation of explosives by the addition of hydrogen or electron donors that produce hydrogen. Water Res. 37: 3499–3507

Adrian NR & Chow T (2001) Identification of hydroxylamino-dinitroso-1,3,5-triazine as a transient intermediate formed during the anaerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine. Environ. Toxicol. Chem. 20: 1874–1877

Adrian NR & Lowder A (1999) Biodegradation of RDX and HMX by a Methanogenic Enrichment Culture Bioremediation of Nitroaromatic and Haloaromatic Compounds B. C. Alleman Columbus, Ohio Battelle Press, pp. 1–6

Balch W, Schoberth S, Tanner R & Wolfe R (1977) *Acetobacterium*, a new genus of hydrogen-oxidizing, carbon dioxide-reducing, anaerobic bacteria. Int. J. Systemat. Bacteriol. 27: 355–361

Beller HR (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

Bhushan B, Halasz A, Spain J, Thiboutot S, Ampleman G & Hawari J (2002) Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-tiazine catalyzed by a nad(p)h: nitrate oxidoreductase from *Aspergillus niger*. Environ. Sci. Technol. 36: 3104–3108

Binks PR, Nicklin S & Bruce NC (1995) Degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (rdx) by stenotrophomonas-maltophilia pb1. Appl. Environ. Microbiol. 61: 1318–1322

Coleman NV, Nelson DR & Duxbury T (1998) 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. 30: 1159–1167

Fiala ES (1977) Investigations into the metabolism and mode of action of the colon carcinogens 1,2-dimethylhydrazine and azoxymethane. Cancer 40: 22436–23445

Greenhouse G (1976) Evaluation of the teratogenic effects of hydrazine, methylhydrazine, and dimethylhydrazine on embryos of xenopus laevis, the south african clawed toad. Teratology 13: 167–177

Hawari J, Halasz A, Beaudet S, Paquet L, Ampleman G & Thiboutot S (2001) Biotransformation routes of octahydro-1,3,5,7-tetranitro- 1,3,5,7-tetrazocine by municipal anaerobic sludge. Environ. Sci. Technol. 35: 70–75

Hawari J, Halasz A, Sheremata T, Beaudet S, Groom C, Paquet L, Rhofir C, Ampleman G & Thiboutot S (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: 2652–2657

Held T, Draude G, Schmidt FRJ, Brokamp A & Reis KH (1997) Enhanced humification as an *in-situ* bioremediation technique for 2,4,6-trinitrotoluene (TNT) contaminated soils. Environ. Technol. 18: 479–487

Hundal LS, Singh J, Bier EL, Shea PJ, Comfort SD & Powers WL (1997) Removal of TNT and RDX from water and soil using iron metal. Environ. Pollut. 97: 55–64.

Kotsyurbenko OR, Simankova MV, Nozhevnikova AN, Zhilina TN, Bolotina NP, Lysenko AM & Osipov GA (1995) New species of psychrophilic acetogens – *Acetobacterium bakii* sp nov, *a. Paludosum* sp nov, *a. Fimetarium* sp nov. Arch. Microbiol. 163: 29–34

Lovanh N & Alvarez PJJ (2004) Effect of ethanol, acetate, and phenol on toluene degradation activity and tod-lux expression in *Pseudomonas putida* tod102: Evaluation of the metabolic flux dilution model. Biotechnol. Bioeng. 86: 801–808

Madigan MT, Martinko JM & Parker J (2000) Brock Biology of Microorganisms Upper Saddle River, New Jersey Prentice-Hall, Inc.

McCormick NG, Cornell JH & Kaplan AM (1981) Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine. Appl. Environ. Microbiol. 42: 817–823

McLellan W, Hartley WR & Brower M (1992) Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) drinking water health advisory: munitions. In: Roberts WC & Hartley WR (Eds) (pp 132–180) Boca Raton, FL Lewis Publishers.

Morgan P & Watkinson RJ (1992) Factors limiting the supply and efficiency of nutrient and oxygen supplements for the *in situ* biotreatment of contaminated soil and groundwater. Water Res. 26: 73–78

Oh BT & Alvarez PJJ (2002) Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degradation in biologically-active iron columns. Water Air Soil Pollut. 141: 325–335

Oh BT, Just CL & Alvarez PJJ (2001) Hexahydro-1,3,5-trinitro-1,3,5-triazine mineralization by zerovalent iron and mixed anaerobic cultures. Environ. Sci. Technol. 35: 4341–4346

Schmelling DC, Gray KA & Kamat PV (1997) The influence of solution matrix on the photocatalytic degradation of TNT in tio2 slurries. Water Res. 31: 1439–1447

Sherburne LA (2003) Biological removal of RDX using selected pure cultures of homoacetogens, Master's Thesis, The University of Iowa

Sheremata TW & Hawari J (2000) Mineralization of RDX by the white rot fungus phanerochaete chrysosporium to carbon dioxide and nitrous oxide. Environ. Sci. Technol. 34: 3384–3388

Skopek TR, Liber HL, Krolewski JJ & Thilly WG (1978) Quantitative forward mutation assay in *Salmonella typhimurium* using 8-azaguanine resistance as a genetic marker. Proc. Nat. Acad. Sci. USA

Testud F, Glanclaude JM & Descotes J (1996) Acute hexogen poisoning after occupational exposure. J. Toxicol.-Clin. Toxicol. 34: 109–111

Wildman MJ & Alvarez PJJ (2001) RDX degradation using an integrated Fe(0)-microbial treatment approach. Water Sci. Technol. 43: 25–33

Zhao JS, Paquet L, Halasz A & Hawari J (2003a) Metabolism of hexahydro-1,3,5-trinitro-1,3,5-triazine through initial reduction to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine followed by denitration in *Clostridium bifermentans* HAW-1. Appl. Microbiol. Biotechnol. 63: 187–193

Zhao JS, Spain J & Hawari M (2003b) Phylogenetic and metabolic diversity of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)-transforming bacteria in strictly anaerobic mixed cultures enriched on RDX as nitrogen source. FEMS Microbiol. Ecol. 46: 189–196