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ORIGINAL PAPER

Biotransformation products and mineralization potential for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in abiotic versus biological degradation pathways with anthraquinone-2,6-disulfonate (AQDS) and *Geobacter metallireducens*

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Abstract This study investigated extracellular electron shuttle-mediated RDX biodegradation and the distribution of ring cleavage metabolites generated by biological degradation (cells) versus the products formed by abiotic degradation (reduced electron shuttles), and when the two pathways were acting simultaneously. All pathways were influenced by pH. Buffered suspensions (pH 6.8/7.9/9.2) were performed with cell-free anthrahydroquinone-2,6-disulfonate as the sole electron donor, cells (*Geobacter metallireducens*) + acetate, or cells/acetate + anthraquinone-2,6-disulfonate as an electron shuttle. The metabolites identified included methylenedinitramine, formaldehyde, nitrous oxide, nitrite, ammonium and carbon dioxide. As pH increased, the rates of RDX reduction by AH_2QDS also increased. Cells alone reduced RDX faster at the lower pH values. However, at all pH the rates of the electron shuttle-mediated pathways were consistently the fastest, and the proportion of carbon present as formaldehyde, which is a precursor to mineralization, was highest in the presence of electron shuttles. Formaldehyde accounted for 45/51/54% of the carbon in electron shuttle amended cell suspensions as opposed to 13/42/45% of carbon without shuttles at the pH 6.8/7.9/9.2, respectively. Approximately 7–20% of RDX was mineralized to CO_2 in the presence of cells at all pH tested; AQDS increased the extent of $^{14}CO_2$ produced. Nitrous oxide and nitrite were end products in the strictly abiotic pathway, but nitrite was depleted in the presence of cells to form ammonium. Understanding the different products formed in the abiotic versus biological pathways and the influence of pH is critical to developing mixed biotic–abiotic remediation strategies for RDX.

Keywords Bioremediation · RDX · Electron shuttling · Fe(III)-reducing microorganisms

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Introduction

The cyclic nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a contaminant of concern at many military sites and live-fire training installations (Spalding and Fulton 1988; Haas et al. 1990; Hawari and Halasz 2002). Aqueous RDX solubility is low ($\sim$40 mg/l) (Talmage et al. 1999); however, once dissolved RDX can migrate with groundwater to contaminate downgradient aquifers. RDX is a possible human carcinogen (lifetime advisory for exposure to RDX in drinking water: 2 μg/l) (Lynch 1988; Lachance et al. 1999) and is a health concern for regulators. Despite reports suggesting that this compound is readily biodegraded, RDX

persists in subsurface environments (Meyers et al. 2007). A recent study reported that RDX remains one of the most significant contaminants at the Camp Edwards Military Reservation despite several years of remediation efforts (Clausen et al. 2004).

Bioremediation is one alternative for explosives contamination in sediment or soil (Adrian et al. 2003; Adrian and Arnett 2004; Sherburne et al. 2005; Thompson et al. 2005; Meyers et al. 2007). Anaerobic biodegradation is an effective strategy for RDX because of the initial nitro → nitroso degradation pathway, the variety of downstream reactions that ensue (Adrian et al. 2003; Crocker et al. 2006), and the fact that subsurface RDX plumes are often anaerobic (Garg et al. 1991; Adrian and Arnett 2004). Direct microbial RDX reduction has been reported (Zhao et al. 2002; Zhao et al. 2003); alternate degradation pathways are possible, but are less easily stimulated in subsurface environments (Pennington and Brannon 2002; Crocker et al. 2006).

Crocker et al. reviewed the pathways by which RDX and its nitroso metabolites can be degraded (Crocker et al. 2006). Hawari et al. (2000a, b) demonstrated that municipal anaerobic sludge can degrade RDX to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), or to ring cleavage products. *Phanerochaete chrysosporium* transformed octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) to octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazocine (1-NO-HMX) plus ring cleavage products in nitrogen-limited medium (Fournier et al. 2004). A facultative, anaerobic bacterium isolated from anoxic sludge, *Klebsiella pneumoniae* strain SCZ-1, degraded RDX by an initial denitration reaction followed by ring cleavage and decomposition in water (Zhao et al. 2002); RDX transformation to nitroso metabolites was minor. A homoacetogen, *Acetobacterium paludosum*, degraded RDX fastest under autotrophic (H_2-fed) conditions when nitrogen sources (e.g. ammonium) were absent. MNX, DNX, and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) were not detected. N_2O accumulated to 64% of the total nitrogen, but RDX was not mineralized to CO_2 (Sherburne et al. 2005).

Cyclic nitramine biodegradation mediated by extracellular electron shuttling (primarily anthraquinone-2,6-disulfonate (AQDS)) and the microorganisms that catalyze these reactions may be another reasonable approach based on the ubiquity of these microbes in subsurface environments (Coates et al. 1998). Recently, the role of fermentative cultures in reductive transformation of explosive compounds was investigated with AQDS (Borch et al. 2005; Bhushan et al. 2006). TNT degradation was stimulated by adding AQDS to fermentative cultures (Borch et al. 2005). A fermentative bacterium, *Clostridium* sp. EDB2, effectively degraded RDX (Bhushan et al. 2006) with AQDS present. Adding AQDS did not significantly increase the RDX reduction rates, but the low pH conditions generated by fermentation may have limited anthrahydroquinone-2,6-disulfonate (AH_2QDS)-mediated RDX reduction. Bradley and Dinicola suggested that Mn(IV) reduction was a dominant pathway for RDX biodegradation, and Mn(IV)-reducing microorganisms are closely related to the known electron shuttle reducers (Bradley and Dinicola 2005).

One past report (Schwarzenbach et al. 1990) and more recent studies (Uchimiya and Stone 2006) demonstrate that reactivity of hydroquinone compounds is pH dependent. Schwarzenbach et al. (1990) demonstrated that the rate of reduction of nitroaromatic compounds by Juglone and lawsone increases with pH, and Uchimiya and Stone (2006) reported that oxidation of 1,2-naphthoquinone-4-sulfonate by 2,6-dimethylhydroquinone was pH-dependent. In the current study, the effect of pH on RDX bioremediation mediated by extracellular electron shuttling compounds was investigated because contaminated subsurface environments with different pH values will influence overall degradation.

Our previous study demonstrated RDX bioremediation by extracellular electron shuttling compounds with two different bacterial species (Kwon and Finneran 2006). However, it did not address the ring cleavage products in extracellular electron shuttle-amended systems. Specifically, what are the reaction products with the abiotic pathway versus the biological pathway? When both pathways are available (abiotic plus biotic, the operationally defined mixed pathway)—what are the dominant products? These data are critical to developing this bioremediation strategy in terms of monitoring and extent of transformation. The strictly abiotic, strictly biological, and mixed pathways were investigated separately at three circumneutral pH values to quantify the ring cleavage products of RDX degradation generated by each. *Geobacter metallireducens* was used as the RDX- and electron shuttle-reducing model microorganism and AQDS was used as the sole electron shuttle.

Material and methods

Chemicals

RDX (97% pure) was provided by the US Army Corps of Engineers, Construction Engineering Research Laboratory (CERL), Champaign, IL. Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX; 99%), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX; 58% pure with 34% MNX and 8% TNX), hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX; >99.9%), methylenedinitramine (MEDINA), and 4-nitro-diazabutanol (NDAB) were purchased from SRI International (Menlo Park, CA, USA). U-[^{14}C]-RDX was purchased from Perkin Elmer (7.7 mCi/mmol) and dissolved in acetone. Anthrquinone-2,6-disulfonate (AQDS) was purchased from Sigma Aldrich (Milwaukee, WI, USA). HPLC-grade methanol was purchased from Aldrich Chemicals. All other chemicals used were of reagent grade quality or higher.

Microorganisms and culturing conditions

Geobacter metallireducens strain GS-15 (ATCC 53774) was maintained using Fe(III)-Citrate or AQDS media described below. The basal anoxic medium consisted of (g l^{-1} unless specified otherwise): $NaHCO_3$ (2.5), NH_4Cl (0.25), $NaH_2PO_4 \cdot H_2O$ (0.6), KCl (0.1), modified Wolfe's vitamin and mineral mixtures (each 10 ml l^{-1}) and 1 ml of 1 mM Na_2SeO_4 (Lovley et al. 1993). Electron acceptors used with the anoxic medium were soluble Fe(III) citrate (45 mM) or anthraquinone-2,6-disulfonate (AQDS—5 mM). The medium was buffered using 30 mM bicarbonate equilibrated with 80:20 (v/v) N_2:CO_2, as previously described (Finneran et al. 2003). Acetate (20 mM) was added as the sole electron donor. Standard anoxic and aseptic culturing techniques were used throughout (Lovley and Phillips 1988).

Pure phase incubations

Biologically reduced AQDS (B-AH_2QDS) was prepared by incubating *Geobacter metallireducens* in AQDS medium (5 mM). The B-AH_2QDS was filtered through a 0.2 μm sterilized PTFE filter into a presterilized, anoxic serum bottle to remove cells. Experimental tubes were prepared by sparging approximately 7.0 ml of 30 mM bicarbonate buffer with anoxic N_2:CO_2 (= 100:0 for pH 9.2, 99.9:0.1 for pH 8.2, 99.6:0.4 for pH 7.9, 96.3:3.7 for pH 7.3, 80:20 for pH 6.8 and 40:60 for pH 6.2). The buffer was sealed under an anoxic headspace of the same mixture. The concentration of reduced AQDS tested was 100 μM for the all incubations with RDX (33 μM–39 μM). Incubations were performed at 30°C. Samples were collected periodically via anoxic syringe and needle; samples were filtered through sterile, 0.2 μm PTFE filters prior to analyses (PALL Life Sciences; *filters did not interfere with nitramine or metabolite quantitation*).

Resting cell suspensions

Geobacter metallireducens was grown anaerobically in freshwater medium with acetate as the sole electron donor and Fe(III) citrate as the sole terminal electron acceptor. One liter of cell culture was harvested during logarithmic growth phase and centrifuged at 5,250*g* for 15 min to form a dense cell pellet. The cell pellet was resuspended in 30 mM bicarbonate buffer under a stream of anoxic gas. The washed cells were centrifuged again at 5,250*g* for 15 min and the resultant biomass was resuspended in 4.0 ml of bicarbonate buffer. Cells were used within 30 min of processing. There was no residual Fe(II) present in the suspension that could act as an abiotic reductant.

Experimental tubes were prepared by sparging approximately 5.0 ml of 30 mM bicarbonate buffer with anoxic N_2:CO_2 as described above for pH 9.2, 7.9, and 6.8 and sealed under an anoxic headspace. Acetate (20 mM) was amended as the sole electron donor. AQDS (100 μM) was the sole electron shuttle added to the cell suspensions. Cells were incubated at 30°C. An aliquot (0.3 ml) of the resting cells (0.27 ± 0.02 mg protein/ml) was added to the sealed pressure tubes to initiate each experiment. The final volume in each tube was 10.0 ml. The starting RDX concentration was approximately 60 μM.

Samples (0.5 ml) were collected periodically via anoxic syringe and needle, and samples were filtered through sterile, 0.2 μm PTFE filters prior to analyses. To minimize sampling volume, glass inserts (250 ul Glass LVI Flat Bottom; Laboratory Supply Distributors, NJ) were used in the autosampler vials. 0.05 ml of sample

was used to quantify RDX and its nitroso metabolites (MNX, DNX, and TNX) at each time point. 0.05, 0.1, 0.1, and 0.1 ml of samples were used to quantify methylenedinitramine (MEDINA), formaldehyde (HCHO), nitrite (NO_2^-), and ammonium (NH_4^+), respectively. No more than five samples were taken from any incubation; therefore, the final volume of each incubation was approximately 7.5 ml at the end of the experiments (75% volume remaining). Nitrous oxide and methanol were monitored by headspace analysis. All experiments were performed in triplicate.

[^{14}C]-RDX study with resting cell suspension

Cell suspension incubations (10 ml) were prepared for $^{14}CO_2$ and $^{14}CH_4$ analysis. pH was controlled with bicarbonate buffer by N_2:CO_2 gas bubbling as mentioned above. Acetate (0.5 mM) was amended as the sole electron donor. AQDS (100 μM) was the only electron acceptor incubated with the cells; the control was cells alone (no electron acceptor). U-[^{14}C]-RDX was amended at the concentration for a final radioactivity of 18,000 dpm/ml. $^{14}CO_2$ and $^{14}CH_4$ were monitored by analysis of headspace samples (1 ml). $H^{14}CO_3^-$ was used to establish $^{14}CO_2$ partitioning between the liquid and gas phase, and was factored in to the final mineralization; the partition coefficients (total dpm $H^{14}CO_3^-$ recovered as $^{14}CO_2$/total dpm added)—were 0.005, 0.027, and 0.135 at pH 9.2, 7.9, and 6.8, respectively. The experimental tubes at pH 9.2 were acidified to convert $H^{14}CO_3^-$ to $^{14}CO_2$ at the end of the experiment (acidified partition coefficient = 0.340). All experiments were performed in triplicate.

Analytical techniques

RDX and its nitroso metabolites were analyzed using high-performance liquid chromatography (HPLC) with a variable wavelength photodiode array (PDA) detector (HPLC/UV, Dionex) at 254 nm as described previously (Fournier et al. 2002). The filtered samples were auto-injected into a Supelcosil LC-CN column (25 cm × 4.6 mm, 5 μm ID) at ambient temperature. A mobile phase consisting of 50% water and 50% methanol was used at a flow rate of 1 ml/min. RDX, MNX, DNX, and TNX were compared to certified analytical standards in acetonitrile at known concentrations. MEDINA and NDAB were analyzed using HPLC at 210 nm as described previously (Zhao et al. 2004). Nitrous oxide was analyzed using gas chromatography (GC) with thermal conductivity detector (TCD, Hewlett-Packard 6890 Series) and Carboxen 1004 stainless steel micropacked column (1/8 inch × 8 feet; Supelco) that was held isothermally at 100°C. Helium was used as the carrier gas at a flow rate of 12.5 ml/min. The inlet and detector temperature were 195°C and 250°C, respectively. Methanol (CH_3OH) was analyzed using a gas chromatograph (GC) with a flame ionization detector (FID, Hewlett-Packard 6890 Series) as described previously (Monteil-Rivera et al. 2005). Nitrite was measured using an ion chromatograph (IC; Dionex 1000) with an AS14A column (250 × 4 mm, Dionex) and isocratic 8 mM Na_2CO_3/1 mM $NaHCO_3$ eluent. Ammonium was determined spectrophotometrically at 650 nm (Rhine et al. 1998). Formaldehyde was measured by a modified version of EPA method 8315A as previously described (Gregory et al. 2004). $^{14}CO_2$ and $^{14}CH_4$ were analyzed using gas chromatography (GC; Hewlett-Packard 6890 Series) with a gas radiochromatography detector (GC-Ram; IN/US system, Tampa, FL) and the same column used for nitrous oxide analysis; however, for $^{14}CO_2$ and $^{14}CH_4$ analyses the oven was held isothermally at 120°C. Helium was used as the carrier gas at a flow rate of 12.8 ml/min. The inlet temperature was 100°C (Finneran and Lovley 2001). The concentration of reduced AQDS was determined spectrophotometrically at 450 nm (Lovley et al. 1996). Total cellular protein was determined by using the DC Protein Assay (Bio-RAD) and a modified Lowry protein assay (Lowry et al. 1951).

Results

pH effects on RDX reduction and AH_2QDS oxidation

As pH increased with AH_2QDS fixed at 100 μM the rates of RDX reduction increased (Fig. 1). Pseudo 1st order rate constants (k_{obs}; h^{-1}) at pH 9.2, 8.2, 7.9, 7.3, 6.8, and 6.2 were 0.428 ± 0.004, 0.168 ± 0.012, 0.118 ± 0.002, 0.054 ± 0.006, 0.019 ± 0.001, and 0.007 ± 0.001, respectively. Comparing the fractional concentration of the monoprotonated hydroquinone (α_1) versus k_{obs} in a log–log plot demonstrates that the rate is

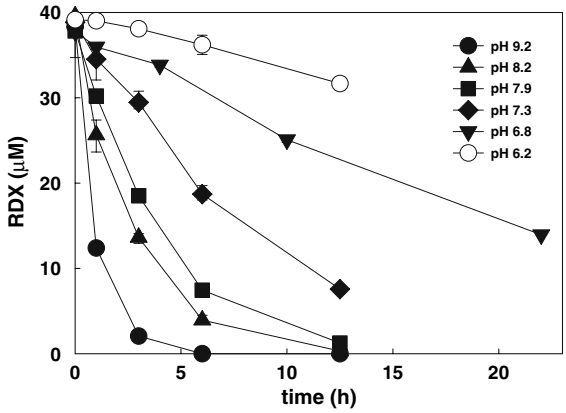

Fig. 1 Abiotic hexahydro-1,3,5-trinitro-1,3,5-triazne (RDX) reduction by AH_2QDS in cell-free incubations at pH 6.2, 6.8, 7.3, 7.9, 8.2, and 9.2. Results are the means of triplicate analyses and bars indicate one standard deviation

proportional to α_1 (the fraction of the monoprotonated form) (Fig. 2). α_1 was calculated as:

$$\alpha_1 = [\text{monoprotonated reduced AQDS}]/C_{\text{total}},$$
$$[\text{monoprotonated reduced AQDS}] = C_{\text{total}}/([H^+]/K_{a1} + 1 + K_{a2}/[H^+]),$$
$$C_{\text{total}} = [\text{diprotonated reduced AQDS}] + [\text{monoprotonated reduced AQDS}] + [\text{unprotonated reduced AQDS}] = 100\mu M,$$

where $K_{a1} = 10^{-8.1}$ and $K_{a2} = 10^{-10.5}$

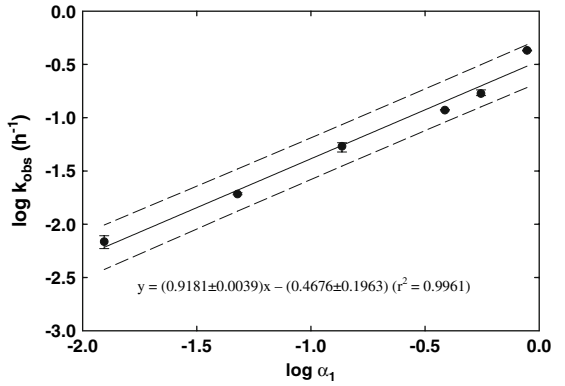

Fig. 2 Correlation between the fractional concentration of monoprotonated reduced AQDS (α_1) and pseudo first order degradation coefficients at pH 6.2, 6.8, 7.3, 7.9, 8.2, and 9.2 (pH increases from left to right along the *x*-axis). The solid line is the linear regression analysis. The dashed lines indicate 95% confidence intervals. Results are the means of triplicate analyses and bars indicate one standard deviation

Transformation products in the abiotic, biological, and mixed abiotic–biological pathways

RDX reduction increased in strictly abiotic suspensions with AH_2QDS (100 μM) as the pH increased from 6.8 to 9.2 (Fig. 3a). HCHO and MEDINA also increased, while MNX production was negligible (Fig. 3b, c; Table 1). The inorganic nitrogen compounds generated were nitrite, nitrous oxide, and ammonium (Fig. 4a–c). $^{14}CO_2$ or $^{14}CH_4$ were not produced from ^{14}C labeled-RDX in any of the abiotic suspensions (Table 1).

In contrast to RDX reduction by the abiotic pathway, cells-alone degraded RDX faster at pH 6.8 and 7.9 than at pH 9.2 (Fig. 3d). HCHO was the primary carbon metabolite and MNX did not accumulate; however, MEDINA was less significant relative to the strictly abiotic pathway and was depleted at pH 6.8 (Fig. 3e, f). Methanol (CH_3OH) was a limited product in the carbon mass balance at pH 9.2 (Table 1). Inorganic nitrogen was predominately ammonium at all pH tested (Fig. 4e). More nitrous oxide did accumulate at lower pH (Fig. 4f). $^{14}CH_4$ was not produced from ^{14}C labeled-RDX in any of the cell suspensions (Table 1), but $^{14}CO_2$ was produced.

Cells + AQDS (the operationally-defined "mixed" pathway) degraded RDX quickly and the rates and extent of RDX reduction were similar at all pH tested (Fig. 3g). HCHO and MEDINA were concurrently produced (Fig. 3h, i); however, MEDINA started to decline at different times for each pH and continued to decline through the final sampling points (an additional data point at hour 66 was not shown) (Fig. 3i). Methanol was also produced at about the same percentage of the mass balance as the cells-alone suspension. $^{14}CH_4$ was not produced from ^{14}C labeled-RDX in any of the cells + AQDS suspensions. $^{14}CO_2$ was produced from ^{14}C labeled-RDX in all cells + AQDS suspensions (Table 1). Nitrite was produced and disappeared quickly at pH 9.2, while nitrite was not detected at pH 7.9 and 6.8 (Fig. 4g); ammonium was the dominant inorganic nitrogen product (Fig. 4h).

Discussion

RDX degrades to a variety of intermediates under anoxic conditions, influenced by the microbial community present and the geochemical conditions

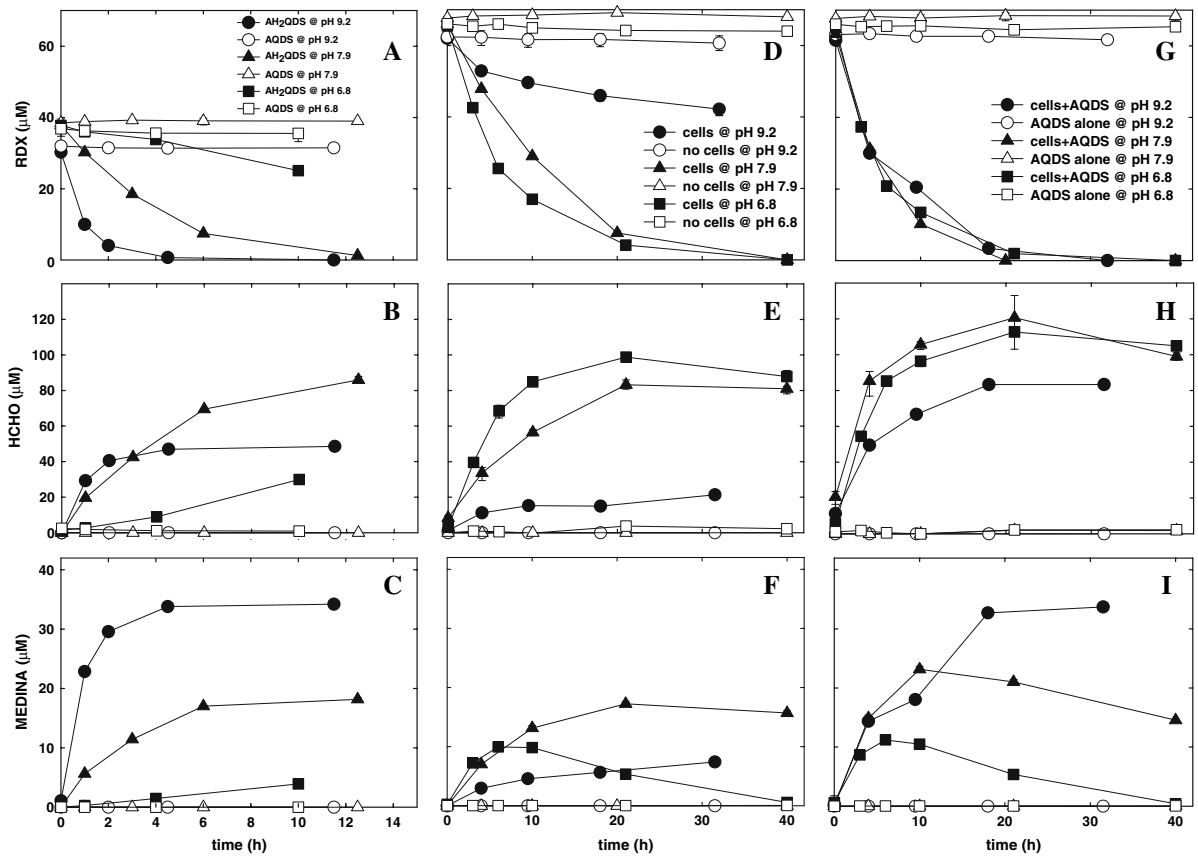

Fig. 3 RDX reduction and HCHO/MEDINA production by abiotic (**a**, **b**, and **c**), biological (**d**, **e**, and **f**), mixed abiotic–biological (**g**, **h**, and **i**) pathways at pH 6.8, 7.9, and 9.2. For biological or mixed abiotic–biological pathways RDX reduction was tested in resting cell suspension of *G. metallireducens* with (**g**, **h**, **i**) or without (**d**, **e**, **f**) AQDS. Acetate was the sole electron donor. Results are the means of triplicate analyses and bars indicate one standard deviation. Note that 40 μM RDX was the starting concentration in the strictly abiotic series, and that 60 μM RDX was the starting concentration in both series with cells

(Crocker et al. 2006). The critical issue for RDX remediation is how fast the ring-cleavage metabolites are produced, and whether they are amenable to further degradation or ultimately mineralization. Formaldehyde and methanol are two desirable carbon intermediates, as neither will persist in subsurface systems. The inorganic nitrogen intermediates are labile, but each can serve as an indicator of the extent of ring-cleavage. Our results demonstrate that electron shuttling compounds (whether in strictly abiotic reactions or in mixed biological-abiotic systems) accelerated formaldehyde production and mineralization relative to pathways devoid of electron shuttling compounds. While this study targets one specific microbial community (extracellular electron shuttle-reducers), it is a reasonable strategy for RDX bioremediation and it demonstrates that the rate and extent of ring cleavage and mineralization can be influenced.

pH influence on RDX reduction in the abiotic, biological, and mixed abiotic–biological pathways

Increasing pH from 6.8 to 9.2 in the abiotic pathways increased the rates and extent of RDX reduction and metabolite formation. Increasing pH in the strictly biological pathway decreased RDX reduction rates and metabolite distribution. However, in the mixed abiotic–biological pathway the rates and extent were similar regardless of pH, which suggests an *optimal*

Table 1 Nitrogen and carbon mass balance (%) at the final sampling point during RDX reduction and metabolite production by abiotic, biological, and mixed abiotic-biological pathways at pH 6.8, 7.9, and 9.2

RDX & degradation products	AH_2QDS alone (abiotic)						Cells alone (biological)					
	N (%)			C (%)			N (%)			C (%)		
	pH 9.2	pH 7.9	pH 6.8	pH 9.2	pH 7.9	pH 6.8	pH 9.2	pH 7.9	pH 6.8	pH 9.2	pH 7.9	pH 6.8
$C_3H_6N_6O_6$ (RDX)	0 ± 0	3.6 ± 0.3	66.3 ± 1.0	0 ± 0	3.6 ± 0.3	66.3 ± 1.0	68.5 ± 2.5	0 ± 0	0 ± 0	68.5 ± 2.5	0 ± 0	0 ± 0
$C_3H_6N_6O_5$ (MNX)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
$CH_4N_4O_4^{2-}$ (MEDINA)	69.1 ± 0.3	34.6 ± 0.8	6.9 ± 0.2	34.5 ± 0.1	17.3 ± 0.4	3.5 ± 0.1	8.0 ± 0.	16.1 ± 0.3	0.6 ± 0.0	4.0 ± 0.1	8.0 ± 0.2	0.3 ± 0.0
HCHO	n/a[a]	n/a	n/a	51.7 ± 0.6	81.9 ± 1.7	26.4 ± 0.2	n/a	n/a	n/a	11.6 ± 0.8	41.5 ± 1.3	45.1 ± 1.6
N_2O	3.9 ± 0.1	4.4 ± 0.2	0 ± 0	n/a	n/a	n/a	0.3 ± 0.3	17.0 ± 0.9	21.7 ± 1.1	n/a	n/a	n/a
NO_2^-	29.7 ± 1.1	5.6 ± 2.4	5.1 ± 0.8	n/a	n/a	n/a	0.3 ± 0.5	0 ± 0	0 ± 0	n/a	n/a	n/a
NH_4^+	7.4 ± 0.3	5.4 ± 0.6	4.2 ± 1.0	n/a	n/a	n/a	20.2 ± 1.3	42.4 ± 0.6	30.1 ± 10.3	n/a	n/a	n/a
CH_3OH	n/a	n/a	n/a	0 ± 0	0 ± 0	0 ± 0	n/a	n/a	n/a	3.6 ± 0.1	0 ± 0	0 ± 0
$^{14}CO^2$	n/a	n/a	n/a	0 ± 0	0 ± 0	0 ± 0	n/a	n/a	n/a	7.4 ± 0.4	13.6 ± 2.4	11.2 ± 3.8
Total	110.2 ± 0.6	53.7 ± 1.5	82.6 ± 1.3	86.2 ± 0.5	102.7 ± 1.7	98.4 ± 5.0	97.3 ± 2.0	75.5 ± 0.9	52.4 ± 11.3	95.0 ± 2.6	63.2 ± 3.7	56.6 ± 5.4

RDX & degradation products	Cells + AQDS (mixed abiotic-biological)					
	N (%)			C (%)		
	pH 9.2	pH 7.9	pH 6.8	pH 9.2	pH 7.9	pH 6.8
$C_3H_6N_6O_6$ (RDX)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
$C_3H_6N_6O_5$ (MNX)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
$CH_4N_4O_4^{2-}$ (MEDINA)	36.5 ± 1.8	15.1 ± 0.2	0.4 ± 0.0	18.3 ± 0.3	7.5 ± 0.1	0.2 ± 0.0
HCHO	n/a	n/a	n/a	45.1 ± 1.3	50.8 ± 1.1	53.8 ± 2.1
N_2O	1.8 ± 0.1	11.6 ± 2.3	24.3 ± 0.5	n/a	n/a	n/a
NO_2^-	0 ± 0	1 ± 0	0 ± 0	n/a	n/a	n/a
NH_4^+	43.1 ± 3.9	44.3 ± 2.1	40.5 ± 2.3	n/a	n/a	n/a
CH_3OH	n/a	n/a	n/a	4.3 ± 0.1	0 ± 0	0 ± 0
$^{14}CO^2$	n/a	n/a	n/a	11.8 ± 1.4	18.9 ± 3.1	11.9 ± 4.4
Total	81.4 ± 3.7	70.9 ± 3.5	65.2 ± 2.1	79.4 ± 3.0	77.2 ± 4.3	65.9 ± 6.5

[a] Not applicable

For biological or mixed abiotic-biological pathways RDX reduction was tested in the resting cell suspension of *G. metallireducens* with or without AQDS. Acetate was the sole electron donor. Results are the means of triplicate analyses with standard deviation

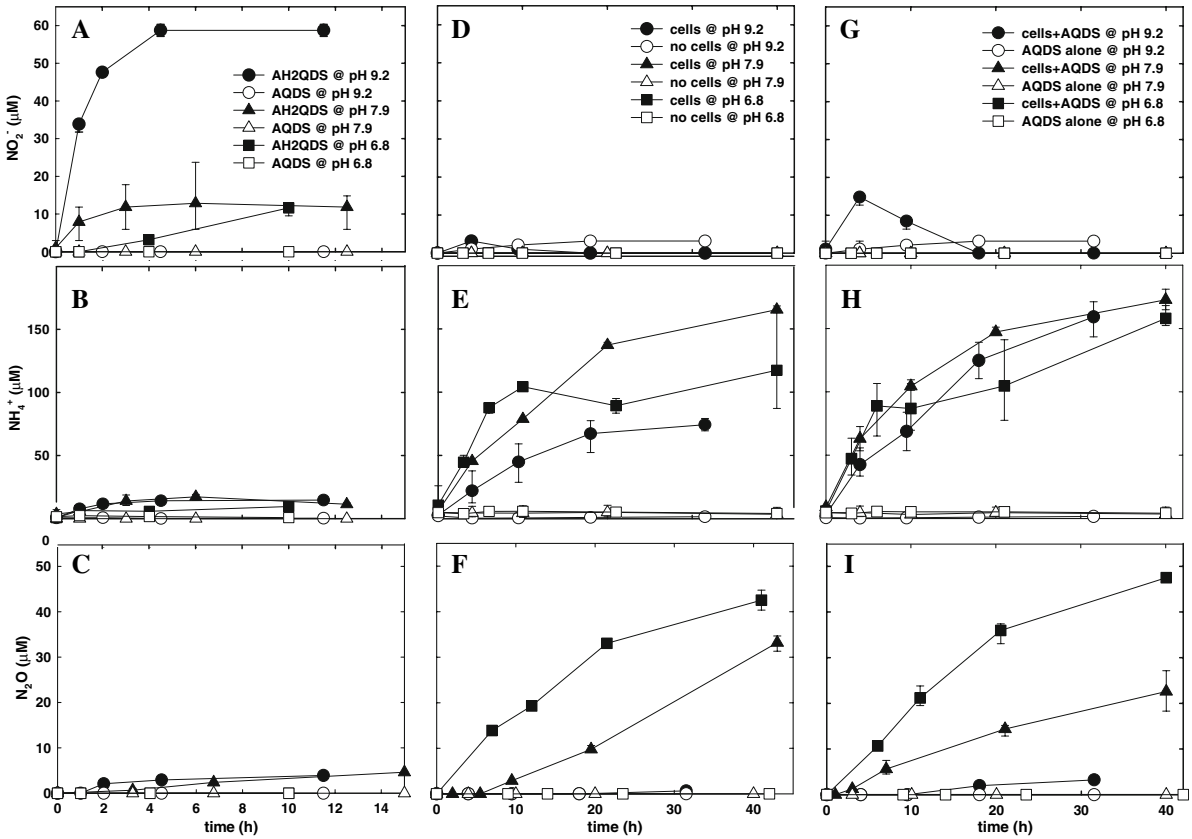

Fig. 4 Production of nitrite, ammonium, and nitrous oxide from RDX by abiotic (**a**, **b**, and **c**), biological (**d**, **e**, and **f**), mixed abiotic–biological (**g**, **h**, and **i**) pathways at pH 6.8, 7.9, and 9.2. For biological or mixed abiotic–biological pathways RDX reduction was tested in the resting cell suspension of *G. metallireducens* with (**g**, **h**, **i**) or without (**d**, **e**, **f**) AQDS. Acetate was the sole electron donor. Results are the means of triplicate analyses and bars indicate one standard deviation. Note that 40 μM RDX was the starting concentration in the strictly abiotic series, and that 60 μM RDX was the starting concentration in both series with cells

pathway in the presence of cells and shuttles. The pH range was selected because it is within the normal tolerance range of *G. metallireducens* (and other AQDS-reducing cells) and was below the pH where alkaline hydrolysis of RDX has been reported (pH $\geq$ 10.5) (Hwang et al. 2006). While *G. metallireducens* is metabolically active from pH 6.8–9.2 its activity is sub-optimal at the higher pH values (pH $\geq$ 8.0).

The pH dependence of RDX reduction by AH_2QDS (abiotic pathway) is consistent with the findings by Schwarzenbach et al. (1990) where they reported pH dependence on the rate of nitroaromatic compound reduction in the presence of juglone and lawsone; as pH increased from 6.1 to 7.8, k_{obs} (s^{-1}) increased. Uchimiya and Stone (2006) also reported that monoprotonated dihydroxybenzene molecules have greater reactivity than diprotonated molecules and that forward rate constants of 1,2-naphthoquinone-4-sulfonate and 1,4-naphthoquinone-2-sulfonate reduction by 2,6-dimethylhydroquinone increase as pH increases (>pH 4.0, >pH 5.0, respectively) (Uchimiya and Stone 2006). They postulated that the relative proportion of the monoprotonated hydroquinone is greater than the diprotonated hydroquinone, which increases quinone-mediated reduction rates. In our current study a fixed concentration of AH_2QDS (100 μM) reduced RDX at a higher rate and extent as pH increased. Between pH 8.5 and 9.2 approximately 90% of the anthrahydroquinone is in the monoprotonated (α_1) form (data not shown). Plotting log-α_1 versus log-k_{obs} (for RDX reduction) demonstrates a linear relationship between the RDX reduction rate and the proportion of the monoprotonated

hydroquinone (Fig. 2). Although some electrons must be transferred from the un-protonated (α_2) form of the hydroquinone, this plot assumes that $\alpha_1 >> \alpha_2$. At values higher than pH 10, alkaline hydrolysis of RDX is a dominant pathway; therefore, the un-protonated form of the hydroquinone is difficult to test.

Transformation products in the abiotic, biological, and mixed abiotic–biological pathways

Formaldehyde was the dominant carbon product in electron shuttle-amended suspensions (abiotic or mixed). Formaldehyde production in the absence of electron shuttling compounds was lower, particularly at the higher pH values tested (pH 7.9 and 9.2). This is critical because formaldehyde is one of the lowest molecular mass metabolites during RDX breakdown, and is a precursor to mineralization (Hawari et al. 2000a, b; Sherburne et al. 2005). MEDINA was also produced, but declined slightly in the presence of cells (Fig. 3). ^{14}C-RDX was mineralized to $^{14}CO_2$ to some extent in all cells-amended incubations. The mass balance for cell-containing suspensions was not closed, primarily at low pH; it is likely that there were unidentified intermediates. Biosorption was considered but little radioactivity (less than 1.0%) was recovered from cell pellets obtained from each suspension in the [^{14}C]-RDX experiments (data not shown). The probable pathways and dominant products/metabolites for the abiotic and mixed pathways are presented in Fig. 5.

Ammonium was the dominant inorganic nitrogen product in cell-containing suspensions at all pH tested, and AQDS increased the extent of ammonium produced (especially at pH 9.2), most likely by stimulating the rate at which nitrite was produced compared to cells alone. Nitrite was a significant end product in the strictly abiotic pathway with AH_2QDS at all pH tested. Nitrite reduction and ammonium production in cell suspensions are not surprising since *Geobacter* can reduce nitrate and nitrite to ammonium (Lovley 2000); nitrite reductase activity in its membrane is well established (Martínez Murillo et al. 1999). Ammonium was produced to a much lower extent in the AH_2QDS-alone suspensions. Nitrous oxide was produced in all suspensions. Nitrous oxide production from RDX, MNX or MEDINA by abiotic and biological pathways has been reported previously (Bhushan et al. 2002; Fournier et al. 2002; Zhao et al. 2002).

MEDINA and 4-nitro-2,4-diazabutanal (NDAB) have also been reported as ring cleavage intermediates (Hawari et al. 2000a, b; Zhao et al. 2003). However, formaldehyde accounted for a greater proportion of carbon than MEDINA in all suspensions suggesting that MEDINA will be less significant than HCHO (Table 1) under electron shuttle-reducing conditions. MEDINA may spontaneously decompose in water to nitrous oxide and formaldehyde or it can be also transformed directly by cells (Halasz et al. 2002), which will be further evaluated for *G. metallireducens*.

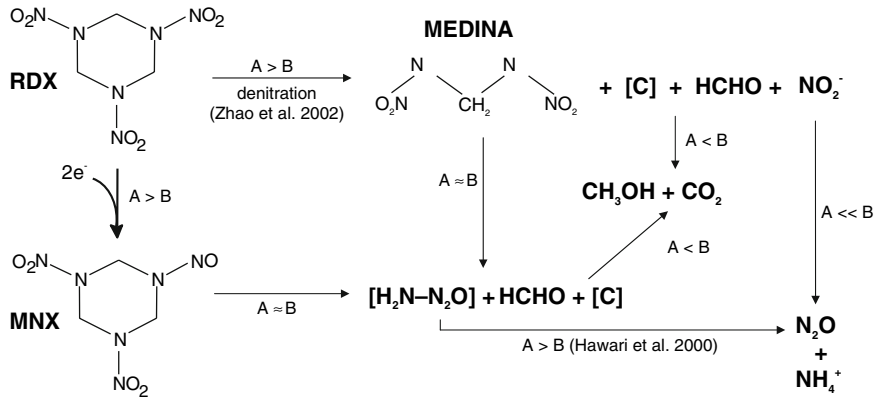

Fig. 5 Probable RDX degradation routes by mixed abiotic–biological pathways based on products identified and reported degradation pathways (references listed on figure) in the presence of AQDS and *G. metallireducens*. Compounds in square brackets were not determined; [C] represents unidentified carbon intermediates. A = abiotic pathway(s), B = biological pathway(s). The >, <, and ≈ indicate whether the products were more significant in the abiotic or biological pathway (based on the mass balance)

Environmental implications

These results demonstrate that electron shuttle mediated RDX transformation led to more low-molecular weight ring cleavage products, formaldehyde in particular, than other conditions we tested. This indicates that in situ this strategy may lead to more rapid and complete RDX mineralization by targeting electron shuttle-reducers and specific biotic–abiotic reactions. The degradation patterns were consistent when electron shuttles were added, whether the pathway was strictly abiotic or mixed biotic–abiotic. Past reports indicate that extracellular electron shuttles are catalytic and therefore in situ applications may only require a low concentration of electron shuttling compounds to stimulate RDX reduction. RDX persists in many subsurface environments despite its perceived "inherently biodegradable" characteristics; more strategies are necessary to promote complete mineralization in situ. pH had a marked effect on RDX reduction and metabolite production in both abiotic and biotic pathways. This must be considered when designing remediation strategies; while extracellular electron shuttling ultimately facilitates RDX degradation, pH variation will impact the rate of RDX reduction and metabolite distribution.

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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(5):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(14):3499–3507

Bhushan B, Halasz A, Spain J, Thiboutot S, Ampleman G, Hawari J (2002) Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine catalyzed by a NAD(P)H: nitrate oxidoreductase from *Aspergillus niger*. Environ Sci Technol 36(14):3104–3108

Bhushan B, Halasz A, Hawari J (2006) Effect of iron(III), humic acids and anthraquinone-2,6-disulfonate on biodegradation of cyclic nitramines by *Clostridium* sp. EDB2. J Appl Microbiol 100(3):555–563

Borch T, Inskeep WP, Harwood JA, Gerlach R (2005) Impact of ferrihydrite and anthraquinone-2,6-disulfonate on the reductive transformation of 2,4,6-trinitrotoluene by a gram-positive fermenting bacterium. Environ Sci Technol 39(18):7126–7133

Bradley PM, Dinicola RS (2005) RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) biodegradation in aquifer sediments under manganese-reducing conditions. Bioremediat J 9(1): 1–8

Clausen J, Robb J, Curry D, Korte N (2004) A case study of contaminants on military ranges: Camp Edwards, Massachusetts, USA. Environ Pollut 129(1):13–21

Coates JD, Ellis DJ, Blunt-Harris EL, Gaw CV, Roden EE, Lovley DR (1998) Recovery of humic reducing bacteria from a diversity of environments. Appl Environ Microbiol 64(4):1504–1509

Crocker FH, Indest KJ, Fredrickson HL (2006) Biodegradation of the cyclic nitramine explosives RDX, HMX, and CL-20. Appl Microbiol Biotechnol 73(2):274–290

Finneran KT, Lovley DR (2001) Anaerobic degradation of methyl tert-butyl ether (MTBE) and tert-butyl alcohol (TBA). Environ Sci Technol 35(9):1785–1790

Finneran KT, Johnsen CV, Lovley DR (2003) *Rhodoferax ferrireducens* sp. nov., a psychrotolerant, facultative anaerobic bacterium that oxidizes acetate with the reduction of Fe(III). Int J Syst Evol Microbiol 53(3): 669–673

Fournier D, Halasz A, Spain J, Firuasek P, Hawari J (2002) Determination of key metabolites during biodegradation of hexahydro-1,3,5-trinitro-triazine with *Rhodococcus* sp. strain DN22. Appl Environ Microbiol 68(1):166–172

Fournier D, Halasz A, Thiboutot S, Ampleman G, Manno D, Hawari J (2004) Biodegradation of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) by *Phanerochaete chrysosporium*. New insight into the degradation pathway. Environ Sci Technol 38(15):4130–4133

Garg R, Grasso D, Hoag G (1991) Treatment of explosives contaminated lagoon sludge. Hazard Waste Hazard Mater 8(4):319–340

Gregory KB, Larese-Casanova P, Parkin GF, Scherer MM (2004) Abiotic transformation of hexahydro-1,3,5-trinitro-1,3,5-triazine by Fe(II) bound to magnetite. Environ Sci Technol 38(5):1408–1414

Haas R, Schreiber I, Low EU, Stork G (1990) Conception for the investigation of contaminated munition plants. Fres J Anal Chem 338:41–45

Halasz A, Spain J, Paquet L, Beaulieu C, Hawari J (2002) Insights into the formation and degradation mechanisms of methylenedinitramine during the incubation of RDX with anaerobic sludge. Environ Sci Technol 36(4): 633–638

Hawari J, Halasz A (2002) Microbial degradation of explosives. In: Bitton G (ed) The encyclopedia of environmental microbiology. John Wiley & Sons Ltd, New York, pp 1979–1993

Hawari J, Beaudet S, Halasz A, Thiboutot S, Ampleman G (2000a) Microbial degradation of explosives: biotransformation versus mineralization. Appl Microbiol Biotechnol 54(5):605–618

Hawari J, Halasz A, Sheremata T, Beaudet S, Groom C, Paquet L, Rhofir C, Ampleman G, Thiboutot S (2000b) 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

Hwang S, Felt DR, Bouwer EJ, Brooks MC, Larson SL, Davis JL (2006) Remediation of RDX-contaminated water using alkaline hydrolysis. J Environ Eng-ASCE 132(2):256–262

Kwon MJ, Finneran KT (2006) Microbially mediated biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine by extracellular electron shuttling compounds. Appl Environ Microbiol 72(9):5933–5941

Lachance B, Robidoux PY, Hawari J, Ampleman G, Thiboutot S, Sunahara GI (1999) Cytotoxic and genotoxic effects of energetic compounds on bacterial and mammalian cells in vitro. Genet Toxicol Environ Mutagen 444(1):25–39

Lovley DR (2000) Dissimilatory Fe(III)- and Mn(IV)-reducing prokaryotes. In: Dworkin M et al (eds) The prokaryotes. Springer-Verlag, New York

Lovley DR, Phillips EJP (1988) Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Appl Environ Microbiol 54(6):1472–1480

Lovley DR, Giovannoni SJ, White DC, Champine JE, Philips EJP, Gorby YA, Goodwin S (1993) *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. Arch Microbiol 159:336–344

Lovley DR, Coates JD, Blunt-Harris EL, Phillips EJP, Woodward JC (1996) Humic substances as electron acceptors for microbial respiration. Nature 382:445–448

Lowry OH, Rosbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

Lynch JM (1988) The terrestrial environment. In: Lynch JM, Hobbie JE (eds) Microorganisms in action: concepts and applications in microbial ecology, vol. 2. Blackwell, Oxford, pp 103–131

Martínez Murillo F, Gugliuzza T, Senko J, Basu P, Stolz JF (1999) A heme-C-containing enzyme complex that exhibits nitrate and nitrite reductase activity from the dissimilatory iron-reducing bacterium *Geobacter metallireducens*. Arch Microbiol 172(5):313–320

Meyers SK, Deng S, Basta NT, Clarkson WW, Wilber GG (2007) Long-term explosive contamination in soil: effects on soil microbial community and bioremediation. Soil Sediment Contam 16(1):61–77

Monteil-Rivera F, Paquet L, Halasz A, Montgomery MT, Hawari J (2005) Reduction of octahydro-1,3,5,7- tetranitro-1,3,5,7-tetrazocine by zero valent iron: product distribution. Environ Sci Technol 39(24):9725–9731

Pennington JC, Brannon JM (2002) Environmental fate of explosives. Thermochimica Acta 384(1–2):163–172

Rhine ED, Sims GK, Mulvaney RL, Pratt EJ (1998) Improving the Berthelot reaction for determining ammonium in soil extracts and water. J Soil Sci Soc Am 62(2):473–480

Schwarzenbach RP, Stierli R, Lanz K, Zeyer J (1990) Quinone and iron porphyrin mediated reduction of nitroaromatic compounds in homogeneous aqueous solution. Environ Sci Technol 24(10):1566–1574

Sherburne LA, Shrout JD, Alvarez PJJ (2005) Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degradation by *Acetobacterium paludosum*. Biodegradation 16(6):539–547

Spalding RF, Fulton JW (1988) Groundwater munition residues and nitrate near Grand Island, Nebraska, USA. J Contam Hydrol 2:139–153

Talmage SS, Opresko DM, Maxwell CJ, Welsh CJ, Cretella FM, Reno PH, Daniel FB (1999) Nitroaromatic munition compounds: environmental effects and screening values. Rev Environ Contam Toxicol 161:1–156

Thompson KT, Crocker FH, Fredrickson HL (2005) Mineralization of the cyclic nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine by *Gordonia* and *Williamsia* spp. Appl Environ Microbiol 71(12):8265–8272

Uchimiya M, Stone AT (2006) Aqueous oxidation of substituted dihydroxybenzenes by substituted benzoquinones. Environ Sci Technol 40(11):3515–3521

Zhao JS, Halasz A, Paquet L, Beaulieu C, Hawari J (2002) Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine and its mononitroso derivative hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine by *Klebsiella pneumoniae* strain SCZ-1 isolated from an anaerobic sludge. Appl Environ Microbiol 68(11):5336–5341

Zhao JS, Paquet L, Halasz A, Hawari J (2003) 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(2):187–193

Zhao JS, Spain J, Thiboutot S, Ampleman G, Greer C, Hawari J (2004) Phylogeny of cyclic nitramine-degrading psychrophilic bacteria in marine sediment and their potential role in the natural attenuation of explosives. FEMS Microbiol Ecol 49:349–357