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ORIGINAL PAPER

Electron shuttle-stimulated RDX mineralization and biological production of 4-nitro-2,4-diazabutanal (NDAB) in RDX-contaminated aquifer material

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Abstract The potential for extracellular electron shuttles to stimulate RDX biodegradation was investigated with RDX-contaminated aquifer material. Electron shuttling compounds including anthraquinone-2, 6-disulfonate (AQDS) and soluble humic substances stimulated RDX mineralization in aquifer sediment. RDX mass-loss was similar in electron shuttle amended and donor-alone treatments; however, the concentrations of nitroso metabolites, in particular TNX, and ring cleavage products (e.g., HCHO, MEDINA, NDAB, and NH_4^+) were different in shuttle-amended incubations. Nitroso metabolites accumulated in the absence of electron shuttles (i.e., acetate alone). Most notably, 40–50% of [^{14}C]-RDX was mineralized to $^{14}CO_2$ in shuttle-amended incubations. Mineralization in acetate amended or unamended incubations was less than 12% within the same time frame. The primary differences in the presence of electron shuttles were the increased production of NDAB and formaldehyde. NDAB did not further degrade, but formaldehyde was not present at final time points, suggesting that it was the mineralization precursor for Fe(III)-reducing microorganisms. RDX was reduced concurrently with Fe(III) reduction rather than nitrate or sulfate reduction. Amplified 16S rDNA restriction analysis (ARDRA) indicated that unique Fe(III)-reducing microbial communities (β- and γ-*proteobacteria*) predominated in shuttle-amended incubations. These results demonstrate that indigenous Fe(III)-reducing microorganisms in RDX-contaminated environments utilize extracellular electron shuttles to enhance RDX mineralization. Electron shuttle-mediated RDX mineralization may become an effective in situ option for contaminated environments.

Keywords Fe(III) reducing microorganisms · Electron shuttling · Cyclic nitramine explosives · Biodegradation · Bioremediation

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Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) biodegradation is one strategy for decontamination of Department of Defense (DoD) or private facilities that use or produce live munitions (Adrian and Arnett 2004; Adrian et al. 2003; Crocker et al. 2005; Meyers et al. 2007; Sherburne et al. 2005; Thompson et al. 2005). Numerous data have been presented that RDX is microbially or chemically reduced, destabilizing the ring structure and leading to spontaneous ring-cleavage (Hawari 2000; McCormick et al. 1981). The ring cleavage products are then available for further metabolism if the appropriate microbial community is present to ultimately mineralize the carbon and/or nitrogen metabolites. One major limitation of RDX

bioremediation is that it often leads to accumulated intermediates, without the compounds ultimately being mineralized to CO_2 or CH_4. The limited mineralization may be a result of targeting microbial communities that cannot oxidize the intermediates as carbon (or nitrogen) substrates either to gain energy or via co-metabolic reactions.

Several enrichment cultures and/or pure cultures have been used to investigate RDX biodegradation. Data have been reported for aerobic conditions (Fournier et al. 2005; Seth-Smith et al. 2002; Thompson et al. 2005), nitrate-reducing conditions (Freedman and Sutherland 1998), sulfate-reducing conditions (Boopathy et al. 1998), methanogenesis (Adrian et al. 2003), and acetogenesis (Adrian and Arnett 2004; Sherburne et al. 2005). Bradley et al. reported in situ mineralization in one contaminated sediment associated with Mn(IV)-reducing conditions (Bradley and Dinicola 2005), which is catalyzed by organisms that may also reduce Fe(III). The work presented here focuses on dissimilatory Fe(III) reduction; the data presented demonstrate that this may be the primary in situ process promoting in situ RDX biodegradation. In addition, stimulating Fe(III) reduction via extracellular electron shuttling compounds increased the overall extent of mineralization, which suggests the process can be accelerated.

Fe(III) reduction is one of the most important biogeochemical processes due to the abundance of ferric minerals (e.g., ferrihydrite, goethite, or phyllosilicate clay minerals) in subsurface environments and its influence on the fate of organic or inorganic contaminants (Kostka et al. 2002; Lovley 1995; Roden et al. 2000; Zachara et al. 2001). Fe(III) reduction is mediated by Fe(III)-reducing microorganisms (Lovley 2000) which are ubiquitous in subsurface environments (Coates et al. 1998). Fe(III)-reducing microorganisms also reduce extracellular electron shuttling compounds, most notably humic substances (HS) or extracellular quinones. Electron shuttling compounds can promote contaminant reduction by accepting electrons in microbial respiration and abiotically transferring the electrons to contaminants (Lovley 2000). The electron shuttles are re-oxidized and available again for microbial respiration; in this manner the shuttles are cycled and only a small concentration would be needed to promote these reactions. Data from our lab and generated by other groups suggest that mixed biotic-abiotic reactions involving Fe(III)-reducing microorganisms, electron shuttling compounds, and reactive Fe(II) degrade nitramine and nitroaromatic explosives effectively (Hofstetter et al. 1999; Schwarzenbach et al. 1990; Bhushan et al. 2006; Borch et al. 2005; Gregory et al. 2004; Kim and Strathmann 2007; Kwon and Finneran 2006; Larese-Casanova and Scherer 2008). However, what remains unknown is: (a) the extent to which this is relevant in RDX-contaminated aquifer material and (b) what microorganisms will catalyze these reactions in the presence and absence of added electron shuttles.

Contaminated subsurface material was used to investigate the extent and metabolite distribution of RDX degradation via extracellular electron shuttling compounds and to determine the dominant biogeochemical process (so called "terminal electron accepting process" or TEAP) that promotes RDX biodegradation. The objectives of this study were to examine to what extent electron shuttles added to contaminated aquifer material will stimulate RDX mineralization (using U-[^{14}C]-RDX) relative to electron donor amendment alone (the typical strategy), and to characterize the microbial communities that develop during RDX biodegradation in the presence and absence of electron shuttles.

Experimental section

Chemicals

RDX (97% pure) was provided by the U.S. 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-2,4-diazabutanal (NDAB) were obtained from SRI International (Menlo Park, CA, USA). Purified humic acid and anthrquinone-2,6-disulfonate (AQDS) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). All other chemicals used were of reagent grade quality or higher.

Sediment and groundwater

Aquifer sediment samples were obtained from the Picatinny Arsenal in New Jersey. The sediment was collected from below the water table and immediately

dispensed into amber glass jars that were then sealed without a headspace. Samples were placed in coolers and shipped via overnight carrier to the laboratory. Anoxic sediment was homogenized in a N_2-filled glove bag prior to processing for individual experiments. Groundwater from the monitoring well at Picatinny Arsenal was sampled with a peristaltic pump. Groundwater samples were collected in 1 l glass bottles that were then sealed without headspace. Groundwater samples were stored at 4°C until processing. The groundwater contained nitrate (27 μM), nitrite (2 μM), chloride (236 μM), bromide (2 μM), and sulfate (144 μM). The porewater was initially contaminated by RDX (~1 μM). The aquifer sediment contained 4.7 mmol/kg of bioavailable (0.5 N HCl-extractable) Fe(III).

Sediment incubations

The aquifer sediments were tested with initial aeration to oxidize native Fe(II) to Fe(III). For initial aeration, the aquifer sediments were aerated with ambient air for 30 min. Approximately 50 g of sediment and 30 ml of groundwater were dispensed into 70 ml serum bottles in an N_2-filled glove bag that were then sealed with a thick butyl-rubber stopper. After removal from the glove bag, the headspace of each bottle was flushed with 95:5 (vol/vol) N_2:CO_2 that had been passed over hot copper filings to remove traces of oxygen. Approximately 55 μM (final concentration) of RDX was added to the aquifer sediment incubations because the initial concentration was too low to quantify significant losses of the parent compound or accumulation of metabolites. Each headspace was flushed with anoxic N_2:CO_2 gases. All amendments were made from sterile, anoxic stock solutions. Acetate was added as a sole electron donor at a final concentration of 10 mM. Although aquifer material already contained nitrate and sulfate, additional nitrate (0.5 mM) and sulfate (1.5 mM) were amended to each bottle to increase the capacity for different terminal electron accepting processes. Electron shuttles used with the sediments included HS (0.15 g/l) and anthraquinone-2,6-disulfonate (AQDS) (100 μM). All subsequent amendments or transfers were made using sterile needles and syringes that had been flushed with anoxic gas.

In order to generate abiotic controls, sediments were autoclaved for 1 h per day for 3 consecutive days (Finneran and Lovley 2001). All bottles were incubated in the dark at 18°C without agitation. Each bottle had an overlying aqueous layer without forming a slurry. Samples (0.6 ml) were collected periodically via anoxic syringe and needle. To minimize sampling volume, glass inserts (250 μl 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), anions (i.e., nitrite, nitrate, and sulfate), and ammonium (NH_4^+), respectively. No more than six samples were taken from any incubation; therefore, the final aqueous volume of each incubation was approximately 26 ml at the end of the experiments (88% volume remaining). Nitrous oxide was monitored by headspace analysis. The aqueous phase pH was measured at each time point in a glove bag. Approximately 1 g of sediment sample was collected at each time point (after headspace samples were analyzed) in an anoxic glove bag to measure total bioavailable iron, which was measured for all incubations at each time point. All liquid and gas samples were taken with a sterile syringe and needle that had been flushed with anoxic gas, and liquid samples were filtered through 0.2 μm PTFE filters (PALL Sciences) prior to analyses (filters did not interfere with any analyses). All experiments were performed in triplicate.

Mineralization with [^{14}C]-RDX

Uniformly radiolabeled- (U-[^{14}C]) RDX (concentration: final radioactivity of 36,000 dpm/ml) was amended at day 32. $^{14}CO_2$ 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 into the final mineralization; the starting partition coefficient (total dpm $H^{14}CO_3^-$ recovered as $^{14}CO_2$/total dpm added) was 0.013, and was adjusted if necessary due to changes in pH. 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/PDA, Dionex) at 254 nm as described previously (Fournier et al. 2002). MEDINA and NDAB were analyzed using HPLC at 225 nm as described previously (Fournier et al. 2004). The peaks of MEDINA and NDAB were confirmed by using a reference standard with the HPLC method and retention times were clearly different between two peaks (MEDINA = 6.6–6.9 min, NDAB = 7.7–7.9 min). In order to double check the production of these compounds, the UV spectrum (200–350 nm) of these products was compared with that of reference standards. Nitrous oxide was analyzed using gas chromatography (GC) with thermal conductivity detection (Kwon and Finneran 2008a). Anions in groundwater (i.e., chlorine, bromide, nitrite, nitrate, and sulfate) were measured using an ion chromatograph (IC; Dionex 1000) with conductivity detection (Kwon and Finneran 2008a). 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) as described previously (Kwon and Finneran 2008a). Aqueous Fe(II) and total solid phase iron concentrations were quantified by the Ferrozine assay as described previously (Lovley and Phillips 1987). pH in aqueous phase was measured by Semi Micro pH probe (Thermo Scientific Inc.).

Microbial community analysis

Approximately 1 g of sediment was collected from each bottle at every time point using a sterile metal spatula in an anoxic glove bag and dispensed into sterile micro-centrifuge tubes. Samples were frozen at −80°C until the experiments were completed. Total genomic DNA was extracted using a Fast DNA SPIN for Soil Kit (MP Biomedicals, LLC.) with a bead-beating apparatus according to manufacturer's directions. Extracted DNA was amplified using universal Eubacterial primers, 338-Forward (338F) (Amann et al. 1995) and 907-Reverse (907R) (Lane et al. 1985). PCR reaction mixtures included primer sets (1 μM each), deoxynucleoside triphosphates (dNTPs) (200 μM), PCR 10× buffers (1×), $MgCl_2$ (2.5 mM), reaction template (extracted DNA), and *Taq* polymerase (2.5 U). The reaction mixture volume for each template was 100 μl. The PCR mixtures were sterilized by UV radiation for 20 min prior to the addition of template and *Taq* polymerase. Templates were amplified using a thermal cycler (an initial denaturation step at 94°C for 4 min, followed by 35 cycles of 94°C (30 s), 50°C (30 s), and 72°C (45 s), with a final extension at 72°C for 7 min).

The initial PCR product was used to generate clone libraries using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's specified instructions. At least 50 clones from the clone library were selected for restriction enzyme analysis. Cloned DNA was re-amplified using clone-specific primers (0.4 μM each), M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) (Invitrogen), dNTPs (350 μM), PCR 10× buffers (1×), $MgCl_2$ (2.5 mM), reaction template (individual clone applied directly into reaction mixture), and *Taq* polymerase (2.5 U). The reaction mixture volume for each template was 50 μl. The thermal cycling program included an initial step of cell lysing and nuclease inactivation at 94°C for 10 min, followed by 30 cycles of 94°C (1 min), 55°C (1 min), and 72°C (1.5 min) with a final extension at 72°C for 10 min). DNA (12 μl) from M13 PCR was digested with two restriction enzymes, *Hha*I and *Msp*I (10 U each) (New England Bio), 10× NE Buffer (1×), and BSA (100 μg/ml) for 16 h at 37°C and 20 min at 60°C. Restriction patterns were visualized on a 3% Metaphor agarose gel (Lonza, Rockland, Maine) using a UV box Gel-Doc system, and unique clones were purified using a QIAquick PCR Purification Kit (QIAGEN Inc.). Purified M13 PCR products were sequenced at the University of Illinois automated sequencing facility with the M13 forward primer. These sequences were analyzed versus 16S rRNA genes in the publicly available database GenBank using BLASTN and SIMILARITY-RANK algorithms. Representative sequences were aligned with the reference sequences using Clustal W (Thompson et al. 1994). The phylogenetic trees were constructed using the maximum-likelihood method with *Geneious* 3.7.0 (Biomatters Ltd.) software.

Nucleotide sequence accession number

Sequences identified in this study were deposited in the GenBank database under the accession numbers EU826721–EU826755.

Results and discussion

RDX reduction and metabolite distribution

RDX was not reduced or transformed in heat-sterilized incubations, or in the absence of acetate (Fig. 1a). Native electron donors were limited in this aquifer material and all activity required substrate amendment. The primary differences between donor alone and donor + shuttles were related to the rate and extent of metabolite formation, the metabolites formed, and eventual mineralization. RDX was reduced in all acetate-amended incubations, the electron shuttles only slightly increased the extent of RDX degraded at each time point (Fig. 1a). The pH remained stable at 7.0 ± 0.2.

Nitroso metabolites accumulated to a lesser extent in the presence of electron shuttles, which is consistent with previous pure culture data (Kwon and Finneran 2006, 2008a, b) (Fig. 1b–d). The nitroso metabolites are toxic and accumulation is a concern (Meyer et al. 2005; Pitot and Dragan 1996). Several reports indicate that the nitroso metabolites are unstable and will spontaneously degrade (Gregory et al. 2004; Oh et al. 2001; Sheremata et al. 2001). TNX has been reported to degrade by an uncharacterized autocatalytic mechanism; however, TNX accumulation has also been reported in pure phase, mixed culture and sediment incubations (Gregory et al. 2004; Larese-Casanova and Scherer 2008). Nitroso metabolite accumulation indicates that the initial steps mediated by the microorganisms with and without electron shuttling compounds was mainly nitro group reduction rather than denitration. Electron shuttles increased the rate of nitroso group transformation (particularly TNX), which was demonstrated previously (Kwon and Finneran 2006).

While autocatalytic decomposition is one pathway for nitroso group degradation these data and other recent reports with iron sulfate and carbonate green rusts (Larese-Casanova and Scherer 2008), complexed ferrous iron (Kim and Strathmann 2007), and zero-valent iron (Naja et al. 2008) suggest that abiotic reduction accelerates nitro reduction and

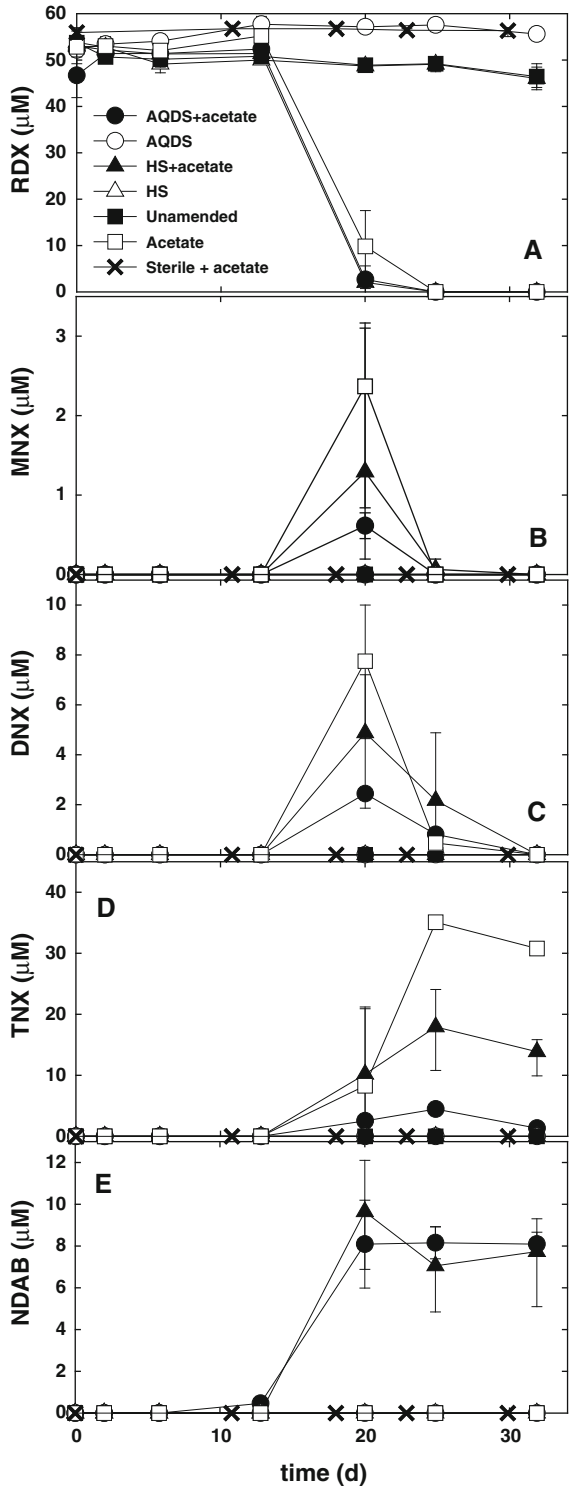

Fig. 1 RDX reduction (**a**) and the production and loss of MNX (**b**), DNX (**c**), TNX (**d**), and NDAB (**e**) in contaminated aquifer material from the Picatinny Arsenal that has been amended with 10 mM acetate plus the electron shuttles AQDS (100 μM) or purified humic substances (HS) (0.15 g/l), or which had only been amended with the electron donor acetate (10 mM). The aquifer material was aerated before beginning the experiments. Results are the means of triplicate analyses and bars indicate one standard deviation

eventual ring cleavage. These recent reports also reinforce the importance of iron in RDX transformation, and support our primary hypothesis that stimulating Fe(III) reduction, and secondary reactions with reduced iron or reduced electron shuttles, will stimulate RDX degradation.

The ring cleavage metabolites HCHO, MEDINA, NDAB, NH_4^+, N_2O, and nitrite were analyzed. HCHO and MEDINA were only produced in AQDS amended incubations; both decreased by day 25 (Data not shown). NDAB increased to approximately 10 μM in electron shuttle-amended incubations and remained stable for the rest of the sampling time (Fig. 1). NDAB has been reported as a ring cleavage intermediate only during *aerobic* RDX transformation such as alkaline hydrolysis of RDX (Balakrishnan et al. 2003), during phytophotolysis of RDX (Just and Schnoor 2004), during photolysis of RDX in aqueous solution (Hawari et al. 2002), and during aerobic biodegradation of RDX (Bhushan et al. 2003; Fournier et al. 2002, 2004, 2005). In fact, a recent paper suggests that NDAB is an "aerobic" RDX intermediate (Jackson et al. 2007). The current incubations were performed under strict anoxic conditions at neutral pH (6.8–7.2) in the dark. NDAB production was unanticipated but demonstrates that there are several possible degradation routes for RDX under anoxic conditions.

NDAB only accounted for 10% of carbon mass balance in the presence of electron shuttling compounds, while ~50% of carbon was present as CO_2. NDAB was a dead-end product, albeit at a limited concentration. HCHO was the likely mineralization precursor; its concentration was low in the electron shuttle amended bottles and HCHO is not particularly recalcitrant. The pathway by which NDAB is converted to HCHO by *Rhodococcus* sp. DN22 is an aerobic metabolism that has been proposed previously (Fournier et al. 2002). It is also reported that NDAB degraded to N_2O and CO_2 by soil bacteria *Methylobacterium* JS178 (Fournier et al. 2005) and by the fungus *Phanerochaete chrysosporium* (Fournier et al. 2004). These are all aerobic biotransformation pathways and are not relevant to the conditions of our study.

The RDX degradation intermediates in this study were considerably different from our previous study (Kwon and Finneran 2008a), because the past experiments were performed with *Geobacter metallireducens* GS-15, which was not the dominant Fe(III) reducer present in the current study (See Fig. 4). Previously GS-15 generated only a small amount of MNX; DNX and TNX were insignificant. Another difference between two studies is that AQDS-amended GS-15 cell suspensions rapidly transformed RDX to HCHO (ca. 50% of the carbon mass balance) and only 7–20% of RDX was mineralized (per the carbon mass balance). Here, HCHO was limited (<6% of the carbon mass balance) and mineralization to CO_2 was significant (~50% of the carbon mass balance) indicating that HCHO oxidation (to CO_2) may be prevalent during stimulated Fe(III) reduction in situ when multiple microbial Genera are enriched.

Since RDX reduction generates a variety of intermediate metabolites, the success of RDX remediation depends on how fast the ring-cleavage metabolites are produced, and whether they are amenable to further degradation or ultimately mineralization (Fournier et al. 2005; Kwon and Finneran 2008a; Thompson et al. 2005). These data demonstrate that extracellular electron shuttling increased the rate and extent of RDX reduction without significant accumulation of unwanted intermediates. Nitroso metabolites were transformed more rapidly in the presence of electron shuttling compounds, and RDX mineralization to CO_2 increased as described below.

[^{14}C-RDX] mineralization

Uniformly labeled [^{14}C]-RDX was amended at day 32 of the non-radiolabeled experiment (the staggered experimental manipulation was due to limited mass of aquifer material). $^{14}CH_4$ was not produced from ^{14}C labeled-RDX in any incubation (Fig. 2). $^{14}CO_2$ production was higher in AQDS- and HS-amended incubations; 40–50% of [^{14}C]-RDX was oxidized to $^{14}CO_2$ (Fig. 2). Acetate-alone incubations mineralized 12% of the RDX. RDX mineralization began without a lag period, indicating there was no acclimation period for the active microorganisms, which at the time was dominated by Fe(III) reducers. The increased mineralization with electron shuttling compounds is likely due to two separate factors: (a) stimulation of the most appropriate community for further oxidation of ring cleavage compounds, and (b) production of HCHO relative to alternate metabolites, which can be oxidized directly.

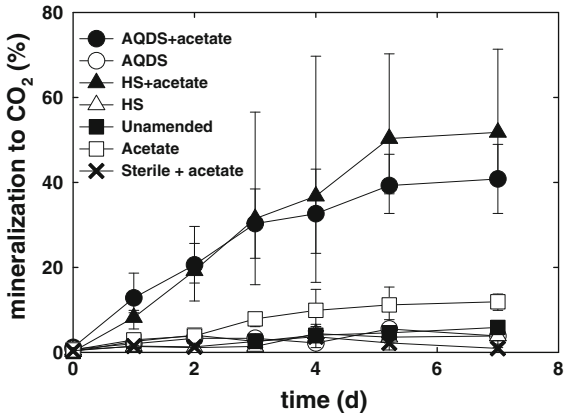

Fig. 2 RDX mineralization in contaminated aquifer material from the Picatinny Arsenal. U-[^{14}C]-RDX was amended at day 32 of non-radiolabeled RDX experiment. No additional amendments were added or re-added at this time point. Results are the means of triplicate analyses and bars indicate one standard deviation

Terminal electron accepting processes (TEAPs) correlated with RDX degradation

RDX reduction and ultimately mineralization were correlated to Fe(III) reduction rather than nitrate reduction, sulfate reduction, or methanogenesis (Fig. 3). Nitrate was reduced in all acetate-amended incubations (acetate alone, and acetate with AQDS or HS) at least 10 days prior to the onset of RDX reduction (Fig. 3a). Nitrate was completely reduced in the sterilized incubations at day 30. It is possible that serial autoclaving does not completely kill all microorganisms in this sediment. However, RDX was not transformed in sterile bottles—only nitrate. Sulfate was not reduced in any incubation within the time frame of the experiments (Fig. 3b) and sulfide was not detected (data not shown). However, reduced iron (% reduced Fe) in AQDS plus acetate incubations rapidly increased to 13% at day 20 and accumulated up to 60% at day 32 (Fig. 3c). The increase in reduced iron in AQDS-amended incubations corresponds directly to RDX reduction and metabolite (e.g., nitroso metabolites and ring cleavage products) production. Reduced Fe (%) in HS-amended acetate incubations increased to 4% by day 32, which was slightly greater than acetate alone (at 1% accumulation by day 32). Reduced Fe (%) did not increase in other treatments. Given the lack of nitrate when RDX reduction began and the lack of sulfate reduction within the timeframe of the experiment, the data indicate that Fe(III) reduction was the dominant process at the onset of RDX reduction and during further transformation of the intermediates.

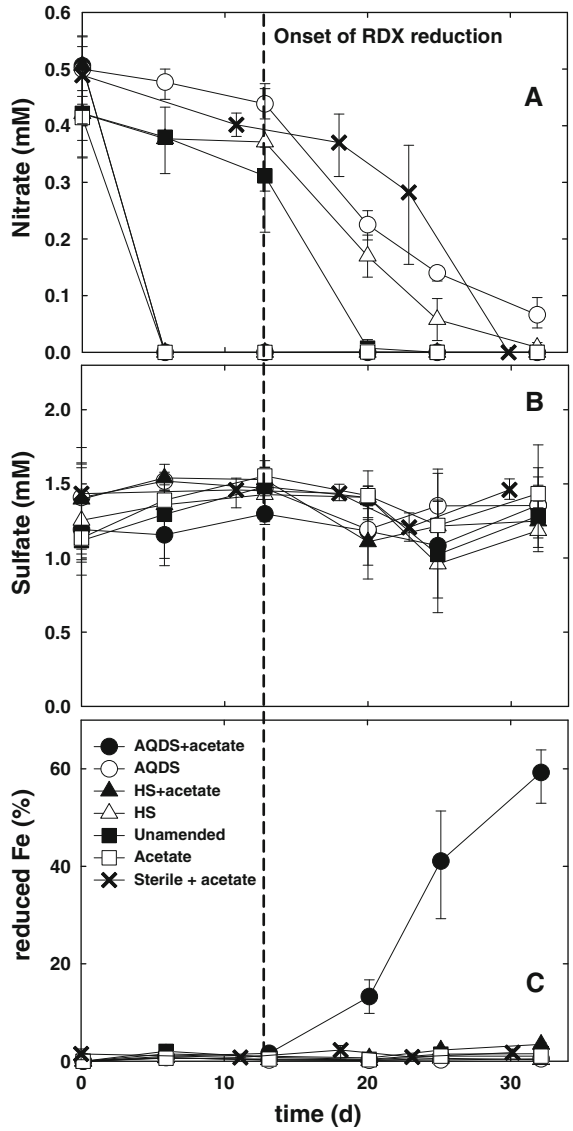

Fig. 3 Reduction of nitrate (**a**), sulfate (**b**), and iron (**c**) as terminal electron acceptors in contaminated aquifer material from the Picatinny Arsenal that has been amended with 10 mM acetate plus the electron shuttles AQDS (100 μM) or purified humic substances (HS) (0.15 g/l), or which had only been amended with the electron donor acetate (10 mM). The aquifer material was aerated before beginning the experiments. The aquifer material was amended with 0.5 mM nitrate and 1.5 mM before beginning the experiments. Results are the means of triplicate analyses and bars indicate one standard deviation. The dashed line indicates the time point at which RDX degradation began (Fig. 1a); approximately 10 days after nitrate was depleted but concurrent with Fe(III) reduction

Reduced Fe(II) reacts directly (and sometimes rapidly) with RDX and its nitroso derivatives. While it has not been definitively shown to reduce the ring cleavage metabolites, it is possible based on speculated degradation pathways (Kwon and Finneran 2008a; Larese-Casanova and Scherer 2008; Naja et al. 2008; Zhao et al. 2003). Only AQDS-amended incubations had a large net increase in reduced Fe(II). The rate of Fe(III) reduction with acetate alone or purified HS is equivalent to the rate of Fe(II)-mediated RDX (and metabolite) reduction. AQDS, on the other hand, increases the rate of Fe(III) reduction sharply as electron transfer to Fe(III) from AH_2QDS is very fast (approximately 11 μmol Fe^{3+} reduced/second (Kwon and Finneran 2008c)). Although Fe(III) reduction dominated in all incubations, the "net increase" in reduced iron was only significant in the AQDS-amended bottles.

Direct electron transfer from reduced electron shuttles to RDX is also a relevant pathway and some proportion of reducing equivalents from reduced electron shuttles will directly reduce RDX and its metabolites (Kwon and Finneran 2006, 2008a). Irrespective of the specific electron transfer mechanism, RDX was ultimately reduced and transformed most completely during Fe(III) reduction. As shown above, the electron shuttles were most critical with respect to RDX mineralization; their use may be warranted on site-specific basis in conjunction with electron donor addition.

Temporal variation of microbial communities

Clone libraries were assembled with 16S rRNA genes amplified from RDX contaminated aquifer material at key time points relative to RDX degradation and geochemical changes. Microorganisms closely related to known Fe(III) reducers and affiliated with in situ Fe(III) and/or U(VI) reduction (which is catalyzed by Fe(III) reducers) predominated after RDX reduction and immediately prior to mineralization (Fig. 4). One interesting characteristic of the Fe(III)- and electron shuttle-reducing community is that it was not dominated by *δ-proteobacteria* or members of the *Geobacteraceae* (they were present but not dominant). This demonstrates that Fe(III)-reducing microbial communities other than *Geobacteraceae* can be stimulated by electron shuttles plus acetate in situ and catalyze the primary reactions of interest.

Initial (time = 0) 16S rRNA gene sequences in the aquifer material were highly similar to *α-proteobacteria* (Genus *Ochrobactrum*) and predominated in all incubations. However, this class decreased over time; 60–24% in AQDS plus acetate incubations, 82–46% in

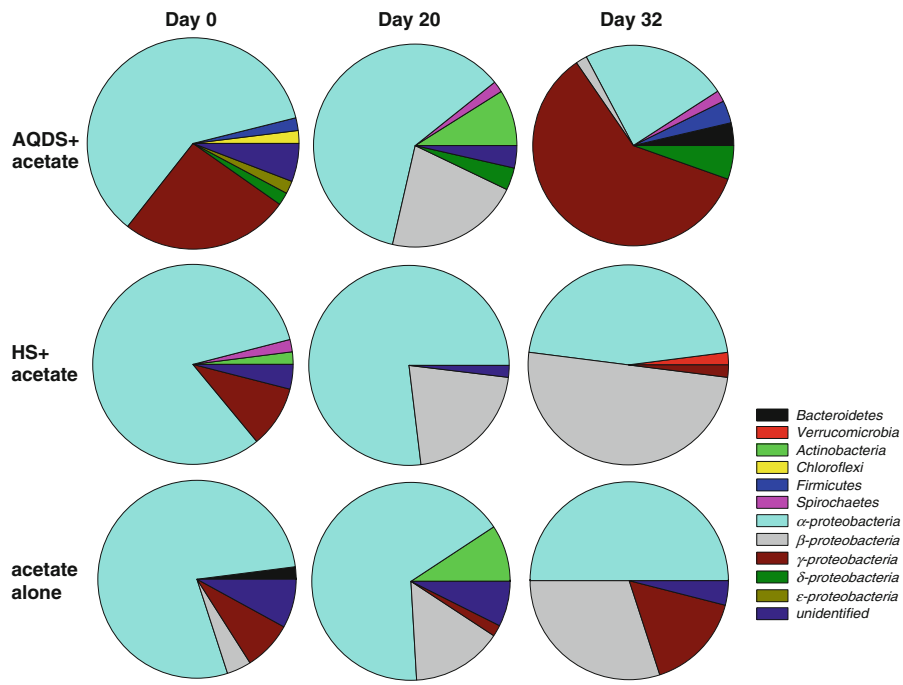

Fig. 4 Temporal variation of microbial community composition in contaminated aquifer material from the Picatinny Arsenal that has been amended with 10 mM acetate plus the electron shuttles AQDS (100 μM) or purified humic substances (HS) (0.15 g/l), or which had only been amended with the electron donor acetate (10 mM). The charts represent the relative proportion of major phyla recovered during the sampling periods

HS plus acetate incubations, and 78–50% in acetate alone incubations. Most of the clones identified in the current study were similar to *Ochrobactrum anthropi* strain CCUG 43892 with more than 99% similarity. The genus *Ochrobactrum* is not reported to reduce ferrihydrite with acetate (Zuo et al. 2008).

In contrast to the sequences at day 0, 16S rRNA gene sequences at day 32 were highly similar to known Fe(III)-reducing microorganisms. AQDS-amended microbial communities included *Pseudomonas* and *Geobacteraceae* that increased from 29% to 67%; with *Pseudomonas* relatives more prevalent than *Geobacteraceae*. In the presence of HS, 52% of sequences at day 32 were identified as *β-proteobacteria* (families *Oxalobacteraceae*). Although *Oxalobacteraceae* isolates are not known Fe(III) reducers, phylotypes similar to these have been recovered in uranium contaminated material during stimulated Fe(III) reduction (Akob et al. 2007, 2008; Li and Krumholz 2008; Reardon et al. 2004). Nitrate was depleted by day 6; therefore, it could not be the terminal electron acceptor promoting microbial activity at day 32. Sequences in acetate-alone incubations were 30% related to *β-proteobacteria* (families *Oxalobacteraceae* and *Comamonadaceae*) and 8% related to *γ-proteobacteria* (Genus *Pseudomonas*).

Pseudomonas and *Geobacteraceae* in AQDS plus acetate incubations were not unexpected because both genera contain well known dissimilatory Fe(III) reducers (Lovley 1997; Lu et al. 2002). However, previous reports would have us predict more presence of phylotypes within the *Geobacteraceae* (Holmes et al. 2002; Snoeyenbos-West et al. 2000). These data suggest that it is unlikely a single group of organisms (*δ-proteobacteria*) can become dominant in every Fe(III)-reducing environment. In this case the *β-proteobacteria* and *γ-proteobacteria* were dominant phylotypes relative to the *δ-proteobacteria*.

Similar *β-proteobacteria* have been enriched in alternate Fe(III)-reducing conditions such as in ethanol and glucose amended nitrate-reducing and Fe(III)-reducing MPN cultures (Akob et al. 2008), in uranium contaminated subsurface sediments (Akob et al. 2007), in enrichment culture of freshwater wetland sediment microorganisms (Weber et al. 2006), and in a specular hematite (Fe_2O_3) medium (Reardon et al. 2004). In particular, Weber et al. (2006) showed that *β-proteobacteria* increase from 3 to 60% when experimental condition shifted from nitrate-reducing to Fe(III)-reducing conditions. Reardon et al. (2004) also reported that the majority of hematite-associated community formed in the pristine area was affiliated with *β-proteobacteria*, and one of the clones was designated as S-E105, which was 98% similar to clones PTA-29 and PTA-31 in the current study (Fig. 5).

β-proteobacteria were also enriched in Tc(VII)-reducing sediments in which Tc(VII) was reduced concurrently with Fe(III) after nitrate was depleted (Li and Krumholz 2008). Li and Krumholz described several clones (M0C22 and M20C4) that proliferated during metal reduction, and both were 98% similar to clones PTA-29 and PTA-31 in the current study (Fig. 5). The clones identified in this study were enriched during Fe(III) reduction (after nitrate reduction and prior to sulfate reduction). Clone PTA-29 was also 97% similar to *Herbaspirillum* sp. K1 (family *Oxalobacteraceae*) which is a facultative bacterium isolated from contaminated groundwater that degrades tetrachlorophenol (Männistö et al. 2001).

Different microbial communities were enriched when AQDS versus HS was used (Fig. 4). At day 20, microbial communities in AQDS-amended incubations were becoming enriched by *γ-proteobacteria* (Genus *Pseudomonas*), while those in HS-amended incubations were *β-proteobacteria* (families *Oxalobacteraceae* and *Comamonadaceae*). However, at day 32 the *γ-proteobacteria* (Genus *Pseudomonas*) and the *β-proteobacteria* (families *Oxalobacteraceae* and *Comamonadaceae*) were dominant in the AQDS and humic amended incubations, respectively. Since many Fe(III)-reducing microorganisms can also reduce humic substances (Coates et al. 1998; Kwon and Finneran 2008b; Lovley et al. 1998), *γ-proteobacteria* and *β-proteobacteria* enriched from AQDS and HS amended incubations, respectively, may utilize both electron shuttling compounds and Fe(III) (directly) as electron acceptors. However, the differences in molecular structure between AQDS (e.g., low molecular weight, simple functional group) and humics (e.g., high molecular weight and multiple functional group) enriched different microorganisms.

Biological NDAB production by Fe(III)-reducing microorganisms

NDAB increased to approximately 10 μM in electron shuttle-amended incubations and remained stable for

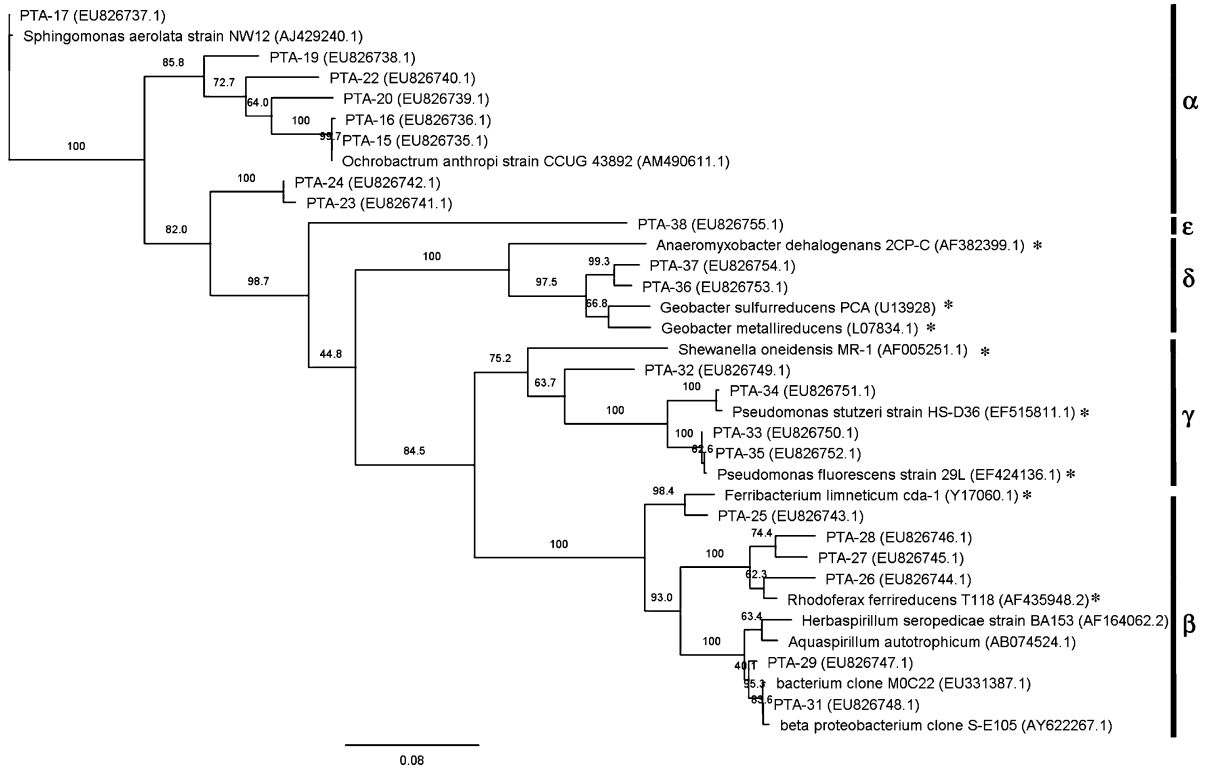

Fig. 5 Phylogenetic tree of proteobacteria based on 16S rRNA gene sequence cloned from aquifer sediment incubations. Branch points were supported by Jukes-Cantor distance and maximum-likelihood methods. (*) indicates known Fe(III) reducing microorganisms. The scale bar indicates 0.08 changes per nucleotide position

the rest of the sampling time (Fig. 1e). As stated above the previous reports of biological NDAB production were strictly aerobic. Zhao et al. reported that NDAB formation from RDX via MNX by *Clostridium bifermentans* HAW-1 was due to an *abiotic reaction*—hydrolysis of MNX, not biological activity (Zhao et al. 2003). Data in Table 1 demonstrate that NDAB was produced biologically from RDX (with MNX as an intermediate), which was tested further using *Shewanella oneidensis* MR1.

We postulated that Fe(III) reducers were responsible for NDAB production under these strictly anoxic conditions. In order to test this, NDAB production from several past experiments (all anoxic systems) was compared (Table 1). NDAB has not been detected in any of the strictly abiotic systems tested. Reduced AQDS (AH_2QDS), surface bound Fe(II), and sterilized sediment incubations did not generate NDAB during RDX degradation. NDAB was only detected in experimental bottles with microbial activity, regardless of the presence or absence of electron shuttling compounds (Table 1).

Previous cell suspensions of *Geobacter metallireducens* and *Shewanella oneidensis* also generated NDAB (Table 1). NDAB (1–5 μM) was produced only when cells were present, irrespective of the presence of electron shuttles. Hydroquinones (AH_2QDS) and reduced humic substances alone did not generate NDAB. In contrast, cell suspensions of an isolate from the sediment used in this study, *Desulfotomaculum* strain MJ1, did not generate NDAB despite rapid RDX transformation (data not shown). *G. metallireducens* and *S. oneidensis* are Fe(III)-reducing microorganisms, whereas strain MJ1 is an obligate fermenter. This suggests that Fe(III) reduction is correlated with biological NDAB formation.

We used resting cell suspensions of *S. oneidensis* to verify whether MNX was further converted to NDAB and if living cells were necessary (Fig. 6). *S.*

Table 1 Summary of NDAB production from current and past experiments

	Abiotic						RDX confidential site #2 (7.5)			
	AH2QDS (6.8)	AH2QDS (7.9)	AH2QDS (9.2)	Fe(II) + FeGel	Fe(II)	FeGel	AQDS + Ac	Ac	Unamended	Sterilized
Starting RDX conc. (μM)	37.8 ± 0.8	37.8 ± 2.7	30.3 ± 0.2	33.9 ± 1.3	35.4 ± 0.1	35.8 ± 0.1	35.8 ± 0.2	46.0 ± 0.5	46.5 ± 0.6	46.0 ± 0.4
Final RDX conc. (μM)	13.9 ± 0.1	1.3 ± 0.1	0.0 ± 0.0	23.0 ± 0.6	34.4 ± 0.3	35.6 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	22.2 ± 2.9	35.1 ± 1.7
% RDX reduced	63.2	96.6	100	32.2	2.8	0.6	100	100	52.3	23.7
Experimental time (h)	22	12.5	11.5	85	85	85	59 d	59 d	59 d	59 d
Final MEDINA conc. (μM)	3.9 ± 0.0	18.2 ± 0.4	34.2 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Final NDAB conc. (μM)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.4 ± 1.5	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
Max. NDAB measured (μM)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.4 ± 1.5	0.5 ± 0.0	0.4 ± 0.0	0.0 ± 0.0
Time at max. NDAB produced (h)	NA	NA	NA	NA	NA	NA	59 d	11 d	11 d	NA

	GS15						MR1 (6.8)		MJ1 (6.8)	
	AQDS + Ac (6.8)	AQDS + Ac (7.9)	AQDS + Ac (9.2)	Ac (6.8)	Ac (7.9)	Ac (9.2)	AQDS + Lc	Lc	AQDS + Ac	Ac
Starting RDX conc. (μM)	65.2 ± 0.5	63.4 ± 0.9	61.5 ± 0.4	65.5 ± 0.7	66.0 ± 0.6	62.0 ± 0.9	61.8 ± 0.5	67.6 ± 2.0	61.8 ± 0.5	64.4 ± 0.1
Final RDX conc.(μM)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	42.1 ± 1.6	3.1 ± 0.8	55.0 ± 0.5	0.0 ± 0.0	0.0 ± 0.0
% RDX reduced	100	100	100	100	100	32.1	95	18.6	100	100
Experimental time (h)	40	40	32	40	40	32	42	42	21	21
MEDINA produced (μM)	0.4 ± 0.0	14.7 ± 0.2	33.7 ± 0.6	0.6 ± 0.0	15.7 ± 0.3	5.2 ± 0.3	55.2 ± 4.3	6.6 ± 1.5	4.7 ± 0.2	4.9 ± 0.2
Final NDAB conc. (μM)	0.6 ± 0.0	0.9 ± 0.0	1.2 ± 0.0	0.9 ± 0.1	2.4 ± 0.0	1.4 ± 0.1	1.1 ± 0.3	4.4 ± 0.3	0	0
Max. NDAB measured (μM)	1.3 ± 0.0	1.4 ± 0.1	1.4 ± 0.0	1.6 ± 0.0	2.7 ± 0.3	1.4 ± 0.1	1.7 ± 0.2	4.4 ± 0.3	0	0
Time at max. NDAB produced (h)	6	21	9.5	6	21	32	2	42	NA	NA

These data are a catalog of NDAB production in experiments performed during one and half years of investigating RDX transformation in Picatinny aquifer material. The data include in situ experiments with Picatinny sediment, alternate site sediment (confidential site), and several pure cultures with corresponding abiotic controls. The purpose of this data analysis was to determine if NDAB had been identified in experiments with and/or without biological activity under anoxic conditions

oneidensis was selected because past data with RDX indicate that it produces more NDAB than *G. metallireducens*. *S. oneidensis* produced approximately 6–7 μM of NDAB from MNX degradation regardless of the presence or absence of AQDS or lactate (Fig. 6a and b). *S. oneidensis* reduced MNX without lactate because the cells utilize decayed cellular biomass as a substrate for respiratory processes (i.e. endogenous respiration), which has been reported in previous studies (Fredrickson et al. 2000; Kwon and Finneran 2008b). Abiotic MNX degradation by AH_2QDS produced a stoichiometric mass of HCHO (1 MNX → 3 HCHO) (Fig. 6c), but did not produce NDAB (Fig. 6b). The rate and extent of AH_2QDS-mediated MNX reduction were faster at the higher pH (8.2) than the lower pH (7.2) which was consistent with the previous results with hydroquinones (Kwon and Finneran 2008a). These sediment and cell suspension data demonstrate that NDAB production is due to anaerobic, biological RDX transformation, with MNX as a key intermediate (Fig. 7).

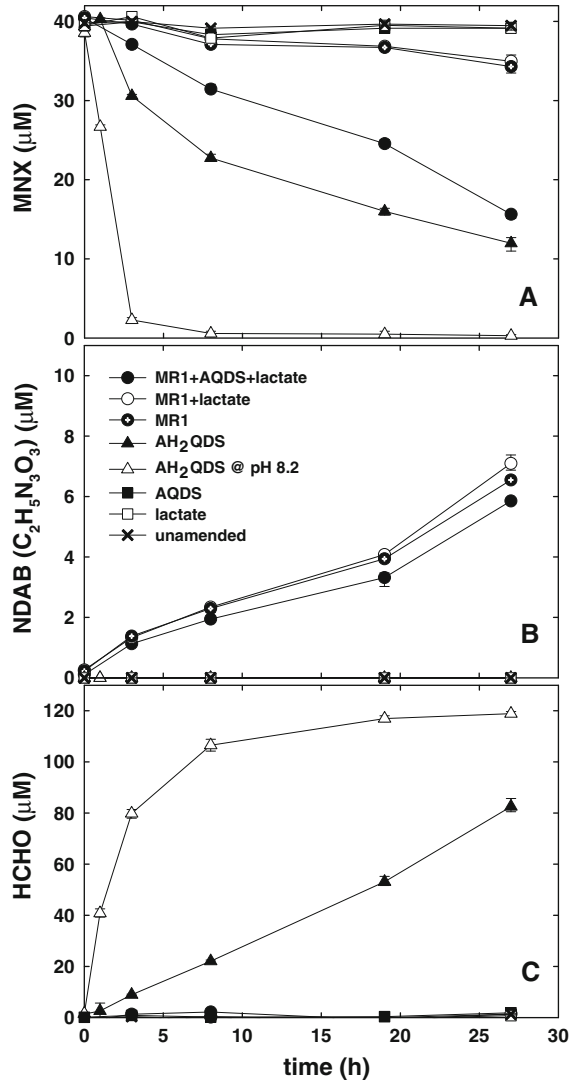

Fig. 6 MNX reduction (**a**) and the production of NDAB (**b**) and HCHO (**c**) in the resting cell suspensions of *Shewanella oneidensis* MR1 that has been amended with the electron shuttles AQDS (100 μM), or which has only been amended with the electron donor lactate (10 mM). Control experiments were performed with AH_2QDS at pH 7.2 and 8.2 or with AQDS alone or lactate alone. Results are the means of triplicate analyses and bars indicate one standard deviation

Environmental relevance

The data suggest that RDX degrades via a variety of intermediates but is ultimately mineralized more quickly and completely with electron shuttling compounds. Adding AQDS or HS prevented accumulation of the nitroso metabolites relative to electron donor alone; *substrate addition* has been the primary strategy for RDX to date. The ring cleavage metabolite NDAB increased with electron shuttles but not in other incubations, indicating that it is a critical carbon intermediate during Fe(III) reduction. These new NDAB data indicate that we cannot assume aerobic conditions when NDAB is quantified in situ, nor can we assume that it is forming abiotically in the absence of oxygen. When correlated to increases in Fe(II) or increases in Fe(III) reducer biomass, the presence of NDAB may be an additional indication of Fe(III)-reducer mediated RDX transformation.

Fe(III) reduction was the dominant "electron accepting process" associated with RDX reduction. Nitrate was depleted approximately 10 days before the onset of RDX reduction in the electron shuttle amended incubations, and sulfate was not reduced (nor was sulfide present) in any of the incubations tested. Targeting Fe(III) and/or electron shuttle reduction opens up a major group of indigenous microorganisms to stimulate these reactions. Fe(III)- and humic-reducing microorganisms are ubiquitous (Coates et al. 1998); therefore, the strategy using electron shuttles and Fe(III)- and humic-reducing microorganisms may work at many contaminated sites.

Finally, Fe(III) reduction in this study was driven by organisms primarily within the *β*- and *γ-proteobacteria*.

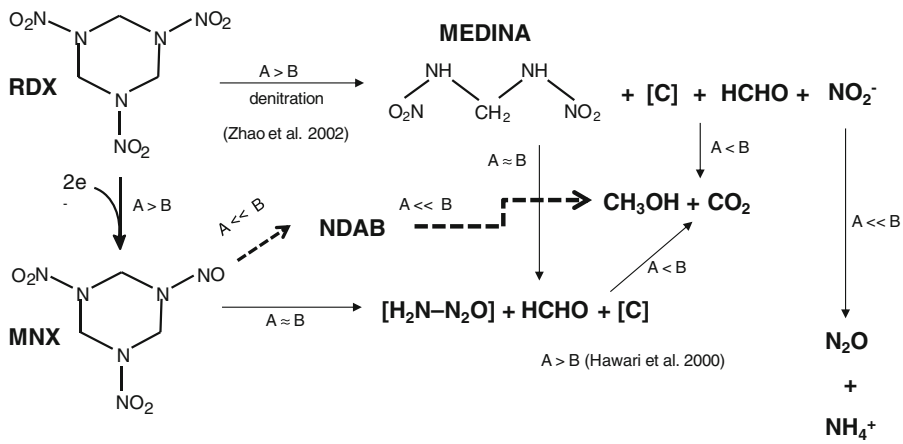

Fig. 7 Probable RDX degradation routes by mixed abiotic-biological pathways based on products identified and reported degradation pathways (references listed on figure) in the presence of AQDS and *G. metallireducens*. The pathway specifically highlights NDAB as a biological intermediate, rather than an abiotic intermediate, under the anoxic/Fe(III) reducing conditions tested. 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

This is vastly different from past reports which suggested that *δ-proteobacteria* are most dominant during Fe(III) reduction, and we intend to follow up by investigating Fe(III) and electron shuttle reduction within genera that were not previously tested for Fe(III)/shuttle reduction, but which clearly were important to Fe(III) reduction in situ.

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