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Biodegradation of RDX by Stimulating Humic Substance- and Fe(III) - Reduction

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The project demonstrated that extracellular electron shuttling compounds and Fe(III)reduction are critical microbially-mediated processes for the biodegradation of the cyclic nitramine compound RDX. Electron shuttles are compounds that promote electron transfer between cells and alternate, extracellular electron acceptors. All electron shuttles increase the rate and extent of Fe(III)reduction; however, until this study, it was unknown whether these molecules could also stimulate RDX reduction and subsequent biodegradation steps. In addition to the electron shuttling data, this study demonstrated that Fe(III) reduction and Fe(III)reducing microorganisms are most likely the dominant process and microbial community, respectively, for complete degradation of RDX in contaminated environments. The project was the first to demonstrate electron shuttle mediated RDX and HMX biodegradation and this strategy serves as the foundation for the future development of technology for the in situ or ex situ biodegradation of RDX.

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Acronyms

AQDS	Anthraquinone-2,6-disulfonate
ATCC	American Type Culture Collection
B-AH_2QDS	Biologically reduced anthraquinone-2,6-disulfonate
C-AH_2QDS	Chemically reduced anthraquinone-2,6-disulfonate
DNX	Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine
DoD	Department of Defense
ESTCP	Environmental Security Technology Certification Program
FZ	Ferrozine
GAC	Granulated activated carbon
GC	Gas chromatography
HMX	Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
HPLC	High-performance liquid chromatography
HS	Humic substances
IHSS	International Humic Substance Society
LAP	Load-Assembly-Package
MNX	Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine
MTBE	Methyl tert butyl ether
NPL	National Priority List
O&M	Operation and maintenance
PDA	Photodiode array
PRB	Permeable reactive barrier
PTFE	Polytetrafluoroethylene
RDX	Hexahydro-1,3,5-trinitro-1,3,5-triazine
RGD	Radiolabeled gas detector
SERDP	Strategic Environmental Research and Development Program
TBA	Tert butyl alcohol
TCD	Thermal conductivity detector
TEAP	Terminal electron accepting process
TNT	Trinitrotoluene
TNX	Hexahydro-1,3,5-trinitroso-1,3,5-triazine
ZVI	Zero-valent iron

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V. Executive Summary

This project demonstrated that extracellular electron shuttling compounds and Fe(III) reduction are critical microbially-mediated processes in the biodegradation of the cyclic nitramine compound hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Electron shuttles are compounds that promote electron transfer between cells and alternate extracellular electron acceptors. All electron shuttles increase the rate and extent of Fe(III) reduction; however, until this study it was unknown whether these molecules could also stimulate RDX reduction and subsequent biodegradation steps. In addition to the electron shuttling data, this study demonstrated that Fe(III) reduction and Fe(III)-reducing microorganisms are most likely the dominant process and microbial community, respectively, for complete degradation of RDX in contaminated environments. Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) was also investigated in less detail, but the reactions identified with RDX were also applicable to HMX, suggesting that this strategy will treat cyclic nitramines in general. This project is the first to demonstrate electron shuttle mediated RDX and HMX biodegradation, and this strategy is the basis for future development of technology for in situ or reactor-based RDX or HMX treatment.

Activities that were completed included all major tasks within the original scope of work, and all milestones associated with the individual tasks were met. The primary findings of the project during the last year of work include the points below. First, electron shuttle mediated RDX biodegradation was more rapid than previously described microbial or chemical processes. Second, biodegradation was more complete in the presence of electron shuttles; there was less formation of toxic nitroso or ring-cleavage metabolites and more formaldehyde generation, which is the precursor to mineralization in mixed systems. Cells alone or Fe(II) alone did not generate comparable amounts of formaldehyde. Third, all Fe(III)-reducing genera that can be considered “model” Fe(III) reducers promoted these reactions, albeit at different rates and to a different extent. Finally, the results were confirmed using contaminated aquifer sediment and the data demonstrated that RDX degradation was stimulated in humics-poor sediment when electron shuttles were added, but sediment with high native humics content had an innate capacity for RDX biodegradation.

The work lays a foundation for a bioremediation strategy for RDX and possibly other explosives that are relevant to the DoD. Electron shuttles are compounds that can be added in situ using groundwater wells or using permeable reactive barriers. This strategy may also have utility in ex situ treatment (e.g. biologically active granular activated carbon or reactors specifically amended with humics rich organic carbon) but these applications were not investigated under the scope of this study. This report details the importance, relevance, and scope of this work and the completion of milestones associated with the original proposal.

VI. Objective

The broad objective of the research project was to characterize the role of Fe(III) reduction, extracellular electron shuttling, and the organisms that mediate these processes in cyclic nitramine biodegradation. The project was a basic science investigation, designed to identify the factors that influence RDX (and HMX within some experiments) degradation in anaerobic, subsurface aquifer material. The results will be used to design in situ or ex situ bioremediation strategies predicated on extracellular electron transfer and Fe(III)-reducing microbial physiology.

The specific technical objectives of this research project were to:

1. Demonstrate that humic substances or alternate electron shuttling compounds added to RDX-contaminated aquifer material will stimulate Fe(III) reduction and promote RDX reduction;

2. Determine if humic substances (natural and synthetic) will transfer electrons directly to RDX;

3. Quantify the rate of RDX reduction catalyzed by Fe(II) versus reduced humic substances; and

4. Identify the dominant microbial community associated with humics and Fe(III) mediated RDX reduction.

The experiments performed during the project tested the following hypotheses:

1. Adding humic substances to nitroaromatic contaminated aquifer material will stimulate Fe(III) reduction and the Fe(II) produced will consequently transfer electrons to RDX;

2. Reduced humic substances will transfer electrons directly to RDX in aquifer material;

3. Direct electron transfer from humic substances reduces RDX faster than electron transfer from reduced humics to Fe(III) → Fe(II) to RDX;

4. Synthetic humic substances will reduce RDX as effectively as natural, purified humic substances; and,

5. *Geobacteraceae* will be enriched in situ during humics mediated RDX reduction.

VII. Background

The Department of Defense (DoD) has identified a need to develop technologies for the treatment of nitroaromatic and cyclic nitramine compounds in groundwater. Both compound classes are used in the manufacturing of explosives and munitions (17, 61). These compounds have been released to the environment through a number of routes, including live fire training and manufacturing. As a result, these classes of compounds have been identified as soil and groundwater contaminants at DoD facilities and include the nitroaromatic trinitrotoluene (TNT), and cyclic nitramines including hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX).

During a recent Navy database search, 117 ordnance-impacted sites at 40 different U.S. Navy facilities were identified, while at least a dozen U.S. Army facilities have documented RDX and/or TNT groundwater impacts. These compounds are cyclic, nitrogen-containing structures, and are moderately resistant to aerobic biodegradation (5, 61). They will biodegrade aerobically in the presence of specific microorganisms, but specialized enrichment conditions are required for these organisms to proliferate (5). In addition, the subsurface environment impacted by nitroaromatic or cyclic nitramine compounds may be anaerobic under natural conditions or affected by co-contaminants that promote anaerobic conditions. The residues of these compounds are somewhat soluble and toxic (17); therefore, efficient strategies to degrade the cyclic nitramines are required for the safety of human health and the environment.

Few cost-effective technologies exist for the treatment of cyclic nitramine compounds in groundwater. Groundwater pump-and-treat systems, while often controlling the migration of contaminant plumes, are typically inefficient for remediation of groundwater, largely because they do not control the source of contamination and because of the large volumes of groundwater that must be treated to achieve both hydraulic control and regulatory compliance (1). In addition, these strategies remove the contaminated groundwater and transfer the contaminants to another medium rather than degrading the contaminants. Permeable reactive barriers (PRB) employing zero-valent-iron (ZVI) show promise in treating RDX and/or HMX, but PRB installation is technically and economically infeasible at sites with deep or wide plumes. Aerobic bioremediation is possible in shallow soil or groundwater that has sufficient oxygen, but is technically difficult in groundwater that has become anaerobic.

The contamination of groundwater, particularly drinking water supply aquifers, with residues of explosives has become a significant problem for the DoD. This condition can occur at ordinance production facilities, arsenals, depots, and live-fire training facilities. In the latter case, control of explosives in groundwater is a critical issue for the continuation of range operations in some areas. The long-term operation of pump-and-treat systems requires significant operation and maintenance (O&M) expenses that may be incurred for decades.

Humic substances are a class of organic compounds that result from the incomplete breakdown of complex organic matter in soil, sediment, surface water, and groundwater (46, 47). They are naturally occurring compounds with no defined molecular formulae or structures other than that they are generally classified as low molecular weight or high molecular weight (45, 48). Low molecular weight humic substances are water-soluble and bind less to subsurface organic

matter or matrices; high molecular weight humic substances bind to subsurface matrices and are not mobile (22, 25). Humic substances can bind smaller molecules, both metals and organic compounds, as chelators (22, 31). However, the most significant property of humic substances with regard to the proposed research is the abundance of quinone moieties present on all humic substance molecules (52, 53).

Quinones are redox active molecules that cycle between oxidized and reduced forms in biological systems such as the electron transport chain of both prokaryotes and eukaryotes (7, 52). Therefore, quinones will accept electrons from lower redox potential electron carriers and transfer the electrons to higher redox potential electron acceptors. These electron transfer reactions can be biological or chemical (52). Recent data indicate that certain Fe(III)-reducing bacteria produce extracellular quinones to transfer electrons to Fe(III) in the terminal respiratory step (20, 46, 49, 50, 55). This indicates that extracellular electron shuttling is an environmentally important process for anaerobic microorganisms.

Several studies to date demonstrate that humic substances serve as electron acceptors in anaerobic, microbial respiration (6, 9, 10, 12, 25, 33). Microorganisms actually conserve energy and grow by respiring humic compounds (33). The humic substances can either be the terminal electron acceptor in the respiratory pathway, or another electron carrier in the electron transfer pathway. In the latter case, the phenomenon is referred to as electron shuttling (34). The humic substances serve as the terminal electron acceptor for the microbial electron transport chain, the humic substance is released from the cell membrane, and the soluble, reduced humic abiotically transfers the electrons to a terminal acceptor – often Fe(III) in subsurface environments.

Electron shuttling increases the bioavailability of metals such as Fe(III) hydroxides. Fe(III) is not freely soluble in subsurface environments at neutral pH; Fe(III) hydroxides (solid ferric iron) typically dominate in these environments (28). Anaerobic microorganisms must physically contact these solids in order to transfer electrons to the Fe(III) (24), which may be occluded from the bacterial cells. Humic substances facilitate Fe(III) reduction by accepting electrons in microbial respiration and abiotically transferring the electrons to Fe(III) (34). The humics are then re-oxidized and available again for microbial respiration. In this manner the humics are catalytic and a small concentration of humic substances stimulates Fe(III) reduction (52, 53). Humic substances are reported to shuttle electrons in this manner to other metals including Mn(IV) and U(VI) (18, 35).

Electron shuttling has applications in bioremediation of organic and metal contaminants in anaerobic, subsurface environments. Several organic compounds are oxidized to CO_2 under Fe(III)-reducing conditions including benzene, toluene, ethylbenzene, methyl tert-butyl ether (MTBE), and tert butyl alcohol (TBA) (14-16). Humic substances added to laboratory incubations increased the rate and extent of contaminant oxidation as Fe(III) reduction increased (14, 42, 43). Uranium (VI) and chromium (VI) are also reported to accept electrons from reduced humic substances (18, 54). These metals are soluble when oxidized and precipitate as less toxic forms when reduced to U(IV) and Cr(III), respectively. In one series of laboratory incubations humic substances did not directly stimulate U(VI) reduction, but the humics did stimulate Fe(III) reduction; the Fe(III)-reducing microorganisms consequently reduced U(VI) to U(IV). Therefore, both direct and indirect pathways may achieve remediation of metals.

Most humics-reducing microorganisms that have been isolated in pure culture, developed in enrichment culture, or identified by molecular analyses, also reduce Fe(III) when it is available for growth (12, 37). Humics-reducing microorganisms are closely related to known Fe(III) reducers, and humics reduction is generally associated with Fe(III) reduction (27). Humics- and Fe(III)-reducing microorganisms are ubiquitous and have been identified in shallow and deep subsurface environments, freshwater and marine sediment, soil, and extreme environments such as volcanic geologic material, deep hydrothermal vents, and permafrost (10). The microorganisms that catalyze these reactions represent Bacteria and Archaea from numerous genera and species (32, 33). This spatial ubiquity and phylogenetic diversity means that bioremediation strategies that rely upon humics and Fe(III) reduction are likely to be successful in any environmental setting.

Acetate is the primary electron donor for Fe(III) and humics reduction in pristine aquifer material (26, 40, 59). In contaminated aquifer material, numerous compounds donate electrons to Fe(III) and humics reduction, but acetate is often an intermediate in the breakdown of more complex organic matter, including contaminants (60). Therefore acetate selects for Fe(III) and humics reduction as do high concentrations of Fe(III) or added humic substances (11). Although other electron donors will promote Fe(III) and humics reduction, data from previous studies demonstrate that acetate narrowly targets microorganisms most closely related to the dominant Fe(III)-reducing Bacteria (56). Acetate has also been reported to promote faster Fe(III) and humics reduction than lactate, formate, or benzoate, which are also intermediates in the breakdown of complex organic matter (11).

Cyclic nitramines, like metals and chlorinated organic compounds, can accept electrons from anaerobic, microbial respiration (2). The nitro functional groups on compounds such as RDX and HMX are electronegative and withdraw electrons from reduced electron carriers or reduced geochemical compounds (2), which is depicted in Figure 1 below. Research to date demonstrates that specific microorganisms catalyze direct RDX or HMX reduction in situ, but the process may be inefficient depending on the initial microbial community.

RDX and HMX will accept electrons from reduced iron, both soluble Fe(II) and magnetite, a mixed Fe(III)/Fe(II) mineral, or Fe(II) adsorbed to the surface of solid iron mineral species (2). Magnetite is a primary end product of Fe(III) reduced by microorganisms within the family *Geobacteraceae* (41, 58). Recent data indicate that biogenic magnetite reduced RDX in pure culture studies (2). The cell suspensions were buffered with bicarbonate, so the pH was balanced for optimum microbial activity (2). The data were promising because *Geobacteraceae* are reported to develop during Fe(III) reduction in several subsurface environments (21, 51, 56). The cell density will be lower in situ, but the concentrations of all compounds, including the cyclic nitramine compounds, will also be lower, and fewer cells may be required to catalyze the reactions. However, Fe(III)- and humics-dependent RDX biodegradation had not been researched under environmentally relevant conditions to that point. The data presented herein are the first to specifically develop an in situ bioremediation strategy focused on electron transfer from reduced humics and Fe(II).

Bioremediation of RDX and HMX to date has focused on strategies similar to those used for chlorinated organic compound (e.g., trichloroethene) remediation. The technology relies upon stimulating a single genus, sometime a single species, of microorganism by adding soluble

substrates (electron donors) to aquifer material. Unlike the dominance of *Dehalococcoides* species in chlorinated solvent remediation; specific RDX-degrading microbial populations are often small, inefficient, and easily out-competed by other microbial communities. The proposed research relies upon stimulating what is arguably the most dominant microbial community in all anaerobic subsurface environments – Fe(III) reducing microorganisms (29). As stated and referenced above, the known Fe(III) reducers also reduce endogenous or exogenous electron shuttling compounds. Unlike targeting a very narrow microbial niche (direct nitro-respiration), this strategy broadly selects for microorganisms that will not be out-competed for available electron donor when sufficient concentrations of Fe(III), Fe(III) plus humic substances, or humic substances alone are available. Fe(III) is often the most prevalent anaerobic electron acceptor in subsurface environments. Therefore, if Fe(III)- and humics-reducers can promote RDX and HMX reduction in situ, this strategy could be widely applied at contaminated sites consistent with the goals of the SERDP and ESTCP programs.

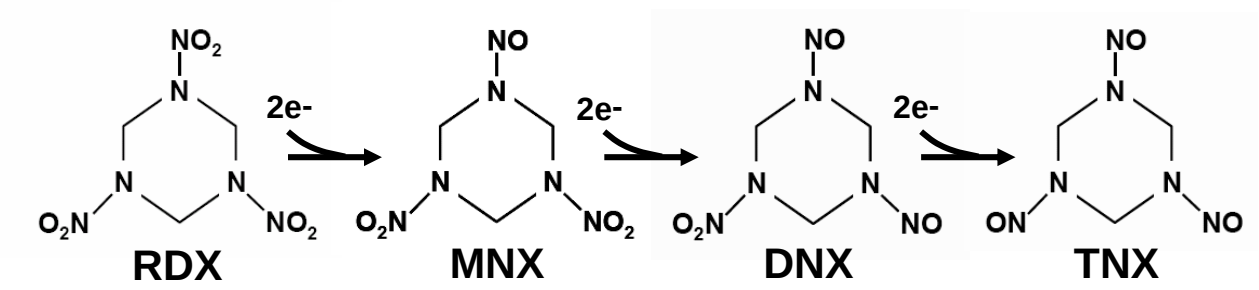

Figure 1. The structure of hexahydro-1,3,5-trinitro-1,3,5-triazne (RDX) and its nitroso metabolites (MNX: hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine, DNX: hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine, TNX: hexahydro-1,3,5-trinitroso-1,3,5-triazine); RDX reduction consists of a series of two-electron transfer steps for each nitro → nitroso reaction.

VIII. Materials and Methods

Sediment and Aquifer Material Sample Collection and Storage

Two types of explosive contaminated material were collected; aquifer sediment and shallow sediment. 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. Anaerobic sediment was homogenized in a N_2-filled glove bag prior to processing for individual experiments. Using a monitoring well installed in the same area from which the sediment was collected, groundwater samples were collected. The samples were collected in 1L glass bottles that were then sealed without headspace. Groundwater samples were stored at 4ºC until processing.

The shallow sediment samples were collected from the unsaturated zone of Joliet Army Ammunition Plant Load-Assembly-Package (LAP) Area [National Priority List (NPL) site], located in Will County, Illinois. The shallow sediment samples were stored at 18ºC before experiments were conducted. The particle size distribution in the soil was 20% gravel (>2 mm), 10% sand (50 μm- 2 mm), 10% silts (2-50 μm) and 60% clays (<2 μm).

Characterization of Contaminated Environmental Samples

Samples were analyzed to determine the dominant terminal electron accepting process (TEAP) using a mixture of solid material and water; both aquifer material and shallow sediment were analyzed in the same manner. Total bioavailable iron (ferric and ferrous) was analyzed using the Ferrozine assay described below. Soluble anion electron acceptors (nitrate and sulfate) and their respective products were measured using ion chromatography as described below. The pH was measured in water and/or porewater using a standard pH probe. Finally, the dominant metabolic state of the material was quantified using the 2-[^{14}C]-acetate assay (30). Briefly, this assay is performed by the addition of radiolabeled 2-[^{14}C]-acetate to sediment incubations under native conditions (no additional stimulatory amendments). One series of incubations is amended with sodium molybdate, which specifically inhibits sulfate reduction. The $^{14}CO_2$ and $^{14}CH_4$ are quantified over time, and the relative extent of $^{14}CO_2$ versus $^{14}CH_4$ in the presence and absence of molybdate indicates the dominant TEAP at the time of collection.

Batch Aquifer Material or Sediment Incubations

Approximately 50 g of sediment and 20 ml of groundwater were dispensed into 160 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 80:20 (vol/vol) N_2:CO_2 that had been passed over hot copper filings to remove traces of oxygen. All sediments were incubated in the dark at 18ºC without agitation. Approximately 60 μM 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. No RDX and HMX were added to the shallow sediment incubations. Each bottle had an overlying aqueous layer without forming a slurry. Each headspace was flushed with anaerobic N_2 gas. All amendments were made from sterile, anaerobic stock solutions. Acetate was added as a sole electron donor at a final concentration of 2 mM. Acetate was added to individual culture vessels using an anaerobic, aseptic technique following sterilization. Bottle headspaces were flushed with the same anaerobic gas and sealed under an anaerobic headspace using a thick butyl rubber stopper fastened with an aluminum crimp. All subsequent amendments or transfers were made using sterile needles and syringes that had been flushed with anaerobic gas.

Electron acceptors/shuttles used with the sediments included humic substances (HS) (0.05 g/L – 0.25 g/L) and anthraquinone-2,6-disulfonate (AQDS) (0.1 mM). AQDS (5 mM) and HS (10 g/L) were prepared in 30 mM bicarbonate buffer and dispensed into a 160mL anaerobic serum bottle. The buffered AQDS and HS were sparged with anaerobic N_2:CO_2 gas and sealed until processing. The aquifer sediments were tested following initial aeration (to oxidize native Fe(II) to Fe(III)) and without aeration. For initial aeration, the aquifer sediments were aerated with ambient air for 30 minutes and capped with a rubber stopper and an aluminum cap. Then, each bottle was flushed with N_2 gas. Unsaturated sediment samples from Joliet were also initially aerated and then flushed with N_2 gas.

To generate abiotic controls, sediments were autoclaved for 1 h per day for 3 consecutive days (14). All liquid samples were taken with a sterile syringe and needle that had been flushed with anaerobic gas, and liquid samples were filtered through 0.2 μm PTFE filters (PALL Sciences) prior to analysis. Samples of sediment were removed from bottles under a flow of N_2 with a sterile metal spatula. Total bioavailable iron and aqueous Fe(II) were quantified for all incubations. All experiments were performed in triplicate and RDX and/or HMX was quantified at each time point.

Pure Cultures Utilized within the Investigation – Growth and Maintenance

Geobacter metallireducens strain GS-15 (ATCC 53774) was originally obtained from the University of Massachusetts at Amherst and maintained using the Ferric Citrate and AQDS media described below. *Anaeromyxobacter dehalogenans* strain K. (no ATCC number) and *Desulfitobacterium chlororespirans* strain Co23 (ATCC 700175) were provided by Dr. Robert Sanford, University of Illinois at Urbana Champaign. *Shewanella oneidensis* strain MR1 (ATCC 700550) was obtained from Dr. Joseph W. Stucki, University of Illinois at Urbana Champaign. *Geobacter metallireducens*, *Anaeromyxobacter dehalogenans*, and *Desulfitobacterium chlororespirans* were maintained using the Ferric Citrate and/or AQDS media, while *Shewanella oneidensis* was maintained on aerobic LB medium. Cultures were transferred to fresh medium prior to the start of any experiments. All media are described below.

The basal anaerobic medium (used for all cultures except *S. oneidensis*) 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.

Electron acceptors used with the anaerobic medium included soluble Fe(III) citrate (45 mM), poorly crystalline Fe(III) oxy(hydroxide) (50 mmol/L), or anthraquinone-2,6-disulfonate (AQDS – 5mM). The medium was buffered using a 30 mM bicarbonate buffer equilibrated with 80:20 (vol/vol) N_2:CO_2, as previously described (13). Culture tubes/bottles were sterilized by autoclaving at 121°C under pressure for 20 minutes. Yeast extract (0.01%) was added to the culture of *A. dehalogenans*. Acetate (20 mM) or lactate (20 mM) was added as the sole electron donor, depending on the specific culture. Yeast extract, acetate, and lactate were maintained as sterile, anaerobic, concentrated stock solutions and were added to individual culture vessels using anaerobic, aseptic technique following sterilization. Standard anaerobic and aseptic culturing techniques were used throughout (38).

Media were sparged with anaerobic gases passed over a heated, reduced copper column to remove trace oxygen from the gas line. Culture tube/bottle headspaces were flushed with the same gas mixture and sealed under an anaerobic headspace using a thick butyl rubber stopper fastened with an aluminum crimp. All subsequent amendments or transfers were made using sterile needles and syringes that had been flushed with anaerobic gas. Cultures were maintained in 160 mL anaerobic serum bottles; individual experiments utilized different culture tubes or bottles, as described below. The aerobic medium was an LB medium that consisted of Bacto tryptone (10.0), Bacto yeast extract (5.0), and NaCl (10.0). The medium was autoclaved prior to inoculation and cultures were transferred in 250-500 mL conical flasks. Individual experiments utilized different culture tubes or bottles, as described below.

Resting Cell Suspensions

G. metallireducens was grown anaerobically in freshwater medium with acetate as the sole electron donor and Fe(III) citrate as the sole terminal electron acceptor. *A. dehalogenans* was grown anaerobically in freshwater medium with acetate as the sole electron donor, Fe(III) citrate as the sole terminal electron acceptor, yeast extract (0.01%) as rich nutrients. *D. chlororespirans* was grown anaerobically in freshwater medium with lactate as the sole electron donor and AQDS as the sole terminal electron acceptor. *S. oneidensis* was grown aerobically in LB medium. One liter of each individual cell culture was harvested during logarithmic growth phase and centrifuged at 5000 rpm for 15 minutes to form a dense cell pellet. Each resultant cell pellet was resuspended in 30 mM bicarbonate buffer under a stream of anaerobic gas. The washed cells were centrifuged again at 5000 rpm for 15 minutes and the resultant biomass was resuspended in 4.0 mL of bicarbonate buffer. Cells were used within 30 minutes of processing.

Experimental tubes were prepared by sparging approximately 5.0 ml of 30 mM bicarbonate buffer with anaerobic N_2:CO_2 and sealing the buffer under an anaerobic headspace. Electron acceptors were amended from concentrated, anaerobic, sterile stock solutions. Electron acceptors incubated with the cells including humic acids (0.25 g/L), poorly crystalline Fe(III) oxide (45 mmol/L), AQDS (5 mM), humic acids plus poorly crystalline Fe(III) oxide, and AQDS plus poorly crystalline Fe(III) oxide. Cells were incubated at 30°C. An aliquot (0.3 ml) of the resting cells was added to the sealed pressure tubes to initiate each experiment.

Samples (0.3 ml) were collected periodically via anaerobic syringe and needle, and samples were filtered through sterile, 0.2 μm PTFE filters prior to analyses. 0.2 ml of the samples was used to quantify RDX or HMX at each time point. 0.1 ml of the samples was used to quantify Fe(II) concentration in poorly crystalline Fe(III) amended incubations. No more than seven samples were taken from any incubation; therefore, the final volume of each incubation was approximately 8 mL at the end of the experiments (80% volume remaining). Total cellular protein was determined by using the DC Protein Assay (Bio-RAD) and a modified Lowry protein assay (44). Cell protein normalized decay rates were calculated based on pseudo first-order degradation coefficients. All experiments were performed in triplicate.

Resting cell suspensions with Ferrozine. Two similar sets of incubations were prepared as described above. Electron acceptors incubated with the resting cell suspension of *G. metallireducens* including poorly crystalline Fe(III) oxide (10 mM) only, AQDS (0.1 mM) plus poorly crystalline Fe(III) oxide, humic acids (0.25 g/L) plus poorly crystalline Fe(III) oxide. RDX and HMX were amended at the concentration of 46 μM and 6 μM, respectively in the resting cell suspension of *G. metallireducens*. Ferrozine reagent (10 mM) as the Fe(II) chelator was added to one set of cell suspension incubations, while the other set was not amended with Ferrozine reagent.

*Mineralization study with resting cell suspension***.** Two sets of cell suspension incubations with different initial volume (20 mL and 10 mL) were prepared for metabolites analysis (nitroso metabolites, nitrous oxide, nitrite, and formaldehyde) and for $^{14}CO_2$ analysis, respectively. Hydrogen (ca. 7 mM) or acetate (20 mM) was amended as the sole electron donor. Electron acceptors incubated with the resting cell suspension of *G. metallireducens* including AQDS (0.1 mM) only, AQDS plus poorly crystalline Fe(III) oxide (10 mM), and cells only. Ferrozine reagent (10 mM) as the Fe(II) chelator was added to one set of AQDS plus poorly crystalline Fe(III) oxide amended incubations. Unlabeled RDX was amended at the concentration of 56 μM in the resting cell suspension. U-[^{14}C]-RDX was amended at the concentration for a final radioactivity of 22,000 dpm/ml. Nitrous oxide, $^{14}CO_2$, and $^{14}CH_4$ were monitored by analysis of headspace samples, 0.5 ml and 1 ml, respectively. A total of 0.6 ml liquid sample was collected at each time point for RDX and metabolites analysis; thus the volume difference between initial volume and final volume at the end of the experiments was 20%.

Pure Phase (Chemical) Suspensions

5 mM AQDS was prepared in 30 mM bicarbonate buffer and dispensed into a 160 mL anaerobic serum bottle. The buffered AQDS was sparged with anaerobic nitrogen gas and sealed until processing. Chemically reduced AQDS (C-AH_2QDS) was prepared by sparging the medium bottle with H_2:CO_2 (80:20, vol/vol) in the presence of palladium-covered alumina pellets as previously described (35). The chemically reduced AQDS was filtered through a 0.2 μm sterilized PTFE filter into a pre-sterilized, anaerobic serum bottle. 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 pre-sterilized, anaerobic serum bottle to remove cells. Biologically reduced Fe(II) was prepared in a similar manner using 45 mM Fe(III) citrate medium in lieu of AQDS medium. The final ferrous iron concentration was checked by Ferrozine assay using a spectrophotometer as described below.

Pure phase incubations of cell free buffer containing B-AH_2QDS, C-AH_2QDS, or reduced Fe(II) were preformed to determine the rate of RDX reduction catalyzed by these electron donors in the absence of microbial biomass. The concentrations of reduced AQDS (chemical or biological AH_2QDS) tested were 500, 300, and 100 μM for the incubations with RDX; 300, 100, and 50 μM for the incubations with hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX); 200, 100, and 50 μM for the incubations with hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX). Incubations were performed at 25°C. The starting RDX concentration was either 100 μM or 50 μM, depending on the specific experimental conditions.

Experiments were initiated by injecting RDX into the buffer with AH_2QDS. Samples were collected periodically via anaerobic syringe and needle; samples were filtered through sterile, 0.2 μm PTFE filters prior to analyses. RDX was quantified at each time point. AH_2QDS concentration was quantified for AH_2QDS/ AQDS incubations, Fe(II) was quantified for ferrous iron incubations. All experiments (except figures noted) were performed in triplicate.

Enrichment Culture Techniques

Sediment samples were suspended in previously described freshwater medium using anaerobic and aseptic techniques. The sediment suspensions were amended with various electron donor-electron acceptor pairs. The electron donors utilized included acetate (20 mM), lactate (20 mM), formate (20 mM), and hydrogen (15 psi at 25°C). The electron acceptors utilized included RDX (25 μM), AQDS (5 mM), and Fe(III) (45 mmol/L). Cultures were monitored by turbidity, color change, and extent of electron acceptor reduced.

One specific enrichment culture (strain MJ1) with lactate + RDX was highly enriched and grew in several successive transfers. It was transferred to lactate + Fe(III) or AQDS, as well as with military smoke dye (Disperse Red #9) as the terminal electron acceptor. It grew with all acceptors tested. The enrichment was streaked on to an anaerobic agar slant with the respective electron acceptors and colonies formed. The colonies were re-suspended in liquid media and the culture is now a purified isolate on AQDS or Fe(III) medium. Further experiments are underway with the isolated culture.

Mulch Humic Substance Extraction Method

The mulch humics extracts were generated using a modified extraction method from the original method developed by the International Humic Substance Society (IHSS) was used to

extract humic substances from mulch. Large fragments of roots and leaves from original material were removed by hand picking and the sample was equilibrated to a pH value between 1 and 2 with 1 *N* HCl at room temperature. The solution volume was adjusted with 0.1 *M* HCl to provide a final concentration that has a ratio of 10 mL liquid/1 g dry sample. The suspension was shaken for 1 h and then the supernatant was separated from the residue by low speed centrifugation. The mulch residue was neutralized with 1 *N* NaOH to pH 7.0 and then 0.1 *M* NaOH was added under an atmosphere of N_2 to give a final extract to sample ratio of 10:1. The suspension was extracted under N_2 with intermittent shaking for a minimum of 4 h. The alkaline suspension was allowed to settle overnight and the supernatant was collected by means of centrifugation. The supernatant was acidified with 6 *N* HCl with constant stirring to pH 1.0 and then the suspension was allowed to stand for 12 to 16 h. The material was centrifuged to separate the humic acid precipitate and fulvic acid fractions. The humic acid fraction was re-dissolved and neutralized with 1 *N* NaOH to pH 7.0 and then 0.1 *N* NaOH was added under an atmosphere of N_2 to give a final extract to sample ratio of 10:1. The extract (humic acid fraction) was filtered through a 0.2 μm sterilized PTFE filter into a pre-sterilized, anaerobic serum bottle to remove impurities including biomass.

Military Smoke Dye Suspensions

These suspensions were performed in an identical manner as the *Geobacter metallireducens* suspensions described above; however, the military smoke dye Red Disperse #9 (1-methylamino anthraquinone) was used as the sole electron shuttling compound. The smoke dye was added in water as a particulate suspension with a concentration range from 1.0 μM to 25.0 μM, the solubility limit.

Analytical Techniques

Aqueous samples from each experiment were filtered through a 0.2 μm sterile PTFE membrane (PALL life science) prior to analysis. RDX and its metabolites, MNX, DNX and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX), and HMX and its metabolite, 1-NO-HMX, 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 (17). The filtered samples were manually injected into a Supelcosil LC-CN column (25cm x 4.6mm, 5μm 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, TNX and HMX, 1-NO-HMX were compared to certified analytical standards in acetonitrile at known concentrations. 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 x 8 feet; Supelco) that was held isothermally at 100ºC. Helium was used as the carrier gas at a flow rate of 12.5 ml/min. The inlet and detector temperature were 195ºC and 250ºC, respectively. Nitrite was measured using ion chromatograph (IC; Dionex 1000) with AS14A column (250 x 4 mm, Dionex) and 8 mM Na_2CO_3/1 mM $NaHCO_3$ as the eluent. Formaldehyde was measured by modified version of EPA method 8315A as previously described (19). $^{14}CO_2$ and $^{14}CH_4$ were

analyzed using gas chromatography (GC; Hewlett-Packard 6890 Series) with radiolabeled gas detector (RGD; IN/US system, Tampa, FL) the same column used for nitrous oxide analysis that was held isothermally at 120ºC. Helium was used as the carrier gas at a flow rate of 12.8 ml/min. The inlet and detector temperature were 195ºC and 250ºC, respectively (14). Aqueous Fe(II) and total solid phase iron concentrations were quantified by the Ferrozine assay as described previously (39). Each 0.1 mL aliquot from Ferrozine reagent (10 mM) amended incubations was diluted in 0.5 *N* HCl and then quantified directly at 562 nm. For the Fe(II) concentration in Ferrozine unamended incubations, each 0.1mL aliquot from the incubations was diluted in 0.5 *N* HCl and 0.1 mL aliquot of the diluted solution was added to 4.9 mL of 1 mM Ferrozine solution, and then quantified at 562 nm.

IX. Results and Accomplishments

Pure Phase Experiments with Reduced Electron Shuttling Compounds or Fe(II)

In order to evaluate if reduced extracellular electron shuttles transfer electrons to the cyclic nitramine RDX, the HS analog AQDS was biologically and chemically reduced, and incubated in cell-free, anaerobic mixtures with RDX. AQDS was used in lieu of purified HS because it is a defined molecule (molecular mass: 366.32 g/mol) and has been well characterized with respect to its electron shuttling capacity. In addition, its color changes from opaque pink to vivid orange as it changes from oxidized to reduced state; therefore it can be quantified spectrophotometrically at 450 nm. Two different concentrations of chemically and biologically reduced AQDS were amended to 50 μM of RDX to determine if the reduction rate of RDX was different with the reduced AQDS produced by different methods (Figure 2). The quantified reduction rate of RDX between chemically and biologically reduced AQDS was not significantly different; therefore, subsequent pure phase incubations were performed with only biologically reduced AQDS.

As previously shown (Figure 1), RDX is reduced by a series of stepwise 2 electron transfers generating nitroso intermediates. Stoichiometry of electron transfer between reduced AQDS and RDX was investigated with three different concentrations of microbially reduced AQDS (Figure 3). 100 μM RDX was completely transformed within 9 hours in the incubations when amended with 500 μM and 300 μM of B-AH_2QDS. However, RDX was reduced to approximately 53 μM with 100 μM of B-AH_2QDS (Figure 3). This result was within range of the expected stoichiometry for oxidation of AH_2QDS coupled to RDX reduction (with TNX as the end product of the reductive pathway). Approximately one-third of the 100 μM RDX was reduced when one third of the stoichiometric equivalent of AH_2QDS was provided, suggesting that RDX was reduced to TNX, with TNX removed by an unidentified reaction pathway. Reduced AQDS transfers two electrons per mole in the coupled oxidation/reduction reaction and RDX accepts six electrons per mole (to TNX.) The half reactions, and full reaction, are as follows:

$C_3H_6N_6O_6$ (RDX) + $6H^+ + 6e^- \rightarrow C_3H_6N_6O_3$ (TNX) + $3H_2O$ (Red. half reaction)

$AH_2QDS \rightarrow AQDS + 2H^+ + 2e^-$ (Ox. half reaction)

$C_3H_6N_6O_6$ (RDX) + $3AH_2QDS \rightarrow C_3H_6N_6O_3$ (TNX) + 3AQDS + $3H_2O$ (Full reaction)

Therefore, three times as much B-AH_2QDS (molar basis) is needed to completely reduce RDX. The two likely reaction pathways would be RDX → MNX, in which case the AH_2QDS would have been oxidized with stoichiometric MNX accumulation, or RDX → TNX, in which case AH_2QDS would be oxidized without MNX or DNX accumulation. MNX, DNX, and TNX did not accumulate; therefore the second pathway is the likely pathway. Lack of TNX accumulation will be discussed below.

The concentration of AH_2QDS decreased coupled to RDX reduction. 500 μM AH_2QDS was oxidized to approximately 200 μM and 300 μM AH_2QDS was oxidized to approximately 90 μM concomitantly with RDX reduction (Figure 3). When AH_2QDS (100 μM) was limiting relative to RDX (at 100 μM), it was completely oxidized within 8 hours.

The metabolites hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) did not significantly accumulate during AH_2QDS-mediated RDX reduction; therefore the individual metabolites were incubated with AH_2QDS to determine if they can be directly reduced. 50 μM MNX was directly reduced by AH_2QDS at all AH_2QDS concentrations tested; however, the extent of reduction differed among the treatments (Figure 4). Excess AH_2QDS (300μM) completely reduced MNX below detection limits within 8 hours. When the stoichiometric equivalent of AH_2QDS (100 μM) was provided, reduction was slower and did not proceed to completion. However, previous experiments suggested that the stoichiometric equivalent completely reduced MNX in a much shorter time frame (Figure 4). 50 μM AH_2QDS reduced approximately 20 μM of MNX (Figure 4), which is within range of the expected stoichiometry.

DNX was also reduced by AH_2QDS; however, the DNX stock was not chemically pure and the reaction did not closely match the expected stoichiometry for DNX (Figure 4). The original stock was composed of approximately 58% DNX, 34% MNX, and 8% TNX. AH_2QDS in excess of the necessary stoichiometry (200 μM) completely reduced the mixture of DNX plus MNX in approximately 30 hours. However, past experiments suggest that MNX and DNX can be reduced in less than 4 hours (Figure 4). MNX and DNX did not accumulate to a significant extent in any AH_2QDS-amended experiments with RDX as the starting material. TNX was transformed as well (Table 2) by an unidentified pathway; most likely ring cleavage and formation of formaldehyde (discussed below). RDX reduction kinetics slowed at 4°C (Figure 5 and Table 1). Although MNX and TNX were measured at low levels, neither of the nitroso intermediates accumulated in the incubations. DNX was not detected at this temperature.

As mentioned, Fe(II) in various forms can abiotically reduce RDX. The initial experiments tested completely soluble Fe(II), as that was the most dominant ferrous iron form in the suspensions. 50 μM RDX was reduced to 19 μM and 39 μM by 1.2 mM and 600 μM of soluble Fe(II), respectively (Figure 6 and Table 1). As suspected, soluble Fe(II) did not transform RDX efficiently, which suggests a solid (mineral) surface is required to facilitate the reactions.

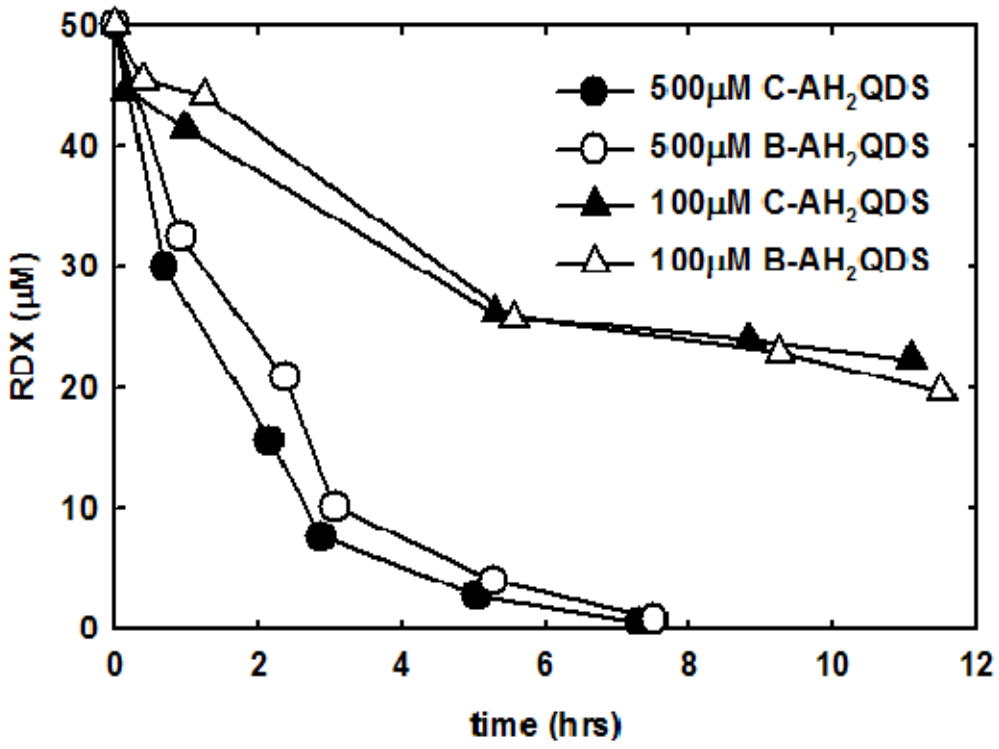

Figure 2. RDX reduction by chemically reduced AQDS (C- AH_2QDS) and biologically reduced AQDS (B-AH_2QDS) in pure phase incubations (no cells). Results are the means of triplicate analyses; standard deviations were presented but analytical error was insignificant (error smaller than figure symbols).

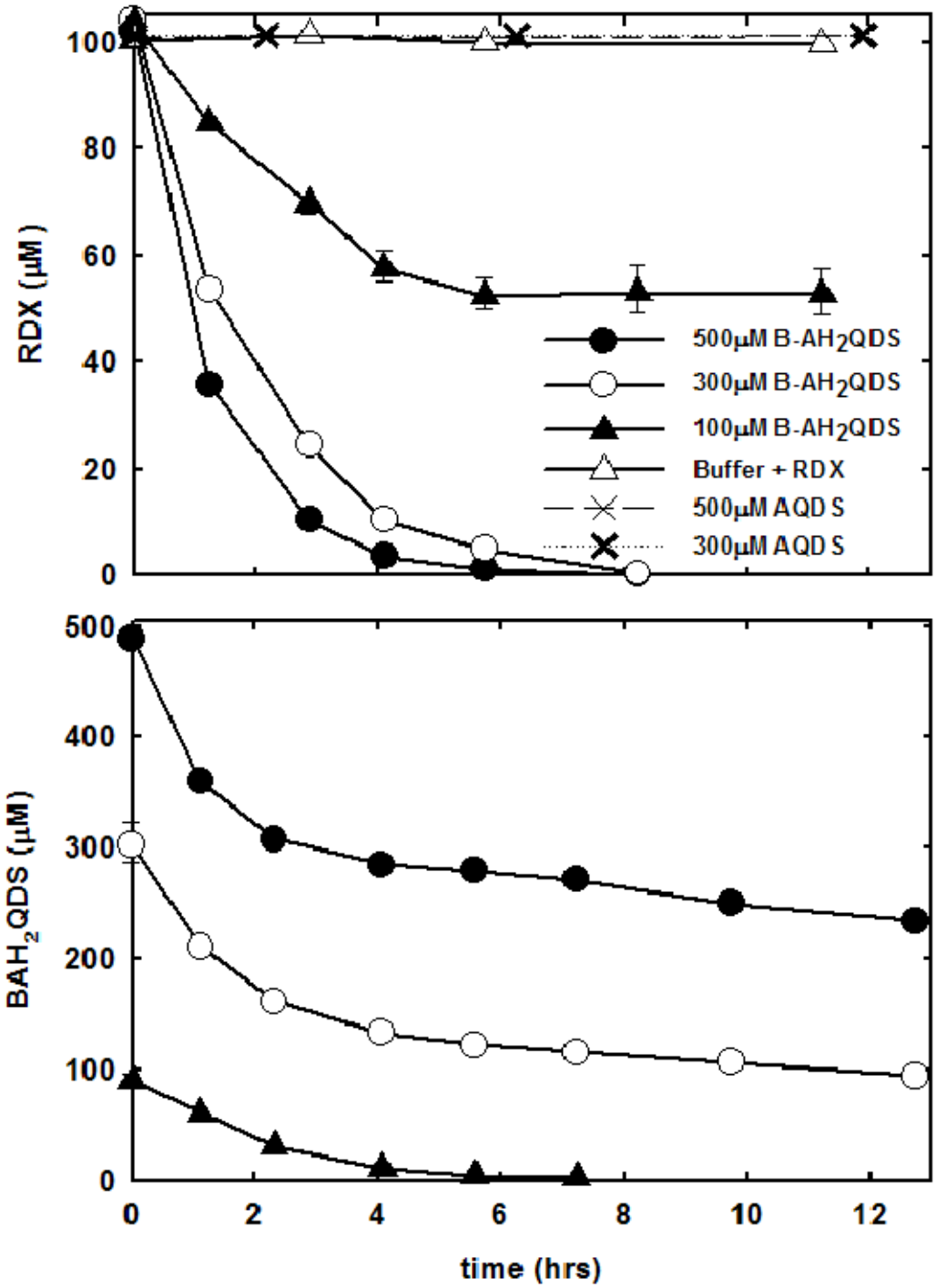

Figure 3. RDX reduction by biologically reduced AQDS (B-AH_2QDS) and AH_2QDS oxidation in pure phase incubations (no cells). Results are the means of triplicate analyses and bars indicate one standard deviation.

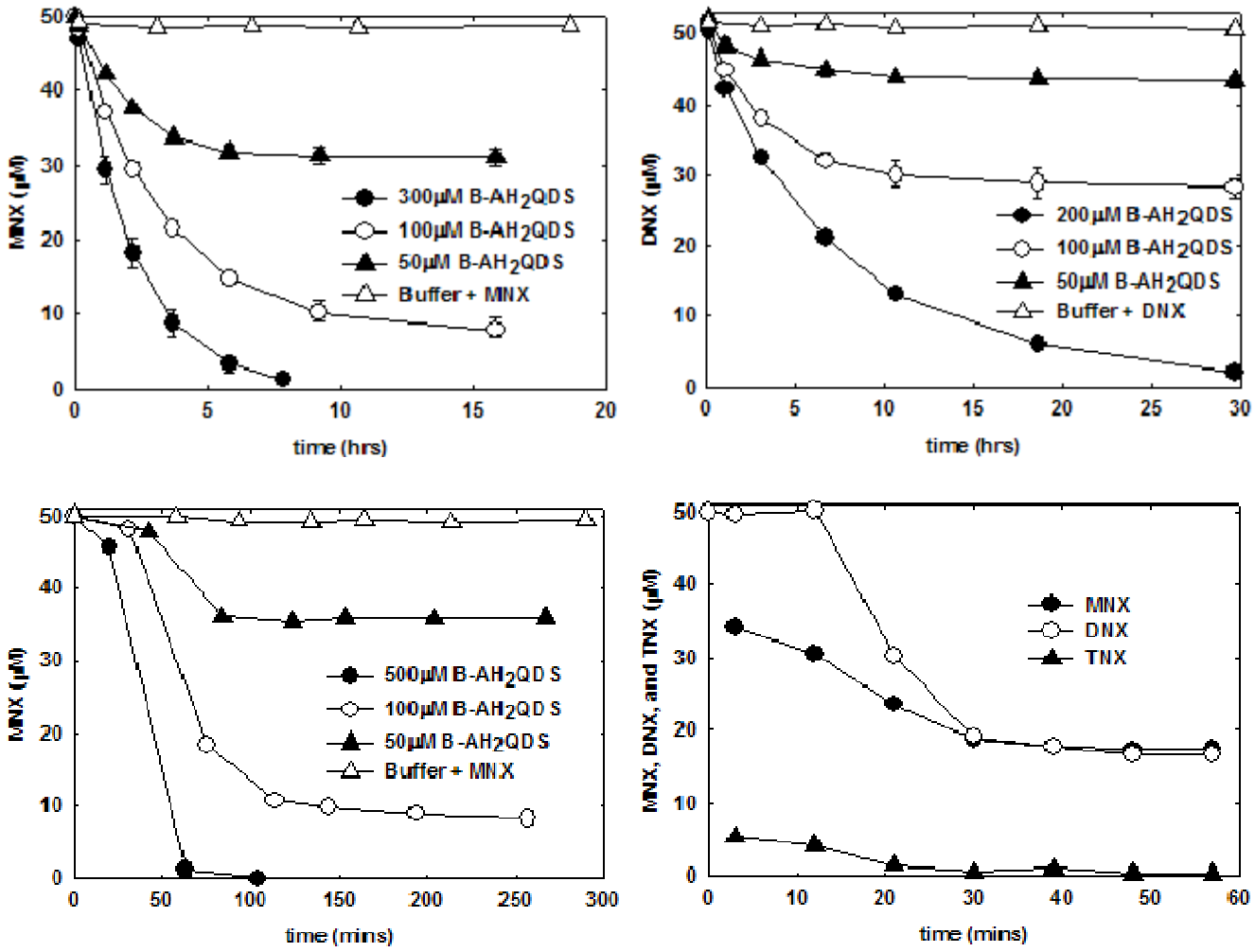

Figure 4. (Upper figures) Stoichiometric reduction of MNX and DNX with three different concentrations of biologically reduced AQDS (B-AH_2QDS) (The DNX stock is not chemically pure; it is approximately 58% DNX, 34% MNX, and 8% TNX); (Lower figures) separate incubations of MNX and DNX with 100 μM of biologically reduced AQDS (B-AH_2QDS) indicating different reduction rates of DNX than later experiments of DNX. Results in upper figures are the means of triplicate analyses and bars indicate one standard deviation; later experiments (lower figures) are the results of single analysis.

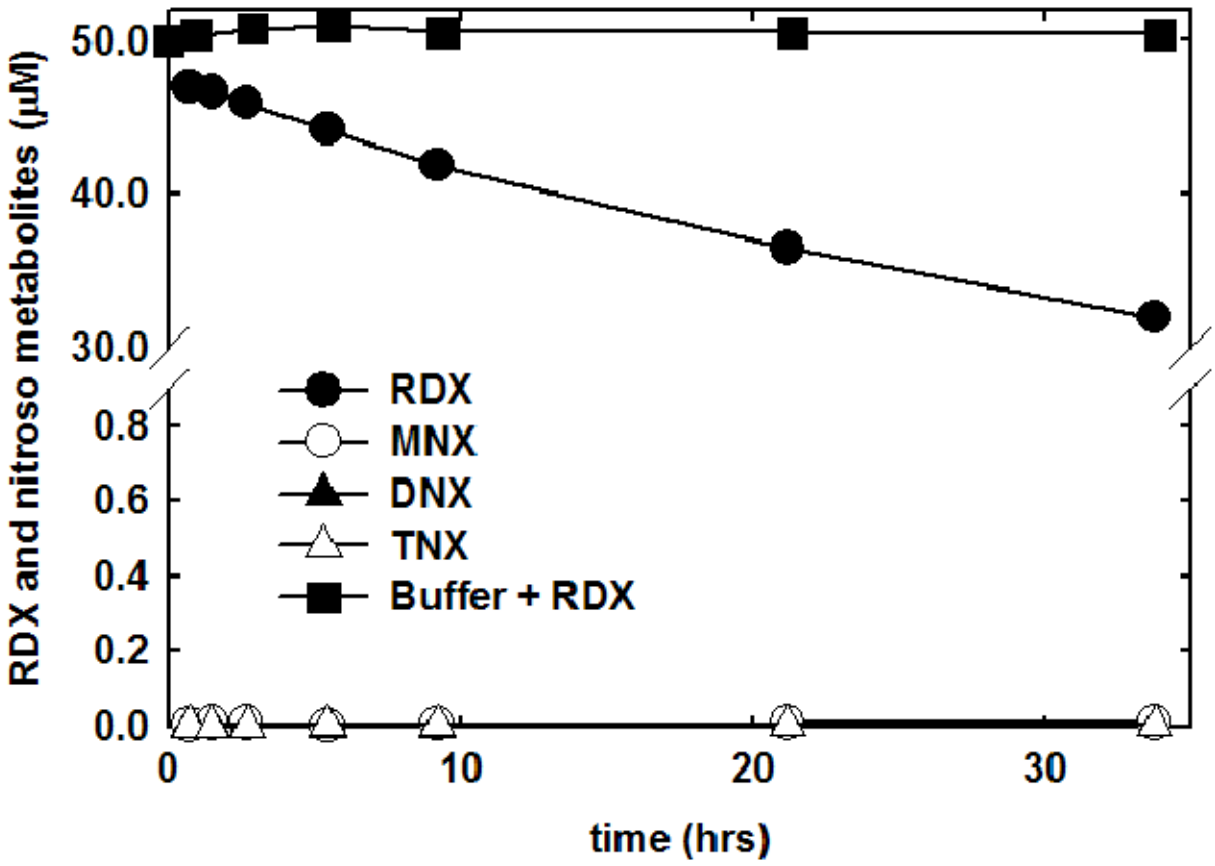

Figure 5. RDX reduction by biologically reduced AQDS (B-AH_2QDS) at 4°C.

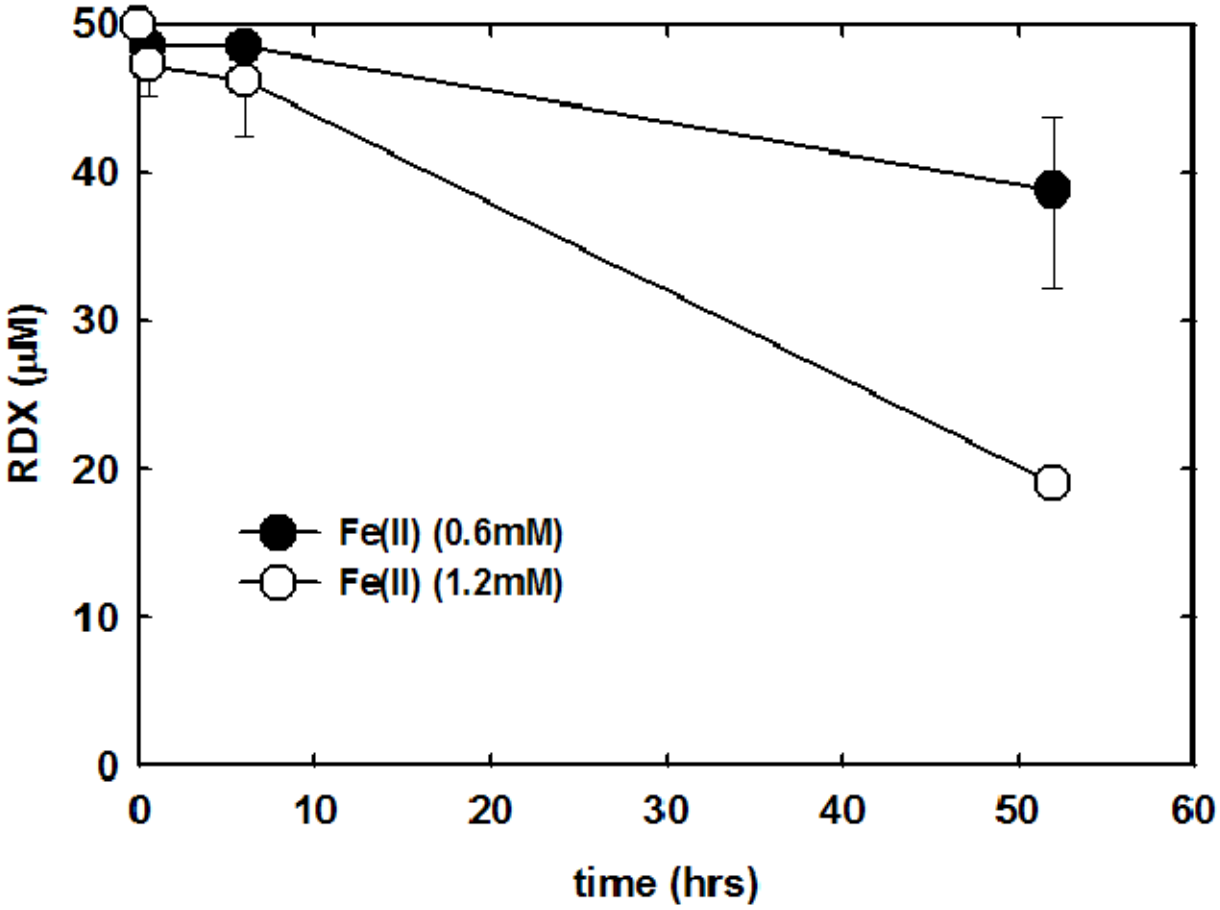

Figure 6. RDX reduction by biologically reduced, soluble Fe(II). Results are the means of triplicate analyses and bars indicate one standard deviation.

Pure phase (no cells) incubations	**Decay rates (μmol RDX hr^{-1} at 100μM AH_2QDS or Fe(II))**	**Temp.**
AH_2QDS alone	4.050	25°C
	0.400	4°C
0.6 mM Fe(II)	0.042	25°C
1.2 mM Fe(II)	0.075	25°C

Cell suspension incubations[a]	**Decay rates (μmol RDX hr^{-1} mg cell $protein^{-1}$)**	
	GS15	**PCA**
cells + AQDS + acetate	0.0312	0.0648
cells + AQDS - acetate	0.0280	0.0565
cells + HS + acetate	0.0079	0.0470
cells + HS - acetate	0.0065	0.0391
cells + Fe(III) + acetate	0.0002	0.0091
cells + Fe(III) - acetate	0.0002	0.0083
cells + AQDS + Fe(III) + acetate	0.0131	0.0008
cells + HS + Fe(III) + acetate	0.0009	0.0058
cells + acetate	0.0150	0.0318
cells - acetate	0.0174	n.d.[b]
aerated cells + acetate	0.0142	n.d.
aerated cells - acetate	0.0150	n.d.

Table 1. Summary rate data for pure phase and pure culture suspensions with RDX and various electron shuttling amendments. Results are the means of triplicate analyses.

Pure Culture Experiments with *Geobacter metallireducens*

RDX was reduced in all experimental incubations containing resting cells; however, the rate and extent of RDX reduction differed greatly among treatments. AQDS stimulated the fastest rate of RDX reduction (Figure 7). RDX was reduced to below detection limits in less than 12 hours regardless of the presence of acetate. The initial degradation rate of RDX with the presence of acetate was faster than that of RDX without acetate. Cells of *G. metallireducens* without AQDS also reduced RDX; RDX was still detectable at 12 hours in cells-only incubations. These are the first data demonstrating RDX reduction by cells within the family *Geobacteraceae*. Later time points demonstrated that cells alone reduced RDX in approximately 50 hours, which was comparable to purified humic substance-mediated RDX reduction. The maximum concentration of MNX quantified during the incubations with AQDS was 0.25 μM and it was no longer detected within 12 hours (Figure 7).

RDX was completely reduced to concentrations below detection within 50 hours with purified humic substances (Figure 7). MNX transiently accumulated to 0.8 μM in humics substance amended incubations, and quickly decreased at approximately 20 hours (Figure 7). RDX reduction was the slowest with poorly crystalline Fe(III) oxide as the sole electron acceptor. RDX slowly decreased from 40 μM to 30 μM with poorly crystalline Fe(III) oxide for 100 hours (Figure 7). MNX accumulated in these incubations, although the concentration was not a stoichiometric increase suggesting partial MNX reduction (Figure 7). MNX concentration eventually decreased below the detection limit with longer incubation times. DNX and TNX did not accumulate significantly within the time frame of these experiments. Heat-killed cells did not reduce RDX in the presence or absence of acetate.

Adding humic acids or AQDS to Fe(III)-containing incubations increased the reduction rates by approximately 5 and 66 times, respectively (Figure 8). These rates of RDX degradation were calculated from the pseudo first-order RDX decay constants (Table 2). Fe(II) concentrations were higher in AQDS and HS amended incubations than poorly crystalline Fe(III) oxide alone (Figure 8). RDX was not reduced in the absence of cells; therefore only the humic amended “no cells” control is presented (results with AQDS were identical.) The rate differences are likely due to the availability of AQDS versus humic substances. AQDS is a small, single quinone molecule that is readily bioavailable. Humics are large, sterically-complex molecules that may not interact as readily with cells and/or contaminants. While AQDS may overestimate the rates compared to natural humics, it is the most consistent analogue for humic substances in many experiments and projects. However, the true “electron shuttle mediated rate” of RDX reduction likely exists somewhere between AQDS (as a high rate) and natural humic substances (as a low rate).

The initial concentration of HMX in the resting cell suspensions of *G. metallireducens* was ca. 7 μM, due to the low solubility of HMX in water. Extracellular electron shuttles stimulated HMX reduction; 7 μM HMX was reduced to approximately 0.5 μM in 35 h in AQDS- or HS-amended incubations (Figure 9). Cells-only incubations reduced HMX to 4 μM in the same time period (Figure 9). Resting cell suspensions with only poorly crystalline Fe(III) reduced HMX to 4.5 μM in 35 h, while adding AQDS to Fe(III) increased HMX reduction rates (Fig. 9 and Table 2). Nitroso metabolites of HMX accumulated in the presence of HS and/or Fe(III), while their

accumulation in AQDS-only amended incubations and cells only incubations was insignificant (Table 2).

Ferrozine is an organic ligand that binds strongly to Fe(II) and stabilizes it as ferrous iron. Ferrozine-bound Fe(II) will not abiotically reduce oxidized compounds, including RDX. Essentially, Ferrozine inhibits Fe(II) electron transfer to RDX, and it was used to block that pathway to isolate the cells only, or the electron shuttle pathway. *G. metallireducens* amended with poorly crystalline Fe(III) oxide (no electron shuttles) reduced RDX to a greater extent in the absence of Ferrozine ((Figure 10 and Table 2). 46 μM RDX was reduced to 35 μM in 52 h (27% reduction) without Ferrozine, while RDX was reduced slowly and stabilized at 40 μM for 52 h (14% reduction) with Ferrozine. RDX was not degraded without cells regardless of the presence or the absence of Ferrozine reagent. Absorbance at 562.0 nm (consistent with Fe(II) production) increased in the Ferrozine amended incubations; the initial absorbance (0.003) increased to 0.009 in 30 h and stabilized for 52 h. Without Ferrozine, absorbance did not increase. Without cells and with Ferrozine, absorbance was constant at 0.005 for 52 h.

Cells incubated with acetate, poorly crystalline Fe(III) oxide, and AQDS reduced RDX to below 4 μM in 52 h (90% reduction) (Figure 10 and Table 2). However, with Ferrozine, RDX was reduced slowly to 30 μM in 52 h (33% reduction). RDX was not reduced without cells regardless of the presence or the absence of Ferrozine reagent. Absorbance at 562.0 nm increased quickly in Ferrozine/AQDS/Fe(III) amended incubations, from 0.010 to 0.032 in 3 h and slowly increased to 0.051 in 52 h. The absorbance did not increase in either cells minus Ferrozine controls or no-cells plus Ferrozine controls.

Cells incubated with HS as an electron shuttle demonstrated a similar trend to AQDS amended incubations; however, the extent of RDX reduction was greater. RDX was reduced below the detection limit in 52 h (100% reduction) without Ferrozine (Figure 10 and Table 2). RDX was reduced slowly to 20 μM (55% reduction) with Ferrozine. RDX was not reduced without cells. Absorbance at 562.0 nm rapidly increased to 0.309 in 52 h in the Ferrozine amended suspensions. The absorbance did not increase in either of the cells minus Ferrozine controls or no-cells plus Ferrozine controls.

HMX reduction was similar to RDX reduction (Figure 11). Cells incubated with poorly crystalline Fe(III) oxides (no electron shuttles) in the absence of Ferrozine reagent reduced HMX to a greater extent than in the presence of Ferrozine (Figure 11 and Table 2). HMX was reduced to 4.7 μM in 53 h (19% reduction) without Ferrozine; however, HMX was reduced only 10% with Ferrozine. HMX was not degraded without cells regardless of the presence or the absence of Ferrozine reagent. Absorbance at 562.0 nm increased in the Ferrozine amended incubations; the initial absorbance (0.005) increased to 0.021 in 53 h. Without Ferrozine, absorbance did not increase. Without cells and with Ferrozine, absorbance was maintained at ca. 0.018 for 53 h.

Cells incubated with AQDS, acetate, and poorly crystalline Fe(III) oxides degraded HMX faster without Ferrozine; 5.7 μM HMX was degraded to 1.5 μM in 53 h (74% reduction) (Figure 11 and Table 2). HMX was reduced slowly to 4.6 μM in 52 h (19% reduction) with Ferrozine. HMX was not reduced without cells regardless of the presence or the absence of Ferrozine reagent. Absorbance at 562.0 nm increased quickly in Ferrozine/AQDS/Fe(III) amended

incubations, from 0.020 to 0.059 in 3 h and slowly increased to 0.083 in 53 h. The absorbance did not increase in either cells minus Ferrozine controls or no-cells plus Ferrozine controls.

Cells incubated with HS were similar to AQDS amended incubations; however, the extent of HMX degradation was greater. RDX was reduced by 96 % without Ferrozine (Figure 11 and Table 2). RDX was reduced slowly to 20 μM (24% reduction) with Ferrozine. RDX was not reduced without cells. Absorbance at 562.0 nm rapidly increased to 0.4 in 53 h. The absorbance did not increase in either of the cells minus Ferrozine controls or no-cells plus Ferrozine controls.

RDX was reduced to its nitroso metabolite, MNX, when Ferrozine was not present and the cells were amended with AQDS plus Fe(III); however, the maximum accumulation of MNX was only 0.1 μM (Table 2). DNX and TNX accumulation was insignificant. RDX was sequentially reduced to its nitroso metabolites, MNX, DNX, and TNX when Ferrozine was not present and the cells were amended with HS plus Fe(III). The maximum accumulation of MNX, DNX, and TNX in HS amended incubations was 5.7 μM (28 h), 3.5 μM (52 h), and 2.5 μM (180 h), respectively. MNX and DNX were completely reduced in 180 h and TNX was completely reduced in 360 h.

HMX was degraded to its one nitroso metabolite, 1-NO-HMX; the maximum accumulation of 1-NO-HMX was 0.4 μM (Table 2) when the cells were amended with AQDS plus Fe(III) in the absence of Ferrozine. HMX was also reduced to its nitroso metabolites, in HS plus Fe(III) amended incubations when Ferrozine was absent. Although analytical standards for 2-NO-HMX, 3-NO-HMX, and 4-NO-HMX were not available, an increase or decrease of the peak area of each compound with time suggested the accumulation or reduction of these metabolites.

The retention times of HMX and 1-NO-HMX measured on the HPLC chromatogram were approximately 13 and 11 minutes, respectively. The peaks of 2-NO-HMX, 3-NO-HMX, and 4-NO-HMX were detected at 9, 7.5, and 6 minutes, respectively. The maximum accumulation of 1-NO-HMX, 2-NO-HMX, 3-NO-HMX and 4-NO-HMX in HS amended incubations was 1.2 μM at 29 h; 0.06 (area units) at 52 h; 0.02 (area units) at 55 h; and 0.07 (area units) at 83 h, respectively. However, nitroso metabolites did not accumulate after 125 h. Nitroso metabolites of RDX/HMX did not accumulate in the presence of Ferrozine regardless of the presence or absence of electron shuttles (Table 2).

In the presence of AQDS and acetate (or H_2), RDX was reduced to below detection limits within 20 h (Figure 12). AQDS plus poorly crystalline Fe(III) incubations that were not amended with Ferrozine also reduced RDX within 20 h (98.5%); however, with Ferrozine the RDX was only reduced to 23 μM in 74 h. Cells alone reduced RDX from 55 μM to 35 μM in 74 h. RDX was not degraded without cells regardless of the presence or absence of Ferrozine.

The concentration of nitrous oxide, a ring cleavage product of RDX, increased to 28 μM within 68 h in the presence of AQDS and when Ferrozine was absent (Figure 12). Nitrous oxide accumulated up to 19 μM within 68 h in AQDS plus poorly crystalline Fe(III) amended incubations without Ferrozine, while nitrous oxide slowly increased to 5 μM when Ferrozine was present in the Fe(III)-containing incubations. In cells-only incubations, nitrous oxide slowly increased to 4 μM in 68 h. Nitrous oxide did not accumulate without cells.

Formaldehyde increased to approximately 100 μM within 20 h in acetate/AQDS and H_2/AQDS incubations (Figure 12) concurrently with RDX reduction. Formaldehyde also increased to 110 μM in acetate/AQDS/poorly crystalline Fe(III) incubations when Ferrozine was absent. However, formaldehyde accumulated slowly in AQDS plus poorly crystalline Fe(III) incubation, when Ferrozine was present, to a final concentration of 52 μM in 74 h. Formaldehyde in cells only incubations was stable at around 18 μM in 74 h. Without cells, formaldehyde did not accumulate over time.

In the presence of AQDS, nitrite concentrations increased to 5 or 11 μM with acetate or H_2, respectively, after 11 h and then decreased to the below detection limit within 21 h (data not shown). Nitrite concentration continuously increased to 13 μM over 74 h in AQDS/acetate/poorly crystalline Fe(III) incubations amended with Ferrozine. 2 μM of nitrite accumulated in cells only incubations by hour 11 and then was completely reduced. Without cells, nitrite did not accumulated within 74 h. $^{14}CO_2$ or $^{14}CH_4$ were not produced from ^{14}C labeled-RDX in any of the suspensions regardless of amendment or the presence or absence of Ferrozine within the 74 h incubation period (data not shown).

Cells amended with either raw humic acid extracted from mulch or the military smoke dye Red Disperse #9 also degraded RDX and HMX. Mulch extracts increased the rate and extent of RDX reduction without significant accumulation of nitroso metabolites (Figure 13.) 5 μM to 25 μM of the smoke dye increased the rate and extent of either RDX (Figure 14) or HMX (Figure 15) reduction. The remaining metabolites (e.g. formaldehyde) were not quantified in these experiments.

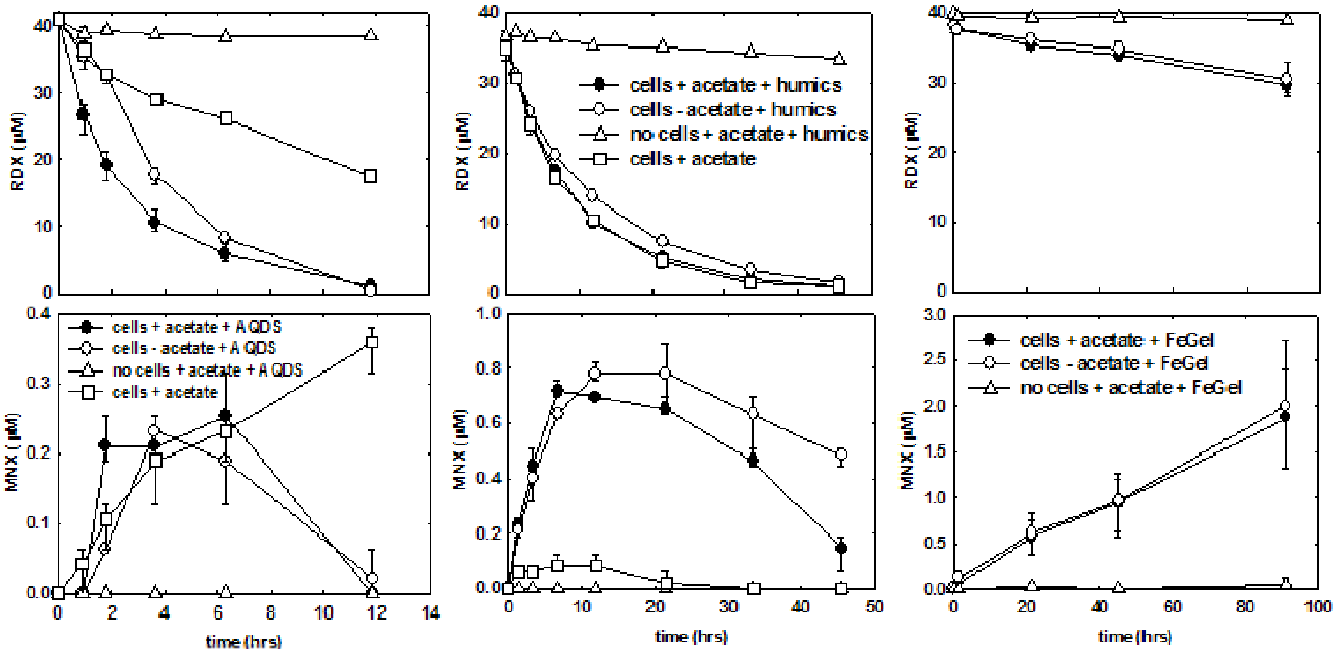

Figure 7. RDX reduction and MNX accumulation with AQDS in cell suspension incubations of *G. metallireducens*; RDX reduction and MNX accumulation with humic substances in cell suspension incubations of *G. metallireducens*; RDX reduction and MNX accumulation with poorly crystalline Fe(III) oxide (FeGel) in cell suspension incubations of *G. metallireducens*; Results are the means of triplicate analyses and bars indicate one standard deviation.

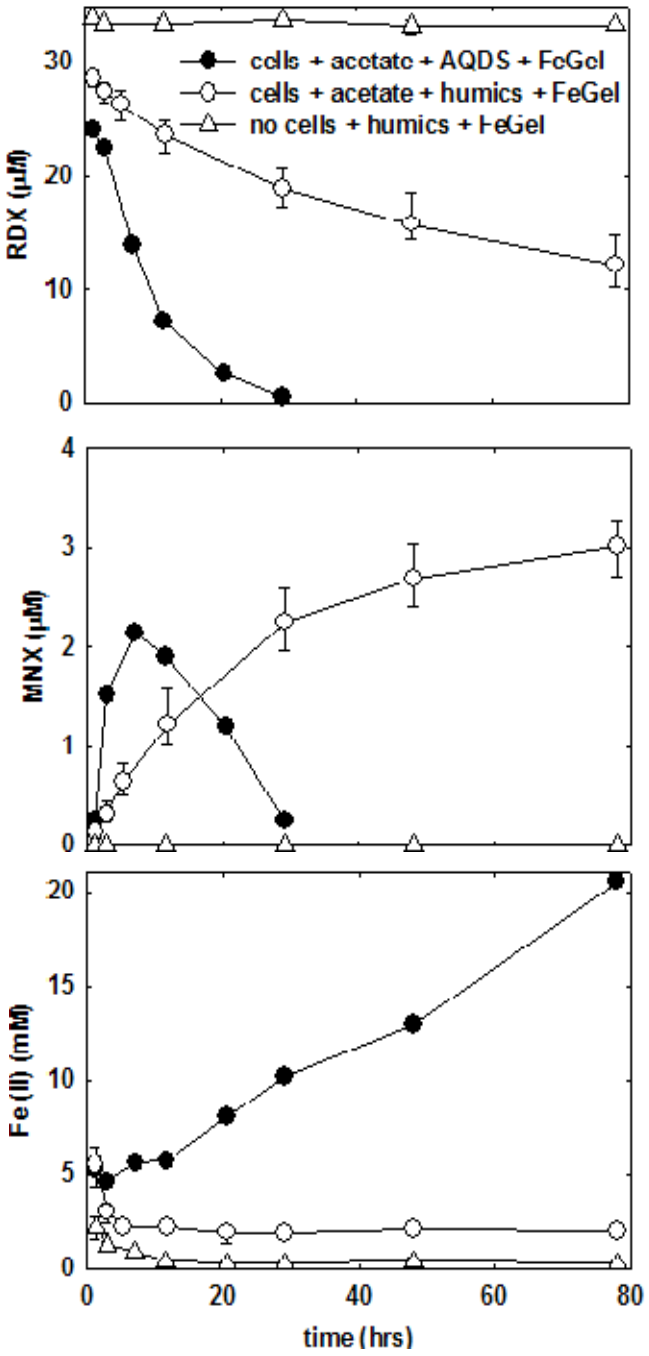

Figure 8. RDX reduction, MNX accumulation, and Fe(II) accumulation with AQDS plus poorly crystalline Fe(III) oxide (FeGel) and humic substances plus poorly crystalline Fe(III) oxide in cell suspension incubations of *G. metallireducens*; Results are the means of triplicate analyses and bars indicate one standard deviation.

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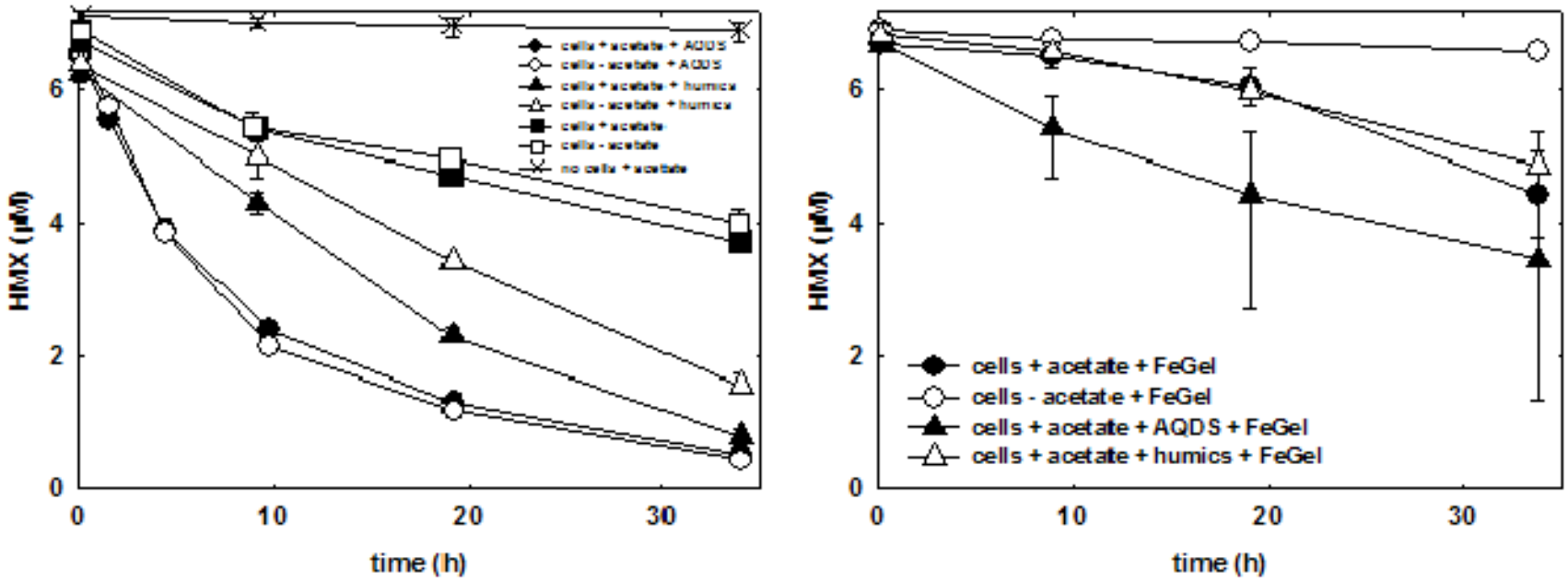

Figure 9. HMX reduction in the resting cell suspensions of *G. metallireducens* with or without extracellular electron shuttling compounds (acetate as the sole electron donor); Results are the means of triplicate analyses and bars indicate one standard deviation.

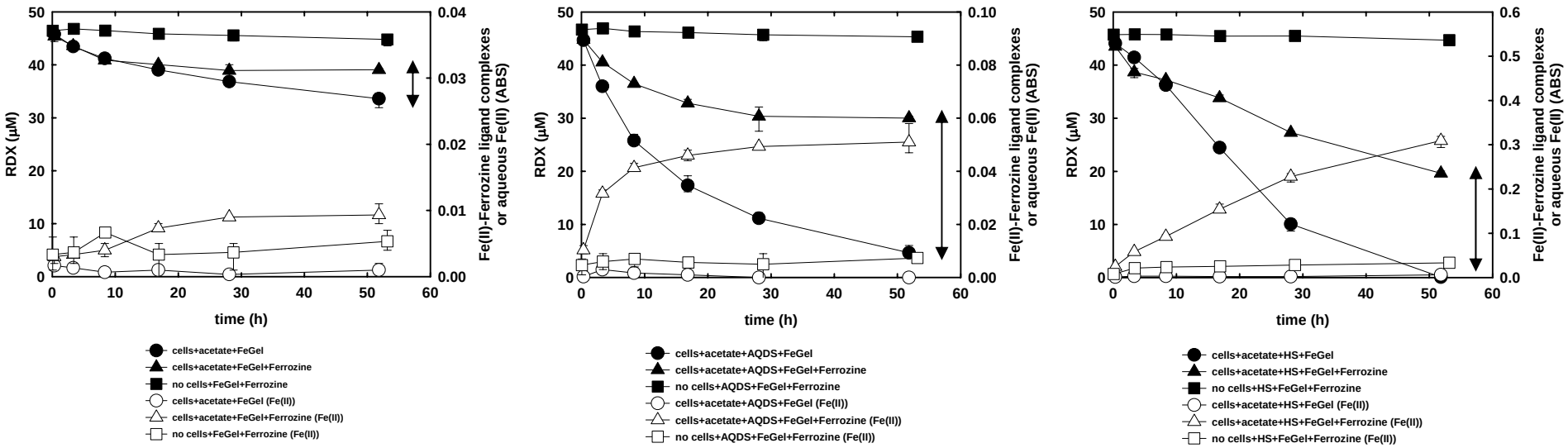

Figure 10. RDX reduction and Fe(II) accumulation with or without Ferrozine reagents in the presence of poorly crystalline Fe(III) hydroxide (FeGel) only, FeGel plus AQDS, or FeGel plus humic substances (HS) in cell suspension incubations of *G. metallireducens*. Arrows indicate differences of the extent of RDX reduction in the presence or absence of Ferrozine. Results are the means of triplicate analyses and bars indicate one standard deviation.

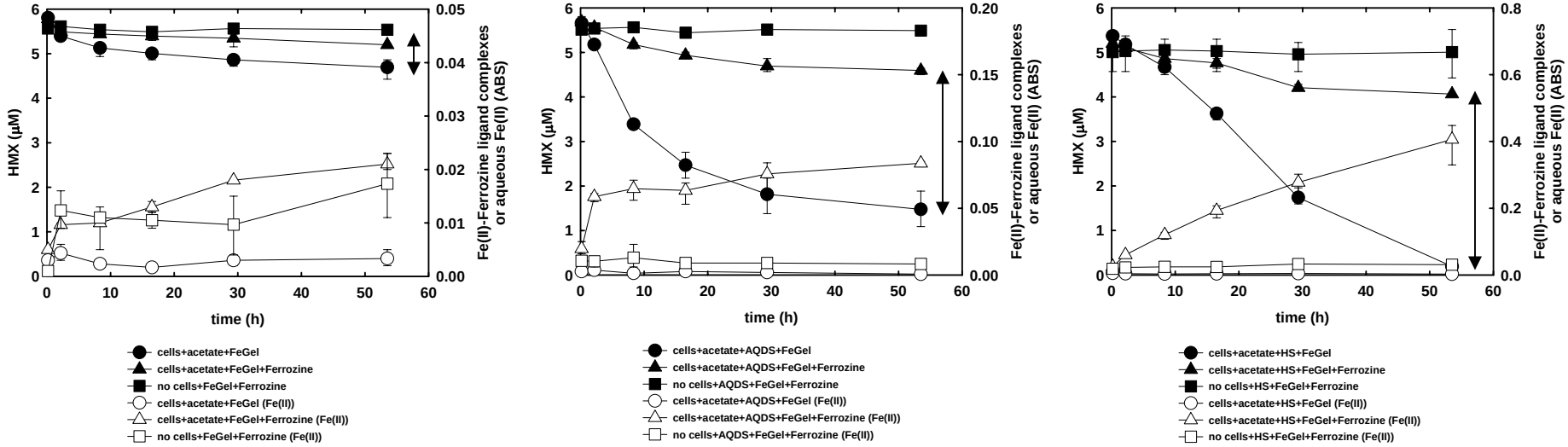

Figure 11. HMX reduction and Fe(II) accumulation with or without Ferrozine reagents in the presence of FeGel only, FeGel plus AQDS, or FeGel plus humic substances (HS) in cell suspension incubations of *G. metallireducens*. Arrows indicate differences of the extent of HMX reduction in the presence or absence of Ferrozine. Results are the means of triplicate analyses and bars indicate one standard deviation.

Cell suspension incubations	Electron transfer pathway	RDX decay rates (μmol/h/mg cell protein)	% reduced at hour 52	Max. accumulation of nitroso metabolites (μM)			HMX decay rates (μmol/h/mg cell protein)	% reduced at hour 52	Max. accumulation of nitroso metabolites (1NO-HMX = μM; others = area)			
				MNX	DNX	TNX			1-NO	2-NO	3-NO	4-NO
cells +donor+AQDS	1,3	0.0312[a]	ND[b]	0.25[a]	NT[a]	NT[a]	0.0068	ND	0.08	NT	NT	NT
cells+donor+HS	1,3	0.0079[a]	ND	0.72[a]	0.06[a]	NT[a]	0.0058	ND	1.00	0.01	0.01	NT
cells+donor	1	0.0150[a]	ND	0.19[a]	NT[a]	NT[a]	0.0016	ND	0.08	NT	NT	NT
cells+donor+Fe(III)	1,2	0.0006	26.6	0.08	NT	NT	0.0005	19.2	0.08	NT	NT	NT
cells+donor+Fe(III)+FZ	1 (2 blocked)	0.0004	13.9	NT[c]	NT	NT	0.0002	10.0	NT	NT	NT	NT
cells+donor+AQDS+Fe(III)	1,2,3,4	0.0043	89.6	0.08	NT	NT	0.0028	73.9	0.41	NT	NT	NT
cells+donor+AQDS+Fe(III)+FZ	1,3 (2,4 blocked)	0.0009	33.0	NT	NT	NT	0.0005	19.1	NT	NT	NT	NT
cells+donor+HS+Fe(III)	1,2,3,4	0.0043	99.8	5.68	3.48	2.46	0.0032	96.4	1.22	0.05	0.02	0.07
cells+donor+HS+Fe(III)+FZ	1,3 (2,4 blocked)	0.0015	54.9	NT	NT	NT	0.0005	24.3	NT	NT	NT	NT

Table 2. Relative comparison of the rates and extent of RDX and HMX reduction, as well as nitroso metabolite accumulation, in the resting cell suspensions of *G. metallireducens* in which the Fe(II) electron transfer pathway was allowed to proceed versus being blocked by Ferrozine (FZ). Results are the means of triplicate analyses.

[a] The results were reproduced from Kwon and Finneran (2006).

[b] ND, not determined.

[c] NT, not detected.

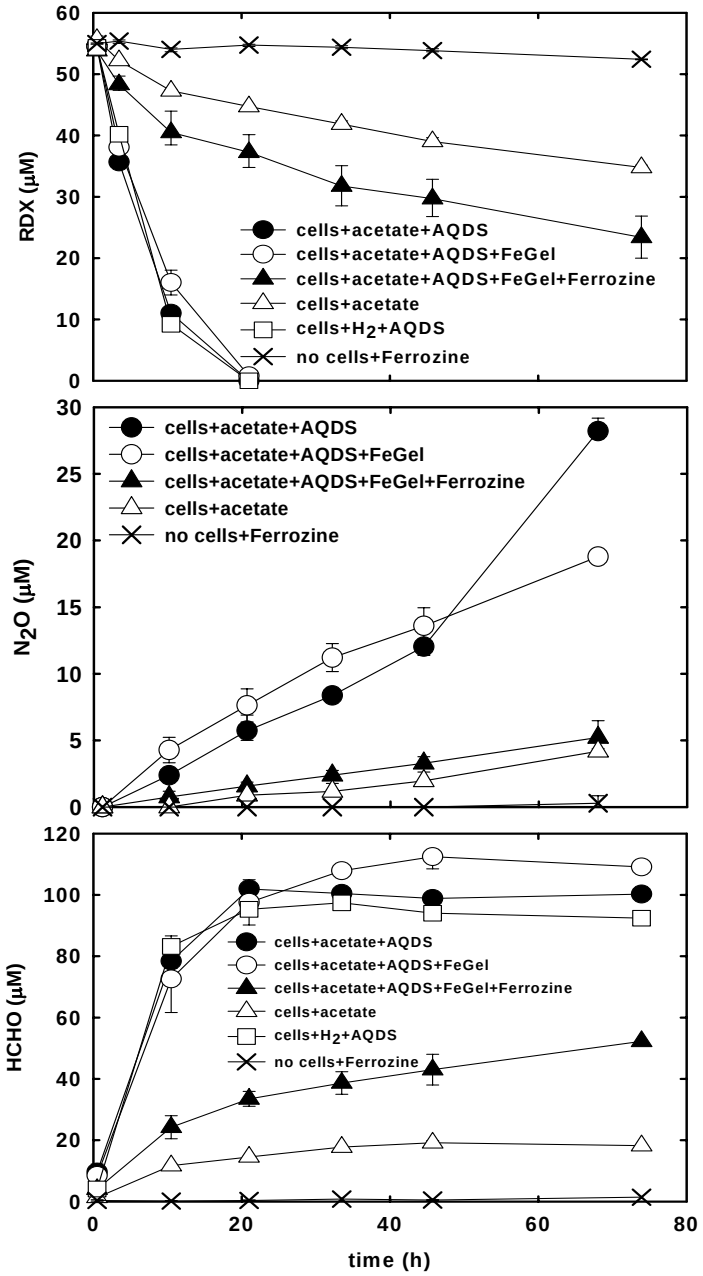

Figure 12. The reduction of RDX, the production of nitrous oxide (N_2O) and the production of formaldehyde (HCHO) in the resting cell suspensions of *G. metallireducens* with or without Ferrozine (AQDS was the sole electron shuttle; acetate or H_2 were added as the electron donors). Results are the means of triplicate analyses and bars indicate one standard deviation.

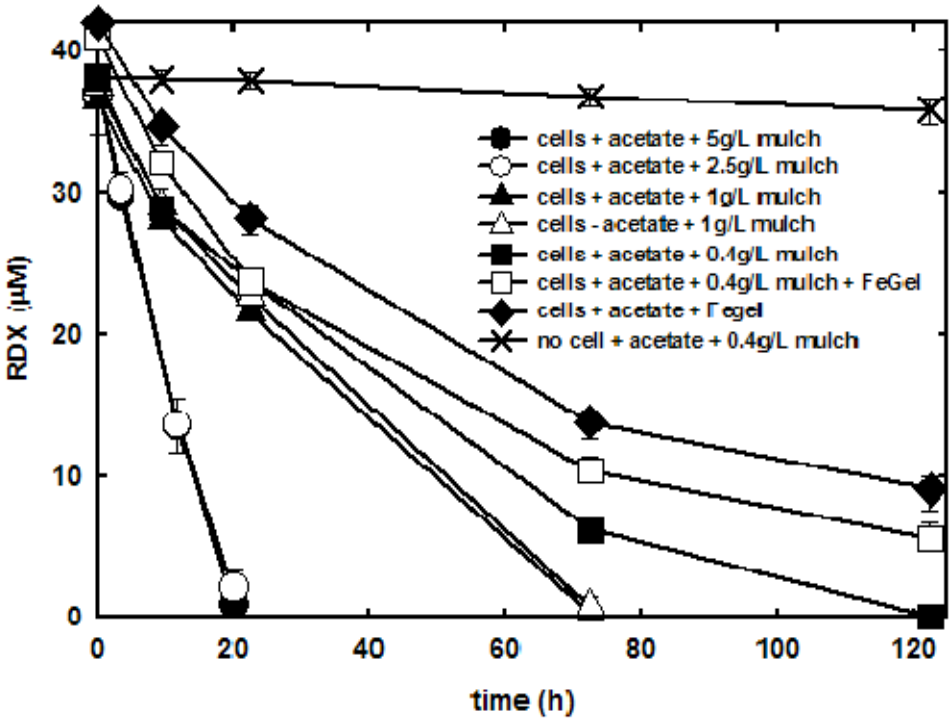

Figure 13. RDX reduction in the resting cell suspensions of *G. metallireducens* amended with different concentrations of raw humic acid extract from mulch material (acetate as the sole electron donor); Results are the means of triplicate analyses and bars indicate one standard deviation.

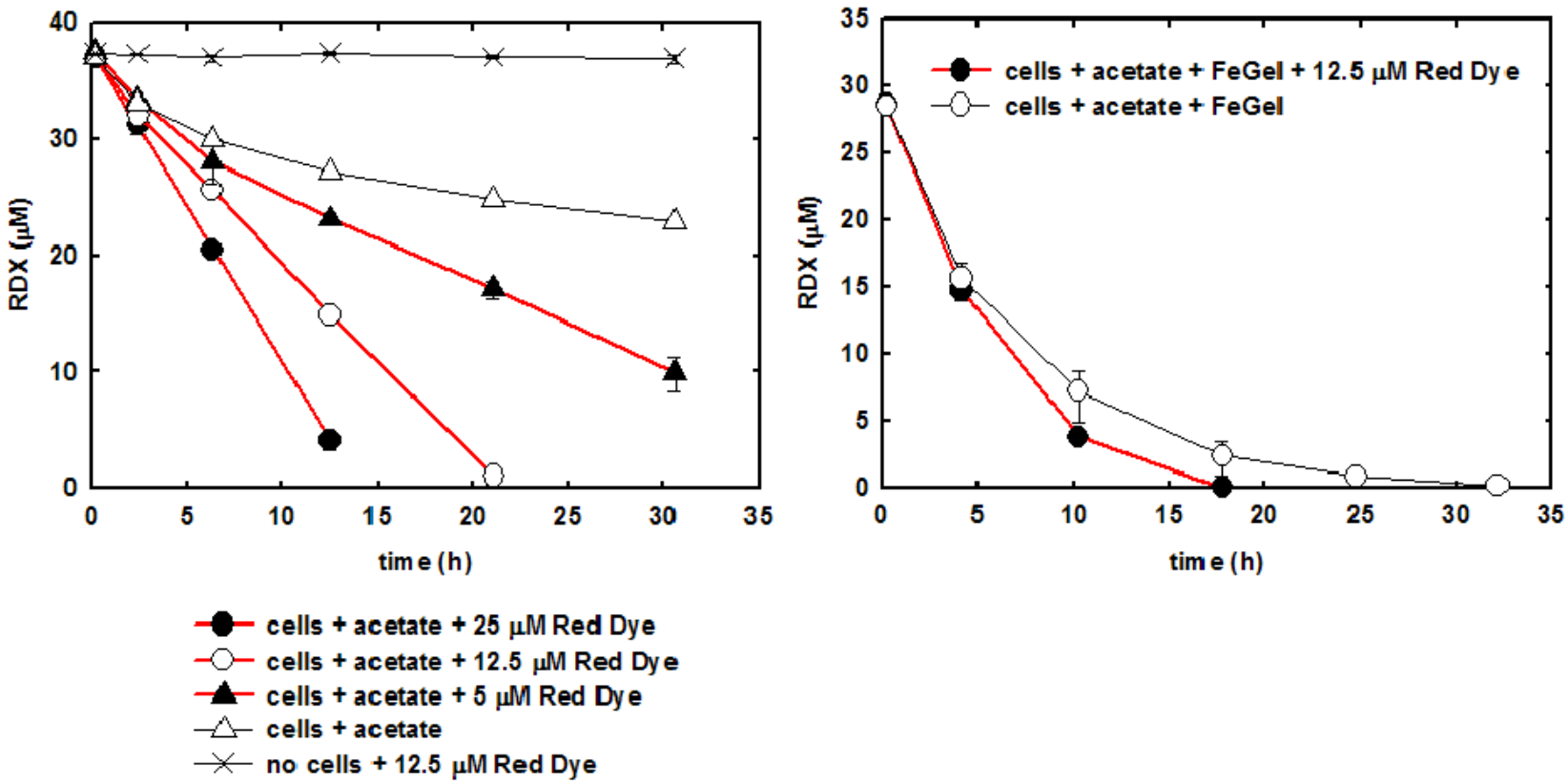

Figure 14. RDX reduction in the resting cell suspensions of *G. metallireducens* amended with different concentrations of the military smoke dye Red Disperse #9 (acetate as the sole electron donor); Results are the means of triplicate analyses and bars indicate one standard deviation.

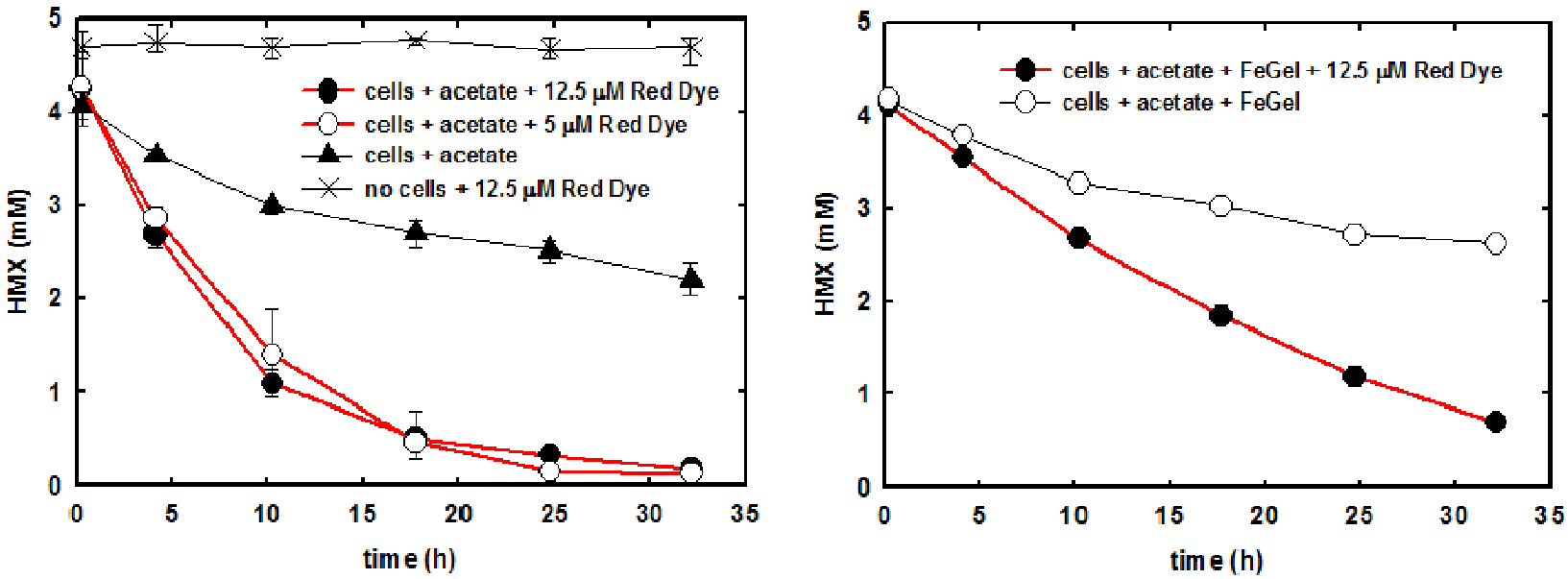

Figure 15. HMX reduction in the resting cell suspensions of *G. metallireducens* amended with different concentrations of the military smoke dye Red Disperse #9 (acetate as the sole electron donor); Results are the means of triplicate analyses and bars indicate one standard deviation.

Pure Culture Experiments with *Anaeromyxobacter dehalogenans, Desulfitobacterium chlororespirans, Geobacter sulfurreducens,* and *Shewanella oneidensis*

Similar results were obtained with resting cell suspensions of *G. sulfurreducens*, another member of the *Geobacteraceae* which has variant physiological properties from *G. metallireducens* (8, 36). RDX was reduced with cell suspensions of *G. sulfurreducens* with several electron acceptors including AQDS, HS, and poorly crystalline Fe(III) oxide. Direct electron transfer from AQDS or HS was faster than Fe(III)-Fe(II) mediated electron transfer to RDX. All *G. sulfurreducens* results are summarized as individual reaction rates in Figure 16 and Tables 3 and 4.

A. dehalogenans reduced RDX most rapidly in the presence of AQDS (Figure 17 and Table 3); however, RDX reduction rates by *A. dehalogenans* were relatively slow compared to the other cultures. RDX (56 μM) was reduced to 5 μM within 71 h with AQDS (data not shown). RDX was reduced to 31 μM within 71 h with HS. RDX was less than 30% reduced when extracellular electron shuttles were not present. *A. dehalogenans* directly reduced RDX (Table 3) slowly relative to electron shuttle amended cells. *A. dehalogenans* did not degrade RDX effectively with poorly crystalline Fe(III) hydroxide regardless of the presence of extracellular electron shuttles (Table 3). MNX, the one nitroso metabolite of RDX, transiently accumulated in cells plus HS incubations and cells only incubations. Nitroso metabolites did not accumulate in AQDS and/or Fe(III) amended incubations (Table 4).

RDX was completely reduced within 3 h in *S. oneidensis* suspensions containing AQDS (Figure 18). RDX was reduced to 5 μM over 65 h in HS amended incubations. HS-amended RDX reduction rates were relatively slow compared to the other cultures (Table 3). *S. oneidensis* also used RDX directly as an electron acceptor. The RDX concentration (40 μM) in lactate-alone incubations decreased to 18 μM in 65 h. RDX degradation rates were similar regardless of the presence or absence of lactate (Table 3). RDX in poorly crystalline Fe(III) amended incubations was reduced to 30 μM in 65 h; however, AQDS and HS increased RDX reduction when Fe(III) was present (Table 3). All but the AQDS-amended suspensions accumulated nitroso metabolites at concentrations higher than other cultures (Table 4).

D. chlororespirans completely reduced RDX within 4 h and 45 h in suspensions that contained AQDS and HS, respectively (Figure 19). RDX was not completely reduced in any other treatment. Cells alone (with lactate as the electron donor) reduced RDX to 20 μM in 43 h, but further reduction was limited. *D. chlororespirans* did not reduce RDX in the absence of electron donor (lactate) (Table 3). RDX was reduced in the presence of Fe(III); however, this rate was increased by adding AQDS or HS to Fe(III) incubations (Table 3). Nitroso metabolites accumulated in the presence of HS and/or Fe(III). Nitroso metabolites did not accumulate in AQDS amended incubations without Fe(III) and cells only incubations (Table 4).

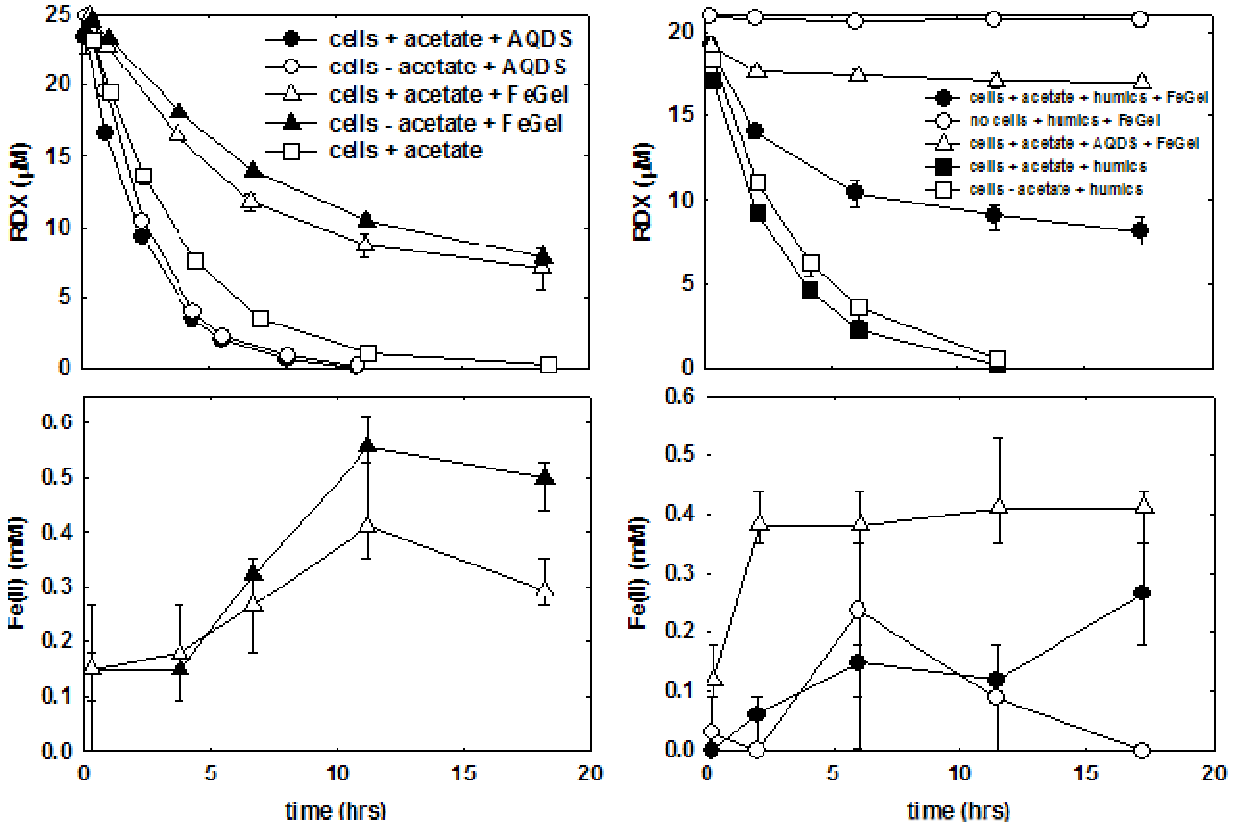

Figure 16. RDX reduction in the resting cell suspensions of *Geobacter metallireducens* strain GS15 (leftmost figures) and *G. sulfurreducens* strain PCA (rightmost figures) with or without extracellular electron shuttling compounds (acetate as the sole electron donor); Results are the means of triplicate analyses and bars indicate one standard deviation.

Cell suspension incubations	Decay rates (μmol/h/mg cell protein)					
	RDX					HMX
	GS15[a]	PCA[a]	AMB.K	MR1	Co23	GS15
cells + AQDS + donor	0.0312	0.0648	0.0074	0.0353	0.2693	0.0068
cells + AQDS - donor	0.0280	0.0565	0.0056	0.0378	0.0009	0.0075
cells + HS + donor	0.0079	0.0470	0.0013	0.0010	0.0211	0.0058
cells + HS - donor	0.0065	0.0391	0.0012	0.0010	0.0021	0.0039
cells + Fe(III) + donor	0.0002	0.0091	0.0003	0.0002	0.0037	0.0005
cells + Fe(III) - donor	0.0002	0.0083	0.0000	0.0002	0.0003	0.0000
cells + AQDS + Fe(III) + donor	0.0131	0.0008	0.0002	0.0016	0.0294	0.0018
cells + HS + Fe(III) + donor	0.0009	0.0058	0.0001	0.0003	0.0071	0.0010
cells + donor	0.0150	0.0318	0.0011	0.0004	0.0042	0.0016
cells - donor	0.0174	ND[b]	0.0010	0.0005	0.0006	0.0014

Table 3. Degradation rates of RDX or HMX in the resting cell suspension of *G. metallireducens*, *G. sulfurreducens*, *A. dehalogenans*, *S. oneidensis*, and *D. chlororespirans* with or without extracellular electron shuttling compounds (acetate or lactate as the sole electron donor). Results are the means of triplicate analyses.

[a] The results were reproduced from Kwon and Finneran (2006).

[b] ND, not determined.

Cell suspension incubations	Max. accumulation of nitroso metabolites of RDX or HMX					
	MNX/DNX/TNX (μM)					1NO(μM)/2NO/3NO/4NO-HMX(area)
	G. metallireducens[a]	*G. sulfurreducens*[a]	*A. dehalogenens*	*S. oneidensis*	*D. chlororespirans*	*G. metallireducens*
cells + AQDS + donor	0.25(6)/NT[b]/NT	NT/NT/NT	NT/NT/NT	NT/NT/NT	NT/NT/NT	0.08(34)/NT/NT/NT
cells + HS + donor	0.72(7)/0.06(78)/NT	0.26(4)/NT/NT	0.30(47)/NT/NT	2.56(30)/0.19(65)/0.86(65)	0.88(15)/NT/NT	1.00(34)/0.01(34)/0.01(34)/NT
cells + Fe(III) + donor	1.88(91)/NT/NT	0.06(17)/NT/NT	NT/NT/NT	4.46(65)/0.15(65)/0.34(65)	1.92(34)/0.06(43)/NT	1.24(34)/0.02(34)/0.01(34)/0.01(34)
cells + AQDS + Fe(III) + donor	3.42(29)/1.64(78)/1.16(78)	NT/NT/NT	NT/NT/NT	5.17(29)/2.72(65)/3.48(65)	1.10(21)/0.13(43)/NT	1.35(34)/0.04(34)/0.02(34)/0.01(19)
cells + HS + Fe(III) + donor	3.01(78)/0.40(78)/NT	0.06(17)/NT/NT	NT/NT/NT	5.06(64)0.27(65)/0.43(65)	4.11(34)/0.39(43)/NT	1.00(34)/0.01(34)/0.01(34)/NT
cells + donor	0.19(7)/NT/NT	NT/NT/NT	0.09(71)/NT/NT	3.34(65)/0.15(65)/1.2(65)	NT/NT/NT	0.08(34)/NT/NT/NT

Table 4. Maximum accumulation of the nitroso metabolites of RDX or HMX in the resting cell suspension of *G. metallireducens*, *G. sulfurreducens*, *A. dehalogenans*, *S. oneidensis*, and *D. chlororespirans* with or without extracellular electron shuttling compounds (acetate or lactate as the sole electron donor). Total incubation times in the resting cell suspensions of *G. metallireducens*, *G. sulfurreducens*, *A. dehalogenans*, *S. oneidensis*, and *D. chlororespirans* were 78 (except cells plus Fe(III) plus donor incubations), 17, 71, 65, and 43 h, respectively. The numbers in the parentheses indicate the time (h) at maximum accumulation of each nitroso metabolites. Results are the means of triplicate analyses.

[a] The results were reproduced from Kwon and Finneran (2006).

[b] NT, not detected.

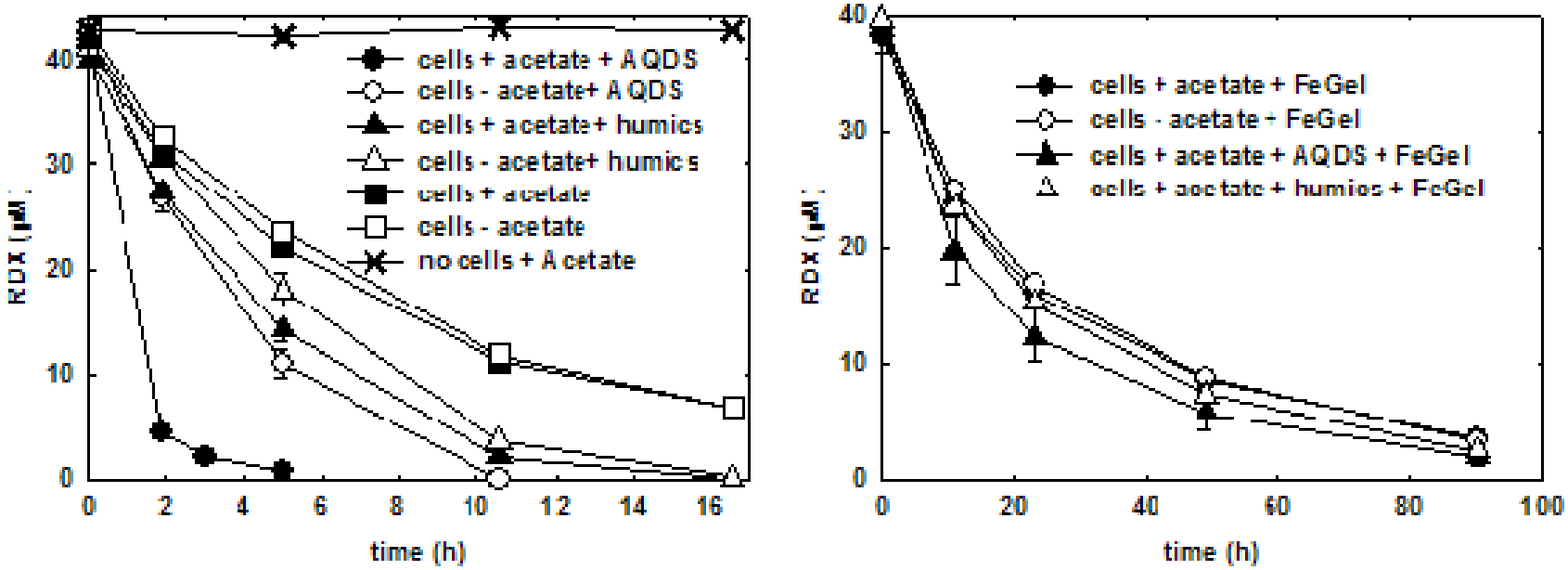

Figure 17. RDX reduction in the resting cell suspensions of *Anaeromyxobacter dehalogenans* strain K with or without extracellular electron shuttling compounds (acetate as the sole electron donor); Results are the means of triplicate analyses and bars indicate one standard deviation.

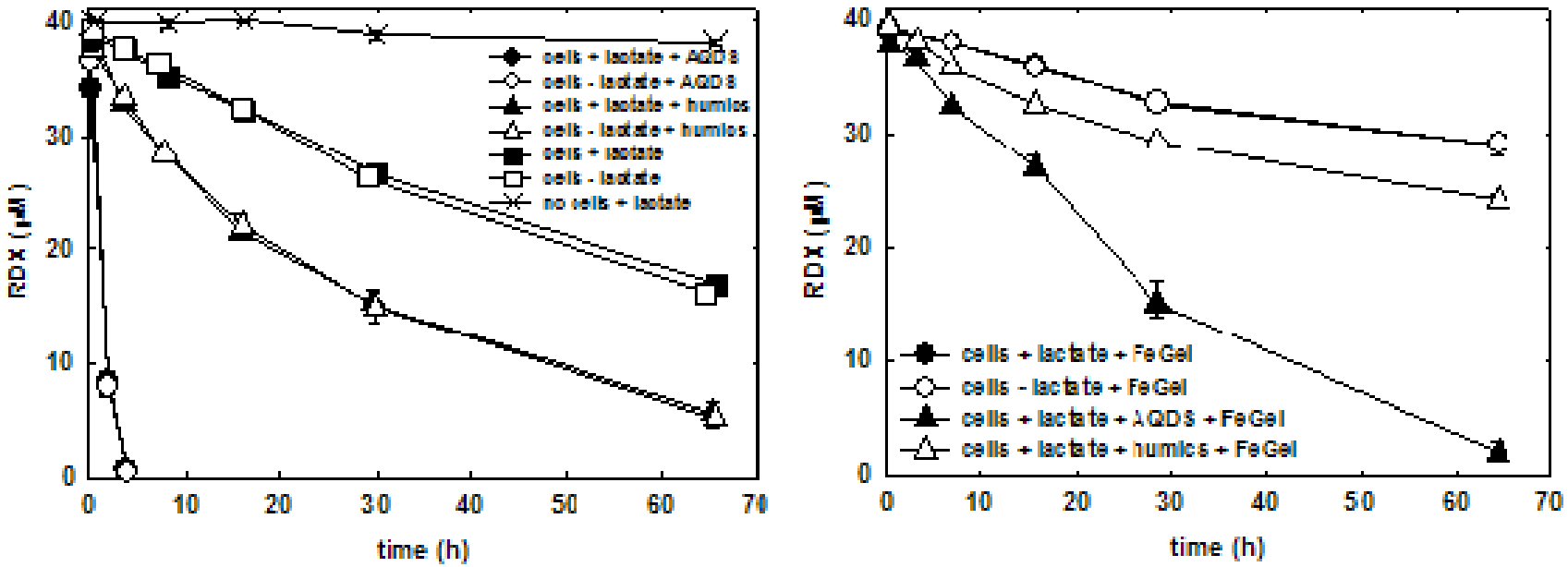

Figure 18. RDX reduction in the resting cell suspensions of *Shewanella oneidensis* strain MR1 with or without extracellular electron shuttling compounds (lactate as the sole electron donor); Results are the means of triplicate analyses and bars indicate one standard deviation.

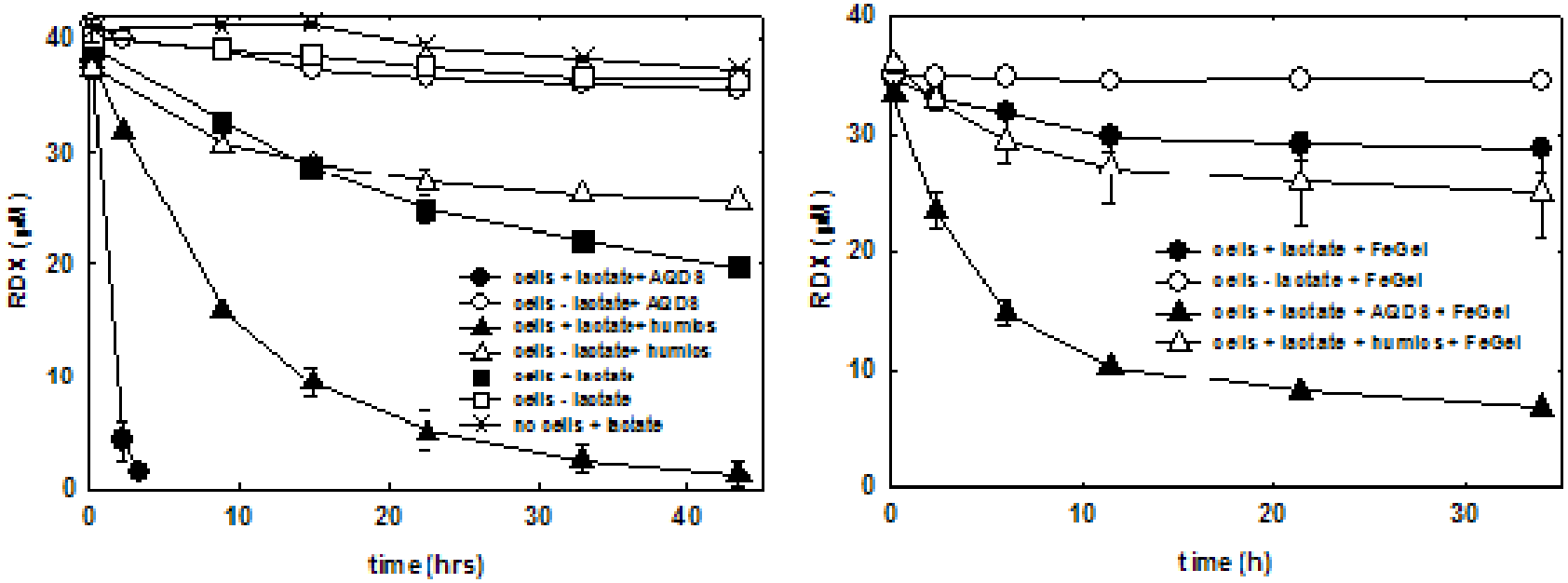

Figure 19. RDX reduction in the resting cell suspensions of *Desulfitobacterium chlororespirans* strain Co23 with or without extracellular electron shuttling compounds (lactate as the sole electron donor); Results are the means of triplicate analyses and bars indicate one standard deviation.

Experiments with Contaminated Sediment or Aquifer Material

The capacity of electron shuttle amendments in sediments to promote biological reduction of RDX was evaluated with RDX-contaminated aquifer material (aerated) from the Picatinny Arsenal in Northwestern New Jersey. RDX was degraded to some extent in all incubations except heat-sterilized incubations; however, transformation rates differed greatly among treatments. The short-term aeration did not limit microbial activity; anaerobic metabolism was almost instantaneous in all bottles. RDX was reduced most quickly in the presence of AQDS and HS; nitroso metabolites did not significantly accumulate in the presence of these electron shuttles. RDX was below detection limits in 20 and 45 days with AQDS and HS, respectively (Figure 20). RDX was not reduced or transformed in heat-sterilized incubations.

Acetate alone did not stimulate the same rate of RDX degradation; RDX remained through 65 days, and the nitroso metabolites accumulated to a greater extent (Figure 21). To date, the primary strategy for remediation of RDX in groundwater has been electron donor amendment to stimulate direct reduction by indigenous microorganisms. However, these data demonstrate that extracellular electron shuttling increases the rate and extent of RDX reduction without significant accumulation of unwanted intermediates; the nitroso metabolites were reduced more rapidly in the presence of electron shuttling compounds (Figure 21).

The difference in reduction rates mediated by AQDS versus HS mirrors what has been reported with pure culture studies (23). It is most likely due to the fact that AQDS is a relatively simple molecule that has readily accessible quinone moieties. HS are large and all quinone functional groups may not be equally as accessible to either the cells or the RDX. Further studies are underway to evaluate “raw” electron shuttles derived from natural organic matter and military smoke dyes, which may be the materials available for in situ application.

The rate of RDX degradation in the presence of AQDS or HS rapidly increased after 6 days and 25 days, respectively (Figure 20). RDX concentration did not decrease significantly in the absence of acetate (Figure 20). Only 20% of the RDX was removed in incubations without acetate during the first 65 days. This suggests that the availability of an electron donor may be a rate-limiting factor to degrade RDX in these sediments. However, the confidence interval was relatively high for the rate of RDX reduction in the acetate alone incubations; the error decreased greatly in the AQDS and HS amended incubations. This indicates that adding catalytic concentrations of electron shuttles stimulated RDX reduction in contaminated sediment, and that targeting a specific group of microorganisms (the Fe(III)/HS reducers) was more efficient than RDX transformation by other mechanisms (possibly including direct RDX reduction.)

The nitroso metabolites including MNX, DNX, and TNX only transiently accumulated in HS and AQDS amended sediment incubations in the presence of acetate (Figure 21). Due to the toxicity of these nitroso metabolites, their accumulation derived from RDX transformation is also of environmental concern. MNX in AQDS plus acetate incubations decreased rapidly after 10 days and the accumulation of DNX and TNX was not significant (Figure 21) as the final data points suggested that the compounds were decreasing (Figure 21). MNX and DNX in HS plus acetate incubations were below the detection limit in 50 and 62 days, respectively (Figure 21). TNX did not accumulate in the electron shuttle amended incubations; TNX concentrations were decreasing after 48 days (Figure 21).

These sequential reductions of RDX and its metabolites indicated that RDX in these incubations degraded progressively from RDX to MNX to DNX to TNX to (putatively) ring cleavage products. The nitroso metabolites accumulated more significantly in the absence of AQDS or HS. Although the MNX and DNX were being reduced in acetate only amendments, TNX was still accumulating after 65 days (Figure 21). These data indicate that electron shuttling decreases the total timeframe for complete nitramine transformation to the intermediate that is the last reductive metabolite prior to mineralization reactions (TNX).

The nitroso metabolites are not often identified in situ, which may be a consequence of the long durations between sampling and analysis in typical environmental monitoring programs. However, these data suggest that the metabolites do not accumulate when electron shuttling compounds are present; therefore, in humic-rich aquifer material it is unlikely that the nitroso metabolites could be quantified even if monitoring were more frequent.

Fe(II) in AQDS plus acetate incubations increased quickly to 4 mM within 20 days (Figure 22). Fe(II) in HS plus acetate incubations increased to 4.3 mM within 62 days. Fe(II) in acetate alone incubations increased to 3 mM with 62 days. Fe(II) did not significantly increase in the other treatments. The sediments contained a high concentration of bioavailable Fe(III) (3.93 mmol/kg) and groundwater samples did not have significant amount of soluble electron acceptors (e.g. SO_4^{2-} and NO_3^-) suggesting that Fe(III) was likely the most prevalent electron acceptor in these incubations.

RDX was reduced most rapidly after 5 days (Figure 22) and prior to the onset of significant Fe(II) accumulation (Figure 22, lower), which suggests that RDX was a) reduced prior to the onset of Fe(III) reduction by reduced extracellular electron shuttling compounds, or b) the Fe(II) generated transferred electrons very rapidly to RDX. Given that past results demonstrated that Fe(II) transfers electrons slowly to RDX (19, 23), and reduced electron shuttles transfer electron very rapidly to RDX (23) these data suggest that the RDX reduction within the first 17 days was primarily due to direct electron transfer from reduced AQDS and after that time RDX may be degraded by indirect electron transfer from Fe(II) as well as direct electron transfer from reduced AQDS (Figure 22). In either mechanism the RDX is ultimately reduced; however, direct electron transfer from electron shuttles indicates that this strategy will be successful in sediment with and without high concentrations of bioavailable Fe(III).

The capacity of sediments to promote chemical reduction of RDX was evaluated with RDX-contaminated aquifer material from the Picatinny Arsenal in Northwestern New Jersey. Initial experiments with active (non-sterilized) and heat-sterilized sediment incubations indicated significant RDX reduction (Appendix Figures). Adsorbed RDX was likely in equilibrium with the aqueous phase, and nitroso metabolites were detected throughout the experiments (data not shown). RDX loss in these incubations was attributed to RDX reduction by chemical and/or biological processes. RDX was reduced faster in the active incubations than in the sterilized incubations; 18 μM RDX was reduced in the heat-sterilized incubations within 12 days, while RDX was below 1 μM in the active incubations. The initial concentration of total Fe(II) was 120 μmol/kg, and the % reduced was 2.4; the reduced Fe(II) was at least partially surface-associated, and past results demonstrate RDX reduction by surface bound Fe(II) (19). These results suggest that indigenous microbial activity as well as chemical reduction capacity in the sediments can reduce RDX. In order to verify this, the aquifer sediments were aerated to remove their chemical

reduction capacity. The sediments were aerated under ambient air for 30 minutes and then incubated under anaerobic conditions. In the heat-sterilized and aerated incubation, RDX was not reduced in 12 days (Appendix Figures), which was consistent with experiments described above. These data suggest that chemical reducing capacity as well as biological activity by indigenous microorganisms in the aquifer sediment reduced RDX.

In order to evaluate the potential of electron shuttles to stimulate RDX and HMX reduction in co-contaminated material, sediments from the Joliet Weapons Depot were incubated under a variety of electron shuttle-amended conditions. The sediment samples contained 150 mg/kg RDX and 28 mg/kg HMX. Initial concentrations of RDX and HMX in equilibrium with groundwater were 180 and 10 μM, respectively.

One of three AQDS amended replicates reduced RDX faster than the other two (Figure 23). An initial concentration of 180 μM RDX degraded to 13μM within 15 days and then RDX decreased slowly through the end of the experiment, which is consistent with the Picatinny sediment data. The remaining two AQDS plus acetate replicates did not reduce RDX in the first 25 days; however, RDX was significantly reduced after 25 days. In the presence of acetate and 0.05 g/L HS approximately 23% RDX was reduced during the first 63 days.

The rates of RDX reduction were similar among the incubations with different concentrations of HS (within error range) suggesting that the HS concentration is not the rate limiting factor; in fact the RDX reduction rates with the lowest HS concentration were statistically indistinguishable from rates with higher HS concentration (Figure 24). 2mM acetate was re-amended to all acetate-containing incubations at day 63. The additional 2mM acetate rapidly increased RDX reduction rates in all incubations within 110 days. This rapid reduction of RDX after additional acetate amendments suggests that electron donor concentration limited RDX reduction.

Although the electron shuttles slightly increased the rate and extent of RDX reduction in this sediment, the microbial community that had been enriched in all incubations within the first 63 days was capable of RDX reduction (either direct or via Fe(II) or reduced electron shuttles) and adding acetate immediately stimulated activity.

In a previous aquifer sediment experiments (Picatinny – presented above), the initial acetate addition (2 mM) was enough to completely degrade the RDX in the sediments and the effects of the electron shuttling amendments were very apparent. However, the initial concentrations of RDX (150 mg/kg) and HMX (28 mg/kg) in the Joliet sediment samples were much higher than their water solubility (RDX: 40 mg/l; HMX: 6 mg/l); therefore, adsorbed mass may have continuously partitioned into the aqueous phase until equilibrium and more electron donor was required to degrade the RDX as it partitioned from the sediment.

The rates of RDX reduction among acetate-alone and electron shuttle amended incubation were similar (within error range), with the exception of the single AQDS-amended replicate, and no significant differences among the incubations with three different concentrations of HS were quantified. These results are likely because the shallow sediment samples already contained high amounts of natural HS relative to the Picatinny material, which were devoid of native HS, based on HS extraction (Figure 25). The Joliet samples were shallow drainage basin sediments which

had surface runoff from nearby soils; soils typically have higher HS content than corresponding aquifer material (57) and HS extracted from soil typically had a greater electron-accepting capacity than HS extracted from aquatic sediments (52).

The shallow sediment samples contained various root fragments which could be HS sources, so although three different concentrations of HS were amended, RDX degradation rates were similar with each other. In addition, RDX degradation rates in two of the three HS amended incubations were not different from the acetate only amended incubations. However, the single replicate with AQDS that was completely reduced within 23 days was comparable in all respects (degradation rates, metabolite dynamics) to the Picatinny sediment, which suggests that electron shuttle amendment is a reasonable strategy to increase cyclic nitramine biodegradation.

The initial HMX concentration in the aqueous phase of incubations was approximately 10 μM (Figures 23 and 24). HMX in all incubations was stable at 10 μM for 75 days despite the additional amendment of acetate at 63 days. HMX was not reduced until RDX was at least 75% removed, indicating that RDX must first be degraded prior to the onset of HMX reduction. It is likely that the same microbial community catalyzes RDX and HMX transformation; however, the additional nitro group on HMX and its decreased solubility likely inhibit reduction when a more accessible molecule (including RDX) is available. In the single replicate that reduced RDX most quickly, HMX degradation began after 25 days once the RDX was removed. HMX degradation stagnated until the second acetate amendment and it was completely reduced at 200 days (Figure 23). HMX reduction in HS amended incubations was also similar. Significant HMX reduction began after RDX reduction (Figure 24). Although RDX decreased after 63 days, HMX decreased after 75 days.

One peak in the HPLC chromatograms was quantified at a shorter retention time than HMX (and therefore assumed to have a lower molecular weight than HMX) and increased during HMX reduction. HMX has a retention time of 16 minutes, while the unknown peak was detected around 13 minutes, which is different from RDX and its nitroso metabolites under the analytical conditions. The peak may have been an HMX transformation product, possibly a nitroso-HMX intermediate metabolite. A certified standard was only available for mono-nitroso HMX, which makes quantification of the entire reductive pathway difficult. Comparison of the chromatograms demonstrated that the unidentified peak was mono-nitroso HMX.

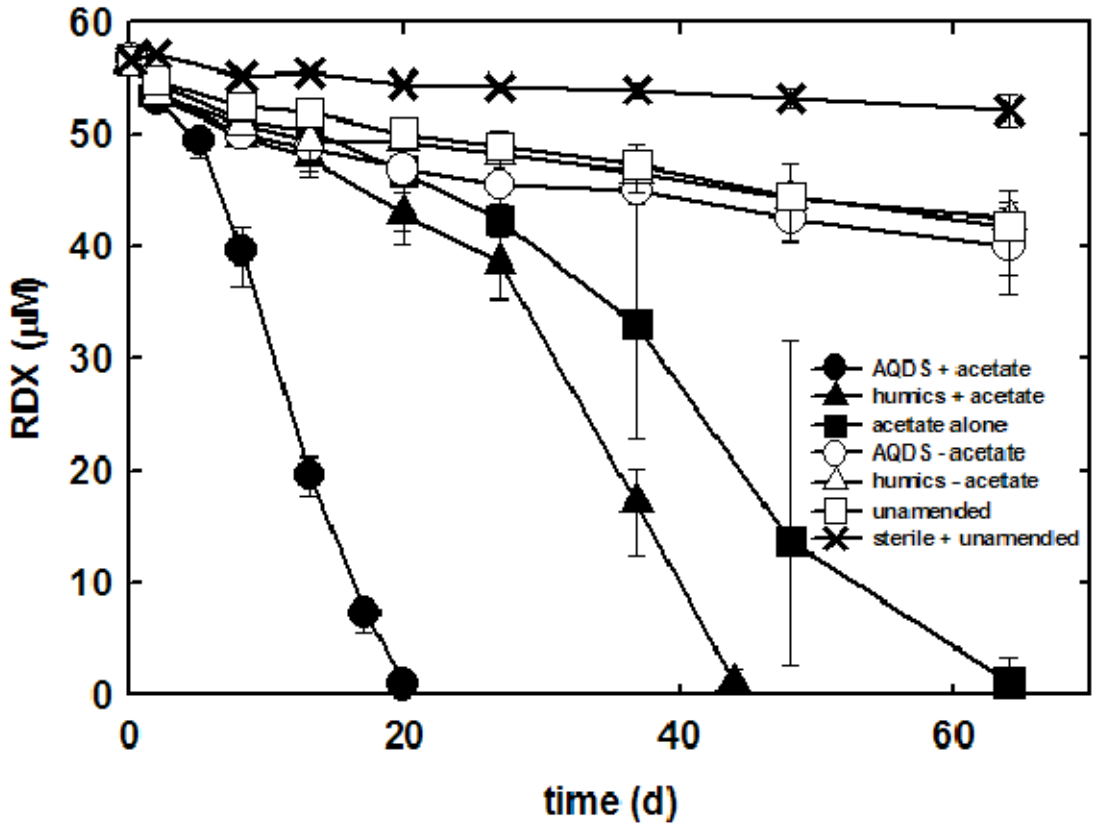

Figure 20. RDX reduction in contaminated aquifer material from the Picatinny Arsenal that has been amended with the electron shuttles AQDS (100 μM) or purified humic substances (HS) (0.15 g/L), or which has only been amended with the electron donor acetate (2 mM). The aquifer material was aerated before beginning the experiments. Results are the means of triplicate analyses and bars indicate one standard deviation.

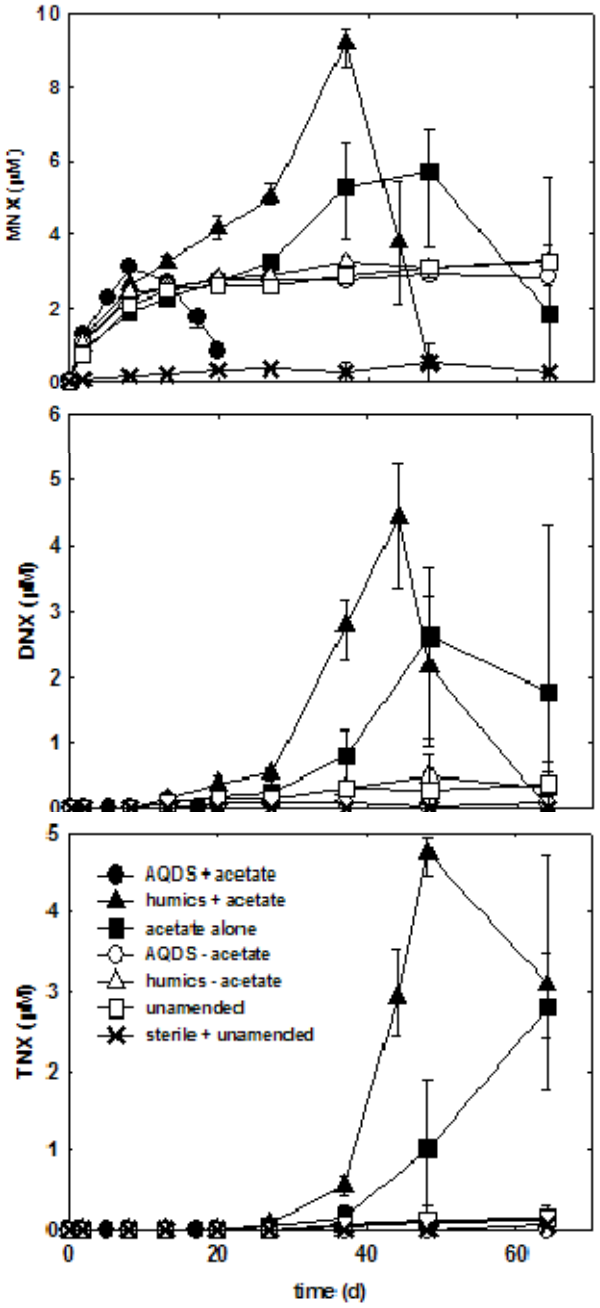

Figure 21. The production and reduction of MNX, DNX and TNX in contaminated aquifer material from the Picatinny Arsenal that has been amended with the electron shuttles AQDS (100 µM) or purified HS (0.15 g/L), or which has only been amended with the electron donor acetate (2 mM). The aquifer material was aerated before beginning the experiments. Results are the means of triplicate analyses and bars indicate one standard deviation.

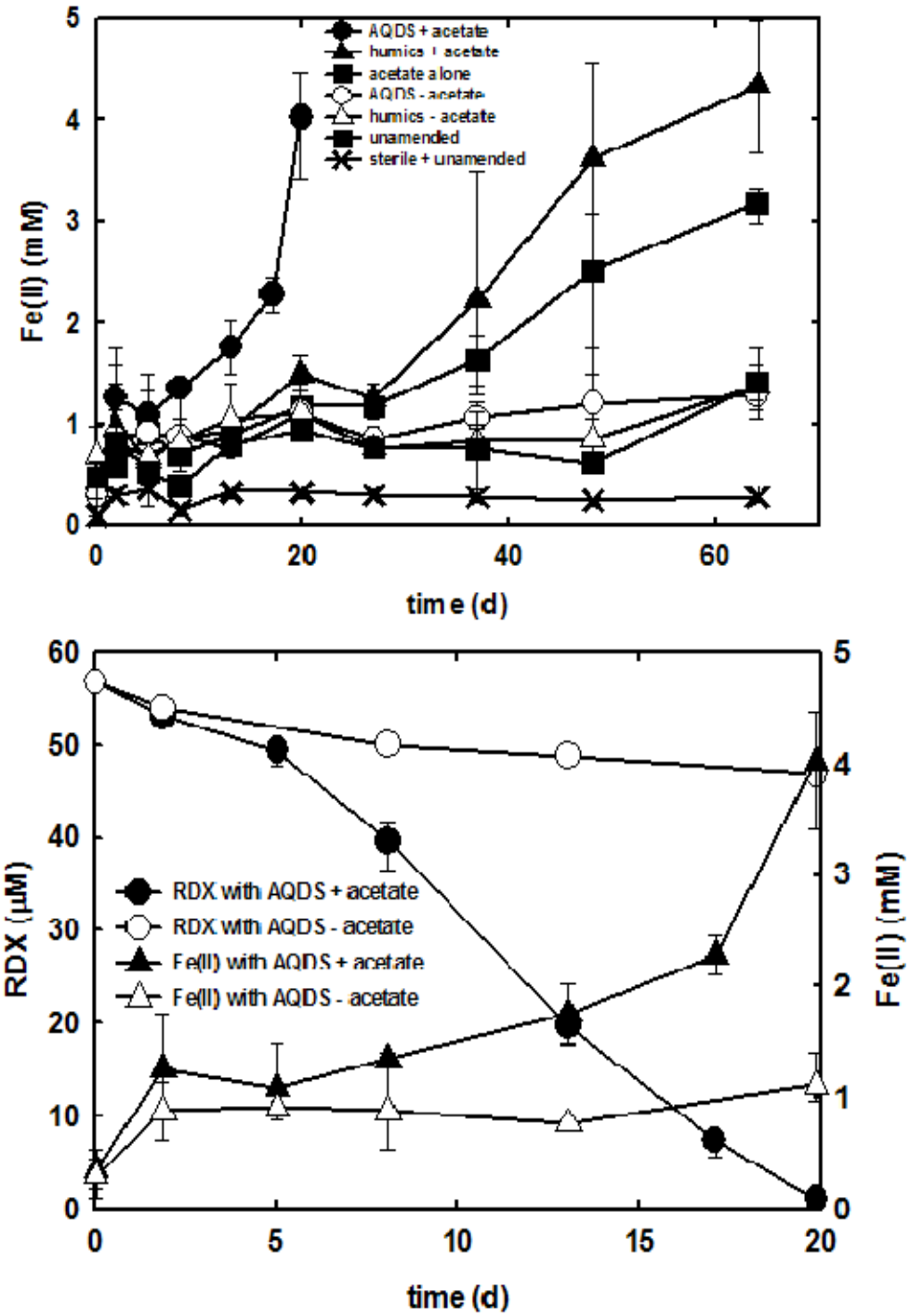

Figure 22. Fe(II) production in contaminated aquifer material; the comparison of RDX reduction and Fe(II) production for the first 20 days in contaminated aquifer material. Results are the means of triplicate analyses and bars indicate one standard deviation.

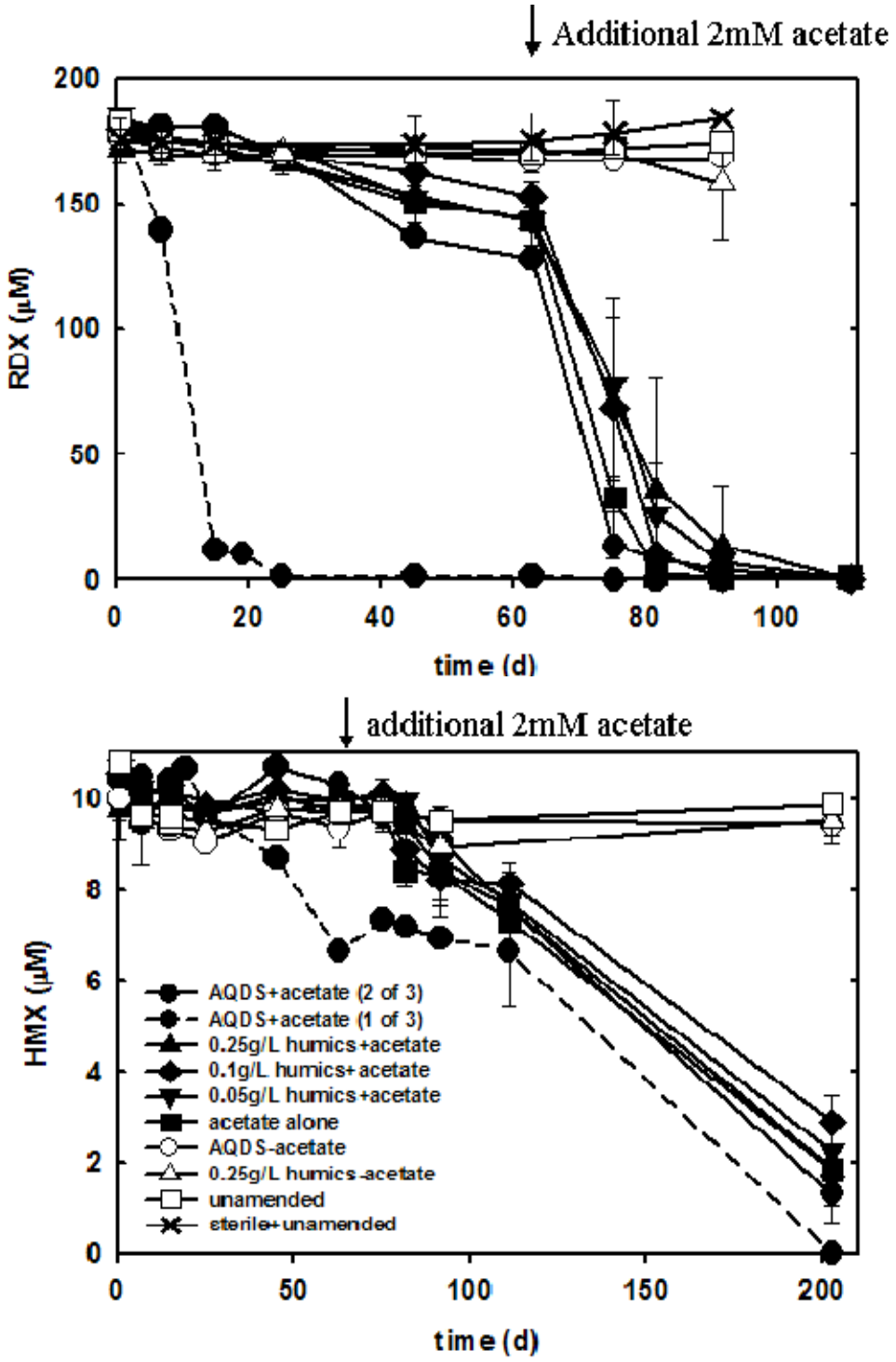

Figure 23. RDX and HMX reduction in co-contaminated sediments from the Joliet Weapons Depot that has been amended with the electron shuttles AQDS (100 μM) or purified HS (0.25, 0.1, and 0.05 g/L) or which has only been amended with the electron donor acetate (2 mM). Additional 2mM of acetate was amended at day 63. Results are the means of triplicate analyses and bars indicate one standard deviation.

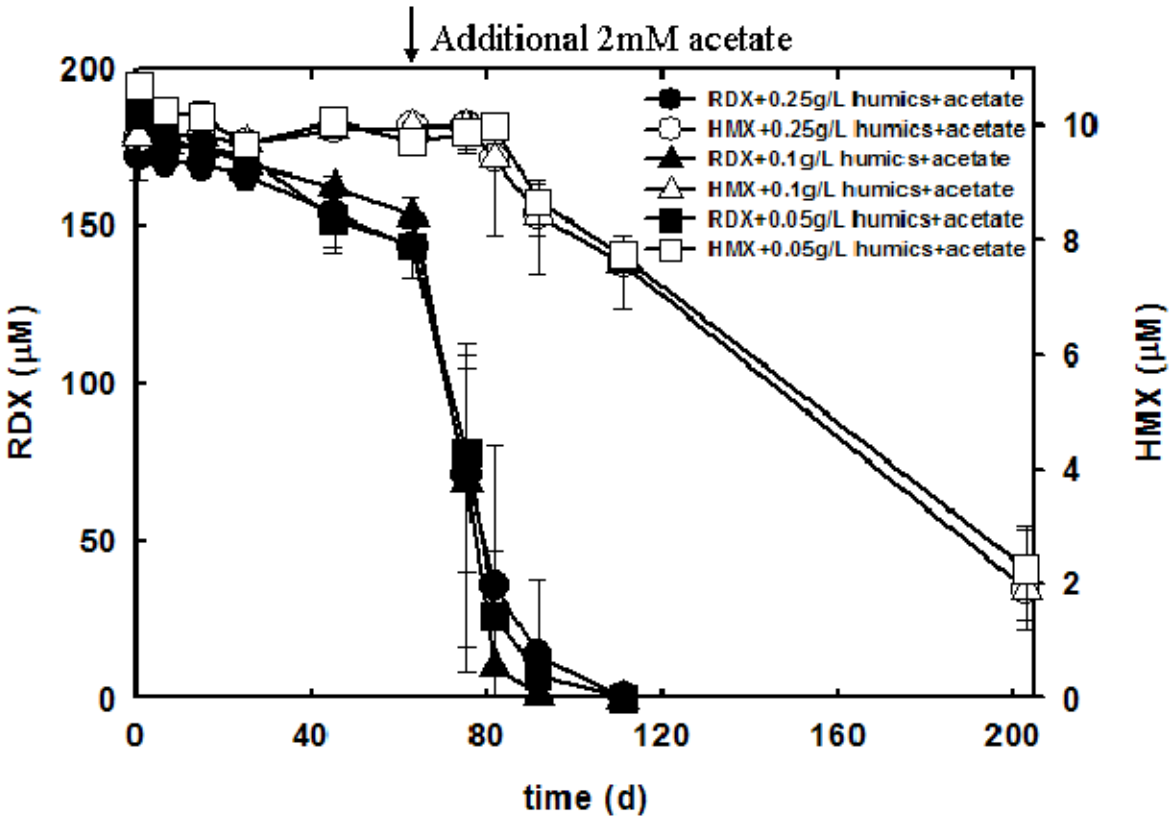

Figure 24. RDX and HMX reduction in co-contaminated sediments from the Joliet Weapons Depot that has been amended with acetate (2 mM) and three different concentrations of purified HS (0.25, 0.1, and 0.05 g/L). Additional 2mM of acetate was amended at day 63. Results are the means of triplicate analyses and bars indicate one standard deviation.

Figure 25. Raw humic substance extracts from the (left) Picatinny Arsenal and (right) Joliet Weapons Depot aquifer material and sediment, respectively. The clear tube on the left demonstrates little or no humics content in the Picatinny material, while the tube on the right represents the typical dark color of high humics content recovered from the Joliet sediment.

X. Conclusions

This project had two primary goals: 1.) understanding the role that Fe(III)- and electron shuttle-reducing microorganisms have in RDX and HMX biotransformation, and 2.) characterizing whether extracellular electron shuttling amendments are a reasonable alternative for in situ cyclic nitramine bioremediation. The experimental data demonstrated that extracellular electron shuttles reduce RDX via its nitroso intermediates and ultimately produce the labile carbon compound formaldehyde. Electron shuttles directly reduced RDX and the nitroso compounds, as well as facilitated RDX reduction via Fe(III) → Fe(II) by accelerating Fe(III) reduction. Electron shuttles were equally efficient in the presence or absence of cell mass, suggesting that it is possible that mixed biotic-abiotic reactions drive RDX reduction in mixed environmental systems.

Fe(III)-reducing microorganisms also reduced RDX. The data presented above are the first reports of RDX reduction by the four genera of "model" Fe(III)-reducing microorganisms. The cells directly reduced RDX at different rates and to different extents, and the nitroso metabolite distribution varied amongst all cells tested. The data suggested that Fe(III)-reducing microorganisms are relevant to RDX biotransformation, and that bioremediation strategies predicated on their metabolism will be successful in several environments, as Fe(III) reducers have been identified in all natural systems (10, 32).

The hypotheses that drove this project all relate to the central, underlying hypothesis that Fe(III) reduction, and the organisms that catalyze the reactions, are the primary process and microbial community with respect to in situ cyclic nitramine biodegradation. These and other SERDP projects have demonstrated that iron geochemistry catalyzes RDX transformation and that Fe(III)-reducing microorganisms are co-localized with RDX biodegradation in sediment. However, this project was the first to specifically conclude that Fe(III) reduction is the driving force, and that accelerating Fe(III) reduction (via electron shuttling) will stimulate RDX and HMX bioremediation. While more work is clearly necessary to further define the role of Fe(III) reduction and electron shuttling in cyclic nitramine bioremediation, this project has demonstrated that it is a critical process under natural conditions, and that stimulating this process is a reasonable strategy to promote RDX and HMX biodegradation.

Summary of Major Accomplishments

1. *Demonstrate that electron shuttling compounds directly reduce RDX and HMX*. This is the first report of electron-shuttle mediated RDX and HMX reduction. These data suggest that abiotic interaction with electron shuttling compounds will limit the in situ lifespan of cyclic nitramines, and lead to innocuous end products such as formaldehyde (which will be readily degraded under prevailing in situ conditions.)

2. *Demonstrate that electron shuttling-compound-stimulated Fe(III) reduction increased the rate and extent of RDX and HMX biotransformation*. Although numerous reports demonstrate that electron shuttling increases the rate and extent of Fe(III) reduction, this is the first project specifically designed to demonstrate that electron-shuttle-mediated

Fe(III) reduction will promote RDX and HMX biodegradation. This allows remediation engineers a tool to manipulate in situ Fe(III) reduction specifically for cyclic nitramine bioremediation.

3. *Demonstrate that multiple electron transfer pathways are functioning simultaneously in electron-shuttle amended systems.* This project identified multiple, overlapping electron transfer pathways that operate simultaneously with respect to RDX biodegradation. This is critical because the redundancies within the electron transfer pathways (e.g. Fe(II) → RDX or reduced shuttle → RDX) all lead to the same outcome, which is RDX biodegradation.

4. *Identify RDX reduction amongst the four "model" Fe(III)-reducing genera*. The four genera studied (*Geobacter*, *Shewanella*, *Anaeromyxobacter*, and *Desulfitobacterium*) are the most widely reported Fe(III)-reducing genera in subsurface environments, and are studied in great detail for their role in the processes associated with iron biogeochemistry and iron-based bioremediation strategies. This study demonstrated activity (RDX biodegradation) amongst all species tested within these genera, and demonstrated that activity can be increased using electron shuttles.

5. *Demonstrate that electron shuttle amendments in contaminated aquifer material increase the rate and extent of RDX degradation*. Contaminated aquifer material amended with AQDS or humic substances resulted in faster and more completely degradation of RDX than aquifer material amended with only electron donors. The latter strategy is the typical remediation alternative, suggesting that electron shuttles may increase the effectiveness of in situ remediation for reducible contaminants. In addition, these results may explain the persistence of RDX in groundwater in humic-poor soils.

6. *Demonstrate that RDX is reduced more rapidly past nitroso intermediates to innocuous end products in electron shuttle-amended systems*. Many "reductive" strategies for RDX end with the nitroso intermediates, which exhibit similar toxicity. Electron shuttles promote rapid production of formaldehyde without accumulating MNX, DNX, or TNX. Formaldehyde is readily mineralized by numerous microorganisms, which suggests that in situ electron shuttles will lead to faster and more complete RDX biodegradation than alternate technologies.

Environmental Relevance

These data suggest that extracellular electron shuttling increases the rate and extent of RDX transformation in contaminated aquifer material. Adding AQDS or HS increased RDX biodegradation and prevented significant accumulation of the nitroso metabolites relative to electron donor alone, which has been the primary strategy to date. In addition, the data demonstrated that material that contains natural humic material has the capacity to degrade RDX and HMX; however, RDX must be at least 75% reduced prior to the onset of significant HMX reduction. All reactions were catalyzed by native microorganisms, which based on alternate experiments were most likely known Fe(III)-reducing microorganisms.

These results are very different than recent data with fermentative microorganisms in which HS and AQDS have been investigated for their role in reductive transformation of explosive compounds (3, 4). The explosive TNT was accelerated by adding AQDS to fermentative cultures; however, TNT reduction is distinctly different than cyclic nitramine biodegradation as it interacts with more reduced compounds than RDX or HMX (3, 4). The data with a fermentative culture and RDX are less clear as the "increase" in reduction due to AQDS amendment is relatively small, and all reactions took several days. Past data associated with the work presented here demonstrated that reduced AH_2QDS directly reduced RDX in less than 24 hours.

Fermentative electron transfer to Fe(III) has been previously reported, and the cited data suggest that this non-respiratory electron transfer mechanism may also stimulate AQDS mediated electron shuttling to the explosives, though not to the same extent as reactions mediated by Fe(III)-reducing microorganisms. Both data are promising and contribute to the potential strategies for explosives residues; however, it is unclear whether the specific fermentative organisms utilized within these experiments are competitive in Fe(III)/electron shuttle-reducing environments contaminated with the explosive compounds. Future work is planned to identify the phylogenetic relatives of the microorganisms mediating the reactions in the environmental samples tested.

The chemical reduction capacity (as Fe(II)) that preexisted in the aquifer sediment promoted RDX reduction, which has implications for natural attenuation of the cyclic nitramines. The data in this study showed that the aquifer sediment samples contained 120 μmol/kg of Fe (II). The preexisting chemical reduction capacity in any aquifer or sedimentary system will strongly depend on in situ conditions and consumed reduction capacity will not be recoverable without additional treatment. Providing exogenous chemical reducing capacity to degrade contaminants will require introduction of artificial and possibly toxic chemicals to subsurface environments, which is unfavorable from a regulatory standpoint. The electron shuttle mediated strategy is more efficient and poses few additional environmental concerns.

Electron shuttle mediated RDX/HMX bioremediation will provide a simple, in situ approach. The results demonstrated that indigenous microorganisms in the sediment samples can reduce RDX/HMX effectively via stimulating electron shuttle and/or Fe(III) reduction. The fact that RDX/HMX were reduced with Fe(III) or HS reduction suggests that a major group of indigenous microorganisms (Fe(III) and/or HS reducing microorganisms) can facilitate these reactions with minimal amendment.

HS are natural compounds and at the extremely low concentrations tested pose no inherent threat to the environment (48). Fe(III)- and humic-reducing microorganisms are ubiquitous (10); therefore, the strategy using electron shuttles and Fe(III)- and humic-reducing microorganisms will work at sites with variant geochemical conditions. Electron shuttling mediated RDX and HMX bioremediation may become a reasonable strategy to be applied to explosives contaminated sediments. In addition, these reactions may help remediation scientists and engineers understand natural attenuation reactions that contribute to contaminant transformation in anaerobic, subsurface environments.

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XII. Appendices

XI-1. Additional Experimental Data

Summary of the Mass Balance Approach using Uniformly Radiolabeled [^{14}C]-RDX

The Waterways Experiment Station (WES) of the U.S. Army Corps of Engineers (USACE) in Vicksburg , MS, performed follow up analytical testing of the sediment experiments described in the text in Figures 19-21. The sediment bottles were spiked with uniformly radiolabeled [^{14}C]-RDX and incubated with the same amendments. Results are presented below in Table XI-1-1 for the summary data as recovery in solid, liquid, or gas. Samples in series 1 and 3 were amended with AQDS and humic substances, respectively. Sample series 5 was unamended; sample series 6 was acetate amended. The control series was a sterile control (heat killed).

The summary data indicate that mineralization was most complete in the AQDS or humics amended incubations. Although there was not 100% recovery of the spiked carbon in most of the sediment incubations, the highest gas-phase ($^{14}CO_2$) recovered was in the electron shuttle amended incubations. These data suggest mineralization was most complete in the presence of electron shuttles, in general agreement with chemical data previously reported.

	$^{14}CO_2$ Recovery (%)			
Sample	**Gas**	**Liquid**	**Soil**	**Total**
Control 1	3.55	81.50	52.38	137.43
Control 2	0.08	52.50	36.42	88.99
Sample 1-1	1.60	5.63	10.54	17.76
Sample 1-2	26.55	3.50	11.35	41.40
Sample 1-3	144.00	4.50	9.94	158.44
Sample 3-1	41.83	4.50	10.20	56.53
Sample 3-2	1.20	2.38	9.52	13.10
Sample 3-3	3.30	NA	9.45	12.75
Sample 5-1	1.73	23.50	6.66	31.88
Sample 5-3	16.35	9.88	8.51	34.73
Sample 6-1	10.73	NA	7.15	17.88
Sample 6-2	13.83	9.88	5.89	29.59
Sample 6-3	0.00	11.75	7.50	19.25

NA = Not Available

Table XI-1-1. Recovery of [^{14}C]-RDX in solid, liquid, or gas phases of spiked sediment bottles.

XI-2. List of Technical Publications

Peer-Reviewed Papers or Manuscripts

1. Kwon, M.J., and K.T. Finneran, 2007, The Influence of Abiotic versus Biotic Degradation Mechanisms on RDX Metabolite Production mediated by Extracellular Electron Shuttling Compounds, manuscript to be submitted to AEM
2. Kwon, M.J., and K.T. Finneran, 2007, Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) Biodegradation Kinetics amongst several Fe(III)-Reducing Genera, manuscript submitted to Soil and Sediment Contamination: An International Journal
3. Kwon, M.J., and K.T. Finneran, 2006, Microbially-Mediated Hexahydro-1,3,5-trinitro-1,3,5-triazine Biodegradation by Extracellular Electron Shuttling Compounds, Appl. Environ. Microbiol., 72(9), 5933-5941

Abstracts or Invited Presentations

1. Mixed Biological-Abiotic Degradation of the Cyclic Nitramine Explosives RDX and HMX, Johns Hopkins, Department of Geography and Environmental Engineering, May 11, 2007
2. Extracellular Electron Shuttle Mediated biodegradation of the Explosives RDX and HMX in Pure Culture and Contaminated Aquifer Material, University of Wisconsin Madison, Department of Civil and Environmental Engineering, November 14, 2006
3. Extracellular Electron Shuttle Mediated biodegradation of the Explosives RDX and HMX in Pure Culture and Contaminated Aquifer Material, Northeastern University, Department of Civil and Environmental Engineering, October 20, 2006
4. Extracellular Electron Shuttling in Bioremediation and Biotechnology, AEHS International Conference on Contaminated Soil, Sediment, and Groundwater, Amherst, MA, October 16 – 19, 2006
5. Kwon, Man Jae, and K.T. Finneran, Multiple Electron Transfer Pathways for RDX and HMX in the Presence and Absence of Bioavailable Fe(III), ASM General Meeting, Toronto, Ontario, Canada, May 20-24, 2007
6. Finneran, K.T. Extracellular Electron Shuttles in Bioremediation and Biotechnology, Battelle Conference on In Situ and On Site Bioremediation, Baltimore, MD, May 6-10, 2007
7. Kwon, Man Jae, and K.T. Finneran, Bioremediation of Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and 1,3,5,7-tetranitroperhydro-1,3,5,7-tetrazocine (HMX)-Contaminated Sediments, AEHS International Conference on Contaminated Soil, Sediment, and Groundwater, Amherst, MA, October 16 - 19, 2006

8. Kwon, Man Jae, S. Drew, and K.T. Finneran, Extracellular Electron Shuttle Mediated Biodegradation of Hexahydro-1,3,5-trinitro-1,3,5-Triazine (RDX) in RDX-Contaminated Aquifer Material, ASM General Meeting, Orlando, FL, May 21-25, 2006
9. Kwon, Man Jae, S. Drew, and K.T. Finneran, Humic Substances Mediated Biodegradation of Hexhydro-1,3,5-trinitro-1,3,5-triazine (RDX) and Other Explosives Residues in Sediment and Water, Battelle International Conference on Recalcitrant and Chlorinated Compounds, Monterey, CA, May 22 – 25, 2006
10. Finneran, K.T. Humic-substance and Iron Mediated Degradation of Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX): the Role of Electron Shuttles in Bioremediation. AEHS Annual West Coast Conference on Contaminated Soils, Sediment, and Water, San Diego, CA, March 13 – 16, 2006
11. Kwon, Man Jae and K.T. Finneran, Humic Substances Mediated Biodegradation of Hexhydro-1,3,5-trinitro-1,3,5-triazine (RDX), AEHS International Conference on Contaminated Soil, Sediment, and Groundwater, Amherst, MA, October 20 – 24, 2005
12. Kwon, Man Jae and K.T. Finneran, Humic Substances Mediated Biodegradation of Hexhydro-1,3,5-trinitro-1,3,5-triazine (RDX), SERDP/ESTCP Partners Meeting, Washington, DC, November 30 – December 1, 2005
13. Kwon, Man Jae and K.T. Finneran, Humic Substances Mediated Biodegradation of Hexhydro-1,3,5-trinitro-1,3,5-triazine (RDX), ISSM/IJSEB Joint International Conference on Subsurface Microbiology, Jackson, WY, August 20 – 26, 2005