FINAL REPORT

Combined Biological and Chemical Mechanisms for Degradation of Insensitive Munitions in the Presence of Alternate Explosives

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The insensitive munitions (IM) 2,4-dinitroanisole (DNAN) and nitroguanidine (NQ) have been used in newly developed explosives as a replacement for the more sensitive munition, 2,4,6-trinitrotoluene (TNT). These new formulations are less sensitive to external shocks, for example heat or strikes, and as such they are safer to store, transport and use in battle conditions. The objective of this research was to quantify the rate and extent to which IM can be transformed by combined biological and chemical reactions with iron and Fe(III)-reducing microorganisms.

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ABSTRACT

Objectives

The insensitive munitions (IM) 2,4-dinitroanisole (DNAN) and nitroguanidine (NQ) have been used in newly developed explosives as a replacement for the more sensitive munition, 2,4,6-trinitrotoluene (TNT). These new formulations are less sensitive to external shocks, for example heat or strikes, and as such they are safer to store, transport and use in battle conditions. The objective of this research was to quantify the rate and extent to which IM can be transformed by combined biological and chemical reactions with iron and Fe(III)-reducing microorganisms.

Technical Approach

The work is an investigation of chemical and biological degradation of DNAN and NQ by ferrous iron or hydroquinones using several metabolically diverse microorganisms: *Geobacter metallireducens* strain GS-15, *Shewanella oneidensis* strain MR-1, *Rhodobacter sphaeroides*, and *Clostridium geopurificans* strain MJ1. Fe(III), as the most common element found in subsurface environment, can be employed for *in-situ* remediation when Fe(III)-reducing microorganisms are active and generating Fe(II). Humic material present in the aquifer can also be used to promote degradation of insensitive munitions, hence quinone/hydroquinone couple was used in the study to represent this group. Fe(III) reducers, such as *Geobacter* and *Shewanella*, are ubiquitous and competitive for available substrates, which makes them suitable for explosives bioremediation.

Results

Experimental results showed that DNAN can be readily reduced by chemical and biological processes at pH 7 within 24 hours, and at pH 8 and 9 in the order of minutes. At pH 6, organic ligands had to be used to stimulate degradation. DNAN and the highly energetic explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) are used together in one of the newly developed explosive formulations and their mixture can be degraded by the same chemical and biological reactions. NQ was less susceptible to reductive degradation and it was most easily degraded at alkaline pH by organically complexed Fe(II) within several days; therefore, NQ may persist in the anoxic subsurface environments.

Benefits

While the new munitions have not yet been detected outside of military test ranges, this work helps to evaluate environmental risks associated with potential contamination. The major benefit is the capacity to use a ubiquitous respiratory process, Fe(III) reduction, as the basis for a remediation strategy that uses both the biological and chemical electron transport processes as mechanisms.

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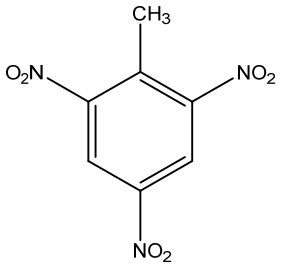
INTRODUCTION

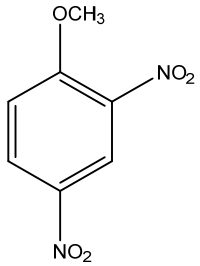
The Department of Defense (DoD) has developed several new explosive composites that contain typical compounds such as the cyclic nitramines but that have been updated with insensitive munitions (IM) including 2,4-dinitroanisole (DNAN), nitroguanidine (NQ), and 3-nitio-l,2,4-triazol-5-one (NTO). Both DNAN and NQ have been proposed as a replacement for 2,4,6-trinitrotoluene (TNT), which has failed to meet up-dated safety requirements. NTO has been proposed as a replacement for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). The use of IM in explosive formulations results in increased stability and improved safety standards for the transport and storage of explosives to avoid accidental detonations. It is crucial to understand their fate and transport in subsurface environments as they can pose a significant hazard to humans and wildlife.

Chemical structures of compounds commonly used in explosives formulations and their physicochemical properties are presented in Table 1.1. DNAN and TNT have two and three nitro groups attached to an aromatic ring structure respectively, while NQ has only one nitro group attached to a nitrogen atom. Highly energetic compounds, RDX, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), and NTO, have additional nitrogen atoms incorporated into ring structure.

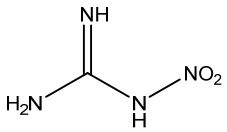
Table 1.1. Molecular formulas, chemical structures, molecular masses, aqueous solubility, and midpoint reduction potentials for the primary explosives and insensitive munitions.[1] Reproduced from DOI: 10.1039/C4EW00062E with permission from the Royal Society of Chemistry.



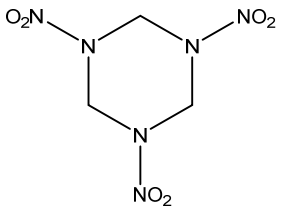
TNT
$C_7H_5N_3O_6$



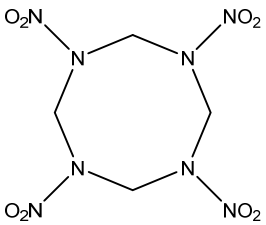
DNAN
$C_7H_6N_2O_5$



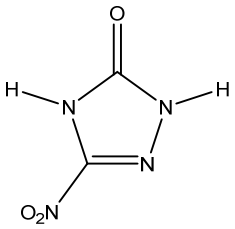
NQ
$CH_4N_4O_2$



RDX
$C_3H_6N_6O_6$



HMX
$C_4H_8N_8O_8$



NTO
$C_2H_2N_4O_3$

Explosive	Molecular mass [g mol^{-1}]	Solubility in water [g L^{-1}]	Reduction potential [V]
RDX	222.12	38.9 mg L-10 (low to negligible)	-0.55
HMX	296.16	6.63 mg L-1 (low)	-0.66
TNT	227.13	Insoluble	N/A
NTO	130.07	12.8	N/A
DNAN	198.13	Sparingly soluble	-0.40
NQ	104.07	3	-0.70

Highly oxidized $-NO_2$ groups have an electron-withdrawing effect and any attack on explosive molecules would preferably take place at these external functional groups. In TNT, four functional groups increase resonance effect of electron cloud around the ring, contributing to the increase in its chemical stability.[2] As such, TNT is resistant to mineralization and its aromatic ring stays unaffected while the transformation occurs only at the functional groups.[2] RDX and HMX are less stable than TNT. When the functional groups on cyclic nitramines are attacked, the inner C-N bonds become very weak and the molecules spontaneously decompose to eventually yield N_2O and CO_2 .[2]

There is an extensive literature on RDX and HMX degradation under both aerobic and anaerobic conditions carried by different microorganisms;[2,3] however, TNT has been reported to transform only under strictly anaerobic conditions.[2,3] Several aerobic bacteria are capable of utilizing RDX and HMX as a nitrogen and carbon source.[2,3] Considering the oxidized state of explosives molecules and the contamination profile in the subsurface, this study focuses on the anaerobic approach as it would be of greater environmental significance.

RDX and HMX demonstrated that these energetic compounds can be degraded via reduction of nitro groups and subsequent ring cleavage.[4-6] Same degradation mechanism, reduction of nitro groups, has been reported for TNT,[2,7,8] except that its aromatic ring stays intact. Nitro functional groups in RDX and HMX are attached to nitrogen atoms incorporated into the ring structure and once the functional groups are being reduced, the ring becomes unstable so that both

compounds can be mineralized to N_2O and CO_2.[2,6,9] TNT, on the other hand, has nitro groups attached to an aromatic ring that remains unaffected after reduction of functional groups; however, aromatic amines and their azo, azoxy, acetyl and phenolic derivatives resulting from TNT degradation can be immobilized from the groundwater through irreversible binding to soil components.[2,7]

Little is known about environmental fate of IM. Several studies show that DNAN is toxic to bacteria,[10-12] and plants.[10] In addition, DNAN can be metabolized by mammals

to 2,4-dinitrophenol,[13] which has high acute toxicity.[14,15] NQ has low toxicity towards freshwater organisms,[16] however its degradation products, nitrosoguanidine, guanidine, cyanoguanidine, melamine, urea, and nitrite can be more toxic than the parent compound.[17] There are no data on IM toxicity to humans; however, nitroaromatic compounds such as explosives have a strong-electron withdrawing effect, which can result in toxicity, carcinogenity, and mutagenicity. None of IM has been identified as a specific groundwater hazard yet but, given their harmful properties, DoD requires crucial information on IM fate in the environment and bioremediation possibilities should they become an issue.

To date there are a few published studies showing DNAN degradation in wastewater streams, using anaerobic fluidized-bed bioreactors,[18] Fe/Cu bimetallic particles,[19] or zero valent iron.[12] Another study demonstrated DNAN transformation by co-metabolism of *Bacillus*, strain 13G, in artificially contaminated soil microcosms.[20] In all cases, DNAN was reduced through 2-methoxy-5-nitroaniline

(MENA) to 2,4-diaminoanisole (DAAN) by sequential reduction of the two nitro groups to amines. Some reactive intermediates from described reduction reactions have been reported to form azo-dimers,[21,22] which, together with DAAN, could bind to soil similarly to TNT degradation products.[7] A bacterial consortium of *Pseudomonas* sp., strain FK357, and *Rhodococcus imtechensis*, strain RKJ300, was able to aerobically degrade DNAN via an intermediate 2,4-dinitrophenol (DNP), which was utilized by strain RKJ300 as a carbon source.[23] Additionally, some studies demonstrate that DNAN can be adsorbed to different types of granular activated carbon (GAC)[24] and lignin.[25]

Fewer reports are available on NQ degradation. Aerobic *Variovorax* strain VC1 can utilize NQ as a sole nitrogen source when an additional carbon source is provided, yielding ammonia, nitrous oxide and carbon dioxide.[26] A different study reports aerobic NQ degradation to nitrourea by microbial cultures enriched from activated sludge, soil and compost.[27] At present, there is no peer-reviewed literature available on anaerobic NQ degradation; therefore, research presented in this report may expand our understanding of its environmental fate.

NTO, as the least studied compound, have been reported to biodegrade aerobically in the mixture with DNAN and NQ by several enrichment cultures[27] and to adsorb to soil.[28] Recent study shows anaerobic degradation of NTO to 3-amino-1,2,4-triazol-5-one (ATO) via reduction of a nitro group by soil microbial communities, followed by aerobic mineralization to NO_2^- and NO_3^-.[29]

Many microorganisms can successfully degrade explosives in *ex-situ* systems, where optimal conditions for mineralization or immobilization of explosives are provided.[2] Given the effectiveness of microbial transformation and the fact that many tested bacteria are soil or wastewater sludge microorganisms,[2] the next step to improve remediation technology is to focus on *in-situ* methods as they are more cost-effective and easier to implement. While several microorganisms are capable of respiring nitramine compounds,[30-32] their effectiveness may be limited in the subsurface, where other environmental factors such as pH, temperature, or availability of nutrients may affect biodegradation. Selected bacteria strains will most likely have poorer performance in the field compared to laboratory conditions; therefore, it is important to promote the growth of naturally occurring microbial communities which can catalyze chemical reactions in the subsurface to degrade contaminants *in-situ*. Combining biological and chemical reactions increases degradation rates compared to the use of only biological or only chemical approaches.[4-6,9] Also, promoting microbial activity of microorganisms that are more widely distributed and less specialized results in development of bioremediation strategies that can be applied for a variety of environments. One of the most recent direction in explosives and IM bioremediation is to focus on coupled biological-chemical reactions mediated by Fe(III)-reducing microorganisms,[4,5] which are common in subsurface environments.[33] These microbes can reduce iron minerals and electron shuttles, such as humic substances, found in abundance in nature. Electron shuttling compounds can accept electrons from microbial respiration and

reduce Fe(III).[34] Resulting ferrous iron can transfer electrons to nitramine and nitroaromatic explosives promoting degradation (Figure 1.1). Re-oxidized shuttles can be recycled so they again accept electrons and transport them among microbes, iron minerals, and contaminants. Both RDX and HMX were successfully degraded by biologically reduced Fe(II) and electron shuttles.[4] Due to the same reductive transformation mechanisms (reduction of nitro group) between nitramine and nitroaromatic explosives, anaerobic biodegradation via electron shuttles has been suggested as an effective strategy for clean-up of future DoD sites contaminated with IM.

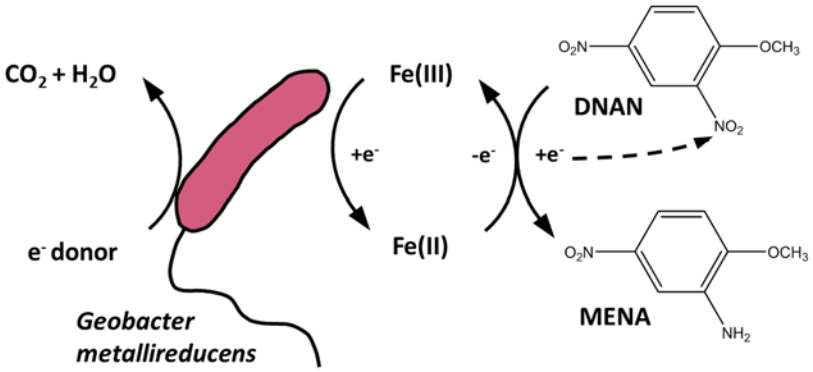


Figure 1.1. Conceptual model of mixed biotic-abiotic reactions between a Fe^{3+} reducing microorganism and DNAN using ferric/ferrous iron as an extracellular electron shuttling molecule. [1] Reproduced from DOI: 10.1039/C4EW00062E with permission from the Royal Society of Chemistry.

This study investigated mechanisms of anaerobic DNAN and NQ degradation by mixed biological-abiotic reactions with ferrous iron and hydroquinone at different pH.

Geobacter metallireducens, strain GS-15, was used as a model Fe(III)-reducing microorganism to simulate in-situ subsurface conditions. Several other metabolically diverse bacteria were investigated to demonstrate the possibility of IM degradation mediated by a variety of microorganisms under different environmental conditions. Finally, I evaluated the composition of microbial communities present in sediments and water samples from detonation tests so that future bioremediation strategies can be tailored to the unique biochemistry of each site. The results of this study may serve as a guidance in designing remediation strategies for groundwater, soil, surface water, and industrial wastewater.

To understand how combined chemical and biological reactions influence degradation of the IM compounds DNAN and NQ, the following specific objectives were addressed in this report:

a) Quantify the kinetics of DNAN and NQ degradation by biological versus chemical transformation mechanisms in the presence and absence of competing molecules found in explosive composites.
b) Determine the intermediates and final products of IM degradation.
c) Characterize the mixed "biological-abiotic" degradation mechanisms in controlled system.
d) Characterize microbial communities present in contaminated aquifer material.

These objectives were met by testing the following hypotheses:

1) Ferrous iron will directly reduce the nitro functional groups on DNAN and NQ.

2) Chemical and biological degradation of DNAN and NQ will proceed under anoxic conditions.
3) Chemical transformation kinetics will be faster than biological transformation kinetics; mixed biotic-abiotic reactions will degrade DNAN and NQ at rates at least double of the rates of individual mechanisms alone.
4) RDX will inhibit DNAN reduction at all conditions.
5) Bacterial communities present in explosives-contaminated aquifers will have the ability to degrade IM.

MATERIALS AND METHODS

Chemicals

DNAN was obtained from Alfa Aesar, MENA and iMENA were provided by Sigma Aldrich, and DAAN by Fluka. The stock solutions of DNAN and its transformation products, due to their low water solubility, were prepared by dissolving analytes in methanol. NQ was obtained from Sigma Aldrich and it was dissolved in water. RDX solution in acetonitrile was provided by US Army Corps of Engineers, Waterways Experiment Station. RDX was recovered from the solution by volatilizing acetonitrile with nitrogen gas and then dissolving solid RDX in water. RDX stock was filtered using a 0.2μm polytetrafluoroethylene (PTFE) syringe filter in order to remove undissolved RDX solids prior to amendment into experimental samples. PTFE filters did not retain neither RDX nor DNAN or NQ. Certified RDX standards were obtained from VWR and used to verify RDX stock concentration. Products of RDX degradation, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), 1,3-dinitroso-5- nitro-1,3,5- triazine (DNX), 1,3,5-trinitroso-1,3,5- triazine (TNX), methylenedinitramine (MEDINA), and 4-nitro-2,4-diazabutanal (NDAB), were sythesized by SRI International. Formaldehyde was obtained from AccuStandard and 4-dinitrophenylhydrazine (DNPH), used for formaldehyde derivatization, was obtained from AmChemteq. Ferrous chloride was provided by Sigma Aldrich. Iron(II) stock solution was prepared in the glove box, filled with gas mix containing 95% N_2 and 5% H_2, by dissolving ferrous chloride in hydrochloric acid at pH 2.5.

Anthraquinone-2,6-disulfonate (AQDS) was obtained from Sigma Aldrich. Reduced AH_2QDS stock was prepared by chemical reduction of AQDS using palladium pellets and sparging the liquid volume with hydrogen gas for one hour. AH_2QDS was added to experimental samples through 0.2µm PTFE syringe filter. HPLC grade methanol was obtained from VWR. All other chemicals are of reagent grade quality or higher.

Abiotic Degradation Study

DNAN and NQ degradation by Fe(II), organically complexed Fe(II), and hydroquinone

Transformation of IM under reducing conditions was tested within a pH range from 6.0 to 9.0. The first phase focused on degradation by ferrous iron alone. Experimental bottles were buffered with 30mM MES at pH 6.0, 30mM HEPES at pH 7.0 and 8.0, and 30mM CHES at pH 9.0. All bottles were degassed with nitrogen. Then 100 µM of DNAN or NQ and 1.5mM of Fe(II) was added to the experimental bottles. Samples aliquots were taken multiple times over the course of the experiments for Fe(II) and IM analysis. Part of aliquot was injected into 0.5N HCl in order to acidify the sample and prevent further Fe(II) oxidation. Then Fe(II) concentration in acidified aliquot was measured using the ferrozine assay.[35] The rest of aliquot was quenched with 200mM ethylenediaminetetraacetic acid (EDTA), which is a strong metal chelating agent, and analyzed for DNAN or NQ. The second phase involved the use of organic ligands which can interact with ferrous and ferric iron to facilitate electron shuttling between iron and the explosives. Several ligands are known to complex Fe(II) and making it a stronger reductant due to their

preferential binding to Fe(III). These ligands decrease the redox potential for the Fe(II)/Fe(III) redox couple and enhance electron shuttling processes.[36] Other ligands can form unreactive complexes with Fe(II) by acting as chelators and thus they prevent IM reduction by Fe(II). The organic ligands used in this study were malic acid, trisodium citrate (NaCit), nitrilotriacetic acid (NTA), EDTA, oxalic acid, tiron, meso-2,3-dimercaptosuccinic acid (2,3-DMSA), 3,4-dihydroxybenzoic acid (3,4-DHBA), 2,3,4-trihydroxybenzoic acid (2,3,4-THBA), and gallic acid. The amount of each ligand required to complex 1.5mM iron(II) was adapted from the literature or it was calculated using Visual Minteq when data on iron-ligand complexation was not previously reported. Visual Minteq is a public domain equilibrium speciation model developed at KTH Royal Institute of Technology in Sweden. Concentrations of ligands varied from 2 to 10mM, based on the literature and calculated values. In the third phase, the effect of humic substances was evaluated for a potential use in IM degradation by using chemically reduced hydroquinone (AH_2QDS) as a model electron shuttling compound with structure similar to humic substances. AH_2QDS oxidation is a two electron transfer reaction compared to one electron transfer in Fe(II) oxidation, therefore only 0.75mM AH_2QDS was required to provide electron equivalents same as by using 1.5mM Fe(II).

DNAN degradation in the presence of RDX

DNAN and RDX are components of several newly developed explosive formulations so it is important to evaluate their degradation in a mixture of both

compounds rather than in single compounds studies. Reduction of both explosives was tested using 0.75, 1.5, and 3.0mM Fe(II) and 0.375, 0.75, and 1.5mM AH_2QDS at pH 7, 8, and 9. Experimental bottles were buffered with 30mM HEPES at pH 7 and 8, and with 30mM CHES at pH 9. After Fe(II) and AH_2QDS were added to bottles, the reactions were initiated with 100µM DNAN and 50µM RDX. Sample aliquots were taken periodically to measure analytes concertation's via HPLC analysis and Fe(II) via ferrozine assay. Aliquots used in HPLC analysis were quenched with 200mM EDTA.

Microbial Degradation Study

Microorganisms

All tested cultures were maintained in anoxic conditions established by sparging growth media with nitrogen and carbon dioxide passed through reduced cooper column to remove trace oxygen. Culture tubes were sealed with a butyl rubber stopper and aluminium crimp and autoclaved for 20 min at 120^{o}C.

Geobacter metallireducens strain GS-15, an iron(III)-reducing bacterium, was obtained from the University of Massachusetts at Amherst and was maintained using ferric citrate media and 20mM acetate as electron donor. A gas mixture of N_2:CO_2, 80:20 (v/v) was used to sparge ferric citrate media and flush headspace.

Shewanella oneidensis strain MR-1 (ATCC 700550), a facultatively aerobic bacterium with diverse respiratory capabilities, was obtained from the University of

Illinois. It was maintained using ferric citrate media and 20mM lactate as electron donor. Media was prepared in the same manner as for *G. metalliraducens*.

Photosythetic *Rhodobacter sphaeroides* ATCC® 17023™ was purchased from ATCC. It was maintained photoheterotrophically with ATCC Medium #550 reduced with 3% cysteine and with 20mM malate as electron donor. The culture was incubated at room temperature and under a tungsten lamp.

Fermentative *Clostridium geopurificans* strain MJ1 was isolated by Dr. Man Jae Kwon, a former member of the research group, from a RDX contaminated aquifer at a live-fire training range in northwest NJ, United States. MJ1 was maintained using TYG media (30g tryptone, 20 g glucose, 10g yeast, at pH 7) sparged with nitrogen gas.

Resting cell suspension experiments

Cultures grown to late exponential growth phase in 1L medium bottle were harvested and centrifuged at 5000 rpm for 20 min to form a dense bacterial pellet. Each pellet was resuspended in 35ml buffer while constantly flushed with oxygen-free gas to maintain anoxic conditions. *G. metallireducens* and *S. oneidensis* were resuspended in bicarbonate buffer and flushed with mixture of 80:20 N_2:CO_2. *C. geopurificans* and *R. sphaeroides* were resuspended in phosphate buffer and flushed with N_2. Cells washed with buffer were centrifuged again at 5000rpm for 20 min. The washing process removes all components of growth medium, leaving only a

dense pallet of cells. Final biomass was resuspended in 4ml of bicarbonate or phosphate buffer accordingly and added immediately to experimental tubes.

Experimental tubes contained 10ml of 30mM bicarbonate buffer for cell suspension experiment with *G. metallireducens* and *S. oneidensis* or 10mM phosphate buffer for *C. geopurificans* and *R. sphaeroides.* Anoxic conditions in experimental tubes were established by sparging liquid and degassing the headspace with the same gas mixture and procedure used as described above for preparing growth medium for each culture.

Table 2.1. Summary of growth conditions and experimental setup for cell suspension experiments

Bacterium	Growth medium	Degassed with	Electron donor	Buffer for cell suspension
Geobacter metallireducens strain GS-15	ferric citrate	N_2:CO_2, 80:20 (v/v)	acetate	30mM bicarbonate
Shewanella oneidensis strain MR-1	ferric citrate	N_2:CO_2, 80:20 (v/v)	lactate	30mM bicarbonate
Clostridium geopurificans strain MJ1	TYG	N_2	glucose	10mM phosphate
Rhodobacter sphaeroides ATCC® 17023TM	ATCC Medium #550	N_2	malate	10mM phosphate

Electron acceptors incubated with cells included AQDS (0.5mM), poorly crystalline Fe(III) hydroxide (FeGel) (1.5mM), and ferric citrate (FeCit) (1.5mM), all

amended from anoxic stock solutions. AQDS could be recycled, undergoing sequential reduction and oxidation more readily than iron, so lower concentration of AQDS were used to achieve IM degradation. FeGel stock was prepared by dissolving ferric chloride in water, adjusting pH to 7, and performing multiple solid centrifugation, washing, and resuspension steps to remove chloride ions to <1mM. According to calculations from Visual Minteq, 30mM of bicarbonate buffer at pH 7 may complex 15% of Fe(II) originated from microbial reduction of Fe(III), while 10mM of phosphate buffer at pH 7 may complex 90% of Fe(II); however, similar experiments run in the past demonstrated that iron complexation by both buffers at these conditions was not significant and it did not affect the reduction of IM. Also, Fe(II) concentration was monitored over the course of the experiment to account for any potential Fe(II) loss due to binding with buffers. To initiate the reaction 0.2ml of resting cells were added to experimental tubes after amendment with either 100μM DNAN, 100μM NQ, or a mixture of 100μM DNAN and 50μM RDX. Samples were collected periodically via anoxic syringe and filtered using 0.2μm PTFE filter prior to analysis.

Table 2.2. Experimental matrix for cell suspension experiments

No. of tubes	Treatment description
1-3	Explosive control
4-6	Explosive+cells
7-9	Explosive+cells+e^- donor
10-12	Explosive+cells+e^- donor+0.5mM AQDS
13-15	Explosive+cells+e^- donor+1.5mM FeGel

16-18 Explosive+cells+e^- donor+0.5mM AQDS+1.5mM FeGel
19-21 Explosive+cells+e^- donor+1.5mM FeCit

Molecular analysis of microbial populations in explosive-contaminated aquifers

Several environmental samples of meltwater, groundwater and soil from sites contaminated with the explosives were screened for 16S rRNA gene to identify microbial communities present at the sites and to recognize specific microorganisms that could be used for *in-situ* bioremediation strategies.

Samples were prepared for DNA screening in the following steps:

DNA extraction. Groundwater samples were filtered through cellulose nitrate membrane filters, 0.2µm, d=47 mm, Whatman. DNA was extracted from filters and soil samples using Fast DNA Spin Kit for Soil from MP Biomedicals.

DNA quantification. Concentrations of DNA extracted from environmental samples was measured in ng/µL using NanoDrop Spectrophotometer to determine the amount of template DNA required for the following steps.

Polymerase Chain Reaction (PCR). For further analysis, DNA extracted from aquifer material was amplified by several orders of magnitude using the PCR technique. Template DNA in the amount of 2.5µl was mixed with master mix solution containing 18.5µl H_2O, 10µl Q, 6µl $MgCl_2$, 5µl 10X, 4µl dNTPs, 1.25µl forward primers 338F (full sequence with Illumina tag: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACTCCTACGG GAGGCAGC-3'), 1.25µl reverse primers 907R (full sequence with Illumina tag: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGTCAATTCCTTTGAGTTT-3'), 1µl

10xBSA, Taq 0.5µl, per one sample DNA. Then, PCR tubes were placed in a thermocycle and run with a program: DNA denaturing at 94°C for 45s, primers annealing at 55°C for 1min, and DNA amplification at 72°C for 30s. The above steps were repeated 35 times.

Gel electrophoresis. Effectiveness of DNA amplification using PRC was tested by a gel electrophoresis method. If DNA template was correctly amplified, a DNA fragment was separated on a gel and verified under UV light. Gel was prepared by mixing approximately 1g of agar with 100ml of TAE buffer and 2µl of ethidium bromide. Then, 5µl of amplified sample DNA was mixed with 2µl of nucleic acid sample loading buffer and placed into separate wells made in gel. Loaded gel was placed in a gel box filled with TAE buffer. Electrophoresis run for 10min at 20V current, and then increased to 80 V for 40min. After the run, the gel was examined under UV light.

DNA purification. To avoid the inhibition of DNA sequencing process by reagents added

in the previous steps, samples were purified using the QIAquick® PCR Purification Kit protocol from QIAGEN®. Concentration of purified DNA was measured using NanoDrop Spectrophotometer.

DNA sequencing. Purified DNA samples were normalized for maximum DNA concentration of 30ng/µl and analyzed via Sanger-style DNA sequencing method by Clemson University Genomics Institute.

Analytical Methods

Liquid samples were analyzed for the explosives by high-performance liquid chromatography (HPLC, Dionex UltiMate 3000).

DNAN was initially analyzed using Acclaim® 120 C18 5µm 120Å (4.6 x 250mm) column. The eluent was a mixture of acetonitrile:water 50:50 (v/v), pumped at 1mL/min. The injection volume was 50µL, and analytical wavelength was 296 nm. At these conditions, DNAN eluted at 9.3 min. Further detection of DNAN together with its transformation products was achieved with Dionex Acclaim® Explosive E1 5µM 120 Å (4.6 x 250mm) column which was more effective for separation of multiple explosives and their derivatives. A mixture of methanol:2mM ammonium acetate at pH 5, 60:40 (v/v) was run at 1 mL/min. Elution times were 7.7min, 5.4min, 4.1min, and 3.6min for DNAN, MENA, iMENA, and DAAN, respectively. The UV detector wavelengths were set to 300nm for DNAN, 254nm for MENA, and 210nm for iMENA and DAAN, or to 210nm for all analytes. Blank water samples and known concentration standards were run periodically to assure high data quality.

NQ was analyzed using AcclaimTM PolarAdvantage II C18 5µm 120Å (4.6x250mm) column. The eluent was 100% water, run isocratically at 0.5 mL/min. The sample injection volume was 50µL, and the detector wavelength was 254 nm. At these conditions, NQ eluted at 10.1min.

RDX and its nitroso intermediates MNX, DNX, and TNX were analyzed using Explosive E1 column and modified DNAN method. A mixture of methanol:2mM ammonium acetate at pH 5, 50:50 (v/v) was run at 1 mL/min and the UV detector was set

to 254nm. For experiments evaluating DNAN and RDX in a mixture, explosives and their immediate degradation products were measured with the above method. The retention times for RDX, MNX, DNX, and TNX were 6.37, 5.66, 4.94, 4.49 min, respectively. The retention times for DNAN, MENA, iMENA, and DAAN were 11.65, 7.25, 4.80, 3.93 min, respectively. MEDINA and NDAB were analyzed via HPLC as described in literature.[37] Formaldehyde was derivatized with DNPH and then analyzed on HPLC using a modified EPA Method 8315A.[38] Nitrate and nitrite were analyzed using ion chromatography (IC, Dionex DX-600) and RFIC™ IonPac® AS14A (4x250mm) column. The mobile phase was a mixture of 8mM Na_2CO_3 and 1mM $NaHCO_3$ run at 1mL/min. Ammonium was quantified spectrophotometrically using an improved Berthelot reaction.[39]

The intermediate of DNAN degradation, 2-HA-4-NAN (2-hydroxylamino-4-nitroanisole), was identified using LC-MS (liquid chromatography- mass spectrometry). Based on retention time of 2-HA-4-NAN in HPLC method, its peak was collected in a separate vial and analyzed using LC-MS by performing a positive and negative scan, as well as product ion scan at collision energy 15V, 20V, and 25V. Positive and negative scans confirmed that the analyte has m/z ratio of 184, which corresponds to molecular weight of 2-HA-4-NAN. Additionally, product ion scan analysis confirmed that the original compound of m/z 184 splits into three fragments of m/z: 168, 138, and 108, which was consistent with predicted 2-HA-4-NAN fragmentation pathway.

Fe(II) concentration was measured using ferrozine assay.[35] In the assay, sample aliquot was first acidified using 0.5N HCl in order to preserve dissolved Fe(II). Then,

acidified aliquot was mixed with ferrozine solution. Ferrozine binds with Fe(II) to develop purple color, for which absorbance can be measured at wavelength 562nm. Ferrozine does not bind with Fe(III), however Fe(III) can be reduced by 6.25N hydroxylamine to Fe(II) and measured again to yield the total amount of iron that represents both Fe(II) and Fe(III) in the sample. Fe(III) concentration can be then calculated as a difference between the total iron and the initial Fe(II) in acidified aliqout.

IRON AND ELECTRON SHUTTLE MEDIATED DEGRADATION OF THE MILITARY NEXT GENERATION INSENSITIVE MUNITION 2,4-DINITROANISOLE (DNAN)

Abstract

The Department of Defense (DoD) has developed next-generation explosives that have been updated with the insensitive munition (IM) 2,4-dinitroanisole (DNAN), to prevent accidental detonations during training and operations. This compound has not been characterized as an environmental hazard, but understanding the fate and transport of DNAN is crucial to assess the risk it may represent to groundwater once new ordnance is used routinely. Previous data demonstrated that hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degrades as a result of electron transfer from iron and humics-based electron shuttles, and this study investigated the potential for DNAN reduction by aqueous phase Fe(II), Fe(II) complexed with organic ligands, the electron shuttle anthraquinone-2,6-disulfonate (AQDS), and the Fe(III)-reducing microorganisms *Geobacter metallireducens,* strain GS-15. Experimental bottles were tested from pH 6 to 9 with an initial DNAN concentration of 100μM. Soluble ferrous iron or anthrahydroquinone-2,6-disulfonate (AH_2QDS) were added directly as reductants. In addition, *G. metallireducens* was added in the presence and absence of Fe(III) and/or AQDS. DNAN was completely reduced by 1.5mM Fe(II) at pH 7, 8, and 9, and rates increased with increasing pH. AH_2QDS reduced DNAN at all pH values tested. DNAN was also reduced in suspensions of GS-15. Cells reduced the compound directly, but both AQDS and Fe(III) increased the reaction rate, presumably via the production of AH_2QDS and/or Fe(II).

DNAN was degraded via intermediates: 2-hydroxylamino-4-nitroanisole (2-HA-4-NAN), 2-methoxy-5-nitroaniline (MENA), and 4-methoxy-3-nitroaniline (iMENA). These date suggest that an effective strategy can be developed for DNAN remediation based on biological-abiotic reactions mediated by Fe(III)-reducing microorganisms.

Introduction

Several newly developed explosive composites, that contain typical compounds such as the cyclic nitramines, have been updated with insensitive munitions including 2,4-dinitroanisole (DNAN). DNAN has been proposed as a replacement for 2,4,6-trinitrotoluene (TNT), which failed to meet safety requirements in the past years. The use of DNAN in new formulations results in increased stability and improved safety standards for explosives transport and storage. Little is known about environmental fate of IM, however several studies prove that DNAN is toxic to bacteria,[10-12] and plants.[10] It can be metabolized in mammal's body to 2,4-dinitrophenol,[13] which has high acute toxicity.[14,15] There is no data on DNAN toxicity to humans; however, nitroaromatic compounds such as explosives have a strong-electron withdrawing effect, which can result in acute toxicity. DNAN has not yet been detected ay any major military installations nor has it been identify as a specific groundwater hazard but, given its harmful properties, DoD requires crucial information on DNAN fate in the environment and bioremediation possibilities should it become an issue.

Reports on DNAN degradation demonstrate that it can be removed from wastewater streams, using anaerobic fluidized-bed bioreactors,[18] Fe/Cu bimetallic particles,[19] or zero valent iron.[12] Another study showed DNAN transformation by co-metabolism of *Bacillus*, strain 13G, in artificially contaminated soil microcosms.[20] In all cases, DNAN was reduced through 2-methoxy-5-nitroaniline (MENA) to 2,4-diaminoanisole (DAAN) by subsequent reduction of two nitro groups to amines. A bacterial consortium of *Pseudomonas* sp., strain FK357, and *Rhodococcus imtechensis*, strain RKJ300, was able to aerobically degrade DNAN via intermediate 2,4-dinitrophenol (DNP), which was utilized by strain RKJ300 as carbon source.[23] Additionally, some studies demonstrate that DNAN can be adsorbed to different types of granular activated carbon (GAC)[24] and lignin.[25]

Reports on currently used cyclic nitramine explosives RDX and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) demonstrated these energetic compounds can be degraded anaerobically via reduction of nitro groups and subsequent ring cleavage.[4-6] While several microorganisms are capable of respiring nitramine compounds,[30-32] their effectiveness may be limited in subsurface, where other environmental factors such as pH, temperature, or availability of nutrients may affect biodegradation. Investigated bacteria strains will most likely have poorer performance in the field compared to laboratory conditions; therefore, it is important to promote the growth of naturally occurring microbial communities to degrade contaminants *in-situ*. More robust approaches focus on coupled biological-chemical reactions mediated by Fe(III)-reducing microorganisms,[4,5] which are

ubiquitous in subsurface environments.[33] These microbes can reduce iron minerals and electron shuttles, such as humic substances, found in abundance in nature. Electron shuttling compounds can accept electrons from microbial respiration and reduce Fe(III).[34] Resulting ferrous iron can transfer electrons to nitramine and nitroaromatic explosives promoting degradation. Re-oxidized shuttles can be recycled so they again accept electrons and transport them among microbes, iron minerals, and contaminants. Both RDX and HMX were successfully degraded by biologically reduced Fe(II) and electron shuttles.[4] Also, several naturally occurring ligands are known to complex Fe(II) making it a stronger reductant due to their preferential binding to Fe(III).[36,40] These ligands decrease the redox potential for the Fe(II)/Fe(III) redox couple and enhance electron shuttling processes, which was showed for degradation of RDX.[40] Due to the same reductive transformation mechanisms (reduction of nitro group) of DNAN and other nitroaromatic explosives, anaerobic biodegradation via electron shuttles has been suggested as an effective strategy for clean-up of future DoD sites.

In this study, we investigated a mechanism of anaerobic DNAN degradation by mixed biological-abiotic reactions with ferrous iron and hydroquinone at different pH. *Geobacter metallireducens*, strain GS-15, was used as a model Fe(III)-reducing microorganism to simulate the in-situ subsurface conditions. The data presented below demonstrate the reaction kinetics, transformation products, and the role of different factors which affect DNAN degradation rates.

Results and Discussion

Ferrous iron alone was able to reduce DNAN at pH 7.0, 8.0, and 9.0; however it didn't reduce DNAN at pH 6.0 (Figure 3.1). At neutral and alkaline conditions DNAN was completely degraded in the presence of ferrous iron within 24 hours for pH 7.0, and 2 hours for pH 8.0 and 9.0. More than 80% of DNAN was reduced within 6.0 hours at pH 7.0. The same effect was achieved for pH 8.0 and 9.0 in less than 1 hour.

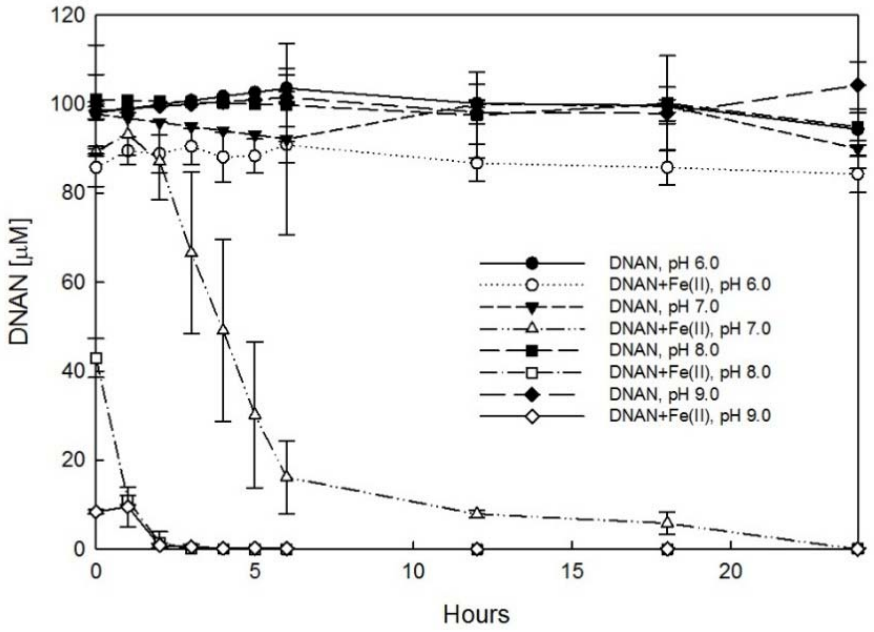


Figure 3.1. DNAN reduction by ferrous iron in pH range from 6.0 to 9.0. Experimental bottles were buffered with 30mM MES, HEPES, and CHES buffers at pH 6.0, 7.0-8.0, and 9.0 respectively. DNAN was amended at 100μM and initial Fe(II) concentration was 1.5mM. The error bars represent the standard deviation with n=3.

Although initial DNAN concentration was 100μM in all samples, the starting data points for ferrous iron treatment at pH 8 and 9 show approximately 40μM and 10μM of DNAN. Detailed investigation of these treatments demonstrated that reduction at alkaline pH occurred within minutes and seconds (Figure 3.3. b-c) and therefore, lower initial DNAN concentrations in Figure 3.1 are due to the fact that by the time samples aliquots were mixed with EDTA to quench Fe(II) and stop the reaction, most DNAN was already degraded. Thus, in further experiments initial data points were taken before addition of Fe(II) for improved DNAN data accuracy .

Almost no DNAN was reduced in the first 2hr at pH 7; however, after this initial lag phase, 90% of DNAN was reduced within next 4hr. The kinetic data of DNAN reduction (Figure 3.2) exhibit similarities to Fe(II) autocatalytic oxidation data[41].

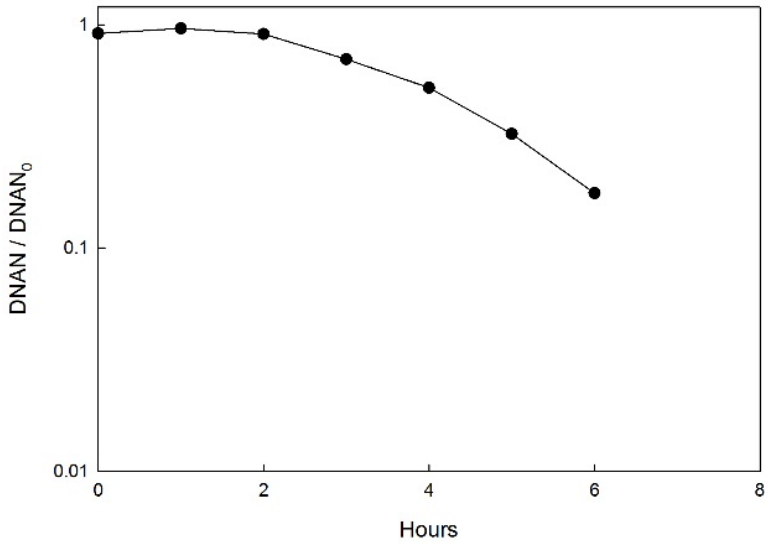


Figure 3.2. Data points represent normalized DNAN concentrations from reduction by ferrous iron at pH 7.0 in the first 6 hours (Figure 2.1), where data follow the pattern for Fe(II) autocatalytic oxidation.[41]

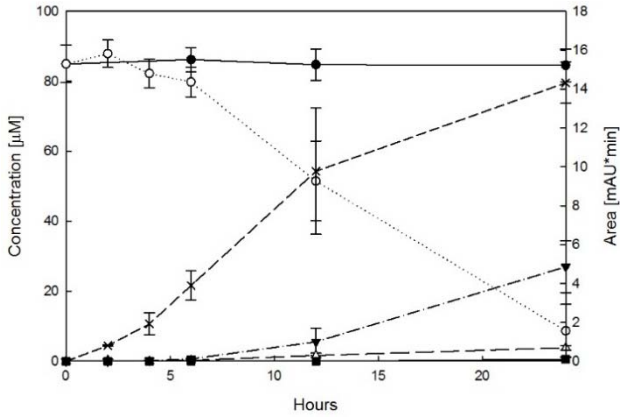
Fe(II), used in the experiments as a reducing agent, transfers one electron to DNAN molecule and it oxidizes to Fe(III):

$$Fe^{2+} \rightarrow Fe^{3+} + e^{-}$$

Then, Fe(III) serves as a catalyst for Fe(II) oxidation in two ways: first, it precipitates out of the solution due to its low solubility and it drives Fe(II) oxidation towards product formation;[41,42] secondly, Fe(II) adsorbs to freshly precipitated Fe(III) hydroxides and undergoes surface oxidation. Also, Fe(II) adsorbed to solid surfaces is more reactive than dissolved Fe(II).[43] The initial 2hr lag phase in DNAN reduction can be thus explained as the time required for sufficient precipitation of Fe(III) to drive iron oxidation[41] and subsequently DNAN reduction forward. Similarities between DNAN degradation kinetics (Figure 3.2) and published autocatalytic oxygenation kinetics of ferrous iron[41] suggest that adsorbed Fe(II) is mostly responsible for DNAN reduction. DNAN did not degrade at pH 6, which can be associated with the absence of reactive solid surfaces as ferrous iron oxidation becomes much slower with decreasing pH.[44]

Iron measurements taken at the beginning and at the end of the experiment showed a total loss of 0.15mM (10%) Fe(II) at pH 6.0, 0.75mM (50%) loss at pH 7.0 and approximately 1.2mM (80%) loss at pH 8.0 and 9.0.

DNAN has been reported to degrade abiotically via reduction of nitro $-NO_2$ functional groups to amine $-NH_2$ group.[17-20] Further investigation on products formed during DNAN reduction by Fe(II) (Figure 3.3) supports this degradation pathway (Figure 3.4).



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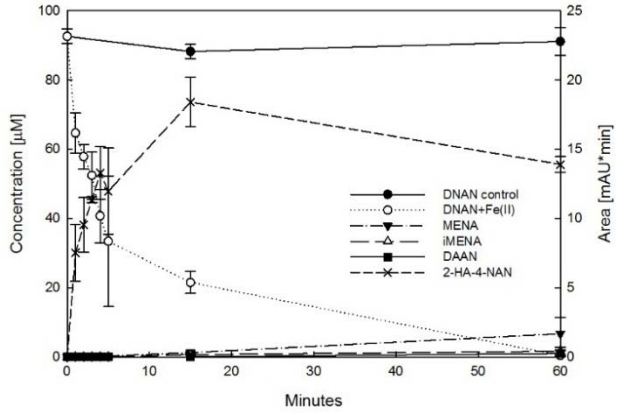

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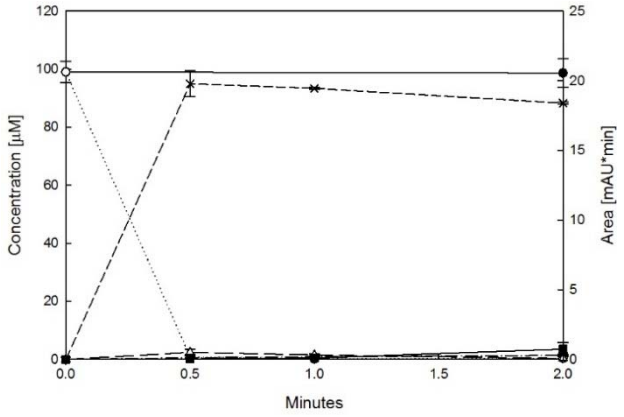


c

Figure 3.3. DNAN degradation by 1.5mM ferrous iron at a) pH 7, b) pH 8, and c) pH 9. Primary y-axis shows concentration of analytes: DNAN, MENA, iMENA, and DAAN. There are no commercially available certified standards of intermediate 2-HA-4-NAN to compare with the amounts detected in experimental samples; therefore, the secondary y-axis shows the peak area of 2-HA-4-NAN rather than concentration. Experimental bottles were buffered with 30mM HEPES at pH 7 and 8, and with 30mM CHES at pH 9. The error bars represent the standard deviation with n=3.

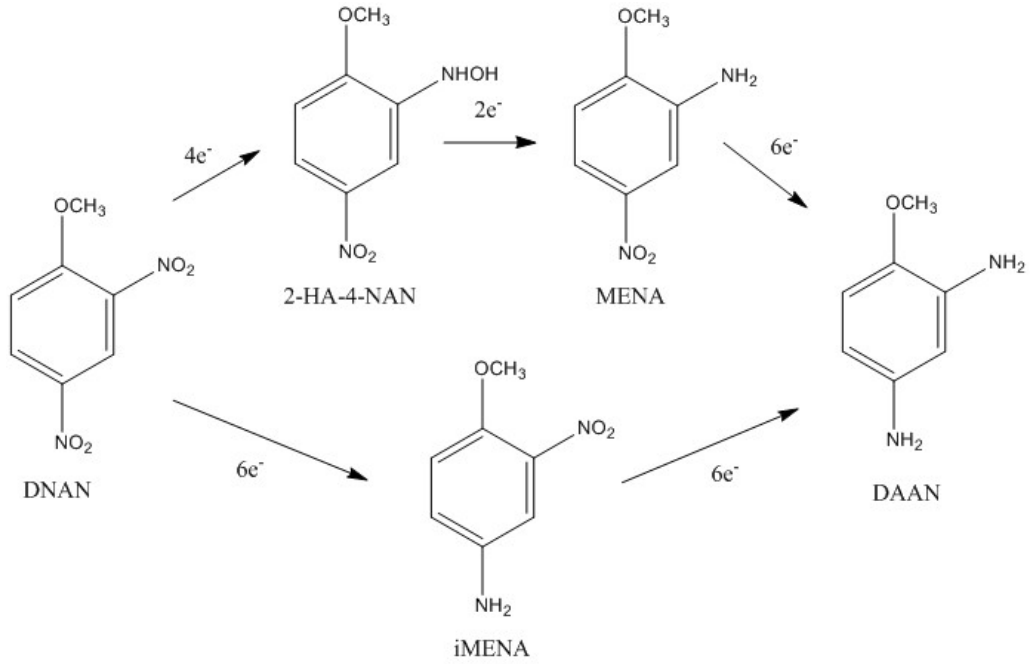


Figure 3.4. DNAN reductive degradation pathway. Complete transformation of one molecule of DNAN to DAAN requires 12 electrons.

Presented data (Figure 3.3) demonstrated that the reduction of $-NO_2$ functional group in ortho position is more thermodynamically favorable under tested conditions. Initial formation of intermediate 2-HA-4-NAN was followed by MENA formation. Further transformation of MENA to DAAN was not detected. At pH 7 (Figure 3.3a) only 25μM MENA was recovered for 90μM DNAN degraded. At pH 8 (Figure 3.3b), 5μM MENA was found when DNAN was completely reduced. No MENA was detected at pH 9 (Figure 3.3c). At all pH values, the intermediate 2-HA-4-NAN was present, suggesting that the reduction of $-NHOH$ to $-NH_2$ may be the rate limiting step in abiotic DNAN degradation. Also, DNAN degradation was slower at

pH 7 compared to the first screening experiment (Figure 3.1) most likely due to the fact that all experimental bottles were placed on a rotary mixer to achieve uniform distribution of reactive iron solids in the sample volume; therefore, mixing may have an effect on Fe(II) adsorption to freshly precipitated Fe(III).

Same experiment, performed with starting 100μM MENA and iMENA (Figure 3.5) showed that both compounds can be reduced with dissolved Fe(II) and that they don't require adsorbed Fe(II) to initiate reaction. The kinetics of MENA and iMENA reduction followed the kinetics for DNAN (Figure 3.1), which was most clear for pH 7 (Figure 3.5.a,b). There was an initial lag phase in the first 3hr, when Fe(II) oxidized to Fe(III) and then precipitated, providing surface for Fe(II) adsorption which drove MENA and iMENA degradation forward. According to the degradation pathway (Figure 3.4), 6 electrons are required to reduce one molecule of MENA or iMENA, which is an equivalent to 0.6mM Fe(II). Although this amount of Fe(II) was used in the experiment, only 50% of MENA and iMENA were reduced at pH 7, while 50% of iMENA and 30% of MENA were reduced at pH 8. DAAN was detected only at pH 8 but total mass balance was not closed. Little or no MENA and iMENA reduction was achieved after 25 hr at pH 7 and after 4 hr at pH 8, while Fe(II) was still present. Fe(II) completely oxidized after 70hr at pH 7 and 24 hr at pH 8 suggesting that electrons from oxidation of the remaining Fe(II) were consumed in other unaccounted reactions.

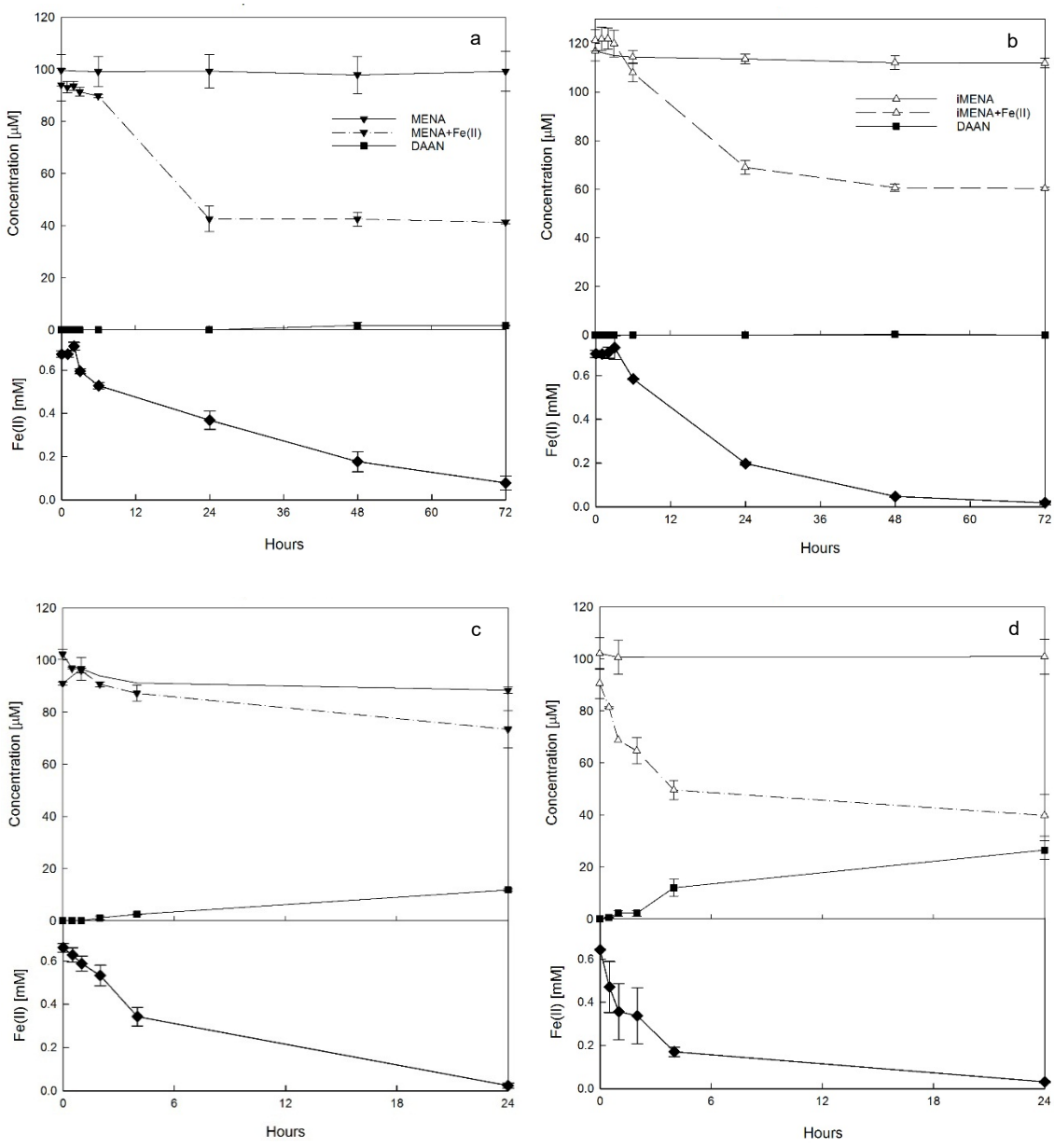


Figure 3.5. MENA and iMENA degradation by 0.6mM ferrous iron at a-b) pH 7, c-d) pH 8. Experimental bottles were buffered with 30mM HEPES. The error bars represent the standard deviation with n=3.

DNAN can be successfully degraded by Fe(II) alone at pH 7 and higher, however not at pH 6 (Figure 3.1). DNAN reduction at this slightly acidic pH can be stimulated by formation of more reactive Fe(II) complexes with several organic ligands that contain catechol or thiol functional groups. These ligands bind preferentially to Fe(III), therefore they lower reduction potential of the redox couple Fe(III)/Fe(II) making organically complexed Fe(II) a stronger reductant.[36, 40]

Screening of several Fe(II)-ligand complexes (Figure 3.6) shows that while some complexes are more reactive at pH 7, there are a few of them capable on reducing DNAN also at lower pH. At pH 6, Fe(II) forms the most reactive complexes with 2,3-DMSA, 2,3,4-THBA and gallic acid, which achieve complete DNAN degradation (Figure 3.6). Also, tiron and 3,4-DHBA showed 90% and 60% DNAN reduction, respectively. These ligands not only increase Fe(II) reactivity but they also bind Fe(III) preventing its precipitation, which may be useful in in-situ remediation projects when precipitated Fe(III) could clog the screen filter of an injection well or when too much precipitation could decrease hydraulic conductivity of an aquifer. L-cysteine and sulfide, which could serve in subsurface as reducing agents alternative to Fe(II), had no effect on DNAN. Some DNAN degradation was achieved in treatments with Fe(II) complexed with NaCit, malic acid and oxalic acid, and it had higher extent at pH 7. Chelating agents NTA and EDTA prevented electron shuttling between Fe(II) and DNAN molecules. These data demonstrate that organically-complexed Fe(II) species may play a key role in natural attenuation of insensitive munitions.

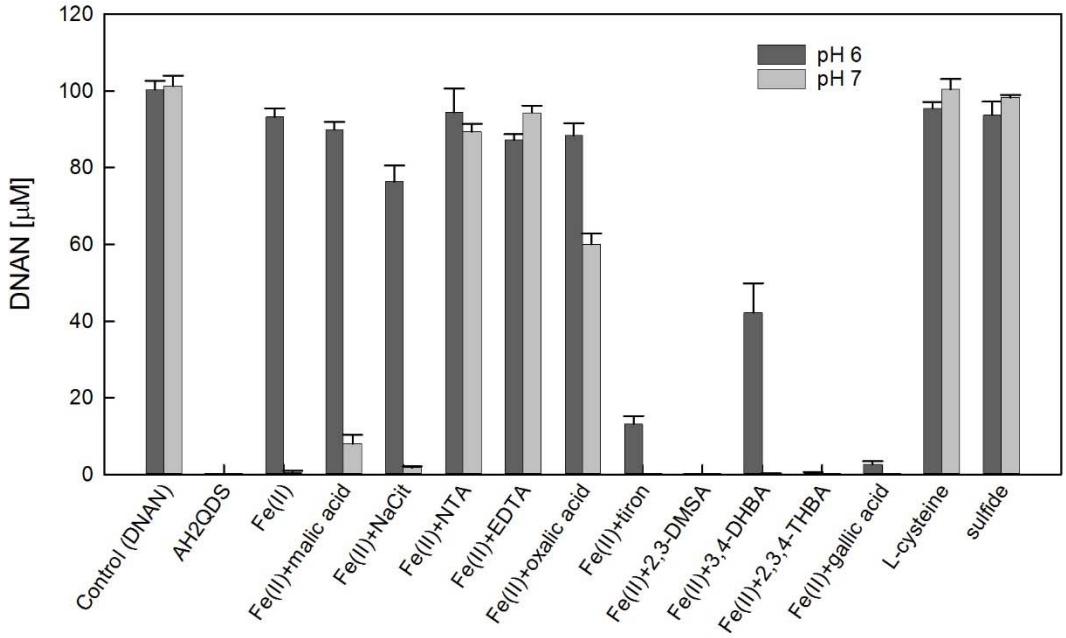


Figure 3.6. Effect of ligands on Fe(II) reaction with DNAN at pH 6 and 7 after 2 days. Experimental conditions: 100μM DNAN, 1.5mM Fe(II), buffered with 30mM MES at pH 6 or 30mM HEPES at pH 7. The error bars represent the standard deviation with n=3.

One of the compounds tested amongst the organic ligands was AH_2QDS which serves as a model moiety used in laboratory studies to mimic the humic substances present in soil. Each molecule of AH_2QDS can transfer 2 electrons:

$$AH_2QDS \rightarrow AQDS + 2e^- + 2H^+$$

A separate experiment run with DNAN and AH_2QDS (Figure 3.7) demonstrated that AH_2QDS was effective in reducing 100μM DNAN within a few minutes at pH 7. The main intermediate detected was 2-HA-4-NAN, similarly to the experiments with Fe(II), however no MENA was measured after disappearance of 2-HA-4-NAN, thus

further products of 2-HA-4-NAN transformation are not known at this point. Instead, approximately 10µM iMENA was recovered, showing that DNAN degradation can be initiated by the reduction of either $–NO_2$ in ortho or in para position.

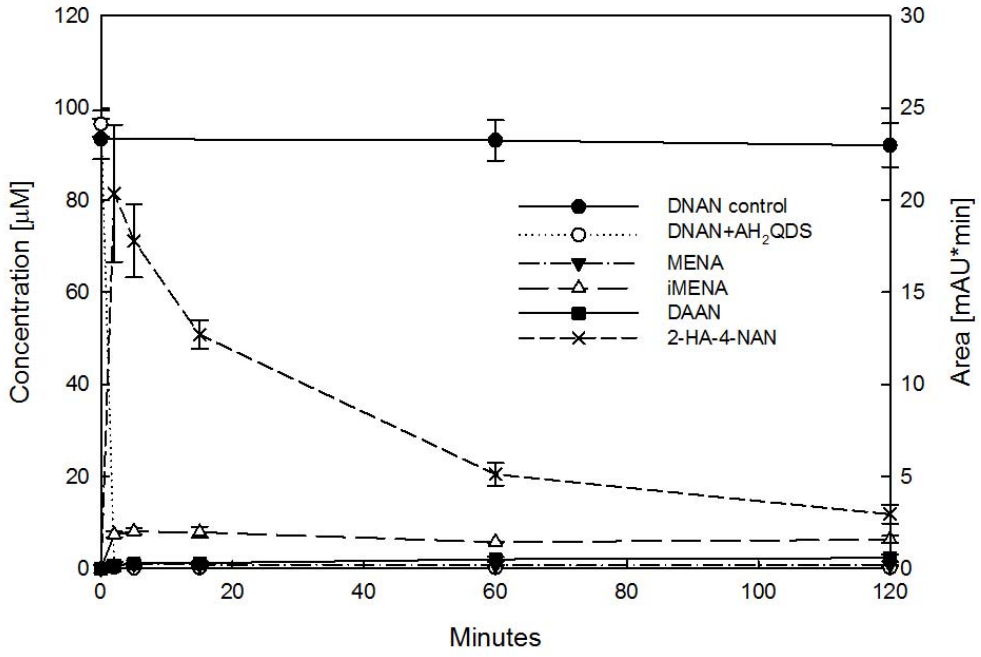


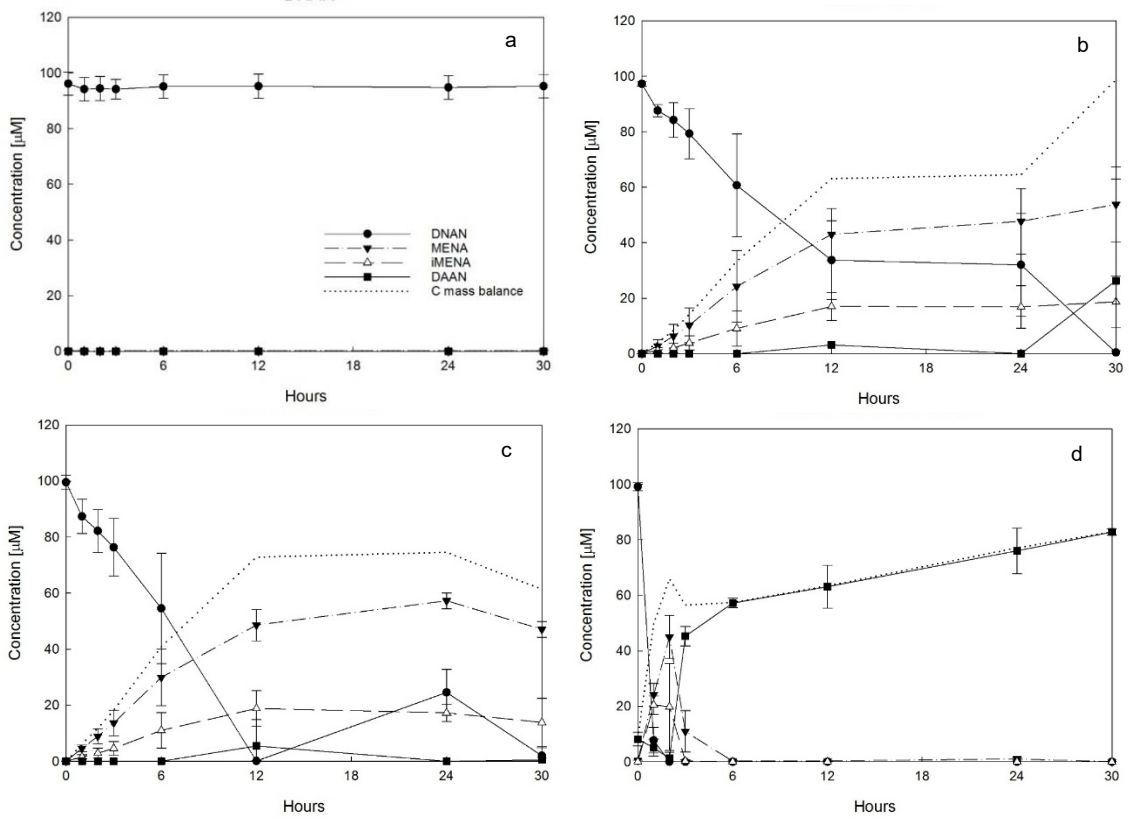
Figure 3.7. DNAN degradation by 600µM AH_2QDS at pH 7, buffered with 30mM HEPES. Primary y-axis shows concentration of analytes: DNAN, MENA, iMENA, and DAAN. The secondary y-axis shows the peak area of 2-HA-4-NAN. The error bars represent the standard deviation with n=3.

The above results show that DNAN can be degraded by iron, its complexes with organic ligands, and by electron shuttles. These processes can be used to design an in-situ bioremediation plan by enabling naturally occurring microorganisms to mediate

reduction of iron and electron shuttles, so that electron shuttling continues in the subsurface.

A model Fe(III)-reducing microorganism *G. metallireducens*, strain GS-15, was capable of reducing DNAN in the presence and absence of extracellular shuttling compounds (Figure 3.8). GS-15 can transfer electrons directly to contaminants or indirectly via shuttles which undergo sequential reduction and oxidation. The electron transfer occurs through the electron transport chain in the outer membrane of GS-15.[45] DNAN was transformed to DAAN mostly via intermediate formation of MENA; however, lower concentrations of iMENA were also detected. In amendments with cells alone (Figure 3.8.b) 100 μM DNAN was transformed to 55μM MENA, 20μM of iMENA, and 25μM DAAN in 30 hours. Treatment with cells and acetate (Figure 3.8.c) gave similar results; therefore, the presence of acetate was not crucial for electron shuttling process. Experimental tubes were buffered with 30mM bicarbonate buffer, thus it is possible that GS-15 used bicarbonate as a carbon source. Reduced AQDS was fastest in transporting electrons from GS-15 to DNAN leading to complete reduction of both nitro groups and formation of DAAN with only short accumulation of intermediates (Figure 3.8.d,f). Poorly crystalline Fe(III) (FeGel) (Figure 3.8.e) demonstrated slower degradation rate than soluble ferric citrate (FeCit) (Figure 3.8g) suggesting that soluble electron shuttles may be more effective than insoluble compounds. Treatment with FeGel (Figure 3.8.e) was the only one to show DAAN disappearance but its further transformation is not known.

Overall, biological DNAN degradation was more complete compared to abiotic experiments with ferrous iron at similar conditions, which is due to microbial ability to quickly reduce electron shuttles and to transfer electrons directly to explosives at the same time. Fe(III) was most readily reduced to Fe(II) when a soluble form of Fe(III) (FeCit) or AQDS were used (Figure 3.8.e-g). Fe(II) measurements confirm that the rate of DNAN degradation depends on the kinetics of electron transport processes.



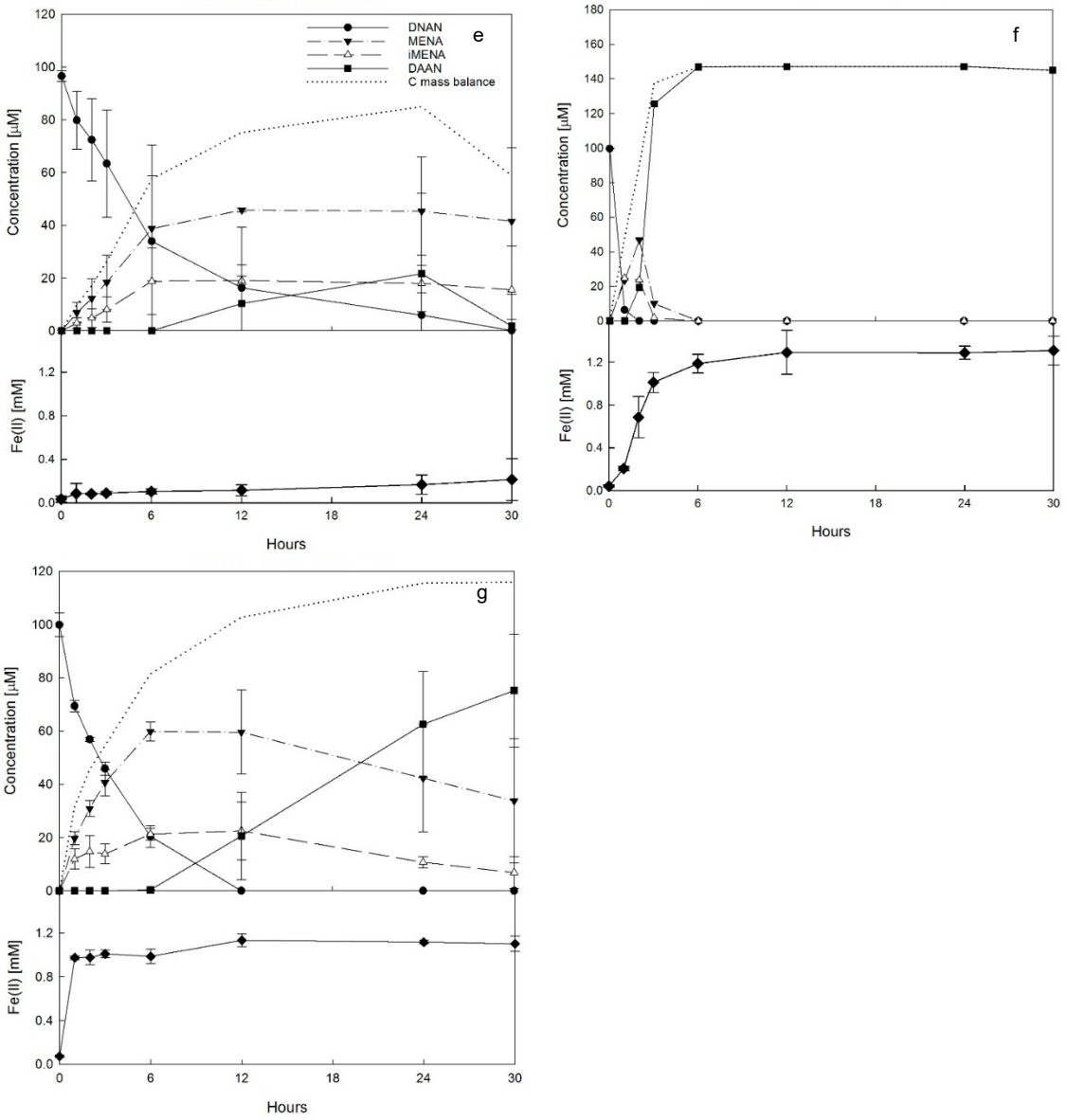


Figure 3.8. DNAN degradation mediated by *Geobacter metallireducens*, strain GS-15. Experimental conditions: 100µM DNAN, 1mM acetate as electron donor where indicated, buffered with 30mM bicarbonate at pH 7. Treatments: a) DNAN control, b) DNAN+cells, c) DNAN+ cells+acetate, d) DNAN+cells+acetate+0.5mM AQDS, e) DNAN+ cells+acetate+1.5mM poorly crystalline Fe(III) (FeGel), f) DNAN+

cells+acetate+0.5mM AQDS+1.5mM Fe(III) as FeGel, g) DNAN+ cells+acetate+1.5mM soluble Fe(III) (FeCit). The error bars represent the standard deviation with n=3.

This study reports for the first time DNAN degradation mediated by ferrous iron and electron shuttles. Previous reports show that RDX was unreactive with dissolved Fe(II)[40,43] and that it required Fe(II) adsorbed to magnetite[43] or Fe(II) complexed with organic ligands[40] to promote degradation. The reaction between DNAN and dissolved Fe(II) may therefore drive RDX reduction by providing freshly precipitated iron solids for Fe(II) adsorption and thus formation of more reactive iron complexes. Additionally, microbially reduced extracellular electron shuttles can effectively reduce not only DNAN but also RDX and HMX,[4] which are commonly used in the old type explosives formulations. These findings facilitate a design of future remediation strategy at sites where several explosives are present in the soil and groundwater, and where active Fe(III)-reducing microorganisms can mediate electron transfer to contaminants.

2,4-DINITROANISOLE (DNAN) DEGRADATION MEDIATED BY FE(III)-REDUCING, PHOTOSYTHETIC, AND FERMENTATIVE BACTERIA

Abstract

The insensitive munition 2,4-dinitroanisole (DNAN) has been used in newly developed explosives as a replacement for the more sensitive munition, trinitrotoluene (TNT). These new formulations are more insensitive to external shocks, for example heat or strikes, in storage, transport, and battle conditions and therefore are safer to handle. It is important to understand the fate of DNAN in the environment and to establish effective remediation methods due to its high toxicity and potential environmental hazards.

DNAN degradation mediated by Fe(III)-reducing, photosynthetic, and fermentative microorganisms was evaluated in resting cell suspension experiments buffered at pH 7 with bicarbonate or phosphate. Cells alone were able to reduce DNAN to 2,4-diaminoanisole (DAAN) through intermediates 2-methoxy-5-nitroaniline (MENA) and 4-methoxy-3-nitroaniline (iMENA). The use of poorly crystalline Fe(III), soluble ferric citrate, and quinone (AQDS) enhanced DNAN degradation by promoting electron transfer in parallel pathways between microorganisms, electron shuttling compounds, and DNAN. *Shewanella oneidensis*, strain MR1, an iron-reducing bacteria, was able to reduce electron shuttles and degrade DNAN most readily with lactate provided as an electron donor. A photosynthetic culture, *Rhodobacter sphaeroides*, reduced DNAN to MENA quickly

when light was present, suggesting that photobiological degradation may be an effective surface water treatment strategy. DNAN was also reduced by *R. sphaeroides* in dark conditions via chemotrophic respiration. Fermentative *Clostridium geopurificans*, strain MJ1, transformed DNAN to DAAN in co-metabolic reactions, where DNAN was used as an electron sink for fermentative growth. In all cases bacteria were able to degrade DNAN within several hours, demonstrating that multiple microorganisms can mediate explosives degradation via extracellular electron shuttling.

Introduction

Several new insensitive explosive composites have been developed by the Department of Defense (DoD) in which unstable 2,4,6-trinitrotolene (TNT) was replaced with more stable 2,4-dinitroanisole (DNAN) to reduce the risk of unwanted detonations. The use of DNAN results in increased stability and improved safety for explosives transport and storage. Few studies are available on DNAN toxicity,[10,11,13] however its toxic and carcinogenic properties can be assumed based on the similarity in chemical characteristics of all explosive compounds.

DNAN, once used regularly for tests and trainings at military facilities and firing ranges, may pose a risk to the environment[10] and humans' health.[13] As such, it may require a remediation plan for the affected sites. Explosives are usually removed from groundwater using pump and treat strategy with adsorption to granular activated carbon (GAC).[9] While the method is effective, it only transfers the contaminant from a liquid

medium to a solid GAC without destroying explosives. GAC laden with highly energetic compounds has to be frequently replaced and safely disposed, which results in high operating costs and huge amounts of wasted GAC. The more sustainable approach for direct, *in situ* degradation of explosives is to use microorganisms naturally present in the subsurface to destroy the contaminants. This strategy lowers the total cost and the carbon footprint by reducing the energy consumption compared to pump and treat method. It also helps to preserve the natural conditions at the site as there is no need to remove big quantities of groundwater.

Explosives and insensitive munitions (IM) can be degraded anaerobically via a reduction of nitro functional groups to amine groups. A number of reductants, such as iron(II), and bacterial nitroreductases are capable of driving this process. Microbially mediated reduction is catalyzed by a specific group of enzymes which promote electron shuttling to the contaminants in non-specific reactions.[2,46] A few reports are available on DNAN degradation by soil and wastewater sludge bacteria;[20,27] however, the exact mechanisms of microbial DNAN reduction have not been identified. RDX, HMX, and TNT can be degraded by a series of reactions mediated by non-specific microorganisms. In these reactions, electrons from microbial respiration are transferred to extracellular shuttling compounds which undergo sequential reduction and oxidation as they further transfer electrons to contaminants.[4-6,8,9]

Extracellular electron shuttling processes may play an important role in natural attenuation of explosives and IM. Future *in-situ* remediation technology could be based on stimulating indigenous microbial communities to mediate degradation by electron

transport in the subsurface. This approach would take advantage of already established communities rather than selected bacterial strains, which may not be competitive for available substrates and thus have poor performance in the field.

Due to the similarities in the structures of DNAN and TNT, lessons learned from reported TNT degradation could be useful in evaluating DNAN transformation, fate and transport in the environment. Some studies show that TNT can be mineralized by different fungi but its decomposition to CO_2 and inorganic nitrogen species has not been reported for bacteria.[2,3] Instead, TNT was biotransformed to 2,4,6-triaminotoluene (TAT). TAT and the reactive intermediates of the $–NO_2$ functional groups reduction form azo, azoxy, hydrazo, phenolic, and acetyl derivatives that irreversibly bind to soil or biomass matrix.[2] This way, TNT can be immobilized from groundwater rather than degraded to innocuous products.[2]

Several bacteria genera that are common in the environment and that could be employed for *in-situ* IM degradation include: *Shewanella, Rhodobacter*, and *Clostridia*. *Shewanella* species have been recently given more attention due to their ability to respire a variety of insoluble substrates using extracellular electron shuttling mechanisms, an ability that makes them attractive for contaminant reduction in bioremediation and wastewater treatment.[47,48] *Rhodobacter* is a metabolically diverse microorganism that can grow via aerobic respiration, anaerobic photosynthesis, and fermentation;[49] therefore, it could be applied in treatment strategies for a wide variety of groundwater conditions. Finally, fermentative *Clostridia* species have been reported to degrade nitroaromatic

compounds, including explosives.[3,50] The contaminants are major electron sinks for fermentative growth resulting in high transformation rates.[3]

In this study, *Shewanella oneidens*is strain MR1, *Rhodobacter sphaeroides*, and *Clostridium geopurificans* strain MJ1 were investigated for their ability to mediate DNAN degradation in the presence and absence of extracellular electron shuttles such as insoluble Fe(III), soluble Fe(III), and humic substances (AQDS).

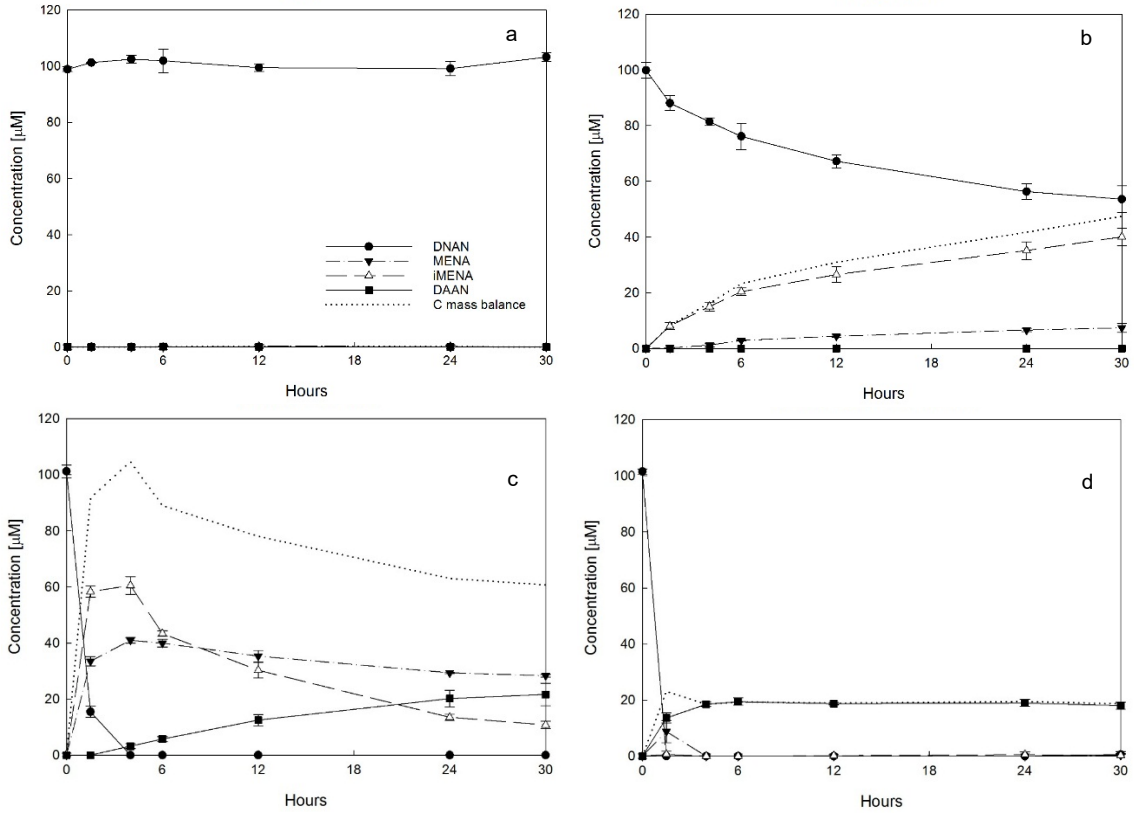
Results and Discussion

Shewanella oneidensis, strain MR1 is a facultative anaerobe capable of respiring diverse compounds.[48] As such, it may play a key role in mediating explosives and IM reduction, especially at the boundary of aerobic and anaerobic conditions in the subsurface environments. *Shewanella* is known for its ability to reduce insoluble metals by the transfer of electrons between the cell and substrate in the process called extracellular respiration or electron shuttling,[47] and it is one of the Fe(III)-reducing microorganisms.

MR1 alone was capable of reducing 50μM DNAN, yielding approximately 40μM iMENA and 5μM MENA (Figure 4.1.b). More complete reduction was achieved with lactate provided as an electron donor (Figure 4.1.c). In this treatment, DNAN was degraded within 4 hrs and the final products detected after 30 hours were 30μM MENA, 10μM iMENA, and 20μM DAAN. Initial higher accumulation of iMENA compared to MENA suggests that MR1 attacks $-NO_2$ group in para position more readily than the $-NO_2$ in ortho position. These findings are consistent with cells

alone incubation. Amendment with AQDS (Figure 4.1.d) demonstrated fast DNAN degradation in 1 hr; however, only 20μM DAAN was detected as the final product. When FeGel (poorly crystalline Fe(III)) was used as an example of insoluble electron shuttle, DNAN was reduced to 30μM DAAN and 20μM MENA (Figure 4.1.e). The addition of AQDS resulted in accumulation of 50μM DAAN due to the increase of electron shuttling between microbes, FeGel, and DNAN (Figure 4.1.f). This suggests that the rate limiting step in DNAN microbially mediated degradation is electron transfer from cell surface to the contaminant. Data shows that the more electron shuttles are available, the faster and more complete DNAN degradation can be achieved. Finally, the treatment with soluble Fe(III) in the form of ferric citrate (FeCit) gave similar results to treatment with FeGel. Although the final mass balance is similar, the intermediates were transformed to DAAN faster when soluble FeCit was used as an electron shuttle (Figure 4.1.g).

Fe(II) data confirms that soluble Fe(III) is most readily reduced by MR1. While significant amount of insoluble FeGel was also reduced, the process was slower. AQDS was able to stimulate FeGel reduction by transferring electrons from cells to FeGel. In all cases, the presence of electron shuttles increased DNAN degradation.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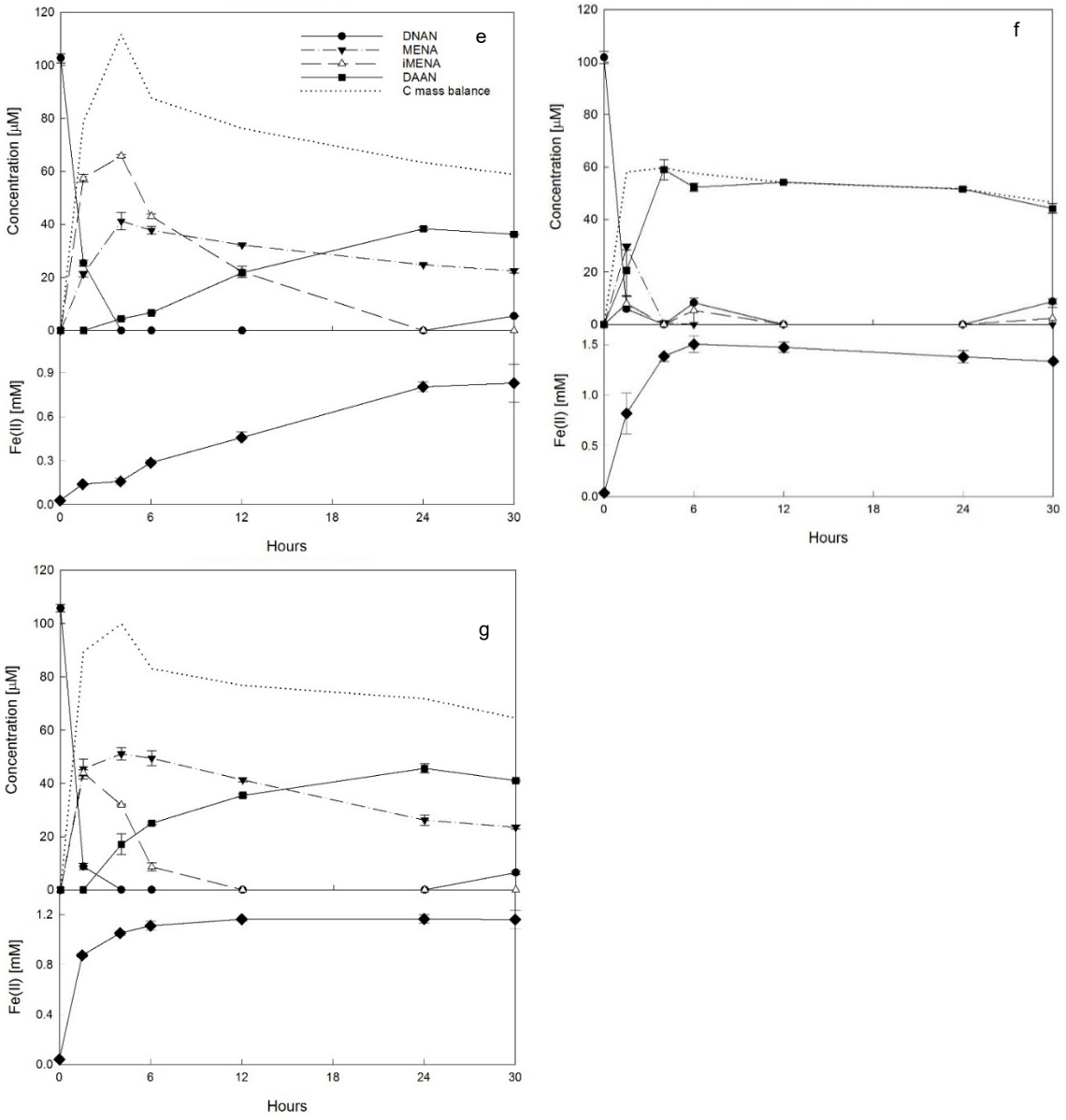


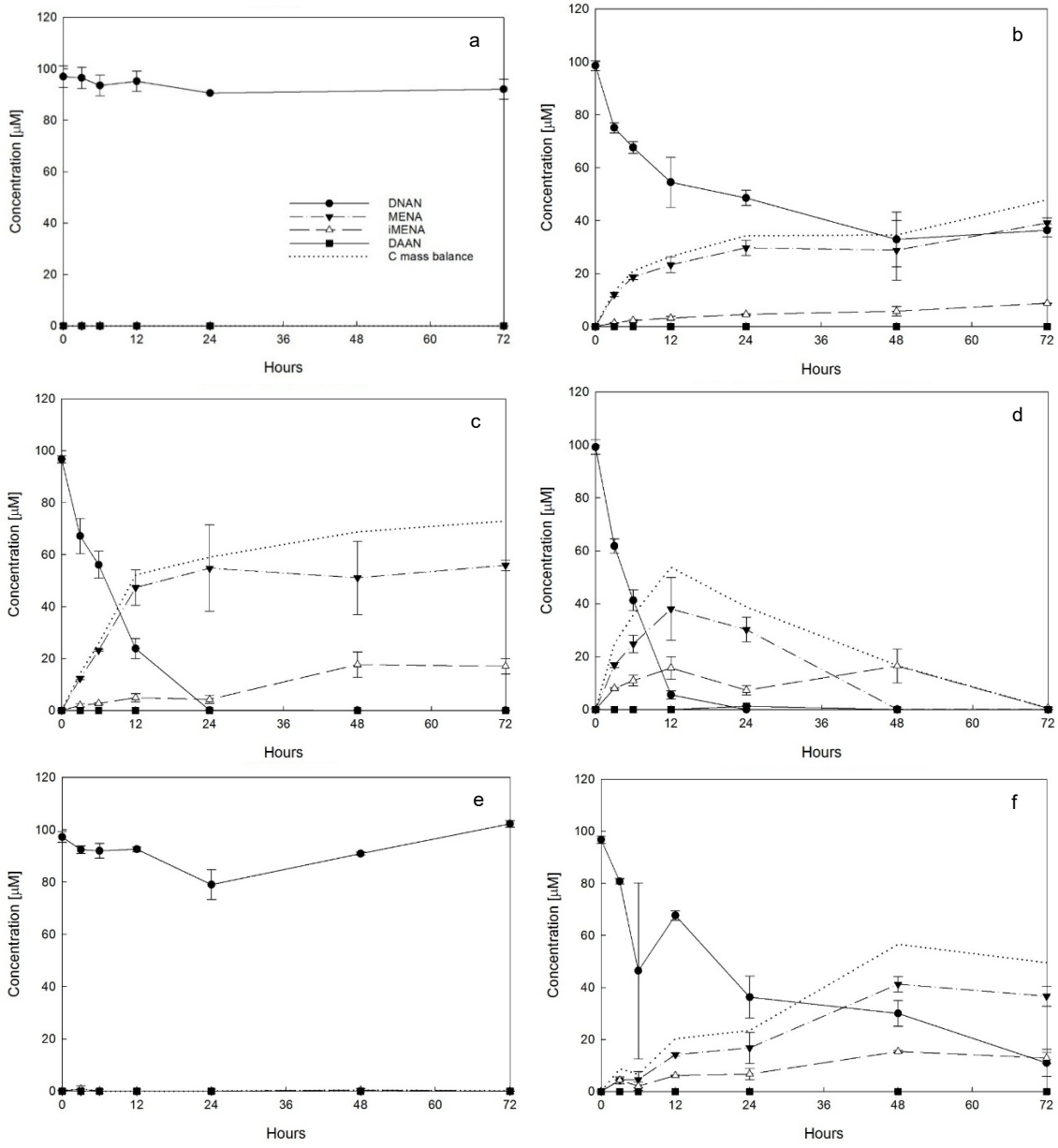
Figure 4.1. DNAN degradation mediated by *Shewanella oneidensis,* strain MR-1. Experimental conditions: 100µM DNAN, 1mM lactate as electron donor where indicated, buffered with 30mM bicarbonate at pH 7. Treatments: a) DNAN control, b) DNAN+cells, c) DNAN+cells+lactate, d) DNAN+cells+ lactate +0.5mM AQDS, e)

DNAN+ cells+ lactate +1.5mM poorly crystalline Fe(III) (FeGel), f) DNAN+ cells+ lactate +0.5mM AQDS+1.5mM Fe(III) as FeGel, g) DNAN+ cells+ lactate +1.5mM soluble Fe(III) (FeCit). The error bars represent the standard deviation with n=3.

R. sphaeroides, a photosynthetic microorganism that could also be employed for bioremediation of explosives due to its diverse metabolic capabilities[49] and environmental relevance, was likewise able to degrade DNAN (Figure 4.2). Degradation occurred via photosynthetic electron transfer in tubes exposed to light, and via chemotrophic respiration in tubes kept in dark. In light conditions, cells alone reduced 60% of DNAN within 72 hours (Figure 4.2.b). The addition of malate as a carbon source stimulated cells activity resulting in complete DNAN degradation within 24 hours (Figure 4.2.c). In both cases, 40-50μM MENA and 10-20μM iMENA were detected as dominant degradation products. Electron shuttling compounds increased rate of reaction (Figure 4.2.d, g-i). Finally, *R. sphaeroides* reduced DNAN in dark conditions (Figure 4.2.f); however, the reaction rate was slower than in the corresponding light conditions (Figure 4.2.c). Due to the diverse metabolic capabilities, *R. sphaeroides* can survive in oxic, micro-oxic, and anoxic conditions, when exposed to light and in the dark. Faster photosynthetic degradation can be explained with more free electrons being produced under light conditions and thus greater potential for electron shuttling. Data suggest that chemotrophic respiration in dark was less thermodynamically favorable and resulted in lower energy production. This led to smaller amount of electrons available for DNAN reduction.

Fe(III) was again most readily reduced to Fe(II) when soluble FeCit was used (Figure 4.2.i). However, after 72 hours, there was no significant difference in the concentration of reduced iron between amendments with soluble and insoluble form of Fe(III). Both FeCit and FeGel were reduced to yield approximately 0.5-0.7mM Fe(II).

R. sphaeroides, due to its metabolic diversity, demonstrated a great potential for designing an in-situ remediation strategy at different subsurface conditions.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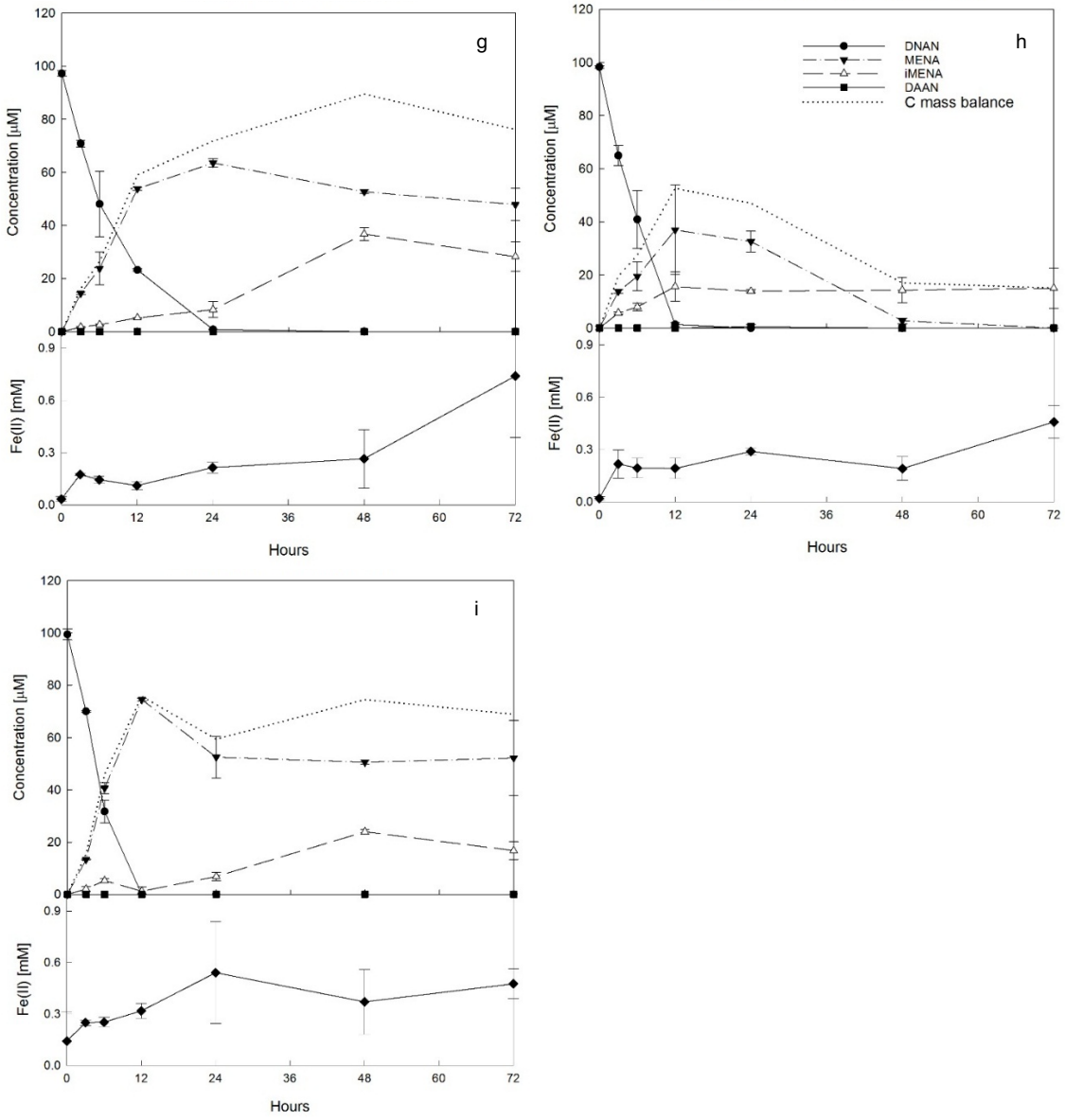
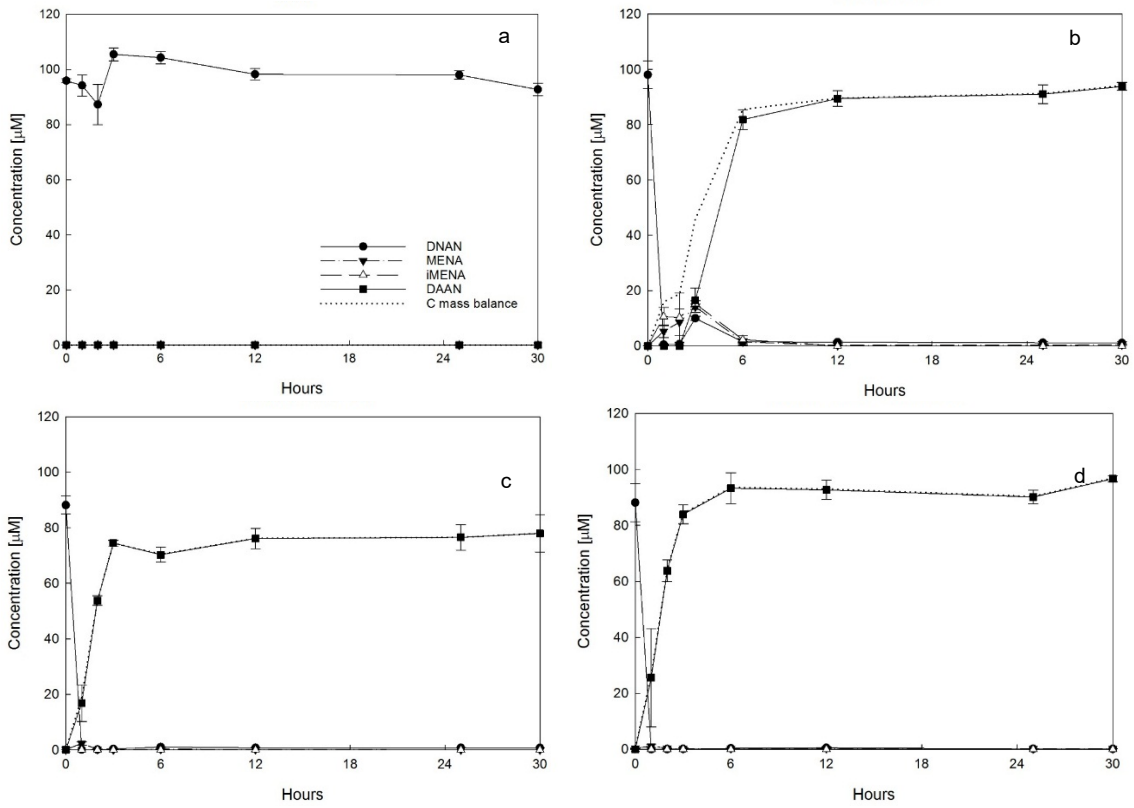


Figure 4.2. DNAN degradation mediated by *Rhodobacter sphaeroides*. Experimental conditions: 100µM DNAN, 20mM malate as electron donor where indicated, buffered with 10mM phosphate at pH 7. Treatments in light conditions (unless indicated otherwise): a) DNAN control, b) DNAN+cells, c) DNAN+cells+malate, d) DNAN+cells+malate+0.5mM AQDS, e) DNAN control in dark conditions, f)

DNAN+cells+malate in dark conditions, g) DNAN+ cells+malate+1.5mM poorly crystalline Fe(III) (FeGel), h) DNAN+ cells+malate+0.5mM AQDS+1.5mM Fe(III) as FeGel, i) DNAN+ cells+malate+1.5mM soluble Fe(III) (FeCit). The error bars represent the standard deviation with n=3.

Fermentative *Clostridium geopurificans*, strain MJ1, transformed DNAN to DAAN within 3-6 hours, with almost no detectable accumulation of intermediates (Figure 4.3). In fermentation, cells obtain energy from substrate-level phosphorylation which utilizes endogenous electron acceptors. This process yields significantly less energy than respiration, where cells obtain energy using exogenous electron acceptors. In anaerobic respiration carried by *Shewanella* and *Rhodobacter,* electron acceptors may compete for electrons with DNAN molecules resulting in slower degradation rates. Fermenters such as *Clostridium* carry their metabolism independent of external electron acceptors and they can transfer electrons to extracellular electron sinks in non-specific reactions catalyzed by redox enzymes.[3,50] RDX and TNT have been reported to serve as electron sinks for fermentative growth of several microorganisms.[3,50] Experimental results (Figure 4.3) suggest that DNAN also may be used as an electron sink that utilizes free electrons fast. Treatments with cells and electron donor (lactate) in the presence and absence of AQDS or FeGel (electron shuttles) showed 80-100% DNAN transformation to DAAN in the first 3 hours (Figure 4.3.c-f). Cells alone degraded DNAN in 6 hours (Figure 4.3.b). Amendments with FeCit (Figure 4.3.g) demonstrated slower DAAN accumulation compared to the other treatments. The stoichiometric amount of DAAN

was detected only after 25 hours, while DNAN was completely reduced in the first hour. This suggests that FeCit might have been used as an alternative electron sink. FeCit was reduced to form 0.9mM Fe(II) and its concentration remained constant during the experiment. Also, twice as much FeCit was reduced compared to FeGel (Figure 4.3.e), which is consistent with previous cell suspensions, where soluble FeCit was more readily reduced than poorly crystalline FeGel. Treatment with FeGel and AQDS showed initial Fe(II) accumulation in the same amount as in treatments with FeCit, 0.9mM Fe(II); however, after 6 hours Fe(II) concentration decreased suggesting that Fe(II) oxidized back to Fe(III) and neither cells nor AQDS were shuttling electrons to further reduce iron. Also, amendment with FeCit was the only one in which DAAN concentration decreased by 50% at 30 hour. It is possible that DAAN adsorbed to reactive iron solids formed during FeCit reduction as TNT degradation products can irreversibly bind to soil components or biomass.[2]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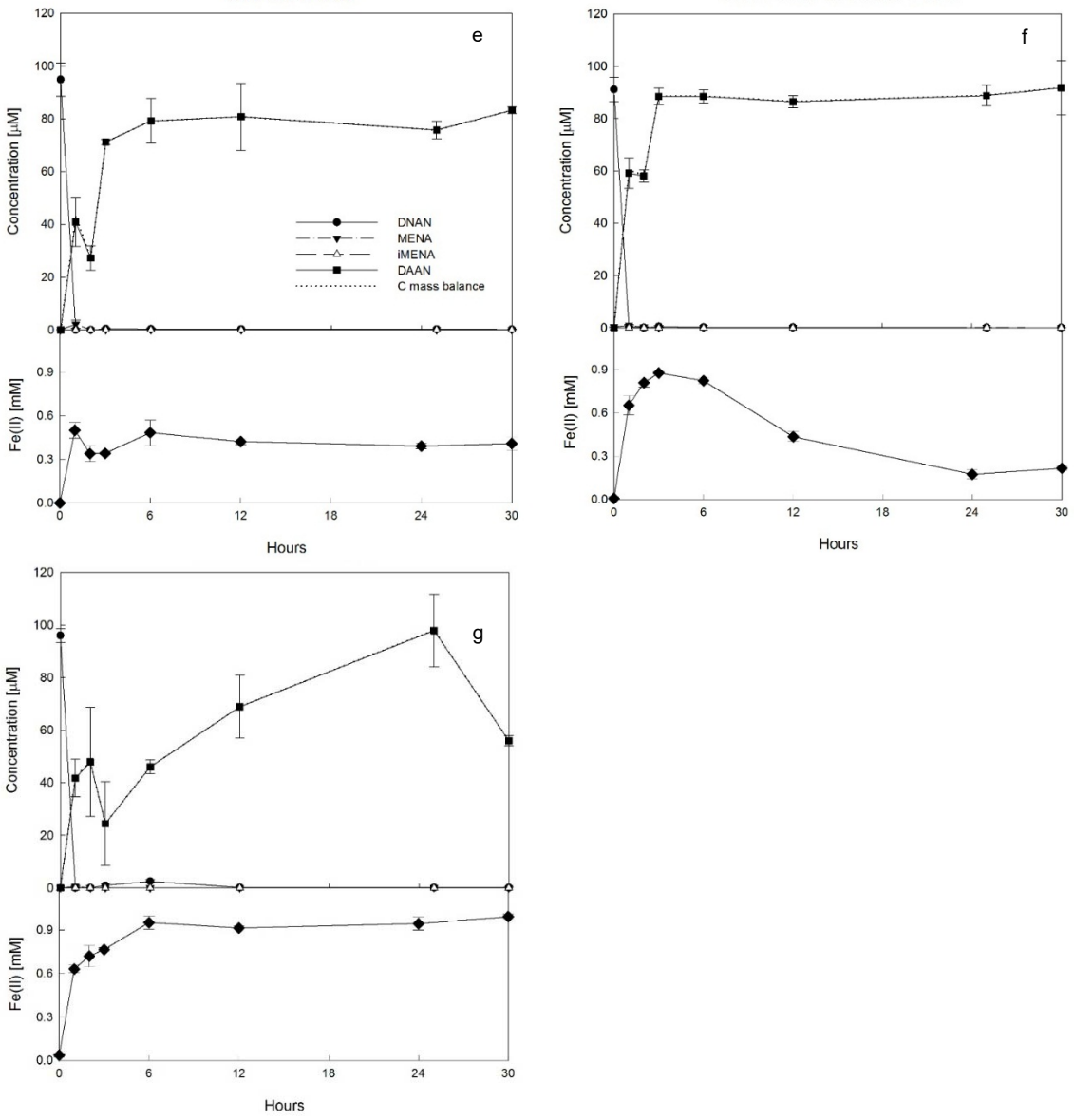


Figure 4.3. DNAN degradation mediated by *Clostridium geopurificans*, strain MJ1. Experimental conditions: 100µM DNAN, 1mM glucose as electron donor where indicated, buffered with 10mM phosphate at pH 7. Treatments: a) DNAN control, b) DNAN+cells, c) DNAN+cells+glucose, d) DNAN+cells+ glucose +0.5mM AQDS, e) DNAN+ cells+glucose+1.5mM poorly crystalline Fe(III) (FeGel), f) DNAN+ cells+glucose+0.5mM AQDS+1.5mM Fe(III) as FeGel, g) DNAN+ cells+glucose

+1.5mM soluble Fe(III) (FeCit). The error bars represent the standard deviation with n=3.

Presented data demonstrate that different microorganisms are capable of mediating DNAN degradation by non-specific electron transfer reactions. All tested bacteria reduced DNAN without an added electron donor most likely by using decayed cells as a substrate for respiration or fermentation, a process called endogenous respiration and endogenous fermentation respectively. In most treatments with *Shewanella* and *Rhodobacter* the total mass balance of detected degradation products was lower than the amount of DNAN reduced suggesting that other intermediates may be also formed. TNT has been reported to form reactive intermediates that can further undergo dimerization and adsorb to solids or biomass.[2] It is possible that during reduction of $-NO_2$ group, DNAN transforms to similar intermediates which are undetectable by analytical methods used for this study. Also, DNAN is reduced by *Shewanella* to DAAN mostly through iMENA intermediate, while *Rhodobacter* mediates degradation through MENA suggesting that different mechanisms may be used by both bacteria. In *Clostridium* cell suspension, all degraded DNAN was recovered as DAAN and only treatment with FeCit showed DAAN disappearance, probably due to adsorption or formation of dimers.

These findings show a potential for stimulating indigenous microbial communities present at contaminated sites to mediate explosives degradation via extracellular electron shuttling. The main advantage of this strategy, compared to ex-situ treatment installations, is that metabolically diverse microorganisms can be employed to

carry similar reduction reactions at sites with different biochemical conditions, making the strategy a robust bioremediation approach.

COMBINED BIOTIC-ABIOTIC 2,4-DINITROANISOLE (DNAN) DEGRADATION IN THE PRESENCE OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE (RDX)

Abstract

Department of Defense has developed new explosives composites in which traditionally used cyclic nitramines such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) have been updated with insensitive munition 2,4-dinitroanisole (DNAN). It is important to understand degradation of both compounds when mixed in a solution as explosives-contaminated sites will contain multiple energetic compounds that may compete with each other in proposed degradation reactions.

DNAN reduction in the presence of RDX was evaluated in abiotic experiments, using substoichiometric, stoichiometric, and above stoichiometric concentrations of ferrous iron and hydroquinone within pH range from 7.0 to 9.0, and in resting cell suspension of *Geobacter metallireducens*, strain GS-15, a model Fe(III)-reducing soil bacteria. Cells were harvested at late growth phase and amended into anoxic tubes buffered at pH 7.0, with an initial 100μM of DNAN and 50 μM of RDX. DNAN was reduced through intermediate 2-methoxy-5-nitroaniline (MENA) or 4-methoxy-3-nitroaniline (iMENA) to 2,4-diaminoanisole (DAAN). RDX was reduced to form (MEDINA), formaldehyde (HCHO), and ammonium (NH_4). Cells were able to reduce both DNAN and RDX most readily in the presence of extracellular electron shuttles. DNAN degradation was faster than degradation of RDX, suggesting that the reduction of insensitive munition will not be inhibited by more energetic compounds.

Introduction

The insensitive munition DNAN and high explosive RDX have been used together in newly developed explosive formulations IMX-104, OSX-12, and PAX-21. DNAN is a more stable replacement for trinitrotoluene (TNT), which failed to meet increasing safety standards. RDX has been used in traditional explosives in the last decades and it is a widespread contaminant at military test and training sites. RDX has been reported to degrade under both aerobic and anaerobic conditions by a variety of microorganisms mostly through co-metabolic reactions.[2,3] A few bacteria were able to use RDX as a sole nitrogen source.[2] Most of up to date studies on DNAN show that it can be degraded anaerobically but it is not mineralized.[12,18,19,21,22,51] Two reports demonstrate that some aerobic bacteria can use DNAN as carbon and energy source.[23,52] Both RDX and DNAN degrade predominantly via reduction of their $–NO_2$ functional groups. When nitro groups in RDX are attacked, the ring structure is destabilized and the molecule undergoes spontaneous decomposition.[2,6] The aromatic ring of DNAN stays intact and only its nitro functional groups undergo reduction to amine groups yielding 2,4-diaminoanisole (DAAN).[12,18,19,21,22,51]

Many reports describe degradation of explosives and insensitive munitions in pure compound studies but only two focus on the mixture of multiple energetic compounds, including both DNAN and RDX. One study demonstrates DNAN and RDX reduction by zero-valent iron[12] while the other reports reductive degradation by Fe/Cu bimetallic particles.[19] No studies have been published to date addressing microbial degradation of mixture of RDX and DNAN specifically. As these compounds are combined in new

formulations, both will be present at explosives-contaminated sites and it is crucial to evaluate their response to proposed decontamination strategy in the presence of one another. Degradation kinetics of DNAN and RDX combined in a solution may be different compared to results for single compounds. Currently investigated mechanisms for *in-situ* explosives degradation focus on non-specific electron shuttling reactions mediated by various indigenous soil bacteria. Coupled biological and chemical reactions increases degradation rates compared to the use of only biological or only chemical approaches.[4-6,9] One group of bacteria especially attractive for *in situ* explosives bioremediation are Fe(III) reducers due to their subsurface prevalence.[33] These microorganisms can reduce iron minerals[34] and electron shuttles, such as humic substances, which can further transfer electrons to nitramine and nitroaromatic explosives by undergoing sequential oxidation and reduction. In this way electrons are cycled between microorganisms, electron shuttles, and explosives. RDX has been reported to degrade by extracellular electron shuttling catalyzed by microbial activity of several Fe(III)-reducing genera.[4] Electron shuttling was facilitated via biogenic Fe(II) and hydroquinone (AH_2QDS), an analog for humic substances, Preliminary results confirmed that DNAN can be degraded by the same reductive reactions.

This study is an investigation of DNAN and RDX degradation by ferrous iron and hydroquinone (AH_2QDS) in abiotic reactions, as well as coupled chemical-biological reactions promoted by a model Fe(III)-reducing bacteria *Geobacter metallireducens*, strain GS-15, in the presence and absence of extracellular electron shuttles.

Results and Discussion

Data presented in Figures 5.1-5.3 show abiotic degradation with ferrous iron and hydroquinone used as reductants. Based on previous experiments and published reports,[5,12] 12 and 6 electrons are required to completely reduce one molecule of DNAN and one molecule of RDX, respectively. Reduction by ferrous iron is a one electron transfer reaction and 1.5mM Fe(II) is required to reduce 100μM DNAN and 50μM RDX. Reduction by hydroquinone is a two electron transfer reaction and only half of that concentration, that is 0.75mM AH_2QDS, is required to reduce the same amount of DNAN and RDX. Although the target RDX concentration in experimental bottles was 50μM, data shows that only approximately 40μM was transferred into the bottles, most likely due to the low RDX solubility in water.

DNAN was completely or almost completely reduced at all Fe(II) concentrations and all pH values tested. When substoichiometric amount of electron shuttle was present (0.75mM Fe(II) or 0.375mM AH_2QDS) RDX concentration remained constant (Figure 5.1.a-b, 5.2.a-b, 5.3.a-b). DNAN transformation to DAAN requires 12 electrons, which gives 1.2mM Fe(II) for 100 μM DNAN. Since only 0.75mM Fe(II) was provided, out of which approximately 0.55mM Fe(II) was oxidized at pH 7 (Figure 5.1.a), DNAN was most likely reduced to one of its intermediates. When ferrous iron and hydroquinone concentration was increased to 1.5mM and 0.75mM, respectively, 20μM RDX was degraded in treatment with Fe(II) and 30μM RDX was degraded by AH_2QDS at pH 8. At pH 9, similar amount of RDX was degraded in treatment with Fe(II) but all RDX was gone in AH_2QDS treatment.

No RDX was degraded at pH 7 for the same reductants concentrations. While DNAN can be reduced by dissolved Fe(II), RDX is unreactive with Fe(II) unless Fe(II) is complexed by ligands[40] or adsorbed to solid phase.[43,53] Iron oxidation is pH dependent and the oxidation rate increases with increasing pH. Lack of RDX degradation at pH 7 with substoichiometric and stoichiometric Fe(II) concentrations compared to pH 8 and 9 could be explained by the faster formation of Fe(III) solids resulting from Fe(II) oxidation at alkaline pH. Also, different iron solids may be formed depending on the pH value and this could affect Fe(II) adsorption and overall explosives degradation. In that sense, DNAN serves as a catalyst for RDX degradation. Once Fe(II) transfers electrons to DNAN and it oxidizes to Fe(III), reactive iron solids are being formed that can promote RDX degradation. It is yet unclear why 0.75mM AH_2QDS did not reduce RDX at pH 7 (Figure 5.1.d) as RDX has been reported to readily undergo hydroquinone reduction at these conditions.[38] When the amount of reductants was increased to double the stoichiometric concentration required for full DNAN and RDX reduction (3mM Fe(II) and 1.5mM AH_2QDS), both explosives were completely degraded at all pH values tested, with RDX disappearance following DNAN removal (Figure 5.1.e-f, 5.2.e-f, 5.3.e-f). It was expected that RDX, as a more energetic compound with a more negative standard electrode potential (E_0 for RDX is -0.55V[54] and for DNAN it is -0.40V[54]) will be degraded faster than DNAN. Nevertheless, results of this study show that DNAN is a more preferred electron acceptor than RDX.

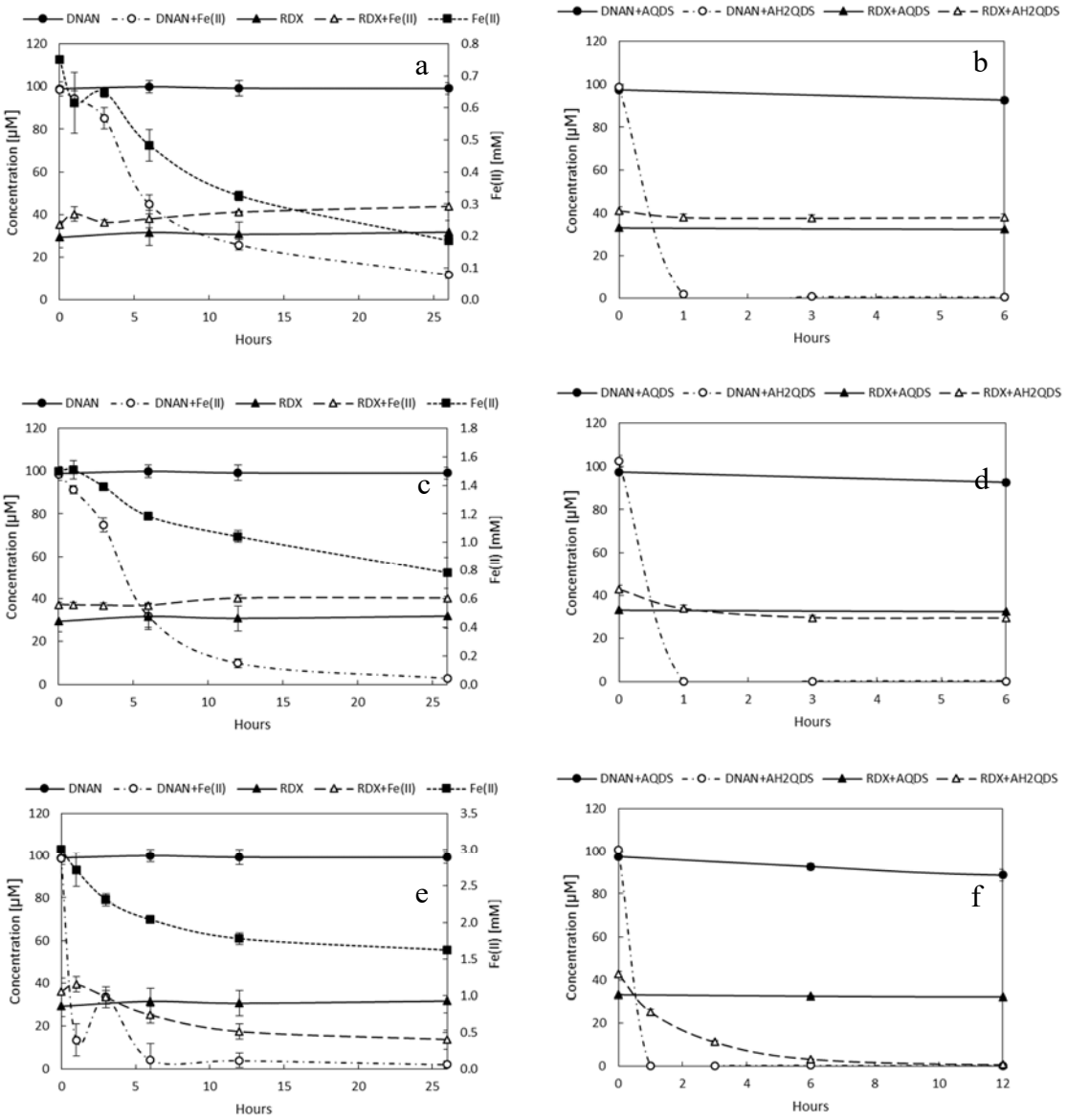


Figure 5.1. DNAN and RDX reduction by Fe(II) and AH_2QDS at pH 7. Experimental conditions: 100µM DNAN, 50µM RDX, buffered with 30mM HEPES. Treatments: a) 0.75mM Fe(II), b) 0.375mM AH_2QDS, c) 1.5mM Fe(II), d) 0.75mM AH_2QDS, e) 3mM Fe(II), f) 1.5mM AH_2QDS. The error bars represent the standard deviation with n=3.

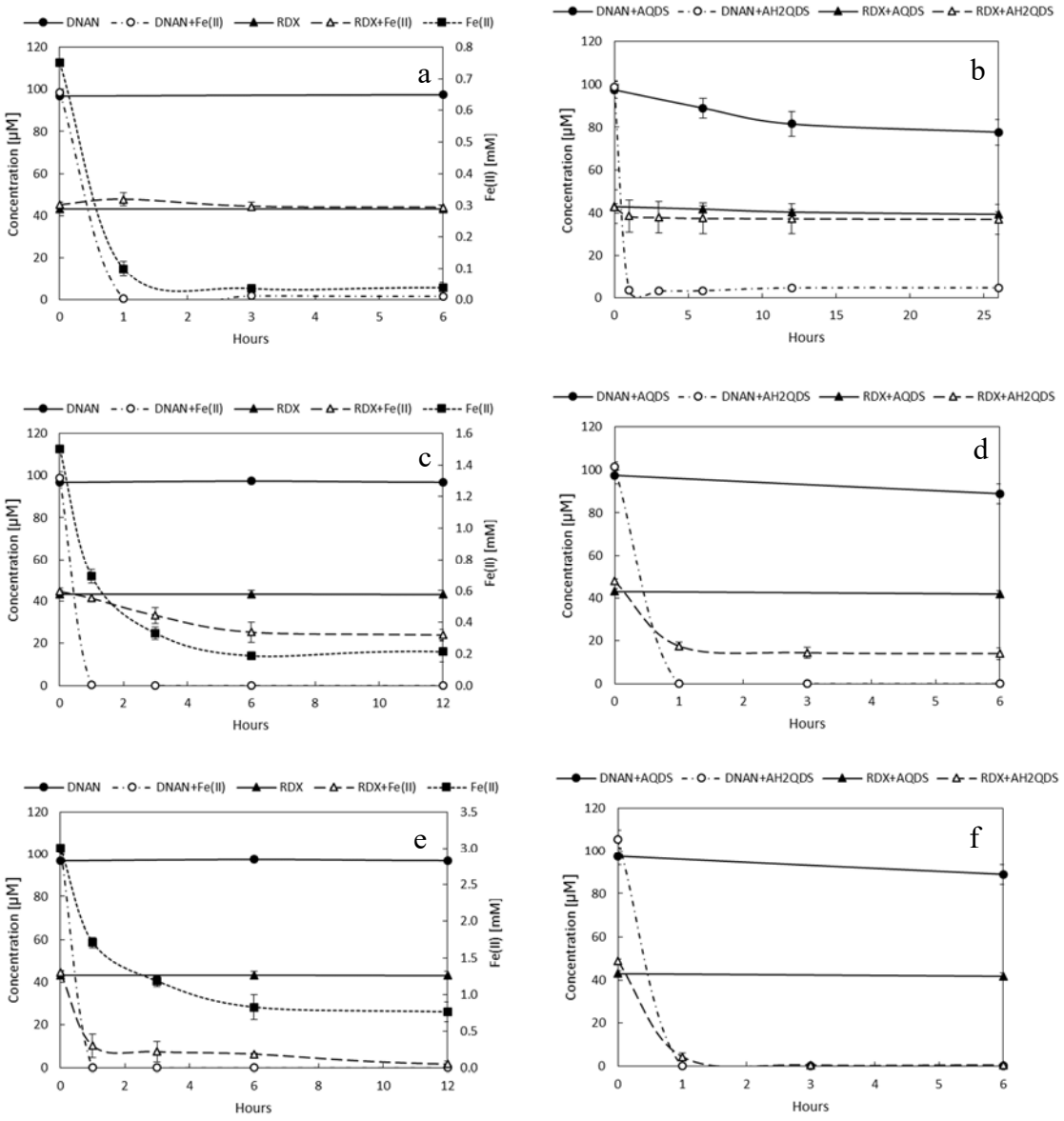


Figure 5.2. DNAN and RDX reduction by Fe(II) and AH_2QDS at pH 8. Experimental conditions: 100µM DNAN, 50µM RDX, buffered with 30mM HEPES. Treatments: a) 0.75mM Fe(II), b) 0.375mM AH_2QDS, c) 1.5mM Fe(II), d) 0.75mM AH_2QDS, e) 3mM Fe(II), f) 1.5mM AH_2QDS. The error bars represent the standard deviation with n=3.

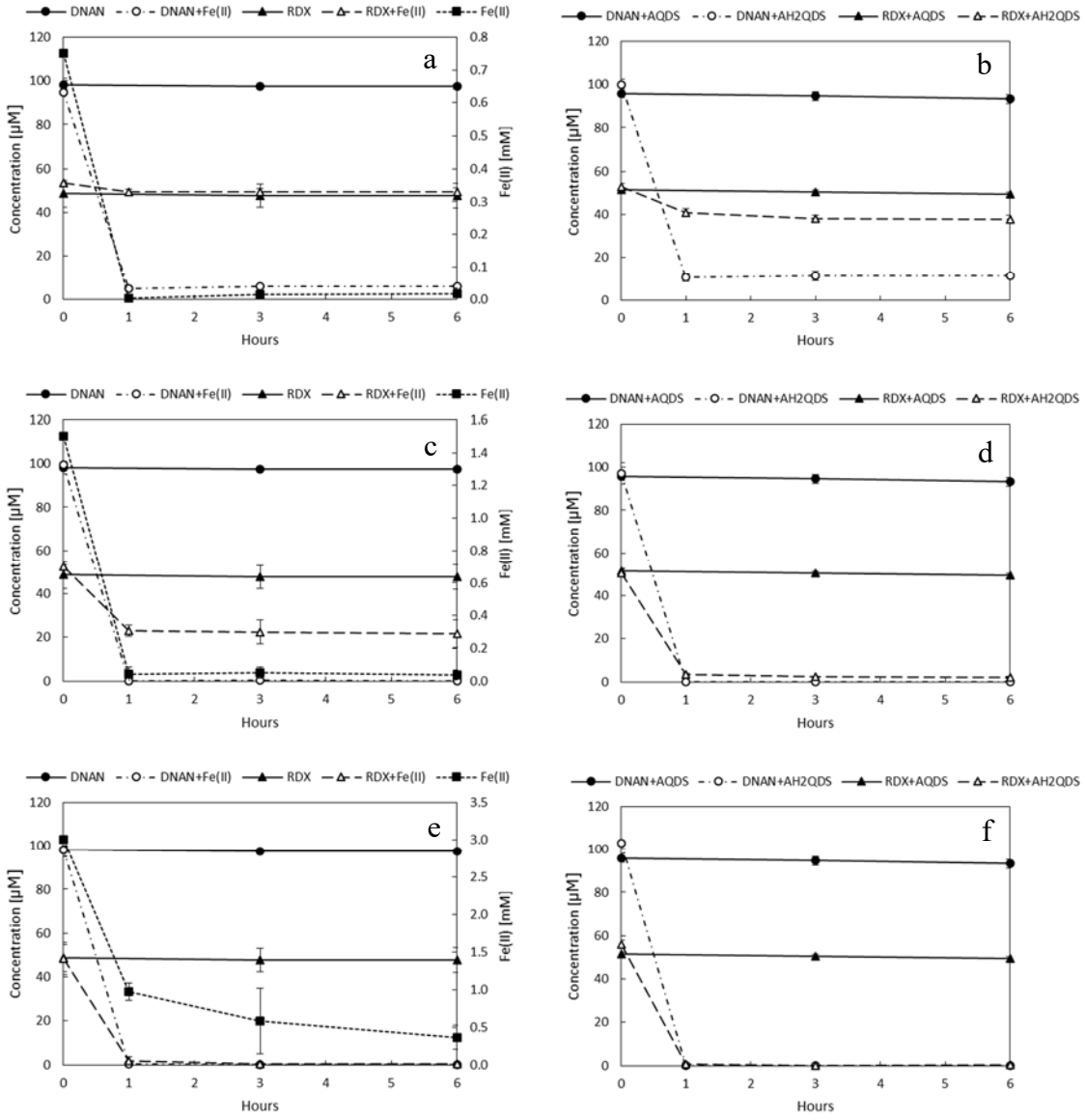
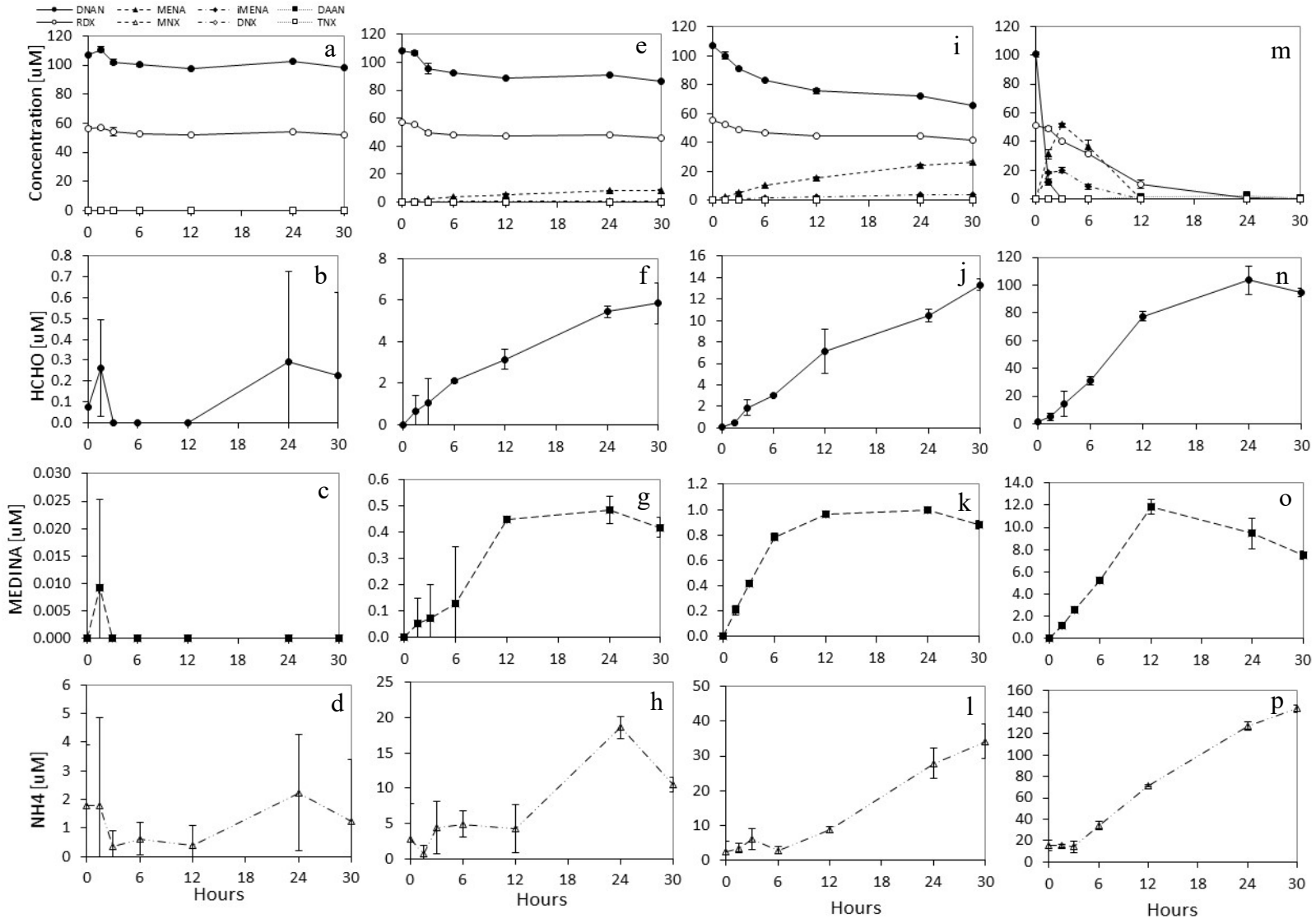


Figure 5.3. DNAN and RDX reduction by Fe(II) and AH_2QDS at pH 9. Experimental conditions: 100µM DNAN, 50µM RDX, buffered with 30mM CHES. Treatments: a) 0.75mM Fe(II), b) 0.375mM AH_2QDS, c) 1.5mM Fe(II), d) 0.75mM AH_2QDS, e) 3mM Fe(II), f) 1.5mM AH_2QDS. The error bars represent the standard deviation with n=3.

The results of cell suspension experiment with *G. metallireducens*, GS-15 show the same pattern in explosives degradation. RDX is being degraded only after DNAN is removed from the solution. DNAN is degraded via intermediates MENA and iMENA to yield DAAN, while RDX is transformed to MEDINA, formaldehyde (HCHO), and ammonium (NH_4). Nitroso intermediates (MNX, TNX, DNX) resulting from the reduction of nitro function groups in RDX were not detected, suggesting that once the molecule is attacked, its ring cleaves immediately to form products of smaller molecular weight.

Cells alone were able to reduce only 20μM DNAN (Figure 5.4.e) and the addition of acetate as electron donor slightly increased DNAN degradation to 30μM (Figure 5.4.i). In both treatments very little of RDX was removed, approximately 5μM. The addition of AQDS significantly increased electron shuttling between microorganism and explosives resulting in complete removal of both DNAN and RDX (Figure 5.4.m). GS-15 did not reduce FeGel readily (Figure 5.4.u); therefore data from this treatment resemble data for incubation with cells and acetate. When both ADQS and FeGel were present (Figure 5.4.v-z), degradation raters were the fastest and FeGel was reduced to Fe(II) within 24 hr due to the additional electron transfer pathway from AQDS. DNAN was degraded to yield 30μM DAAN and RDX degraded to 70μM HCHO, 12μM MEDINA, and 120μM NH_4. Also in treatment with FeCit, cells reduced iron immediately in the first hour (Figure 5.4. aa-ae). DNAN was reduced within 12 hr to form 50 μM MENA and 10 μM iMENA, while 30 μM RDX was reduced to 35 μM HCHO, 3μM MEDINA and 30μM NH_4.


DNAN
RDX
MENA
MNX
4MENA
DNX
DAAN
TNX
Concentration [uM]
HCHO [uM]
MEDINA [uM]
NH4 [uM]
Hours
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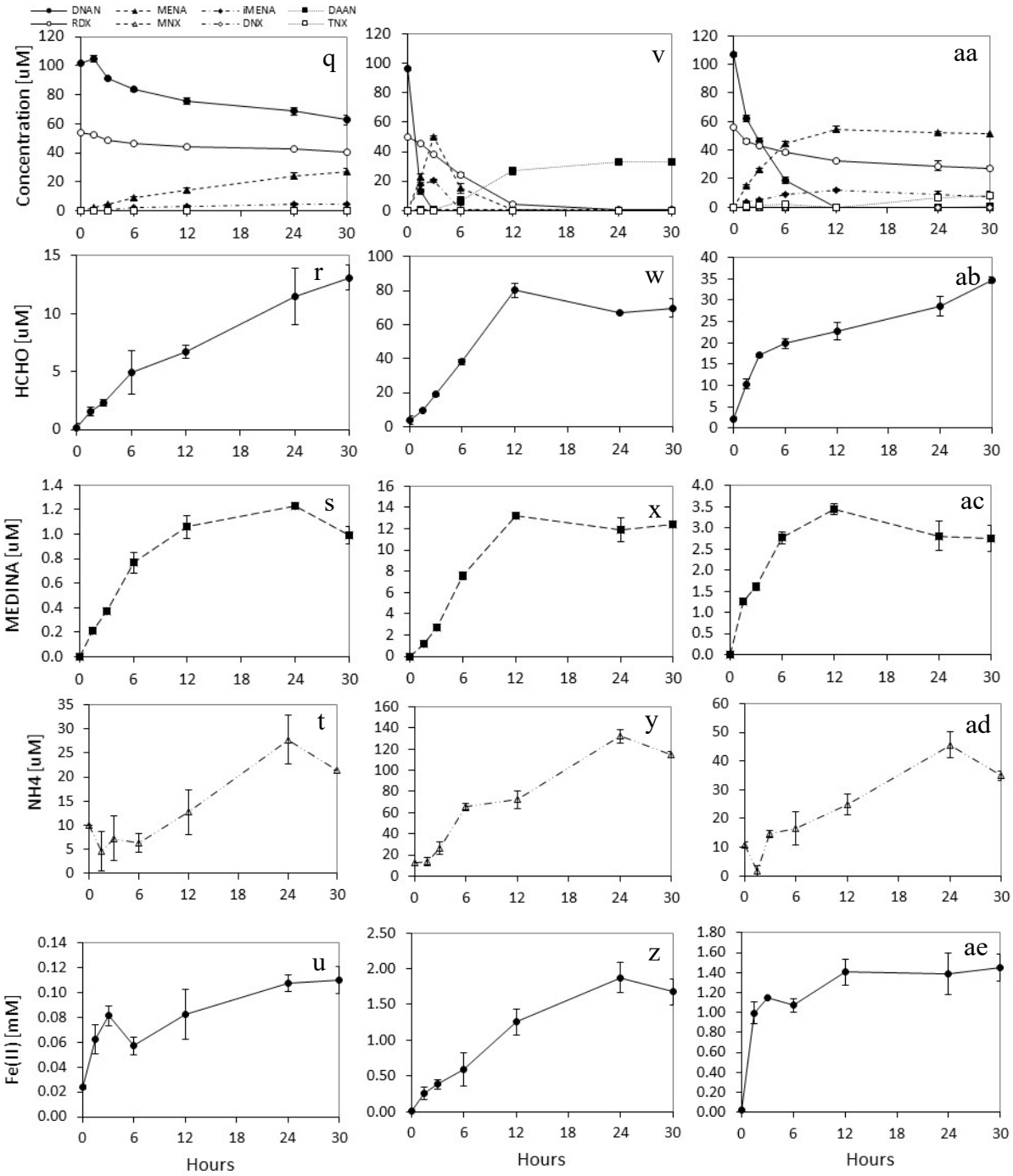

Figure 5.4. DNAN and RDX degradation mediated by *Geobacter metallireducens*, strain GS-15. Experimental conditions: 100µM DNAN, 50µM RDX, 1mM acetate as electron donor where indicated, buffered with 30mM bicarbonate at pH 7. Treatments: a-d)

DNAN and RDX control, e-h) DNAN+RDX+cells, i-l) DNAN+RDX+cells+acetate, m-p) DNAN+RDX+cells+acetate +0.5mM AQDS, q-u) DNAN+RDX+cells+acetate +1.5mM FeGel, v-z) DNAN+RDX+cells+acetate+0.5mM AQDS+1.5mM FeGel, aa-ae) DNAN+RDX+cells+acetate+1.5mM FeCit.

This data suggests that RDX affects the distribution of DNAN degradation products. While RDX itself does not inhibit DNAN reduction, it competes for electrons with MENA and iMENA. DNAN alone in GS-15 resting cell suspension was more completely transformed to DAAN than it was shown for the mixture of DNAN and RDX. Electron shuttles increased transformation of both explosives and data suggests that the rate limiting step was electron transfer from microbes to shuttles.

Both DNAN and RDX can be effectively reduced by extracellular electron shuttling mediated by GS-15; therefore, this strategy may prove useful for *in situ* bioremediation applications.

REDUCTIVE TRANSFORMATION OF NITROGUANIDINE (NQ) BY FERROUS IRON AND ELECTRON SHUTTLES

Abstract

Department of Defense has recently developed new insensitive munitions in which trinitrotoluene (TNT) has been replaced with more stable compound, nitroguanidine (NQ), to avoid accidental detonations. NQ has not yet been identified as an environmental contaminant of concern but it can be mobile in the subsurface due to its high water solubility; therefore, it is important to understand its fate and transport in the environment and to develop effective remediation strategy should it become an issue.

This study investigates reductive NQ degradation via electron transfer from iron and humics electron shuttles. NQ was degraded by Fe(II) complexed with trihydroxybenzoic acid (THBA) at pH 7, 8, and 9. Fe(II) alone was able to reduce NQ only at pH 9. Anthraquinone-2,6-disulfonate (AQDS), a model compound for humic substances, did not react with NQ. Also, NQ was not degraded in resting cell suspensions of a Fe(III)-reducing microorganisms *Geobacter metallireducens,* strain GS-15, and a fermentative bacteria *Clostridium geopurificans*, strain MJ1. The addition of Fe(III) and AQDS as electron shuttles did not stimulate microbial degradation.

These data suggest that NQ may persist in the environment and it may be resistant to the currently developed bioremediation strategies to treat explosives-contaminated sites via microbially mediated extracellular electron shuttling processes; therefore, NQ may require a different approach for contamination control.

Introduction

Nitroguanidine (NQ) is one of the insensitive munitions used in new explosive formulations in place of 2,4,6-trinitrotoluene (TNT). It improves chemical stability of new formulations to avoid accidental detonations. Environmental fate and transport of NQ has not been fully evaluated but considering its high water solubility (3g/L[1]) it may become a contaminant of concern. While NQ itself is not toxic,[26] some of its decomposition products are.

There are only a few reports available on NQ degradation. One study showed NQ transformation to nitrosoguanidine by anaerobic activated sludge and no degradation under aerobic conditions.[55] Another group reported NQ mineralization by aerobic bacteria *Variovorax* strain VC1 to yield ammonia, nitrous oxide, and carbon dioxide.[26] Also, NQ can be removed from wastewater in a reactor containing bimetallic particles.[19]

NQ has been reported to undergo biodegradation by indigenous bacteria in three soil samples though the exact mechanisms of transformation has not been identified.[27] Once NQ is released to the environment, it will be one of many energetic compounds present in explosives plumes. Various soil microorganisms have been reported to mediate non-specific extracellular electron shuttling reactions, using iron and quinone/hydroquinone (AQDS/AH_2QDS) couple as electron shuttles, that were successful in degrading high explosives such as RDX and HMX;[4,38] therefore, since this strategy may be applied to treat contaminated sites, it is important to evaluate NQ response to the same type of redox reactions.

This study investigates anaerobic NQ degradation by ferrous iron alone, ferrous iron complexed with 2,3,4-trihydroxybenzoic acid (THBA), and hydroquinone at different pH. Two microorganisms commonly found in soil *Geobacter metallireducens*, strain GS-15, and *Clostridium geopurificans*, strain MJ1, were used to stimulate electron shuttling to degrade NQ in a manner similar to naturally occurring subsurface redox processes.

Results and Discussion

NQ degradation has been evaluated based on the assumption that its $-NO_2$ functional group can be reduced under anaerobic conditions, which has been reported as a mechanism of NQ transformation by anaerobic activated sludge.[55] Screening of ferrous iron alone or ferrous iron complexed with organic ligand THBA at pH values from 6 to 9 shows that NQ is most readily degraded at neutral and alkaline pH by THBA-Fe(II) complex as well as Fe(II) alone at pH 9 (Figure 6.1). THBA makes Fe(II) a stronger reductant due to the fact that it binds preferentially to oxidized Fe(III) and it lowers redox potential of Fe(II)/Fe(III) couple.[36,40] Organically complexed Fe(II) was reported to degrade contaminants which were not reactive with Fe(II) alone.[40] No NQ degradation was detected at pH 6 (Figure 6.1.a). THBA-Fe(II) reduced 50μM NQ after 30 days at pH 7 and 9 (Figure 6.1.b, d). At pH 8, 70μM NQ was degraded in 2 days and it was almost completely removed after 30 days (Figure 6.1.c). At pH 9, 80μM NQ was reduced by Fe(II) alone only after 2 days

and after 5 days all NQ was gone. This is most likely related to fast Fe(II) oxidation at pH 9 and formation of reactive iron solids.

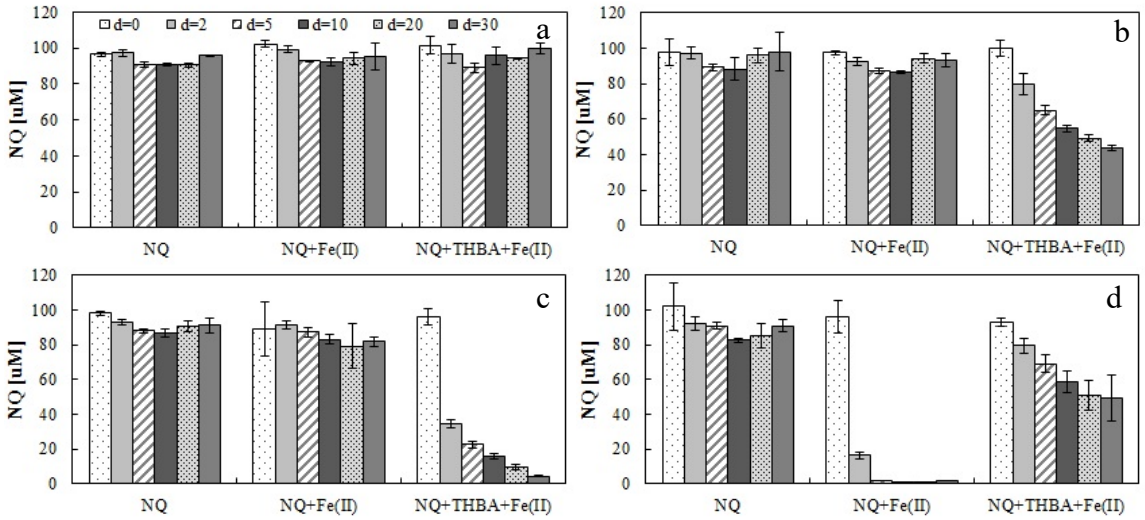

Figure 6.1. NQ reduction by Fe(II) alone and Fe(II) complexed with THBA at pH range 6-9. Experimental conditions: 100μM NQ, 1.5mM Fe(II), 3mM THBA, buffered with 30mM a) MES at pH 6, b) HEPES at pH 7, c) HEPES at pH 8, and d) CHES at pH 9. The error bars represent the standard deviation with n=3.

Given that NQ can be degraded by Fe(II), another compound evaluated for future remediation strategies was hydroquinone, a model moiety for humic substances that constitute soil. Despite the effective reduction of other explosive compounds by hydroquinone,[5,6,38] NQ was not degraded neither at pH 7 nor at pH 8 (Figure 6.2) even when relatively high concentration of 4mM AH_2QDS was used. This data suggests that NQ may require the presence of reactive solids, which are formed during iron-mediated degradation but not using AH_2QDS.

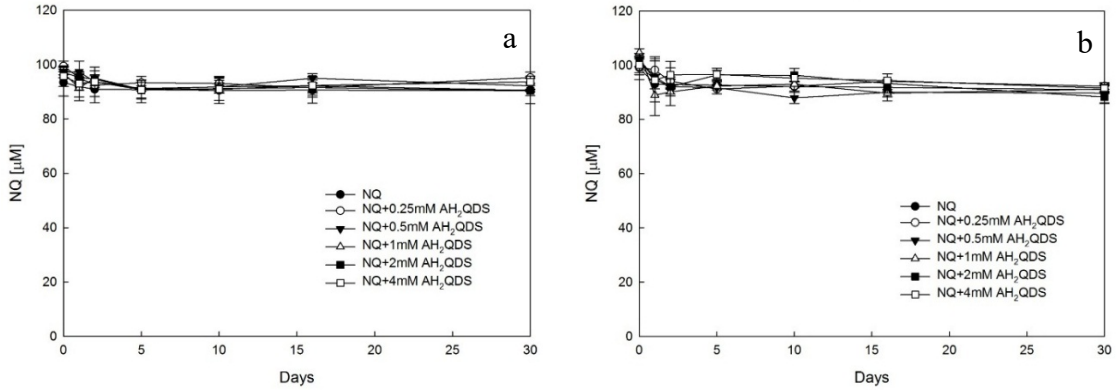

Figure 6.2. NQ reduction by AH_2QDS. Experimental conditions: 100µM NQ, buffered with 30mM HEPES at pH a) 7 and b) 8. The error bars represent the standard deviation with n=3.

Reduction of Fe(II) and electron shuttling can be promoted in the subsurface environment by indigenous microorganisms.[33,47,48] Mixed biological-chemical processes lead to more complete explosive degradation compared to only chemical reactions.[9] Although high explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) was reported to undergo degradation by *Geobacter metallireducens*, strain GS-15[9,38] and *Clostridium geopurificans*, strain MJ1,[50] NQ was not degraded by any of the mentioned bacteria, neither in the absence or presence of electron shuttles (Figures 6.3 6.5). Fe(II) measurements for GS-15 cell suspension experiment (Figure 6.4) show that little Fe(III) was reduced to Fe(II). Out of 1.5mM Fe(II) added, only 80 µM was recovered as Fe(II). Tubes run as controls without NQ demonstrated higher Fe(III) reduction but the final Fe(II) concentration was still low, only 140 µM. GS-15 did not reduce poorly crystalline Fe(III) even when AQDS was used to facilitate

electron transfer. These results show that mixed biological-chemical processes that are effective for degradation of nitroaromatic or nitramine explosives may not work for NQ.

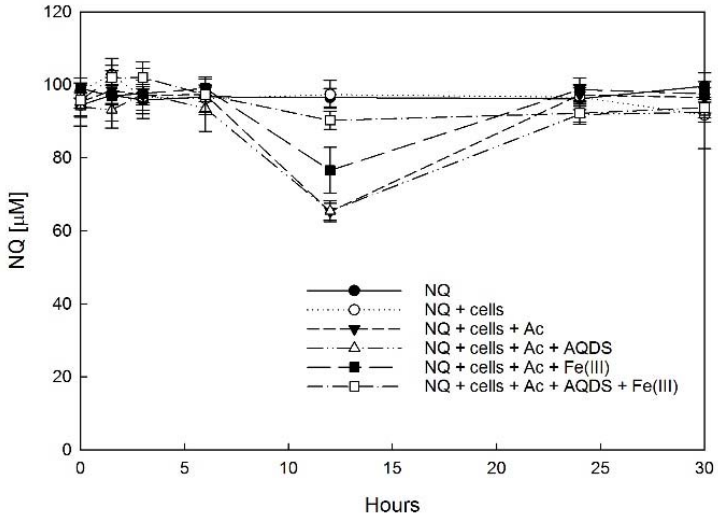

Figure 6.3. NQ in the cell suspension of *Geobacter metallireducens*, strain GS-15. Experimental conditions: 100μM NQ, 1.5mM poorly crystalline Fe(III) (FeGel), 0.5mM AQDS, buffered with 30mM bicarbonate at pH 7. Electron donor was 1mM acetate. The error bars represent the standard deviation with n=3.

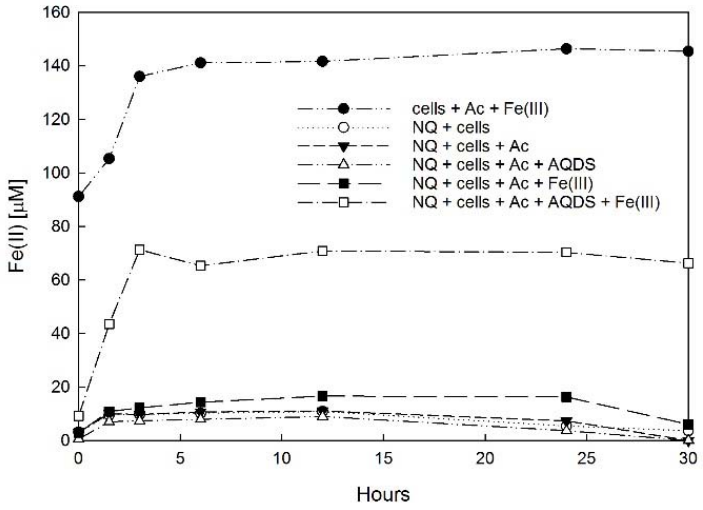

Figure 6.4. Reduction of Fe(III) to Fe(II) by *Geobacter metallireducens*, strain GS-15 in the presence of 100µM NQ. Experimental conditions same as in Figure 6.3.

NQ degradation mediated by MJ1 was studied with Fe(III) concentration increased to 5mM (Figures 6.5 and 6.6) in order to exclude the potential inhibition effect of too low electron shuttle concentration on NQ degradation within 30hr timeframe of cell suspension experiment. MJ1 with electron donor reduced 0.4mM Fe(III) to Fe(II) and AH_2QDS increased reduction to 1mM Fe(II), out of initial 5mM Fe(III) in both treatments (Figure 6.6). This suggests that the increase in electron shuttle concentration would not have an effect on NQ degradation, especially since NQ was resistant to proposed decontamination strategy.

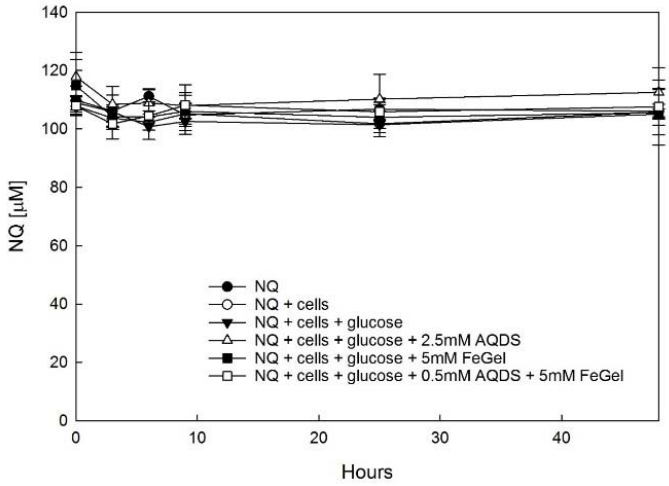

Figure 6.5. NQ in the cell suspension of *Clostridium geopurificans*, strain MJ1. Experimental conditions: 100µM NQ, 5mM Fe(III) in poorly crystalline (FeGel), 0.5mM or 2.5mM AQDS, 10mM phosphate buffer at pH 7. Electron donor was 1mM glucose. The error bars represent the standard deviation with n=3.

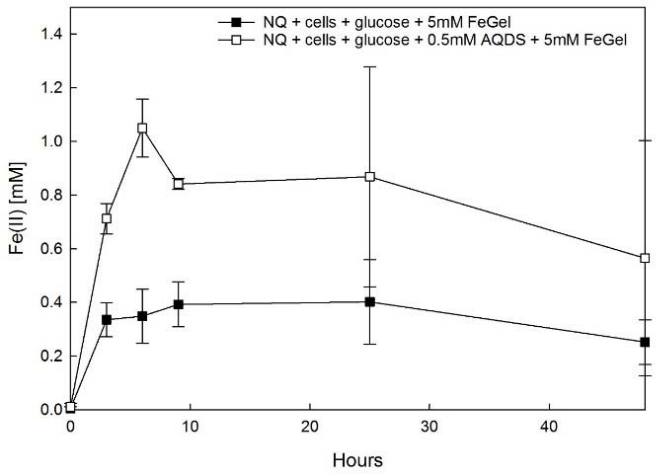

Figure 6.6. Reduction of Fe(III) to Fe(II) by *Clostridium geopurificans*, strain MJ1. Experimental conditions same as in Figure 6.5.

Results show that NQ can be resistant to reductive degradation and it may become a serious groundwater contaminant considering its mobility in the subsurface. NQ was not affected by non-specific electron shuttling processes mediated by soil microorganisms at circumneutral pH; however, it was degraded by organically complexed Fe(II) at pH 7, 8, and 9 in the order of a few to several days. NQ is the only explosive compound without a ring structure and thus it may require a different approach for bioremediation due to its chemical stability. Another insensitive munition considered for TNT replacement is 2,4-dinitroanisole (DNAN). DNAN was readily degraded in suspensions of Fe(III)-reducing, photosynthetic, and fermentative bacteria, both in the presence and absence of electron shuttles abundant in soils. The findings of this study are important for decision making process in regard to the use of DNAN versus NQ in newly developed explosive formulations and possible environmental and health implications.

MICROBIAL COMMUNITY SCREENING AT EXPLOSIVES-CONTAMINATED SITES

Abstract

Soil and water samples from explosives-contaminated military test sites in Alaska, Virginia, and North Carolina were screened for the dominant microbial populations. The results were obtained by Illumina sequencing of 16s ribosomal RNA (rRNA) gene in DNA extracted from the samples. Explosive compounds pose a threat to public health if they migrate to groundwater and enter water supply system. Identification of indigenous microorganisms will provide a guidance in developing *in-situ* bioremediation strategies that use natural geochemical processes mediated by active soil bacteria. Screening of microbial communities showed that the most dominant orders of microorganisms potentially capable of degrading explosives at evaluated sites include *Clostridiales, Burkholderiales, Actinomycetales, Myxococcales,* and *Rhodospirillales.*

Introduction

Explosives are common contaminants at army training sites and live-fire ranges. Nitroaromatic and nitramine compounds such as explosives are carcinogenic and mutagenic so they pose threat to human health and the environment. Many microorganisms have been reported to degrade explosives in laboratory conditions[2,3] and some of them were isolated from aquifer material at contaminated military sites; however, specialized strains may not be effective in the subsurface as they will have to compete for nutrients and space with already established microbial communities. Recently, the use of non-specific extracellular electron shuttling reactions has been proposed as an *in-situ* bioremediation strategy that can be mediated by metabolically diverse indigenous microorganisms at sites of different geochemistry.

One study demonstrated through 16s rRNA gene sequencing that microorganisms from *Comamonas, Pseudomonadaceae, Acinetobacter*, and *Rhodococcus* phylotype were most abundant after degradation of high explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in sediments from a detonation area at a naval base.[56] Stable isotope probing (SIP) study using labeled (^{13}C and ^{15}N) RDX showed that *Pseudomonadaceae* were a dominant phylotype to uptake labelled atoms, which suggests that they were primary responsible for RDX degradation.[56]

In this study, several samples from three army detonation test sites were analyzed for 16s rRNA gene sequences to identify the dominant microbial communities that could be employed for *in-situ* explosives bioremediation. Microorganisms were classified based on their taxonomic order.

Results and Discussion

The biggest group of microorganisms present in meltwater from snow sample collected at test site in Alaska was *Clostridiales* (Figure 7.1, Table 7.1). These bacteria grow via fermentation and several species have been so far reported to degrade explosives[2,3] due to the fact that energetic compounds act as electron sinks for fermentative growth.[3] Another group that may be significant for bioremediation applications are *Burkholderiales*. Microorganisms from that order were enriched in sediments following RDX degradation[56] suggesting that their metabolic capabilities may be important for natural attenuation of explosives. Also, *Burkholderiales* were reported to degrade antibiotics and they were suggested for potential use in bioremediation operations to degrade pesticides.[57]

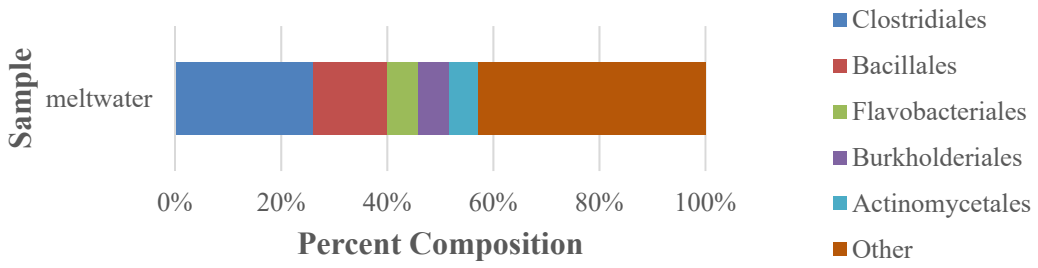

Figure 7.1. Microbial community composition in meltwater sample from snow collected at military site in Alaska from IMX-101 and IMX-104 detonation test. Dominant groups were operationally described as >5% of all 16srRNA gene copies in the sample. Non-dominant groups were combined in a category "Other".

Table. 7.1. Percentage composition of dominant microbial communities in meltwater from Alaska test site.

Order	Sample meltwater
Clostridiales	26.06%
Bacillales	13.94%
Flavobacteriales	5.86%
Burkholderiales	5.77%
Actinomycetales	5.56%
Other	42.83%

Samples from test site in North Carolina (Figure 7.2, Table 7.2) also demonstrated well established presentence of *Burkholderiales*. Additionally, significant content of *Actinomysetales* and *Clostridiales* may be beneficial in promoting *in situ* degradation. Representatives of *Actinomycetales* order were previously reported to degrade RDX.[58] The presence of *Acidobacteriales* in pore water sample suggest that the site conditions may be acidic, which could inhibit the activity of target microorganisms as well as subsurface abiotic reactions leading to explosives degradation. Also, the combination of aerobic (*Xanthomonadales, Actinomycetales*) and anaerobic (*Burkholderiales, Clostridiales*) bacteria in the samples implies that site conditions may shift temporarily from oxic to anoxic. Finally, *Myxococcales*, detected in water and soil samples, produce extracellular enzymes to access insoluble organic substrates and they could be one of the target community to stimulate explosives degradation via extracellular electron shuttling.

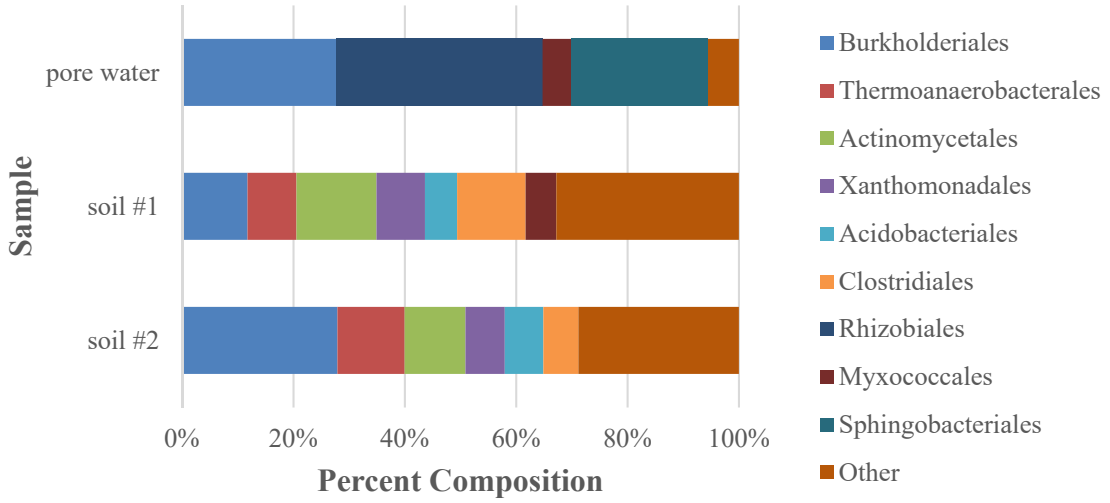

Figure 7.2. Microbial community composition in pore water and soil samples collected at detonation test site in eastern North Carolina. Dominant groups were operationally described as >5% of all 16srRNA gene copies in the sample. Non-dominant groups were combined in a category "Other".

Table. 7.2. Percentage composition of dominant microbial communities in samples from North Carolina test site.

Order	Sample		
	pore water	soil #1	soil #2
Burkholderiales	27.74%	11.75%	27.90%
Thermoanaerobacterales	0.00%	8.79%	12.06%
Actinomycetales	0.00%	14.36%	10.92%
Xanthomonadales	0.00%	8.71%	7.09%
Acidobacteriales	0.00%	5.78%	6.92%
Clostridiales	0.00%	12.29%	6.29%
Rhizobiales	36.99%	4.13%	3.84%
Myxococcales	5.14%	5.53%	3.25%
Sphingobacteriales	24.58%	2.82%	0.00%
Other	5.55%	32.77%	28.83%

The last set of soils samples, provided from Naval Surface Warfare Center (NSWC) Dahlgren Churchil testing range (Figure 7.3, Table 7.3), shows significant presence of microorganism that could potentially be used for bioremediation and that include orders *Actinomycetales, Clostridiales, Myxococcales*, and *Rhodospirillales*. The last group represents purple nonsulfur bacteria that can grow by photosynthesis. One representative of purple bacteria was reported to degrade RDX[59] and DNAN; therefore other microorganisms with similar metabolic capabilities will most likely encode same genes responsible for explosives degradation.

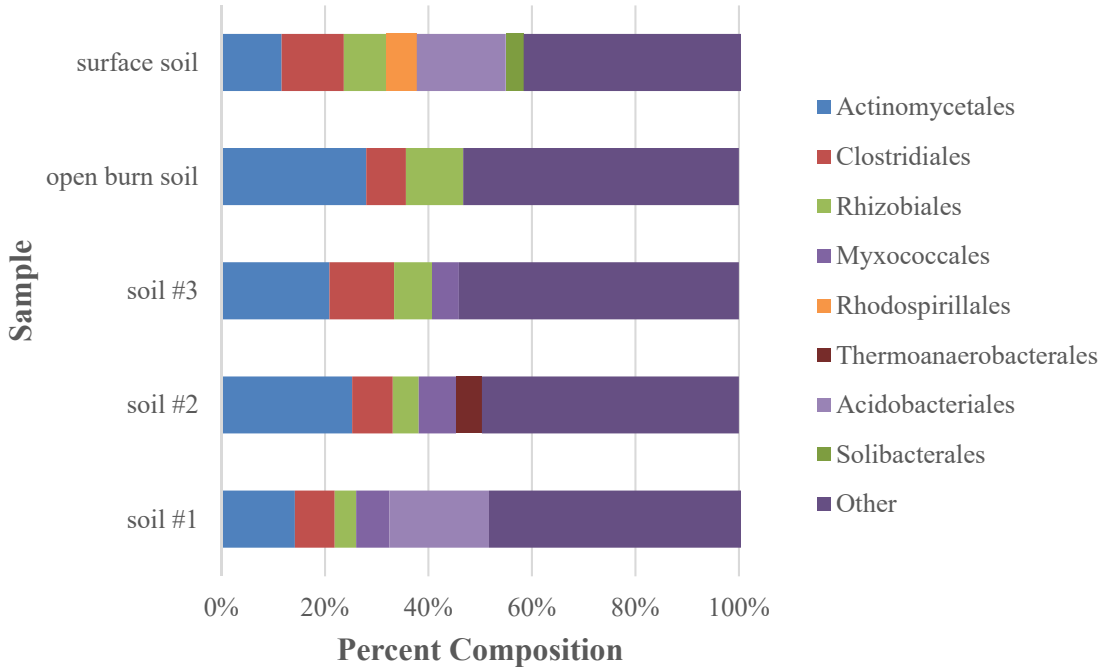

Figure 7.3. Microbial community composition in soil samples collected from detonation tests at NSWC Dahlgren Churchil range in Virginia. Dominant groups were operationally described as >5% of all 16srRNA gene copies in the sample. Non-dominant groups were combined in a category “Other”.

Table. 7.3. Percentage composition of dominant microbial communities in samples from Virginia test site.

Order	Sample				
	soil #1	soil #2	soil #3	open burn soil	surface soil
Actinomycetales	14.18%	25.25%	20.88%	28.00%	11.64%
Clostridiales	7.71%	7.86%	12.50%	7.65%	12.02%
Rhizobiales	4.16%	5.01%	7.33%	11.07%	8.16%
Myxococcales	6.43%	7.21%	5.19%	0.00%	0.00%
Rhodospirillales	0.00%	0.00%	0.00%	0.00%	5.94%
Thermoanaerobacterales	0.00%	5.01%	0.00%	0.00%	0.00%
Acidobacteriales	19.23%	0.00%	0.00%	0.00%	17.17%
Solibacterales	0.00%	0.00%	0.00%	0.00%	3.47%
Other	52.45%	49.66%	54.10%	53.28%	45.06%

Analysis of microbial communities in samples from explosive-contaminated sites demonstrated that microorganism capable of degrading energetic compounds were present in significant amounts; therefore, bioremediation using indigenous soil bacteria may be a reasonable approach. Further characterization of sites biochemical conditions' would have to be performed to evaluate environmental factors, such as pH or nutrient availability, that may inhibit microbial activity. Additionally, explosives degradation could be verified in sediment incubations to provide experimental evidence of microbially mediated degradation at military sites.

CONCLUSIONS

Data presented in this work demonstrate that DNAN can be readily degraded via extracellular electron shuttling promoted in the subsurface by metabolically diverse microorganisms. DNAN degradation is not inhibited by RDX, which is important as multiple energetic compounds are present at contaminated sites and they may affect degradation kinetics of one another. Dissolved ferrous iron was effective at reducing DNAN; however, once Fe(II) oxidizes to Fe(III) and precipitates, further degradation is achieved by Fe(II) adsorbed to freshly formed iron solids. Biological transformation kinetics were faster than chemical reactions kinetics, but combined biological-chemical processes degraded DNAN at even faster rates than individual mechanisms alone. In all tested conditions, DNAN was degraded by a reduction of $–NO_2$ functional group to $–NH_2$.

Non-specific co-metabolic reactions carried by active microbial communities are advantageous for bioremediation compared to treatment methods that employ a single bacterial strain. Although specialized strains or consortia can degrade DNAN to innocuous products and use them as carbon and energy source,[23,52] these bacteria may not be competitive enough with microbial communities established at military facility sites. Non-specific co-metabolic reactions that result in electron transfer and reductive explosives degradation are mediated by multiple soil microorganisms and can therefore be used to decontaminate sites of different biochemistries. All bacteria tested in this study were able to degrade DNAN and their unique capabilities to strive in different conditions show that extracellular electron shuttling can be applied to many different environments.

Geobacter metallireducens and *Shewanella oneidensis* are representatives of Fe(III)-reducing genera and they will be most active once Fe(III)-reducing conditions in the subsurface are established. *Clostridium geopurificans* demonstrates the ability of fermenters to use explosives as electron sinks. Fermentative bacteria will dominate anaerobic plumes with methanogenic conditions. *Rhodobacter sphaeroides* and *S. oneidensis* can grow both aerobically and anaerobically using multiple substrates and they will prevail at the oxic/anoxic boundary as well as in surface waters. The diversity of microorganisms capable of promoting electron shuttling makes the suggested strategy a robust bioremediation approach for nitroaromatic and nitramine energetic compounds. Iron minerals and humic substances are effective electron shuttles and they can increase degradation rates significantly.

NQ was more resistant to reductive degradation and it did not degrade in resting cell suspensions of *G. metallireducens* or *C. geopurificans*. Extracellular electron shuttling strategies that can be successfully applied to RDX, HMX, TNT,[4-6,8] and DNAN will not work for NQ; therefore, it is an important information for DoD in regard to environmental implications of the further use of NQ in newly developed explosives formulations. Both NQ and DNAN have been proposed as a replacement for TNT. Considering the ease of DNAN degradation and persistence of NQ, it would be recommended to substitute NQ use with complete switch to DNAN.

This study reports for the first time that insensitive munitions can be degraded by promoting naturally occurring subsurface processes which will play a key role in natural attenuation of energetic compounds and in developing *in-situ* bioremediation methods.

FUTURE RESEARCH NEEDS

While this report furthers understanding of insensitive munitions fate and transport in the environment, there are still several questions that need to be addressed:

1. Does DAAN undergo any further transformations?

   Although DNAN was showed to degrade via reduction of nitro functional groups to form DAAN, the fate and transport of DAAN in the environment has not yet been investigated. TNT degradation products have been reported to form insoluble dimers and to irreversibly bind to soil or biomass matrix so that they are immobilized from groundwater. It is possible that DAAN could also adsorb to solids, based on the similarities in degradation mechanisms for TNT and DNAN that lead to formation of amino products. Further evaluation of DAAN transformations would provide a full picture of degradation pathway.

2. Are there any azo, azoxy, acetyl or phenolic derivatives formed during DNAN degradation as it was reported for TNT?

   The distribution of DNAN degradation products in performed experiments suggests that degradation using ferrous iron and hydroquinone yields different intermediates. Other researchers reported more intermediates than MENA and iMENA before the final formation of DAAN;[21,22] however, they employed more sensitive detection method with the use of mass spectrometry, which was not available for this research. The presence of another intermediates is also suggested by the incomplete mass balance in many experiments.

3. Can microbial communities established in sediment samples from explosives-contaminated sites degrade DNAN?

   Samples were analyzed for 16s rRNA gene sequences to identify the most dominant bacteria order but no degradation experiments were performed on the provided aquifer material.

4. How NQ degradation could be enhanced at neutral pH?

   Only the complex of Fe(II)-THBA was able to degrade NQ at pH 7, which is the most relevant pH for the environmental applications. NQ was resistant to microbially mediated extracellular electron shuttling so the alternate strategy will have to be developed. Fe(II) adsorbed to mineral surfaces has been reported to degrade nitramine explosive RDX when Fe(II) alone was not effective; therefore, the suggested additional study could investigate if NQ can be degraded by more reactive, surface-bound Fe(II).

5. What are degradation kinetics for RDX, HMX, NTO, DNAN, and NQ combined in a solution?

   There is no study up to date to address degradation of the mixture of all mentioned explosives. There are a few reports on degradation of RDX combined with HMX, or the solution of NTO, DNAN, and NQ; however, all of these compounds will be present at contaminated sites or in the wastewater streams and it is important to understand their interactions.

APPENDICES

Appendix A

Supporting information for Chapter V

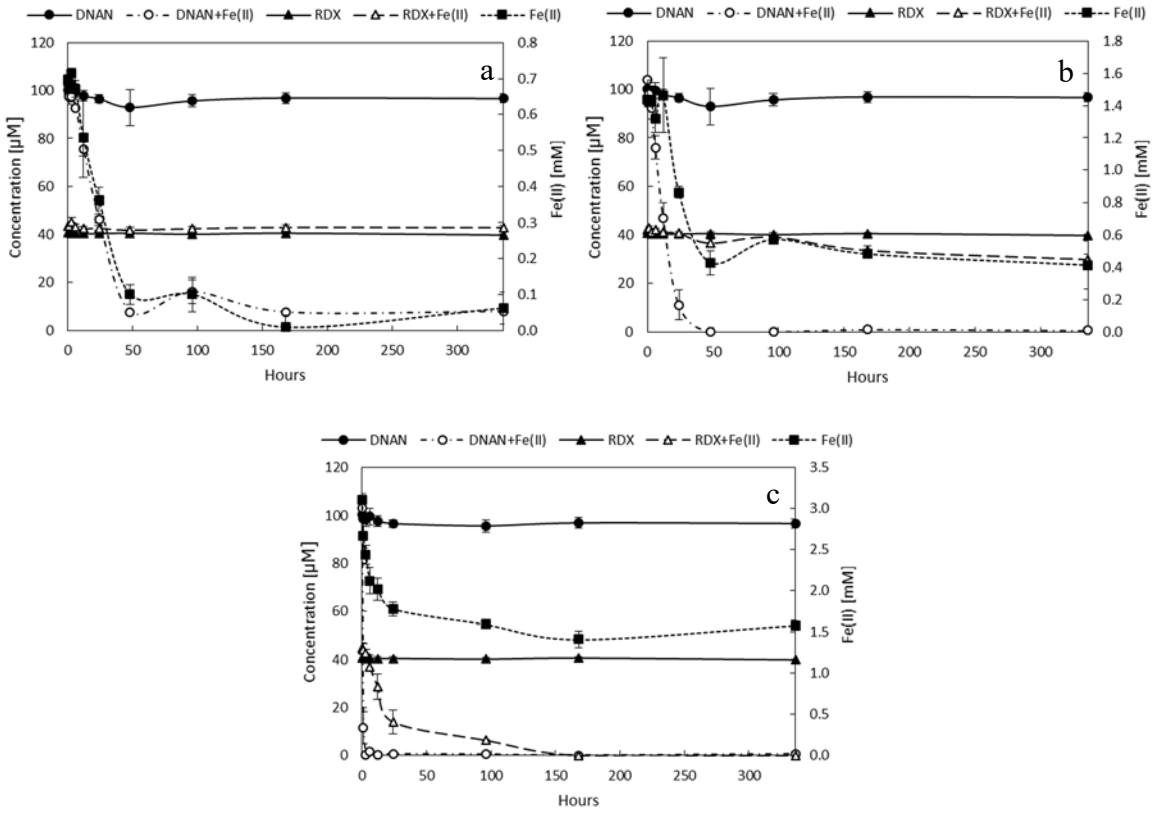

Figure A-1: DNAN and RDX reduction by Fe(II) at pH 7. Experimental conditions: 100µM DNAN, 50µM RDX, buffered with 30mM HEPES. Treatments: a) 0.75mM Fe(II), b) 1.5mM Fe(II), c) 3mM Fe(II). The error bars represent the standard deviation with n=3.

The above data was collected to confirm results presented in Figure 5.1, which shows DNAN and RDX degradation at pH 7 within 26 hours. After monitoring the reaction for 14 days, the same extend of explosives degradation was measured; therefore, the results from Figure A-1 support findings presented in Figure 5.1.

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