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Novel Electron Shuttling Compounds

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NOVEL ELECTRON SHUTTLING COMPOUNDS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Environmental Engineering and Earth Science

by
Neeraja Ramasubramanian
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Accepted by:
Dr. Kevin T. Finneran, Committee Chair
Dr. David Freedman
Dr. Brain Powell
ABSTRACT

Electron mediator’s usage in remediation technologies might be an excellent alternative to increase the rate of transformation of various contaminants such as nitroamine, polyhalogenated compounds and many others. Employing electron shuttles open up different pathways for the contaminant transformation and the reaction goes to completion at a much faster rate. Anthraquinone-2,6-disulfonate (AQDS) has been known to be a model electron shuttle with good shuttling capacity followed by humic acids. But the necessity for identifying electron shuttles suitable for field applications is important. As remediation technologies will command application of the shuttles on a large scale, tracing shuttles which are cheap, easily available and non-toxic is vital. This study mainly aimed at studying the efficiency of military smoke dyes as electron shuttles. Dispersed red 11 and quinoline yellow were the two military smoke dyes tested.

This study demonstrates that the smoke dyes act as electron shuttles for transforming poorly crystalline Fe(III) oxide to reactive Fe(II). Higher extent of transformation could be observed in amendments with the electron shuttles and disperse red 11 was found to be as effective as AQDS in increasing the transformation. Increase in concentration proportionately increased the shuttling ability as well. The shuttling ability of quinoline yellow was found to be lower than the other shuttles. Electron mediated transformation was also investigated in amendments with royal demolition explosive (RDX) as the electron acceptor. The military smoke dyes did not transfer electrons directly to RDX. The experiments were also carried at two different pH conditions to study the variation in shuttling ability with the change in pH conditions. A
higher pH condition did not bring a significant increase in Fe(III) transformation in amendments though a minor increase could be observed in the Fe(II) concentration. The pH variation had no impact on the RDX amendments. Though the electron shuttles exhibited limited capacity to transform RDX directly, their potential to transform Fe(III) to Fe(II) suggests that they can be exploited to reduce RDX by a Fe(II) mediated pathway.
DEDICATION

This thesis is dedicated to my brother Mr. Arun Shrvivats Ramasubramanian, who was my greatest propulsive force for bringing in confidence and to my parents Mr. and Mrs. Ramasubramanian. Their encouraging words and support were the sole reasons which helped me to overcome my obstacles during these two years. I cannot thank them enough for bringing this great positive change in my life.
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I would like to thank my colleagues Kay Dunnett Millerick, Jovan Popovic, Christopher Weber and Jolanta Niedzwiecka for providing me with support and assistance. I would also like to thank my lab manager Anne H. Cummings for her readiness to help me trouble shoot issues related to analytical instruments whenever needed. I am also obligated to thank all other staff members of the environmental engineering department. I would like to extend my gratitude to all my well-wishers and friends in the department who never hesitated to provide a hand of help at the time of need. A special mention about Ademola Bakenne for the kind of wonderful person he had been and for all the help and kindness which he had rendered throughout my period of stay here. Last but definitely not the least, I thank almighty god for blessing me with this wonderful opportunity.
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LIST OF ABBREVIATIONS

AQDS - Anthraquinone-2,6-disulfonate
CERL - Construction Engineering Research Laboratory
CL-20 - 2,4,6,8,10,12-hexaazaisowurtzitane
ES - Electron Shuttle
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMX - Octogen
HNB - Hexanitrobenzene
HPLC - High Performance Liquid Chromatography
RDX - Royal Demolition Explosive
RM - Redox Mediators
MNX - Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine
PTFE - Polytetrafluoroethylene
TNT - 2,4,6-trinitrotoluene
TNX - Hexahydro -1,3,5-Trinitroso-1,3,5-Triazine
CHAPTER 1
INTRODUCTION AND BACKGROUND

The chemical and petrochemical industrial sector and munitions manufacturing units release thousands of compounds into the environment. Many of these compounds pave way for contaminations in various realms. The global market of these industries is huge and the prominence of especially the petrochemical industry is quite intense in the United States. Over a past couple of years, the sales in the USA have summed up to $110.4 billion for 26 companies in the chemical sector (Vanderzee et al., 2009). Linked to the great economic benefits, large volumes of waste water containing toxic and recalcitrant chemicals are being discharged. Some of the compounds released during this process are unaffected during aerobic waste water treatment. When the treated waste water is released into the environment, they accumulate in different areas leading to soil and groundwater pollution (Vanderzee et al., 2009). Though these contaminants undergo reductive transformation under anaerobic conditions, the mechanism involved is slow due to electron transfer limitations (Rodgers et al., 2001; Vanderzee et al., 2001).

The problem of effective contact between the contaminant molecule and the cells involved in degradation is another limitation encountered in case of biotic transformation involving microorganisms. To overcome these drawbacks, the usage of electron shuttles might prove out to be an effective solution.

Electron shuttles (also referred to as redox mediators (RM)) are organic molecules that can be reversibly oxidized and reduced. These molecules have the capacity to serve as electron carriers in multiple redox reactions. RM accelerate reactions by lowering the
activation energy of the total reaction and their presence might be the prerequisite for some reactions to take place (Cervantes et al., 2004; 2006). Reduction of electron shuttles can be promoted by reactions involving reductants commonly found in anaerobic environments such as sulfide, cysteine and Fe(II) and various other specific electron donors used in remediation technologies.

As reduction of electron shuttles might also be connected with the oxidation of organic substrates involving microbes, humic substances and their quinoid analogues often play a vital role (Schwarzenbach et al., 1990; Dror et al., 2004). In the recent studies conducted by Kwon and Finneran (2006; 2008), various compounds which are effective as electron shuttles have been evaluated and initiatives to determine cost effective electron shuttles are still being conducted. Though all quinone containing molecules have the potential to shuttle electrons, identifying cost effective shuttles is important which will increase their usability in field applications. The present study of interest looks at the shuttling capacity of specific smoke dyes to enhance the transformation rates of RDX. Military smoke dyes are dyes which are used for signaling purposes in the military areas. These dyes being easily available might provide a good solution for replacing the shuttles which are not viable for field applications. Moreover, these dyes might already be present in the contaminated military zones. This might mean that no addition of the dye from an external source will be required to treat the contamination in these zones resulting in reduced remediation cost.
Research Objectives

In this study, military smoke dyes have been evaluated as electron shuttles for enhancing the reduction rate of the cyclic nitramine compound – Royal Demolition Explosive (RDX) and poorly crystalline iron(III) oxide. The smoke dyes of interest include disperse red 11 and quinoline yellow. Fe(III) transformation was also evaluated so as to assess indirect reduction mechanisms to transform RDX. The experiments carried out aimed at determining:

- The capacity of disperse red 11 and quinoline yellow to bring about the direct transformation of RDX which involves the transfer of electrons from the reduced smoke dyes to the RDX without the involvement of a mediating pathway;
- The ability of the smoke dyes to transform poorly crystalline Fe(III) oxide and the effect of the electron shuttle (ES) concentrations on increasing the extent of transformation; and
- The effect of pH on the electron shuttling ability of the smoke dyes.
CHAPTER 2
LITERATURE REVIEW

Cyclic nitramines constitute a group of nitrate explosives made of a ring structure comprised of alternating carbon and nitrogen atoms. Amines and nitro groups are substituted in these molecules at the N position. They are produced by the direct nitration of hexamine with ammonium nitrate and nitric acid in an acetic/acetic anhydride solvent. Various anthropogenic activities including military operations and industrial manufacturing processes result in the release of the cyclic nitramines into the environment (Agency for Toxic substances and Disease Registry 1995; Agency for Toxic substances and Disease Registry, 2010). These compounds are toxic and have hence drawn the attention of researchers for remedial activities as they might be carcinogenic and mutagenic. Cyclic nitramines are persistent in the environment. They exhibit low solubility and exist as particulate matter in the soil and air.

The concentration of cyclic nitramine compounds in polluted area depends on the proximity of the region to the source of contamination. The nitramines are released from various sources including waste leaks from storage points, sewage discharges, explosive detonation zones and discharge of pink water from industrial units. Pink water formation results when water used for cleaning the explosive manufacturing units is released after the cleaning process, carrying residual amounts of RDX. The nitramine compounds have long half-lives which depict their presence in any environment for a long period of time as they are resistant to degradation.
2.1 Physical and Chemical Properties of Cyclic Nitramines

Cyclic nitramines are classified as primary or secondary depending upon their susceptibility to ignition which depends upon their ignition capacity. The structures of the commonly known explosives compounds are presented in Figure 2.1.

![Figure 2.1 Structures of explosive compounds.](image)
Table 2.1: The physical and chemical properties of RDX, HMX, TNT  (Data sourced from Material safety data sheets)

<table>
<thead>
<tr>
<th>Name</th>
<th>RDX</th>
<th>HMX</th>
<th>TNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical Formula</td>
<td>C₃H₆N₆O₆</td>
<td>C₄H₈N₈O₈</td>
<td>C₇H₅N₃O₆</td>
</tr>
<tr>
<td>Melting point (°C)</td>
<td>204</td>
<td>276-280</td>
<td>80-82</td>
</tr>
<tr>
<td>Solubility in water (mg/L)</td>
<td>42</td>
<td>5</td>
<td>130</td>
</tr>
<tr>
<td>Mass density (g/cm³)</td>
<td>1.82</td>
<td>1.96</td>
<td>1.5-1.6</td>
</tr>
<tr>
<td>Vapour Pressure (1 bar, 20°C)</td>
<td>5.3 x 10⁻¹²</td>
<td>4.3 x 10⁻¹⁷</td>
<td>7.2 x 10⁻⁹</td>
</tr>
<tr>
<td>Henry’s Law constant: k_H (bar m³ mol⁻¹)</td>
<td>6.3 x 10⁻⁸ to 1.96 x 10⁻¹¹</td>
<td>2.6 x 10⁻¹⁵</td>
<td>4.57 x 10⁻⁷ to 1.1 x 10⁻⁸</td>
</tr>
<tr>
<td>Octanol/water partitioning coefficient log(k_{OW})</td>
<td>0.86</td>
<td>0.061</td>
<td>1.86</td>
</tr>
<tr>
<td>Appearance and Odor</td>
<td>White or Gray powder, odorless</td>
<td>White or Gray powder, odorless</td>
<td>Yellow flakes with bitter almond taste</td>
</tr>
</tbody>
</table>
2.2 Toxicity of RDX and TNT

Cyclic nitramines are classified as class C carcinogens, which refers to compounds having suggestive cancer potential. The compounds pose a risk when they come in contact via inhalation, direct contact with skin and ingestion (Eaton, 2011). Plants exhibit tolerance to explosive compounds till specific levels. Specific plant species tend to accumulate TNT in their roots and bioaccumulate RDX. Due to this mechanism of bioaccumulation, RDX was noticed in the edible portions of the plant in a few cases (Pennington and Brannon, 2002). Explosives exhibit minimal amount of toxicity to animal species. Effects of exposure were observed to be sub lethal at a dosage of 6 mg/kg/day (Agency for Toxic Substances and Disease Registry, 1995).

2.3 Environmental Behavior of RDX and HMX

The degradation rate of explosives is very slow in environmental systems. The process often does not proceed beyond the reduction of the nitro groups to amines. With RDX, reduction of the three nitro groups yields hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) and hence accumulation of mineralization products is low in the contaminated zones (Pennington and Brannon, 2002). Reduction occurs more readily under anaerobic conditions than aerobic conditions. Cyclic nitramines are non-volatile and have poor water solubility. The magnitude of adsorption to soil of RDX, HMX and other cyclic nitramines is dependent upon the nature of the soil. Presence of high clay content promotes greater degree of adsorption (Ryon and Pal, 1984). The main sinks of nitramine compound accumulation are soil, sediment and water.
2.3.1 Sorption/Desorption

The movement of cyclic nitramine compounds in the subsurface environments is influenced by the sorption/desorption processes. These mechanisms are governed by the amount of organic carbon content present in the soil ($f_{oc}$) apart from the type of clay minerals present in the contaminated zone. The amount of contaminant retained by the soil is calculated by the linearized adsorption isotherm given by the following relation:

$$C_s = K_d C_l$$

where $C_s$ is the amount of compound retained by the soil (mg/kg), $C_l$ is the compound concentration (mg/l) in the aqueous phase and $K_d$ is the adsorption coefficient (cm$^3$/g).

The value of the adsorption coefficient is calculated based on the carbon partition coefficient and the organic carbon concentration in a specific area (Robert and Robin, 1998) which is given by the following relation:

$$K_d = f_{oc} \times k_{oc}$$

where $f_{oc}$ gives the fraction of organic carbon present in the soil and $k_{oc}$ gives the ratio of the mass of the chemical that is adsorbed in the soil per unit mass of the organic carbon present in the soil per the equilibrium chemical concentration in a solution.

2.3.2 Photolysis

The photolytic degradation of HMX and RDX has been studied in various areas where the concentration of the contaminant has been found to decrease upon light exposure. The degradation rate of the contaminants is described by the following relation:

$$\ln C = \ln C_s - kt$$
where \( C_0 \) (mg/l) gives the initial concentration, \( k \) (time\(^{-1}\)) represents the degradation constant and \( C \) gives the concentration of the compound at the time of measurement. The photolytic degradation of the nitramine compounds have been found to increase with lesser turbidity and depths. At lesser turbidity and depth conditions, more amount of light energy tends to penetrate through a given area thus increasing the rate of transformation (Robert and Robin, 1998).

2.3.3 Biotic Transformation of Cyclic Nitrarnines

Biotic transformation involves the degradation of contaminants into a less complex form by the activity of microorganisms present in the region of contamination. The microbes have specific enzymes which facilitates their intake of contaminants. Cyclic nitramine compounds fall into the major class of organic compounds which serve as both an electron donor (carbon source which serves as the base for cell components synthesis) as well as an electron acceptor in the electron transport chain which help the microbes to derive energy (Eaton, 2011). Nitramine compounds are degraded by both aerobic and anaerobic microorganisms.

The aerobic microbes degrade the pollutants in the presence of oxygen while the anaerobic microbes utilize other electron donors such as nitrate (NO\(_3^-\)), sulfate (SO\(_4^{2-}\)) and metals such as iron (Fe(III)) and manganese (Mn(IV)). Due to the ability of these anaerobic microbes to use different donors, they are found in numerous environments with a varied electron donor conditions. Due to this characteristic, the anaerobes degrade various pollutant molecules as well.
2.3.3.1 Aerobic Degradation of RDX

Past experimental results indicate the potential of aerobic degradation mechanisms to mineralize these explosive compounds (Binks et al., 1995; Coleman et al., 1998; Hawari et al., 2000). Several bacterial species have been known to degrade RDX when supplied as the sole nitrogen source. Nitrite was found to be a prime product formed when RDX was used as a nitrogen source. The intermediates and the enzymes involved in this transformation mechanism have not been identified. Studies conducted with white rot fungi indicated hexahydro-1-nitroso-3,5-dinitro-3,5 dinitro-1,3,5-triazine (MNX) to be the only intermediate in this pathway of transformation (Hawari et al., 2000).

Another study using Rhodococcus soil isolate indicated the formation of nitrous oxide as a product of RDX degradation under aerobic conditions. Though different intermediates have been formed in this pathway, conclusive results have not been determined. The metabolites formed, the enzymes and the microbial species involved in the aerobic degradation pathway are still under research. When compared to anaerobic mechanisms, aerobic mechanisms bring a lesser degree of transformation of the nitroaromatic compounds as they degrade more slowly under aerobic conditions and specific microbial populations are required for this degradation.

2.3.3.2 Anaerobic Transformation of RDX

Cyclic nitramine compounds tend to accumulate in the sediments more than in water or air systems owing to their high hydrophobic and low vapor pressure values (Eaton, 2011). As these sediment areas contaminated with the explosive compounds tend
to be anoxic, anaerobic bacteria often play a prime role in bringing about the transformation of these compounds. In case of anaerobic degradation pathways, different reducing conditions might be present and the electron acceptors in these conditions will aid the degradation of the contaminant. Some of the electron acceptors found in these environments include nitrate, sulfate and Fe(III) (Kwon and Finneran, 2006). In nitrate reducing condition, a high percentage of RDX mineralization was observed in the native soil environment in the study conducted by Bradley et al (2005). Poor removal of RDX has also been recorded under nitrate reducing conditions depicting the inhibitory effect of nitrate on RDX transformation (Freedman and Sutherland, 1998). The onset of RDX degradation was observed in this system only when the nitrate concentration dropped to low levels. Under manganese reducing conditions, mineralization of RDX was seen in aquifer sediment microcosms (Bradley and Dinicola, 2005).

Sulfate reducing conditions have also been identified to be effective for bringing transformation of explosive compounds. Sulfate being widely present in subsurface anaerobic environments, it can be exploited for the transformation of explosive compounds. Previous studies conducted with a specific sulfate reducing species resulted in the removal of about 75 to 95% of RDX and MNX present within a time span of about 21 days (Boopathy et al., 1998). Species of Desulfovibrio have proved to have the ability of degrading RDX when used as the main carbon source (Adrain and Arnett, 2009). Sulfate reducing bacteria can thus play a main role in transforming RDX compounds.

Though the reducing environments discussed above are considered for manipulating RDX transformation, Fe(III) reducing conditions have been given the
utmost importance as Fe(III) forms are the most widely present electron acceptors in the subsurface, anoxic environments (Lovely et al., 1995). One of the viable methods under investigation for bringing about transformation of RDX under Fe(III) reducing conditions involves the use of extracellular electron shuttling compounds (redox mediators).

2.4 Redox Mediators

Redox mediators are capable of transferring electrons between a variety of compounds including both organic and inorganic. The reduction process proceeds both in a biotic and abiotic manner. In case of an abiotic mechanism, the reaction is triggered by using specific reductants commonly found in nature such as sulfide, cysteine and iron. These reductants are mainly prevalent in anaerobic environments (Schwarzenbach et al., 1990; Curtis and Reinhard, 1994). The reaction with electron mediators can also be initiated using other specific donors used in remediation technologies like zero valent iron (ZVI), Ti(III)/citrate (Dror and Schlautman, 2004; Keum and Li, 2004).

Anaerobic oxidation of organic substrates by microorganisms also paves way for the reduction of redox mediators by biological means. Redox mediators involved in these reactions include humic substances and their quinoid analogues (Cervantes et al., 2004). The other organic substrates might also include short fatty acid chains, monosaccharides and alcohols (Lovely et al., 1996). Reoxidation of redox mediators in a redox reaction results in the transfer of electrons from the reduced mediators to contaminant molecules.

As Fe(III) reducing organisms are widespread, some decontamination strategies are based on the physiology of these microbes. In order to explore their effectiveness many studies have been conducted to observe the transformation of contaminants in the
presence of various Fe(III) forms and the associated microbial community present. One such study involving RDX reduction tailored in the presence of poorly crystalline Fe(III) was slow and the amount of metabolites accumulated was also of lower magnitude (Kwon and Finneran, 2006). Addition of humics and AQDS (anthraquinone-2,6-disulfonate) however increased the rate of transformation indicating the potential role played by these molecules as electron shuttles. Therefore a strategy involving the native iron reducing community, Fe(III) forms present in the given environment and electron shuttling compounds might bring about faster rates of degradation. This is because various pathways come into play which ultimately results in the degradation of RDX. The likely pathways are as follows:

Pathway 1: Direct reduction of RDX by the Fe(III) reducing community;

Pathway 2: Reduction of Fe(III) by the Fe(III) reducers present and the transformation of RDX by the reactive Fe(II) formed;

Pathway 3: Reduction of the electron shuttling compound by the Fe(III) reducers and the transformation of RDX by the reduced shuttles; and

Pathway 4: Reactive Fe(II) formation by the reduced shuttles which in turn reduces the RDX.

Though the electron shuttling compounds have the potential for enhancing degradation rate, delineating electron shuttles which are suitable for field application is an important aspect of this strategy.
2.5 Compounds with Electron Shuttling Capacity

All compounds having the quinone functional group have the capacity to shuttle electrons. Previous studies by Finneran and colleagues have involved the use of humics extract and military smoke dye - red 9 to estimate their capacity to shuttle electrons. These compounds possess quinone functional groups and were expected to have shuttling capacity. The aim was to compare their capacity to AQDS which is known to be a model electron shuttle and hence their potential to be used for in-situ remediation strategies. Raw humics extract (refers to humic substances extracted from natural materials like leaf litter, plant and wood compost) was found to increase RDX reduction. The rates of reduction were found to vary proportionately with the concentration of the humics extract. RDX concentration dropped below detection limit with a concentration of 0.04 g/l of raw humics extract within a span of 120 hours. The degradation was much higher with 0.1 g/l of humics acid and went below detectable concentration within 72 hours. Addition of humics to Fe(III) amended incubations was found to increase the degradation rate more (Kwon, 2009). Addition of red 9 was also found to increase the rate of RDX degradation and the increase in rates was found to be proportional to the concentration.

For in-situ treatment strategies, the electron shuttles might have to be used in a large scale and hence has to be inexpensive and readily available. Though AQDS and humic substances have been known to exhibit good electron shuttling activity, using them for field applications is not viable due to the cost associated with AQDS and the extensive extraction procedure associated with the humic substances. Military smoke dyes on the other hand, do not involve any extensive preparation procedure before
application. They are cheap and do not pose any potential environmental hazards. There are a wide range of smoke dyes apart from red 9 and exploring the capacity of these dyes might provide viable options for using these dyes as electron shuttles. Before tailoring any remediation strategy using these smoke dyes, analyzing their electron shuttling property in biotic and abiotic systems involving metal mediators will be a required step. Structures of the electron shuttles used in this study are presented below in Figures 2.2, 2.3 and 2.4.

The smoke dyes tested in this experiment (Disperse Red 11 and quinoline yellow) are quinone bearing compounds which is depicted by the structures presented above. Disperse red 11 also known as solvent violet 26 is a red dye which is obtained from anthraquinone. It is in the form of a dark black powder
with a density of 1.42 g/cm$^3$ and has a flash point of 322.4°C. It is soluble in alcohols but exhibits a low solubility in water. It is used in textile and plastic industries for dyeing and also in violet and red colored smoke formulations to produce smoke bombs for signaling purposes in military. Quinoline yellow also referred to as the food yellow 13 is in the form of yellow granules. It is soluble in water and sparingly soluble in ethanol. It is a commonly used food dye and is also used in smoke grenade production like disperse red 11. The smoke grenades made out these dyes are used for signaling and combat exercises in military zones.
CHAPTER 3

ABIOTIC TRANSFORMATION OF POORLY CRYSTALLINE Fe(III)OXIDE IN THE PRESENCE OF ELECTRON SHUTTLING COMPOUNDS

Metal mediated remediation is a widely used option for in-situ cleaning up of contaminants. Metal mediated remediation involves the use of specific metal ions as mediators to transfer electrons from the microbes to the contaminant or between the reducing agent and the contaminant molecule. Among the widely used metal mediators, usage of various iron forms is the most prevalent. Fe(III) reduction is an important biogeochemical process. Ferric minerals are present in various forms (e.g. ferrihydrite, goethite, magnetite) in subsurface environments and often act as catalysts for bringing about various degradation processes.

Due to the abundance of Fe(III) reducing microorganisms in most of the subsurface zones, the process of using iron transformation methods serves to be effective for degrading different compounds in natural environments (Lovely et al., 1998). Factors controlling the rate and magnitude of Fe(III) reduction are of interest as iron reduction dictates the geochemistry in a variety of subsurface mechanisms. Presence of humic substances significantly increase the reduction of Fe(III) compounds coupled with the oxidation of organic substrates (Anderson and lovely, 1999; Lovely et al., 1996; Lovely et al., 1998). Humic substances stimulate Fe(III) reduction due to their property of shuttling electrons. The shuttling property exhibited by these substances is because of the presence of quinone moieties in their structure.
During the reduction process, electrons produced due to the oxidation of substrates are transferred to the quinone moieties which results in their transformation to hydroquinones. The hydroquinones in turn reduce the Fe(III) oxides present in the subsurface. Apart from humics there are many other compounds which contain the quinone groups and exhibit good electron shuttling capacities. One good example is the anthraquinone-2,6-disulfonate (humic acid analogue). Due to their property to switch between their oxidized and reduced states, these electron shuttling compounds enhance the degradation rate of the target molecule. When applied in iron reducing conditions, they act as intermediates to aid electron transfer to the Fe(III) form present in the environment. Past experimental data suggests that mixed biotic-abiotic reactions involving Fe(III) reducing organisms, electron shuttling compounds and Fe(II) formed aid in RDX degradation (Kwon and Finneran, 2006; Kim and Strathman, 2007).

Application of electron shuttles in a contaminated environment with iron reducing conditions might transform the target contaminant at a faster rate. The pathway of degradation noticed in these environments revolves around the transfer of electrons from the iron reducers to the iron forms present in the environment. The reactive Fe(II) formed in turn donates electrons to the target contaminant.

With electron shuttles, the rate is much faster as the shuttles keep getting regenerated in the process and leads to a higher concentration of Fe(II) and hence a higher magnitude of the contaminant transformation. Though application of electron shuttles appears to be a viable option for clean up strategies, determining cost effective
electron shuttles for the process is equally important. Compounds containing quinone groups are a suitable option for effective shuttling but not all quinone containing compounds are suited for field applications.

The purpose of this study was to evaluate smoke dyes as electron shuttles in an abiotic transformation mechanism. The system tested was abiotic and involved the use of cysteine and ascorbic acid as electron donors with poorly crystalline Fe(III) oxide as the sole terminal electron acceptor. Smoke dyes tested include disperse red 11 and quinoline yellow. The data demonstrated that the smoke dyes used will serve as effective electron shuttles from the donor to Fe(III) as the acceptor. This might be exploited in natural iron reducing environments containing contaminants to bring about transformation.

3.1 Materials and Methods

All materials obtained were of analytical grade and commercially obtained from authentic sources. The smoke dye disperse red 11 was obtained from U.S Army Corps of Engineers, Construction Engineering Research Laboratory (CERL). Quinoline yellow was obtained from Sigma Aldrich. Cysteine ≥ 97% purity, ascorbic acid ≥ 99% purity, ferric Chloride and anthraquinone-2,6-disulfonate (AQDS) were obtained from Sigma Aldrich.

3.1.1 Preparation of Stock Solutions

Amorphous Fe(III) solution (iron gel) was prepared by adding 10 N NaOH to a solution of ferric chloride (108 g/l) and the pH was adjusted to 7. The solution obtained was transferred to centrifuge tubes (150 ml) for centrifugation (Evolution RC, Sorvall) at
5000 rpm for 20 minutes. After each centrifugal run, the supernatant was disposed and the solid residue re-suspended in distilled water. This procedure of washing was repeated nine times so as to remove the predominant amount of chloride present. After the ninth wash, the residue was re-suspended in distilled water and mixed well (Lovely and Elizabeth, 1986). In order to measure the concentration of the iron gel (Fegel) stock, 0.1ml of the stock solution was acidified using 4.7 ml of 0.5N HCl and 0.2 ml of hydroxylamine HCl and was left overnight.

The concentration was measured by using a UV visible spectrophotometer at a wavelength of 562 nm. Hydroxylamine HCl being a strong reductant, reduces the Fe(III) present to Fe(II) which can be measured by the ferrozine assay (Stookey, 1970). The concentration of the measured ferrous ions was used to quantify the concentration of the Fegel stock. The stock solution used for the experiment was obtained by diluting the mother stock prepared and quantified by the above method.

AQDS and quinoline yellow stock solutions were prepared in distilled water by measuring out the required weight of the chemicals to obtain the stock solution of appropriate concentration. Disperse red 11 stock solution was prepared by dissolving disperse red 11 in ethanol. Ethanol was used as a solvent for disperse red 11 as it yields the highest magnitude of solubility only in alcohols. The stock solutions were degassed with nitrogen for a span of 30 minutes for the liquid medium and 7 minutes for the head space. The degassing was done to maintain the anaerobic conditions. The AQDS and quinoline yellow stock solutions were sterilized by autoclaving for a time period of 20 minutes at a temperature of 121°C. Ferrozine reagent used for ferrozine assay was
prepared by adding HEPES and ferrozine (Fe(II) reagent) to distilled water. The solution was brought up to its final volume after adjusting the pH to 7 using 10N NaOH.

3.1.2 Experimental Sample Design

Experimental samples were set up in 10 ml glass tubes. The experimental tubes were prepared by sparging the tubes with N₂ & CO₂ in the ratio of 95:5 for experiments conducted at a lower alkaline pH and with 100% nitrogen for experiments conducted at a higher alkaline pH. For maintaining the lower pH (7.5-8), 30 mM bicarbonate/5% CO₂ system was used. For higher alkaline pH conditions (9.2-9.3), 30 mM bicarbonate/5mM carbonate system was used. The experimental tubes were prepared in triplicates and contained a total sample volume of 10 ml. In order to maintain anoxic conditions, the tubes were sealed with thick butyl rubber stoppers and crimped with aluminum caps after degassing.

The reductants (electron donors) used in this experiment reduces Fe(III) by the following reactions:

\[ 2\text{C}_3\text{H}_7\text{NO}_2\text{S} \rightarrow \text{C}_6\text{H}_{12}\text{N}_2\text{O}_4\text{S}_2\ + 2\text{H}^+ + 2e^- \text{(Ox. half reaction of Cysteine)} \]  

\[ \text{Fe}^{3+} + e^- \rightarrow \text{Fe}^{2+} \text{(Red.half reaction)} \]  

\[ 2\text{C}_3\text{H}_7\text{NO}_2\text{S} + 2\text{Fe}^{3+} \rightarrow \text{C}_6\text{H}_{12}\text{N}_2\text{O}_4\text{S}_2 + 2\text{Fe}^{2+} + 2\text{H}^+ \text{(Full reaction)} \]  

\[ \text{C}_6\text{H}_8\text{O}_6 \rightarrow \text{C}_6\text{H}_6\text{O}_6 + 2\text{H}^+ + 2e^- \text{(Ox.half reaction of Ascorbic acid)} \]  

\[ \text{Fe}^{3+} + e^- \rightarrow \text{Fe}^{2+} \text{(Red.half reaction)} \]
In order to achieve complete transformation of 2 mM poorly crystalline Fe(III) oxide by cysteine and ascorbic acid, 2 mM and 1 mM concentration of the reductants was required, respectively. Cysteine and ascorbic acid were injected from sterile and anoxic stock solutions so as to obtain a final concentration of 1.4 mM and 0.7 mM in the samples, respectively. Only 70% of the stoichiometric concentration of the reductants was amended so as to delineate the differences in transformation by using the electron shuttles under donor deficient conditions. The Fe gel was injected into the sample tubes before sparging by using a pipette to obtain a final concentration of 2 mM in each tube. This concentration was used so as to look at a reasonable concentration of Fe(II) measurable by the ferrozine assay after acidification. Fe(II) produced during the experiment might change the surface properties of the Fe(III) amended by surface adsorption resulting in a decrease in reaction kinetics due to the change in the Fe(III) form. This aspect was also taken care of by not amending a high concentration of Fe(III) which might result in a higher Fe(II) formation. The electron shuttles concentrations were selected based on previous studies using reduced AQDS as an electron shuttle which demonstrated good shuttling ability at a concentration of 100μM-300μM (Kwon, 2009). This study was taken as a basis as the smoke dyes are also quinone bearing compounds like AQDS. Biotic iron transformation experiments were also used as a basis for selecting the concentrations where the extent of transformation was observed to be more pronounced in the experiment with a shuttle concentration of 100μM. These results indicated that a base concentration of 100μM might be reasonable to amend as the
starting concentration. Data plots demonstrating the results for the biotic experiments are included in the appendix.

Reductants and the smoke dyes were amended by using anaerobic syringes and needles. The experiments were initiated by injecting the reductant into the samples containing the Fegel and the electron shuttles. Samples for Fe(II) analysis was collected every one hour for five hours by using anaerobic syringes and needles. The samples for the initial time point was withdrawn as soon as the reductant was injected with a time lag of a minute. Incubation time of five hours was considered as the reductants used are good reducing agents and this period was assumed to be reasonable to allow sufficient transformation for delineating the extent of transformation as well as provide enough time period for sampling without overlapping in sampling times between amendments. The anaerobic conditions were maintained by using a gassing station. Eight different types of amendments considered for all the experiments are specified below:

Table 3.1: Treatment Description for Iron Transformation Experiment

<table>
<thead>
<tr>
<th></th>
<th>Treatment Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fegel + Reductant + AQDS</td>
</tr>
<tr>
<td>2</td>
<td>Fegel + Reductant + Disperse Red 11</td>
</tr>
<tr>
<td>3</td>
<td>Fegel + Reductant + Quinoline Yellow</td>
</tr>
<tr>
<td>4</td>
<td>Fegel + Reductant</td>
</tr>
<tr>
<td>5</td>
<td>Fegel + AQDS</td>
</tr>
<tr>
<td>6</td>
<td>Fegel + Disperse Red 11</td>
</tr>
<tr>
<td>7</td>
<td>Fegel + Quinoline Yellow</td>
</tr>
<tr>
<td>8</td>
<td>Fegel</td>
</tr>
</tbody>
</table>
3.1.3 Ferrozine Assay

The extent of transformation of poorly crystalline Fe(III) oxide present in the sample was quantified by measuring the concentration of ferrous ions produced over time. Samples were withdrawn every one hour for five hours using anaerobic syringes and needles. The sample withdrawn was acidified using 0.5 N HCl to prevent the oxidation of ferrous ions formed. After an hour of incubation time, to extract all the HCl extractable iron, the samples were analyzed for ferrous ion concentration. An aliquot of the extract was added to HEPES buffer containing Fe(II) reagent ferrozine and the absorbance at 562 nm was quantified to measure the Fe(II) concentration.

3.2 Results and Discussion

Experiments were designed to observe the variation in the extent of electron shuttling by differing shuttle concentrations and pH conditions. Results presented in this section are demonstrated by using a higher and lower value for the electron shuttle concentrations (0.1 mM, 0.5 mM) and the pH conditions (7.5-8; 9.2-9.3) for amendments.

3.2.1 Transformation of Fe(III) in Amendments with Lower Shuttle Concentration

With a 0.1 mM concentration of electron shuttle, the difference in extent of Fe(III) transformation in samples administered with reductants + electron shuttle(ES) and the reductant only control was observed but the extent of Fe(III) transformation was less between these amendments.

Figure 3.1 (A, B) shows the concentration of the Fe(II) produced over time when cysteine was used as a reductant. The Fe(II) concentration increased above 0.3 mM in all
the samples within a minute except for the shuttle/fegel only controls which recorded no transformation. The highest extent of transformation was recorded by the samples amended with disperse red 11 as the electron shuttle. About 0.453 mM concentration of Fe(II) was produced at the first sampling time point. Though a comparatively higher transformation was observed in the sample with disperse red 11, the concentration of Fe(II) produced in the cysteine + AQDS amendments started to overlap with that of the disperse red 11 after an hour of incubation.

The accumulation of Fe(II) in samples containing quinoline yellow as an electron shuttle was less compared to the samples administered with other electron shuttles. The initial difference in Fe(II) concentrations demonstrated by the data indicates that the reactions occurred within 1 min and the addition of reductant coupled with the shuttling ability of the ES resulted in rapid Fe(III) transformation within a short time frame. From the trend demonstrated by the data, it is observed that the reaction reaches a threshold within a short time span. This might be due to the inhibition caused by the adsorption of Fe(II) formed onto the Fe(III) present retarding further transformation. Figure 3.1C shows the relative Fe(II) accumulation in the cysteine and the electron shuttle amended samples. It depicts the amount of Fe(II) produced at the initial and final time points and gives an idea about the difference in magnitude of transformation due to the amendment of different shuttles. From Figure 3.1C, it can be observed that the extent of transformation on using disperse red 11 as an electron shuttle is nearly the same as that of AQDS. The concentration of Fe(II) formed in the sample administered with quinoline yellow has produced no enhanced transformation due to electron shuttling activity.
Figure 3.1: Fe(II) accumulation over time for amendments using cysteine as the electron donor and 0.1 mM (A) AQDS & disperse red 11 as electron shuttles (B) AQDS and quinoline yellow as electron shuttles; (C) Concentration of Fe(II) accumulated for the initial and final time points for amendments with reductants + ES. Error bars represent standard deviation for triplicate samples.
In treatments using ascorbic acid as reductant, the same trend was observed as well. Figure 3.2 (A, B) shows the variation in Fe(II) concentration over the course of this experiment amended with ascorbic acid. The trend noticed in this experiment is similar to the trend noticed in the cysteine amended experiment. Samples having ascorbic acid + disperse red 11 had a higher transformation of Fe(III) while the transformation difference between the samples with ascorbic acid + quinoline yellow as an electron shuttle compared to other reductant+ ES amendments was less. This difference is well explained by the data presented in Figure 3.2 C which clearly depicts the lower shuttling ability of quinoline yellow. From both the experiments, relatively higher formation of Fe(II) was seen in amendments with (AQDS; disperse red 11) + reductant than in samples with the reductant alone indicating that the shuttles increase the extent of transformation. The amendment with the reductant+quinoline yellow was an exception.
Figure 3.2: Fe(II) accumulation over time for amendments using ascorbic acid as the electron donor and 0.1 mM (A) AQDS & disperse red 11 as electron shuttles (B) AQDS and quinoline yellow as electron shuttles; C) Concentration of Fe(II) accumulated for the initial and final time points for amendments with reductants + ES. Error bars represent standard deviation for triplicate samples.
3.2.2 Transformation of Fe(III) in Amendments with a Higher Shuttle Concentration

This experiment was conducted using a higher concentration of the electron shuttles to delineate the difference in extent of transformation due to shuttle concentrations. A 0.5mM concentration of shuttles was used. The experiments conducted with cysteine as the electron donor demonstrated a higher concentration of Fe(II) produced in the all the reductant +ES amendments within the same experimental phase than the previous experiment (Figure 3.3(A,B)). This indicates the increase in shuttling ability of the ES with increase in concentration. Amendments containing the electron shuttles and having ascorbic acid as an electron donor also demonstrated a greater extent of Fe(III) reduction in the present study compared to what was observed in amendments with lower shuttle concentration (Figure 3.4(A,B)).

The trend of disperse red 11 having a higher or equivalent transformation ability to AQDS was prominent in the amendments with both reductants while quinoline yellow produced less transformation. The overall increase in transformation might be due to the higher concentration of quinone moieties present in the amendment paving way for a higher shuttling ability. Figures 3.3C and 3.4C demonstrates the extent of reduction due to the different shuttles used and the transformation due to the reductant alone. The amount of Fe(II) produced in case of both reductants + ES amendments indicate disperse red 11 to be very effective with a high shuttling ability. Figure 3.5(A,B) gives an idea about the differences in amount of Fe(III) reduced at the initial and at the final time point due to the different ES concentrations amended. From this figure, it is observed that administering higher ES concentrations results in greater transformation.
Figure 3.3: Fe(II) accumulation over time for amendments using cysteine as the electron donor and 0.5mM (A) AQDS & disperse red 11 as electron shuttles (B) AQDS and quinoline yellow as electron shuttles; (C) Concentration of Fe(II) accumulated for the initial and final time points for amendments with reductants + ES and the reductant alone. Error bars represent standard deviation for triplicate samples.
Figure 3.4: Fe(II) accumulation over time for amendments using ascorbic acid as the electron donor and 0.5mM of (A) AQDS & disperse red 11 as electron shuttles (B) AQDS and quinoline yellow as electron shuttles; C) Concentration of Fe(II) accumulated for the initial and final time points for amendments with reductants + ES and the reductant alone. Error bars represent standard deviation for triplicate samples.
Figure 3.5: Fe(II) accumulation in amendments with 0.1mM & 0.5mM electron shuttles at the initial and final hour time points and (A) Cysteine as the reductant (B) Ascorbic acid as the reductant. Error bars represent standard deviation for triplicate samples.
3.2.3 Effect of pH on Transformation of Fe(III)

The effect of smoke dyes as electron shuttles was studied at a higher pH condition of 9.5. The pH of the experiment dropped to 9.3 and varied between 9.2-9.3 over the course of the experiment. Figures 3.6&3.7 (A, B) demonstrate the concentration of Fe(II) produced over time when cysteine and ascorbic acid were used as electron donors. Slightly greater magnitude of Fe(III) was transformed at the initial time point compared to that reduced in the experimental conditions with pH varying from 7.5-8.0. As the difference in transformation magnitude was less under both conditions, student’s $t$-test was performed to test the level of significant difference. There was no significant difference in Fe(II) concentration at the higher pH based on the student’s $t$-test ($\alpha=0.05$). Highest transformation of Fe(III) was seen in the disperse red 11 + reductant amendments in case of both electron donors. Figures 3.6 & 3.7 (C) gives an idea about the relative concentration of Fe(II) accumulated at the initial and final hour time points outlining the transformation ability between shuttles. The smoke dye disperse red 11 was observed to be an effective electron shuttle with a shuttling capacity equivalent or greater than AQDS. Quinoline yellow exhibited a lesser ability to shuttle under all conditions. This might be due to structural differences showing a lesser tendency to accept and donate electrons. pH can be another reason for quinoline yellow to have showed lesser capacity as the effective pH ranges for shuttles differ and enough data is not available to prove the same\textit{(Fu and Viraraghavan,2003)}.

Though the extent of shuttling was expected to increase at higher pH levels, the change observed in the case of this experiment was not significant. The Fe(II)
accumulated at the initial and final time points in the amendments at the two pH conditions are shown in Figure 3.8(A,B). This shows the relative differences in Fe(III) transformation caused due to varying pH.
Figure 3.6: Fe(II) accumulation over time for amendments using cysteine as the electron donor and 0.5mM (A) AQDS & disperse red 11 as electron shuttles at pH 9.2 (B) AQDS and quinoline yellow as electron shuttles at pH 9.2; (C) Concentration of Fe(II) accumulated at the initial and final time points for amendments with reductant and ES. Error bars represent standard deviation for triplicate samples.
Figure 3. 7: Fe(II) accumulation over time for amendments with ascorbic acid as the electron donor and 0.5 mM (A) AQDS & disperse red 11 as electron shuttles at pH 9.2 (B) AQDS and quinoline yellow as electron shuttles at pH 9.2; C) Concentration of Fe(II) accumulated for the initial and final time points for amendments with reductant and ES. Error bars represent standard deviation for triplicate samples.
Figure 3.8: (A) Fe(II) accumulation in amendments with the electron shuttles at the initial and final time points and (A) Cysteine as the reductant (B) Ascorbic acid as the reductant at different pH conditions. Error bars represent standard deviation for triplicate samples.
3.3 Conclusion

The trend demonstrated by the data clearly indicates that the smoke dyes shuttle electrons between the donor (Reductant) and poorly crystalline Fe(III) oxide. A considerably higher concentration of Fe(II) is produced with higher concentration of electron shuttles. A higher alkaline pH did not produce a significant increase in the extent of transformation of Fe(III). Though the extent of shuttling was expected to increase at higher pH levels, the change observed in the case of this experiment was not significant but there was a minor increase in the mean concentration values. Better shuttling capability is expected as the compounds become deprotonated at higher pH conditions and hence become more reactive. Enough information to explain the effect of pH and the shuttling ability of these compounds was not available as their pKa values could not be determined. The two smoke dyes tested, exhibit a good electron shuttling capacity with disperse red 11 having a greater capacity. Thus, these military dyes can be a good option for large scale applications in remediation strategies carried out under iron reducing conditions.
CHAPTER 4

ABΙΤΙC TRANSFORMATION ΟF HEXAHYDRO-1,3,5,-TRINITRO-1,3,5
TRIAZINE IN THE PRESEΝCE OF ELECTRON SHUTTLING COMPOUNDS

Royal demolition explosive (RDX) also referred to as Hexahydro-1,3,5-Trinitro-1,3,5 Triazine is a class C carcinogen. RDX provides a major challenge for cleanup mechanisms at munitions manufacturing units due to its resistance to (bio)degradation, low capacity to volatilize and its tendency to be transported from the source of contamination within aquifers (Byung et al., 2001). Though there are many ex-situ treatments for transforming RDX, most of these strategies result in displacing the RDX contamination from one zone to another with accumulation of daughter products with equivalent or greater toxicity and have low economic feasibility. Alternatively, in-situ technologies are more cost effective.

Biotic as well as abiotic mechanisms viable for the degradation process can be easily exploited in in-situ treatment methods (Byung et al., 2001). Transformation involving biotic mechanisms is supported by microbially mediated processes. Abiotic mechanisms involve reducing agents either naturally present in the subsurface or by the construction of permeable reactive barriers.

In-situ anaerobic strategies employing biotic methods involve the reduction of the nitro functional groups in the cyclic structure by native microbial population to produce nitroso metabolites. Biotic strategies can be adopted only when specific microbial population are present which bring about the transformation of RDX. When the microbial
population responsible for transformation is not widespread, the reactions are slow and
do not go to completion, hindering the yield of the final metabolite. Employing electron
mediators might be a viable option to increase the reaction rate. Electron shuttling
involves the transformation of the shuttles into its reduced form which in turn brings
about the degradation of the contaminant. As the electron shuttles have the capacity to
cycle between their oxidized and reduced states, applying even small concentrations of
the shuttle might bring large magnitudes of transformation. This shuttling mechanism
also eliminates the need for the cell to come into direct contact with the contaminant
molecule and other solid phase minerals which often act as intermediate donors in the
reduction pathway (Lovely et al., 1996).

Utilizing the electron shuttling mechanism for RDX transformation might be a
viable option. Electron shuttles used can mediate the transfer of electrons either between
the cells and the contaminant or between the cells and the solid mineral surfaces present
in the contaminated subsurface which in turn serves as an electron donor to the
contaminant molecule. This opens up two different pathways for the transformation to
occur (Kwon and Finneran, 2006; Kwon and Finneran, 2008).

Remediation strategies that stimulate Fe(III) reducing organisms via extracellular
electron transfer is a good option as the mechanism does not depend upon organisms
which transfer electrons directly to RDX (Lovely and Philips, 1986; Kwon and Finneran,
2009). The rates of transformation differ appreciably in environments supported by
electron shuttle mediation and the concentration of various Fe(III) forms present.
The functional groups present in these shuttling compounds influence their effectiveness as an electron shuttle. Compounds containing thiol and quinone groups have been known to be effective electron shuttles. Among the compounds having quinone groups, AQDS has been found to be very effective as a shuttle but usage of AQDS for field applications will not be cost effective (Kwon, 2009). The need for exploring other compounds for their shuttling capacity arises if these compounds have to be applied for in-situ remediation methods. Previous data suggested that humics extract and Red 9 can promote nitro group reduction by acting as an electron acceptor in microbial respiration and in turn transfer electrons to the nitramine compounds (Kwon and Finneran, 2006; Kwon and Finneran, 2008). Reduced humics extract and Red 9 showed electron donating capacity but the capacity for the latter compound was relatively less. Fe(III) and nitrate were observed to be better electron acceptors than RDX in the study conducted with the reduced shuttles.

In abiotic experiments conducted with reduced Red 9, no transformation occurred and this was attributed to kinetic limitations in the system (Kwon, 2009). Biotic experiments demonstrated red 9 to be an effective electron shuttle and the rate was found to be more pronounced in experimental systems having poorly crystalline Fe(III) oxides. This is likely due to the transformation occurring because of the reactive Fe(II) formed by the microbially reduced shuttles and by direct electron transfer to RDX from the microbially reduced shuttles (Kwon and Finneran, 2008). This data suggests that smoke dyes containing quinone groups could be a viable alternative for the other shuttles.
exhibiting good electron shuttling capacity. This formed a good basis for the experiments conducted in this study.

The main purpose of this study was to evaluate the effectiveness of two smoke dyes—disperse red 11 and quinoline yellow as electron shuttling compounds. Experiments were conducted to observe the effectiveness of the direct electron transfer pathway which involves the transformation of RDX by abiotically reduced electron shuttles. The objective was to determine whether the smoke dyes have the capacity to transfer electrons directly to RDX and will promote the degradation of RDX by the direct transformation pathway.

4.1 Materials and Methods

All materials obtained were of analytical grade and commercially obtained from authentic sources. The smoke dye disperse red 11 and stock solution of RDX in acetonitrile was purchased from U.S Army Corps of Engineers, Construction Engineering Research Laboratory (CERL). Quinoline yellow was obtained from Sigma Aldrich. Cysteine ≥ 97% purity, ascorbic acid ≥ 99% purity, ferric chloride and anthraquinone-2,6-disulfonate (AQDS) were obtained from Sigma Aldrich.

4.1.1 Preparation of Stock Solutions

AQDS and quinoline yellow stock solutions were prepared in distilled water by measuring out the required weight of the chemicals to obtain the stock solution of appropriate concentration. Disperse red 11 stock solution was prepared by dissolving disperse red 11 in ethanol. Ethanol was used as a solvent for disperse red 11 as it yields
the highest magnitude of solubility only in alcohols. The stock solutions were degassed with nitrogen for a time span of 30 minutes for the liquid medium and 7 minutes for the head space. The degassing was done so as to maintain the anoxic conditions. The AQDS and quinoline yellow stock solutions were sterilized by autoclaving for a time period of 20 minutes at a temperature of 121°C.

RDX stock solution was prepared by taking 0.2ml of the concentrated RDX stock (obtained from CERL) in acetonitrile and gas dried so as to vaporize the acetonitrile. The solid residue obtained was dissolved in distilled water and stirred for four hours at a temperature of 30°C. This temperature was maintained so as to increase the solubility of RDX in water.

4.1.2 Experimental Sample Design

The experimental tubes were prepared by sparging the tubes with N₂ & CO₂ in the ratio of 95:5 for experiments conducted at a lower pH and with 100% nitrogen for experiments conducted at a higher alkaline pH. For maintaining the pH at around 7.5, 30 mM bicarbonate/5% CO₂ system was used. For higher alkaline pH conditions (around 9.5), 30 mM bicarbonate/5mM Carbonate system was used. The experimental tubes were prepared in triplicates and contained a total sample volume of 10 ml. In order to maintain anoxic conditions, the tubes were sealed with thick butyl rubber stoppers and crimped with aluminum caps after degassing. RDX, electron shuttles and the reductants were injected using anaerobic syringes and needles from the concentrated, anaerobic stock
Solutions. Twelve different amendments used for both the experiments conducted at the two pH conditions are specified below:

<table>
<thead>
<tr>
<th></th>
<th>Treatment Description for RDX Transformation Experiment</th>
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<tbody>
<tr>
<td>1</td>
<td>40μM RDX+240 μM Cysteine+500 μM AQDS</td>
</tr>
<tr>
<td>2</td>
<td>40 μM RDX + 240μM Cysteine+500 μM Disperse Red 11</td>
</tr>
<tr>
<td>3</td>
<td>40μM RDX+240μM Cysteine+500μM Quinoline Yellow</td>
</tr>
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<td>4</td>
<td>40μM RDX+120 μM Ascorbic acid+500 μM AQDS</td>
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<tr>
<td>5</td>
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<td>40μM RDX+120μM Ascorbic acid+500μM Quinoline Yellow</td>
</tr>
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<td>7</td>
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<tr>
<td>10</td>
<td>40μM RDX +500 μM Disperse Red 11</td>
</tr>
<tr>
<td>11</td>
<td>40μM RDX +500 μM Quinoline Yellow</td>
</tr>
<tr>
<td>12</td>
<td>40μM RDX</td>
</tr>
</tbody>
</table>

4.1.3 Analytical Methods

Samples were withdrawn and sacrificed for analysis at the 0th, 2nd, 4th, 6th, 8th and the 24th hour time points. The last sample was withdrawn after thirty and nine days for the amendments at the lower and higher alkaline pH conditions respectively. Aliquots of sample withdrawn from the experimental tubes amended with the reductants, ES and RDX were filtered using 0.2μM PTFE filters so as to remove any un-dissolved solid particles of the smoke dyes getting into the HPLC sample vials during analysis. Concentrations of RDX was measured using high performance liquid chromatography (HPLC) with UV detection (Dionex) at a wavelength of 254nm coupled
with Supelcosil LC-CN column (Supelco 4.6 x 25mm x 5μm) with a guard column and an isocratic mobile phase (50% water, 50% methanol, 1.0ml/min).

The RDX transformation experiment was conducted at two different pH conditions. A higher shuttle concentration was employed for these experiments as they were found to work better for the iron transformation studies. Stoichiometric concentrations of the reductants were used for the experiments according to the following reactions:

\[
C_3H_6N_6O_6 + 6H^+ + 6e^- \rightarrow C_3H_6N_6O_3(TNX) + 3H_2O \quad \text{(Red. half reaction)} \quad (7)
\]

\[
2C_3H_7NO_2S \rightarrow C_6H_{12}N_2O_4S_2 + 2H^+ + 2e^- \quad \text{(Ox. half reaction of Cysteine)} \quad (8)
\]

\[
C_3H_6N_6O_6 + 6C_3H_7NO_2S \rightarrow C_3H_6N_6O_3(TNX) + 3H_2O + 3C_6H_{12}N_2O_4S_2 \quad \text{(Full reaction)} \quad (9)
\]

\[
C_6H_8O_6 \rightarrow C_6H_6O_6 + 2H^+ + 2e^- \quad \text{(Ox. half reaction of Ascorbic acid)} \quad (10)
\]

\[
C_3H_6N_6O_6 + 3C_6H_8O_6 \rightarrow C_3H_6N_6O_3(TNX) + 3H_2O + 3C_6H_6O_6 \quad \text{(Full reaction)} \quad (11)
\]

Hence 6 times the concentration of Cysteine and 3 times the concentration of Ascorbic acid are required to completely reduce RDX to TNX (Hexahydro-1,3,5-Trinitroso-1,3,5-Triazine). A stoichiometric concentration of 240μM of Cysteine and 120μM ascorbic acid was used for this experiment to ensure the concentrations did not become the rate limiting factor. The experiments were initiated by injecting the reductants into the samples amended with RDX and the electron shuttles.
4.2 Results and Discussion

4.2.1 Transformation Experiment at Lower Alkaline pH Conditions

The transformation of RDX when observed under lower pH conditions (around 7.5-8) was less for all the ES (Electron shuttle) + reductant amendments except for the amendment containing AQDS. The RDX in the amendment with Cysteine + AQDS decreased to a concentration of 12.14 μM from an initial concentration of 42.22 μM in a thirty day time frame (Figure 4.1A). In case of ascorbic acid, the final value was determined to be 29.09 μM from an initial concentration value of 37.21 μM which was much lesser when compared to Cysteine amendment with AQDS (Figure 4.1 B).

The other amendments having the reductants + ES and the reductant only amendment depicted a decrease in RDX concentration over the thirty day time frame but the reduction was less and could not be proved to be occurring as a result of RDX transformation (Figure 4.1(A,B)). The controls containing only RDX and RDX+ ES did not show any considerable transformation except for minor inconsistency in values which might be due to analytical issues due to carryover of the analyte.
Figure 4.1: (A) Reduction of RDX in cysteine + electron shuttle amendments at a lower alkaline pH (B) Reduction of RDX in ascorbic acid+ electron shuttle amendments at a lower alkaline pH. Error bars represent standard deviation for triplicate samples.
4.2.2 Transformation Experiment at Higher Alkaline pH Conditions

This experiment was imitated at a higher alkaline pH of around 9.5 to determine whether higher pH conditions will increase the electron donating capacity of the electron shuttles used. The pH varied between 9.2-9.3 during the experiment. The experimental design was the same with only changes in pH conditions. Significant reduction of RDX was seen only in amendments with the reductants and AQDS as electron shuttles. In the amendment with Cysteine about 36.79 μM concentration of RDX was reduced to 1.60 μM in nine days (Figure 4.2 A). There was observed to be a significant reduction in concentration by the end of the eighth hour in these amendments. The same trend was observed in amendments containing AQDS and ascorbic acid as well. About 38.85 μM RDX was reduced to 10.68 μM in nine days (Figure 4.2 B). The reduction was observed to be evident within a period of six hours in these samples.

The rate of reduction in the AQDS + reductant amendments was found to be higher than the previous experiment conducted at a lower alkaline pH. This might be due to the fact that electron shuttles have a greater shuttling capacity at an alkaline pH (Uchimiya and Stone, 2006). The other amendments containing the reductants + smoke dyes as shuttles recorded no significant decrease in the concentration of RDX (Figure 4.2 A,B). This might denote the inability of the smoke dyes to donate electrons directly to RDX. The control samples exhibited no activity except for the initial inconsistency in the RDX+AQDS control. This could have been due to the detection issues in the UV detector which might have resulted in lower detection of initial concentrations.
The wide time variation in the final sampling periods for both the experiments was due to the analytical issues associated with the column. A couple of analysis aspects had to be taken care of before the column could be used for continuing the analysis during the experimental run at lower alkaline pH. From both the experiments, it was observed that the smoke dyes exhibited limited electron shuttling capacity and were not able to transfer electrons directly to RDX to a large extent to bring about significant transformation. No nitroso metabolites were detected as there was transformation only in the amendments with the reductant and AQDS. In case of AQDS mediated transformation of RDX, the prime metabolite formed is formaldehyde which was not quantified by the HPLC analysis. The model electron shuttle, AQDS could reduce RDX to a large extent. Further experimentation will be required to test the effectiveness of these smoke dyes as electron shuttles for direct RDX transformation.
Figure 4.2: (A) Reduction of RDX in cysteine + electron shuttle amendments at a higher alkaline pH (B) Reduction of RDX in ascorbic acid + electron shuttle amendments at higher alkaline pH. Error bars represent standard deviation for triplicate samples.
4.3 Conclusion

The experimental results obtained clearly indicate that the electron shuttling capacity of the smoke dyes in reducing RDX directly is limited. Both the smoke dyes brought negligible or no transformation in the amendments tested. However, the reaction pathway in this system can be modified by using a metal mediator. This will pave way for bringing about the transformation of RDX by an indirect mechanism. If iron reducing conditions are considered for the indirect mechanism, the reduced shuttles will transfer electrons to the iron form present which in turn will produce reactive Fe(II). The reactive Fe(II) might act as effective electron donors to reduce RDX. From the previous experiments involving iron transformation, the electron shuttles have demonstrated to be effective under iron reducing conditions. This mechanism can be exploited to bring the transformation of RDX using these shuttles.
CHAPTER 5

SUMMARY AND RECOMMENDATIONS

Smoke dyes as electron shuttling compounds increased the extent of poorly crystalline Fe(III) oxide transformation. The iron transformation study also demonstrated that increasing the concentration of the shuttles accelerated the rate at which the iron was transformed within the same time period. This might be due to the fact that more number of quinone moieties were present to reduce the Fe(III) present. Increasing the pH condition did not significantly increase the extent of Fe(III) transformation though there was observed to be an increase in mean concentration values indicating that the pH maintained might have been lesser than the pKa value of the compound and increasing the pH value further up can bring higher extent of transformation.

Under all the conditions tested for Iron transformation using the electron shuttles, disperse red 11 showed an equivalent or higher capacity compared to AQDS to shuttle electrons. The magnitude of Fe(II) accumulation was found to be the highest in the amendments containing the reductants and disperse red 11. Quinoline yellow demonstrated a lower capacity to shuttle electrons compared to the other electron shuttles under all conditions. The addition of smoke dyes as electron shuttles was found to increase the transformation of Fe(III) more than using the electron donor alone. As iron reducing organisms are widespread in most of the subsurface zones, electron shuttles effective for increasing Fe(III) reduction rates will be a good option for decontamination strategies. Moreover, the smoke dyes tested might already be present in contaminated areas near army zones and hence no external addition of the dyes might be required for
executing this strategy. This will make this strategy very economical as no additional cost of adding electron shuttles is involved.

In the experiments carried out with RDX as the electron acceptor, the smoke dyes did not exhibit the capacity to transfer electrons directly to RDX. No significant reduction was seen in the samples amended with the reductants and smoke dyes. An appreciable decrease in RDX concentration was seen only in the amendments containing the reductants and AQDS as an electron shuttle. This signifies that the smoke dyes are not as effective as AQDS in transferring and bringing the direct transformation of RDX. Though they exhibit a low capacity to donate electrons directly to RDX, it does not eliminate the option of exploiting smoke dyes to bring about RDX transformation. Metal mediated degradation pathways might be implemented for degrading RDX using Fe(III) forms that are already present in the subsurface as the mediators.

The data from this study clearly shows the capacity of the smoke dyes to transform Fe(III). This pathway can be exploited to form reactive Fe(II) which in turn can donate electrons to reduce RDX. Previous studies done have shown the metal mediation pathway to be effective for transforming RDX (Kwon and Finneran, 2006). A combination of reactions involving biotic and abiotic degradation mechanisms involving iron reducing conditions, electron shuttles and reactive Fe(II) formed can be employed for degrading RDX by an indirect mechanism using Fe(III) as the intermediate electron acceptor (Kwon and Finneran, 2006). The smoke dyes considered here may be suitable for field applications and might be appropriate for remediation strategies using flow
controlled pumping systems to supply donor and the shuttle in the zone of contamination. Moreover, these smoke dyes might already be present in the military zones which might provide an extra advantage of implementing this strategy with no external ES addition. The two main aspects which has to be explored further is the effectiveness of these shuttles in metal mediated mechanisms and the tolerance of the microbial community to use these shuttle as electron acceptors. If these shuttles pose no potential toxicity effects to the microbes, they will act as a suitable alternative for increasing the rate of contaminant transformation in (Bio) remediation strategies.

Though this research provides a good idea about the shuttling ability of the electron shuttles for bringing about RDX and Fe(III) transformation, some recommendations for future work include:

- The evaluation of the transformation of RDX by an indirect metal mediated pathway involving Fe(III) as the mediator in the presence of electron shuttles.
- Study which involves the testing of the same strategy including both direct and indirect transformation of RDX by the electron shuttles in a biotic mechanism where cells are employed as electron donors instead of the reductants.
APPENDIX

Biotic iron transformation using a) 2.5 μM concentration of AQDS & disperse red 11 as electron shuttles b) 100 μM concentration of AQDS & disperse red 11 as electron shuttles.
REFERENCES


