EX SITU BIODEGRADATION OF EXPLOSIVES USING PHOTOSYNTHETIC BACTERIA AND BIOLOGICAL GRANULAR ACTIVATED CARBON SYSTEMS

BY

KAYLEIGH ANNE MILLERICK

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Environmental Engineering in Civil Engineering in the Graduate College of the University of Illinois at Urbana-Champaign, 2014

Urbana, Illinois

Doctoral Committee:

Professor Charles Werth, Chair
Associate Professor Kevin Finneran
Associate Professor Timothy Strathmann
Professor David Freedman of Clemson University
Military explosives have been identified globally as anthropogenic groundwater contaminants. Communication with the US Army indicates that ex situ pump and treat, a strategy that intercepts contaminated groundwater and pumps it aboveground for treatment, will continue to be the default treatment approach for explosives-laden groundwater in the foreseeable future. This project investigates potential ex situ treatment strategies that could be used as alternatives to the unsustainable activated carbon systems currently in use. Part I examines current activated carbon practices and tests experimentally whether GAC pre-adsorbed with the high explosive RDX can be biologically regenerated. Part II evaluates photosynthetic *Rhodobacter sphaeroides* as a potential ex situ treatment option for explosives, to be used in lieu of activated carbon. Both focus on biotransformation by bacterial pure cultures. Experiments were conducted as small-scale batch reactors with amendments of both chemicals and bacteria (suspended cells and growing transfers).

Reduction of adsorbed RDX was investigated using three different electron sources: biological, chemical, and a mixed biological-chemical system. Each experimental system was capable of ≥ 97.0% adsorbed RDX transformation. Formaldehyde (daughter product) was produced rapidly and was stoichiometric in chemical systems, which operated more rapidly than systems with bacteria. All systems were able to transform adsorbed RDX within 90 hours. This is the first study to successfully demonstrate biological transformation of RDX adsorbed to GAC. Following experimentation, GAC could be re-used for removal of RDX from water. These data suggest the masses of GAC waste currently produced by activated carbon at RDX remediation sites can be minimized.

Photosynthetic studies demonstrate that *Rhodobacter sphaeroides* can biologically transform RDX and novel insensitive munitions (IMs), which will replace the explosive TNT in many next generation explosives. *R. sphaeroides* degraded RDX and IMs within 72 hours under light conditions. Photosynthetic electron transfer is identified as the degradation mechanism. Additional experiments with RDX showed that succinate and malate were the most effective reducing equivalents for photosynthetic reactions; however, biodiesel-derived waste glycerol was
also utilized as an electron donor. RDX was transformed irrespective of the presence of carbon dioxide. Electron shuttling compounds increase degradation kinetics in the absence of CO₂. In conditions where CO₂ was present (and growth possible), the electron shuttling compound had no stimulatory effect. End products indicated that much of the RDX carbon became CO₂ and biomass, but a fraction became a soluble, aqueous metabolite, characterized using 14C-labeled RDX. End products for IMs were consistent with reduction metabolites present in the literature. The presence of high explosive RDX did not inhibit photosynthetic growth of R. sphaeroides, and growing cultures consistently degraded spikes of RDX over 480 hours. These data are the first to suggest that photobiological RDX and insensitive munitions degradation is possible.
This thesis is dedicated to Mom, Dad, Nick, Harrison, and especially Sean for their love and support.
ACKNOWLEDGEMENTS

The results presented here could not have been accomplished without the help of financial resources. My work was funded by a combination of the Department of Education’s Graduate Assistance in Areas of National Need (GAANN) program, a US EPA Science to Achieve Results (EPA STAR, FP-917130) fellowship, and a research assistantship provided by Professor Kevin Finneran. My studies would not have been possible without this, and I am extremely thankful.

I needed more than monetary support to complete my dissertation. The University of Illinois, Urbana-Champaign has provided many opportunities for me. Professor Charlie Werth helped me develop my research project. Professor Timothy Strathmann provided suggestions that have improved its overall quality. Mary Pearson and Joan Christian coordinated graduation logistics. Shaoying Qi assisted with all things lab-related. Professor Vern Snoeyink made me a better public speaker. Fourth floor companions Aimee Gall, Kaitlin Mallouk, Francisco Mena, and Theresa Vonder Haar kept me connected with the department, even when I was far away.

Much of the work presented here was completed at Clemson University, and I am very grateful to its community and specifically the Environmental Engineering and Earth Sciences Department for welcoming me as one of their own. Mary Shirley and Jan Young coordinated the many logistics associated with my stay. Kenneth Dunn addressed all issues mechanical. A special thanks to Anne Cummings, whose knowledge of analytics rescued my project on a weekly (if not daily) basis. Fellow graduate students Onur Apul, Francisco Barajas, Nathan Conroy, Diana Delach, Yogendra Kanitkar, Adam Mangel, Meric Selbes, Megan Smith, Muriel Steele, and Rong Yu each offered advice and ideas that strengthened this project. Professor David Freedman kindly agreed to participate in my dissertation committee. Thank you, Tigers!

Most of my support came from within the Finneran laboratory at Clemson and Illinois. Hossain Azam, Annie Haluska, Priya Jacob, Andres Jurado, Neeraja Ramasubramania, Christopher Weber, XiaoFeng Ye, and Xinyu Zhang each offered assistance and suggestions. Five undergraduate research assistants directly and indirectly contributed to this work: Jillian Goodlove, Erin Grubbs, Kathryn Fauerby, Jessica Bush, and Trevor Johnston. The work of Man
Jae Kwon provided the foundation for this project. Jolanta Niedźwiecka provided invaluable assistance with the insensitive munitions work, as well as a friendly smile to improve a rainy day. Na Wei is a model of patience, poise, and persistence, and I look up to her daily.

Finally, I would like to thank my advisor, Professor Kevin Finneran, for the opportunity to conduct research under his guidance. He has been an excellent role model. I entered graduate school with no laboratory or research experience; all I have accomplished is a result of him.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>INTRODUCTION AND OVERVIEW</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>ELECTRON-SHUTTLE MEDIATED BIOTRANSFORMATION OF RDX ADSORBED TO GRANULAR ACTIVATED CARBON (GAC)</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>PHOTOBIOLOGICAL DEGRADATION OF CYCLIC NITRAMINES AND INSENSITIVE MUNITIONS (IM) USING RHODOBACTER SPHAEROIDES</td>
<td>44</td>
</tr>
<tr>
<td>4</td>
<td>ESTABLISHING A FRAMEWORK FOR THE APPLICATION OF PHOTOSYNTHETIC RHODOBACTER SPHAEROIDES IN EXPLOSIVES REMEDIATION</td>
<td>71</td>
</tr>
<tr>
<td>5</td>
<td>CONCLUSIONS AND FUTURE RESEARCH</td>
<td>91</td>
</tr>
<tr>
<td>A</td>
<td>SUPPORTING INFORMATION FOR CHAPTER 2</td>
<td>97</td>
</tr>
<tr>
<td>B</td>
<td>SUPPORTING INFORMATION FOR CHAPTER 3</td>
<td>113</td>
</tr>
<tr>
<td>C</td>
<td>ANAEROBIC, REDUCTIVE TRANSFORMATION OF NITROGUANIDINE (NQ) USING IRON(II) SPECIES</td>
<td>123</td>
</tr>
<tr>
<td>D</td>
<td>DATA SUPPORTING FUTURE RESEARCH</td>
<td>143</td>
</tr>
<tr>
<td>E</td>
<td>SAMPLE STANDARD CURVES FOR ANALYTES</td>
<td>145</td>
</tr>
<tr>
<td>F</td>
<td>MOLECULAR STRUCTURES OF SELECTED CONTAMINANTS</td>
<td>152</td>
</tr>
<tr>
<td>G</td>
<td>PERMISSION (ACS) TO REPRINT PUBLISHED MATERIALS</td>
<td>154</td>
</tr>
</tbody>
</table>
1.1 State of Practice in Explosives Remediation

One of the most widely used military high explosives, the cyclic nitramine RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine, chemical structure provided in Appendix F) has been identified globally as an anthropogenic groundwater contaminant (Garg et al., 1991; Pennington et al., 2002; Steuckart et al., 1994). Originally produced during World War II, RDX has entered the environment due to the discharging of manufacturing wastewaters to surface waters and lagoons, operational spills, landfill leaching, open burning and detonation of obsolete munitions, and activities at firing and testing ranges (Morley et al., 2006; Pennington et al., 2002). A report from the Massachusetts Military Reservation (MMR) located in Sandwich, MA, indicated that RDX was identified in a groundwater plume at concentrations as high as 0.370 mg/L, more than 150 times its recommended lifetime health advisory of 2 µg/L (Etnier et al., 1990). MMR is the most studied of contaminated military facilities (Clausen et al., 2004), but is only one of many. It is estimated that the US Department of Defense (DOD) alone has over 317,500,000 kg of high explosives to treat, and continued dismantling of Cold War weapons, as proposed by agendas such as the STRategic Arms Reduction Treaties (START I and II), will add an additional 50,000 kg of RDX waste per year (Wilkie, 1996). In short, the dismantling process has generated large volumes of problematic, RDX-contaminated wastewaters, and contaminated groundwater is often large volumes of water containing low concentrations of RDX.

Significant progress has been made in RDX research over the last 15 years. Formerly considered recalcitrant and immune to many remediation approaches, the literature is now filled with reports of successful chemical oxidation (Adam et al., 2006; Chokejaroenrat et al., 2011), reduction via zero valent iron (Albano et al., 2010; Halihan et al., 2012), alkaline hydrolysis (Balakrishnan et al., 2003; Heilmann et al., 1996), and biotransformation within soil (discussed in Section 1.2), to name a few. What is absent, however, is large-scale field implementation of these. The scientific community is filled with various promising in situ technologies, which do not require removal of the contaminated groundwater from its native environment. Yet personal communication with the US Army, Camp Edwards (Massachusetts) indicates that pump and
treat, an ex situ strategy that intercepts contaminated groundwater and pumps it aboveground for treatment, will continue to be the presumptive treatment for explosives-laden groundwater for the foreseeable future.

Carbon adsorption is the most widely employed method of treatment for RDX-contaminated waters because it concentrates the explosive within the pores of carbon granules, effectively removing it from groundwater. Pump and treat followed by granular activated carbon (GAC, the most common form of porous carbon) adsorption is still the “de facto” treatment strategy utilized in RDX remediation (Hwang et al., 2006). However, adsorption of RDX to GAC can create an explosive hazard, and carbon must be replaced before dangerous levels of RDX accumulation on the granules can occur. With other contaminants, spent activated carbon granules may be regenerated thermally. However, the highly reactive nature of explosives makes this practice unsafe, and air quality legislation prohibits intentional detonation of the compound for cleanup purposes (Bernstein et al., 2012). As a result, spent carbon is usually disposed of as hazardous waste (Wilkie, 1996). The safe transport and landfilling of RDX-spent GAC requires considerable labor and cost; potentially explosive hazardous waste must be handled carefully, and appropriate landfills are often far from cleanup sites.

The US Department of Defense is currently developing novel explosive formulas containing insensitive munitions (IMs), designed to prevent unintentional detonations. These IMs will be regulated as Class 4 explosives—not Class 1 like high explosives TNT and RDX—and will be safer and less costly to transport. The novel formulas in development, such as IMX-104 and PAX-12, are intended to eradicate TNT use in the United States, as the US has not manufactured it in 25 years (personal communication with Bob Winstead, Holston Army Ammunition Plant). They will not replace RDX, as the proposed formulas contain both RDX and IMs (Taylor et al., 2013; Walsh et al., 2013). Therefore, it is reasonable to assume that as these new formulas are implemented, detection of RDX in soils and waters surrounding military facilities and firing ranges will continue, and the new IMs may emerge as novel contaminants.

Ex situ approaches involve continuous pumping and may require extensive engineered systems. As a result, they tend to be expensive and require considerable time to address large groundwater
plumes of contamination. Current studies on RDX frequently acknowledge these problems with pump and treat systems and use them as an argument for the further development of in situ technologies. However, pump and treat can continuously treat pumped water, can prevent contamination from migrating to sensitive receptors such as drinking water, and can be 100% effective at addressing plume contamination. For these reasons, regulatory agencies often favor this approach. If both state and federal regulators favor the current pump and treat systems, it is reasonable to assume that this technology will continue to be utilized, and may be utilized for both RDX and IMs in the future. Therefore, as an alternative to drastically altering current remedial practices, improved, sustainable, yet science-based ex situ techniques for explosives should be explored.

The sections to follow provide a brief introduction to the topics discussed in this document. These subjects (particularly *Rhodobacter sphaeroides*) have been featured in journal articles, review articles, and stand-alone textbooks and can be easily researched, if more information is desired. The intention of the summaries, rather than to saturate with information, is to shape the scope of work presented in this paper.

1.2 Biodegradation of RDX

Biodegradation—using microorganisms or other forms of biota to transform or destroy a contaminant—is a technique that can be utilized in lieu of harsh chemicals or extreme conditions to address groundwater contamination. It is a proven technique to address many undesirable compounds, both organic and inorganic. Evidence first suggesting biodegradation of RDX was published in 1981 (McCormick et al., 1981); since then, a variety of biota capable of RDX transformation have been identified, including higher organisms. Fungi such as white rot *Phanerochaete chrysosporium* have been proven to biodegrade RDX using the explosive as the main source of nitrogen (Sheremata et al., 2000). Phytoremediation of RDX using poplar trees has also been documented (Brentner et al., 2010; Thompson et al., 1999; Yoon et al., 2006). RDX uptake occurs through the roots and can be transformed, stored in the leaves of the trees, or biodegraded by phytosymbiotic culture *Methylobacterium* sp. (Van Aken et al., 2004). While
innovative, limitations in both structure and depth of roots restrict such approaches to shallow groundwater plumes.

Most RDX biotransformation research has focused upon respiratory bacteria; aerobic, anaerobic, and facultative degraders have all been identified. Isolates have been enriched from wastewater sludge (McCormick et al., 1981; Zhao et al., 2002; Zhao et al., 2003), explosives-contaminated soils (Coleman et al., 1998; Seth-Smith et al., 2002), and marine sediment (Zhao et al., 2004). The isolates themselves are quite diverse. They can use various carbon sources including acetate (Kwon et al., 2008a), ethanol (Adrian et al., 2007), propylene glycol (Adrian et al., 2007), hydrogen (Adrian et al., 2003; Beller, 2002) as an electron donor, and even RDX as the sole carbon source (Arnett et al., 2009; Thompson et al., 2005).

RDX biodegradation can occur under a wide range of terminal electron accepting processes; acceptors include oxygen (Fournier et al., 2002), sulfate (Arnett et al., 2009), and carbon dioxide (Beller, 2002). In addition to these acceptors, bacteria can also use redox-active small molecules generated outside the cell to shuttle electrons between reduced and oxidized compounds; these extracellular electron shuttles (EES) provide an alternative to direct-contact reduction (Hernandez et al., 2001). Common electron shuttles include anthraquinone-2,6-disulfonate (AQDS), a model for humic substances found naturally in the subsurface that can naturally donate and accept electrons (Lovley et al., 1996; Lovley et al., 1999). Complete RDX reduction using reduced AH2QDS is rapid (Kwon et al., 2006), and EES-mediated degradation of RDX is faster than direct microbial reduction and can be conducted using a wide variety of microorganisms, including Geobacter metallireducens, Geobacter sulfurreducens, Anaeromyxobacter dehalogenans strain K, Defulfitobacterium chlororespirans strain Co23, and Shewanella marina strain MR1(Kwon et al., 2008b).

Degradation routes and products for many isolates have not been identified (Arnett et al., 2009; Cho et al., 2008), but common intermediates include nitrosos MNX (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine), TNX (1,3,5-trinitroso-1,3,5- triazine), and DNX (1,3-dinitroso-5- nitro-1,3,5- triazine), the organic acids MEDINA (methylenedinitramine) and NDAB (4-nitro-2,4-diazabutanal), formaldehyde, formate, methanol, carbon dioxide, methane, nitrite, nitrate,
ammonia, and nitrous oxide and are described in detail in a recent review paper (Singh et al., 2012). RDX degradation pathways most pertinent to the research proposed here are summarized in Figure A.4 of Appendix A.

Aerobic denitrification (the most reported form of biotransformation) has been linked to the enzyme P450, encoded by the gene \textit{XplA} (Rylott et al., 2006). \textit{XplA} promotes denitrification with NADPH as an electron donor (Indest et al., 2007), and is partnered with \textit{XplB}, an NADH utilizing reductase. The “RDX gene” has been most widely studied in \textit{Rhodococcus} (Rylott et al., 2011), but has also been detected in \textit{Gordonia} (Indest et al., 2010). An alternative to enzymatic P450 pathways, anaerobic reduction via two-electron transfer has also been linked to cytochromes, and such bacteria do not require \textit{XplA/B} (Perreault et al., 2012).

1.3 Purple, nonsulfur bacterium \textit{Rhodobacter sphaeroides}

Phototrophic organisms use light energy (photons) from the sun or other light sources to generate ATP (cellular energy) and biomass (Madigan et al., 2009). While the most recognizable example of this is plant photosynthesis, phototrophic bacteria are both ubiquitous and extremely diverse (Dubbs et al., 2004). Purple bacteria are gram-negative prokaryotes that convert light energy into chemical energy via photosynthesis in the absence of oxygen (McEwan, 1994). Although certain phototrophic bacteria can utilize water as reducing equivalents (electrons) to do this, purple, non-sulfur bacteria cannot; they use organic materials or metals as reducing equivalents for photosynthesis, which is perpetuated by sunlight excitation. \textit{Rhodobacter sphaeroides} is the most widely studied of the purple photosynthesizers, and its genome has been completely mapped (http://www.rhodobacter.org/).

Metabolically, \textit{Rhodobacter sphaeroides} is very versatile. It can survive in oxic, micro-oxic, and anoxic environments, either in the dark or in the light (Sistrom, 1977). \textit{R. sphaeroides} is facultative, and both aerobic and anaerobic respiratory (dark) mechanisms have been extensively studied (McEwan, 1994). Most acceptors used for respiration are inorganic; common acceptors include oxygen, nitrate, and nitrite (Laratta et al., 2002). Organic acceptors DMSO (dimethyl sulfoxide) and TMAO (trimethylamine oxide) can also be utilized (McCrindle et al., 2005). It is
also a producer of dihydrogen gas (Fang et al., 2005) from acetate and butyrate. It behaves photosynthetically as a nitrogen fixer, reducing N₂ to ammonia (Kranz et al., 1995), but \textit{R. sphaeroides} also contains denitrification genes (Laratta et al., 2002), and at least one strain can catalyze the complete denitrification of nitrate to dinitrogen gas (Satoh et al., 1983).

As a phototroph, \textit{Rhodobacter sphaeroides} cycles carbon as both a carbon dioxide assimilator (Tabita, 1988) and a consumer of reduced organic compounds. When purple, nonsulfur bacteria grow photosynthetically using organic carbon as their electron donor, excess reducing equivalents must be removed or phototrophic growth is impossible. Under conditions that generate excess reducing potential, caused by either increases of reductant into the system or a shortage of electron acceptor, photosynthetic \textit{Rhodobacter} expresses genetic pathways that consume reductant, including expression of the \textit{cbb} genes (carbon fixation) \textit{nif} genes (nitrogen fixation), and increased use of the electron transport chain (Dubbs et al., 2004). The preferential means to remove or sequester excess reducing equivalents is through use of CO₂ as an electron sink (Wang et al., 1993); in the absence of CO₂, other acceptors can be utilized. Oxygen cannot be used an electron sink under photosynthetic conditions; its presence disables the photosystem (Eraso et al., 1995). In addition to carbon dioxide, photosynthetic \textit{Rhodobacter sphaeroides} is capable of oxidizing many small organic compounds, including formaldehyde (Barber et al., 1998; Barber et al., 1996; Wilson et al., 2008), and using them as carbon and energy sources.

Electron transfer theories within photosynthetic reaction complexes have been extensively developed. \textit{R. sphaeroides} is known to contain two terminal cytochrome oxidases that accept electrons from the cytochrome \textit{bc1} complex, via a soluble cytochrome \textit{c2} or a membrane-bound \textit{cy} during respiratory growth under oxic (Daldal et al., 2001) and DMSO (Eraso et al., 1995) accepting conditions. Respiration of nitrate, nitrous oxide, and DMSO all require soluble cytochrome \textit{c2} (McEwan, 1994).

\textit{Rhodobacter sphaeroides} has been the focus of intensive study under the US Department of Energy’s Genomics to Life program, whose interest in the bacteria has focused upon gene expression and regulation. Most application-based studies on \textit{Rhodobacter} have focused upon carbon fixation and production of hydrogen gas (Fang et al., 2005; Zhu et al., 1999), for potential
implementation in the biofuel industry. Few publications linking \textit{R. sphaeroides} to remediation could be located, but the metabolic diversity of \textit{R. sphaeroides} suggests that it may be applicable as a treatment strategy to a wide variety of groundwater conditions.

1.4 Approach and Scope of this Project

This project focuses on technologies that could be utilized in an ex situ capacity. The reason for this is pragmatic; most sites with explosives contamination are military, and the Department of Defense has chosen to utilize pump and treat systems to address them. This is not dismissing the potential to treat explosives in situ, nor is pump and treat appropriate for all clean up sites. The decision to focus on ex situ treatment stems from the belief that, for eventual implementation, it may be easier to retrofit or “tweak” what is already being done in the field than to start afresh.

In general, the project can be divided into two parts. Part I (Chapter 2, plus Appendix A) focuses on current activated carbon practices and tests experimentally whether GAC with pre-adsorbed RDX can be biologically regenerated and reused. Part II (Chapters 3 and 4, plus Appendices B, C, and D) evaluates \textit{Rhodobacter sphaeroides} as a potential ex situ treatment approach for explosives, to be used in lieu of activated carbon. The work in each of these chapters was conducted with a specific focus on biotransformation by bacterial pure cultures, although some of the work in Chapter 2 uses AQDS as an abiotic reductant because of its association with iron-reducing bacteria. For both parts, experiments were conducted as small-scale batch reactors with specific amendments of both chemicals and bacteria (suspended cells and growing transfers).

Because of its history as a contaminant and likelihood that it will continue to be a concern, this project focuses primarily on the high explosive RDX. However, some of the photobiology work explores the two most common insensitive munitions, 2,4-dinitroanisole (Chapter 3) and nitroguanidine (Chapter 4), that are likely to be contaminants alongside RDX in the future. Standard curves and chemical structures of these are provided in Appendices E and F. In addition to the discussion provided in Chapter 4, alternate reductants for nitroguanidine are discussed in Appendix C. The work in Appendix C is non-biological and is more appropriate for in situ treatment, but the results complement the discussion of Chapter 4.
1.5 Specific Project Objectives

The main objectives of this study were to:

1. Evaluate whether the model humic substance anthrahydroquinone-2,6-disulfonate reduces RDX adsorbed to activated carbon granules. Different amounts (super-stoichiometric, sub-stoichiometric) of AQDS were added to batch reactors containing GAC with pre-adsorbed RDX. Formaldehyde products were identified, and final adsorbed RDX was extracted and quantified.

2. Determine the extent to which Geobacter metallireducens can reduce RDX adsorbed to activated carbon granules, with and without AQDS. \(^{14}\text{C}\)-RDX was used for these studies to assess the length of time for complete transformation to occur and to quantify the metabolites produced.

3. Examine the role that photosynthetic bacterium Rhodobacter sphaeroides can play in biotransformation of RDX. Rhodobacter cells were suspended under light conditions in tubes containing reductant and explosives. Tests were conducted in the presence and absence of CO\(_2\), and first order rates of decay were calculated.

4. Provide a framework for which phototrophy can be used in environmental restoration of explosives contaminated water. Cells were suspended in the presence of insensitive munitions and atrazine, used as an alternate triazine. Rhodobacter cells were also tested under growth conditions. Tests were conducted to see if Rhodobacter was capable of degrading multiple, sequential spikes of RDX over 480 hours.

A detailed description of these objectives and the methods used to evaluate them are described in Chapters 2, 3, and 4.

1.6 References


CHAPTER 2: ELECTRON-SHUTTLE MEDIATED BIOTRANSFORMATION OF RDX ADSORBED TO GRANULAR ACTIVATED CARBON (GAC)

2.1 Abstract

Granular activated carbon (GAC) effectively removes hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) from groundwater but generates RDX-laden GAC that must be disposed of or regenerated. Batch reactors containing GAC to which RDX was pre-adsorbed were used in experiments to test the potential for adsorbed RDX reduction and daughter product formation using (i) chemically reduced anthrahydroquinone-2,6-disulfonate (AH$_2$QDS) (ii) resting Geobacter metallireducens strain GS-15, and (iii) a combined system containing AQDS and GS-15. Approximately 97.0% of the adsorbed RDX was transformed in each of these experimental systems. Chemically reduced AQDS (AH$_2$QDS) transformed 99.2% of adsorbed RDX; formaldehyde (daughter) was produced rapidly and was stoichiometric (3 mol HCHO per mol RDX). Geobacter metallireducens also reduced RDX with and without AQDS present. This is the first study to demonstrate biological transformation of RDX adsorbed to GAC. Formaldehyde increased then decreased in biological systems, suggesting a previously unreported capacity for G. metallireducens to oxidize formaldehyde, which was confirmed with resting cell suspensions. These data suggest the masses of GAC waste currently produced by activated carbon at RDX remediation sites can be minimized. Alternatively, this strategy may be used to develop a Bio-GAC system for ex situ RDX treatment.

2.2 Introduction

Granular activated carbon (GAC) adsorption is a common treatment strategy for waters contaminated with the toxic high explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), a contaminant at military facilities and firing ranges (Heilmann et al., 1996; Morley et al., 2006a; Wujcik et al., 1992). RDX adsorbs well to GAC despite its moderate solubility and low absorption coefficient for natural organic matter.(Morley et al., 2006b; Talmage et al., 1999), and

---

1 Reproduced in part from Millerick, K.A., Drew, S.R., Finneran, K.T. *Environmental Science and Technology.* 2013, 47(15): 8743-8750. Reproduced with permission from authors and ACS (approval included in Appendix G)
GAC is used as a treatment for pumped groundwater, leaving clean water that can be returned to the subsurface or sent to municipal wastewater facilities (Morley et al., 2005; Wujeck et al., 1992). Pump and treat with GAC has become the preferred remediation strategy at a number of explosives contaminated sites (Personal Communication, Federal Remediation Technology Roundtable).

Carbon adsorption transfers the explosive from the aqueous to a solid phase surface; it does not attenuate or destroy the contaminant (Aktas et al., 2007; Morley et al., 2006a). GAC has limited capacity for explosives, and the United States Environmental Protection Agency (USEPA) recommends that no more than 10% (mass/mass) be adsorbed to carbon granules to reduce the risk of explosion (Crockett et al., 1999). Activated carbon is thus continually replaced, which results in high operating and maintenance costs and creates explosive-laden solid granules that must be safely disposed (Morley et al., 2005; Morley et al., 2006a; Morley et al., 2006b).

Explosives-spent activated carbon is generally reactivated by thermal processes or disposed of in landfills. The process requires shipment to licensed facilities, the replenishment of the reactivated carbon with virgin carbon, or in the latter case, long-term liability associated with disposal. The direct burning of activated carbon has been banned due to more stringent regulations (Heilmann et al., 1996). Alternate carbon regeneration approaches that transfer contaminants into the liquid phase are available, but they also have the problem of safe disposal (Aktas et al., 2007). A strategy that ultimately destroys RDX adsorbed to GAC is needed.

There are few published reports regarding RDX treatment after GAC adsorption. Heilmann et al reported transformation of sorbed RDX using alkaline hydrolysis (Heilmann et al., 1996). Operational parameters were extreme (pH 12, temperature of 80°C) and high salinity produced required specialized on-site handling. Alternate reports indicate that hydrogen sulfide transformed RDX rapidly to formaldehyde and other products in the presence of black carbon, including GAC (Kemper et al., 2008; Xu et al., 2010). The sulfide was not biogenic, although the authors indicated that microbially generated sulfide would promote the same reaction. While reactions are similar between these two studies, sulfate-reducing microbes and Fe(III)-reducing microbes (discussed below) have different physiologies, and will proliferate under different
conditions both in situ and ex situ.

No studies have been published to date assessing direct biotransformation of RDX adsorbed to GAC or any other form of black carbon. Morley et al. postulated that direct biological GAC regeneration was limited by slow RDX desorption kinetics, and that physio-chemical methods increase diffusion rates, making RDX more accessible to microorganisms (Morley et al., 2005; Morley et al., 2006a). Ethanol (5% vol:vol) desorbed RDX in a column study, and the authors speculated that this ethanol solution could then support a bacterial community as in previous studies (Adrian et al., 2007), but there were no data supporting this mechanism. Biological transformation of desorbed RDX using ethanol-based reactors has been reported (Chiou et al., 1997). The approach required initial desorption with ethanol (≥50%), dilution, and transformation in a separate packed bed bioreactor.

Several reports have demonstrated RDX biotransformation using aerobic (Andeer et al., 2009; Crocker et al., 2006; Fournier et al., 2002) and anaerobic (Kwon et al., 2008; Perreault et al., 2012; Zhao et al., 2008; Zhao et al., 2003) bacteria in aqueous systems. Biodegradation strategies are advantageous because they can produce innocuous products (Fournier et al., 2002; Fournier et al., 2005) or mineralize (Arnett et al., 2009; Zhao et al., 2002) RDX. One reported reductive biodegradation mechanism is the sequential 2-electron reduction of N-N₂O functional groups followed by ring cleavage (Perreault et al., 2012); this is analogous to the abiotic pathways reported using chemically-reduced anthrahydroquinone-2,6-disulfonate AH₂QDS (Kwon et al., 2008) and biologically-reduced Fe(III) (Williams et al., 2005). Biogenic extracellular electron shuttles such as humic acids, AQDS, and Fe(II)/Fe(III) (Hernandez et al., 2001) have been reported to degrade aqueous-phase RDX (Bhushan et al., 2006; Kim et al., 2007; Kwon et al., 2006; Kwon et al., 2009; Kwon et al., 2010), and such compounds accelerate degradation kinetics in the presence of the Fe(III)-reducing bacterium Geobacter metallireducens (Kwon et al., 2006; Kwon et al., 2008).

We report for the first time biological transformation of adsorbed RDX; our experiments utilized G. metallireducens strain GS-15. We also report chemical transformation of adsorbed RDX using AH₂QDS as an electron donor. Batch experiments were conducted to investigate
transformation of GAC-adsorbed RDX using chemical, biological, and mixed chemical-biological systems mediated by quinones/hydroquinones. Specific goals were to (i) identify whether reduced electron donor AH$_2$QDS was capable of transforming adsorbed RDX, (ii) determine if electron shuttles could be combined with bacteria to continuously reduce adsorbed RDX and (iii) characterize the kinetics and major transformation products of these reactions.

2.3 Materials and Methods

*Chemicals.* RDX (dissolved in acetonitrile for transport) was obtained from the US Army Corps of Engineers Waterways Experiment Station (WES). Acetonitrile was removed by a direct stream of nitrogen gas until only solid RDX remained, which was then dissolved in ultrapure water, filtered using 0.2µm polytetrafluoroethylene (PTFE) filters, and quantified using certified standards (VWR; Bridgeport, NJ). No residual acetonitrile was detected. Uniformly labeled $^{14}$C-RDX (7.7mCi/mmol dissolved in acetone) was obtained from PerkinElmer (Boston, MA). This was purified by HPLC prior to experimentation to remove residual acetone; purity following treatment exceeded 99.0%. Purified 2,4-dinitrophenylhydrazine (DNPH; for formaldehyde derivatization) was provided by AmChemteq, Inc. (Port Matilda, PA). Methylene dinitramine (MEDINA) and 4-nitro-2,4-diazabutanal (NDAB) were obtained from SRI International (Menlo Park, CA). Anthraquinone-2,6-disulfonate (AQDS) and HPLC grade ethanol (≥99.8% purity) were purchased from Sigma Aldrich (Milwaukee, WI). HPLC-grade methanol and acetonitrile were purchased from VWR (Bridgeport, NJ). All other chemicals used were of reagent grade quality or higher.

*GAC Studies.* All GAC experiments used AquaCarb 1230C high activity 12x30 mesh virgin coconut shell granular activated carbon (US Filter Corporation; Snellville, GA). Prior to use, granules were rinsed with DI water to remove any fines, baked overnight at 60 °C, and weighed on a precision microbalance. ‘Equilibrium’ for all GAC experiments was operationally defined as three consecutive sampling points over three days where the total change in aqueous RDX was less than 0.05% of the initial mass.
Experimental vials were prepared as follows: Activated carbon (0.0115 ± 0.0005g) was added to bottles containing 30mL of 30mM bicarbonate buffer at pH 7.0. Aqueous phase controls (for comparative purposes) contained no activated carbon. Bottles were degassed with anoxic N₂-CO₂ (80:20 [vol/vol]), sealed under the same headspace, and autoclaved. RDX was then amended and allowed to adsorb to carbon granules until equilibrium; the final mass loading on granules was 23mg RDX per g GAC (mg g⁻¹). This preloading stage took approximately 350 hours (Figure A.2). The aqueous phase was decanted and the carbon dried after the experiments ended; it was then weighed at ambient temperature (Heilmann et al., 1996). Ethanol (60mL) was added to desorb RDX, as suggested in previous reports (Morley et al., 2006a). This GAC/ethanol ratio yielded approximately 97-99% recovery of RDX (Figure A.1 and A.2). After equilibrating, residual RDX in ethanol was quantified. Vials were mixed at 150rpm and were maintained at 30 °C for the duration of the experiment unless indicated otherwise. Water alone was not used to desorb RDX because preliminary experiments using very high water volume to GAC mass ratios demonstrated that ethanol was required to desorb RDX from GAC (Table A.1). Additional information detailing the full adsorption/desorption preliminary studies and subsequent experiments are available in Appendix A.

Abiotic AH₂QDS Transformation Study. Chemically-reduced AH₂QDS was prepared as previously described (Kwon et al., 2008). A 100mL, 5mM AQDS solution in 30mM bicarbonate buffer was added to 10g palladium pellets and heated to 50 °C to catalyze reduction. During heating, the solution was degassed with anoxic H₂-CO₂ (80:20 [vol/vol]) for 1 hour and sealed under the same headspace. The solution was incubated overnight at 30 °C and double-filtered into a bottle degassed with 100% N₂ using 0.2µm PTFE filters to remove residual palladium. The hydroquinone concentration was calculated prior to amendment. Hydrogen was not present in the filtered AH₂QDS stock solution. AQDS solutions (not reduced) were prepared in DDI water and were degassed using 100% N₂.

[^{14}C]-RDX Transformation Study. All radiolabeled experiments were prepared as described in GAC Studies except were preloaded with 0.0478µmol acetone-free U-[^{14}C]-RDX (radioactivity of 14,000 dpm mL⁻¹) plus non-radiolabeled RDX for a final mass loading of 23mg g⁻¹, 4% as ^{14}C-RDX. RDX was allowed to adsorb until equilibrium was established. Immediately before
transformation, cells, electron donor (acetate, 20mM), and electron shuttle (AQDS, 150µM) were added. Following amendments, liquid aliquots were withdrawn periodically. Liquid aliquots were analyzed for RDX, metabolites MNX, TNX, and DNX, organic acids methylenedinitramine (MEDINA) and 4-nitro-2,4-diazabutanal (NDAB), formaldehyde, methanol, and inorganic nitrogen species ammonium, nitrate and nitrite. Total aqueous radioactivity measured as dpm mL⁻¹ was also monitored at selected time points. Organic acid and aqueous radioactivity samples were analyzed immediately after sampling; all others were stored at 4 °C until analysis.

¹⁴CO₂ and ¹⁴CH₄ were monitored by analysis of headspace samples (1 mL); H¹⁴CO₃⁻ was used to establish pH-dependent ¹⁴CO₂ partitioning (Kwon et al., 2008), and partition values were confirmed by acidification. Vials were maintained at 30 °C during sorption/desorption phases and at ambient temperature (22 °C) during transformation (a 30 °C incubator was not available in the building housing the gas radiochromatography detector).

**Microbial Growth and Experiments.** *Geobacter metallireducens* strain GS-15 (ATCC 53774) was maintained using ferric citrate medium amended with 20mM acetate (Kwon et al., 2006; Kwon et al., 2008). Cells were harvested for resting cell suspensions as described previously (Kwon et al., 2006; Kwon et al., 2008; Nevin et al., 2002). Briefly, one liter of cell culture was harvested during late logarithmic growth phase and centrifuged at 5,000g for 20 min to form a dense cell pellet. This pellet was resuspended in 30 mM bicarbonate buffer under anoxic conditions and centrifuged again at 5,000g for 20 min. The resultant pellet was resuspended in 4.0 mL of buffer, and this was amended into experimental vials at 2% (v/v) within 15 min of processing. Cell suspensions were conducted for 90 hours (¹⁴C transformation study; aqueous/headspace samples taken at 0, 12, 18, 26, 36, 64, and 88 hrs) and for 68 hours (nitrogen mass balance, aqueous samples taken at 0 and 68 hrs).

**Analytical Methods.** Aqueous samples were collected using an anoxic syringe and needle and filtered prior to analysis. RDX and nitroso metabolites MNX, TNX, and DNX were analyzed using high performance liquid chromatography (HPLC; Dionex UltiMate 3000) with a previously described method (Kim et al., 2007; Kwon et al., 2006). The U-[¹⁴C]-RDX stock was filtered prior to use via HPLC injection using an LC-CN column (Supelco, 4.6 x 250mm, 5µm) and ultrapure water (1.5mL min⁻¹) as the mobile phase; RDX eluted at 10 minutes, and this peak
was captured manually. $^{14}$CO$_2$ and $^{14}$CH$_4$ were monitored via gas chromatography (GC; Shimadzu 8A) with a gas radiochromatography detector (GC-RAM; IN/US system) (Kwon et al., 2008). 1mL aqueous radioactivity samples were added to 10mL scintillation cocktail and analyzed on a liquid scintillation analyzer (Tri-Carb 2910TR; PerkinElmer). MEDINA and NDAB were analyzed by HPLC as previously described (Zhao et al., 2004) at 210 nm. Formaldehyde (HCHO) was analyzed using a modified version of EPA Method 8315A. Aliquots were derivatized with DNPH for 1 h at 30 °C, and the derivative was analyzed by HPLC with a C18 Acclaim RP column (Dionex, 4.6 x 150mm, 5µm, 120A) and an isocratic mobile phase (50% acetonitrile, 50% water, 0.8mL min$^{-1}$). Certified standards of formaldehyde (Accustandard; New Haven, CT) were used for quantification. Nitrate and nitrite were measured using ion chromatography (IC; Dionex DX-600) using an AS-14 column (Dionex, 4 x 250mm) with a mobile phase (1mL/min) of 8mM Na$_2$CO$_3$/1mM NaHCO$_3$. Methanol was measured using a gas chromatograph equipped with a flame ionization detector (GC-FID) as described previously (Monteil-Rivera et al., 2005). Ammonium was determined spectrophotometrically at 650nm (Rhine et al., 1998).

2.4 Results and Discussion

*Transformation of sorbed RDX using AH$_2$QDS in the absence of cells.* Abiotic reduction of adsorbed RDX consisted of three steps: (i) Preloading RDX on to GAC, (ii) transforming RDX with hydroquinone, and (iii) ethanol extraction to confirm extent of transformation. Figure 2.1 (top) demonstrates the time course for RDX degradation in the presence of AH$_2$QDS in the presence and absence of GAC. GAC-free suspensions were run as controls, even though these data have been reported previously (Bhushan et al., 2006; Kwon et al., 2006; Kwon et al., 2008). RDX transformation rates could not be calculated for experimental series containing activated carbon (<99% of RDX was adsorbed). Our aqueous controls were consistent with previous reports in the literature (Kwon et al., 2006); the pseudo-first-order degradation coefficient ($k_{obs}$; h$^{-1}$) for RDX in the aqueous system at pH 7.0 amended with 150µM AH$_2$QDS was 0.222 ± 0.054.
RDX is reduced by a series of stepwise two-electron transfers generating nitroso intermediates MNX, DNX, and TNX (McCormick et al., 1981). These intermediates are transformed in aqueous systems in the presence of AH2QDS (Kwon et al., 2006; Kwon et al., 2008), producing formaldehyde (HCHO). Previous literature reports cited formaldehyde as the predominant carbon product formed from this reaction in aqueous systems; as expected, formaldehyde was the dominant product in the GAC-containing incubations. Alternate products are discussed below. Formaldehyde was produced in all of our GAC systems where RDX was not recovered after ethanol extraction (Figure 2.1, bottom).

Formaldehyde (HCHO) production was independent of GAC. Figure 2.1 (bottom) demonstrates the time course for formaldehyde production in solutions containing RDX, quinones versus hydroquinones, and GAC. Final HCHO:RDX ratios in both systems containing excess hydroquinone were similar (approximately 3:1), suggesting that GAC does not affect the overall extent of the reaction. HCHO formation follows a pseudo-first-order rate law; the pseudo-first-order degradation coefficients (kobs; h⁻¹) for formaldehyde production in the GAC system with complete RDX removal was 0.0415 ± 0.0031(150µM AH2QDS + GAC) compared to the aqueous control (0.138 ± 0.021). This difference in the regeneration (GAC) and homogeneous (no GAC) data suggests a diffusion or electron transfer limitation at the beginning of the experiment. Heilmann et al. reported similar limitations when monitoring for formate in their regeneration work (Heilmann et al., 1996), but this differs from the findings of Xu et al., whose work suggested that black carbons accelerated RDX destruction (Xu et al., 2010).

Approximately 1.0% of RDX was recovered from GAC that had been in solutions containing stoichiometric-excess hydroquinone, demonstrating functionally complete removal of adsorbed RDX (Figure 2.2). RDX mass was nearly unchanged (statistically identical recovery) in controls without quinone or with AQDS (Figure 2.2). Decreasing the concentration of AH2QDS to 30µM limited RDX transformation suggesting that there was an insufficient concentration of reducing equivalents (electrons as AH2QDS); less than 21.2% was transformed in the hydroquinone-limited systems.

Nitroso intermediates were not detected in GAC amended systems; they either adsorbed to GAC
as previously reported (Morley et al., 2010), or were transformed. These compounds were not detected in the extractions steps. Relative rates of degradation of nitroso compounds proceeds as follows: TNX > DNX > MNX >> RDX (Kim et al., 2007). The full theoretical reaction between AH$_2$QDS and RDX (if the nitroso intermediates accumulate) is as follows:

\[
\text{C}_3\text{H}_6\text{N}_6\text{O}_6 \text{ (RDX)} + 3\text{AH}_2\text{QDS} \rightarrow \text{C}_3\text{H}_6\text{N}_6\text{O}_3 \text{ (TNX)} + 3\text{AQDS} + 3\text{H}_2\text{O} \text{ (Equation 2.1)}
\]

Electrons are in excess in the GAC incubations containing 150µM AH$_2$QDS, and RDX is in excess in the GAC incubations containing 30µM AH$_2$QDS. TNX is unstable and can form formaldehyde. Formaldehyde production in the 30µM AH$_2$QDS + GAC corresponded with this mechanism (approximately 1/3 of the stoichiometric amount was expected); the rate-limiting step was most likely RDX degradation, and given that RDX was in excess in this system not all was transformed.

AQDS is a “model” quinone electron shuttle (at least at this stage it has strong precedent in the literature with Fe(III)-reducing microorganisms); it can easily shift from its oxidized (AQDS) and reduced (AH$_2$QDS) forms and is regenerated with external electron donor and acceptor. AH$_2$QDS served as only an electron donor in these experiments; it was not continuously regenerated, and it was not acting as a shuttle. Total quinone concentration (irrespective of oxidation state) decreased at ~96 hours in the GAC systems, which was well beyond the duration of the actual transformation reactions. This suggested that AQDS adsorbed to GAC (based on visual inspection; AQDS/AH$_2$QDS has distinct color phases that are easily monitored in solution). AH$_2$QDS has the potential to both adsorb to activated carbon granules and donate electrons to electron-accepting functional groups on the surface of GAC granules. Cervantes et al. demonstrated that AQDS can be adsorbed to anion exchange resins (AER); capacities reported were 2.20 and 1.87mM AQDS per gram resin, and the linking functional group between the AER and AQDS was the sulfonate group on the hydroquinone (Cervantes et al., 2010).

While no studies directly supporting sorption of AQDS onto GAC could be located, the porous nature, high surface area, and unique surface chemistry of activated carbon suggest that quinone or hydroquinone adsorption is likely, and AQDS has been shown to adsorb to functional groups on glassy carbon electrodes (Chen et al., 1996). The functional groups on activated carbon have been reported to serve as electron acceptors, and redox mediators and have been reported to
catalyze reactions with $E_0'$ values ranging from -430 to +770mV (Van der Zee et al., 2003).

The specific surface chemistry of activated carbon granules differ based on pretreatment, but certain types can contain surface quinone (carbonyl) structures with concentrations up to a few millimoles per gram (Mangun et al., 1999). This is significant because of the potential of adsorbed quinones to transform RDX, as done with azo dyes (Mezohegyi et al., 2007), but a previous RDX-GAC study has discredited this as a likely sorbed RDX degradation pathway (Kemper et al., 2008). Graphite has been reported to transfer electrons from thiol reductants to adsorbed RDX (Oh et al., 2009); however, studies using graphite, hydrogen sulfide, and an electrochemical cell suggests that RDX reduction cannot be explained by electron transfer through graphite (Xu et al., 2010).

The potential for AH$_2$QDS to adsorb to activated carbon granules and transfer electrons to GAC was tested in batch experiments with GAC + quinone (full methods and results are described in detail in Appendix A). Data indicated that 40.0% of 30µM AQDS and 25.2% of 100µM AQDS were removed from solution at 47 hours using the same aqueous volumes and GAC masses as the RDX transformation experiments (Figure A.3). Quinone/hydroquinone adsorption was apparently slower than electron transfer reactions, given that RDX was transformed in less than 20 hours. Values shown in the figure are total quinone (AQDS), but removal rates for reduced quinone (AH$_2$QDS) are identical. Systems containing AH$_2$QDS did not produce measurable amounts of AQDS in aqueous solution, suggesting that if electrons were transferred to GAC, sorption likely prevented the capacity to measure AQDS in solution. However, the alternative possibility is that AH$_2$QDS did not transfer electrons to the GAC. Additional studies are in progress to determine if reduced quinones participate in redox reactions with activated carbon to transform RDX. However, the potential for GAC-mediated electron transfer was not a goal of this specific study, and was not investigated further.

*Transformation of sorbed RDX by Geobacter metallireducens with and without AQDS.* Biological transformation experiments were conducted similar to the strictly abiotic investigation. The major differences were the use of 4% [$^{14}$C]-RDX (to account for $^{14}$CO$_2$ if produced) and that the transformation phase utilized resting (non-growing) GS-15 cells with and
without quinones. Abiotic controls were run for comparison; these experiments were to assess the capacity for biological transformation of adsorbed GAC.

Figure 2.3 (top) indicates the change in aqueous radioactivity (DPM x 1000 mL⁻¹) during the 88-hour cell suspension duration. Aqueous radioactivity increased in GAC-amended systems at 36.5 (AH₂QDS-alone and Cells + AQDS) and 88 hours (cells alone); all expected radioactivity was recovered. HPLC chromatograms confirm that this was not RDX but instead represented a soluble, radiolabeled transformation product. Pseudo-first-order rate coefficients (k_{obs}; h⁻¹) for this production are 0.0877 ± 0.0200 (hydroquinone) and 0.0915 ± 0.0137 (mixed series).

Production of the soluble compound is identical in both the AH₂QDS-alone and AQDS + cells incubations, suggesting that the quinone (irrespective of cells) is the dominant factor with respect to RDX transformation kinetics. This is consistent with previous reports with Fe(III)-reducing microbes, but without GAC.

Initial concentrations of AQDS (150μM) greatly exceeded those of RDX (33μM). It is likely that AQDS transferred electrons once from the bacteria to RDX in the mixed system. In this case we cannot refer to this as “electron shuttling”. It does still demonstrate that redox active molecules are important in RDX transformation, and they may be critical in GAC-containing systems, because soluble products were not detected at similar quantities until 88 hours in the GS-15-alone incubations.

Approximately 93% of radioactivity was recovered after a 64-hour lag period in the cells-alone experimental series (confirmed with additional samples). The reason for this lag is currently unknown, but it is related to granular activated carbon (there was no lag in previously published GAC-free reactions between GS-15 and RDX) (Kwon et al., 2006). This figure suggests that AQDS may play a significant role in reducing lag periods observed in strictly biotic activated carbon systems. We postulate that cells may be physically occluded from RDX adsorption sites similar to what was reported by Nevin and Lovley using alginate beads with GS-15 (Nevin et al., 2000). The lag phase may be due to electron transfer of biological origin via activated carbon granules, as reported for another species of Geobacter (Van der Zee et al., 2003), although this specific mechanism was not tested within this study. The previous study also investigated
concurrent adsorption and transformation (Van der Zee et al., 2003), whereas our study investigated only pre-adsorbed RDX (similar to a retrofitted pump and treat-GAC unit). This would signify that reduction of sorbed RDX might in fact be indirect, where RDX is being reduced by the redox-active functional groups of the GAC. Since this was a cell suspension, all necessary biomolecules required for RDX transformation (i.e. cytochromes) were present at the onset of the experiment given the cell growth conditions. Further investigation is required to determine why there is a lag, but this lag does not hinder the use of these cells as a remediation strategy; the flow rates would merely need to be adjusted to accommodate the lag in initial activity.

Final formaldehyde concentrations in the abiotic controls (starting with only AH$_2$QDS) were similar irrespective of the presence of GAC and were nearly stoichiometric in both cases (Figure 2.3, bottom), which agree with previous studies and the data reported above. There was a lag in the GAC-containing control, but the final HCHO recovered was similar to the GAC-free control. The reason for this lag was not identified, as it was not a goal of this study. It was not apparently due to HCHO adsorption, formaldehyde + GAC controls did not adsorb formaldehyde (data not shown). Results were very different when cells were present.

Formaldehyde accumulated for the first 26.5 hours before sharply decreasing in any incubation with cells. It was partially recovered at 64.5 hours (GAC + GS-15 + AQDS) and at 88 hours (GAC + GS-15 system only), both shown in Figure 2.3, bottom. Although abiotic HCHO production is faster, several reports demonstrate that HCHO has been produced in the presence of cells (Kwon et al., 2006; Kwon et al., 2008; Zhao et al., 2002). In the cells + AQDS suspensions, it is likely that the abiotic reaction predominated, with respect to HCHO production. HCHO recoveries did not include paraformaldehyde; however, to date this compound has not been recovered in our experiments or others reported in the literature.

Minimal (≤2.0%) RDX was detected following ethanol extraction in all incubations containing GS-15, so it was not a lack of RDX transformation that accounted for the fluctuations in HCHO concentrations. Alternate carbon products were analyzed, and that is described below. As detailed below any discrepancies between RDX and total $^{14}$C extracted from GAC suggested that
a second RDX metabolite (such as daughter nitroso compounds) would account for only a very small fraction (<2.8%) of total carbon.

The pattern of formaldehyde production then loss in systems containing GS-15 (Figure 2.3, bottom) strongly supported HCHO biotransformation. HCHO can be easily oxidized to CO$_2$ by many prokaryotes, as reported for other RDX-degrading bacteria (Fournier et al., 2002). Oddly, we could not find any reports specifically related to whether GS-15 transforms HCHO. Cell suspensions of GS-15 buffered at pH 7 with only formaldehyde as the electron donor and soluble Fe(III) as the electron acceptor transformed HCHO in 18.5 hours as long as both electron donor (HCHO) and electron acceptor (Fe(III)) were present (Figure 2.4). Formaldehyde loss in sterile tubes was minimal (13.2% ± 7.1; Figure 2.4). These are the first data demonstrating HCHO transformation by GS-15 to the best of our knowledge. More importantly, they indicate that HCHO will not accumulate in remediation systems predicated on this strategy, which is important to having it accepted by regulators.

Transformation Products in the Presence and Absence of Cells. Table 2.1 indicates the distribution of $^{14}$C carbon metabolites produced in GAC incubations and compares them with radiolabeled metabolites produced from GAC-free controls. A schematic of reported RDX transformation pathways has been provided in the Appendix A for reference (Figure A.4). Nitrogenous products in the “Cells Alone” series were analyzed at 68 hours; the other series were analyzed at 90 hours – accounting for the difference in the final amount of RDX remaining amongst the treatments.

Formaldehyde concentrations correlate well with total carbon recovered in Table 2.1; total carbon recovered exceeded 90% in systems with high overall formaldehyde production (abiotic systems) and was less than 40% in the biotic systems showing minimal formaldehyde production. This is consistent with our suggestion that formaldehyde is being produced and degraded in suspensions with cells. $^{14}$CO$_2$ was recovered and values ranged from 5.41-16.04% (Table 2.1); acidified liquid aliquots confirmed that the unidentified aqueous radioactivity in the biological experiments shown in Figure 2.3 were not bicarbonate species missed in the $^{14}$CO$_2$ analyses. GS-15 may be incompletely oxidizing formaldehyde to another soluble carbon
intermediate, such as formate, which needs to be further tested. It is also possible that GS-15 needs more time to transform HCHO, but cell suspensions have a limited timeframe before activity diminishes.

NDAB and MEDINA, which are known transformation products of RDX, were also detected in different experimental systems. NDAB was present when cells were present, consistent with past data (Kwon et al., 2008). MEDINA was produced in AQDS or AH$_2$QDS containing experimental systems, which is consistent with the previously described pathway (Figure A.4). In all cases these compounds were recovered at higher percentages in the GAC-free controls, most likely indicating that ethanol was a poor solvent for desorbing them from GAC, as it is less likely that there were differences in the actual transformation pathway(s) based on the presence of GAC.

Nitrogen transformation products were also compared amongst the different experimental systems and controls (Figure 2.5, N$_2$O not tested). Ammonium and nitrite were the dominant nitrogen compounds produced for all reactions studied and correlated well between systems with GAC and the GAC-free controls (Figure 2.5). Only RDX was detected in both the sorbed and aqueous phases; all other nitrogen-bearing compounds were identified in the aqueous phase only.

Findings from this work may have relevance to pump and treat systems currently treating RDX-contaminated groundwater at military facilities as well as treatment of wastewater at production facilities and demilitarization operations. In addition, the on-site regeneration of GAC would greatly decrease the carbon footprint of the treatment technology. This is the first study to successfully demonstrate biological transformation of RDX adsorbed to GAC either directly or indirectly via quinone electron transfer molecules. The combination of cells and soluble molecules like AQDS may be more suitable for treating GAC on-site because they operate at neutral pH, ambient temperature, and can be continuously regenerated and recycled through the reactors. Alternatively, cells alone (no quinones) may be the actual remediation technology in a reactor because of expense, and this is a viable option to explore. Treatment of RDX contamination via reductive pathways has the added benefit of transforming the contaminant to low molecular mass metabolites or intermediates, many of which are labile relative to the parent
compound RDX.

Preliminary data exploring the potential for GAC reuse show that GAC still has a high capacity for RDX adsorption following the treatment approaches discussed in this paper (Table A.2). These data suggest the volumes of waste currently produced by activated carbon technology could be minimized. Personal communication with the US Army, Camp Edwards (Massachusetts) indicates that GAC will continue to be the presumptive treatment for explosives-laden groundwater for the foreseeable future. The strategies proposed may eliminate expenses associated with proper handling, transport, reactivation, or landfilling of RDX-contaminated GAC and would enhance, not replace, current systems nationwide.
Table 2.1 Radiolabeled carbon (\(^{14}\)C) and carbon (Total C) mass balance (%) following RDX reduction and metabolite production by abiotic, biological, and mixed abiotic-biological pathways with and without GAC

<table>
<thead>
<tr>
<th>RDX and degradation products</th>
<th>AH(_2)QDS Alone (abiotic)</th>
<th>Cells alone (biological)</th>
<th>Cells + AQDS (mixed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GAC (^2)</td>
<td>No GAC (^1)</td>
<td>GAC (^3)</td>
</tr>
<tr>
<td>(\text{C}_2\text{H}_6\text{N}_6\text{O}_6) (RDX), \textit{aqueous}</td>
<td>0.00</td>
<td>1.10</td>
<td>0.00</td>
</tr>
<tr>
<td>(\text{CH}_2\text{N}_4\text{O}_4) (MDNA)</td>
<td>10.70</td>
<td>4.50</td>
<td>0.62</td>
</tr>
<tr>
<td>(\text{C}_2\text{H}_3\text{N}_3\text{O}_3) (NDAB)</td>
<td>0.00</td>
<td>0.08</td>
<td>0.65</td>
</tr>
<tr>
<td>HCHO</td>
<td>84.80</td>
<td>87.70</td>
<td>16.10</td>
</tr>
<tr>
<td>(\text{CH}_2\text{OH})</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(^{14}\text{CO}_2)</td>
<td>0.00</td>
<td>0.00</td>
<td>13.07</td>
</tr>
<tr>
<td>(\text{C}_2\text{H}_4\text{N}_6\text{O}_6) (RDX), \textit{extracted}</td>
<td>0.30</td>
<td>N/A</td>
<td>2.00</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>95.80</strong></td>
<td><strong>93.38</strong></td>
<td><strong>32.44</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phase</th>
<th>(^{14}\text{C} (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AH(_2)QDS Alone (abiotic)</td>
</tr>
<tr>
<td></td>
<td>GAC</td>
</tr>
<tr>
<td>Liquid</td>
<td>91.30</td>
</tr>
<tr>
<td>Headspace</td>
<td>0.00</td>
</tr>
<tr>
<td>GAC (Extracted)</td>
<td>2.80</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>94.10</strong></td>
</tr>
</tbody>
</table>

Mass balances conducted at \(^1\)26.5 hrs, \(^2\)64.5 hours, and \(^3\)88 hours. \(^4\)Methanol detection limit is 30uM.
Figure 2.1 RDX reduction by chemically reduced AQDS (AH$_2$QDS) (top; inset shows concentrations in the presence of GAC) and production of formaldehyde (bottom; dashed line represents theoretical stoichiometry). The results are the means of triplicate analyses, and the bars indicate one standard deviation.
Figure 2.2  Sorbed RDX recovery after transformation using chemically reduced AQDS (AH$_2$QDS) The results are the means of triplicate analyses, and the bars indicate one standard deviation.
Figure 2.3 RDX degradation and production of soluble, radiolabeled metabolites (top) and formaldehyde (bottom). The results are the means of triplicate analyses, and the bars indicate one standard deviation. Initial DPM added was 485,711; therefore, max DPM per mL expected was 16,190.
Figure 2.4 Change in formaldehyde concentrations over time under experimental conditions (30mM bicarbonate buffer, 30°C) with soluble Fe(III) present as an electron acceptor, in the presence and absence of active but non-growing suspensions of Geobacter metallireducens. The results are the means of triplicate analyses, and the bars indicate one standard deviation.
Figure 2.5 Nitrogen mass balance at the final sampling point during reduction and metabolite production by abiotic, biotic, and mixed abiotic-biotic pathways for both GAC-sorbed and aqueous RDX.
2.5 Acknowledgements

This publication was developed under STAR Fellowship Assistance Agreement no. FP-917130 awarded by the U.S. Environmental Protection Agency (EPA). It has not been formally reviewed by EPA. The views expressed in this publication are solely those of the authors, and EPA does not endorse any products or commercial services mentioned in this publication.

2.6 References


15. Kemper, J. M., E. Ammar and W. A. Mitch. Abiotic degradation of hexahydro-1,3,5-
trinitro-1,3,5-triazine in the presence of hydrogen sulfide and black carbon. 
*Environmental Science & Technology* **2008**, *42* (6), 2118-2123.

16. Kim, D. and T. J. Strathmann. Role of organically complexed iron(II) species in the 
reductive transformation of RDX in anoxic environments. *Environmental Science &

17. Kwon, M. J. and K. T. Finneran. Microbially mediated biodegradation of hexahydro-
1,3,5-trinitro-1,3,5-triazine by extracellular electron shuttling compounds. *Applied and
Environmental Microbiology* **2006**, *72* (9), 5933-5941.

for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in abiotic versus biological degradation 
pathways with anthraquinone-2,6-disulphonate (AQDS) and *Geobacter metallireducens*.

Reduction Is Concurrently Mediated by Direct Electron Transfer from Hydroquinones 
and Resulting Biogenic Fe(II) Formed During Electron Shuttle-Amended 

biological production of 4-nitro-2,4-diazabutanal (NDAB) in RDX-contaminated aquifer 

fibers: Effect on pore size, surface chemistry, and adsorption properties. *Chemistry of 


Effective anaerobic decolorization of azo dye acid orange 7 in continuous upflow packed-


CHAPTER 3: PHOTOBIOLOGICAL DEGRADATION OF CYCLIC NITRAMINES AND INSENSITIVE MUNITIONS (IM) USING RHODOBACTER SPHAEROIDES

3.1 Abstract

This chapter investigated bacterial photosynthesis as a strategy for ex situ treatment of explosives-laden groundwater, using light as the primary energy source for driving the reactions. The objective of this work was to characterize the ability of photosynthetic Rhodobacter sphaeroides to biologically transform the high-energy military explosive RDX and the insensitive munition 2,4-dinitroanisole (DNAN), which will replace TNT in many next generation explosives. R. sphaeroides degraded both RDX and DNAN within 40 hours under light conditions. DNAN was fully biodegraded in the dark within 72 hours. RDX was not fully transformed in the dark, suggesting that photosynthetic electron transfer was its degradation mechanism. Additional experiments with RDX showed that succinate and malate were the most effective reducing equivalents for photosynthetic reactions; however, biodiesel-derived waste glycerol was also utilized as an electron donor. RDX was transformed irrespective of the presence of carbon dioxide. The electron shuttling compound anthraquinone-2,6-disulfonate (AQDS) increased degradation kinetics in the absence of CO$_2$, when presumably the cells had excess NADPH that needed to be re-oxidized. In conditions where CO$_2$ was present (and growth possible), the cells generated biomass, and AQDS had no stimulatory effect. End products indicated that much of the RDX carbon became CO$_2$ and biomass, but a fraction became a soluble aqueous metabolite, characterized using $^{14}$C-labeled RDX. These data are the first to suggest that photobiological RDX and insensitive munitions degradation is possible.

3.2 Introduction

The cyclic nitramine hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and 2,4-dinitroanisole (DNAN) are key constituents of novel, insensitive explosive formulas designed for the defense industry (Ahn et al., 2011; Walsh et al., 2013); chemical structures for these compounds are provided in Appendix F. RDX is already a well-characterized groundwater contaminant (Hawari et al., 2000; McCormick et al., 1981). The environmental fate of new insensitive munitions (IM)
like DNAN, a TNT replacement, is the subject of current study (Dodard et al., 2013; Liang et al., 2013; Olivares et al., 2013). Although still in development, the new formulas are expected to increase explosive residues on firing ranges (Walsh et al., 2013), and the potential release of these contaminants into groundwater should be addressed.

Despite promising advances in developing in situ remedial strategies for RDX (Bradley et al., 2005; Hatzinger et al., 2004; Oh et al., 2001; Zhao et al., 2003), many state and federal regulators prefer pump and treat approaches, if contaminated groundwater is in close proximity to sensitive receptors (Personal Communication, Federal Remediation Technology Roundtable). The most common ex situ approach currently in use for RDX is adsorption to granular activated carbon (GAC), and both RDX and DNAN adsorb moderately well to GAC surfaces (Boddu et al., 2009; Morley et al., 2005). However, carbon adsorption transfers munitions from groundwater to the solid phase surface but does not attenuate the contaminants (Morley et al., 2006), and continuously replacing spent GAC makes this approach unsustainable. Alternatively, dissolved nitro-bearing compounds can be degraded using ex situ strategies that treat water in aboveground reactors. The reactors may have physical, chemical, biological, or a combination of mechanisms that allow for complete transformation (Hwang et al., 2006; Platten et al., 2010).

Biodegradation strategies are advantageous because they are environmentally friendly and can produce innocuous products without the need of harsh chemicals or extreme conditions. Ex situ bioremediation strategies are most reasonable if they can be constructed easily or retrofitted to existing pump and treat units. The cells utilized must be robust, easily maintained, and preferably grow on simple substrates with stable metabolic activity that withstands perturbations of flow, temperature, pH, and/or substrate availability. One such group of organisms that has been underutilized in remediation applications is the anoxygenic photosynthetic bacteria, of which *Rhodobacter sphaeroides* is the most widely reported.

*R. sphaeroides* grows using light energy with organic reducing equivalents (e.g. malate, succinate) providing the electrons to generate ATP, reduced anabolic electron carriers (e.g. NADPH), and biomass (Madigan et al., 2009a). It also has non-photosynthetic respiratory metabolism, and *R. sphaeroides* cells harbor several membrane bound cytochromes for electron
transport to terminal acceptors such as dimethyl sulfoxide (DMSO) (McCrindle et al., 2005; McEwan, 1994), and cytochrome $c_2$ functions as a mobile electron carrier in both aerobic and photosynthetic electron transport chains (Donohue et al., 1986). They are similar in this manner to Fe(III) reducing bacteria, which use membrane bound cytochromes for electron transfer to ferric iron and other compounds (Mehta et al., 2005). Recent data suggest that reduction of RDX by Fe(III) reducers is also mediated by these cytochromes (Perreault et al., 2012). Given this similarity, it is reasonable that *R. sphaeroides* will reduce explosives like RDX and alternate nitro-group bearing contaminants in a similar manner - utilizing light energy as the reducing power.

In this study, we investigated the potential for ex situ biodegradation of groundwater containing RDX and DNAN using photosynthetic *Rhodobacter sphaeroides*. Our studies utilized resting and growing cells of *R. sphaeroides* with different reducing equivalents. Data demonstrate that RDX and DNAN can be reduced via bacterial photosynthesis. Additional RDX studies show that extracellular electron transfer molecules accelerate degradation reactions with resting cells. These results provide the first evidence of photobiological RDX and DNAN degradation, or explosives degradation by *R. sphaeroides*.

3.3 Materials and Methods

*Chemicals:* RDX (0.5g in 20mL acetonitrile) was obtained from the US Army Corps of Engineers Waterways Experiment Station (Andrewes et al.). Acetonitrile was removed by a direct stream of nitrogen gas until only solid RDX remained, which was then dissolved in ultrapure water, filtered using 0.2um polytetrafluoroethylene (PTFE) filters, and quantified using certified standards (VWR; Bridgeport, NJ). No residual acetonitrile was detected. Uniformly-labeled $^{14}$C-RDX (7.7mCi/mmol dissolved in acetone) was obtained from PerkinElmer (Boston, MA). This was dissolved in ultrapure water and purified prior to experimentation to remove residual acetone. 2,4-dinitroanisole (DNAN) and 2-methoxy-4-nitroaniline (MENA) were obtained from Sigma Aldrich and dissolved in methanol prior to use. Purified 2,4-dinitrophenylhydrazine (DNPH; for formaldehyde derivization) was prepared by AmChemteq, Inc. (Port Matilda, PA). Methyleneedinitramine (MEDINA) and 4-nitro-2,4-diazabutanal
(NDAB) were provided by SRI International (Menlo Park, CA). Anthraquinone-2,6-disulfonate (AQDS) was purchased from Sigma Aldrich (Milwaukee, WI). HPLC-grade methanol and acetonitrile were purchased from VWR (Bridgeport, NJ). All other chemicals used were of reagent grade quality or higher.

**Microorganisms:** *Rhodobacter sphaeroides* ATCC® 17023™ was purchased from the ATCC and maintained photoheterotrophically using ATCC Medium #550 pre-reduced with cysteine. The carbon source in this medium was malate. Strains were incubated at ambient temperature (~25°C) under a tungsten lamp (~270W/m²) (Argun et al., 2010). Relative cellular growth was measured as optical density (OD) at 680nm (Cohen Bazire et al., 1957). *Rhodobacter sphaeroides* strain SW-102, a mutant strain possibly deficient for formaldehyde-oxidation was obtained from the University of Wisconsin-Madison and maintained using SMM media amended with succinate (Donohue et al., 1986). Strain 17023 was utilized for all experiments except when stated otherwise.

**Resting cell suspension incubations:** *R. sphaeroides* was grown as described above, and 1L of cells were harvested at late log phase (OD₆₈₀ 1.5 to 1.7) and centrifuged at 5,000xG for 20 minutes. The supernatant was discarded and cells were washed with 10mM phosphate buffer, resuspended, and re-centrifuged. Cells were suspended in 6mL of the same phosphate buffer before amendment into experimental tubes (0.2mL cell suspension per 10mL buffer). All photosynthetic (light) experiments were conducted under a tungsten lamp at ambient temperature; tubes were randomly positioned and moved during experimentation to distribute light equally amongst them. All respiratory experiments were incubated at 30°C in darkness. Tubes were autoclaved prior to amendments. All experiments contained controls with cysteine but without cells to monitor for phototransformation caused by the lamp. Tubes for suspensions (except for experiments testing the effects of carbon dioxide) were prepared at pH = 6.8 to 7.0 with 10mM PO₄³⁻ buffer, 0.03% cysteine, and 5% CO₂/95% N₂. Experiments testing the effects of CO₂ were conducted at pH 7.0 and contained either 10mM PO₄³⁻ buffer, degassed with 100% N₂ (CO₂-free experiments) or 10mM HCO₃⁻ buffer, degassed with N₂/CO₂ (CO₂ experiments).

**Growth experiments:** Growth experiments were conducted using *Rhodobacter sphaeroides*
ATCC® 17023™. A modified version of ATCC Medium #550 was used, containing (all concentrations in g/L except as specified): NH₄Cl (1.01), MgCl₂ (0.165), CaCl₂ • 2H₂O (0.70), EDTA (0.02), KH₂PO₄ (0.60), K₂HPO₄ (0.90), Metals “44” solution (1mL) (Cohen Bazire et al., 1957), and vitamin solution (7.5mL). The solution was bubbled with anoxic N₂-CO₂ (95:5 [vol/vol]), sealed under the same headspace, and autoclaved at 121ºC for 20 minutes. Donor and amendments were added following autoclaving using purged syringe and needles. Cells (2% [vol/vol]) were added during late log phase, 55-60 hours into growth. Series were conducted in quadruplicate.

**Analytical Methods:** Aqueous samples were collected using an anoxic syringe and needle and filtered prior to analysis. RDX, nitroso metabolites MNX, TNX, and DNX, and organic acids NDAB and MEDINA were analyzed using high performance liquid chromatography (HPLC) with previously described methods (Kim et al., 2007; Kwon et al., 2008). DNAN and MENA were analyzed by HPLC with a C18 Acclaim RP column (Dionex, 4.6 x 250mm, 5µm, 120A) and an isocratic mobile phase (50% acetonitrile, 50% water, 1.0mL/min) at 296nm (DNAN) and 210nm (MENA). Formaldehyde was analyzed using a modified version of EPA Method 8315A. Aliquots were derivatized with DNPH for 1 h at 30 °C, and the derivative was analyzed by HPLC with a C18 Acclaim RP column (Dionex, 4.6 x 150mm, 5µm, 120A) and an isocratic mobile phase (50% acetonitrile, 50% water, 0.8mL/min) at 360nm. Certified standards of formaldehyde (Accustandard; New Haven, CT) were used for quantification. ¹⁴CO₂ and ¹⁴CH₄ were monitored via gas chromatography (GC; Shimadzu 8A) with a gas radiochromatography detector (GC-RAM; IN/US system) (Kwon et al., 2008). Cellular uptake of radiolabeled material was measured using a liquid scintillation counter. 1mL of sample was centrifuged at 5,000xG for 10 minutes. The resulting cell pellet was washed three times with DDI water (no residual radioactivity was detected in DDI), suspended in scintillation cocktail, and soniccated for 90 minutes before analysis. For aqueous radioactivity, samples were filtered through a 0.22um PTFE filter, and 1mL of filtrate was added to 10mL scintillation cocktail and analyzed on the liquid scintillation counter.

3.4 Results
**RDX biodegradation by R. sphaeroides strain 17023.** RDX was degraded in all incubations with *Rhodobacter sphaeroides* ATCC® 17023™ under light conditions (Figure 3.1, open symbols); the rate and extent of RDX reduction were similar amongst all incubations (k_{obs} between 5.11 and 7.68 × 10^{-2}/hr). RDX was reduced by 90% in 33 hours in the presence of either malic acid (electron donor) or AQDS, an added extracellular electron shuttle that can facilitate RDX reduction (Bhushan et al., 2006). The degradation rates of RDX in the presence of malic acid (k_{obs} = 7.45 × 10^{-2}/hr without AQDS; k_{obs} = 5.11 × 10^{-2}/hr with AQDS) were slower than that of RDX without malic acid (k_{obs} = 7.68 × 10^{-2}/hr). RDX was below detection limits in these suspensions at 40 hours. *R. sphaeroides* can reduce RDX with and without AQDS. Final concentrations of RDX in the 33-hour experimental timeframe were similar, regardless of the shuttle; however, the degradation rate was highest in the incubations containing resting photosynthetic cells, AQDS, and RDX, without an additional donor.

RDX was not transformed in abiotic light controls (Figure 3.1, dashed lines), indicating that the tungsten lamp alone does not photodegrade RDX, with or without AQDS. Approximately 37% (cells, donor, and AQDS) and 29% (cells plus donor) of RDX was transformed in the two series maintained in the dark (Figure 3.1, closed symbols). First order rate constants for these are 1.35 × 10^{-2}/hr (cells, donor, AQDS) and 8.10 × 10^{-3}/hr (cells, donor). MNX accumulated only transiently in the resting cell suspensions and decreased below the detection limits with longer incubation times. MNX was not detected in either the two “no cell” control incubations; DNX and TNX were not detected.

**DNAN biodegradation by R. sphaeroides strain 17023.** DNAN was degraded in less than 30 hours with *R. sphaeroides* strain 17023 under light conditions using malate as the carbon source and 0.03% cysteine as the reducing equivalent source (Figure 3.2). Cells also degraded DNAN under dark conditions, but rates were slower (k_{obs} = 5.21 × 10^{-2}/hr in dark, compared with 3.07 × 10^{-1}/hr with light) and DNAN reduction was not complete until 72 hours. The primary metabolite was 2-methoxy-4-nitroaniline (MENA), which has been identified in other putative biological DNAN degradation pathways (Liang et al., 2013; Olivares et al., 2013). MENA did not significantly degrade within the timeframe tested. 2,4-diaminoanisole (DAAN), another
terminal reduction product of DNAN, was not tested. DNAN was not transformed in abiotic light controls, indicating that the tungsten lamp alone does not photodegrade DNAN.

**RDX degradation with different reducing equivalent sources.** Table 3.1 shows the screening results for RDX reduction using resting cells and several reducing equivalents, versus cells alone, at 30 hours. Cells alone reduced nearly all RDX within 30 hours, which is not uncommon for resting biomass. Glucose, succinate, and malic acid also promoted RDX degradation using resting cells. The viability of operationally defined waste reducing equivalents such as sterilized septic wastewater and glycerol (byproduct of biodiesel production) was also investigated. RDX reduction was the least complete when wastewater was used. No discernable difference in extent of degradation between filtered and autoclaved wastewater was observed, and RDX reduction in non-sterile wastewater was only slightly improved. Septic wastewater was not explored further. In contrast, RDX was completely reduced below detection limits within 30 hours in the presence of resting cells and glycerol.

These donors (excluding wastewater, but with the addition of formaldehyde) were re-tested with 2% transfers of growing *R. sphaeroides* into media. Degradation was fastest and most complete with succinate and malic acid (Figure 3.3). Observed rate constants for these reactions were $1.35 \times 10^{-2}$/hr (malic acid) and $1.28 \times 10^{-2}$/hr (succinate) and are not significantly different. Only 61% of RDX was reduced using glucose as a substrate before transformation stalled at 250 hours, and this reaction ($k_{obs} = 4.08 \times 10^{-3}$/hr) was slower than that for malic acid and succinate. Unlike resting cells, growing cells had a lag period of 175 hours was before glycerol amended *R. sphaeroides* began to degrade RDX. The lag period was followed by steady decreases in RDX concentrations to non-detect but could fitted to a zero order reaction ($R^2 = 0.985$). Minimal biomass increases (Figure B.1, Appendix B) or RDX losses (Figure 3.3) were quantified in abiotic controls, experiments lacking reducing equivalents, or experiments with formaldehyde (as a possible reducing equivalent source). OD$_{680}$ measurements exceeding 1.500 were obtained using glycerol, glucose, malate, and succinate as electron donors.

**RDX degradation in the absence of carbon dioxide.** All data presented above were in phosphate buffered medium, with 5% CO$_2$ in the headspace. *R. sphaeroides* is capable of carbon dioxide
fixation; this contributes to biomass increase and alters electron transfer pathways, even though the cells were started as “resting” suspensions. This potential for growth may change electron transfer dynamics and was investigated further. Figure 3.4 demonstrates the differences in RDX degradation time courses using cell suspensions of *Rhodobacter sphaeroides* ATCC® 17023™ under strictly CO₂-free conditions (top panel; no growth possible) and conditions permitting autotrophic growth (bottom panel).

AQDS increased the rate and extent of RDX degradation in phosphate buffered, CO₂-free cell suspensions (Figure 3.4, top). Approximately 91% was reduced within 28 hours in CO₂-free suspensions containing AQDS, compared to approximately 70% reduced at 60 hours in suspensions that did not have AQDS. The suspensions containing AQDS followed first order kinetics, \( k_{\text{obs}} = 1.11 \times 10^{-2}/\text{hr} \). AQDS had no impact on RDX reduction in suspensions that contained CO₂; each of the three triplicate series were similar (Figure 3.4, bottom).

**Preliminary metabolite investigation.** Cell suspensions utilizing uniformly-labeled \(^{14}\)C-RDX were conducted to quantify the fractions going to RDX mineralization, carbon assimilation, and aqueous metabolites. Analytes measured included RDX, liquid phase radioactivity (measured as dpm/mL), \(^{14}\)CO₂, \(^{14}\)CH₄, and radioactivity (dpm/mL) in washed, lysed cells (measuring \(^{14}\)C assimilation). Figure 3.5 is a mass balance of \(^{14}\)C after 72 hours of suspension, in the absence (top) and presence (bottom) of carbon dioxide. \(^{14}\)CH₄, was not detected; however, \(^{14}\)CO₂ was produced in all experiments containing *R. sphaeroides*, confirming mineralization of RDX. Total production of carbon dioxide (operationally defined as the total radioactivity (%) measured as CO₂/bicarbonate + recovery in biomass, since any \(^{14}\)CO₂ generated can be incorporated into biomass) is similar between buffers; for instance, in the “cells alone” series, this represents 45.1% (phosphate) and 49.1% (bicarbonate buffer) of total carbon.

A large fraction of \(^{14}\)C was recovered as soluble, aqueous organic metabolite(s). Its identity is unknown, but this compound elutes within 4 minutes on the LC-CN column (see Figure B.2, B.3, and discussion in Appendix B). For both buffer systems, production of this metabolite is greatest in the systems that contain malic acid.
Each of the major known RDX pathways are summarized in detail by Singh et al. (Singh et al., 2012) and produce transient production of nitroso compounds (see Figure B.4 of Appendix B for MNX time courses; MNX did not accumulate) and significant quantities of formaldehyde. Formaldehyde was not detected in any of our experiments. *R. sphaeroides* can oxidize formaldehyde to carbon dioxide (Barber et al., 1998), suggesting the potential of RDX mineralization. To test this, we obtained *Rhodobacter sphaeroides* strain SW-102, a mutant theoretically incapable of formaldehyde oxidation based on a deletion in the glutathione formaldehyde activating enzyme (*gfa*), from the University of Wisconsin-Madison and ran suspensions similar to those previously conducted with strain 17023 (Wilson et al., 2008). RDX degradation rates and patterns using SW-102 were consistent with previous experiments, and formaldehyde did not accumulate. Table 3.2 presents mass recoveries of carbon using SW-102. Almost all carbon recovered was from untransformed RDX, not from suspected metabolites. It is possible that the lack of *gfa* alone does not negate HCHO oxidation (Wilson et al., 2008).

To test the influence of nitrogen gas (presence/absence of nitrogen fixation) on *Rhodobacter sphaeroides*’ ability to transform RDX, Figure 3.4 was conducted using helium gas instead of nitrogen. Results using helium are presented in the Appendix (Figure B.5) and closely resemble the trends presented in Figure 3.4.

3.5 Discussion

RDX was readily degraded by resting and growing cells of *R. sphaeroides*; to the best of our knowledge this is the first report of direct photosynthetic RDX degradation by anoxygenic photosynthetic microorganisms. Cells required light energy to completely transform RDX, as only a limited concentration of RDX was reduced in cultures suspended in the dark. This suggests that the light reactions of photosynthetic metabolism are more critical to *R. sphaeroides* mediated RDX transformation than the dark reactions, or its respiratory metabolism. Reports indicate that several *Rhodobacter* species can reduce nitrate during light-mediated growth and light-free respiratory reactions (Ferguson et al., 1987). Nitro functional group reduction is less widely reported, but *Rhodobacter capsulatus* can reduce nitrophenol compounds during light-mediated growth (Roldan et al., 1998; Witte et al., 1998). Enzymes that catalyze the reduction
of nitro groups on aromatic compounds are termed nitroreductases. Azoreductase (AZR) from \textit{R. sphaeroides} possesses nitroreductase activities, and AZR can catalyze destruction of TNT, when the protein is cloned, purified, and assayed overnight (Liu et al., 2008).

DNAN and other IM compounds are becoming more critical to the Department of Defense (Dodard et al.) as it moves away from first generation munitions and explosives and towards new formulations like IMX-101 and PAX-21 (Taylor et al., 2013; Walsh et al., 2013). Although these IM compounds have not yet been identified as a soil or groundwater problem, the DoD is being proactive with respect to understanding their degradation both in situ and ex situ. DNAN was degraded within a similar timeframe as RDX by resting cell mass. Unlike RDX, however, DNAN was degraded to detection limits by dark-incubated cells. This suggests one of two things. One possibility is that DNAN is degraded during anaerobic respiration by \textit{R. sphaeroides}. Given its metabolic flexibility, it has multiple electron transfer pathways and can use standard respiration (not light-mediated reactions) to gain energy. It is not likely that \textit{R. sphaeroides} can grow via DNAN reduction; it is most likely an electron sink reaction (often referred to as co-metabolic reduction) similar to those between cells and RDX (Perreault et al., 2012). The other possibility is that reduced intracellular electron transfer compounds such as NADPH are at a high enough concentration in the biomass that DNAN is reduced by light grown cells as the NADPH is re-oxidized to NADP\(^+\). If this were the case we would have expected similar results with RDX, but RDX does have one additional nitro group, which may change the extent of reduction under dark conditions. Reduction of DNAN is also more thermodynamically favorable than RDX, based on one-electron standard reduction potentials (Uchimiya et al., 2010).

The metabolite 2-methoxy-4-nitroaniline (MENA) accumulated to approximately 60\% of the initial carbon (as DNAN) in the lightmediate reactions. MENA also accumulated in the dark reactions, but at a much slower rate, which was comparable to the rate of DNAN reduction. These data suggest that biological IM transformation is possible under both dark and photobiological conditions, and \textit{R. sphaeroides} may be a viable inoculum for reactor systems used to treat IM contaminated groundwater or production water.
The capacity to use different electron donors for photobiodegradation is critical to flexibility in field applications. Malate and succinate were most efficient, but use of waste substrate glycerol (from biodiesel) is advantageous because it is a relatively inexpensive substrate and is widely available. Glucose was not an appropriate substrate; RDX was never fully degraded. *Rhodobacter* is capable of photofermentation and produces organic acids that are immediately utilized by the cell from glucose (Han et al., 2012). Under growth conditions in media, this would produce additional biomass but not excess electrons for RDX reduction. It is likely was photofermented in this manner, which limited RDX transformation.

When present, carbon dioxide is the preferred electron sink (Wang et al., 1993) for *Rhodobacter*; in the absence of CO₂, other acceptors can be utilized. When carbon dioxide is absent, *Rhodobacter* cells utilize external electron sinks to remove excess reducing equivalents, and these may accelerate degradation kinetics if they can transfer electrons to RDX. AQDS facilitated RDX transformation when CO₂ was absent, consistent with trends observed in respiratory bacteria (Kwon et al., 2006; Kwon et al., 2008; Kwon et al., 2009; Kwon et al., 2010). No previous reports suggesting reduction of AQDS or humic substances by *Rhodobacter sphaeroides* could be found. It is unique that AQDS accelerates biodegradation rates with this culture when carbon dioxide is absent, since most other AQDS degraders can also reduce iron. While purple bacteria can photosynthetically oxidize iron, they cannot facilitate ferric iron reduction (Madigan et al., 2009b). The presence of AQDS affected kinetics but did not affect extent of RDX degradation.

During growth, NADPH and ATP are generated during the light reactions, and the energy and electrons are used to synthesize biomass from CO₂. Therefore the NADPH is oxidized by making biomass. In the absence of biomass synthesis, the NADPH must still be oxidized to maintain NADP⁺ in the cells; there is a surplus of reducing equivalents that must be expended. We believe that in the CO₂-free systems, AQDS served as the electron sink for NADPH, and the AH₂QDS was available to transfer electrons to RDX. When CO₂ was present, the proportion of electrons available to reduce AQDS was much smaller, if not absent. Therefore, AQDS did not influence the relative rate and extent of RDX degradation with growing cells. These data are important for eventual field applications, because they demonstrate that the rate and extent of
RDX degradation can be modulated by electron shuttles based on growth (CO\textsubscript{2}) versus complete non-growth (CO\textsubscript{2}-free) conditions. In the field it is reasonable to predict that both conditions may be encountered, depending on the prevailing environmental conditions.

The fraction of carbon dioxide assimilated by cells verses carbon dioxide present as CO\textsubscript{2}/aqueous carbonate species differed between the two buffer treatments; more \textsuperscript{14}C was recovered as biomass in the phosphate (CO\textsubscript{2}-free) buffer. This makes sense; the only available CO\textsubscript{2} present is \textsuperscript{14}CO\textsubscript{2} generated during RDX degradation. Once generated, the cells assimilated it to produce biomass. In the bicarbonate buffer, non-radiolabeled CO\textsubscript{2} is always present for biomass, so more \textsuperscript{14}CO\textsubscript{2} generated remained outside of the cells. However, the relative proportion of carbon recovered in the biomass/mineralization fractions was the same.

Photobiological DNAN transformation produced large quantities of reduction product MENA, which is consistent with previous reports (Liang et al., 2013; Olivares et al., 2013). We are still investigating the ultimate fate of MENA, and whether conditions can be met to further degrade it to a less hazardous compound. The identity of the dominant aqueous RDX transformation product(s) is unknown. Although it was not definitively identified, our \textsuperscript{14}C total radioactivity fractionation experiments (Figures B.2 and B.3, Appendix B) suggest it is a low molecular mass product. We are currently seeking an analytical chemistry partner to identify this product; this will be reported in future work.

The presence of an additional carbon source (e.g. malate) produced greater quantities of the unknown aqueous metabolite, as compared to series that did not. It is our hope that identification of this metabolite will help us understand why. As mentioned previously, the primary carbon product generated during alternative (respiratory) degradation – formaldehyde – did not accumulate under these conditions, and \textit{R. sphaeroides} can further oxidize HCHO to CO\textsubscript{2} (Wilson et al., 2008). Additionally, formaldehyde inhibited growth, which is interesting because \textit{R. sphaeroides} is a documented formaldehyde oxidizer (Barber et al., 1998; Barber et al., 1996; Wilson et al., 2008).

Unlike many aerobic bacteria (Singh et al., 2012), RDX photobiodegradation does not require
nitrogen-limiting conditions. This may indicate that *Rhodobacter* is not using RDX as a nitrogen source, and a Blast search reveals that *R. sphaeroides* does not contain the *XplA* gene present in many RDX degraders capable of denitrification (Rylott et al., 2011). This is significant because RDX is often present in the environment in plumes containing other nitrogen-bearing compounds. Data presented in the Appendix support that RDX degradation is independent of the presence of nitrogen; product speciation did not differ when helium was substituted for nitrogen in the headspace (Figure B.5), and nitrogen fixation occurred simultaneously to RDX degradation (Figure B.6). Time courses showing nitrite accumulation are presented in Figure B.7.

Transformation of explosives by photosynthetic *Rhodobacter sphaeroides* is significant for two reasons: 1) There are no previous reports in the literature suggesting photobiological transformation of either RDX or DNAN, and 2) Photobiology in general is a relatively unexplored area of bioremediation. *Rhodobacter sphaeroides* has been the focus of intensive study under the US Department of Energy’s Genomics to Life program, whose interest in the bacteria has focused upon gene expression and regulation. Most application-based studies on *Rhodobacter* have focused upon carbon fixation and production of hydrogen gas (Fang et al., 2005; Zhu et al., 1999), for potential implementation in the biofuel industry. This is the second reporting of degradation of a triazine compound by *R. sphaeroides*; it can resist (Brown et al., 1988) and degrade (Zhang et al., 2012) the pesticide atrazine. *R. sphaeroides* may be a viable photosynthetic candidate for a wide variety of remediation applications.
Table 3.1 Effect of substrate on extent of RDX reduction over 30 hours using resting *Rhodobacter sphaeroides*

<table>
<thead>
<tr>
<th>Substrate (20mM)</th>
<th>RDX (%) Remaining&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Donor</td>
<td>6.8 ± 6.6</td>
</tr>
<tr>
<td>Malic Acid</td>
<td>20.9 ± 13.5</td>
</tr>
<tr>
<td>Unfiltered Wastewater&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.7 ± 2.6</td>
</tr>
<tr>
<td>Filtered Wastewater&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.3 ± 5.6</td>
</tr>
<tr>
<td>Autoclaved Wastewater&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.4 ± 2.6</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.2 ± 2.2</td>
</tr>
<tr>
<td>Control (no cells)</td>
<td>89.1 ± 2.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>For wastewater, COD (units = mg/L) was measured and converted to mM by assuming 1 mg COD = 1 mg CH2O (so 30 g/mol), Wastewater was diluted with water until it was 20 mM as CH2O.

<sup>b</sup>Initial concentration is 40 μM.
Table 3.2 Carbon recovery during RDX biodegradation and metabolite production using formaldehyde-oxidizing deficient mutant SW-102

<table>
<thead>
<tr>
<th>RDX &amp; degradation products</th>
<th>Total carbon mass (umol)</th>
<th>0 hrs</th>
<th>8 hrs</th>
<th>20 hrs</th>
<th>36 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDX (C$_3$H$_6$N$_6$O$_6$)</td>
<td>2.05</td>
<td>1.84</td>
<td>1.30</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>MNX (C$_3$H$_6$N$_6$O$_5$)</td>
<td>0.00</td>
<td>0.01</td>
<td>0.02</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>DNX (C$_3$H$_6$N$_6$O$_4$)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>TNX (C$_3$H$_6$N$_6$O$_3$)</td>
<td>None Detected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDNA (CH$_4$N$_4$O$_4$²⁻)</td>
<td>None Detected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDAB (C$_2$H$_3$N$_3$O$_2$⁻)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Formaldehyde (HCHO)</td>
<td>0.00</td>
<td>0.02</td>
<td>0.01</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

umol C recovered  | 2.05  | 1.86  | 1.34  | 0.29   |

% C recovered     | 100.0 | 90.6  | 65.3  | 14.0   |
Figure 3.1 RDX biodegradation by *R. sphaeroides* wildtype strain in buffer (10mM PO$_4$$^{3-}$, 0.03% cysteine, 5% CO$_2$/95% N$_2$). Solid shapes represent experimental series conducted in the dark; all others were maintained under a tungsten lamp. Dashed lines show cell-free controls. Error bars represent one standard deviation.
Figure 3.2 2,4-dinitroanisole (DNAN) biodegradation (solid shapes) and production of reduction product MENA (open shapes) by *R. sphaeroides* wildtype strain in buffer (10mM $\text{PO}_4^{3-}$, 0.03% cysteine, 5% CO2/95% N$_2$). Triangles represent experimental series conducted in the dark; all others were maintained under a tungsten lamp. Error bars represent one standard deviation.
Figure 3.3 RDX biodegradation by growing *R. sphaeroides* wildtype strain in modified SMM media. ‘No donor’ represents a triplicate series where RDX is the sole carbon source added to media. ‘RDX control’ does not contain cells. The final carbon concentration for each donor is 20mM carbon. Values shown are averages of quadruplicates.
Figure 3.4 RDX photobiodegradation by resting *R. sphaeroides* when CO₂ is absent (top, phosphate buffer) and present (bottom, bicarbonate). Error bars represent one standard deviation.
Figure 3.5 $^{14}$C mass balance after 72 hours using resting *R. sphaeroides* 17023 when CO$_2$ is absent (top, phosphate buffer) and present (bottom, bicarbonate). Error bars represent one standard deviation.
3.6 Acknowledgements

We thank Tim Donohue at the University of Wisconsin-Madison for providing *Rhodobacter sphaeroides* strain SW-102. Clemson Ph.D. student Jolanta Niedźwiecka provided assistance with quantifying DNAN and MENA. This publication was developed under STAR Fellowship Assistance Agreement no. FP-917130 awarded by the U.S. Environmental Protection Agency (EPA). It has not been formally reviewed by EPA. The views expressed in this publication, are solely those of the authors, and EPA does not endorse any products or commercial services mentioned in this publication.

3.7 References


CHAPTER 4: ESTABLISHING A FRAMEWORK FOR THE APPLICATION OF PHOTOSYNTHETIC RHODOBACTER SPHAEROIDES IN EXPLOSIVES REMEDIATION

4.1 Abstract

Purple, nonsulfur bacterium *Rhodobacter sphaeroides* has been explored extensively in fundamental microbiology as a model for photosynthesis and in bioenergy research as a producer of hydrogen gas. *R. sphaeroides* is highly versatile and adaptive, making it desirable in groundwater treatment applications, but photobiology has little precedent in remediation. The objective of this work is to provide a context for which phototrophy can be used in environmental restoration of explosives contaminated water. Batch experiments tested the ability of *R. sphaeroides* to reduce explosive compound nitroguanidine (NQ) and the pesticide atrazine. Partial degradation of atrazine was observed, but *R. sphaeroides* was able to completely degrade NQ under photosynthetic conditions, and first order decay rates were compared to those previously obtained for other military explosives. The presence of high explosive RDX did not inhibit photosynthetic growth of *R. sphaeroides*, and growing cultures were able to consistently degrade spikes of RDX over 480 hours. Work in progress will explore a continuously flowing photobioreactor system containing *R. sphaeroides* in anoxic conditions with an artificial light source, modeling a potential ex situ reactor configuration. It will be used to assess whether *Rhodobacter sphaeroides* can effectively treat the explosive RDX continuously.

4.2 Introduction

Bioremediation, broadly defined as the use of microorganisms to detoxify and degrade contaminants of concern to human health, can address polluted groundwater in the subsurface or aboveground in bioreactors (Alexander, 1999). Treating groundwater aboveground is often advantageous because it continuously addresses contaminants, preventing migration to sensitive receptors. Pump and treat is currently—and will likely continue to be—the default cleanup strategy for dissolved explosives in groundwater (personal communication with Camp Edwards,
MMR). Bioreactors can successfully treat explosives-laden waters (Ederer et al., 1997; Esteve-Nunez et al., 2001; Zoh et al., 2002), and the bacteria within these systems are typically classified as aerobic, anaerobic, or facultative, defined by the terminal electron acceptors they utilize in respiration. Phototrophic bacteria in bioreactors treating explosives have not been reported.

Photosynthetic bacteria are capable of treating a variety of contaminants in water. They have been linked to hydrocarbon oxidation (Harwood et al., 1988) and treatment of odorous swine wastewater (Kim et al., 2004). *Rhodobacter sphaeroides* is the most widely published of the purple photosynthetic bacteria and has been linked to cadmium reduction in wastewater (Bai et al., 2008) and soils (Fan et al., 2012). It can also uptake heavy metals in contaminated shrimp ponds (Panwichian et al., 2011). *R. sphaeroides* can degrade pharmaceutically active compounds in wastewater via a photobiological reactor, although few reactor details were disclosed (Madukasi et al., 2011; Madukasi et al., 2010). Our previous work (Chapter 3) demonstrates the ability of *R. sphaeroides* to degrade the high explosive RDX and novel munition 2,4-dinitroanisole (DNAN) at laboratory scale. Given its metabolic diversity, it is likely that *R. sphaeroides* is capable of biotransforming a wider variety of contaminants than those described above and could be very useful in groundwater cleanup.

In general, the potential role of photobiological bacteria in systems (such as bioreactors) with direct remedial applications is not well defined. Algal photobioreactors for wastewater treatment is a current research area of interest (Bordel et al., 2009; Munoz et al., 2006); however, most of these systems focus on oxic photosynthesizers, and configurations would not be appropriate for *R. sphaeroides*. The role of *Rhodobacter sphaeroides* in photobioreactors for generating bioenergy has been discussed in detail, as *R. sphaeroides* is a well-defined producer of dihydrogen gas. Reactors for H₂ production can be quite complex (Gilbert et al., 2011), and a review paper details many potential configurations for photobioreactors designed for H₂ production (Dasgupta et al., 2010). Such systems are fermentative batch reactors but give a good overview of operating parameters (light intensity, pH limitations) that that would be important to any system containing *Rhodobacter sphaeroides*.
While we have already demonstrated the ability of *R. sphaeroides* to reduce RDX and 2,4-dinitroanisole (DNAN, future TNT replacement), our previous work focused on the phenomenon of photobiodegradation of only these specific munitions, and in single-amendment tubes. This work explores photobiodegradation broadly, examining a wider range of contaminants and studying more continuous systems. It is designed to provide a practical framework for which photobiology could be utilized. Specific goals were to (i) identify whether reduction rates of other triazine or nitro-bearing compounds resembled those obtained for RDX, (ii) determine whether the presence of high explosive RDX would inhibit photosynthetic growth of *R. sphaeroides*, and if growing cultures could consistently degrade repeat spikes of RDX over 480 hours (iii) assess whether *Rhodobacter sphaeroides* can effectively treat the explosive RDX in a continuous flow system.

4.3 Materials and Methods

**Chemicals:** RDX (0.5g in 20mL acetonitrile) was obtained from the US Army Corps of Engineers Waterways Experiment Station (WES). Acetonitrile was removed by a direct stream of nitrogen gas until only solid RDX remained, which was then dissolved in ultrapure water, filtered using 0.2um polytetrafluoroethylene (PTFE) filters, and quantified using certified standards (VWR; Bridgeport, NJ). No residual acetonitrile was detected. Atrazine and nitroguanidine (NQ) were obtained from Sigma Aldrich and dissolved in DDI prior to use. HPLC-grade methanol and acetonitrile were purchased from VWR (Bridgeport, NJ). All other chemicals used were of reagent grade quality.

**Microorganisms:** *Rhodobacter sphaeroides* ATCC® 17023™ was purchased from the ATCC and maintained photoheterotrophically using ATCC Medium #550 pre-reduced with cysteine. The carbon source in this medium was malate. Strains were incubated at ambient temperature (~25°C) under a tungsten lamp (~270W/m²) (Argun et al., 2010). Relative cellular growth was measured as optical density (OD) at 680nm (Cohen Bazire et al., 1957).

**Resting cell suspension incubations:** *R. sphaeroides* was grown as described above, and 1L of cells were harvested at late log phase (OD₆₈₀ 1.5 to 1.7) and centrifuged at 5,000xG for 20
minutes. The supernatant was discarded and cells were washed with 10mM phosphate buffer, resuspended, and re-centrifuged. Cells were suspended in 6mL of the same phosphate buffer before amendment into experimental tubes (0.2mL cell suspension per 10mL buffer). All photosynthetic (light) experiments were conducted under a tungsten lamp at ambient temperature; all respiratory experiments were incubated at 30ºC in darkness. Tubes were autoclaved prior to amendments. All experiments contained controls with cysteine but without cells to monitor for phototransformation caused by the lamp. Tubes for suspensions were prepared at pH = 6.8 to 7.0 with 10mM PO$_4^{3-}$ buffer, 0.03% cysteine, and 5% CO$_2$/95% N$_2$.

**Batch Reactor Studies:** Reactor studies containing *Rhodobacter sphaeroides* ATCC® 17023™ were conducted in 160mL bottles and maintained beneath a tungsten lamp. Reactors contained a modified version of ATCC Medium #550 composed of (all concentrations in g/L except as specified): NH$_4$Cl (1.01), MgCl$_2$ (0.165), CaCl$_2$ • 2H$_2$O (0.70), EDTA (0.02), KH$_2$PO$_4$ (0.60), K$_2$HPO$_4$ (0.90), Metals “44” solution (1mL) (Cohen Bazire et al., 1957), and vitamin solution (7.5mL). Media was degassed with anoxic N$_2$-CO$_2$ (95:5 [vol/vol]), sealed under the same headspace, and autoclaved at 121ºC for 20 minutes. Carbon source, reductant (cysteine), and amendments were added following autoclaving using purged syringe and needles. Cells (2% [vol/vol]) were added during late log phase, 55-60 hours into growth. Series were conducted in quadruplicate. Reactors were maintained at room temperature for the duration of the study (480 hours). RDX was continuously monitored and re-spiked into reactors following complete degradation.

**Continuous Flow Studies:** The continuous flow studies will be conducted in three sealed borosilicate glass columns (25mm [inner diameter] × 250mm). Columns contain glide fit end caps with ports located at the inlets and outlets for sampling. 1mL of cells grown to late log phase will be inoculated at the column base. Columns will be initially filled with SMM media amended with cysteine. 48 hours after inoculation, flow will be turned on. Sterile feed will continuously pass through columns in an upflow direction. Feed, designed to mimic natural groundwater, will contain malate, RDX, and (g/L): MgSO$_4$·7H$_2$O, 0.06; NaBr, 0.0013; KCl, 0.0019; NaHCO$_3$, 0.212; CaCl 0.012. Bromide will be used as a conservative tracer at a concentration of 1mM. A tungsten lamp (~100W/m$^2$) is the light source unless otherwise stated.
Initial parameters: 0.15mL/min flow, 4uM RDX, 1mM malate.

Analytical Methods: Aqueous samples were collected using an anoxic syringe and needle and filtered prior to analysis. RDX was analyzed using high performance liquid chromatography (HPLC; Dionex UltiMate 3000) with an LC-CN column at 254nm (Kim et al., 2007; Kwon et al., 2008). Atrazine was analyzed by HPLC with a C18 Acclaim RP column (Dionex, 4.6 x 250mm, 5µm, 120A) and an isocratic mobile phase (60% acetonitrile, 40% water, 1.0mL/min) at 222nm. Nitroguanidine was analyzed by HPLC with an Acclaim Polar Advantage II column (Dionex, 4.6 x 250mm, 5µm) and ultrapure water (1.0mL min\(^{-1}\)) as the mobile phase; nitroguanidine eluted at 10.1 minutes at 254nm. NQ and atrazine were quantified using standards prepared in DDI.

4.4 Results

NQ biodegradation by R. sphaeroides strain 17023: Nitroguanidine (structure in Appendix F) was degraded in incubations with Rhodobacter sphaeroides ATCC\textsuperscript{®} 17023\textsuperscript{TM} under light conditions (Figure 4.1). 73.9% of nitroguanidine was transformed in the 72-hour experimental window. No transformation of nitroguanidine was observed at or before 12.5 hours. Reduction of nitroguanidine after this lag period can be modeled as a first order reaction (\(k_{\text{obs}} = 2.79 \times 10^{-2}/\text{hr}\), \(R^2 = 0.977\)). NQ losses in the abiotic light control series and for the biological series maintained in the dark were minimal and not statistically different.

Deviation between samples increased with time in the series containing R. sphaeroides under light conditions. This increase in deviation can be correlated with NQ degradation; deviation first exceeded 5% at 23 hours, when NQ concentrations first decreased, and subsequent deviations became incrementally bigger. This is because two of the triplicates actively degraded NQ, while the third showed little activity.

At 72 hours, a second peak was observed in the HPLC chromatograms, eluting at approximately 7.3 minutes. Although it has not been concretely identified, it has been putatively identified as nitrosoguanidine, a reduction product of nitroguanidine (Kaplan et al., 1982). This is discussed in detail in Appendix C. The peak was observed in chromatograms for the two active triplicates
that degraded NQ under light conditions; the average peak area for these two peak areas was 148.3 mAU. It was also observed in the biological tubes maintained in the dark, but the average peak area, 3.95 mAU, was considerably lower.

*Atrazine biodegradation by R. sphaeroides strain 17023:* Atrazine was partially degraded in incubations with *Rhodobacter sphaeroides* ATCC® 17023™ under light and dark conditions (Figure 4.2). 16.0% of atrazine was transformed in the first 2.5 hours under light conditions and was further degraded to 60.5% of its initial concentration after 72 hours total. This can be modeled as a first-order equation ($k_{obs} = 6.63 \times 10^{-3}$/hr ), but the model does not fit well ($R^2 = 0.842$) due to the sharp decrease in atrazine concentrations within the first 2.5 hours, followed by stepwise decreases. Under dark conditions, 19.1% of atrazine was transformed in the first 2.5 hours under light conditions and was further degraded to 76.5% of its initial concentration after 72 hours total. 81.3% of the observed atrazine transformation occurred in the first 2.5 hours, and it was not possible to fit this data to a kinetic model. No abiotic degradation of atrazine from the lamp was observed.

*Batch Reactor Studies:* Using malate as a carbon source, RDX degradation occurred concurrent with cellular growth (Figure 4.3). Cells reached stationary phase at 60 hours and at an OD$_{680}$ of approximately 1.8, consistent with previous transfers. Growth rates obtained for the experimental series containing 20mM carbon substrate, RDX, and *R. sphaeroides* (black diamonds) are identical to growth rates obtained for the experimental series containing 20mM carbon substrate and *R. sphaeroides* but without RDX (dark X markers). Optical density values did not increase in series that did not contain malate as a carbon source, and a maximum OD$_{680}$ of 0.23 was obtained for both series that contained 1mM of carbon source.

No change in RDX was observed in the two sterile series (dashed lines). Gradual decreases occurred in the series without malate as a carbon source, the series with 1mM malate, and the series with 1mM malate and 1mM RDX. Complete RDX degradation was observed only in bottles containing 20mM carbon substrate, RDX, and *R. sphaeroides*. When RDX concentrations dropped below HPLC detection limits, it was re-amended. RDX was amended into these bottles a total of four times; each time, it was degraded.
Results using succinate as the carbon source (Figure 4.4) closely resemble the results obtained using malate, except RDX decreases are more rapid. Initial RDX decay did not fit kinetic models well, but the three subsequent re-spikes at 149, 219, and 363 hours could be modeled as a first-order decay for succinate: 0.163 ($R^2 = 0.983$), 0.0864 ($R^2 = 0.908$), and 0.0479/hr ($R^2 = 0.920$), respectively. Corresponding first order reaction rate constants for malate are 0.0223 ($R^2 = 0.994$), 0.0239 ($R^2 = 0.912$), and 0.0255/hr ($R^2 = 0.984$).

Little change in RDX was observed in bottles containing glucose as a substrate. Cells reached stationary phase at 100 hours and at an $OD_{680}$ of approximately 1.6. RDX decreased to 89.0, 35.1, and 36.5% of its initial concentration in bottles amended with 0, 1, and 20mM glucose. RDX decay could not be modeled to a first order reaction.

**Continuous Flow Studies:** As of December 15th, 2013, we still had not received from suppliers the fitted end pieces needed to assemble the continuous flow-through column set up, so no results are presented here. The discussion section outlines what we hope to accomplish with the columns.

### 4.5 Discussion

One of several proposed insensitive munitions, nitroguanidine ($\text{CH}_4\text{N}_4\text{O}_2$, NQ) differs from many classical explosives because it is aliphatic, has relatively high water solubility (2.6 ± 0.1 g/L) and is more persistent (Haag et al., 1990). Nitroguanidine was degraded by resting cells of *Rhodobacter sphaeroides* under light conditions. To the best of our knowledge, this is the first report of NQ degradation under anoxic conditions by a bacterial pure culture as well as the first report of direct photosynthetic NQ degradation by anoxygenic photosynthetic microorganisms. This is significant because of its recalcitrance under typical anoxic conditions.

NQ is the third nitro-bearing compound we have successfully been able to degrade using *R. sphaeroides* and is the least thermodynamically favorable in reduction potential. Degradation rates and extent of degradation for the three nitro-bearing compounds using *R. sphaeroides*
correlate with thermodynamic one-electron potentials (Uchimiya et al., 2010); two-electron reduction potentials could not be located for the insensitive munitions. DNAN, the most favorable for reduction \((E_{\text{theoretical}}^o = -0.39V)\), was degraded the fastest \((k_{\text{obs}} = 3.07 \times 10^{-1}/\text{hr})\) under light conditions and was the only compound completely degraded in the dark \((k_{\text{obs}} = 5.21 \times 10^{-2}/\text{hr})\), discussed in Chapter 3. RDX, \((E_{\text{theoretical}}^o = -0.54V)\) not as favorable as DNAN, degraded under light conditions more slowly \((k_{\text{obs}} = 7.45 \times 10^{-2}/\text{hr})\) and was only partially degraded under dark conditions. NQ \((E_{\text{theoretical}}^o = -0.70V)\) did not degrade at all under dark conditions and its rate of degradation \((k_{\text{obs}} = 2.79 \times 10^{-2}/\text{hr})\) under light conditions was an order of magnitude lower than that for DNAN.

While photobiological transformation pathways have not been developed, the intermediates we have observed using \textit{R. sphaeroides} are similar to those observed during reductive transformation using anaerobic, respiratory bacteria and abiotic reductants. With DNAN, the nitro functional groups, bound to carbon molecules on the anisole ring, become amines. MENA, the product of this, has been detected in our photosynthetic experiments in light and dark (Chapter 3), in anoxic microcosms (Liang et al., 2013; Olivares et al., 2013), and using AH\(\text{2QDS}\) and ferrous iron as reductants (discussion with Jolanta Niedźwiecka of Clemson University, data unpublished). With RDX and NQ, nitro groups are bound to cyclical (RDX) and alkane (NQ) nitrogen molecules and become nitroso (N-NO) groups when reduced, forming unstable intermediates. Our experiments with \textit{R. sphaeroides} and RDX (Chapter 3) show transient production of nitroso MNX, which is consistent with reports using anoxic bacteria and using AH\(\text{2QDS}\) and ferrous iron as reductants. The only published metabolite of anoxic NQ reduction is nitrosoguanidine, which is unstable. This has been observed in sediment incubations (Kaplan) and is our suspected HPLC metabolite eluting at 7.3 minutes in studies using ferrous iron (Appendix C) and photobiology. This may be utilized as a tool for future work to predict how \textit{Rhodobacter sphaeroides} may attack particular contaminants of concern.

The formulas for insensitive the Department of Defense is currently developing (IMX-101, PAX-21, etc…) are not composed of a single explosive compound (Taylor et al., 2013; Walsh et al., 2013). Preliminary reports suggest that these mixtures will contain a combination of RDX, DNAN, and NQ. Therefore, it is imperative that potential treatment systems be able to address
all of these compounds. The ability of *Rhodobacter sphaeroides* to transform each of these strengthens it as a viable and pragmatic remediation strategy for groundwater contaminated with munitions.

The limited transformation of atrazine by *Rhodobacter sphaeroides* was surprising because of its literature precedent. We had hypothesized that transformation of atrazine would be similar to what we observed with RDX, and the intention of this experiment was to compare rates of degradation for the respective triazine compounds. However, our strain of *Rhodobacter sphaeroides* was the wildtype from the ATCC®, while strains of *R. sphaeroides* demonstrating the ability to completely degrade atrazine have been isolated from agricultural fields exposed to years of atrazine usage (Brown et al., 1988; Zhang et al., 2012). While certain strains of *Rhodobacter* are triazine-resistant, this resistance is due to genetic modification and is not naturally-occurring (Spitz et al., 2005).

RDX concentrations in the growth experiments did not follow first order decay while cells were in logarithmic growth. This makes sense; cells were active at this time but reducing equivalents were utilized to also generate additional biomass, and RDX was not serving as the sole electron sink. For subsequent RDX spikes at 149, 219, and 363 hours, rate did follow first order decay but differed between malate and succinate. The average rate constants for RDX decay for these spikes were 0.0991/hr ± 0.0585 (succinate) and 0.0239/hr ± 0.0016 (malate). Degradation rates with succinate as a carbon source were quicker than those obtained for malate, but with more variation. The exact reason for the higher variation is unknown, but it may be because decay of RDX in the presence of malate was slower, meaning more data points were gathered, and decay models became better (and more accurate) fits than for succinate. Han et al. compared H₂ production using malate and succinate as carbon sources in batch reactors under conditions similar to the ones studied here. They found that less H₂ is produced from succinate than malate (Han et al., 2012), which may mean systems with succinate contain more excess electrons available for external acceptors such as RDX.

Glucose was intended as a negative control for this series of experiments, and RDX decreased in this system less rapidly (and was overall less complete) than systems containing malate and
succinate as carbon sources. This is consistent with our previous work with glucose (Chapter 3) and is because fermentation of glucose does not produce excess electrons for external acceptors.

The presence of RDX did not affect the overall growth rate of *Rhodobacter sphaeroides* and optical density values obtained, which differs from results obtained with other photosynthetic cultures and triazine structures (Gonzalez-Barreiro et al., 2006), and as well as results obtained with *Rhodobacter sphaeroides* and atrazine (Zhang et al., 2012). While RDX has been shown to have a toxic effect on other bacteria, it does not appear to affect growth of *R. sphaeroides*, which is highly advantageous for field implementation.

We were only able to re-spike our systems with RDX three times. Any additional spikes would have dropped the biomass concentrations to less than 50% of their initial maximum. Due to its low solubility, the concentration of the RDX stock for re-amendment was 200uM, meaning that bottles needed to be diluted with 15-16% stock with each amendment. It is this dilution that causes optical density values to drop at 149, 219, and 363 hours in the respiked series. Rates of decay using succinate decreased over time (from 0.163/hr, to 0.0864/hr and finally to 0.0479/hr). We do not know if this was because cells started to lag in activity over the 480 hr experimental window, and it is difficult to draw conclusions based on three spikes. Additional spikes of RDX would have indicated if this trend would continue.

Our hope is that the continuous flow system will address whether the cells lose activity over time in reactors. The preliminary design for the continuous flow system is based on simplicity; upflow columns are the easiest and most basic configuration to modulate when changes are needed, and they have considerable precedent in remediation. In reviewing the literature for photosynthetic reactors, light intensity and biofilm fixation both have a considerable effect on overall reaction efficiency (Grobbelaar et al., 2003; Munoz et al., 2009). It is hoped that once supplies arrive, light intensity can be modulated and glass beads added to glass columns to maximize reactor potential.
Figure 4.1 Nitroguanidine biodegradation by *R. sphaeroides* wildtype strain in buffer (10mM \( \text{PO}_4^{3-} \), 0.03% cysteine, 5% \( \text{CO}_2/95\% \text{N}_2 \)). Solid shapes represent experimental series conducted in the dark; all others were maintained under a tungsten lamp. Dashed lines show cell-free controls. Error bars represent one standard deviation.
Figure 4.2 Atrazine biodegradation by *R. sphaeroides* wildtype strain in buffer (10mM PO$_4^{3-}$, 0.03% cysteine, 5% CO$_2$/95% N$_2$). Solid shapes represent experimental series conducted in the dark; all others were maintained under a tungsten lamp. Dashed lines show cell-free controls. Error bars represent one standard deviation.
Figure 4.3 Concentrations of RDX (top) and relative cellular growth (bottom) in batch reactors with media amended with malate (carbon source). RDX was re-amended into reactors when concentrations dropped below detection limits. X represents controls not amended with RDX. Initial concentrations of malate were 20mM unless indicated otherwise; * indicates a concentration of 1mM (malate or RDX). Results are the average of quadruplicate samples.
Figure 4.4 Concentrations of RDX (top) and relative cellular growth (bottom) in batch reactors with media amended with succinate (carbon source). RDX was re-amended into reactors when concentrations dropped below detection limits. X represents controls not amended with RDX. Initial concentrations of succinate were 20mM unless indicated otherwise; * indicates a concentration of 1mM (succinate or RDX). Results are the average of quadruplicate samples.
Figure 4.5 Concentrations of RDX (top) and relative cellular growth (bottom) in batch reactors with media amended with glucose (fermentative carbon source). X represents controls not amended with RDX. Initial concentrations of glucose were 20mM unless indicated otherwise; * indicates a concentration of 1mM (glucose or RDX). Results are the average of quadruplicate samples.
4.6 Acknowledgements

Clemson Ph.D. student Jolanta Niedźwiecka provided assistance with developing the HPLC method used to quantify nitroguanidine. This publication was developed under STAR Fellowship Assistance Agreement no. FP-917130 awarded by the U.S. Environmental Protection Agency (EPA). It has not been formally reviewed by EPA. The views expressed in this publication, are solely those of the authors, and EPA does not endorse any products or commercial services mentioned in this publication.

4.7 References


CHAPTER 5: CONCLUSIONS AND FUTURE RESEARCH

5.1 Summary of Work

RDX can be reduced while absorbed to GAC granules, without the need of an initial desorption step. It is reduced using reduced electron shuttle AQDS (model for naturally-occurring humic substances and an electron acceptor for iron-reducing bacteria) as a chemical electron donor, using iron reducing bacterium *Geobacter metallireducens* strain GS-15 as a biological electron source, or using a combination of the two. Each experimental system was capable of ≥ 97.0% RDX transformation. Systems using the electron shuttle as an electron donor produce formaldehyde (daughter product) rapidly, and extent of formaldehyde production correlated well with equivalent amount of electron donor added. Systems containing electron shuttle (the chemical system and the mixed chemical-biological system) transformed RDX more rapidly than systems containing only bacteria. All systems were able to transform adsorbed RDX within 90 hours.

This is the first study to demonstrate successful biological transformation of adsorbed RDX without pre-desorption. GS-15 was selected because of its previous demonstrated ability to transform RDX and the electron shuttle AQDS in aqueous solution, and because other members of the *Geobacter* genus have been shown to reduce organic compounds adsorbed to activated carbon granules. Following transformation with GS-15, GAC could be re-used for removal of RDX from water, with ≥ 95.0% of its initial capacity to adsorb RDX, which is similar to recoveries reported for thermal regeneration techniques. These data suggest the masses of GAC waste currently produced by activated carbon at RDX sites can be minimized.

Photosynthetic studies demonstrate that *Rhodobacter sphaeroides* can biologically transform RDX and novel insensitive munitions (IM) 2,4-dinitroanisole and nitroguanidine, which will replace the explosive TNT in many next generation explosives. *R. sphaeroides* degraded RDX and the IMs within 72 hours under light conditions. Photosynthetic electron transfer was identified as the degradation mechanism. Additional experiments with RDX showed that succinate and malate were the most effective reducing equivalents for photosynthetic reactions;
however, biodiesel-derived waste glycerol was also utilized as an electron donor. RDX was transformed irrespective of the presence of carbon dioxide. Electron shuttling compounds increase degradation kinetics in the absence of CO₂. In conditions where CO₂ was present (and growth possible), the electron shuttling compound had no stimulatory effect. End products indicated that much of the RDX carbon became CO₂ and biomass, but a fraction became a soluble aqueous metabolite, characterized using ¹⁴C-labeled RDX. End products for 2,4-dinitroanisole and nitroguanidine were consistent with reduction metabolites present in the literature.

The presence of high explosive RDX did not inhibit photosynthetic growth of *R. sphaeroides*, and growing cultures were able to consistently degrade spikes of RDX over 480 hours. These data are the first to suggest that photobiological RDX and insensitive munitions degradation is possible, and are the first steps in developing a practical framework for which photobiology could be utilized in remediation.

### 5.2 Areas for Future Research

Our studies focused exclusively upon pure culture systems with *Geobacter metallireducens* strain GS-15 (activated carbon) and *Rhodobacter sphaeroides* ATCC® 17023™ (photobiology). These cultures were chosen deliberately, as they seemed the most likely candidates for success. In addition, both of these cultures have been studied extensively at the genetic level, which was helpful in identifying potential cellular mechanisms responsible for the phenomena we observed.

Using well-understood bacterial cultures and controlled environments removed many variables associated with cellular growth and metabolism, allowing us to focus upon biologically mediated explosives transformation, our goal. However, not all of our observations have been definitively explained. To do so would have extended beyond the scope of this project and available equipment. Some of these questions (such as identifying the aqueous metabolite *Rhodobacter sphaeroides* produces from RDX) are straightforward and in progress. Others are more in-depth, requiring longer studies and are suggested as topics for future research. Examples of questions to address in future studies include:
Do activated carbon granules accept electrons via quinolic surface functional groups, and then act as electron shuttles themselves, facilitating sorbed RDX reduction? Previous reports are inconsistent regarding the role of quinolic functional groups in electron transfer through carbonaceous materials. Recently, quinolic surface groups were determined to not influence in sorbed RDX transformations using sulfides (Xu et al., 2013), but this was linked to a nucleophilic reaction, not reduction. In our study, it seems likely that surface quinolic groups would play a role, as our experiments used both AQDS (quinone) and Geobacter metallireducens (a well-known reducer of quinones). We attempted to test this by reducing activated carbon granules with H₂ and heat prior to use (this would specifically reduce quinolic groups), but our results were inconclusive. A more mechanistic study is required. This could be studied concurrently with the next research question, which is…

Do physiological attributes of GS-15 allow the bacterium to reduce RDX and activated carbon granules? Certain species of Geobacter are capable of extracellular electron transfer via conductive pili, referred to as microbial nanowires (Reguera et al., 2005). Are these pili facilitating i) direct reduction of sorbed RDX molecules that may be otherwise inaccessible to cells, or ii) reduction of the activated carbon granules, similar to what has been reported with graphite electrodes in fuel cells (Reguera et al., 2006)? In our studies, we observed that while both GS-15 and 17023 can reduce RDX in aqueous solution, only GS-15 can transform adsorbed RDX (all sorbed RDX was recovered via extraction after a 72-hour suspension containing 17023, but RDX was degraded in controls that did not contain GAC).

Does GS-15 oxidize formaldehyde to formate? This has not been previously reported, as discussed in Chapter 2. Geobacter species are capable of facilitating many redox reactions. As formaldehyde is a regulated compound, this would be an additional attribute to a group of bacteria already significant in bioremediation.

To what extent does the presence of more favorable electron acceptors inhibit photobiological reduction of contaminants? Other reducible compounds may divert electrons from the reduction of explosives, potentially preventing contaminant transformation. A screening experiment was
conducted to see if RDX transformation would be inhibited using nitrate, nitrite, DMSO, and fumarate (data presented in Figure D.1 and D.2, Appendix D). RDX decreases were observed, despite the presence of alternate electron acceptor. This indicates that either the alternate electron acceptors were not accepting electrons from the photosystem (therefore not affecting RDX degradation), or that both RDX and acceptor were receiving electrons. After re-examining our experimental setup, we believe this is due to excess electrons already present in the highly concentrated biomass within each experimental tube (ATCC® literature confirms this may occur). This would have allowed for electron flow to both the alternate acceptor and to RDX. A different experimental approach is required to study this further. Serial dilutions of biomass simultaneous monitoring of reductant concentrations may be a good way to start.

Can the photobiological degradation of explosives observed in this work be replicated in continuous flow systems? This work explored photosynthetic bacteria as a potential ex situ remedial approach, but experiments were limited to batch reactors. It is reasonable to assume that a field reactor would be a continuous-flow system. Unfortunately, we were not able to set up a continuous-flow system for this work, but it is in progress (discussed in Chapter 4), likely to be operated by a current MS student at Clemson University. Establishment of a successful, continuous-flow photobioreactor is imperative for eventual field application and acceptance as a treatment strategy.

Can other forms of photobiology be utilized in a similar manner (in lieu of Rhodobacter sphaeroides)? Rhodobacter sphaeroides was the only phototroph we examined. It was selected because it i) certain strains are triazine-tolerant (Brown et al., 1988), which is important because triazines are toxic/inhibitory to many bacteria, ii) is capable of cytochrome-based reduction reactions similar to those expected for RDX, and iii) contains genetic material for nitroreductase activity (Liu et al., 2008). However, it requires an external electron source (we used cysteine and malate) and operates photosynthetically under anoxic conditions exclusively. From a field/implementation perspective, cyanobacteria may be appealing, because they operate under oxic conditions and use energy from water instead of an external source. Exploring cyanobacteria and/or other forms of photobiology for application in explosives remediation would be interesting, but would also constitute an entirely separate study. As our photobiology
work had little literature precedent, choosing a bacterial strain with a high likelihood for success was the simplest and most pragmatic way to explore photobiological as a potential remedial strategy.

5.3 Potential Applications

As scientists, we are trained in numbers and objectivity; all statements should be factually supported. One of the difficulties I have had with this project is that most explosives-contamination in the United States is on land owned by the Department of Defense, and the DoD often restricts the publication of material related to military firing ranges. Much of my practical knowledge comes from discussions with Army Corps Engineers and not peer-reviewed literature. Other scientists frequently ask me to quantify the amount of money spent on explosives-spent activated carbon disposal, as a way to justify the utility of my project. I do not believe this information is available to the general public. However, conversations with field engineers assure me that my work is pragmatic and ultimately desirable to the remediation industry.

‘Cradle to grave’ is an expression frequently cited in remediation, and it seems only appropriate that this project was conducted with that mentality. Although conducted at bench-scale using small model ‘reactors’, both the GAC and photobiology work were conducted with the vision of eventual field application, and the objectives of this project were designed to address some of the concerns that field engineers may have. Pilot-scale implementation of these technologies is beyond the scope of a single Ph.D. project. The intention is to patent the ideas described, in hopes that industry will recognize the value and push forward towards implementation. A joint patent between Clemson University and the University of Illinois, Urbana-Champaign has already been filed for the activated carbon work, and one remediation firm has already expressed interest in purchasing the intellectual property. The photobiological studies have not been published yet, but the hope is that it too will be tested at larger scale.

5.4 References


**Table A.1** RDX percentage recovered after sorption and desorption in water versus ethanol extraction at 30 °C and 36-38 °C

<table>
<thead>
<tr>
<th>#</th>
<th>Desorbant</th>
<th>RDX (%) recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Not sorbed (remaining in solution)</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>98.10 ± 0.10</td>
</tr>
<tr>
<td>2</td>
<td>Ethanol</td>
<td>2.24 ± 1.56</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>89.61 ± 1.73</td>
</tr>
<tr>
<td>4</td>
<td>Ethanol</td>
<td>1.51 ± 0.07</td>
</tr>
<tr>
<td>5</td>
<td>5.55 L/g Water</td>
<td>1.14 ± 0.39</td>
</tr>
<tr>
<td>6</td>
<td>55.5 L/g Water</td>
<td>1.19 ± 0.46</td>
</tr>
<tr>
<td>7</td>
<td>555.5 L/g Water</td>
<td>0.70 ± 0.13</td>
</tr>
</tbody>
</table>

Series 1 and 3 (in **bold**) represent control experiments conducted in the absence of GAC. Series 1 and 2 were conducted at 30 °C; all others were maintained at 36-38 °C. “L g⁻¹” units represent liters of desorbing (ethanol or water) per gram GAC.

**Objective:** The objective was to demonstrate that RDX transformation techniques that depend upon water as the sole solvent for desorption are not reasonable for field applications. To do this, we used different volumes of water and elevated temperatures versus 100% ethanol, which is a known solvent for RDX desorption.

**Methods:** Each of the seven series within Table A.1 was conducted in triplicate and utilized AquaCarb 1230C high activity, 12x30 mesh, virgin coconut shell granular activated carbon. Prior to use, granules were rinsed with DI water to remove any fines, baked overnight at 60 °C, and weighed on a precision microbalance. Experimental vials were prepared as follows: Activated carbon (0.0115 ± 0.0005g) was added to bottles containing 30mL of 30mM ethanol.
bicarbonate buffer at pH 7.0. Bottles were degassed with anoxic N_2:CO_2 (80:20 [vol/vol]), sealed under the same headspace, and autoclaved. Controls (Series 1 and 3) were treated identically except contained no activated carbon. Bottles were amended with 40µM RDX and were placed in an incubator (150 rpm, 30 °C) for preloading. RDX was preloaded onto carbon granules at 23mg g\(^{-1}\), consistent with experiments presented in the main text. The different temperatures are the result of the experiment having been run at different laboratories, which at the time(s) it happened were beyond our immediate control.

Preloading took 350 hours. RDX was then desorbed as follows:

- **Series 1** was maintained at 30 °C for 5 days (120 hrs).
- **Series 2** was decanted, and the buffer in the vials was replaced with 60mL of ethanol. This was returned to the 30 °C incubator for 5 days.
- **Series 3** was maintained at 37 °C for 23 days.
- **Series 4** was maintained at 37 °C for 18 days. After 18 days, the buffer was decanted, and the buffer in the vials was replaced with 60mL of ethanol. This was returned to the 37 °C incubator for an additional 5 days.
- **Series 5** was decanted, and the buffer in the vials was replaced with 5.55 L g\(^{-1}\) of DI water. This was maintained at 37 °C for 18 days. After 18 days, the DI water was decanted, and the buffer in the vials was replaced with 60mL of ethanol. This was returned to the 37 °C incubator for an additional 5 days.
- **Series 6** was decanted, and the buffer in the vials was replaced with 55.5 L g\(^{-1}\) of DI water. This was maintained at 37 °C for 18 days. After 18 days, the DI water was decanted, and the buffer in the vials was replaced with 60mL of ethanol. This was returned to the 37 °C incubator for an additional 5 days.
- **Series 7** was decanted, and the buffer in the vials was replaced with 555.5 L g\(^{-1}\) of DI water. This was maintained at 37 °C for 18 days. After 18 days, the DI water was
decanted, and the buffer in the vials was replaced with 60mL of ethanol. This was returned to the 37 °C incubator for an additional 5 days.

Each series was continually agitated at 150rpm during desorption. Liquid aliquots were taken immediately after amendments, before decanting, and periodically during desorption phases. These were analyzed for RDX as described in the main text (HPLC-UV).

Results and Discussion: RDX mass recovered in Series 1 and 2 (maintained at 30 °C) both exceeded 95%. Mass recoveries for the five series maintained at 37 °C were lower: 80.21 to 92.03%. Low RDX recoveries from Series 3 (89.61%, no GAC) and Series 4 (92.03%, ethanol as desorbant), as compared to Series 1 and 2, suggested hydrolysis, the result of prolonged incubation (23 days) at the elevated temperature. Hydrolysis of RDX has been reported at higher temperatures but was not expected at 37 °C; as a result, we did not monitor for hydrolysis products.

Less than 3.3% of RDX mass was recovered at 18 days using DI water as a desorbant at 37 °C, consistent with reports of limited or no desorption in soils,(Douglas et al., 2011; Hatzinger et al., 2004) Series 5 and 6 (5.55 and 55.5 liters of water per gram of activated carbon) both reached equilibrium within 15 days; daily measurements of aqueous-phase RDX between Day 16-18 remained constant. In contrast, the 555.5L g⁻¹ series (Series 7) did not come to equilibrium, likely due to the shaking platform used in these studies being insufficient at completely mixing GAC in such large volumes of water. RDX recovery using 555.5L g⁻¹ was still marginal at days 17 and 18, just above HPLC detection limits and is likely why the amount desorbed using 555.55 L g⁻¹ DI water was less than 55.5 L g⁻¹ DI water.

These data indicate that, to recover even small amounts without organic solvents, large volumes of water, high temperatures, and long contact times with mixing are required for water mediated-desorption. This is undesirable for field applications and demonstrates the need for sorbed RDX treatment. It also suggests that adsorbed RDX was directly reduced by either cells or AH₂QDS, as the volumes of water in each experimental series were not sufficient to desorb any significant mass of RDX.
Table A.2 Capacity for GAC to re-adsorb RDX following the different treatments discussed in text. All RDX removal values indicated below indicate the capacity for GAC that has been treated with AH₂QDS to continue adsorbing RDX at least one additional time

<table>
<thead>
<tr>
<th>Series</th>
<th>Pretreatment</th>
<th>RDX Removal in 350 hours</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No pretreatment (virgin carbon)</td>
<td>99.22%</td>
<td>0.12%</td>
</tr>
<tr>
<td>2</td>
<td>Ethanol extraction (120 hrs)</td>
<td>95.89%</td>
<td>3.98%</td>
</tr>
<tr>
<td>3</td>
<td>Hydroquinone (210 hrs)</td>
<td>95.88%</td>
<td>0.77%</td>
</tr>
<tr>
<td>4</td>
<td>Hydroquinone (90 hrs), followed by ethanol extraction (120 hrs)</td>
<td>97.08%</td>
<td>1.28%</td>
</tr>
<tr>
<td>5</td>
<td>RDX (350 hrs), followed by ethanol extraction (120 hrs)</td>
<td>98.49%</td>
<td>0.31%</td>
</tr>
<tr>
<td>6</td>
<td>RDX (350 hrs), followed by hydroquinone (90 hrs), followed by ethanol extraction (120 hrs)</td>
<td>97.54%</td>
<td>0.78%</td>
</tr>
</tbody>
</table>

Objective: The objective was to identify whether the treatment approaches discussed in the main text would affect the reuse potential of GAC for RDX adsorption.

Methods: This experiment was conducted identically to the experimental conditions presented in the main text, with additional RDX amended at a second, later time point. Each of the triplicates described above utilized AquaCarb 1230C high activity, 12x30 mesh, virgin coconut shell granular activated carbon. Prior to use, granules were rinsed with DI water to remove any fines, baked overnight at 60 °C, and weighed on a precision microbalance. Experimental vials were prepared as follows: Activated carbon (0.0115 ± 0.0005g) was added to bottles containing 30mL of 30mM bicarbonate buffer at pH 7.0. Bottles were degassed with anoxic N₂:CO₂ (80:20 [vol/vol]), sealed under the same headspace, and autoclaved. Vials were mixed at 150rpm and were maintained at 30 °C for the duration of the experiment.

Pretreatment took 560 hours. Specific series were pretreated as follows:

- **Series 1** was left in the incubator unamended for 560 hrs.
- **Series 2** was left in the incubator unamended for 440 hrs. At 440 hrs, the buffer in the vials was replaced with 60mL of ethanol for the remaining 120 hrs.
• **Series 3** was left in the incubator unamended for 350 hrs. At 350 hrs, AH$_2$QDS was amended to a final concentration of 150µM for the remaining 210 hrs.

• **Series 4** was left in the incubator unamended for 350 hrs. At 350 hrs, AH$_2$QDS was amended to a final concentration of 150µM for 90 hrs. At 440 hrs, the buffer in the vials was replaced with 60mL of ethanol for the remaining 120 hrs.

• **Series 5** was left in the incubator unamended for 90 hrs. At 90 hrs, RDX was amended to a final concentration of 40µM and allowed to adsorb to carbon granules for 350 hours; the final mass loading on granules was approximately 23mg RDX per g GAC (mg g$^{-1}$), same as experiments presented in the main text. At 440 hrs, the buffer + any residual RDX in the vials was replaced with 60mL of ethanol for the remaining 120 hrs.

• **Series 6** was amended immediately with RDX (final concentration = 40µM) and allowed to adsorb to carbon granules for 350 hours; the final mass loading on granules was approximately 23mg RDX per g GAC (mg g$^{-1}$). At 350 hrs, AH$_2$QDS was amended to a final concentration of 150µM for 90 hrs. At 440 hrs, buffer in vials was replaced with 60mL of ethanol for the remaining 120 hrs. Series 6 was designed to mimic the experimental conditions presented in the text.

The aqueous phase for all six series was decanted following pretreatment. Vials + GAC were placed in a separate 60 °C oven to bake for three days to ensure complete removal of ethanol residual. Vials were re-amended with 30mL of 30mM bicarbonate buffer at pH 7.0, degassed with anoxic N$_2$:CO$_2$ (80:20 [vol/vol]), sealed under the same headspace, and left in the incubator (150 rpm, 30 °C) overnight before they were tested for residual ethanol. None was detected. All series were then amended with 40µM RDX and returned to the incubator for an additional 350 hours.

*Results and Discussion:* Series 1 demonstrated 99.22% RDX removal following pretreatment. This is similar to what was obtained under experimental conditions in the main text and indicates that the 560 hours in the incubator, followed by decantation and 3 days at 60 °C did not affect GAC capacity for RDX adsorption. This series served as a positive control.
Series 6 (designed to mimic the experimental conditions presented in the text) was able to remove 97.54% of RDX in solution after 350 hours. Reduced removal efficiency of Series 6 (97.54% removal, 90 hrs with hydroquinone) as compared to Series 5 (98.49%, no hydroquinone) suggests competition for adsorption sites between the hydroquinone and RDX. AH$_2$QDS may be either competing directly for adsorption sites, or (as a larger, more bulkier molecule) blocking access to such sites. This is supported by Series 3, which allowed for 210 hours of contact between AH$_2$QDS and GAC. This series had the lowest percent removal (95.88%) of RDX following pretreatment. Additional studies are needed to fully understand the effects of competitive adsorption that may occur between AH$_2$QDS and RDX.

Removal efficiency of Series 6 (97.54% removal, 350 hrs with RDX) as compared to Series 4 (97.08%, no RDX) suggests RDX itself does not appear to affect reuse potential, provided it is desorbed prior to re-adsorption. The lower removal efficiency of Series 4 as compared with Series 6 was unexpected but is minimal.

Both series that had 350 hours of pretreatment with RDX also had the ethanol extraction step. It is likely that this extraction step may be affecting GAC capacity more than pre-adsorption with RDX; the ethanol may be altering surface functional groups. We were not able to run a series pretreated with RDX but without ethanol extraction; for safety reasons, we kept the RDX:GAC m/m ratio below 3% (10% is the EPA explosive limit). Series 2 shows that ethanol alone reduces the capacity of GAC for RDX adsorption (amount removed was 95.89%), but the standard deviation in this series (3.98%, much greater than for any of the other series studied) suggests that experimental error (one of the triplicates had a much lower recovery than the other two) may have lowered this value. If that replicate is removed the mean is near the other bottles.

Secondary RDX removal exceeded 95% for all of the experimental bottles tested. This is much higher than thermal regeneration processes, which typically reduce GAC capacity by 10-20%. Although Series 2-6 had slightly reduced RDX adsorption, each still had a relatively high capacity for RDX adsorption following our treatment approaches. These data indicate that the techniques discussed could be incorporated into current GAC treatment systems and that GAC reuse following RDX adsorption is viable. However, our reuse test was a single time RDX re-
application, and prior to any potential field studies this test would need to be run for several subsequent RDX re-amendments.
Figure A.1 Screening experiment to assess $^{14}$C-RDX desorption over time in 100% ethanol. Units are gram GAC mass per liter of ethanol. The dashed line at the top represents maximum theoretical recovery of $^{14}$C-RDX. Experiments were conducted in single vials.

**Objective:** The objective was to identify an ethanol-to-GAC ratio that would result in high recovery of adsorbed RDX to assist in mass balances.

**Methods:** Different masses (0.00, 0.18, 0.25, 0.54, 0.93, 1.88, and 2.90 g L$^{-1}$) of AquaCarb® carbon were added to vials containing U-$[^{14}\text{C}]$-RDX (final radioactivity of 40,000 dpm mL$^{-1}$) in pH = 7.0 nanopure water, sealed, and continuously agitated at ambient temperature. Aqueous dpm counts were taken at ~20 hour intervals. At 101 hours, the bulk liquid was decanted, and the carbon was added to ethanol. Liquid aliquots of ethanol were taken daily and analyzed for radioactivity (as described in the main text) until equilibrium was established (aqueous phase radioactivity stabilized). Glassware and the butyl stoppers used to seal bottles were analyzed for residual radioactivity; none was detected.
Results and Discussion: Sorption profiles for different GAC masses amended with a fixed amount of U-[\(^{14}\)C]-RDX became uniformly asymptotic after 40 hours. The percent of initial radioactivity remaining in the aqueous phase after 101 hours of contact with activated carbon was 14.0\% \pm 2.3 for all GAC masses, higher than previously reported.\(^{105}\) (Morley et al., 2005; Morley et al., 2006) This was the result of residual acetone that was in the \(^{14}\)C-stock. We were not aware our \(^{14}\)C stock contained acetone until we used it this first time and generated this irregular-looking sorption curve and then realized it was due to the residual acetone. These sorption curve data were discarded, as stocks containing percent levels of acetone will inevitably affect sorption. But, this experiment did leave us with \(^{14}\)C-RDX adsorbed to activated carbon, and we knew the precise masses adsorbed. We did not want to waste the pre-adsorbed \(^{14}\)C-RDX (\(^{14}\)C-RDX is extremely costly). We instead decanted the aqueous phase and made sure any residual acetone was removed and used the adsorbed \(^{14}\)C-RDX for the extractions shown in Figure A.1. These are the only data generated using the \(^{14}\)C-RDX stock that initially contained acetone; all subsequent experiments utilized HPLC-purified \(^{14}\)C-RDX.

Final mass loadings on activated carbon granules were (mg g\(^{-1}\)): 23.1 (0.18 g GAC), 16.7 (0.25 g GAC), 7.7 (0.54 g GAC), 4.4 (0.93 g GAC), 2.2 (1.88 g GAC), and 1.4 (2.90 g GAC). Figure S1 demonstrated that \(^{14}\)C-RDX in ethanol stabilized after approximately one day for all GAC-to-ethanol ratios; vials were sampled for an additional week without additional changes. Granules preloaded with 23.1 mg g\(^{-1}\) had the highest recovery of radiolabeled material at 98.0\%; lower recovery rates from other ratios were operationally defined as unsatisfactory for use in further experiments because of the lower RDX recoveries following ethanol amendment.

For subsequent experiments that required radiolabeled RDX, we filtered the \(^{14}\)C-RDX stock via HPLC and checked purity prior to use to ensure that <99.0\% of \(^{14}\)C present was RDX, and that no residual acetone was present.
Figure A.2  RDX adsorption curve using AquaCarb GAC under typical experimental conditions (30mM bicarbonate buffer, 30°C, 0.18g GAC per liter of buffer) (A), and recovery with ethanol (B).  1.00 in the lower plot (B) represents maximum theoretical RDX recovery.  The results are the means of triplicate analyses, and the bars indicate one standard deviation.

Objective:  RDX transformation has been reported during desorption, predominantly at elevated pH and temperature.(Heilmann et al., 1996) To ensure that the recovered aqueous radioactivity
obtained in Figure S1 was RDX and not transformation products, the experiment was replicated in triplicate for radiolabeled and non-radiolabeled RDX and known transformation products.

Methods: This study was conducted as close as possible to the experimental conditions presented in the main text.

Each of the series described above was conducted in triplicate and utilized AquaCarb 1230C high activity, 12x30 mesh, virgin coconut shell granular activated carbon. Prior to use, granules were rinsed with DI water to remove any fines, baked overnight at 60 °C, and weighed on a precision microbalance. Activated carbon (0.0115 ± 0.0005g) was added to bottles containing 30mL of 30mM bicarbonate buffer at pH 7.0. Bottles were degassed with anoxic N₂-CO₂ (80:20 [vol/vol]), sealed under the same headspace, and autoclaved.

GAC was amended with 0.0478µmol acetone-free U-[¹⁴C]-RDX (radioactivity of 14,000 dpm mL⁻¹) plus non-radiolabeled RDX for a final mass loading of 23mg g⁻¹, 4% as ¹⁴C-RDX. RDX was allowed to adsorb. Vials were mixed at 150rpm and were maintained at 30 °C for the duration of the experiment. Liquid aliquots were periodically taken and analyzed for both total radioactivity and RDX until equilibrium was established. ‘Equilibrium’ for all GAC experiments was operationally defined as three consecutive sampling points over three days where the total change in aqueous RDX was less than 0.05% of the initial mass.

After equilibrium was established, the buffer in the vials was replaced with 60mL of ethanol. This was returned to the 30 °C incubator for an additional 5 days. Liquid aliquots were taken daily and analyzed for both total radioactivity and RDX, as described in the main text.

Results and Discussion: First order rate constants for sorption are 0.0126 hr⁻¹ (total radioactivity) and 0.0139 hr⁻¹ (RDX); RDX adsorption/desorption curves are provided in Figure A.2. No intermediates or hydrolysis products were detected. The mass recoveries for total radioactivity and RDX (97.7% ± 4.6 and 96.0% ± 3.2, respectively) were similar. The desorption time frame in these screening tests are consistent with the literature while RDX recovery is increased, 96.0% instead of 92.8%. (Morley et al., 2006) This was attributed to the elevated desorption temperature (30 °C instead of ambient) and the different activated carbon type (Morley et. al used AquaCarb 830, 8 x 30 mesh).
**Figure A.3** AH$_2$QDS adsorption curve using AquaCarb GAC under typical experimental conditions (30mM bicarbonate buffer, 30°C, 0.18 g GAC per liter of buffer) at various mass loadings. The results are the means of triplicate analyses; bars indicate one standard deviation.

**Objective:** At pH 7 and in clear buffer, solutions containing AQDS are colored (AQDS is a pale yellow; AH$_2$QDS is a bright color). We visually observed a color change in our GAC experiments in controls containing activated carbon and AH$_2$QDS that was unrelated to RDX transformation. We postulated that this color change was likely due to either i) AH$_2$QDS adsorbing to activated carbon granules, or ii) AH$_2$QDS donating electrons to activated carbon granules. The objective was to identify which (or both) may be occurring.

Batch experiments were conducted as described in GAC Studies and amended with quinone in lieu of RDX to test the potential of AQDS to adsorb to activated carbon granules or to transfer electrons to GAC. Experiments were conducted with both AQDS and reduced AH$_2$QDS. We also reduced the surfaces of activated carbon with hydrogen gas to determine if this affected electron transfer through activated carbon granules.
*Methods:* Sorption curves using different concentrations of quinone (30, 75, 100, 250, and 500µM) were generated. Additionally, a 500µM AQDS (no GAC) series served as a control. Each series was conducted in triplicate.

Activated carbon experiments utilized AquaCarb 1230C high activity, 12x30 mesh, virgin coconut shell granular activated carbon. Prior to use, granules were rinsed with DI water to remove any fines, baked overnight at 60 °C, and weighed on a precision microbalance. Activated carbon (0.0115 ± 0.0005g) was added to bottles containing 30mL of 30mM bicarbonate buffer at pH 7.0. Bottles were degassed (see below) and sealed under the same headspace prior to amendment with quinone. Vials were mixed at 150rpm and were maintained at 30 °C for the duration of the experiment.

Three experiments were conducted. They were:

- **Series 1:** Experimental vials were degassed with anoxic N₂:CO₂ (80:20 [vol/vol]), and quinone was amended as AQDS.

- **Series 2:** Experimental vials were degassed with anoxic N₂:CO₂ (80:20 [vol/vol]), and quinone was amended as AH₂QDS.

- **Series 3:** Experimental vials were degassed with anoxic H₂:CO₂ (80:20 [vol/vol]) for 60 minutes, and quinone was amended as AH₂QDS.

Both total quinone (AQDS + AH₂QDS) and reduced quinone (AH₂QDS alone) were measured. Total quinone was measured spectrophotometrically under oxic conditions at 225nm. AH₂QDS was measured using an electron shuttling assay. (Ye et al., 2011) Briefly, liquid samples containing AH₂QDS were mixed with ferric citrate under anoxic conditions. This resulted in the production of ferrous iron, 2 moles of Fe²⁺ for every mole of AH₂QDS. The AH₂QDS/ferric citrate mixture was added to the organic ligand ferrozine, which turns purple in the presence of Fe²⁺. This purple product is measured spectrophotometrically at 562nm.
Results and Discussion: Figure A.3 demonstrates sorption curves of different amounts of AH$_2$QDS, degassed with N$_2$:CO$_2$. Values in the figure are total quinone (AQDS), but loss data for reduced quinone (AH$_2$QDS) are identical, indicating that all quinone in aqueous solution is present as AH$_2$QDS. There were no differences for solutions amended with AQDS versus AH$_2$QDS (data not shown), suggesting that AQDS adsorbs independent of quinone protonation.

We degassed vials with H$_2$:CO$_2$ instead of N$_2$:CO$_2$ in an attempt to reduce functional groups (believing that modified functional groups may adsorb AH$_2$QDS differently). Still no statistically different data was obtained - sorption curves again looked identical, and no AQDS was detected in the aqueous phase (data not shown). This indicated that AQDS adsorbs independent of the presence of hydrogen gas, and that electron transfer to GAC (if occurring) cannot be monitored by aqueous measurements of AQDS/AH$_2$QDS. It is possible that only a fraction of the reducible functional groups on GAC are reduced by sparged molecular H$_2$. These initial data suggest that adsorption was the dominant mechanism relative to electron transfer, but admittedly this experiment was merely a baseline characterization of a complex series of processes.

At this point it was determined that a more in-depth analysis of our activated carbon system was required, and the amount of work this would entail would constitute an entire study in itself. Because of this, we did not run any experiments testing the biological potential to transfer electrons through activated carbon. We believe that understanding electron transfer specifically by GAC is beyond the scope of this study and will be readdressed in future work. Additional studies are in progress for a better understanding of how reduced quinones mediate RDX transformation in the presence of activated carbon, but data with cells + GAC (without AQDS) indicated that less RDX was transformed. This at least suggests that the electron shuttling molecules are important to RDX transformation, and likely have the capacity to act as a redox mediator.
**Figure A.4** RDX degradation routes by mixed abiotic–biological pathways based on products identified and reported degradation pathways (references listed on figure) in the presence of AQDS and *G. metallireducens*.

Compounds in square brackets were not determined; [C] represents unidentified carbon intermediates. $A = $ abiotic pathway(s), $B = $ biological pathway(s). The $<, >, $ and $\approx$ indicate whether the products were more significant in the abiotic or biological pathway (based on the mass balance).


Reproduced with permission of authors.

**References**


Figure B.1 Relative growth of *Rhodobacter sphaeroides* in modified SMM media amended with RDX and the donors listed in legend. This figure was generated in conjunction with Figure 3, reported in the main text. For our experimental conditions, we operationally defined the culture as ‘fully grown’ at OD$_{680}$ 1.8. ‘No donor’ represents a triplicate series where RDX is the sole carbon source added to the media. ‘RDX control’ does not contain cells. The final carbon concentration for each donor is 20mM carbon. Values shown are averages of quadruplicates, and the error bars represent one standard deviation.
Figure B.2 Radioactivity recovered via HPLC effluent trapping using a Supelcosil LC-CN column (4.6 x 250mm). Injection volumes were 100μL. This figure was generated in conjunction with Figure 5 of the main text; the ‘Control’ column of Figure 5 is not reported here. The x-axis represents trapping intervals, listed as time (min) after sample injection. HPLC effluent was captured manually in scintillation vials containing 10mL of scintillation cocktail and
was analyzed on the scintillation counter following capture. Error bars on the figures represent one standard deviation between triplicates. $^{14}$C recovery (%) was calculated as the sum of the net dpm collected for each triplicate over 15 minutes, divided by the total dpm of 100uL sample injected directly into cocktail. This is reported in the top right of each frame as the average of the triplicates, plus/minus one standard deviation.
Figure B.3 $^{14}$C radioactivity elution windows for radiolabeled bicarbonate and RDX stocks. Radioactivity was recovered over a 10-minute window via HPLC effluent trapping using a Supelcosil LC-CN column (4.6 x 250mm). The x-axis represents the start time of each capture interval, listed as time (min) after sample injection. HPLC effluent was captured manually in scintillation vials containing 10mL of scintillation cocktail and was analyzed on the scintillation
counter following capture. $^{14}$C recovery (%) was calculated as the sum of the net dpm collected over the interval stated in the panel, divided by the total dpm collected over the 10-minute capture interval.

**Discussion of Figures B.2 and B.3**: Our objective with using the $^{14}$C sample trapping technique was to identify the elution time of the unknown compound(s). Since no corresponding metabolite peak has been observed on HPLC chromatograms, we concluded that this compound cannot be detected with UV$_{254}$ or was present at levels too low for UV$_{254}$ detection. By identifying the elution time of this compound, we hoped to draw conclusions that would assist with identifying it. The capture window plots presented in B.3 are for direct comparison against the sample capture window plots presented in B.2, since samples contain RDX and bicarbonate, in addition to the unknown metabolite.

Elution windows were designed as follows:

- 0 to 3 minutes: Prior to water injection
- 3 to 4 minutes: Water injection
- 4 to 6 minutes: Retained metabolite that is not RDX or nitroso
- 6 to 7.7 minutes: Nitroso metabolites (MNX, TNX, DNX)
- 7.7 to 9.1 minutes: RDX
- Windows following 9.1 minutes: Unknown

Most of the sample radioactivity (after accounting for residual RDX and bicarbonate species) for the unknown product eluted within the first three minutes. This indicated that the compound could not be retained on the LC-CN compound. Bicarbonate elution corresponded with water injections on the HPLC, which elute between 3.4 and 3.8 minutes. Bicarbonate did not account for all the radioactivity recovered in samples between 3-4 minutes; metabolites partially eluted within this window too. RDX and MNX were identified in the experimental series containing cells and AQDS, which is likely why radioactivity in these samples was recovered between 6 to 9.1 minutes.
The early elution window indicates that the products formed from photobiodegradation of RDX are likely small, miscible low-weight organic compounds. Elution prior to the injection peak was unexpected, and may indicate that this product is an alcohol, which would travel through this column faster than water. If an alcohol, the most likely candidate is methanol. These samples were tested for methanol, but none was observed above detection levels (which were 30uM for our FID). Also, *Rhodobacter sphaeroides* readily transforms methanol into CO$_2$ under photosynthetic conditions.

We attempted to replicate the $^{14}$C sample trapping experiments with a second column, designed to retain organic acids. The objective with this study was to further gain insight on this metabolite’s identity. However, RDX eluted after 187 minutes on the organic acid column. No pulse of radioactivity was detected in this window, just a slow trickle over time. Results overall were too dilute for accurate measurements (scintillation vials could hold a max window of 4 minutes, and radioactivity detection, even when present, were often at or just above background levels).
Figure B.4 Production of MNX in suspensions containing resting *R. sphaeroides* and RDX under strictly CO$_2$-free conditions (top) and autotrophic conditions (bottom). This figure was generated in conjunction with Figure 4 of the main text. Error bars represent one standard deviation between triplicates. MNX was the only metabolite detected in significant quantity. MNX, MEDINA, NDAB, and formaldehyde were detected at the final time point but cumulatively composed less than 5% of the initial carbon amended as RDX. Methanol and formate were not detected.
Figure B.5 Nitrogen-limiting, $^{14}$C mass balance after 72 hours with resting *R. sphaeroides* under strictly CO$_2$-free conditions (top) and autotrophic conditions (bottom). This experiment was conducted at pH 7.0 and contained either 10mM PO$_4^{3-}$ buffer, degassed with 100% He (CO$_2$-free, top) or 10mM HCO$_3^-$ buffer, degassed with He/CO$_2$ (CO$_2$, bottom). This experiment is analogous to that of Figure 5 of the main text, except that helium was used in lieu of nitrogen gas. Error bars represent one standard deviation between triplicates.
Figure B.6  Production of ammonium in suspensions containing resting *R. sphaeroides* and RDX under strictly CO2-free conditions (top) and autotrophic conditions (bottom). This figure was generated in conjunction with Figure 4 of the main text. Ammonium was measured colorimetrically at 650nm using a previously described method (Rhine et al., 1998). Ammonium levels measured were super-stoichiometric; controls indicated that ammonium production was linked to nitrogen fixation regardless of the presence of RDX. Error bars represent one standard deviation between triplicates.
Figure B.7 Production of nitrite in suspensions containing resting *R. sphaeroides* and RDX in bicarbonate buffer. This figure was generated in conjunction with the bottom panel of Figure 4 of the main text. Nitrite was measured via ion chromatography using a previously described method (See Chapter 2). Nitrite produced in bicarbonate was the only nitrogen-containing compound generated in significant quantity that could be correlated with RDX degradation. Nitrate (same method, data not shown) detected in bicarbonate buffer composed less than 1% of the initial nitrogen amended as RDX. Monitoring for nitrite or nitrate under strictly CO$_2$-free conditions could not be conducted because of interference of the phosphate buffer. Error bars represent one standard deviation between triplicates.

References

APPENDIX C: ANAEROBIC, REDUCTIVE TRANSFORMATION OF NITROGUANIDINE (NQ) USING IRON(II) SPECIES

C.1 Introduction

One of several proposed insensitive munitions (IM), nitroguanidine (CH₄N₄O₂, NQ) differs from many classical explosives because it is aliphatic, has relatively high water solubility (2.6 ± 0.1 g/L) and is more persistent (Haag et al., 1990). NQ has historically been utilized as an oxidizer in triple base propellants (Jenkins et al., 2006), but it is now being integrated into novel explosive formulas such as IMX 101, IMX 104, and PAX 21, developed by the defense industry (Taylor et al., 2013; Walsh et al., 2013). At this time, nitroguanidine is not a significant environmental concern at military firing ranges, but its projected increased use, combined with its documented leaching from IM crystals (Taylor et al., 2013), suggest that release into the environment is likely (Mulherin et al., 2005).

Nitroguanidine is generally resistant to degradation, but it does occur. Williams et al. observed biotransformation of NQ within biologically-active, aerobic water columns (Williams et al., 1989). NQ has also been reported to degrade in surface waters, in the presence of UV light or aerobic bacteria (Haag et al., 1990). Only one pure culture capable of NQ degradation has been identified. Aerobic *Variovorax* strain VC1 is capable of NQ mineralization; it was isolated in soils that originated from a military live fire training range (Perreault et al., 2012).

Anaerobic transformation of NQ is not widely reported. The only published study demonstrating biodegradation under anoxic conditions is from 1982 (Kaplan et al., 1982), and it shows reduction of NQ to unstable nitrosoguanidine within activated sludge. Two additional studies attempted to study NQ reduction under anoxic conditions via soil microcosms but were unsuccessful, as NQ concentrations did not decrease in a reasonable amount of time (Haag et al., 1990; Perreault et al., 2012). However, its relatively high solubility and low octanol-water partitioning coefficient suggest that NQ has the potential to leach through surface soils and contaminate underlying groundwater aquifers (Mulherin et al., 2005), which would likely be anoxic. Identification of an in situ transformation mechanism for nitroguanidine is needed.
Reduction of NQ is not as thermodynamically favorable as other explosives (Uchimiya et al., 2010), but reduced iron may be a viable reductant for nitroguanidine transformation. Cited as personal communication, zero valent iron (ZVI) has been linked to NQ reduction (Perreault et al., 2012). Fe/Cu bimetallic particles also reduce NQ under acidic conditions (Koutsospyros et al., 2012). While ZVI is not naturally abundant and bimetallic particles are synthetic, natural forms of iron minerals have been shown to reduce other explosives (Larese-Casanova et al., 2008; Oh et al., 2008). Ferrous iron specifically has been demonstrated to transform the high explosive RDX, but reactive forms of Fe(II) are complexed (example groups include hydroxyl groups on mineral surfaces and dissolved catechols) and is not dissolved Fe$^{2+}$ (Gregory et al., 2004; Kim et al., 2007). These complexed forms of iron(II) are stronger reductants than dissolved Fe$^{2+}$ alone and can catalyze reactions that the latter cannot.

Our work investigated NQ reduction using dissolved Fe$^{2+}$, ferrous iron complexed with the organic ligand 2,3,4-trihydroxybenzoic acid (2,3,4-THBA), and anthrahydroquinone-2,6-disulfonate (AH$_2$QDS, a model compound representing naturally-occurring humic substances) as reductants, and we report successful abiotic reduction of NQ using Fe(II). Batch experiments were conducted to characterize reactions between NQ and reductant. Specific goals of the study were to i) determine if dissolved Fe$^{2+}$, ligand-bound Fe(II), and AH$_2$QDS were capable of reducing NQ, ii) characterize the kinetics of these reactions, and iii) determine how reaction rates and extent of reaction are influenced by pH and ligand/reductant concentrations.

C.2 Methods and Materials

**Chemicals.** Nitroguanidine, FeCl$_2$ • 4H$_2$O, MES buffer, HEPES buffer, CHES buffer, sodium bicarbonate, EDTA (ethylenediaminetetraacetic acid), AQDS (anthraquinone 2,6-disulfonate), hydroxylamine hydrochloride, iron(II) ethylenediammonium sulfate tetrahydrate, and 2,3,4-THBA (2,3,4-trihydroxybenzoic acid) were obtained from Sigma-Aldrich. HCl and NaOH were purchased from Fisher Scientific. RDX was obtained as previously described (Chapter 2 and 3).
Experimental Setup. Experiments were conducted as batch experiments inside an anoxic glovebox (95% N₂, 5% H₂, Coy Laboratory Products) at room temperature (21-23°C). Palladium catalysts were baked prior to the start of each experiment and changed weekly to maintain anoxic conditions. A beaker of DDI water (pH 10) was placed inside the glovebox and pH tested daily. A decrease in its pH would indicate the presence of carbon dioxide, but none was observed. All glassware was rinsed with 0.5N HCl and allowed to equilibrate overnight prior to use. Solid-phase FeCl₂ • 4H₂O was stored permanently inside the glove box to prevent oxidation. Solutions were prepared with deionized water (18MΩ-cm resistivity) that was boil-degassed and sparged with ultrahigh purity nitrogen for 1 hour before transfer to the glove box, as previously described (Kim et al., 2007). All ligand, NQ, and iron amendments were conducted in the glove box. Experiments were conducted in 50mL borosilicate glass bottles sealed with thick blue, butyl stoppers. Bottles and solutions were stored in covered boxes when not in use to prevent light-mediated reactions.

AH₂QDS Screening Experiment. Experiments in 30mM buffered solutions of HEPES (pH 7.0 and 8.0) CHES (pH 9.0) and bicarbonate (pH 7.0, 8.0, and 9.0) tested the potential of nitroguanidine transformation using anthraquinone 2,6-disulfonate (AH₂QDS) as a reductant. Bicarbonate bottles were sparged with N₂:CO₂ (=80:20 for pH 7.0, =99:1:0.1 for pH 8.0, and =100:0:0 for pH 9.0) and sealed prior to transfer to glove box. A chemically-reduced stock of 30mM AH₂QDS was prepared as described (Ye et al., 2011) and amended to a concentration of 500uM. Nitroguanidine was amended to 100uM; RDX (positive control for quinone-mediated reduction) was amended to 40uM. Series were conducted in triplicate.

Iron-Ligand Experiments. Experiments buffered in organic solutions of 30mM HEPES tested the potential of nitroguanidine reduction at different pH values using Fe(II) and THBA-Fe(II) complexes as reductants. For each pH, solutions of HEPES and HEPES amended with 4mM THBA were prepared. pH values were adjusted to pH 7.5 and 8.5 and allowed to equilibrate overnight before diluted to appropriate THBA concentrations in vials. A concentrated stock of FeCl₂ • 4H₂O in 0.5N HCl was amended into bottles to a final concentration of 1mM; this was allowed to equilibrate with buffer and ligands (if present) overnight prior to amendment with
nitroguanidine to a final concentration of 100uM NQ. Series were conducted in quadruplicate; the fourth vial of each series was not amended with NQ.

*Iron Experiment, pH 8.0.* HEPES was prepared at pH 8.2. A concentrated stock of FeCl$_2$ • 4H$_2$O in 0.5N HCl was amended into bottles to appropriate concentrations, and the pH for each was adjusted with 0.5N HCl to pH 8.0 ± 0.05. Bottles were prepared without addition of NaOH, minimizing Fe(II)-hydroxide formation. Bottles equilibrated overnight, and the pH was confirmed again prior to amendment to a final concentration of 100uM NQ. Series were conducted in quadruplicate; the fourth vial of each series was not amended with NQ.

*Iron Analysis.* Iron sampling was conducted using a modified version of the Ferrozine assay (Stookey, 1970) and a previously described method (Boparai et al., 2010). Aqueous samples were collected via syringe in the glove box, filtered, and diluted 1:3 (Fe < 2mM) or 1:10 (Fe ≥ 2mM) in 0.5mM HCl and mixed via vortex. This sample:acid mix was diluted 1:50 in Ferrozine solution and measured at 562nm; these readings were operationally defined as dissolved Fe(II). Additional aqueous samples were collected via syringe in the glove box (these were not filtered), diluted as before in HCl, mixed via vortex, and removed from the glove box. An aliquot of this sample:acid mix was diluted 1:50 in Ferrozine and measured at 562nm; these readings were operationally defined as total Fe(II). The remaining sample:acid mix was amended with 4% 6.25N hydroxylamine HCl, mixed via vortex, and stored overnight at room temperature in the dark. The following day, this was diluted 1:50 in Ferrozine and measured at 562nm; these readings were operationally defined as total Fe. Iron concentrations were quantified using known standards in acid. Reported values for solid ferrous iron is the difference between total Fe(II) and dissolved Fe(II); reported values for ferric iron is the difference between total Fe and total Fe(II) divided by 0.96 (to account for hydroxylamine dilution).

*Analytical (HPLC).* Aqueous samples were collected using an anoxic syringe and needle and filtered prior to analysis. Nitroguanidine was analyzed using high performance liquid chromatography (HPLC; Dionex UltiMate 3000) using an Acclaim Polar Advantage II column (Dionex, 4.6 x 250mm, 5µm) and ultrapure water (1.0mL min$^{-1}$) as the mobile phase; nitroguanidine eluted at 10.1 minutes and was quantified at 254nm. NQ was quantified using
standards prepared in DDI. The unknown peak eluted at approximately 7.1 minutes; this unknown is reported in PA units.

C.3 Results and Discussion

The results presented below are incomplete. These data will be combined with figures generated by Jolanta Niedźwiecka of Clemson University to formulate conclusions on NQ transformation. Her work is still in progress and is not included, but key discussion points are below the figures.

AH$_2$QDS Screening Experiment. No statistically significant transformation of NQ was observed in any treatment containing buffer, NQ, and reduced anthrahydraquinone-2,6-disulphonate (Table C.1). Two buffer types (organic and inorganic) were tested for each pH; AQDS rates of reduction for some contaminants (data not shown) vary between buffers. RDX was used as a positive control, and RDX was completely transformed in each series within 24 hours (data shown is for 7 days), indicating functioning electron donor. A slight decrease in NQ in CHES buffer at pH 9.0 with AH$_2$QDS was observed but was not considered significant, given the experimental length. AH$_2$QDS is effective at reducing other explosives (Bhushan et al., 2006; Borch et al., 2005; Kwon et al., 2010) but is not effective at transforming NQ.

Iron-Ligand Experiments. Partial NQ degradation was observed over 30 days but was incomplete.

Iron Experiments, pH 8.0. Fe(II) was able to transform NQ at pH 8.0. Transformation rates and extent of transformation were proportional to the amount of Fe(II) initially amended. Due to the long experimental time frame, solids formed in bottles, even though they were sealed and maintained in the glove box. This is believed to have been unavoidable, given the experimental length and elevated pH. Solids are mixed valence, but the exact identity of solids is unknown. Suspensions that exhibited the greatest and most rapid amount of NQ degradation had the highest amount of these solids present. Transformation of NQ may be accelerated when Fe(II)/Fe(III) solids are available for use as a catalytic surface. A similar trend was observed with RDX(Boparai et al., 2010).
Transformation products of NQ reduction. Identifying transformation products was not a specific goal of this study, as most metabolites are either unknown or extremely unstable in aqueous solution (Haag et al., 1990; Kaplan et al., 1982; Perreault et al., 2012; Williams et al., 1989). However, chromatograms for batch experiments that showed a decrease in nitroguanidine concentrations showed the concurrent formation of an unknown peak eluting at 7.1 minutes at 254 and 264nm. We suspect this is nitrosoguanidine. Formation of nitrosoguanidine has a literature precedent (Kaplan et al., 1982) under anoxic conditions and corresponds with the amount of Fe(III) formed by our systems. Nitrosoguanidine is not available commercially; we were not able to purchase standards to verify the identity of this peak. However, nitrosoguanidine typically elutes 2-3 minutes earlier than nitroguanidine on reverse-phase C18 columns, consistent with what we are observing. Nitrosoguanidine is unstable in water and decays to varying forms of urea, which cannot be detected via HPLC at UV$_{254}$. This may explain why the peak we have observed decays over time. This same peak was observed in suspensions conducted with *Rhodobacter sphaeroides* (Chapter 4).
Table C.1 Observed removal (C/C₀) of Nitroguanidine (NQ) in incubations with anthrahydroquinone 2,6-disulfonate (AH₂QDS) after 7, 15, and 30 days. RDX served as a positive control for reduction.

<table>
<thead>
<tr>
<th>Buffer  (30mM)</th>
<th>Munition  (100uM NQ, 40uM RDX)</th>
<th>pH</th>
<th>Shuttle  (500uM)</th>
<th>Type</th>
<th>7 days C/C₀</th>
<th>St. Dev.</th>
<th>15 days C/C₀</th>
<th>St. Dev.</th>
<th>30 days C/C₀</th>
<th>St. Dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>NQ</td>
<td>7.0</td>
<td>Hydroquinone</td>
<td>Expt</td>
<td>100.4%</td>
<td>4.0%</td>
<td>100.7%</td>
<td>4.8%</td>
<td>96.0%</td>
<td>1.7%</td>
</tr>
<tr>
<td>HEPES</td>
<td>NQ</td>
<td>7.0</td>
<td>Quinone</td>
<td>Control (-)</td>
<td>103.7%</td>
<td>8.4%</td>
<td>104.3%</td>
<td>9.5%</td>
<td>93.5%</td>
<td>6.0%</td>
</tr>
<tr>
<td>HEPES</td>
<td>RDX</td>
<td>7.0</td>
<td>Hydroquinone</td>
<td>Control (+)</td>
<td>0.0%</td>
<td>0.0%</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>NQ</td>
<td>7.0</td>
<td>Hydroquinone</td>
<td>Expt</td>
<td>106.5%</td>
<td>5.0%</td>
<td>107.2%</td>
<td>4.6%</td>
<td>105.2%</td>
<td>3.7%</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>NQ</td>
<td>7.0</td>
<td>Quinone</td>
<td>Control (-)</td>
<td>100.3%</td>
<td>4.5%</td>
<td>105.6%</td>
<td>5.5%</td>
<td>105.0%</td>
<td>5.5%</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>RDX</td>
<td>7.0</td>
<td>Hydroquinone</td>
<td>Control (+)</td>
<td>0.0%</td>
<td>0.0%</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>HEPES</td>
<td>NQ</td>
<td>8.0</td>
<td>Hydroquinone</td>
<td>Expt</td>
<td>101.2%</td>
<td>3.9%</td>
<td>101.1%</td>
<td>4.2%</td>
<td>92.8%</td>
<td>1.2%</td>
</tr>
<tr>
<td>HEPES</td>
<td>NQ</td>
<td>8.0</td>
<td>Quinone</td>
<td>Control (-)</td>
<td>98.6%</td>
<td>2.2%</td>
<td>106.9%</td>
<td>4.4%</td>
<td>93.6%</td>
<td>0.5%</td>
</tr>
<tr>
<td>HEPES</td>
<td>RDX</td>
<td>8.0</td>
<td>Hydroquinone</td>
<td>Control (+)</td>
<td>0.0%</td>
<td>0.0%</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>NQ</td>
<td>8.0</td>
<td>Hydroquinone</td>
<td>Expt</td>
<td>95.4%</td>
<td>1.8%</td>
<td>95.5%</td>
<td>2.4%</td>
<td>92.2%</td>
<td>3.7%</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>NQ</td>
<td>8.0</td>
<td>Quinone</td>
<td>Control (-)</td>
<td>96.6%</td>
<td>1.5%</td>
<td>99.3%</td>
<td>2.7%</td>
<td>97.1%</td>
<td>1.8%</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>RDX</td>
<td>8.0</td>
<td>Hydroquinone</td>
<td>Control (+)</td>
<td>0.0%</td>
<td>0.0%</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CHES</td>
<td>NQ</td>
<td>9.0</td>
<td>Hydroquinone</td>
<td>Expt</td>
<td>96.8%</td>
<td>1.2%</td>
<td>91.3%</td>
<td>0.5%</td>
<td>87.3%</td>
<td>0.3%</td>
</tr>
<tr>
<td>CHES</td>
<td>NQ</td>
<td>9.0</td>
<td>Quinone</td>
<td>Control (-)</td>
<td>95.8%</td>
<td>1.8%</td>
<td>92.6%</td>
<td>0.6%</td>
<td>88.7%</td>
<td>1.9%</td>
</tr>
<tr>
<td>CHES</td>
<td>RDX</td>
<td>9.0</td>
<td>Hydroquinone</td>
<td>Control (+)</td>
<td>0.0%</td>
<td>0.0%</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>NQ</td>
<td>9.0</td>
<td>Hydroquinone</td>
<td>Expt</td>
<td>93.3%</td>
<td>1.6%</td>
<td>88.9%</td>
<td>2.7%</td>
<td>85.9%</td>
<td>2.7%</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>NQ</td>
<td>9.0</td>
<td>Quinone</td>
<td>Control (-)</td>
<td>104.1%</td>
<td>5.7%</td>
<td>102.0%</td>
<td>5.9%</td>
<td>97.3%</td>
<td>1.1%</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>RDX</td>
<td>9.0</td>
<td>Hydroquinone</td>
<td>Control (+)</td>
<td>0.0%</td>
<td>0.0%</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
**Figure C.1** Reduction of nitroguanidine at pH 7.3 (A) and 8.0 (B). Filled symbols represent THBA concentrations that have completely complexed Fe(II). Error bars represent one standard deviation.

Trends observed:
- NQ decreased in all series containing NQ, iron, and THBA
• No change in NQ was observed at pH 7.3 in the series containing iron, NQ, but no THBA
• In contrast, a change in NQ was observed at pH 8.0 in the series containing iron, NQ, but no THBA
• For either pH, no difference was observed in series containing 0.5, 0.75, and 1mM THBA
• The greatest extent of degradation occurred in the series at pH 8.0 with 4mM THBA. In this series, NQ degradation was almost complete.
• These results suggest that NQ reduction by complexed Fe(II) may be effective especially at higher pH values.
Figure C.2 Iron concentrations measured at each THBA concentration for pH 7.3 (A) and 8.0 (B). Error bars represent one standard deviation.

Trends Observed:

- A decrease in iron was observed at pH 8.0 in the series containing iron, NQ, but no THBA.
- Fe(II) measurements in samples containing 2,3,4-THBA did not change therefore we cannot draw any conclusions about iron(II) transformation in these samples. The presence of 2,3,4-THBA affects the Ferrozine assay, preventing accurate iron(II) measurements (confirmed with additional testing).
Figure C.3  Peak areas of unknown soluble metabolite eluting on HPLC at 7.1 minutes for pH 7.3 (A) and 8.0 (B).  Error bars represent one standard deviation.

Trends Observed

• A peak appeared in HPLC chromatograms, eluting at 7.1 minutes. It was not stable and decreased over time.

• The peak did not change in the series at pH = 7.3 that did not contain THBA. This is the same series that showed no NQ degradation.
Figure C.4 Initial and final pH values for Fe-ligand experiments starting at pH 7.3 (A) and 8.0 (B). Error bars represent one standard deviation. Controls represent single bottle experiments that did not contain nitroguanidine.

Trends Observed

- No significant change or trends in pH was observed at pH 7.3
- In most series, the pH at 8.0 dropped over time. This occurred in vials with and without NQ.
Figure C.5 Reduction of nitroguanidine at pH 8.0 using Fe$^{2+}$. Filled symbols represent series where nitroguanidine was completely transformed. Error bars represent one standard deviation.

Trends Observed

- No change in NQ was observed in the absence of Fe(II)
- NQ was partially degraded in series containing 0.5, 0.75, and 1mM Fe. Extent of degradation was proportional to the amount of Fe(II) added.
- NQ was completely degraded in series containing 2, 3, 4, and 5mM Fe. Results fit first order decay. Reaction rates increase with increasing amounts of Fe.
Figure C.6  Iron speciation at 0, 5, 10, and 30 days in series with initial Fe\(^{2+}\) concentrations of 0.25 (A), 0.50 (B), 0.75 (C) and 1.0mM (D). In these series, nitroguanididine reduction was incomplete. Time points marked with an asterisk (*) represent iron speciation in single bottle controls that do not contain nitroguanididine. Error bars represent one standard deviation in total iron measured.

Trends Observed

- Formation of some Fe(III) and Fe(II) solids; in general, these increased over time.
- For each series, Fe(III) formation was greatest after 30 days in vials containing NQ
Figure C.7  Iron speciation at 0, 5, 10, and 30 days in series with initial Fe$^{2+}$ concentrations of 2.0 (A), 3.0 (B), 4.0 (C) and 5.0mM (D). In these series, nitroguanidine reduction was complete. Time points marked with an asterisk (*) represent iron speciation in single bottle controls that do not contain nitroguanidine. Error bars represent one standard deviation in total iron measured.

Trends Observed

• Formation of considerable Fe(III) and Fe(II) solids. These increased over time.
• Formation of Fe(II) and Fe(III) solids was greatest in bottles containing NQ. Considerably fewer solids of either species were formed in
• For each series, Fe(III) formation was greatest after 30 days in vials containing NQ
Figure C.8  Peak areas of unknown soluble metabolite eluting on HPLC at 7.1 minutes for series where nitroguanidine was incomplete (A) and complete (B). Error bars represent one standard deviation.

Trends Observed

- The unknown peak appeared in all samples
- Peak areas were much bigger for 2-5mM Fe(II) concentrations than for the lower concentrations
- Peak areas incrementally increased for amendments containing 0-1mM Fe(II)
- Peak areas increased and then decreased for series containing 2-5mM Fe(II), consistent with the belief that this peak may be nitrosoguanidine
Figure C.9 Initial and final pH values for Fe$^{2+}$ experiments. Error bars represent one standard deviation. Controls represent single bottle experiments that did not contain nitroguanidine.

Trends Observed

- pH dropped in all samples over time
- The pH drop was greatest in samples containing NQ; samples that did not contain NQ did not drop as much in pH
- Even though all samples started at approximately the same pH, the pH decrease was inversely proportional to the amount of Fe(II) amended
C.4 Acknowledgements

Clemson Ph.D. student Jolanta Niedźwiecka provided assistance with developing the HPLC method used to quantify nitroguanidine. She ran concurrent NQ-ligand experiments, generating figures that showed a strong pH-dependence on degradation kinetics (data not presented here). This publication was developed under STAR Fellowship Assistance Agreement no. FP-917130 awarded by the U.S. Environmental Protection Agency (EPA). It has not been formally reviewed by EPA. The views expressed in this publication, are solely those of the authors, and EPA does not endorse any products or commercial services mentioned in this publication.

C.5 References


Figure D.1  RDX concentrations at 0, 24, 36, and 48 hours when suspended with resting cells of *Rhodobacter sphaeroides* and alternate electron acceptors nitrate (top) and nitrite (bottom). Acceptor concentrations were 5mM. Patterned bars represent incubations with biomass; solid bars are abiotic controls. Error bars represent one standard deviation.
Figure D.2 RDX concentrations at 0, 24, 36, and 48 hours when suspended with resting cells of *Rhodobacter sphaeroides* and alternate electron acceptors fumarate (top) and DMSO (bottom). Acceptor concentrations were 1mM. Patterned bars represent incubations with biomass; solid bars are abiotic controls. Error bars represent one standard deviation.


**Figure E.1** Sample HPLC standard curves for RDX and MNX using LC-CN column at 254nm. Units for standards is uM.
Figure E.2 Sample HPLC standard curves for DNAN and MENA using C18 column at 296nm. Units for standards is uM.
Figure E.3 Sample HPLC standard curves for nitroguanidine (NQ, top) and atrazine (bottom). NQ quantification was conducted using an Acclaim Polar Advantage II column at 254nm; atrazine quantification was conducted using a C18 column at 222nm. Units for standards is uM.
Figure E.4  Sample HPLC standard curves for organic acids NDAB (top) and MEDINA (bottom) using ION-ICE column at 210nm. Units for standards is uM.
Figure E.5  Sample HPLC standard curve for formaldehyde using a C18 column at 360nm. Formaldehyde is derivitized with 2,4-dinitrophenylhydrazine (DNPH) for 1 hour prior to analysis. This process generates trace amounts of formaldehyde in standards, which is detected in blanks at a peak area of between 6-9. For this reason, formaldehyde standards do not intercept the origin. Units for standards is uM.

\[ y = 1.7597x - 7.5174 \]

\[ R^2 = 0.99988 \]

**Formaldehyde Standards, after derivization with DNPH**

**HPLC Peak Area**
Figure E.6 Sample ion chromatography (IC) standard curves for nitrite (top) and nitrate (bottom) using Dionex AS-14A column and a 43mA current. Units for standards is uM.
Figure E.7 Sample standard curves for AH₂QDS (top), ammonia (middle), and Fe(II) (bottom). These compounds were measured colorimetrically as described in text and analyzed spectrophotometrically at 450, 650, and 562nm respectively. Units for standards is μM.
Figure F.1 Molecular structure of RDX (Hexahydro-1,3,5-trinitro-1,3,5-triazine).
Figure F.2 Molecular structure of 2,4-dinitroanisole, nitroguanidine, and atrazine.
APPENDIX G: PERMISSION (ACS) TO REPRINT PUBLISHED MATERIALS

Title: Electron Shuttle-Mediated Biotransformation of Hexahydro-1,3,5-trinitro-1,3,5-triazine Adsorbed to Granular Activated Carbon
Author: Kayleigh Millerick, Scott R. Drew, and Kevin T. Finneran
Publication: Environmental Science & Technology
Publisher: American Chemical Society
Date: Aug 1, 2013
Copyright © 2013, American Chemical Society

PERMISSION/LICENSE IS GRANTED FOR YOUR ORDER AT NO CHARGE

This type of permission/license, instead of the standard Terms & Conditions, is sent to you because no fee is being charged for your order. Please note the following:

- Permission is granted for your request in both print and electronic formats, and translations.
- If figures and/or tables were requested, they may be adapted or used in part.
- Please print this page for your records and send a copy of it to your publisher/graduate school.
- Appropriate credit for the requested material should be given as follows: "Reprinted (adapted) with permission from (COMPLETE REFERENCE CITATION). Copyright (YEAR) American Chemical Society." Insert appropriate information in place of the capitalized words.
- One-time permission is granted only for the use specified in your request. No additional uses are granted (such as derivative works or other editions). For any other uses, please submit a new request.