8-2015

# BIODEGRADATION OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE (RDX) USING PHOTOSYNTHETIC BACTERIA

Sarah Galloway
*Clemson University*, shgallo@g.clemson.edu

# BIODEGRADATION OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE (RDX) USING PHOTOSYNTHETIC BACTERIA

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A Thesis
Presented to
the Graduate School of
Clemson University

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

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by
Sarah Galloway
August 2015

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Accepted by:
Dr. Kevin Finneran, Chair
Dr. Cindy Lee
Dr. David Ladner

## ABSTRACT

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is an emerging contaminant according to the Environmental Protection Agency (EPA). RDX was introduced as a secondary explosive during World War II. It is still used in many explosive such as hand grenades.

RDX enters the environment mostly through the manufacturing process or from the use of explosives. RDX is a suspected carcinogen and can also affect the nervous system of humans. Therefore, RDX has become a chemical of concern across many United States military bases and open and closed manufacturing plants.

The goal of this research was to evaluate the biodegradation of RDX via two phototrophic bacteria: *Rhodobacter sphaeroides* ATCC® 17023™ and *Synechocystis* sp. PCC 6803. The ability to degrade RDX via a phototrophic bacteria could make remediation more passive. A passive remediation option could be an easier and more cost effective way to remediate RDX. Biodegradation of RDX has been successful with other bacteria, but *Synechocystis* sp. PCC 6803 was studied because it is robust and grows well in aerobic environments.

The specific objectives for this research were to:

1. Determine if electron acceptors nitrate (1 mM), sulfate (10 mM), and perchlorate (1 mM) influence *R. sphaeroides'* ability to degrade RDX under ideal conditions (growth conditions with succinate as electron donor).
2. Determine if *R. sphaeroides* can degrade RDX with oxygen present.
3. Determine if *Synechocystis* can degrade RDX and if so, under what conditions.

The work conducted showed that:

1. Adding electron acceptors to incubations with *R. sphaeroides,* electron donor, and RDX while in the presence of light did not significantly change the time required to degrade RDX. After 49 hours RDX was degraded 100% in samples with no electron acceptor and with perchlorate, 99% in samples with sulfate, and 94% in samples with nitrate.
2. Exposing *R. sphaeroides* to air drastically inhibited the degradation of RDX. After 19 days, 40% of RDX still remained in the samples. The same samples in anaerobic conditions degraded RDX in only 49 hours.
3. BG-11 media degraded RDX significantly under a cool-white fluorescent light.
4. *Synechocystis's* growth was affected when BG-11 nutrient stock concentration was degraded.
5. *Synechocystis* completely degraded RDX with and without an electron shuttle and in anaerobic environments and in the presence of air.
6. *Synechocystis* reduced RDX more efficiently when placed in incubations with growth media as opposed to being placed in incubations with HEPES buffer.

RDX degradation via a bacteria, *Synechocystis,* in aerobic conditions has not been published. The work conducted showed that RDX can be degraded by *Synechocystis* and *R. sphaeroides* (in the presence of air and electron acceptors). However, more research needs to be conducted. Reduction of RDX by *R. sphaeroides* in field conditions needs to be examined. Also, the mechanisms of *Synechocystis* that degrade RDX need to be further studied.

DEDICATION

To my parents, Gene and Ernie, for their never ending love and support.

I would not be where or who I am today without their encouragement.

ACKNOWLEDGMENTS

Thank you to my advisor, Kevin Finneran, for giving me the opportunity to work on this project. With his help, I have learned a great deal about working in a laboratory and biological processes over the past two years.

I would also like to extend a thank you to Jolanta Niedźwiecka for all the invaluable lessons she taught me in the lab. My other lab mates (Jovan Popovic, Amanda Kunkle, Paul Vecchiarelli, Courtney Thompson, Nicholas Hotzelt and Alexander Rogier) also helped me in many ways and I am very grateful for all their guidance and advice. Kay Millerick provided the foundation for my research, and I am grateful for all she taught me. Finally, I am very thankful for Anne Cummings. She was always willing to help and to teach me about the lab equipment I needed to use.

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LIST OF ABBREVIATIONS

| | |
|---|---|
| DOD | U.S. Department of Defense |
| DNX | hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine |
| GAC | Granular Activated Carbon |
| HCHO | formaldehyde |
| HCOOH | formic acid |
| HPLC | high performance liquid chromatography |
| MDNA | methylene dinitramine |
| MNX | hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine |
| NDAB | 4-nitro- 2,4-diazabutanal |
| $NH_3$ | ammonia |
| $NO_2^-$ | nitrite |
| $N_20$ | nitrous oxide |
| Polytetrafluoroethylene | PTFE |
| RDX | Hexahydro-1,3,5-trinitro-1,3,5-triazine |
| *R. sphaeroides* | *Rhodobacter sphaeroides* (strain ATCC® 17023™) |
| *Synechocystis* | *Synechocystis* sp. PCC 6803 |
| TNX | hexahydro- 1,3,5-trinitroso-1,3,5-triazine |

# CHAPTER ONE: INTRODUCTION AND OBJECTIVES

## 1.1 Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a secondary explosive used by the U.S. Department of Defense (DOD). A secondary explosive is a booster explosion that ignites the main bursting charge (EPA, 2014). Production and use of RDX began during World War II (Adam et al., 2006) and is still extensively used by DOD (EPA, 2014). The United States produced approximately 15,200,000 kg per month of RDX during World War II (Urbanski, 1967). Holston Army Ammunition Plant in Kingsport, Tennessee, is the only remaining manufacturer of RDX in the US (EPA, 2014). RDX is found in 4,000 DOD munitions including small ignitors to large bombs (US Department of Defense, 2011). Although RDX is mostly used in DOD applications, RDX is also used and found in industrial activities. RDX can be used for underwater blasting, mining, and as a precursor of synthetic leather and dyestuff (Bhosale, 2015).

In the early 1980s RDX groundwater contamination was first reported near munition manufacturing plants in Nebraska (Spalding and Fulton, 1988). RDX enters the environment through four main pathways: (1) training activities, (2) loading and assembly, (3) manufacturing, and (4) maintenance and demilitarization (Brannon and Pennington, 2002). Manufacturing and training activities are the most prevalent pathways in which RDX enters the environment. During the early years of RDX manufacturing, the majority of soil and groundwater contamination stemmed from waste (water or material) disposal in unlined ditches and lagoons (EPA, 2014). Although disposal of RDX manufacturing waste does not go into unlined ditches and lagoons in

modern manufacturing plants, RDX can still find a way into the environment during the manufacturing process via wastewater effluent (Bhosale, 2015). During DOD training activities fractions of RDX are not consumed during the explosion, thereby depositing RDX residue on the soil surface (Kuperman et al. 2013). RDX can contaminate the subsurface by percolating down into the unsaturated zone and then may dissolve into the groundwater.

RDX contamination is found at many DOD sites throughout the US. Approximately 15 million acres of land is contaminated with explosive material with the most common explosive material being RDX. (Kuperman et al., 2013).

RDX is a white crystal that is a solid at ambient temperature and has a fairly low water solubility of 59.7 mg/L (EPA, 2014 and Kuperman et al., 2013). It also has a low soil sorption coefficient (Log $K_{oc}$ = 1.8), but the amount of RDX that is retained in soils is very dependent on the composition of the soil (EPA, 2014).

RDX is a suspected human carcinogen with a lifetime advisory exposure in drinking water of 2 µg/L (Kwon et al., 2008). When exposed to large amounts of RDX, human and animal nervous systems are affected (EPA, 2014). RDX is also known to cause liver damage in humans and can have adverse affects on aquatic environments (Bhosale, 2015). Possible human exposure routes to RDX are dermal and inhalation (EPA, 2014).

RDX degradation can be biotic or abiotic and can follow two pathways (Paquet et al., 2011). The most common pathway is a nitro to nitroso degradation (reduction of nitro groups) pathway (Kwon et al., 2008). RDX can transform into hexahydro-1-nitroso-3,5-

dinitro-1,3,5-triazine (MNX) then into hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) and then into hexahydro- 1,3,5-trinitroso-1,3,5-triazine (TNX) (Zhao et al., 2004) (Figure 1.1).

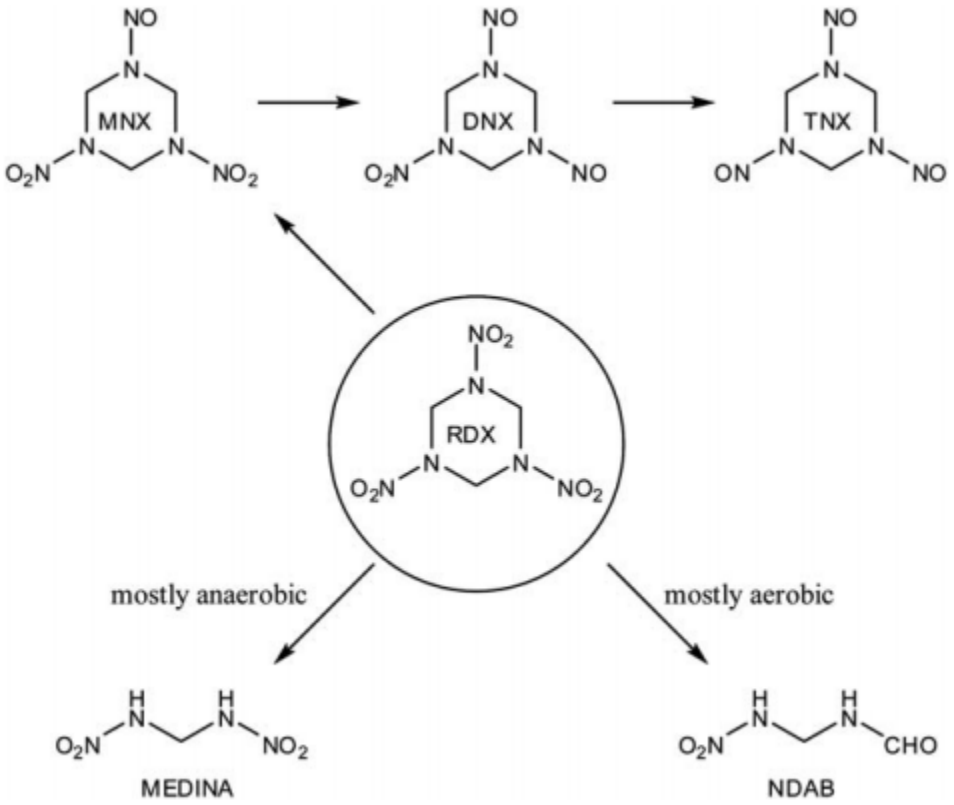


**Figure 1.1** - RDX Transformation Products

The other pathway RDX can follow when it degrades is ring cleavage of the N-$NO_2$ bonds. The main products of this cleavage is methylene dinitramine (MDNA) and 4-nitro- 2,4-diazabutanal (NDAB) (Figure 1.1). MDNA and NDAB are not naturally occurring chemicals, and therefore, are evidence that RDX contamination (past or present) has occured. MDNA has never been detected in field samples because it is

unstable in water. NDAB has only been found in soil samples at a handful of sites. Ring cleavage of the N-$NO_2$ bonds eventually produce products such as formaldehyde (HCHO), nitrous oxide ($N_20$) nitrite ($NO_2^-$), ammonia ($NH_3$) and formic acid (HCOOH) (Paquet et al., 2011).

Once RDX dissolves in groundwater it becomes mobile which can lead to significant groundwater contamination. For example, RDX contaminated 6.5 $km^2$ of groundwater near Cornhusker Army Ammunition Plant in Nebraska (Spalding and Fulton, 1988). RDX has a low vapor pressure ($1.0 \times 10^{-9}$ mm Hg) resulting in RDX remaining in the solid or dissolved phase (EPA, 2014). Because of the chemical properties described above, the vadose zone tends to be a source area of RDX and is a constant source for groundwater contamination (Adam et al., 2006).

In recent years, research to remediate RDX from the unsaturated zone and groundwater has made substantial progress. Some of the current remedial approaches include (1) in situ chemical oxidation (Albano et al., 2010), (2) ozone treatment (Adam et al., 2006), (3) electron shuttle-stimulated RDX biodegradation (Kwon and Finneran, 2010), (4) adsorption by granular activated carbon (GAC) (EPA, 2014) and (5) photosynthetic biodegradation (Millerick et al., 2015).

The main purpose of this document is to discuss biodegradation of RDX via two phototrophic bacteria, *Rhodobacter sphaeroides* (strain ATCC® $17023^{TM}$) and *Synechocystis* sp. PCC 6803. The ability to degrade RDX via a phototrophic bacteria could make remediation more passive. A passive remediation option could be an easier and more cost effective way to remediate RDX.

*Rhodobacter sphaeroides* (strain ATCC® 17023™) (*R. sphaeroides)* is a purple phototrophic proteobacteria and can grow via fermentation, photosynthesis, aerobic respiration or anaerobic respiration (Mackenzie et al., 2007). ATCC® owns and maintains *R. sphaeroides*. *R. sphaeroides* can live in a variety of environments because of its wide range of growth mechanisms (Mackenzie et al., 2007). For this this thesis, *R. sphaeroides* was grown anaerobically under a tungsten lamp (details in 2.1.2). Under anaerobic conditions, *R. sphaeroides* gains chemical energy by photosynthesis (McEwan, 1994). *R. sphaeroides* has been studied extensively for production of biofuel (Fang et al., 2005) and has been found to degrade aromatic compounds containing nitrogen (Millerick et al., 2015)

*Synechocystis* sp. PCC 6803 (*Synechocystis)* is a cyanobacteria and grows using oxygenic photosynthesis, similar to a plant (Knoop et al., 2010). *Synechocystis* is a very tough bacteria and can grow in a variety of carbon dioxide levels, temperatures, pH levels, and UV light exposure times. *Synechocystis* has the capability to grow both phototrophically and hetetrophically (Anderson et al., 1991). *Synechocystis* grows phototrophically by utilizing $CO_2$ and photons (Yu et al., 2013) and hetetrophically by utilizing 5 to 15 minutes of light and a carbon source (commonly gloucose) (Plohnke et al., 2015). *Synechocystis* is the center of extensive research in the field of biofuels, because it is an easily renewable source of biomass and it has a high lipid content (Kim et al., 2011).

**1.2 Objectives**

The goal of this research was to evaluate biodegradation of RDX via two bacteria: (1) *R. sphaeroides* and (2) *Synechocystis*. Most RDX contamination is associated with DOD. DOD utilizes pump and treat systems for the majority of RDX remediation sites. All experiments were performed as bench scale experiments with the goal of scaling up the experimental setup to retrofit existing pump and treat systems. Experiments with *R. sphearoides* were conducted to determine if RDX would still degrade if certain aspects were not at ideal degradation conditions. *Synechocystis* experiments were performed to determine if RDX degradation would occur.

The main objectives of this study were to:

1. Determine if electron acceptors nitrate (1 mM), sulfate (10 mM), and perchlorate 1 mM) influence *R. sphaeroides* ability to degrade RDX under ideal conditions (growth conditions with succinate as electron donor).
2. Determine if *R. sphaeroides* can degrade RDX with oxygen present.
3. Determine if *Synechocystis* can degrade RDX and if so, under what conditions.

*R. sphaeroides* and *Synechocystis* experiments are described and discussed in chapter three and four respectively of this document.

RDX degradation via photosynthetic bacteria could revolutionize the way RDX is remediated because remediation could become passive.

# CHAPTER TWO: MATERIALS AND METHODS

## 2.1 Materials

### 2.1.2 Chemicals

US Army Corps of Engineers Waterways Experiment Station provided 0.5 g of RDX dissolved in 20mL of acetonitrile. RDX stock solution was prepared from the RDX dissolved in acetonitrile by removing acetonitrile via a direct stream of nitrogen gas onto the acetonitrile/RDX solution (under a vented hood) until only solid RDX remained. Immediately the solid RDX was dissolved in deionized ultrapure water. RDX was filtered using a 0.2µm polytetrafluoroethylene (PTFE) filter to remove all solid undissolved RDX before it was used in an experiment. RDX stock solution concentration was verified by VWR certified standards.

RDX daughter products MNX, TNX, DNX, MDNA, NDAB, Nitrate, Nitrite, methanol, ammonium, and formaldehyde were analyzed by comparing chromatograms to standard curve chromatograms. 10mg of MNX, TNX, and DNX was obtained from SRI International. The 10mg of MNX, TNX, and DNX was dissolved in 25mL of Accetronitrile so standards could be produced for MNX, TNX, and DNX. MDNA and NDAB was purchased from SRI International and was dissolved in 25mL of DDI so standards could be produced. Formaldehyde standards were purchased from AccuStandard. Methanol standards were produced from HPLC-grade methanol purchased from VWR. Nitrate and nitrite standards were produced from sodium nitrate and sodium nitrite, respectively. Finally, ammonium standards were produced from ammonium chloride.

HPLC-grade methanol was obtained from VWR. Anthraquinone-2,6-disulfonate (AQDS) was obtained from Sigma Aldrich. All chemicals used were reagent grade quality (Millerick et al., 2015).

2.1.2 Microorganisms

*2.1.2.1 Rhodobacter sphaeroides* ATCC® 17023™

*Rhodobacter sphaeroides* ATCC® 17023™ was obtained from ATCC®. *R. sphaeroides* was grown under a tungsten lamp (~270W/$m^2$) at ambient temperature of approximately 25°C for 24 hours a day in an anoxic environment (Argun et al., 2010). *R. sphaeroides* was photoheterotrophically sustained by adding late log phase. *R. sphaeroides* cells (20% by volume). Late log phase *R. sphaeroides* are cells with a 680nm optical density of 1.6 or higher. AQDS and cysteine (each 10% by volume) to pre-prepared tubes of ATCC® Medium #550 (Appendix A). Cellular growth was measured by optical density (OD) at 680 nm at approximately three days (Appendix B) (Millerick et al., 2015).

2.1.2.2 *Synechocystis* sp. PCC 6803

*Synechocystis* was obtained from Dr. David Ladner at Clemson University who obtained the culture from Dr. Bruce Rittman's lab at Arizona State University in 2010. *Synechocystis* was grown under a cool-white flourecent lamp for 12 hours a day (Maeda et al., 2005) at approximately 25°C. Air was sparged into *Synechocystis* 24 hours a day during growth. Sparging $CO_2$ enhanced air can produce faster growth rates (Lea-Smith et al., 2014), but was not utilized. *Synechocystis* was photoheterotrophically sustained by adding 10% by volume of late phase (730nm optical density 4.5 or higher) (Joseph, et al.,

2014) *Synechocystis* to fresh autoclaved BG-11 media (Appendix C) (Varman et al., 2013). After approximately 10 to 14 days of growth, 4.5 $OD_{750}$ cellular growth was obtained (Joseph, et al., 2014).

## 2.2 Methods

### 2.2.1 Resting Cell Suspension

#### 2.2.1.1 *Rhodobacter sphaeroides ATCC® 17023™*

Two experiments were conducted using *R. shpaeroides* cell suspensions: (1) RDX degradation using *R. sphaeroides* under aerobic conditions versus anaerobic conditions and (2) the affect electron acceptors (1 mM nitrate, 10 mM sulfate, and 1 mM perchlorate) have on RDX degradation with *R. shpaeroides* and succinate versus RDX degradation with only *R. shpaeroides* and succinate (electron donor). A cell suspension inhibits the cells ability to grow, but they are still living.

As described in section 2.1.2.1, 200 mL of late log phase *R. sphaeroides* was centrifuged at 5,000xG for 20 minutes at 4°C. The excess liquid was discarded and the cells were washed in 10 mM phosphate buffer (10 mM phosphate buffer used in experimental test tubes) and decanted in 35 mL of 10 mM phosphate buffer. The resuspended *R. sphaeroides* cells were centrifuged again at 5,000xG for 20 minutes at 4°C. The excess liquid was discarded and the cells were washed in 10 mM phosphate buffer and resuspended in 1.5 mL of 10 mM phosphate buffer resulting in approximately 2 mL of *R. sphaeroides* cells. During cell suspension, cells were kept in ice and kept under $N_2$ gas to minimize disruption to cells. The suspended *R. sphaeroides* (0.2 mL,

10% by volume) were added to 10 mL tubes of phosphate buffer. Appendix D contains the flow chart of cell suspension process.

During the experiment which examined the effects of air being present in the system, 0.2 mL of suspended cells (condensed from 200 mL to 2 mL) was added to 9.8 mL of 10 mM phosphate buffer ($NaH_2PO_4 \cdot 1H_20$) which contained 50 µM of RDX, and in some test tubes, 20 mM succinate as an electron donor. Anaerobic test tubes were sealed with blue, butyl stoppers and degassed with $N_2$. Aerobic test tubes were capped with a loose sleeve to allow air to move freely in and out of the test tube. Both anaerobic and aerobic test tubes were placed on a shaker table set at 150 rpm to ensure mixing. This also ensured that aerobic test tubes had ample air movement in and out of the test tubes. The shaker table containing the test tubes was placed under a tungsten lamp (~270W/$m^2$) for 24 hours a day and was kept at ambient temperature of approximately 25°C.

When examining the affects that electron acceptors had on RDX degradation, three electron acceptors were compared (1 mM nitrate, 10 mM sulfate, and 1 mM perchlorate). For these experiments, 0.2 mL of suspended cells (condensed from 200 mL to 2 mL) was added to 9.8 mL of 10 mM phosphate buffer ($NaH_2PO_4 \cdot 1H_20$) which contained 50 µM of RDX, 20 mM succinate as an electron donor, and 1 mM nitrate, 10 mM sulfate, or 1 mM perchlorate. All test tubes were anaerobic and sealed with blue, butyl stoppers and degassed with $N_2$. The test tubes were placed under a tungsten lamp (~270W/$m^2$) for 24 hours a day and were kept at ambient temperature of approximately 25°C.

2.2.1.2 *Synechocystis* sp. PCC 6803

Two *Synechocystis* experiments were conducted using a cell suspension. The purpose was to examine RDX degradation with *Synechocystis* in HEPES buffer. A cell suspension inhibits the cells ability to grow, but they are still living.

As described in section 2.1.2.2, 200 mL of late log phase *R. Synechocystis* was centrifuged at 5,000xG for 20 minutes at 4°C. The excess liquid was discarded and the cells were washed in 10 mM HEPES buffer (10 mM HEPES buffer used in experimental test tubes) and decanted in 35 mL of 10 mM HEPES buffer. The resuspended *Synechocystis* cells were centrifuged again at 5,000xG for 20 minutes at 4°C. The excess liquid was discarded and the cells were washed in 10 mM HEPES buffer and resuspended in 1.5 mL of 10 mM HEPES buffer resulting in approximately 2 mL of *Synechocystis* cells. During cell suspension, cells were kept in ice to minimize disruption to cells. The suspended *Synechocystis* (0.2 mL, 10% by volume) were added to 10 mL tubes of HEPES buffer. Appendix D contains a flow chart of the cell suspension process.

RDX degradation using *Synechocystis* was examined. For this experiment, 0.2 mL of suspended cells (condensed from 200 mL to 2 mL) was added to 9.8 mL of 10 mM HEPES buffer that contained 50 µM or 5 µM of RDX and, in some test tubes, 2 mM AQDS as an electron shuttle. Test tubes were sealed with blue, butyl stoppers but not degassed. Test tubes were placed on a shaker table set at 100 rpm to ensure mixing. The shaker table containing the test tubes was placed under a cool-white fluorescent lamp for 12 hours a day and was kept at ambient temperature of approximately 25°C.

2.2.3 *Synechocystis* sp. PCC 6803 Growth Media

2.2.3.1 *Synechocystis* Growth in BG-11 vs. Modified BG-11

*Synechocystis* growth in BG-11 (Appendix C) and modified BG-11 were compared. Modified BG-11 was prepared in the same manner as BG-11 (as prescribed in Appendix C) except 0.1mL of citric acid, ferric ammonium citrate, $K_2HPO_4 \cdot 3H_2O$, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, and $Na_2CO_3$ was added to the media instead of 1mL. Thus reducing the nutrient stock by 90%. *Synechocystis* was grown in both media as described in 2.1.2.2.

2.2.3.1 *Synechocystis* sp. PCC 6803 RDX degradation in Modified BG-11

RDX degradation with *Synechocystis* in modified BG-11 media was examined. Late log phase *Synechocystis* (5 mL) was added to 5mL of modified BG-11 media containing 5 µM RDX. In investigations analyzing RDX degradation daughter products 14 mL of late log *Synechocystis* was added to 14mL of modified BG-11 media containing 5 µM RDX. Test tubes were sealed with blue, butyl stoppers. Some test tubes were degassed with $N_2$ but others were not degassed. Test tubes were placed on a shaker table set at 100 rpm to ensure mixing. The shaker table containing the test tubes was placed under a cool-white fluorescent lamp for 12 hours a day and was kept at ambient temperature of approximately 25°C.

2.2.3 Analytical Methods

Anoxic syringe and needle were used to collect aqueous samples. All samples containing cells were filtered using 0.2 µm PTFE filters before running analyses were performed to ensure no cells remained in the sample.

RDX and metabolites MNX, DNX, and TNX were analyzed in the aqueous form using a high performance liquid chromatography (HPLC) with a variable wavelength

photodiode array detector at 254 nm (Kwon et al., 2008). A Supelcosil LC-CN column (Kim et al., 2007) at 30° C was utilized to determine RDX, MNX, DNX, and TNX. The influent consisted of 50% methanol and 50% distilled deionized water at a flow rate of 1ml/min (Kwon et al., 2008). RDX peaks were produced around 6.5 minutes. RDX concentrations were obtained by comparing curve areas to those of the curve areas of VWR standards. MNX, DNX, and TNX concentrations were obtained through an external calibration using standards. Examples of RDX, MNX, DNX, and TNX standard curves can be seen in Appendix E.

MDNA and NDAB were analyzed in the aqueous form using a high performance liquid chromatography (HPLC) with a variable wavelength photodiode array detector at 210 nm (Zhao et al, 2004). The influent consisted of 100% hydrochloric acid at a flow rate of 0.6 mL/min (Millerick et al., 2015). Formaldehyde was analyzed using an HPLC with a C18 Acclaim RP column. The influent consisted at 50% acetonitrile and 50% DDI at 0.8mL/min. Formaldehyde was derivatized with NDPH for 1 hour at 30$^{o}$C (Millerick et al., 2015). Methanol was analyzed with a flame ionization gas chromatograph (Monteil-Rivera et al., 2005). Spectrophotometer at a wavelength of 650 nm was used to determine ammonium concentration (Rhine et al., 2008).

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CHAPTER THREE: RESULTS

## 3.1 RDX degradation with *R. sphaeroides* and electron acceptors

RDX was degraded in all incubations with *R. sphaeroides* under tungsten light conditions (Figure 3.1).

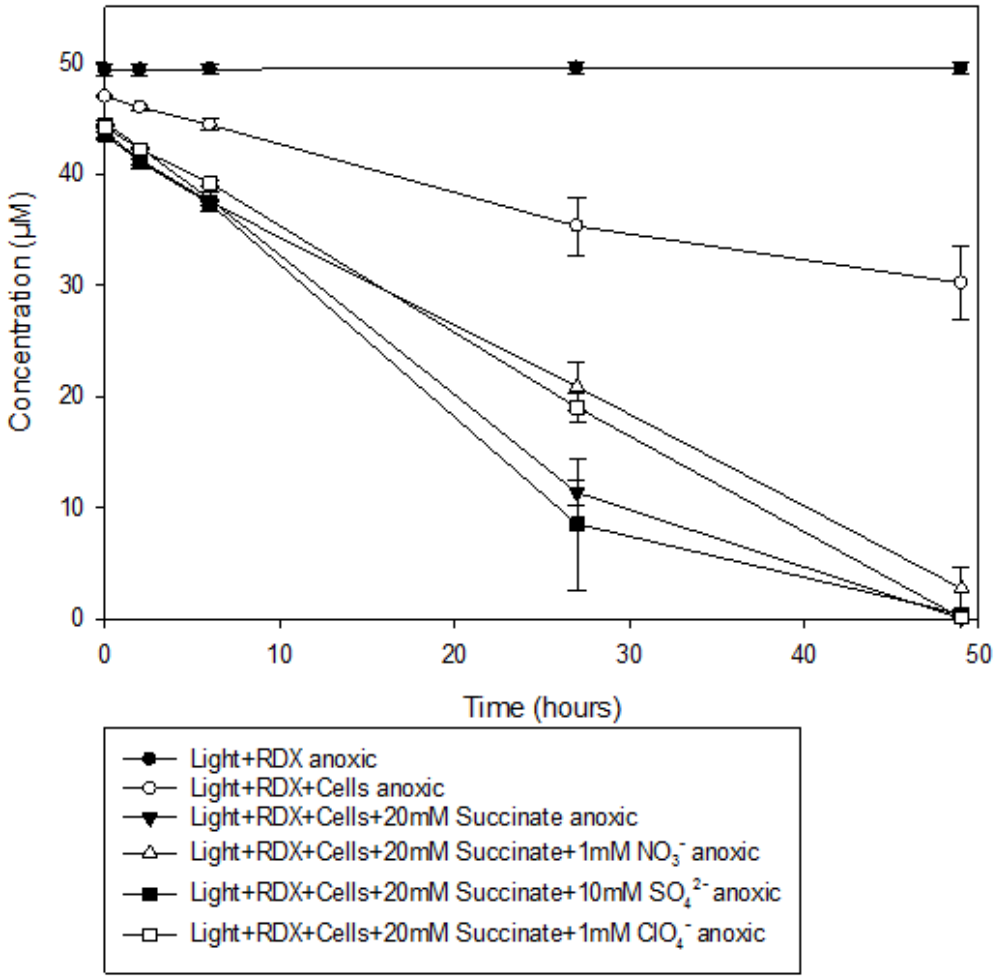



**Figure 3.1 -** Average RDX concentration in anoxic conditions. Error bars represent standard deviations. Three samples (n=3) for each incubations.

After 49 hours, 100% of RDX was reduced in tungsten light anaerobic incubations that contained *R. sphaeroides* and 20 mM succinate (electron donor). RDX was completely degraded in samples containing RDX, 20 mM succinate, and 1 mM of perchlorate (electron acceptor). After 49 hours, 99% and 94% of RDX was degraded in samples containing RDX, 20 mM succinate, and 10 mM of sulfate (electron acceptor) and 1mM of nitrate (electron acceptor), respectively. RDX degradation without an electron acceptor was faster than those incubations with an electron acceptor. The degradation rate for samples containing RDX, *R. sphaeroides* and 20 mM succinate in tungsten light was a zero order decay rate where $k_{obs}$ equaled 0.95 µM/hr. The degradation rate for samples that contained electron acceptors also followed a zero order decay rate, but the rate constants were slightly lower. Samples with 10 mM of sulfate, 1 mM of perchlorate and 1 mM nitrate had a $k_{obs}$ of 0.93 µM/hr, 0.91 µM/hr, and 0.82 µM/hr, respectively.

At 49 hours, incubations containing only RDX and *R. sphaeroides* in tungsten light degraded 36% of RDX and at 456 hours (19 days), 93% of RDX was degraded. Samples in tungsten light containing *R. sphaeroides* and RDX had a first order decay rate ($k_{obs}$ = 0.006/hr). RDX was not degraded by tungsten light alone.

### 3.2 RDX degradation with *R. sphaeroides* under aerobic conditions

RDX was degraded in all incubations with *R. sphaeroides* under tungsten light conditions (Figure 3.2).

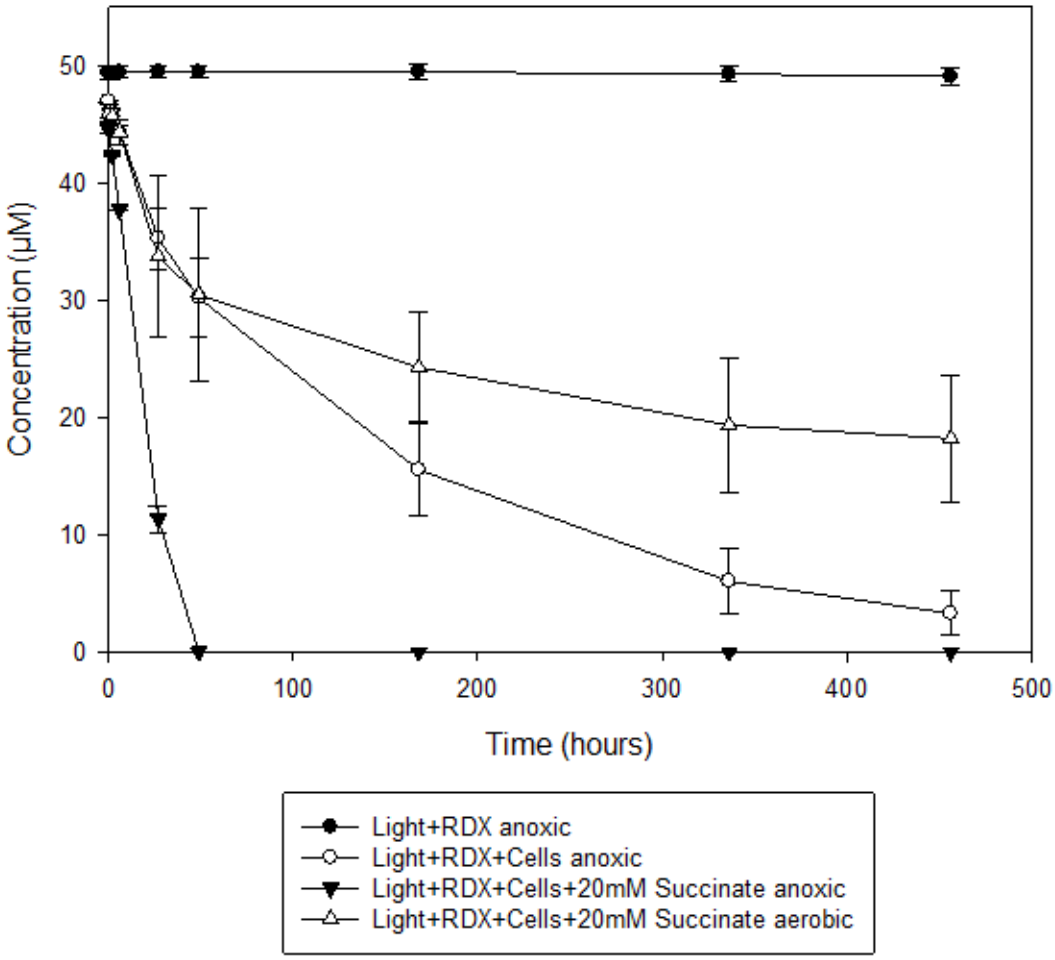



**Figure 3.2** Average RDX concentration in anoxic and aerobic conditions. Error bars represent standard deviations. Three samples (n=3) for each incubations.

Anoxic samples in tungsten light containing *R. sphaeroides* and 20 mM succinate (electron donor) completely degraded RDX after 49 hours and had a zero order decay rate ($k_{obs}$ = 0.95 µM/hr). Aerobic samples in tungsten light containing only *R. sphaeroides* and 20 mM succinate (electron donor) only degraded 60% of RDX after 19 days. Anoxic

incubations in tungsten light containing only RDX and *R. sphaeroides* degraded 93% of RDX after 19 days.

Anoxic incubations in tungsten light containing *R. sphaeroides* and RDX and aerobic incubations in tungsten light containing *R. sphaeroides*, 20 mM succinate (electron donor), and RDX both followed a first order decay rate and had a $k_{obs}$ of 0.006/hr and 0.002/hr, respectively. RDX was not degraded by tungsten light alone.

**3.3 RDX degradation in BG-11 media**

BG-11 in the presence of cool-white fluorescent light alone degraded RDX (Figure 3.3).

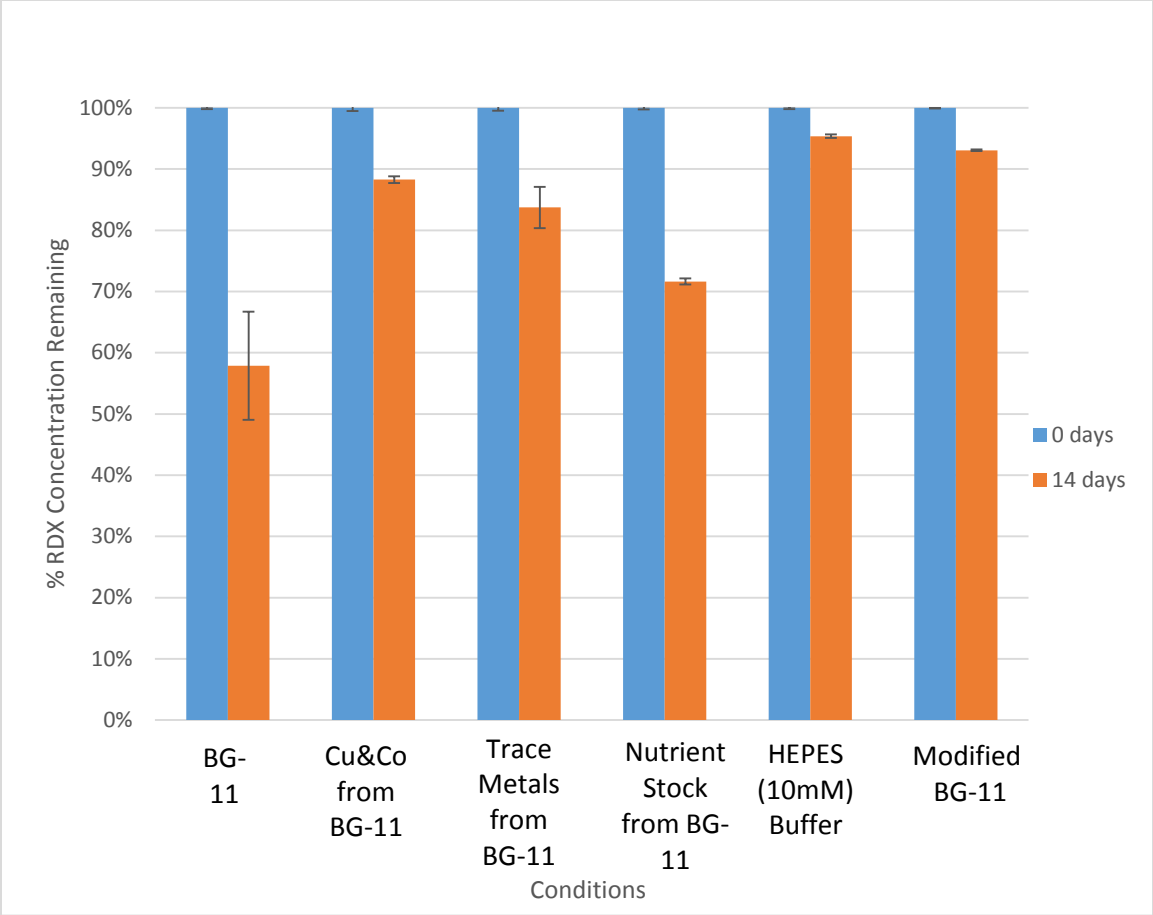



**Figure 3.3** Percent of RDX remaining after exposure to cool-white fluorescent light under various conditions with no bacteria present. Error bars represent standard deviations. Three samples (n=3) for each incubations.

After 14 days concentration of RDX was reduced by 42% in BG-11 without cells. Modified BG-11 under cool-white fluorescent light (BG-11 prepared as prescribed in Appendix C except 0.1mL of citric acid, ferric ammonium citrate, $K_2HPO_4 \cdot 3H_2O$, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, and $Na_2CO_3$ instead of 1mL was added to the media) showed the least amount degradation with only a 5% reduction in concentration after 14

days. RDX in 10 mM HEPES buffer was degraded to 7% of the original concentration under cool-white fluorescent light. These results were confirmed in samples from other experiments (section 3.5). In HEPES buffer when RDX had an initial concentration of 50 µM, RDX was decreased by 6% in 14 days and RDX was decreased by 7% in 14 days when the initial concentration was 5 µM.

The copper and cobalt components of BG-11 (at same concentration found in BG-11) degraded 12% of the total RDX concentration after 14 days. The trace metals stock (as prescribed in Appendix C, includes copper and cobalt) of BG-11 degraded more RDX in 14 days than the copper and cobalt. After 14 days, the trace metals stock had 84% of the original RDX remaining. The nutrients stocks of BG-11 degraded the greatest amount of RDX at 28% in 14 days. When RDX was added to each of the three components independently the total reduction of RDX in all three components was 56% after 14 days. When RDX was add to BG-11 (all three components combined) the total reduction after 14 days in RDX was 42%.

### 3.4 *Synechocystis* sp. PCC 6803 Growth Experiment

*Synechocystis* growth in BG-11 and modified BG-11 (as explained in section 2.2.3.1) was compared. At time zero *Synechocystis* in BG-11 and modified BG-11 look the same (Figure 3.4a).


**Figure 3.4a** *Synechocystis* sp. 6803 Growth in BG-11 (left) vs. BG-11 modified (right) at time zero.

*Synechocystis* biomass is visually larger in BG-11 when compared to modified BG-11 (Figure 3.4b).


**Figure 3.4b** *Synechocystis* sp. 6803 Growth in BG-11 (left) vs. BG-11 modified (right) after 14 days.

Comparing Figure 3.4a and Figure 3.4b shows *Synechocystis* grown in modified BG-11 did acquire biomass. However, after transferring *Synechocystis* grown in modified BG-11 into modified BG-11, *Synechocystis* growth significantly decreased and after several transfers from modified BG-11 into modified BG-11 *Synechocystis* growth became minimal.

### 3.5 RDX degradation with *Synechocystis* in HEPES buffer

3.5.1 RDX (50 µM) degradation with *Synechocystis* in HEPES buffer

Samples (capped with air) in fluorescent light that contained RDX, *Synechocystis*, and 2 mM AQDS degraded 97% of RDX (44.9 µM to 1.4 µM) in 35 days (Figure 3.5a).

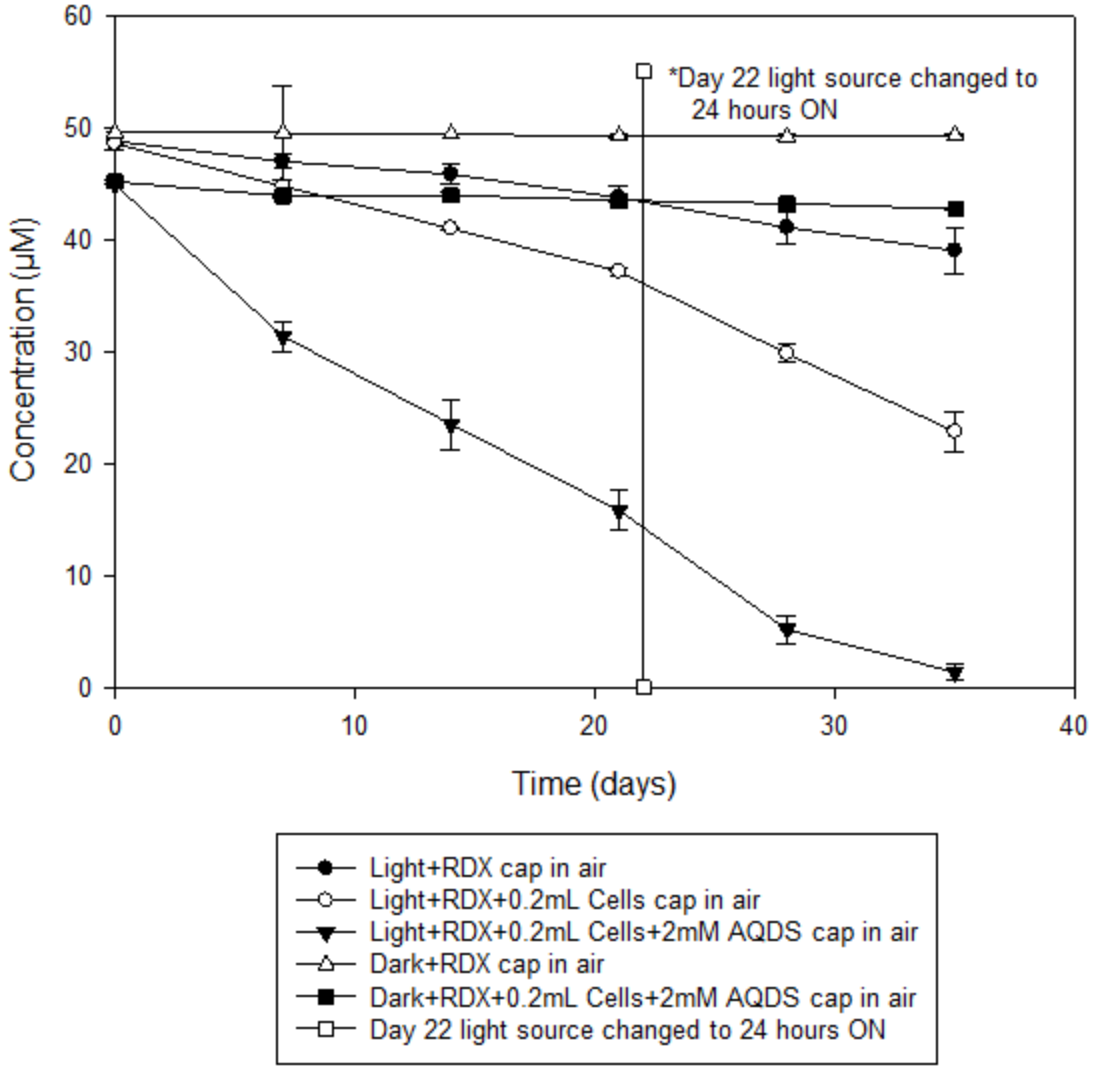



**Figure 3.5a** Average RDX concentration in sample in 10 mM HEPES buffer. Error bars represent standard deviations. Three samples (n=3) for each incubations.

Whereas, samples (capped with air) in the dark that contained RDX, *Synechocystis*, and 2 mM AQDS degraded 5% of RDX. Samples (capped with air) in light that contained RDX, *Synechocystis*, and 2 mM AQDS had a zero order decay rate where $k_{obs}$ = 1.24

μM/day. Incubations in the cool-white fluorescent light and capped in air that contained only RDX and *Synechocystis* degraded 53% of original RDX concentration after 35 days. These samples exhibited a zero order decay ($k_{obs}$ = 0.72 μM/day).

Capped with air incubations in the dark with only RDX had no degradation of RDX after 35 days. Samples (capped with air) in the light with only RDX followed trends discussed in section 3.3 where 94% of RDX remained after 14 days. After 35 days, 80% of RDX remained in samples (capped with air) in the light with only RDX.

At day 22 of the experiment the light source was altered to be continuously on for twenty-four hours a day to determine if the rate of degradation would increase. The rate of degradation did increase once the light source was changed to being on for 24 hours.

3.5.2 RDX (5 μM) degradation with *Synechocystis* in HEPES buffer

Anaerobic incubations in light consisting of only RDX and *Synechocystis* degraded the quickest (Figure 3.5b).

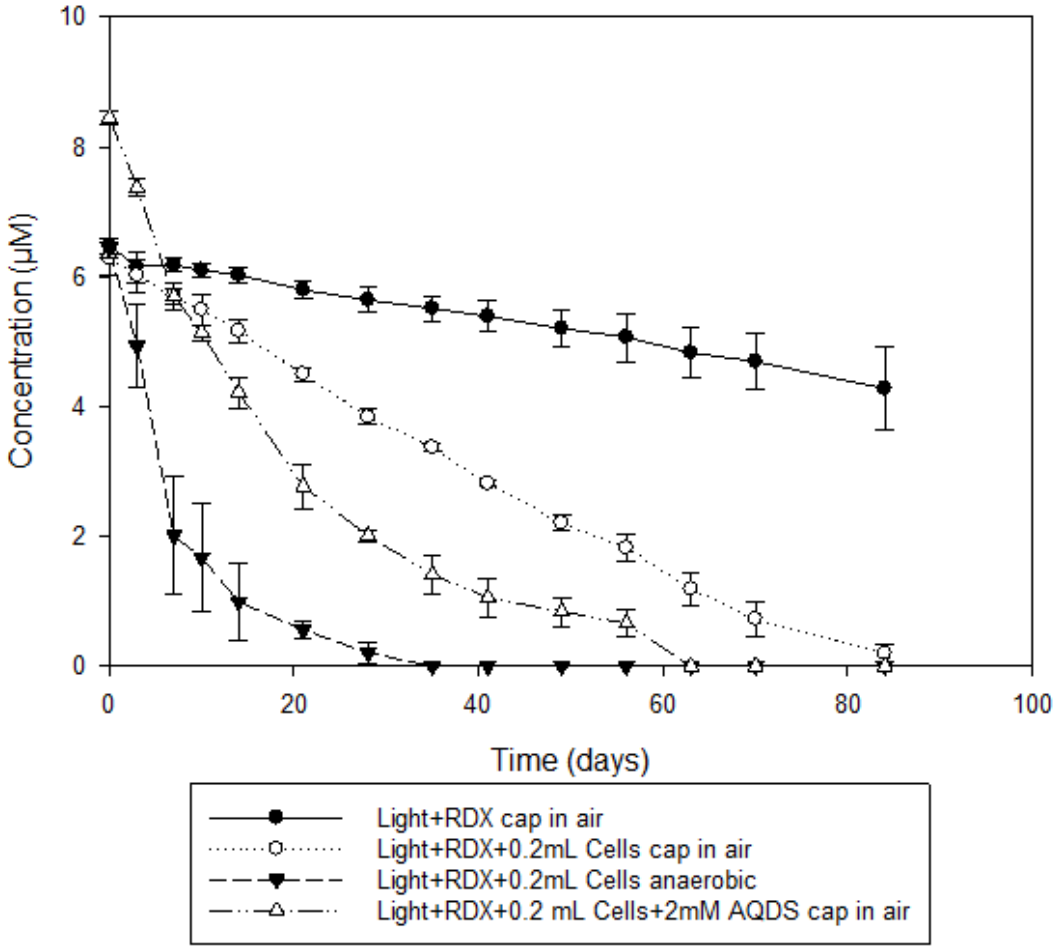



**Figure 3.5b** Average RDX concentration in 10 mM HEPES buffer. Error bars represent standard deviations. Three samples (n=3) for each incubations.

In 35 days, 100% of RDX was degraded. Lighted samples (capped with air) that contained RDX, *Synechocystis*, and 2 mM AQDS degraded all of the initial RDX in 63 days. Both sets of samples had a first order decay. Anaerobic samples in light consisting of only RDX and *Synechocystis* had a decay rate constant of 0.12/day ($k_{obs}$ = 0.12/day)

and samples (capped with air) in light that contained RDX, *Synechocystis*, and 2 mM AQDS had a decay rate constant of 0.047/day ($k_{obs}$ = 0.0.47/day).

Lighted samples (capped with air) that contained RDX and *Synechocystis* degraded 97% of original RDX concentration (6.28 µM to 0.14 µM) in 84 days with a zero order decay rate constant of 0.077 µM/day ($k_{obs}$ = 0.077/day). Lighted incubations (capped with air) that only contained RDX in 10 mM HEPES buffer degraded to 66% of the original RDX concentration after 84 days.

### 3.6 RDX degradation with *Synechocystis* in modified BG-11 media

RDX was degraded completely in both anaerobic and capped with air samples in light containing RDX and *Synechocystis*. Incubations (anaerobic) in light with RDX and *Synechocystis* degraded 99% of RDX in 27 days and completely degraded RDX by 34 days (Figure 3.6a).

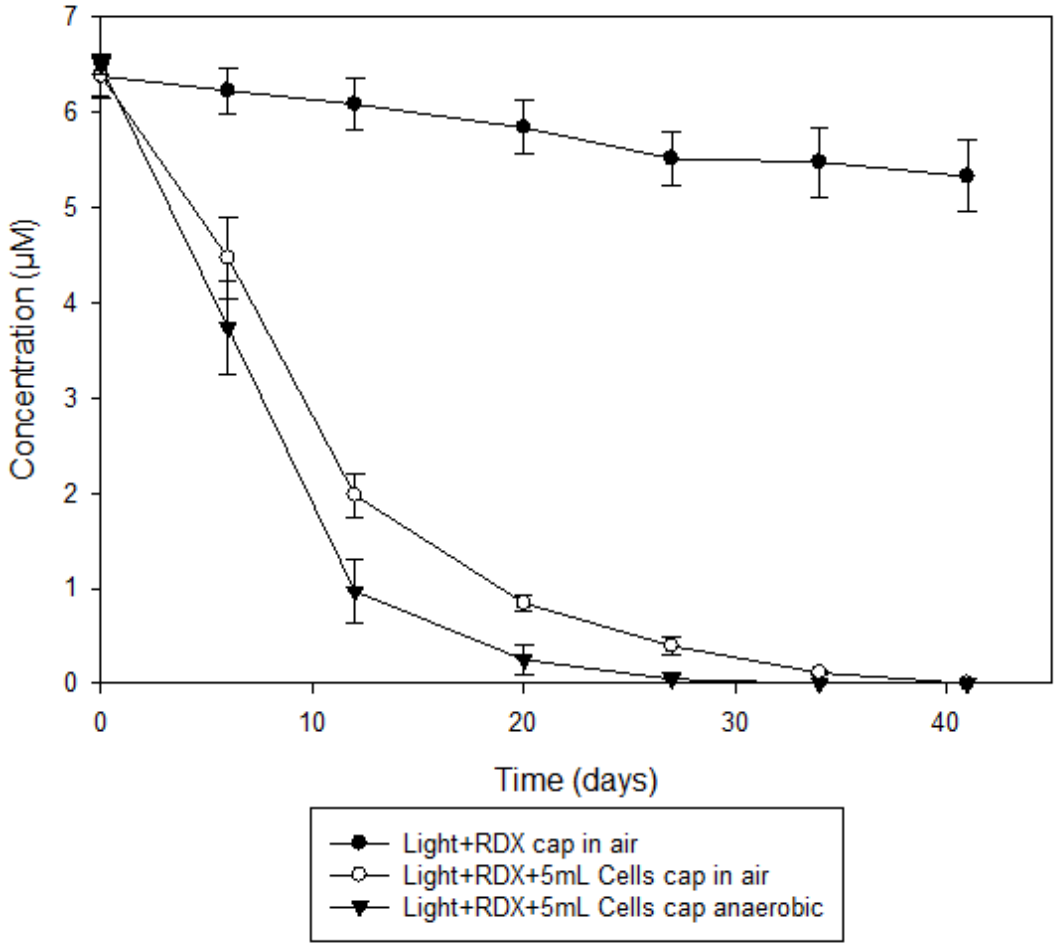



**Figure 3.6a** Average RDX concentration in modified BG-11. Error bars represent standard deviations. Three samples (n=3) for each incubations.

Anaerobic samples in light with RDX and *Synechocystis* had a first order of decay rate constant of 0.17/day ($k_{obs}$ = 0.17/day).

Incubations (capped with air) in light with RDX and *Synechocystis* degraded 98% of RDX in 34 days and completely degraded RDX by 41 days. A first order of decay rate

was followed in lighted samples (capped with air) that contained RDX and *Synechocystis* and had a first order decay constant of 0.11/day ($k_{obs}$ = 0.11/day). Lighted samples (capped with air) that contained only RDX degraded 16% of RDX in 41 days.

### 3.7 RDX daughter products with *Synechocystis* in modified BG-11 media

During the degradation of RDX, RDX intermediate metabolites were detected (Figure 3.6a, Figure 3.7b, and Figure 3.7c).

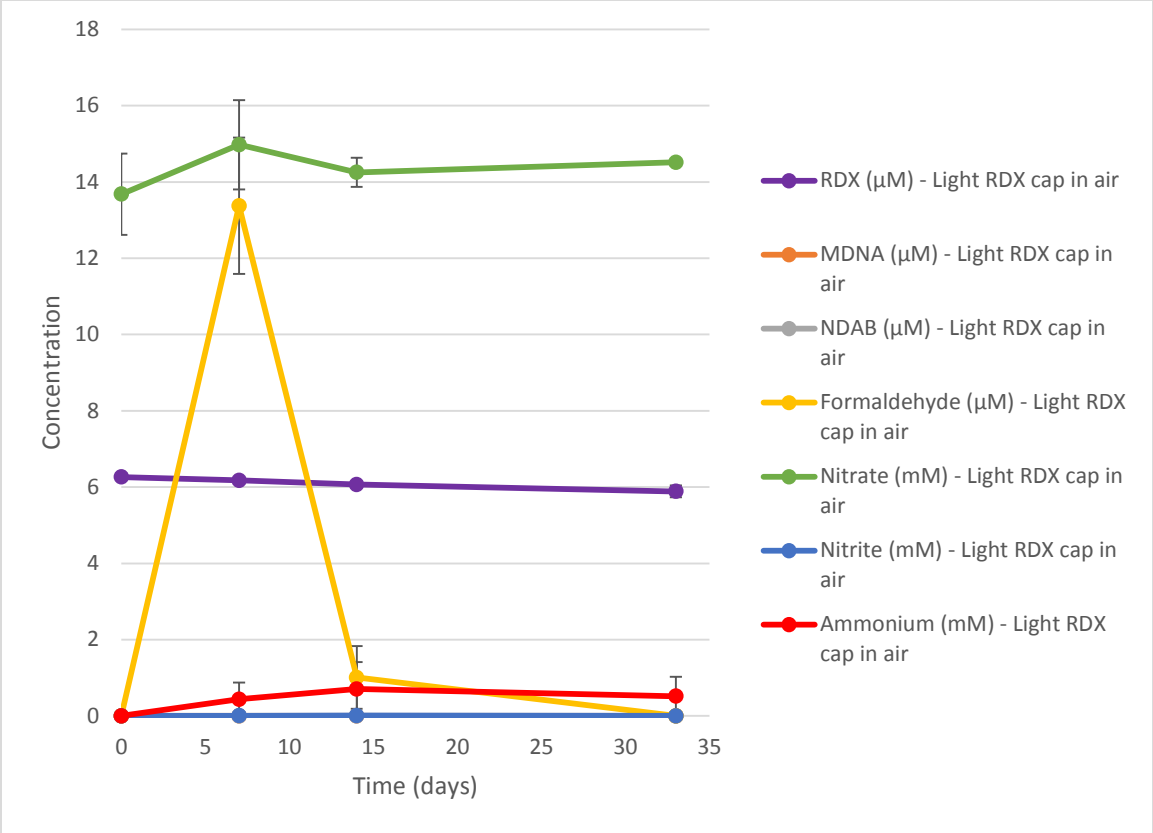



**Figure 3.7a** Average RDX, MDNA, NDAB, formaldehyde, nitrate, nitrite, and ammonium concentration in modified BG-11. Error bars represent standard deviations. Three samples (n=3) for each incubations.

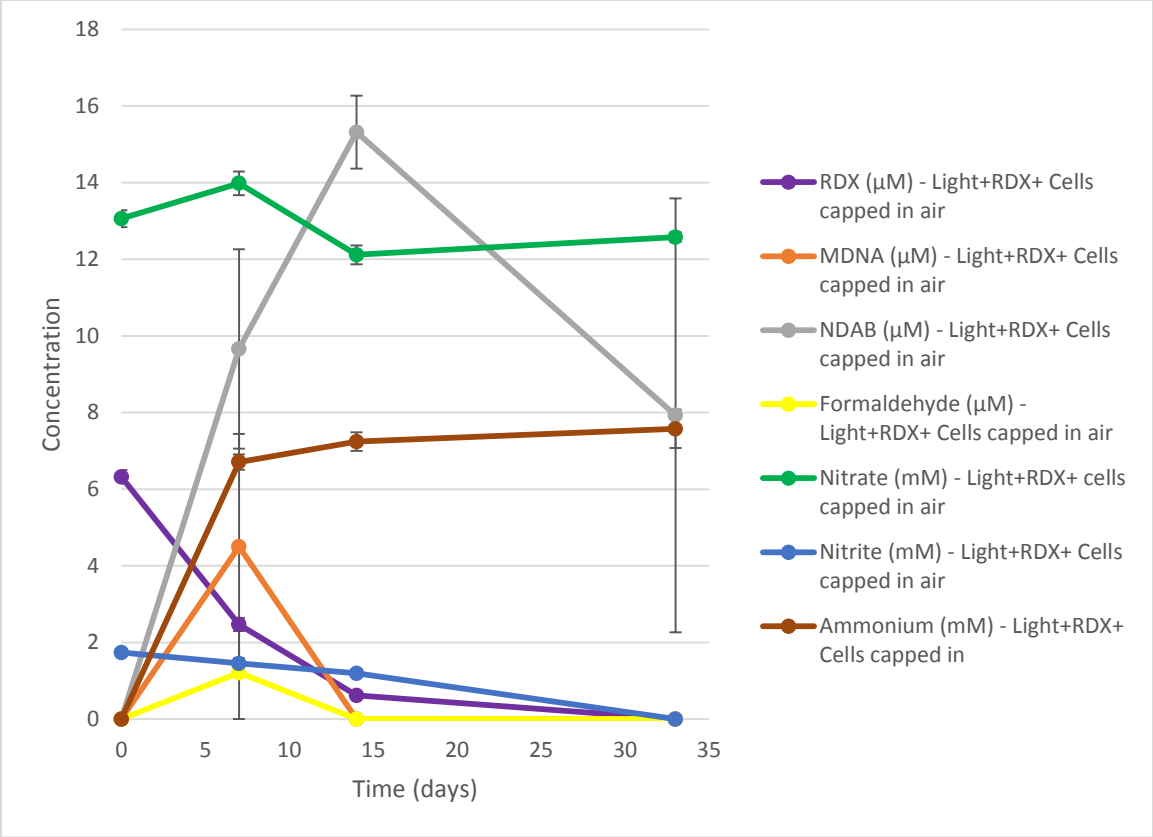



**Figure 3.7b** Average RDX, MDNA, NDAB, formaldehyde, nitrate, nitrite, and ammonium concentration in modified BG-11. Error bars represent standard deviations. Three samples (n=3) for each incubations.

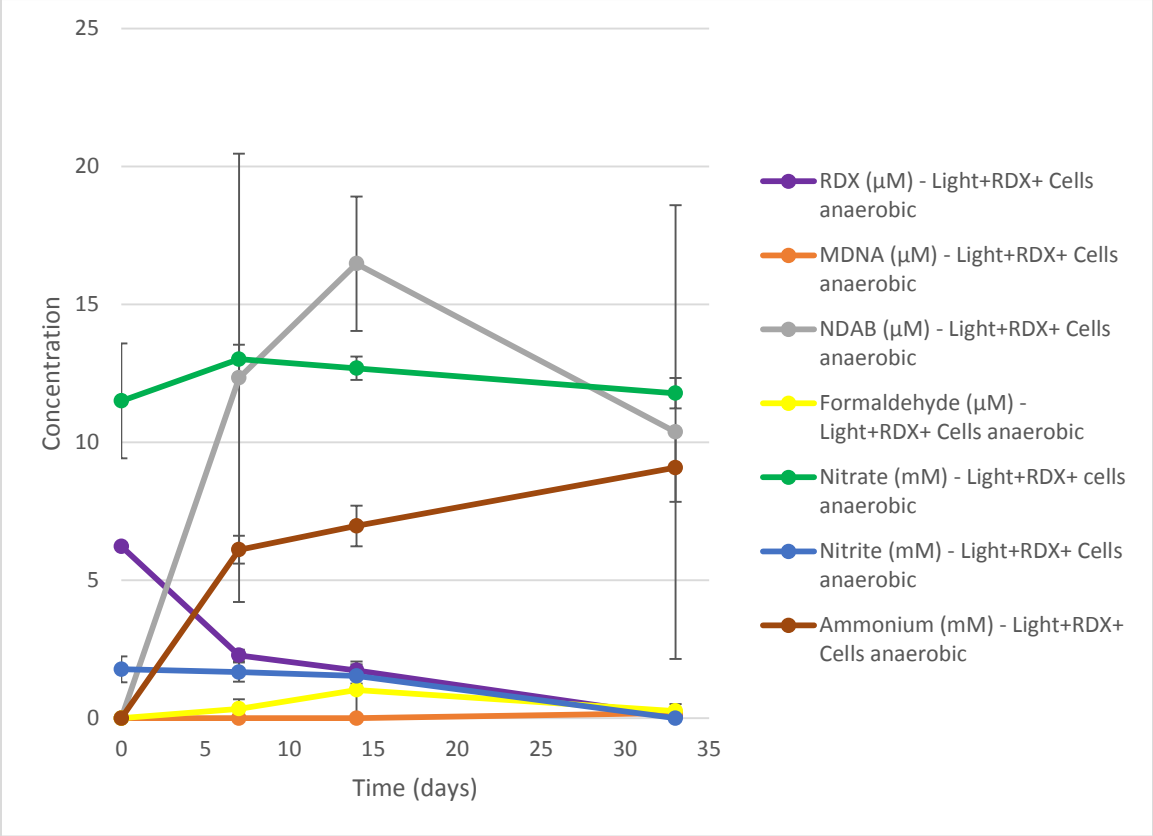



**Figure 3.7c** Average RDX, MDNA, NDAB, formaldehyde, nitrate, nitrite, and ammonium concentration in modified BG-11. Error bars represent standard deviations. Three samples (n=3) for each incubations.

Figure 3.6a shows RDX daughter products' concentration in samples containing light and RDX capped in air. Ammonium and formaldehyde were produced. Figure 3.6b shows RDX intermediate metabolites for samples in light containing RDX and *Synechocystis* (capped in air). NDAB, MDNA, formaldehyde, and ammonium were produced. Anaerobic samples in light containing RDX and *Synechocystis* (Figure 3.6c) displays

RDX daughter products NDAB, Formaldehyde, and ammonium. Table 3.6 shows the percent of carbon recovered in RDX and RDX degradation products compared to the initial concentration.

**Table 3.6** Percent of carbon in RDX and daughter products compared to initial RDX concentration.

| total C (%) | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | #1-3 Light + RDX | | #4-6 Light + RDX + cells capped in air | | #7-9 Light + RDX + cells anaerobic | |
| RDX and degradtion products | | 0 hr | Day 33 | 0 hr | Day 33 | 0 hr | Day 33 |
| RDX | C3H6N6O6 | 100% | 94% | 100% | 0% | 100% | 1% |
| MDNA | CH4N4O4 | 0% | 0% | 0% | 0% | 0% | 0% |
| NDAB | C2H5N3O3 | 0% | 0% | 0% | 84% | 0% | 111% |
| Methanol | CH3OH | 0% | 0% | 0% | 0% | 0% | 0% |
| Formaldehyde | CH2O | 0% | 0% | 0% | 0% | 0% | 1% |
| total | | 100% | 94% | 100% | 84% | 100% | 113% |

# CHAPTER FOUR: DISCUSSION

## 4.1 RDX degradation with *R. sphaeroides* and electron acceptors

RDX degradation using *R. sphaeroides* with an electron donor such as succinate was previously proven (Millerick et al., 2015). Sulfate, nitrate, and perchlorate, all electron acceptors, were added to samples to examine if they would affect *R. sphaeroides'* ability to degrade RDX. Of the three electron acceptors used, sulfate has the lowest redox potential and perchlorate has the highest redox potential. RDX transformations occur via co-metabolism (Millerick et al., 2015). In *R. sphaeroides* growth RDX is used as a weak electron acceptor (Perreault et al., 2012). The addition of electron acceptors was thought to disrupt RDX's ability to be an electron acceptor. Even with the presence of perchlorate (a high redox potential) RDX was still reduced in the same time frame as if no electron acceptor was present. At 2 and 6 hours into the reduction, all samples containing succinate had relatively the same RDX concentration.

## 4.2 RDX degradation with *R. sphaeroides* aerobic

*R. sphaeroides* optimal growing conditions are aerobic chemoheterotrophic (in dark) or anaerobic photorophically (Mackenzie et al., 2007). The experiment examined if *R. sphaeroides* would degrade RDX under lighted aerobic conditions. RDX was degraded in aerobic tubes, but the presence of air did inhibit the degradation when compared to those same incubations in anaerobic conditions. Aerobic samples (in light) containing *R. sphaeroides*, RDX, and succinate degraded less RDX than those anaerobic samples (in light) containing RDX and *R. sphaeroides* alone. These results follow *R. sphaeroides*

were expected, because the growing conditions in aerobic conditions was chemoheterotrophically.

In another study, aerobic incubations in the dark with *R. sphaeroides*, RDX, and succinate did not reduce RDX. In aerobic dark reactions, *R. sphaeroides* is chemohetrotrophic, but is not utilizing RDX.

**4.3 RDX degradation in BG-11 media**

RDX can be degraded by UV light alone when dissolved in water (Bose et al., 1998). Cool-white fluorescent bulbs produce a trace amount of UV light. Therefore, minimal degradation of RDX was expected in samples containing only RDX in BG-11 media under the cool-white fluorescent light. However, BG-11 media and fluorescent light degraded 42% of RDX after only 14 days, suggesting that RDX reduction was due to BG-11 ingredients.

BG-11's ingredients were divided into three components (Cu & Co, trace metals stock, and nutrient stock). BG-11 stock ingredients can be found in Appendix A. Together these three components make BG-11. Each of the three components was prepared with the same final concentrations that would be found in the final BG-11 media, but did not include the other two components.

All components of BG-11 media was found to degrade BG-11. The nutrient stock ($K_2HPO_4 \cdot 3H_2O$, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, and $Na_2CO_3$) of BG-11 had the greatest affect on RDX degradation.

HEPES buffer and RDX under the cool-white fluorescent light had a 6% reduction of RDX after 14 days showing that cool-white fluorescent light alone does

degrade RDX. RDX in modified BG-11 was examined and the reduction of RDX was similar to that of HEPES buffer with 7% reduction of RDX after 14 days. Therefore, modified BG-11 was decided to be an appropriate medium to conduct experiments in because RDX reduction in modified BG-11 was comparable to that of HEPES buffer.

### 4.4 *Synechocystis* sp. PCC 6803 Growth Experiment

Because of *Synechocystis'* long growth cycle (when compared to other bacteria), experiments were planned in BG-11 so that *Synechocystis* would have the necessary nutrients to grow as opposed to a cell suspension where a dense volume of cells are added to a buffer. Because BG-11 significantly degrades RDX in the presence of cool-white fluorescent light, an extensive literature review was conducted to find an alternative growth media for *Synechocystis.* The review revealed no other known *Synechocystis* growth medium. Therefore, BG-11 was modified (only 10% of BG-11 nutrient stock solutions were added to media). As discussed in the previous sections, modified BG-11 did not degrade RDX as readily as BG-11 and RDX degradation in modified BG-11 was comparable to degradation in HEPES buffer.

Although modified BG-11 was an acceptable medium for conducting RDX experiments, *Synechocystis'* ability to grow in modified BG-11 was unknown. The first transfer of *Synechocystis* (grown in BG-11) to the modified BG-11, showed that biomass accumulation when compared to BG-11 grown *Synechocystis*, was adequate, as seen in Figure 3.4. However, by the third transfer of *Synechocystis* grown in modified BG-11 to modified BG-11, biomass took four weeks to accumulate to the density observed by day 3 in BG-11 grown *Synechocystis.*

### 4.5 RDX degradation with *Synechocystis* in HEPES buffer

RDX degradation with an initial concentration of 50µM and 5µM did occur with *Synechocystis* (after cell suspension) in HEPES buffer capped in air and in anaerobic incubations.

In the experiment with 50µM (ended at day 35) as the initial RDX concentration, RDX was reduced in four of the five incubations. RDX was degraded in sample groups 1, 2, 3, and 5 of the five listed below:

**Table 4.5a** Percent RDX degradation with *Synechocystis* in HEPES buffer

| Incubations (50µM ended at day 35) | % reduced at 35 days |
|---|---|
| 1. Fluorescent Light + RDX cap with air | 7% |
| 2. Fluorescent Light + RDX +0.2 mL *Synechocystis* cap in air | 50% |
| 3. Fluorescent Light + RDX + 0.2 mL *Synechocystis* + 2 mM AQDS cap in air | 97% |
| 4. Dark + RDX cap in air | 0% |
| 5. Dark + RDX + 0.2 mL *Synechocystis* + 2 mM AQDS cap in air | 5% |

In the experiment with 5 µM (ended at day 84) as the initial RDX concentration, RDX was reduced in all four sample groups listed below:

**Table 4.5b** Percent RDX degradation with *Synechocystis* in HEPES buffer

| Incubations (5µM ended at day 84) | % reduced at 35 days |
|---|---|
| 6. Fluorescent Light + RDX cap with air | 7% |
| 7. Fluorescent Light + RDX +0.2 mL *Synechocystis* cap in air | 50% |
| 8. Fluorescent Light + RDX +0.2 mL *Synechocystis* anaerobic | 98% |

| | | |
|---|---|---|
| 9. | Fluorescent Light + RDX + 0.2 mL *Synechocystis* + 2 mM AQDS cap in air | 83% |

In both samples 1 and 6, RDX was reduced around 17% at 35 days demonstrating RDX is reduced marginally by cool-white fluorescent light when dissolved in water.

AQDS and RDX in fluorescent light alone does not reduce RDX (Kwon and Finneran, 2008). RDX reduction in samples 3, 5, and 9 were due to the cells. Samples 3 and 9 were the same except for the initial concentration of RDX. RDX was reduced by 97% in sample 3 at day 35 and only 83% in sample 9. The light source of the 50µM experiment was changed to twenty-four hours (to see if the rate of degradation would increase) on at 22 days which is most likely why more RDX was reduced quicker in the sample 3 when compared to sample 9. At day 21, the RDX reduction in both sample 3 and 9 was around 65%. The reduction, 5% reduction of RDX, in sample 5 was not expected because there was no light source. *Synechocystis* can incorporate nitrogen from a variety of sources into required metabolites for growth (Kolodny et al., 2006). RDX reduction via bacteria has been previously reported as mostly co-metabolism (Perreault et al., 2012), so the RDX reduction in dark incubations could be a result of *Synechocystis* using RDX as a nitrogen source.

In samples 2, 7, and 8 RDX reduction did occur without an electron shuttle. As suspected, at day 35 sample 2 (50µM) and 7 (5µM) had similar RDX reduction of around 50%. RDX reduction was more efficient in sample 8, anaerobic conditions. Sample 8 (anaerobic with no electron shuttle) degraded RDX 28 days faster than sample 9 (capped in air with electron shuttle), showing that the anaerobic system degraded RDX quicker.

Appendix F shows that different mechanisms for samples 7, 8, and 9 were taking place when degrading RDX.

### 4.6 RDX degradation with *Synechocystis* in modified BG-11 media

RDX degradation by *Synechocystis* in modified BG-11 was significantly faster than RDX degradation *Synechocystis* in HEPES buffer. RDX reduction with only *Synechocystis* in BG-11 was faster by two fold than that of only *Synechocystis* in HEPES buffer.

RDX degradation in anaerobic conditions was more efficient than degradation in incubations capped in air. *Synechocystis* does not have the ability for fully effective nitrogen fixation (Turner and Huang, 2001). Because *Synechocystis* is degrading RDX quickly in incubations with only nitrogen, it could be possible that nitrogen fixation is occurring making anaerobic samples more efficient than those with air.

*Synechocystis* degradation of RDX created NDAB in both samples; therefore ring cleavage of RDX occurred (Paquet et al., 201). Ammonium and NDAB had the greatest concentration. Whereas, RDX degradation in incubations containing only fluorescent light and RDX produced formaldehyde and ammonium.

Analyzing RDX degradation products in this experiment showed that RDX transformed mostly into NDAB and ammonium in samples containing *Synechocystis* (capped in air and anaerobic). Low concentrations of formaldehyde were produced. Nitrate concentrations were unchanged. Nitrite concentrations decreased to zero by day 33.

Table 3.6 shows that the carbon mass was accounted for during RDX transformation.

RDX degradation an aerobic photosynthetic bacteria (*Synechocystis*) has never been observed before. Additionally, *Synechocystis* degraded RDX without an amendment.

# CHAPTER FIVE: CONCLUSTIONS AND RECCOMMENDATIONS

## 5.1 Conclusion

The following conclusions can be made as a result of this thesis research.

1. Adding electron acceptors to incubations with *R. sphaeroides,* electron donor, and RDX in tungsten light did not significantly change the time required to degrade RDX. Samples with 10 mM of sulfate, 1 mM of perchlorate and 1 mM nitrate had a $k_{obs}$ of 0.93 µM/hr, 0.91 µM/hr, and 0.82 µM/hr respectively.
2. Exposing *R. sphaeroides* to oxygen drastically inhibited the degradation of RDX. After 19 days, 40% of RDX still remained in the samples. The same samples in anaerobic conditions degraded RDX in only 49 hours.
3. BG-11 media alone degraded RDX significantly under a cool-white fluorescent light.
4. *Synechocystis's* growth was affected when BG-11 nutrient stock concentration was modified.
5. *Synechocystis* completely degraded RDX with and without an electron shuttle and in aerobic and anaerobic conditions.
6. *Synechocystis* reduced RDX more efficiently when in incubations with growth media as opposed to incubations with HEPES buffer.

Both bacteria degraded RDX, but because *Synechocystis* degraded RDX without an amendment and in aerobic environments *Synechocystis* appears to have the greatest potential as a RDX bioremediation technique. RDX degradation via a bacteria, *Synechocystis,* in aerobic conditions has not been published.

## 5.2 Recommendations

The following recommendations are offered as a result of this thesis research.

1. Additional experiments should be performed to examine the affects of the presence of electron acceptors at different concentrations the ability of on *R. sphaeroides* to degrade RDX.
2. *R. sphaeroides* did degrade RDX under aerobic conditions. A batch reactor should be engineered to determine if *R. sphaeroides* can efficiently degrade RDX in a non-ideal conditions (paralleling field conditions).
3. RDX degradation in BG-11 under a tungsten light should be examined.
4. RDX degradation in water under cool-white fluorescent light should be examined.
5. *Synechocystis's* mechanisms should be studied. Examine why *Synechocystis* degraded RDX in dark reactions and more efficiently degraded RDX under anaerobic incubations.
6. Examine if *Synechocystis* can degrade RDX in a batch reactor in ideal and field conditions.

# APPENDICES

## APPENDIX A: ATCC® #550 Medium

2.5 g Yeast Extract (BD 212750).....1.0 g

(NH4)2SO4.....1.25 g

MgSO4 . 7H2O .....0.2 g

CaCl2 . 2H2O .....0.07 g

Ferric citrate.....0.01 g

EDTA.....0.02 g

KH2PO4 .....0.6 g

K2HPO4 .....0.9 g

Trace Elements (see below).....1.0 ml

Vitamin Solution (see below).....7.5 ml

Neutralize malic acid with NaOH and adjust the pH of the comfpleted medium to 6.9.

Distilled water to.....1.0 L

Add solution to test tubes with anaerobic conditions (80% $N_2$ – 20% $CO_2$).

Autoclave at 121°C for 15 minutes.

Trace Elements:

Ferric citrate.....0.3 g

MnSO4 . H2O .....0.002 g

H3BO3 .....0.001 g

CuSO4 . 5H2O .....0.001 g

(NH4)6Mo7O24 . 4H2O.....0.002 g

ZnSO4 .....0.001 g

EDTA.....0.05 g

$CaCl_2 . 2H_2O$ .....0.02 g

Distilled water.....100.0 ml

Vitamin Solution:

Nicotinic acid.....0.2 g

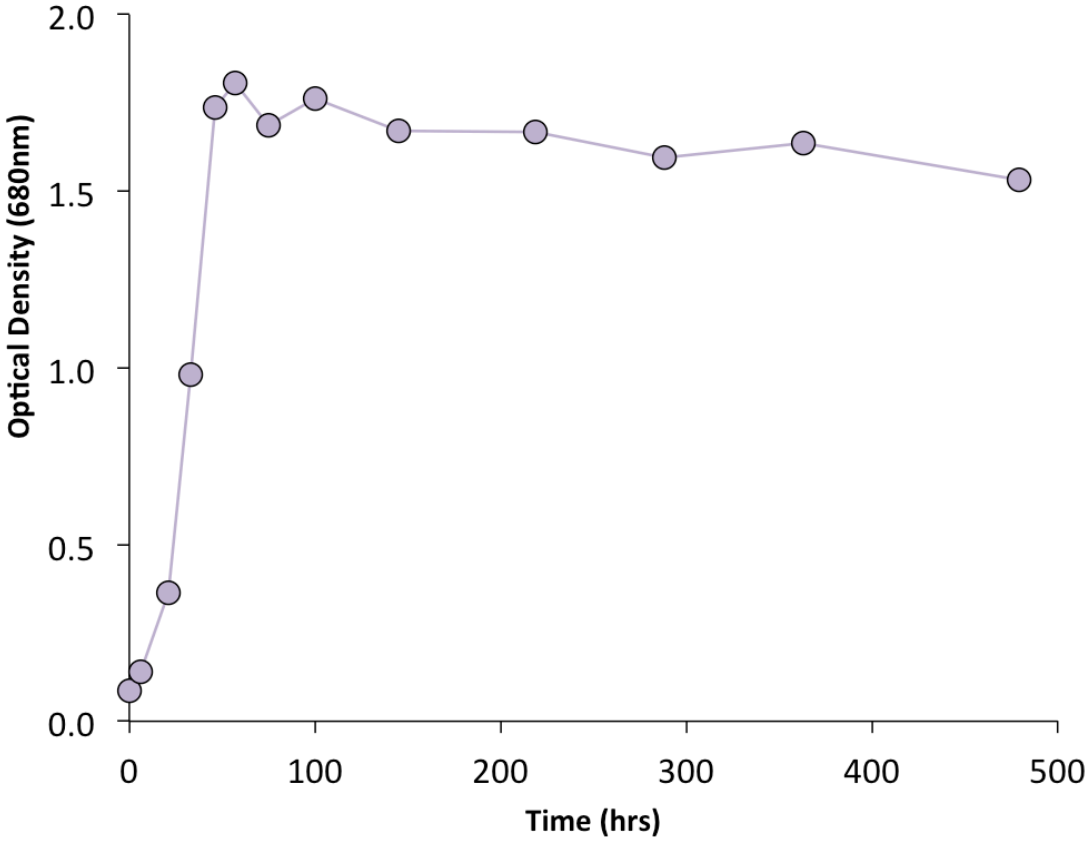
Nicotinamide.....0.2 g

Thiamine . HCl.....0.4 g

Biotin.....0.008 g

Distilled water.....1.0 L

## APPENDIX B: *R. sphaeroides* OD 680nm




(from Kay Millerick presentation)

## APPENDIX C: BG-11 Medium

BG-11 media preparation Updated 5-26-2011

For the culture of freshwater, soil, thermal and marine cyanobacteria. Nitrate and phosphate levels are exceptionally high in this medium. After stock solutions are prepared, only step 3 is required for media preparation.

Materials

- Chemical components
- DDI water
- Scale (0.0001g)
- Weigh boats, spatula
- Volumetric flasks
- Storage containers

Procedures

1. Prepare trace metals solutions
    1.1 Prepare chelating solution: 1g/L $MgNa_2EDTA \cdot H_2O$
    1.2 Prepare primary stocks for copper and cobalt as follows:

| Compound | Concentration (g/L) |
|---|---|
| $CuSO_4 \cdot 5H_20$ | 79.0 |
| $Co(NO_3) \cdot 6H_20$ | 49.4 |

1.3 Prepare final trace metals stock solution as follows and bring to 1L:

| Compound | Mass (g) |
|---|---|
| $H_3BO_3$ | 2.860 g |
| MnCl2 · 4H2O | 1.810 g |
| ZnSO4 · 7H2O | 0.220 g |
| Na2MoO4 · 2H2O | 0.391 g |
| Cu 1° Stock | 1 mL |
| Co 1° Stock | 1 mL |

1.4 Store solution in plastic bottle at 4°C

*NOTE: EDTA can be added directly to the trace metals stock solution*

2. Prepare Nutrient stock solutions as follows:

| Compound | Concentration (g/L) |
|---|---|
| Citric acid | 6 |
| Ferric ammonium citrate | 6 |
| $K_2HPO_4 \cdot 3H_2O$ | 40 |
| $MgSO_4 \cdot 7H_2O$ | 75 |
| $CaCl_2 \cdot 2H_2O$ | 36 |
| $Na_2CO_3$ | 20 |

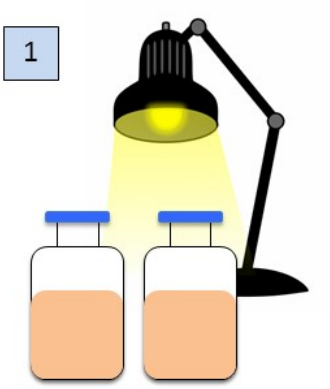
*NOTE: Citric acid and ferric ammonium citrate may be prepared as a single stock solution.*

3. To prepare 1L of BG-11 media, dissolve 1.5g of $NaNO_3$ into 900mL of $dH_2O$ and add 1mL each of (6) stock solutions from step 2 and 1mL of trace metals solution from step 1. Autoclave, if required.
   *NOTE: final pH should be 7.4.*

**APPENDIX D: Cell Suspension Flow Chart**

## Preparing 2mL of *R. sphaeroides* for suspension:

1. 200mL of cells are grown to late log phase in media under light conditions

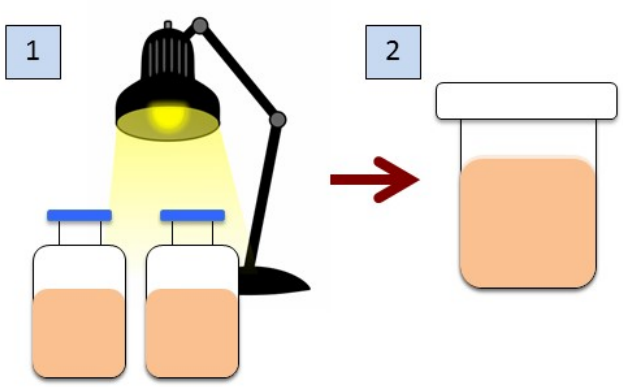



Cell harvest conducted quickly under $N_2$ gas and on ice to minimize disruption to cells

(from Kay Millerick PowerPoint Presentation)

## Preparing 2mL of *R. sphaeroides* for suspension:

2. Cells are transferred to a 250mL centrifuge tube and centrifuged for 20 minutes

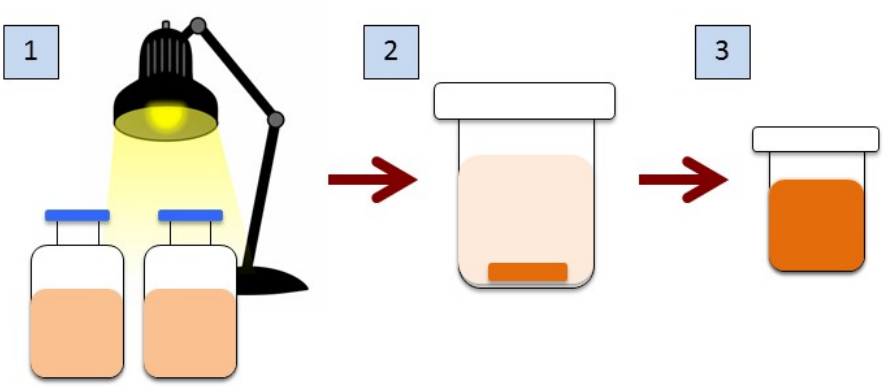



Cell harvest conducted quickly under $N_2$ gas and on ice to minimize disruption to cells

(from Kay Millerick PowerPoint Presentation)

## Preparing 2mL of *R. sphaeroides* for suspension:

3. Liquid is decanted, 35 mL of phosphate buffer is added, cells + buffer are transferred to a smaller centrifuge bottle and centrifuged for 20 minutes

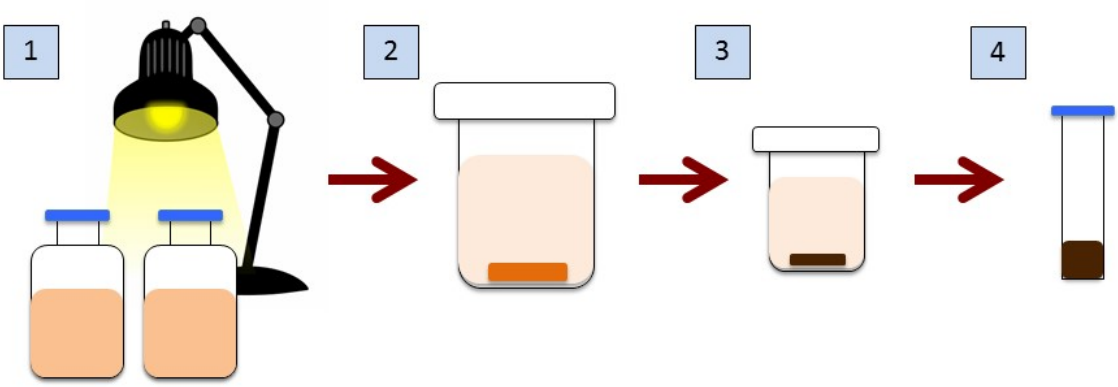



Cell harvest conducted quickly under $N_2$ gas and on ice to minimize disruption to cells

(from Kay Millerick PowerPoint Presentation)

## Preparing 2mL of *R. sphaeroides* for suspension:

4. Liquid is decanted, 1.5 mL of phosphate buffer is added, cells + buffer are transferred to an anoxic tube on ice. Cells + buffer is approximately 2mL.

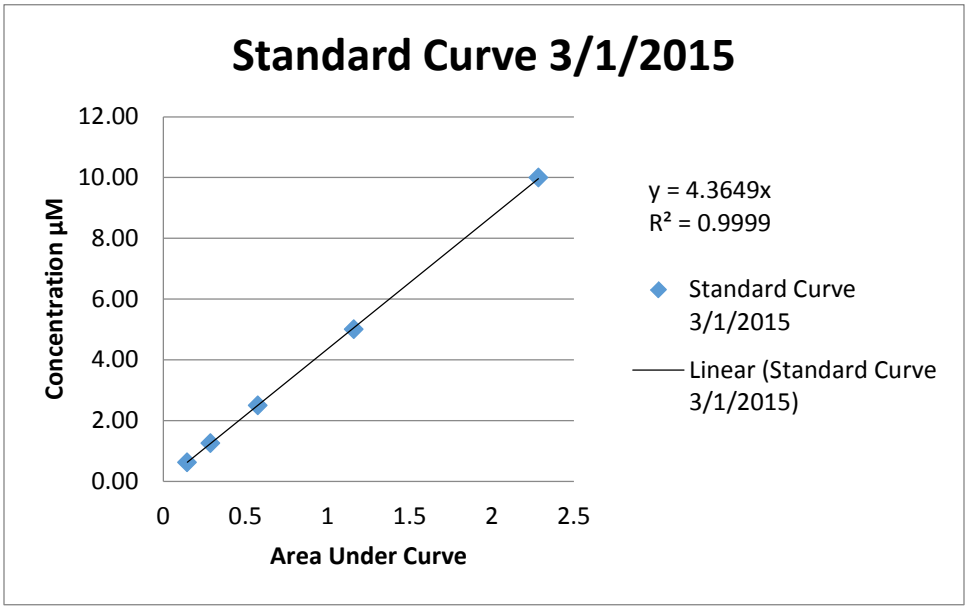



Cell harvest conducted quickly under $N_2$ gas and on ice to minimize disruption to cells

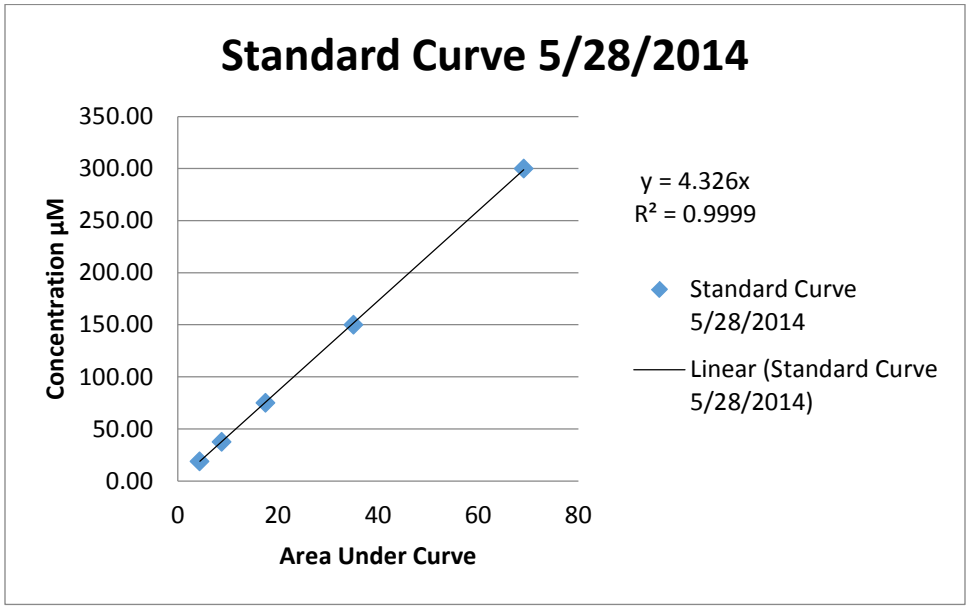
(from Kay Millerick PowerPoint Presentation)

## APPENDIX E: Sample Standard Curves for RDX, MNX, DNX, and TNX

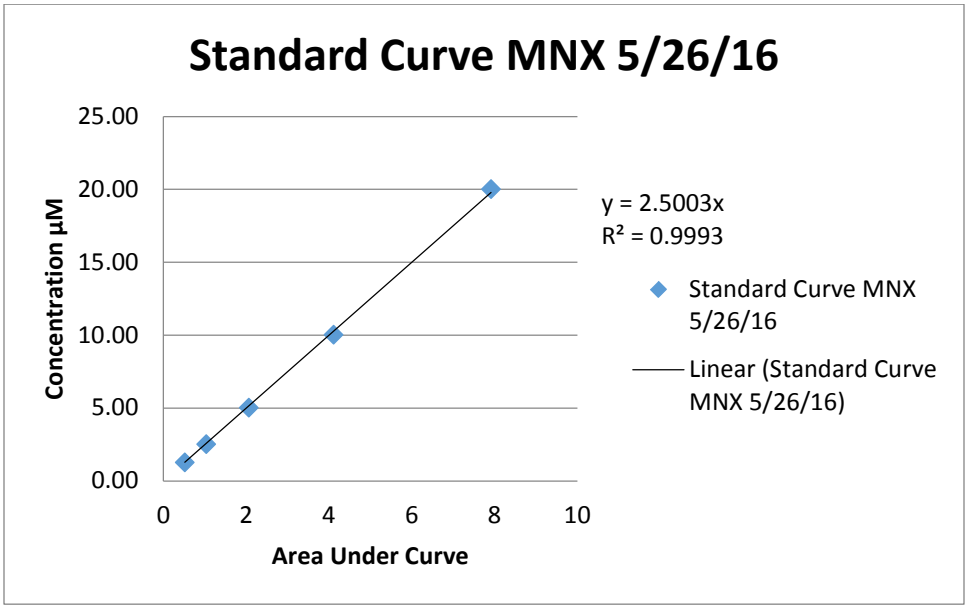
| Standard Curve 3/1/2015 | |
|---|---|
| Standards µM | Area Under Curve |
| 0.63 | 0.1449 |
| 1.25 | 0.2879 |
| 2.50 | 0.576 |
| 5.00 | 1.1592 |
| 10.00 | 2.2829 |



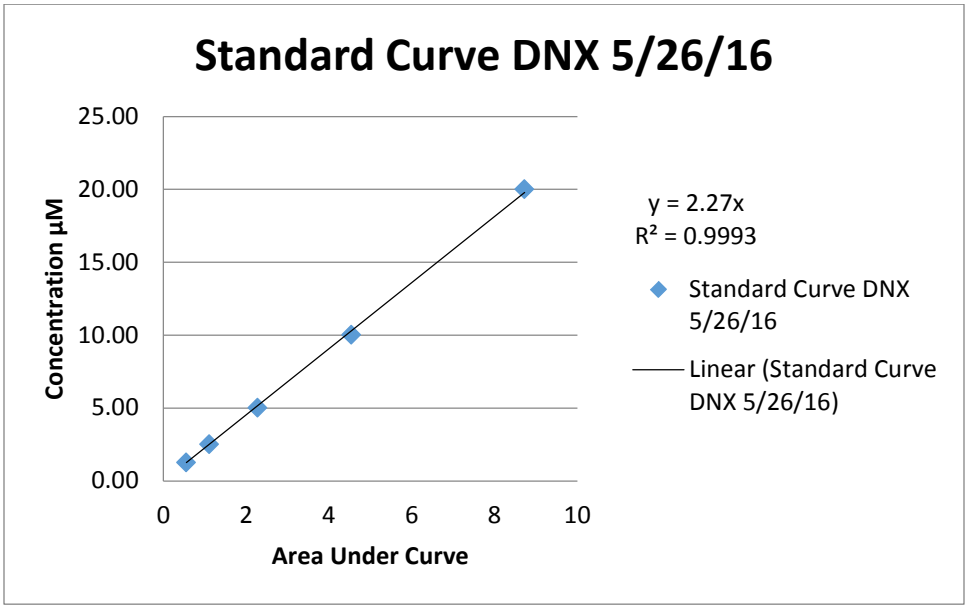
| Standard Curve 5/28/2014 | |
|---|---|
| Standards µM | Area Under Curve |
| 18.75 | 4.3608 |
| 37.50 | 8.731 |
| 75.00 | 17.486 |
| 150.00 | 35.087 |
| 300.00 | 69.0909 |



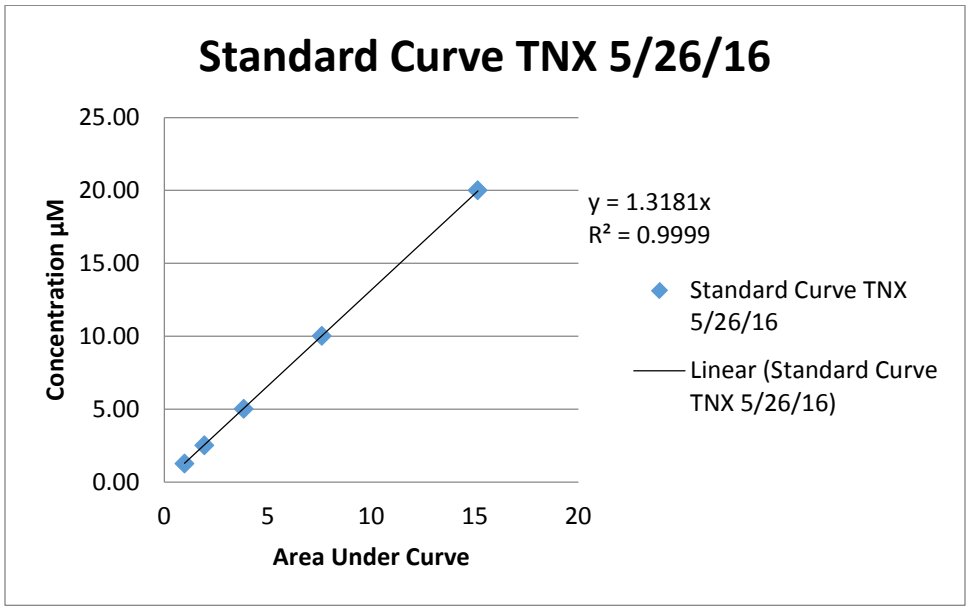
| Standard Curve MNX 5/26/16 | |
|---|---|
| Standards µM | Area Under Curve |
| 1.25 | 0.5173 |
| 2.50 | 1.0377 |
| 5.00 | 2.0662 |
| 10.00 | 4.1142 |
| 20.00 | 7.9159 |



| Standard Curve DNX 5/26/16 | |
|---|---|
| Standards µM | Area Under Curve |
| 1.25 | 0.5517 |
| 2.50 | 1.109 |
| 5.00 | 2.2752 |
| 10.00 | 4.5357 |
| 20.00 | 8.7227 |



| Standard Curve TNX 5/26/16 | |
|---|---|
| Standards µM | Area Under Curve |
| 1.25 | 0.9739 |
| 2.50 | 1.9422 |
| 5.00 | 3.8429 |
| 10.00 | 7.6107 |
| 20.00 | 15.1406 |



**APPENDIX F: Picture of Experimental Test Tubes at Day 7 of RDX (5µm) Degradation with Synechocystis in 10mM HEPES**

Picture Experimental Test Tubes at day 7 of RDX (5µM) Degradation with *Synechocystis* in 10mM HEPES Buffer experiment. Left 3 test tubes (milky white) are light + RDX + 0.2mL *Synechocystis* capped in air; middle 3 test tubes (dark green) are light + RDX + 0.2mL *Synechocystis* anaerobic; right 3 test tubes (yellow, orange) are light + RDX + 0.2mL *Synechocystis* + AQDS capped in air.

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