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Uptake and fate of hexahydro-1,3,5-trinitro-1,3,5-triazine by *Chrysopogon zizanioides*

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UPTAKE AND FATE OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE BY
CHRYSOPOGON ZIZANIOIDES

By

Claire M. Doskey

A THESIS

Submitted in partial fulfillment of the requirements for the degree of

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(Biological Sciences)

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2012

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This thesis, "Uptake and Fate of hexahydro-1,3,5-trinitro-1,3,5-triazine by *Chrysopogon zizanioides*," is hereby approved in partial fulfillment of the requirements for the Degree of MASTER OF SCIENCE IN BIOLOGICAL SCIENCES

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Abstract

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a nitramine compound that has been used heavily by the military as an explosive. Manufacturing, use, and disposal of RDX have led to several contamination sites across the United States. RDX is both persistent in the environment and a threat to human health, making its remediation vital. The use of plants to extract RDX from the soil and metabolize it once it is in the plant tissue, is being considered as a possible solution. In the present study, the tropical grass *Chrysopogon zizanioides* was grown hydroponically in the presence RDX at 3 different concentration levels: 0.3, 1.1, and 2.26 ppm. The uptake of RDX was quantified by high performance liquid chromatography (HPLC) analysis of media samples taken every 6 hr during the first 24 hr and then daily over a 30-day experimental period. A rapid decrease in RDX concentration in the media of both controls and plant treatments was seen within the first 18 hours of the experiment with the greatest loss in RDX over time occurring within the first 6 hours of exposure. The loss was similar in both controls and plant exposures and possibly attributed to rapid uptake by the containers. A plant from one treatment at each of the three concentrations was harvested at Day 10, 20 and 30 throughout the experiment and extracted to determine the localization of RDX within the tissue and potentially identify any metabolites on the basis of differing retention times. Of the treatments containing 0.3, 1.1, and 2.26 ppm RDX, 13.1%, 18.3%, and 24.2% respectively, was quantified in vetiver extracts, with the majority of the RDX being localized to the roots. All plants not yet harvested were harvested on Day 30 of the experiment. A total of three plants exposed to each concentration level as well as the control, were extracted and analyzed with HPLC to determine amount of RDX taken up, localization of RDX within the plant tissue, and potentially identify any metabolites. Phytotoxicity of RDX to vetiver was also monitored. While a loss in biomass was observed in plants exposed to all the different concentrations of RDX, control plants grown in media not exposed to RDX showed the greatest biomass loss of all the treatments. There was also little variation in chlorophyll content between the different concentration treatments with RDX. This preliminary greenhouse study of RDX uptake

by *Chrysopogon zizanioides* will help indicate the potential ability of vetiver to serve as a plant system in the phytoremediation of RDX.

Chapter 1

Introduction and Objectives

1.1 RDX Contamination and Toxicity

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a nitramine compound that was once used heavily by the military as an explosive (Table 1.1). It has also been used commercially in coal mining (Chen et al. 2011). Such heavy use and therefore production of RDX has contributed to sites that are heavily contaminated with RDX, among other energetic compounds, such as TNT and HMX (Yoon 2005). RDX is highly soluble in water, which contributes to its ability to leach into the groundwater from the soil (ATSDR 1995). Several military sites in the U.S. have high levels of RDX contamination that surpass the maximum contaminant level for drinking water, 0.1 mg/L (Etinier 1989). RDX contamination largely results from the manufacturing process and from improper disposal (Best et al. 1999; Rao et al. 2009; Chen et al. 2011). RDX is persistent in soil and groundwater and is considered toxic, affecting the central nervous system, gastrointestinal and renal system in humans (Etinier 1989). Such environmental persistence and threat to human health has led to a need for remediation of the contaminated sites, which are considered 16 on the national priority list for superfund cleanup sites across the U.S. (ATSDR 1995).

1.2 Past Methods of RDX Remediation

Past methods of remediating explosive-contaminated soil include: open burning/open detonation (OB/OD), adsorption onto activated carbon or resin, advanced photooxidation (UV/O₃), biodegradation, composting, and chemical treatment (Card and Autenrieth et al. 1998; Schnoor et al. 2006). Phytoremediation is a promising alternative to these past remediation methods as it is economical, environmentally friendly, and is thought to be a particularly effective method for removing low concentrations of contaminants that are spread over a large area, which matches as a good remediation method for explosives contamination because it is wide-spread, diffuse and heterogenous

within the contamination sites (Schnoor et al. 2006). Some existing concerns with phytoremediation as a clean-up method include: how long the contaminants remain in the plant tissue as well as whether or not they are metabolized or degraded. In some instances the metabolites and degradation products of the contaminants are equally or more toxic than the original contaminant and this could have an impact on both human and environmental health.

1.3 Phytoremediation of RDX

1.3.1 “Green Liver” Model

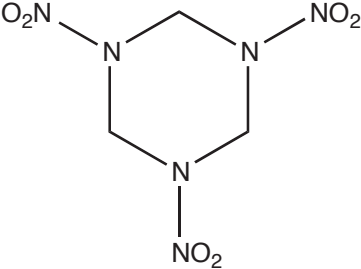
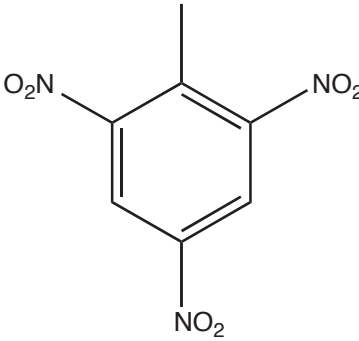
Previous research indicates plants are able to uptake several contaminants, such as metals, organic compounds and explosives from soil and groundwater. The “Green Liver” model is a concept developed to describe the transformation process of xenobiotic pollutants once they are taken up from the soil by plants. It is proposed that the process of how plants deal with contaminants is similar to how the human liver metabolizes toxicants. The 3 steps proposed in this model include: initial transformation or “activation” of contaminant by several reactions such as, oxidation, reduction, or hydrolysis, followed by conjugation of activated compounds with plant molecules such as D-glucose, glutathione, or amino acids to produce soluble or insoluble substances, which are subsequently sequestered in cellular compartments of the plant for storage and compartmentalization (Schnoor et al. 2006; Yoon et al. 2005). The soluble compounds are stored in vacuoles or as cell wall material, whereas the insoluble compounds are likely incorporated into the cell wall material (Yoon et al. 2005). Several plant enzymes are responsible for these processes and will be discussed in following sections.

1.3.2 Physical and Chemical Properties of RDX and TNT

The ability of plants to uptake and potentially metabolize xenobiotics and pollutants from the soil is largely dependent on the physical and chemical properties of those specific compounds. While phytoremediation of RDX has not been extensively studied, phytoremediation and subsequent plant metabolism of TNT has been studied in

great detail and it is assumed that phytoremediation of RDX will be similar. Table 1.1 compares the physical and chemical properties of RDX and TNT that are largely related to a plant's ability to uptake the compounds from soil. In order for a compound to be taken up by a plant it must first be able to pass through the membrane of the roots, which is largely dependent on the logarithm of the compound's octanol water partition coefficient, K_{OW} (Yoon et al. 2005). Various studies have looked at this relationship and have indicated that hydrophilic compounds, those with $\log K_{OW}$ of less than 1.8, are not able to pass through the lipid-rich membranes of roots, while hydrophobic compounds with $\log K_{OW}$ greater than 3.8 will be taken up into the roots, but will not be translocated to the shoots (Yoon et al. 2005). The $\log K_{OW}$ of RDX and TNT differ, with RDX having half the $\log K_{OW}$ of TNT (Table 1.1). The water solubility of TNT is more than double that of RDX (Table 1.1). A major difference in the two energetic compounds' properties is in the logarithm of their soil organic carbon-water coefficient (K_{OC}), as the $\log K_{OC}$ of TNT is over a hundred-fold greater than RDX (Table 1.1). For this reason, TNT will more strongly adsorb to other organic matter in the soil, whereas RDX mainly moves deeply through the soil to the groundwater (Kalderis et al. 2001).

Table 1.1 Physical and Chemical Properties of RDX and TNT (USEPA 2011)

Property	RDX	TNT
State at Room Temperature	White crystalline solid	Yellow, odorless solid
Molecular Weight (g/mol)	222	227
Water solubility (mg/L)	42 (at 20°C)	130 (at 25°C)
Octanol-water partition coefficient log(K _{ow})	0.87	1.6
Soil organic carbon-water coefficient log(K _{oc})	1.80	300
Vapor pressure at 25°C (mm Hg)	4.0 x 10 ⁻⁹	1.99 x 10 ⁻⁴
Henry's Law Constant (atm·m ³ /mol)	1.96x10 ⁻¹¹ (at 25°C)	4.57x10 ⁻⁷ (at 20°C)
Molecular Structure		

1.3.3 Uptake of RDX by Plants, Localization, and Metabolism

As shown in Table 1.1, RDX and TNT differ in molecular structure, with RDX being a nitramine and TNT a nitroaromatic compound. Such structural differences have led to differences in their uptake rates and fates in plants (Yoon et al. 2005). Past studies of the phytoremediation of RDX and TNT from hydroponic systems have shown that 95% of TNT was removed from a hydroponic system within the first 24 hours of exposure, whereas 71% of RDX was removed within 7 days of the initial exposure (Thompson et al. 1998; Thompson et al. 1999). In addition, uptake of pollutants from soil is expected to be much slower than from a hydroponic system because the pollutant is less available when in soil (Yoon et al. 2005). The RDX was found to be translocated to the leaves of plants after it is taken up from the soil (Schnoor et al. 2006). Some of the transformation products found in plants include de-nitrated compounds of RDX such as

hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazazine (TNX), along with other products such as: 4-nitro-2,4-diazabutanal, formaldehyde, methanol, nitrous oxide, and nitrite, and higher molecular weight and polar metabolites that have not yet been identified (McCormick et al. 1981; Schnoor et al. 2006). These unidentified transformation products could be the result of conjugation during the plant's detoxification process (Schnoor et al. 2006). Some conjugated products following plant uptake of RDX have been found in reed canary grass, using mass spectral analysis (Just and Schnoor 2004). RDX was transformed in plants by 3 main mechanisms: chemical reduction, degradation into intermediate metabolites, and complete mineralization to CO₂ (Schnoor et al. 2006). Figure 1.1 shows the proposed mechanism of RDX transformation and degradation.

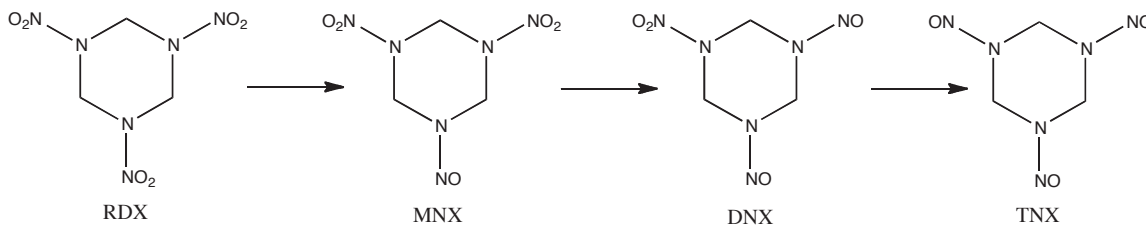


Figure 1.1 Reduction of RDX to nitroso intermediates MNX, DNX, and TNX. Each of these products can undergo further anaerobic degradation, ring cleavages, and decomposition to methanol (Adapted from McCormick et al. 1981).

Figure 1.2 is a conceptual model of RDX partitioning within a plant. The compound must first be taken up into the roots of the plant where it can be translocated into the shoots and more apical tissues of the plant. Each tissue has a storage capacity for the compound defined by K_{OC} . If the compound is metabolized by the plant or lost through the transpiration stream or decomposed due to photolysis, the plant will take up additional RDX from solution. It may also be possible for the plant to excrete the compound back into the soil or hydroponic media.

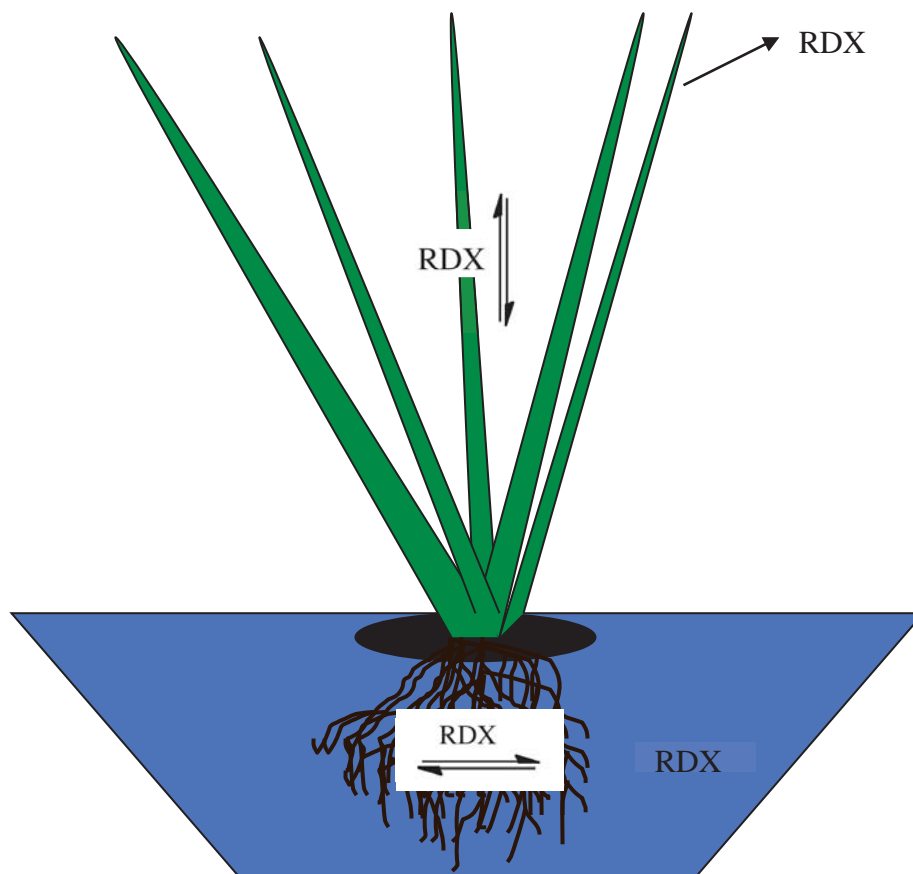


Figure 1.2 Conceptual model of RDX partitioning in plants.

1.3.4 Enzymes involved in RDX Degradation

Nitroreductases are responsible for catalyzing the reduction of the nitro groups in RDX into compounds that may be easier to degrade by the plants and could be more or less toxic in general (Schnoor et al. 2006). These enzymes have also been indicated in the reduction of nitro groups in both HMX and TNT (Schnoor et al. 2006). Other enzymes that might be involved in the activation step of metabolism and/or degradation of RDX include Cytochrome P450 mono-oxygenases and peroxidases, which are responsible for the catalysis of oxidation in HMX, RDX and TNT and also the catalysis of oxidation in the reduced derivatives (Schnoor et al. 2006). Glutathione S-transferases are involved in the conjugation step of explosive contaminant metabolism, as they catalyze the

conjugation of the activated derivatives of these explosives to forms that are much less toxic (Schnoor et al. 2006).

1.4 *Chrysopogon zizanioides*

Chrysopogon zizanioides (vetiver) is a tropical grass that has been previously used in several phytoremediation studies due to its large biomass, marked by its expansive root system (extending 3 meters deep) and ability to grow in a wide range of extreme soil conditions (Makris et al. 2007). Vetiver is particularly tolerable to extreme environmental conditions: frost, heat, sodic, and saline conditions (Makris et al. 2007). In particular, Vetiver is both a hydrophyte and a xerophyte, meaning that is not affected by flood or drought, respectively (Makris et al. 2007). Such adaptability to grow under numerous conditions is ideal for phytoremediation, as many contamination sites are not ideal for plant growth. Vetiver has been successful in the uptake of TNT from hydroponic media in several lab and greenhouse experiments, and it has therefore been proposed for phytoremediation of RDX, due to the similarity in chemical structures (Table 1.1).

1.5 Phytotoxicity of RDX

Schnoor et al. 2006 tested whether exposure to RDX would be detrimental or show toxic effects to poplar. When exposed to 50 mg/L RDX for 24 hours, no visible toxicity was observed (Schnoor et al. 2006). The metabolism and degradation of RDX in plants varies between different species, so toxicity of RDX exposure will also vary among different plant species (Yoon et al. 2005). Phytotoxicity might also be sensitive to the length of exposure and should be investigated (Yoon et al. 2005).

1.6 Objectives

Vetiver has never been used for phytoremediation of RDX. The overall objective of the research was to demonstrate the ability of vetiver to uptake RDX from a hydroponic solution containing nutrients and RDX. This work will serve as a "proof of principle" for future research that will investigate uptake from a soil matrix. Within this

objective, was the goal to quantify the uptake of RDX at three different concentration conditions: 0.3, 1.1, and 2.26 ppm, by measuring RDX in media sampled from the treatments at different times throughout a 30-day exposure to RDX. The sampling schedule was designed to provide information on the rate of RDX uptake. Also included within this objective was to quantify RDX within the plant tissue. Vetiver was periodically harvested, extracted, and the RDX quantified by HPLC with UV detection. The distribution and location of RDX in vetiver was determined from plant extracts of roots and the lower, middle and top third of shoots. Another objective was to examine phytotoxicity of RDX to vetiver and sensitivity to RDX level in the hydroponic solution. Phytotoxicity of RDX to vetiver was monitored through observation of toxicity symptoms, biomass measurements, and chlorophyll content determination.

Chapter 2

Materials and Methods

2.1 Growth of Vetiver

Chrysopogon zizanioides were obtained from Floraland Farms and Nurseries (St. Cloud, FL) as bare root divisions. They were planted immediately upon arrival in Sunshine Professional Growing Mix 1, which does not contain added nutrients. Plants were grown in the soil for 72 days, extracted from the soil, the roots washed to remove soil particles, and then placed in the hydroponic media.



Figure 2.1 Vetiver growing in soil in greenhouse

Fifty-seven plants were cleaned of soil and rinsed with distilled deionized water to remove the majority of soil from the roots. The plants were then weighed and shoots were cut so that each plant had a mass of about 40g each, for a total mass of 120g (3 plants) per container in each hydroponic system.

Plants acclimatized in hydroponics system, containing 3 liters of half-strength Hoagland's solution for 14 days before treatment with RDX began.

2.2 Hydroponics Set-up

The experiment was located in the greenhouse partitioned by plastic sheeting. Temperatures averaged 70-90°F and a 16-h light/ 8-h dark schedule was used. Six-liter plastic Sterilite© containers were covered in black plastic to limit photolysis of RDX in the hydroponic media. A small hole was drilled into the side of the container to fit a piece of aquarium tubing, to which an aquarium pump was connected. Three 2-in diameter holes were drilled into the lid of the container to expose shoots of the vetiver. Each system contained 3 L of half-strength Hoaglands solution for the nutrient media.



Figure 2.2: Hydroponics set-up.

2.3 RDX Treatments

2.3.1 Chemicals

1000 $\mu\text{g}/\text{mL} \pm 5\%$ Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in acetonitrile was purchased from ChemService (99% purity). HPLC Grade solvents were used during the HPLC analyses.

2.3.2 Treatment Concentrations

A 1-L stock solution of 40 ppm RDX was prepared with distilled deionized water that was filtered through the MilliQ system. The RDX was purchased as a 1000 $\mu\text{g mL}^{-1}$ solution (99% purity) in acetonitrile (ChemService, Westchester, PA). Concentrations of 2.26, 1.1, and 0.3 ppm were used for the RDX treatments, all of which were prepared from the 40 ppm RDX stock solution by adding the appropriate amount of RDX stock solution to the Hoagland's nutrient media. The 30-day experimental period began upon addition of the 40 ppm RDX stock to the hydroponics system. The RDX treatments were prepared with fresh Hoagland's nutrient media day 1 of the experimental period. Similar concentrations of RDX treatments have been used in previous hydroponic studies of RDX remediation.

2.3.3 Replicates

Three replicates of each treatment were sampled. Three replicates of a control containing no RDX was also included along with a control at each of the RDX concentrations with no plants. Another control containing acetonitrile without RDX was sampled to evaluate the toxicity of acetonitrile on the plant. The same quantity of acetonitrile that was in the 0.3, 1.1, and 2.26 ppm RDX treatment was added to the acetonitrile controls: 18.75, 75, and 150mL 40ppm acetonitrile respectively. The treatments were arranged so that a randomized block experimental design was achieved.

2.4 Media Samples

2.4.1 Sample Collection

Nutrient media (2 mL) was collected from each treatment and the control according to the following schedule: every 6 h for the first 72 h, - every 12 h for days 4-10, and every 24 hours for the remainder of the 30-d exposure period. Samples were stored at 4°C until HPLC analysis.

2.4.2 Sample Preparation for HPLC

Immediately prior to HPLC analysis, 1 mL of HPLC grade acetonitrile (Fisher Scientific, Pittsburgh, PA) was added to 1mL of media sample. Samples were shaken for about 1 min and filtered through a 0.45 µm nylon syringe filter (Fisher Scientific). Filtered samples were stored in microcentrifuge tubes immediately prior to analysis.

2.5 Plant Samples

2.5.1 Harvesting Vetiver

Plants were harvested by removing them from the appropriate RDX treatment. The plants were rinsed with DDI Millipore water to remove RDX residues, blotted dry, and weighed. The length of the shoots and roots of each plant were recorded. The roots were removed from the shoots and weighed separately. Root tissue was stored in 50-mL centrifuge tubes at -80°C. Plant shoots were cut into thirds and stored in 50-mL centrifuge tubes at -80°C.

2.5.2 Plant Sample Processing and Extraction

To extract RDX from plant tissue, the frozen plant samples were allowed to come to room temperature. Plants samples were prepared for extraction by cutting the plants into small pieces, freeze-drying with liquid nitrogen, and grinding the pieces to a fine dust with a mortar and pestle. The ground plant tissue was weighed to obtain the dry mass of the plant sample. A 2 g sub-sample of the ground plant tissue was suspended in 8

mL of HPLC grade acetonitrile and mixed for 1 min using a Vortex mixer. Samples were stored overnight at 4°C, sonicated the next day at 25°C for 17 hours, and centrifuged at 2500 rpm for 5 min in preparation for sample cleanup.

Plant extracts (2 mL) were eluted through Pasteur pipettes containing 0.25 g Florisil and 0.25 g Alumina, which were activated at 130°C, to remove chlorophyll and plant pigments that interfere with UV detection of RDX. The RDX was eluted from column with 2 mL HPLC grade acetonitrile and the extract filtered through a 0.45µm Nylon syringe filter.

2.5.3 HPLC Analysis of Media and Plant Samples

Hydroponic media samples and plant samples were analyzed on a Beckman Coulter System Gold HPLC (Beckman Coulter, Brea, CA) with a 125 Solvent module and 166 UV detector. A 5 µm LC-18-DB Supelcosil column (25 cm × 4.6 mm ID)) was used for the analyses (Supelco, St. Louis, MO). Organic species were resolved with an isocratic elution method using a 50:50 mixture of HPLC-grade methanol and DDI Millipore filtered water as the mobile phase. The RDX absorbance at 254 nm was measured with a UV detector. A 5-pt calibration curve was generated each day to quantify the RDX in media and plant samples. Calibration standards of RDX in acetonitrile were prepared from the stock solution (1000 µg mL⁻¹) at levels of 0.5, 1, 2, 3, and 4 ppm.

2.6 Plant Growth and Phytotoxicity Analysis

Growth parameters (i.e., plant biomass and root and shoot length) were measured and recorded as a means to determine plant health. Length, fresh weight and dry weight of roots and shoots were measured on days 0, 10, 20, and 30 for plants harvested during the experiment. Initial and final measurements of the growth parameters were recorded for all plants in the experiment. Spots of necrosis and chlorosis that indicate phytotoxic reactions were recorded and documented with photographs.

Whole plant chlorophyll content was determined by processing the middle third of a shoot. The shoot was ground to a fine powder and a 30-mg sub-sample was added to 10 mL of 80% acetone in DDI Millipore water. The sample was then centrifuged at 3000x g for 5 minutes. Chlorophyll was measured spectrophotometrically at 645 and 663 nm and quantified using the following equation:

$$\text{Total Chlorophyll} = (20.2 \times A_{645} + 8.02 \times A_{663}) \times \text{dilution factor (Vila et al. 2007)}$$

Chapter 3

Results and Discussion

3.1 RDX loss from Hydroponic Media

The RDX was rapidly taken up from media containing vetiver within the first 18 hours of exposure. Levels of RDX in the 2.26 ppm, 1.1 ppm, and 0.3 ppm RDX treatments were reduced to 0.68 ± 0.059 , 0.32 ± 0.039 , and 0.10 ± 0.0014 ppm respectively, during the first 18 hours of exposure (Figure 3.3). The greatest decrease in concentration was observed during the first 6 hours of RDX exposure. Concentrations of RDX in the 2.26 ppm, 1.1 ppm, and 0.3 ppm RDX treatments were reduced to 0.97 ± 0.027 , 0.61 ± 0.021 , and 0.20 ± 0.043 ppm respectively, (Figure 3.3). An approximate 50% loss in RDX from the initial amount in the hydroponic media is indicated by comparing HPLC chromatograms from the 2.26 ppm treatments at 0 h exposure and 6 h exposure (Figures 3.1, 3.2). However, a similar rapid decrease in RDX concentration was also seen in the controls, which did not contain plants (Figure 3.3-6).

After the rapid initial decrease in RDX concentration during the first 18 h, losses of RDX from the media are more gradual, becoming constant, and then increasing after 4 days (Figure 3.7). Media was not added during the first 10 d of the experiment, and thus, increases in RDX levels are attributed to evaporation of hydroponic media. The RDX behaved similarly in controls at all RDX exposure concentrations (Figure 3.7-10). Concentrations of RDX in the hydroponic media continued to increase over the remainder of the 30 d experiment in treatments with and without vetiver (Figure 3.11-14). Figure 3.15 shows the results of variation in RDX concentration in the hydroponic media at all of the concentrations, with and without plants, over the entire experimental period.

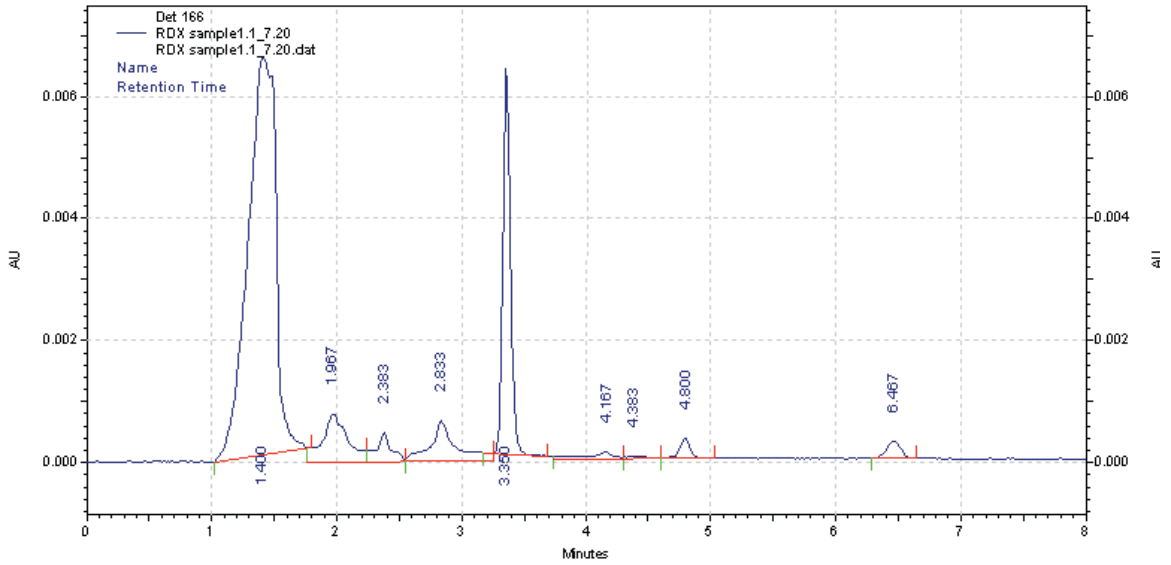


Figure 3.1 Chromatogram of media sample taken at 0 h from 2 ppm RDX treatment. The measured concentration was 2.32ppm.

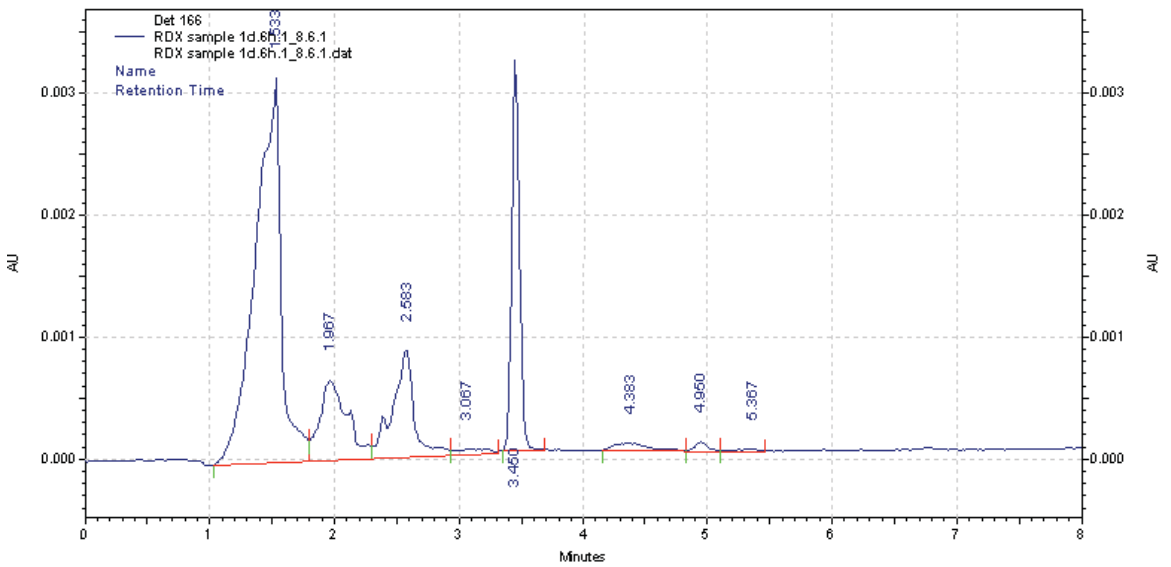


Figure 3.2 Chromatogram of media sample taken at 6 h from 2 ppm RDX treatment. The measured concentration was 0.928 ppm.

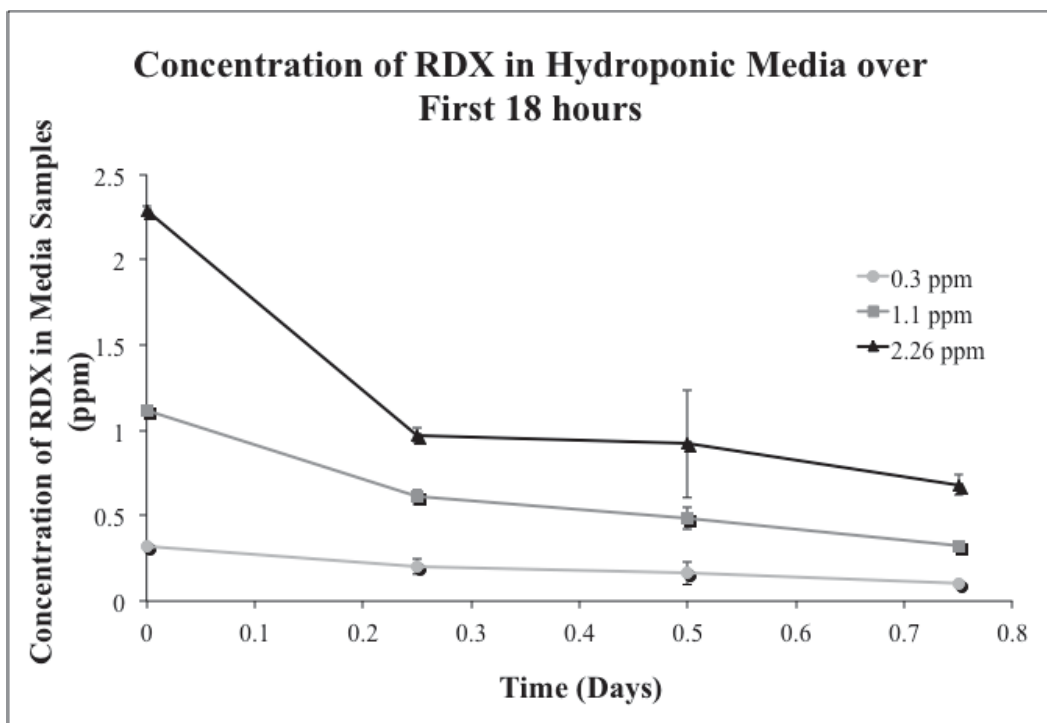


Figure 3.3 Measured concentrations of RDX in hydroponic media during the first 18 h of the experiment. Error bars represent the standard deviation for triplicate media samples.

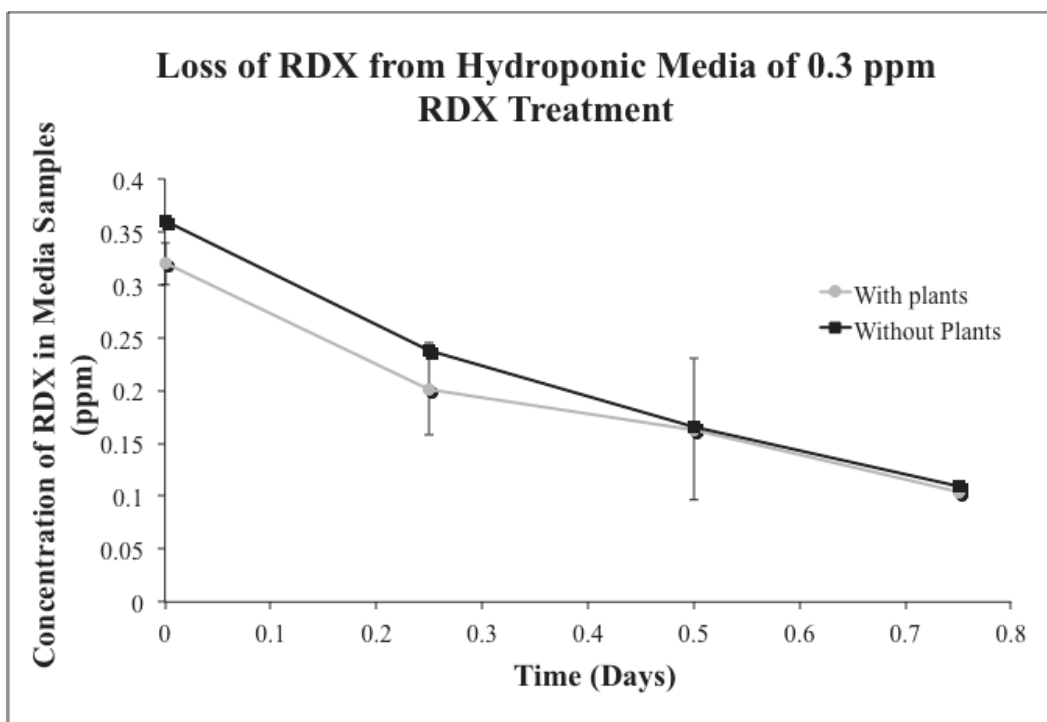


Figure 3.4 Concentrations of RDX in hydroponic media containing 0.3 ppm RDX treatments with and without plants. Error bars represent the standard deviation for triplicate media samples.

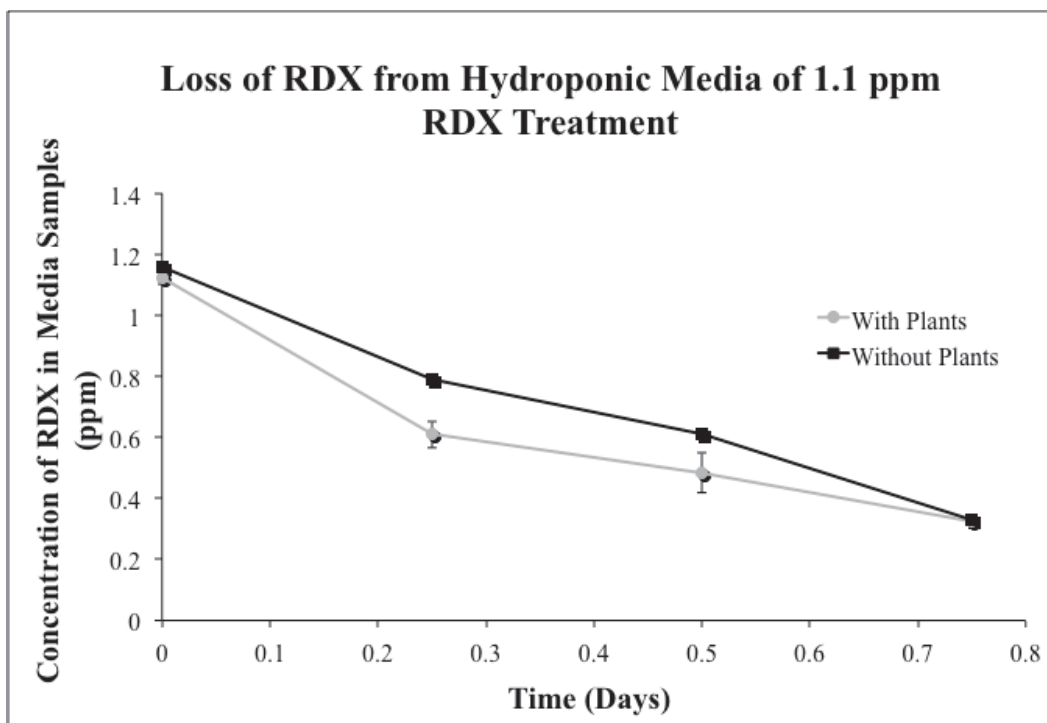


Figure 3.5 Concentrations of RDX in hydroponic media containing 1.1 ppm RDX treatments with and without plants. Error bars represent the standard deviation for triplicate media samples.

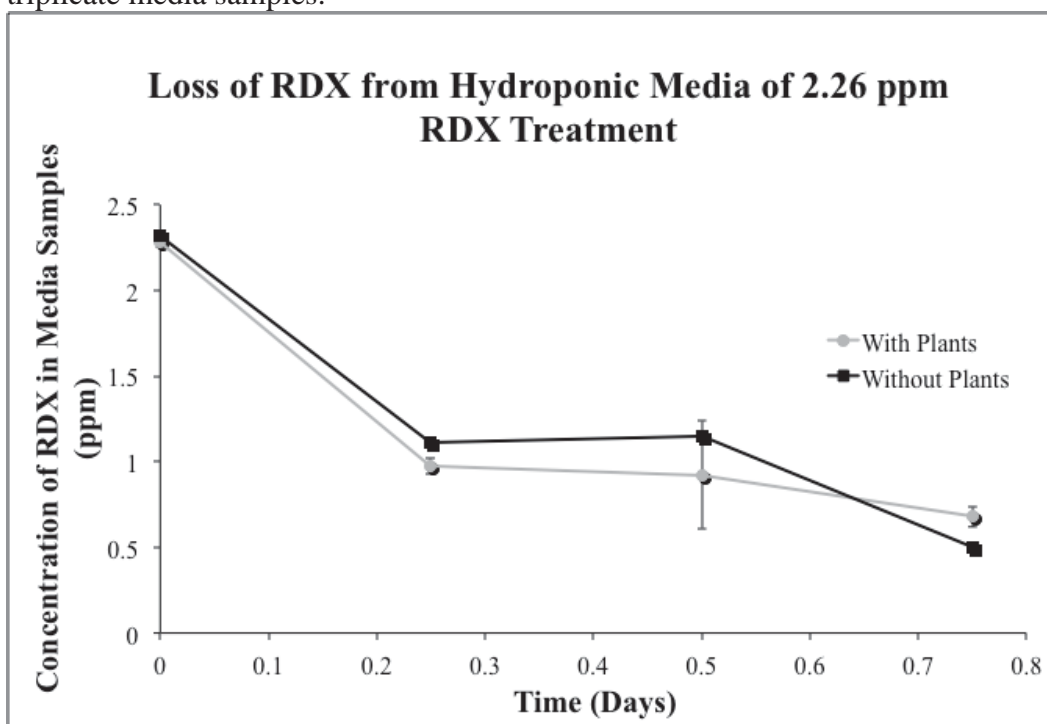


Figure 3.6 Concentrations of RDX in hydroponic media containing 2.26 ppm RDX treatments with and without plants. Error bars represent the standard deviation for triplicate media samples.

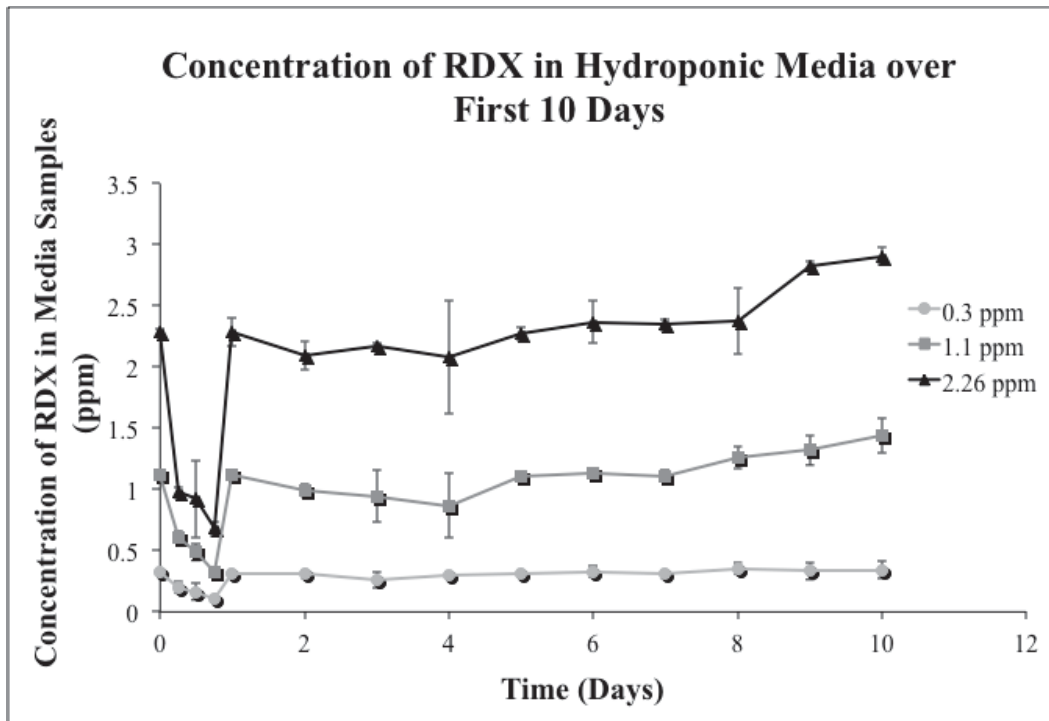


Figure 3.7 Concentrations of RDX in media samples from day 1 through day 10. Error bars represent the standard deviation for triplicate media samples.

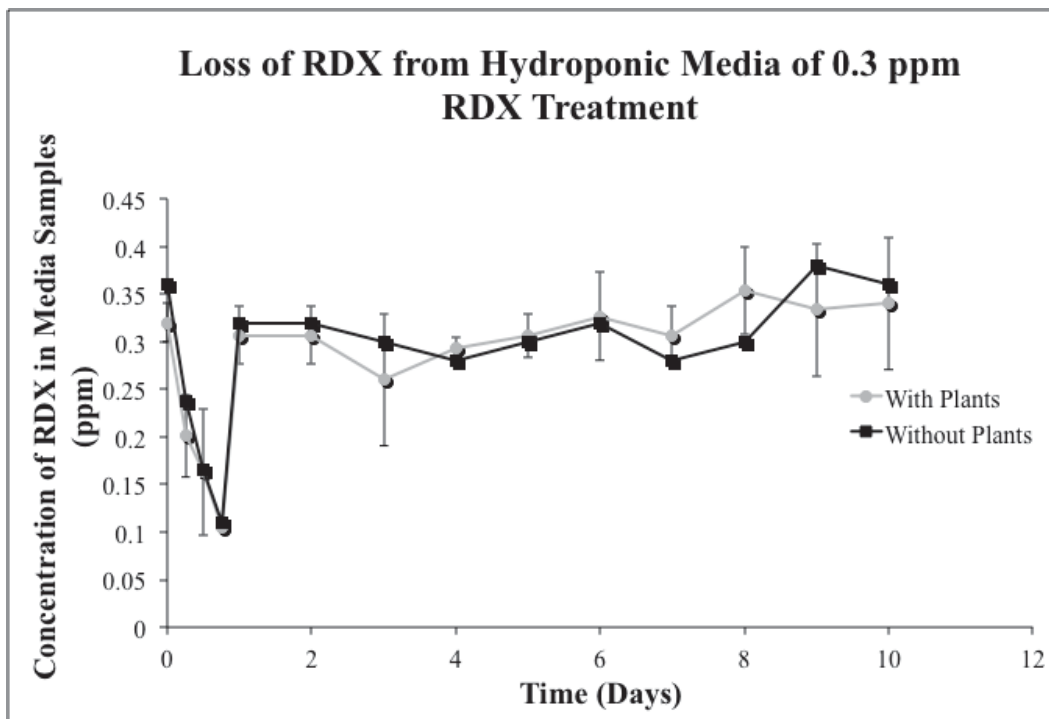


Figure 3.8 Loss of RDX from Hydroponic Media of 0.3 ppm RDX Treatment. Error bars represent standard deviation between triplicate media samples.

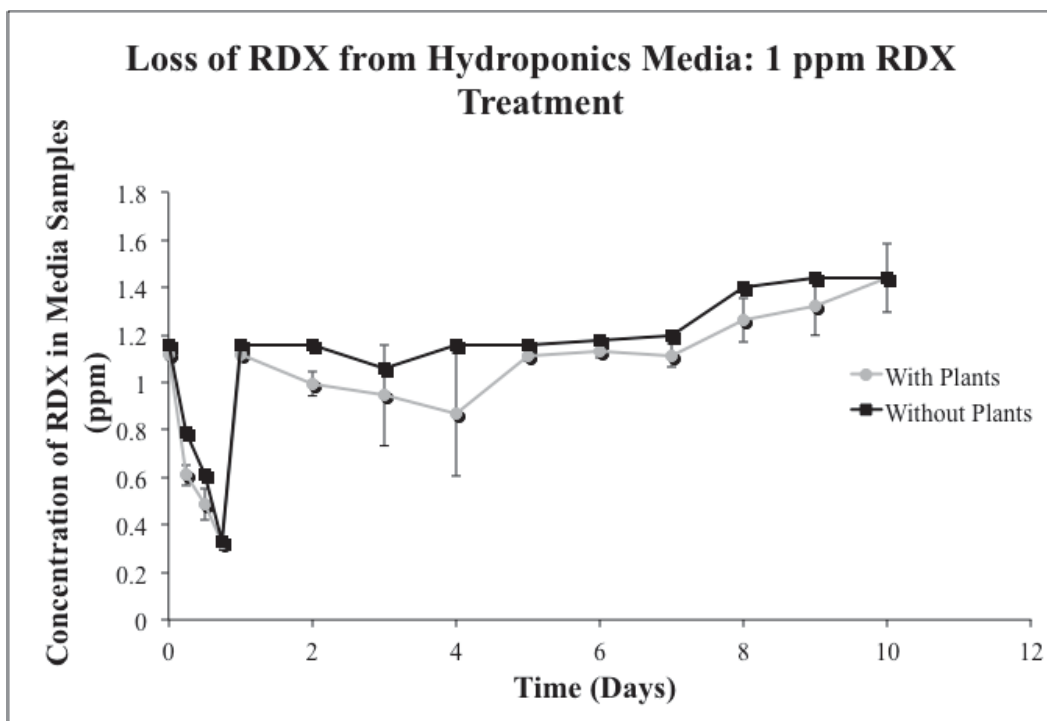


Figure 3.9 Loss of RDX from Hydroponic Media of 1.1 ppm RDX Treatment. Error bars represent standard deviation between triplicate media samples.

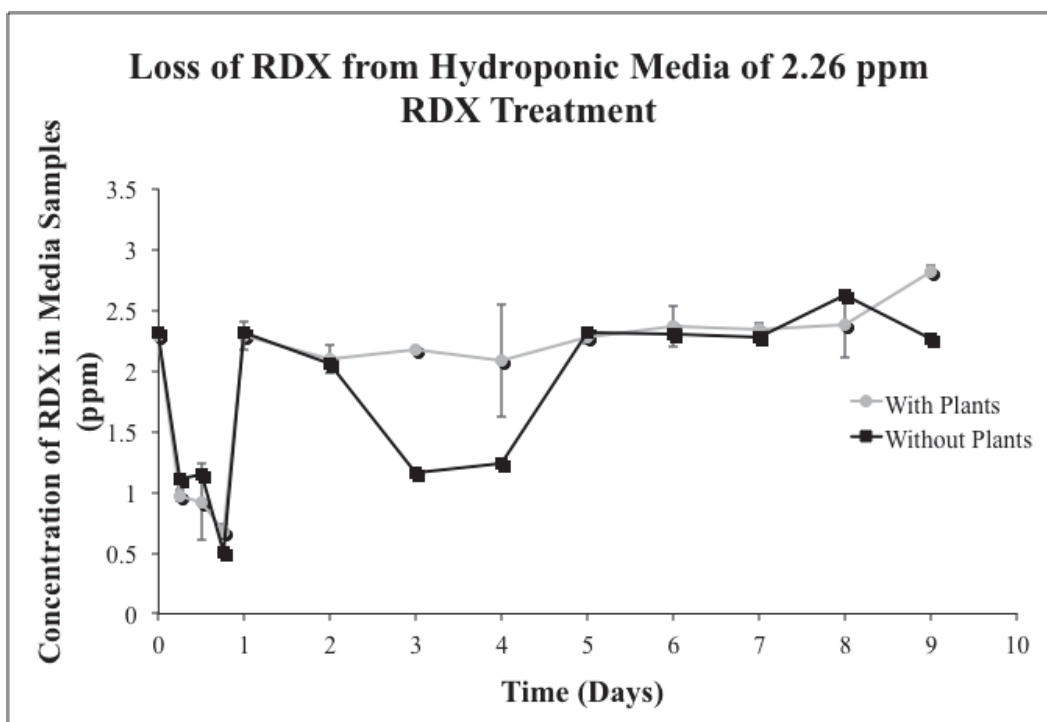


Figure 3.10 Loss of RDX from Hydroponic Media of 2.26 ppm RDX Treatment. Error bars represent standard deviation between triplicate media samples.

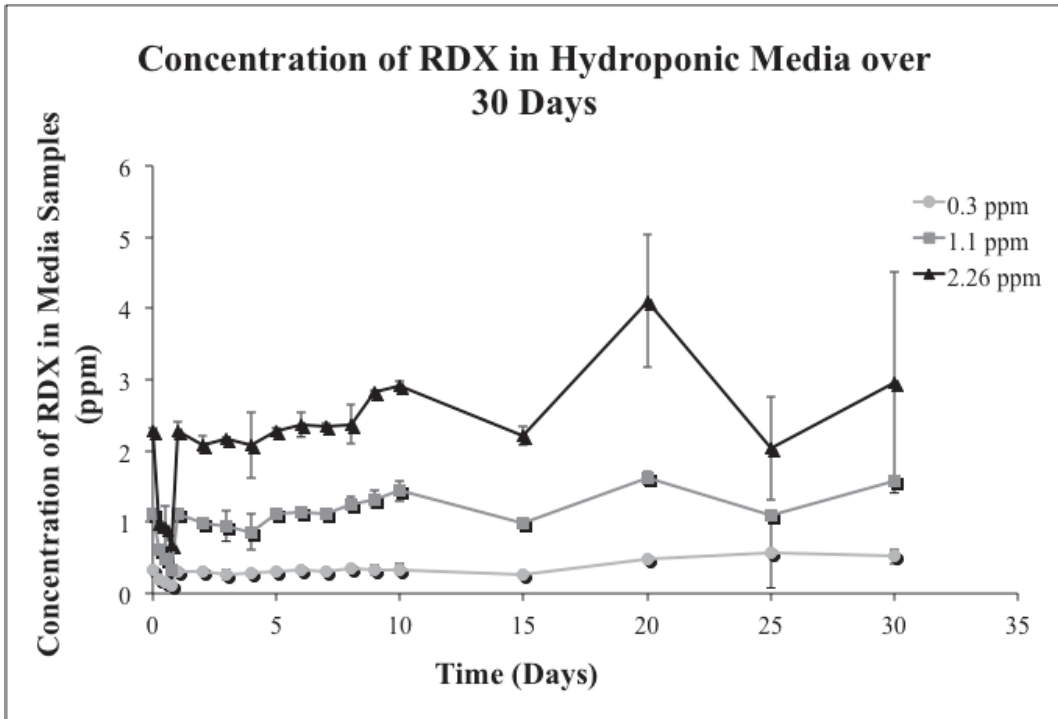


Figure 3.11 RDX in Media Samples Day 1-30. Error bars represent the standard deviation between duplicate samples.

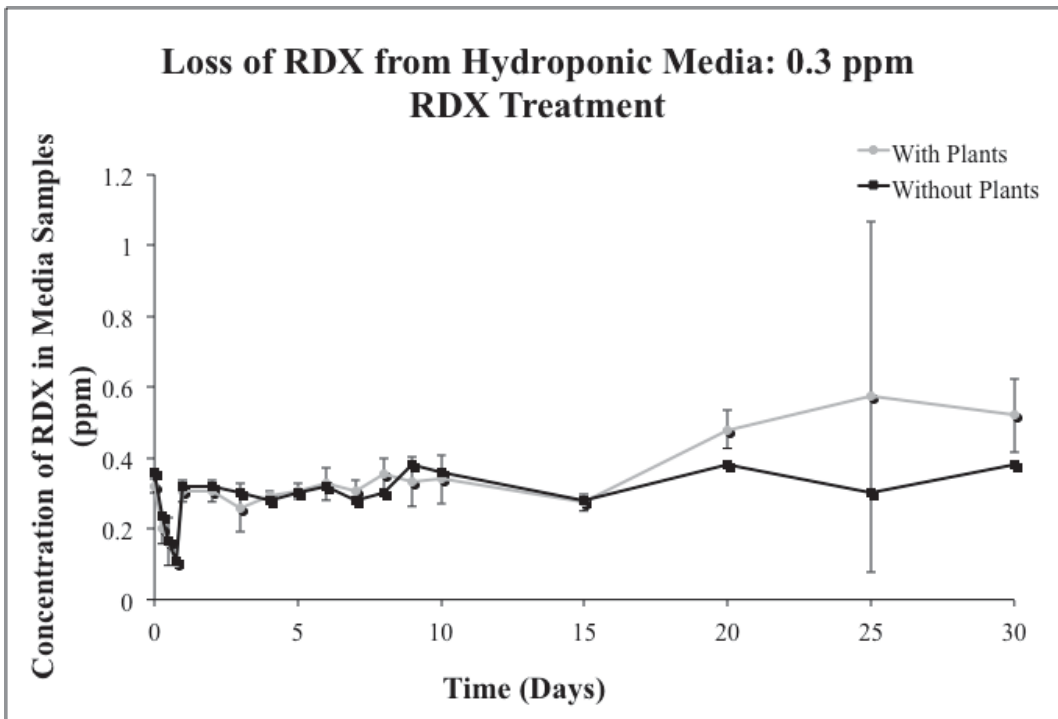


Figure 3.12 Loss of RDX from Hydroponic Media of 0.3 ppm RDX Treatment. Error bars represent standard deviation between duplicate media samples.

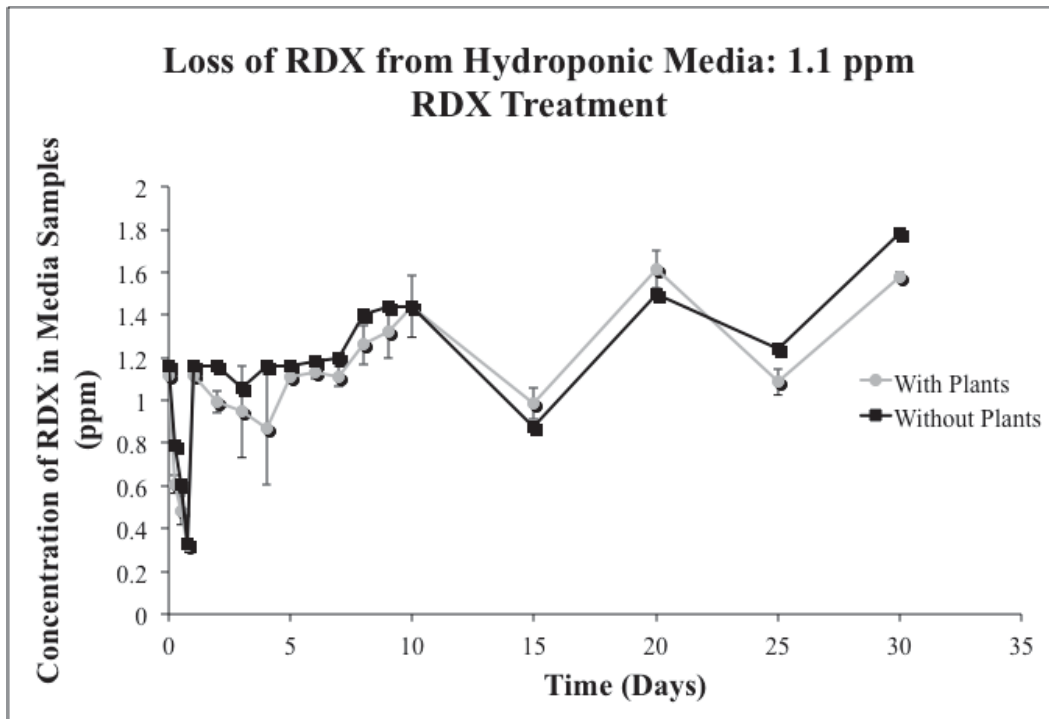


Figure 3.13 Loss of RDX from Hydroponic Media of 1.1 ppm RDX Treatment. Error bars represent standard deviation between duplicate media samples.

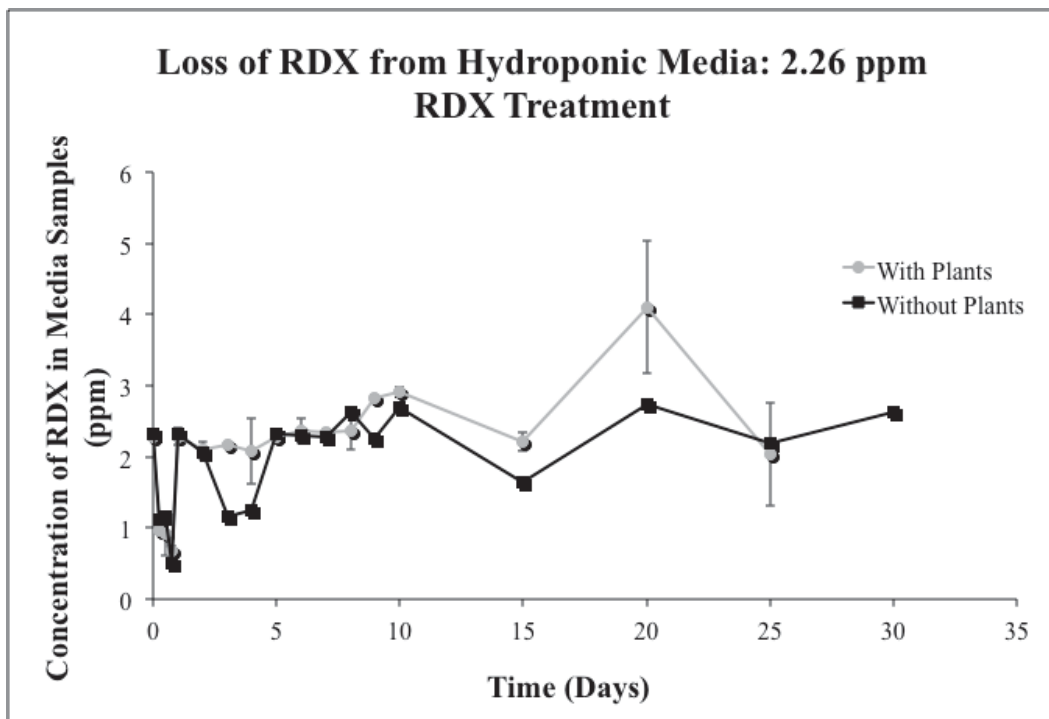


Figure 3.14 Loss of RDX from Hydroponic Media of 2.26 ppm RDX Treatment. Error bars represent standard deviation between duplicate media samples.

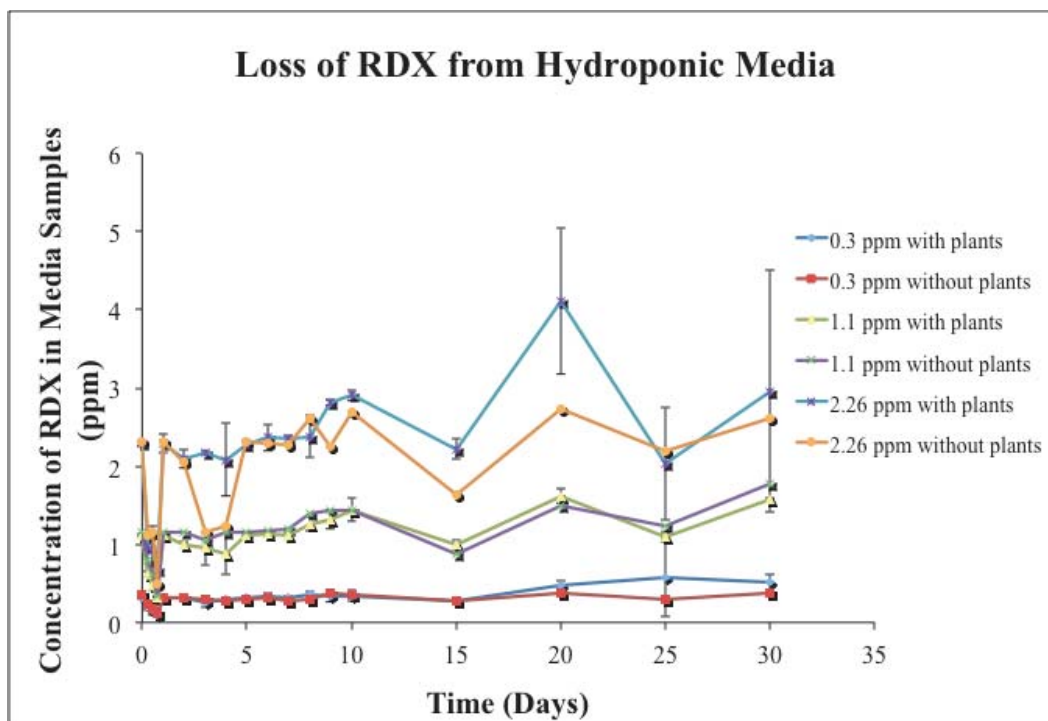


Figure 3.15 Compiled Results of RDX Loss from Hydroponic Media. Error bars represent standard deviation between triplicate media samples for days 1-10 and duplicate media samples for days 10-30.

Henry's law constants for RDX and water at 25°C are $2.0 \times 10^{-11} \text{ atm}\cdot\text{m}^3/\text{mol}$ and $5.63 \times 10^{-7} \text{ atm}\cdot\text{m}^3/\text{mol}$, respectively, indicating that water is more volatile than RDX, and thus, the concentration of RDX in water will actually increase as water evaporates (ATSDR 1995; Thomas 1982). By assuming RDX did not evaporate from the hydroponic media, levels of RDX in the plant tissue were used to calculate the theoretical loss of RDX from the hydroponic media (Figure 3.16; Table 3.1). The values were calculated by dividing the mass of RDX in the plant at 10, 20, and 30 d by 3L (i.e., initial volume of hydroponic media) and subtracting this value from the initial RDX concentration of the hydroponic media (Table 3.1).

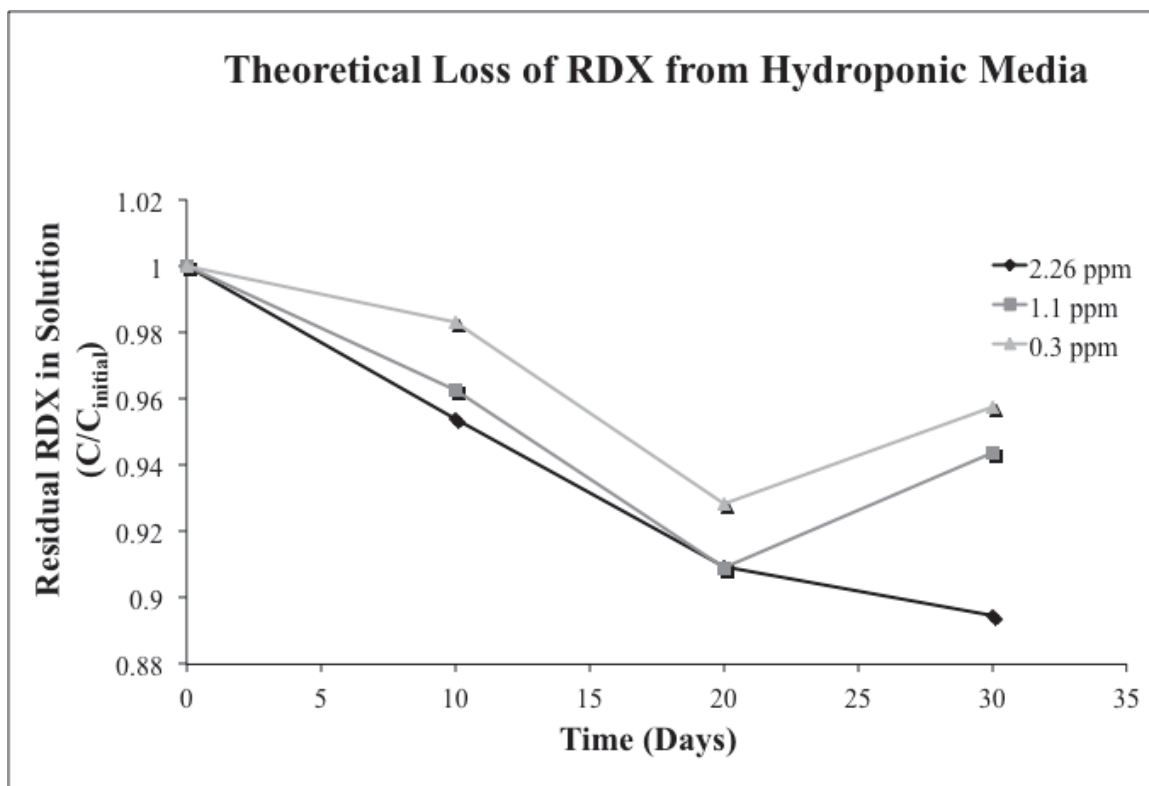


Figure 3.16 Theoretical Loss of RDX from Hydroponic media.

Table 3.1: Theoretical Uptake Values

Days	0.3 ppm RDX		1.1 ppm RDX		2.26 ppm RDX	
	mg of RDX in Plant	Calculated Concentration RDX remaining in Media (ppm)	mg of RDX in Plant	Calculated Concentration RDX remaining in Media (ppm)	mg of RDX in Plant	Calculated Concentration RDX remaining in Media (ppm)
10	0.01517	0.2949	0.1208	1.059	0.3141	2.155
20	0.06445	0.2785	0.2987	1.000	0.6150	2.055
30	0.03849	0.2872	0.1857	1.038	0.7131	2.022

3.2 RDX in Vetiver

3.2.1 Plant Uptake of RDX

Evaporation of the hydroponic media precluded quantification of the uptake of RDX via loss from the hydroponic media. Instead, uptake of RDX was quantified through measurement of RDX in the harvested plants. The large peak eluting at 3.550

min in the HPLC chromatogram was identified as RDX through addition of an RDX standard to a media sample (Figure 3.17). The two smaller peaks eluting at 4.317 and 5.050 min were only observed in plant extracts and might be degradation products of RDX, which are less polar than RDX and expected to elute later. Mass spectral analysis of the vetiver extracts would be necessary for positive identification of degradation products.

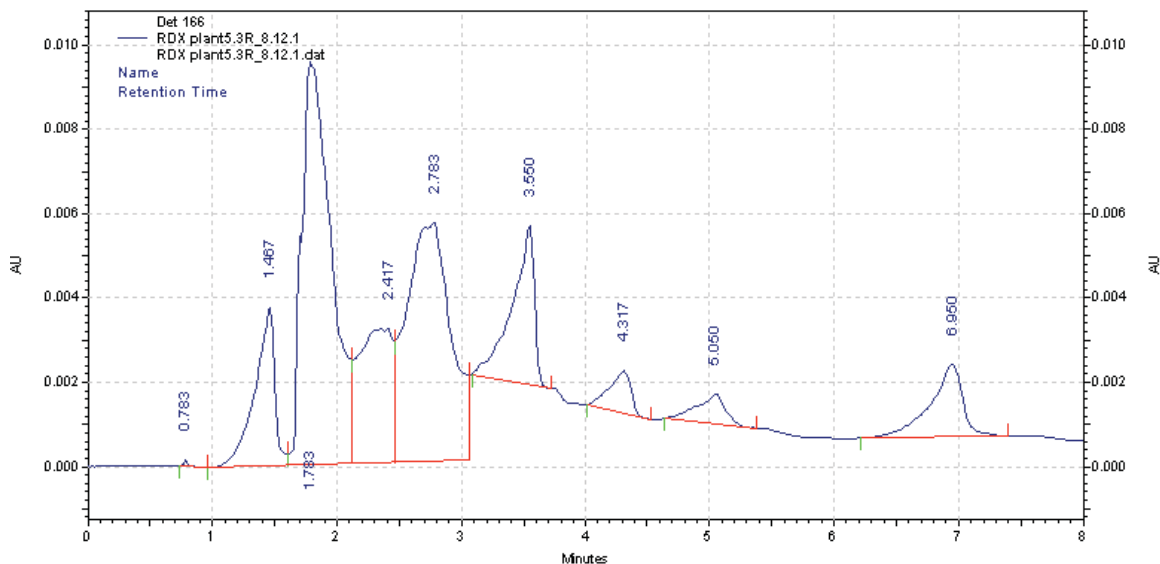


Figure 3.17 Chromatogram of root extract of Vetiver after 30 d exposure to 2.26 ppm RDX. Retention time of RDX is 3.550 min.

3.2.2 Location of RDX in Plant

The amount of RDX in plants harvested at 10, 20, and 30 d the was located in roots and shoots in nearly equal amounts (Figure 3.18). Greater than 75% of the RDX in plants harvested after 10 d of exposure was located in the roots and lower third of vetiver for each RDX treatment (Figure 3.19). Very little RDX made it to the upper third of the vetiver shoots (Figure 3.19). For plants harvested on day 20, the majority of RDX was located in the roots and lower third of vetiver shoots; however, more RDX was found in the middle and upper portion of the shoot tissue than was found in the 10 and 30 d exposures (Figure 3.20). Plants harvested after 30 d exhibited very similar results to plants exposed for 10 d (Figure 3.21).

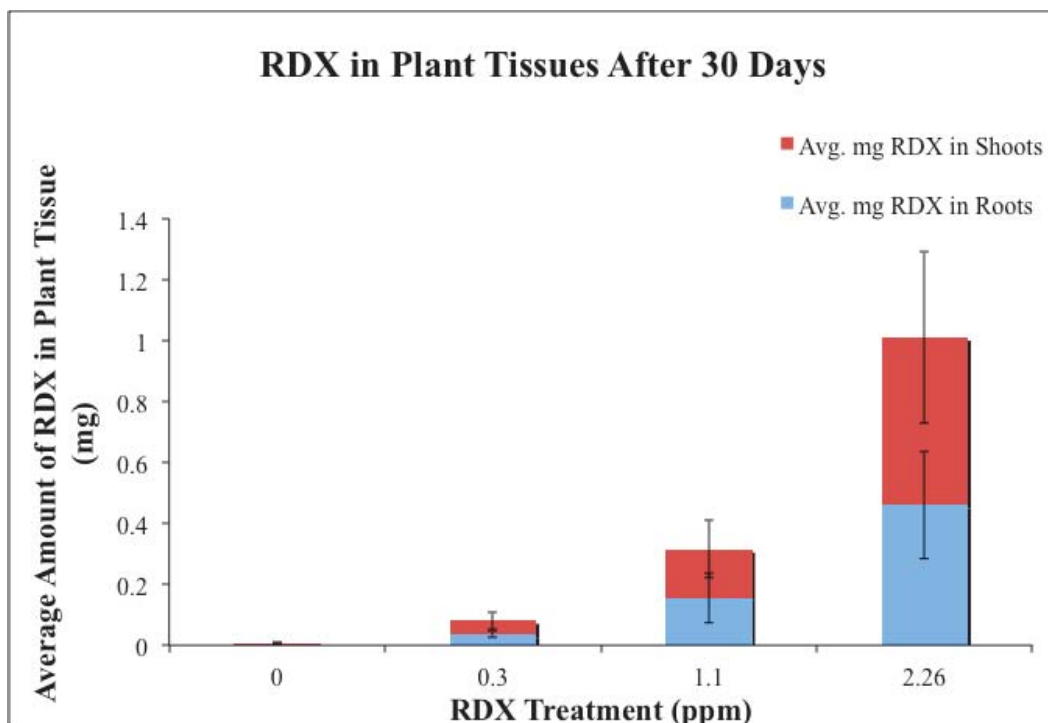


Figure 3.18 RDX in Plant Tissue after 30 Days. Error bars represent standard deviation between triplicate plant samples

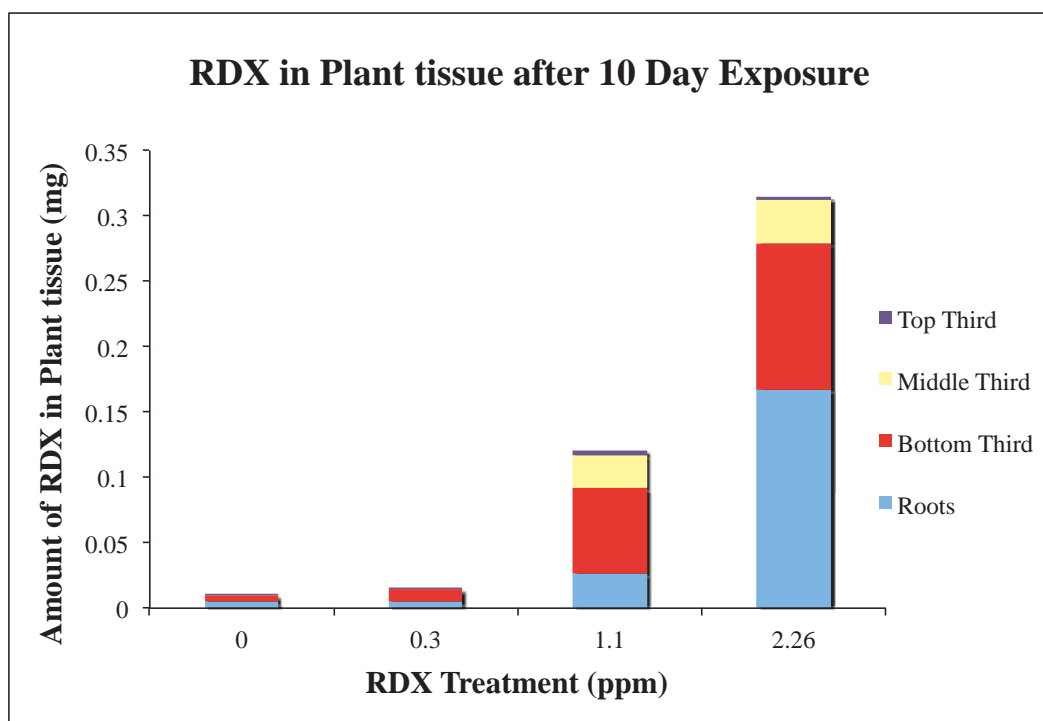


Figure 3.19 Mass Balance of RDX in Plant Tissue after 10-Day Exposure. One plant from each concentration was harvested and analyzed at this time point.

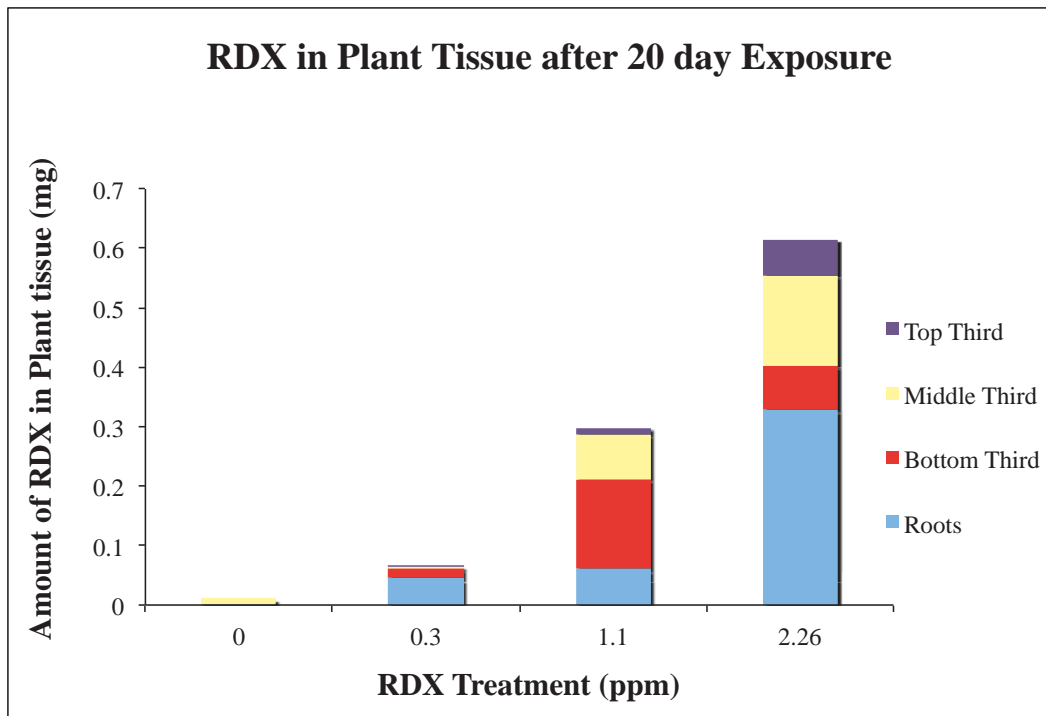


Figure 3.20 Mass Balance of RDX in Plant Tissue after 20-Day Exposure. One plant from each concentration was harvested and analyzed at this time point.

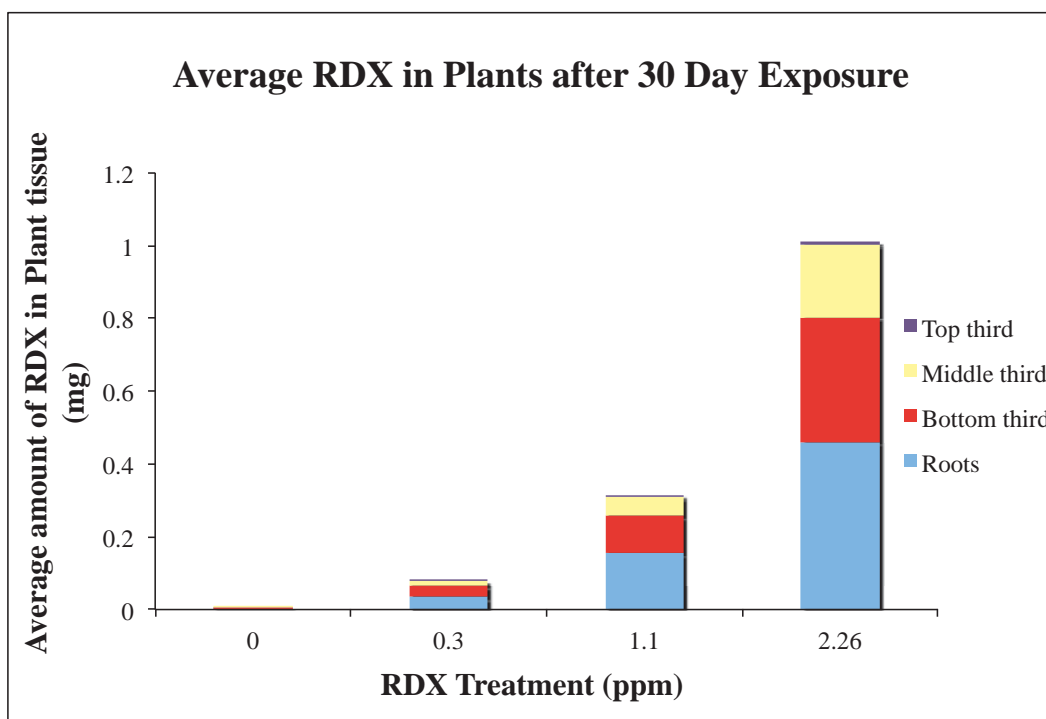


Figure 3.21 Mass Balance of RDX in Plant Tissue after 30-Day Exposure Three plants from each concentration were harvested and analyzed at this time point.

3.2.3 Mass Balance of RDX

The ratio, expressed as a percentage, of the mass of RDX that was taken up by the plants relative to the mass of RDX initially present in the hydroponic media after 10, 20, and 30 days of exposure is reported in Tables 3.2, 3.3, and 3.4. At 30 d of exposure to the 0.3, 1.1, and 2.26 ppm treatments 13.12%, 18.34% and 24.22%, respectively, of the initial RDX in the hydroponic media was found in plant tissue (Table 3.5). Three plant replicates from different containers of each treatment were harvested on day 30 and the averages and standard deviation of the RDX (%) in the plant tissues are presented in Table 3.6.

Table 3.2: 10 Day RDX Exposure

Initial RDX Concentration in Media (mg/L)	mg of RDX in treatment	Total mg RDX in Plant Extracts	% RDX in Plant Tissues
0.300	0.900	0.01517	1.686
1.10	3.30	0.1208	3.6559
2.26	6.78	0.3141	4.634

Table 3.3: 20 Day RDX Exposure

Initial RDX Concentration in Media (mg/L)	mg of RDX in treatment	Total mg RDX quantified in Plant Extracts	% RDX in Plant Tissues
0.300	0.900	0.06445	7.161
1.10	3.30	0.2987	9.051
2.26	6.78	0.6150	9.071

Table 3.4: 30 Day RDX Exposure

Initial RDX Concentration in Media (mg/L)	mg of RDX in treatment	Total mg RDX quantified in Plant Extracts	% RDX in Plant Tissues
0.300	0.900	0.03848	4.275
1.10	3.30	0.1856	5.626
2.26	6.78	0.7131	10.52

Table 3.5: Total Uptake of RDX from Treatments Harvested at 10, 20, and 30 Days

Initial RDX Concentration in Media (mg/L)	mg of RDX in treatment	Total mg RDX quantified in Plant Extracts	% RDX in Plant Tissues
0.300	0.900	0.1181	13.12
1.10	3.30	0.6051	18.34
2.26	6.78	1.642	24.22

Table 3.6: Average 30 Day RDX Exposure

Initial RDX Concentration in Media (mg/L)	mg of RDX in treatment	Total mg RDX quantified in Plant Extracts	% RDX in Plant Tissues \pm stdev
0.320	0.960	0.08046	8.288 \pm 3.801
1.12	3.36	0.3147	9.358 \pm 5.199
2.29	6.86	1.009	14.66 \pm 6.104

3.2.4 Bioconcentration Factor

The bioconcentration factor (BCF) is a measure of the accumulation of a chemical in the plant tissue relative to the concentration in the surrounding environment. The BCF was calculated by dividing the concentration of RDX in the plant tissue by the concentration in the hydroponic media at harvest. The BCFs for all treatments at 30 d were relatively low, with the 2.26 ppm treatment exhibiting the lowest BCF of 0.098 (Figure 3.22).

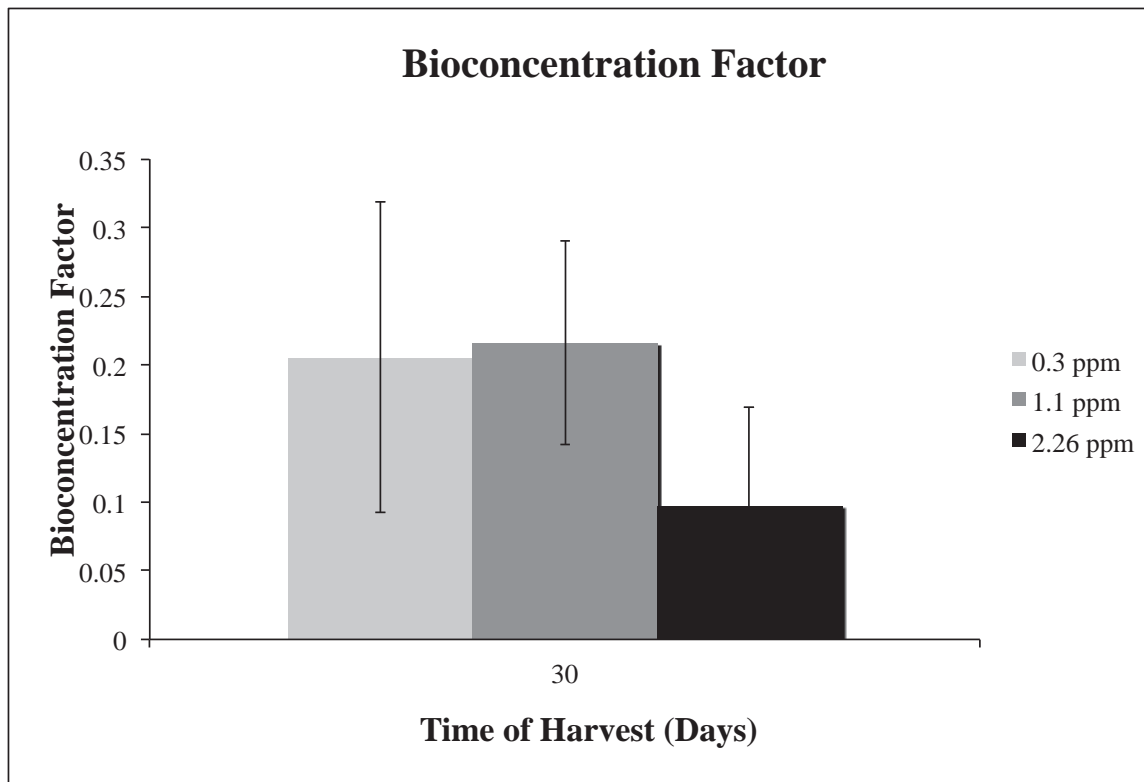


Figure 3.22 : Bioconcentration Factor of RDX in vetiver tissue harvested at 30 days. Error bars represent ± 1 standard deviation between triplicate plant samples.

3.2.5 Translocation Index

The translocation index (TLI) of RDX, expressed as a percent, is the mass of RDX located in the shoots of vetiver relative to the mass of RDX in the entire plant. The TLIs for all treatments at 30 d is presented in Figure 3.23. The TLIs for the treatments were similar and were 53.2%, 49.9%, and 53.4% for the 0.3, 1.1, and 2.26 ppm RDX treatments, respectively (Figure 3.23).

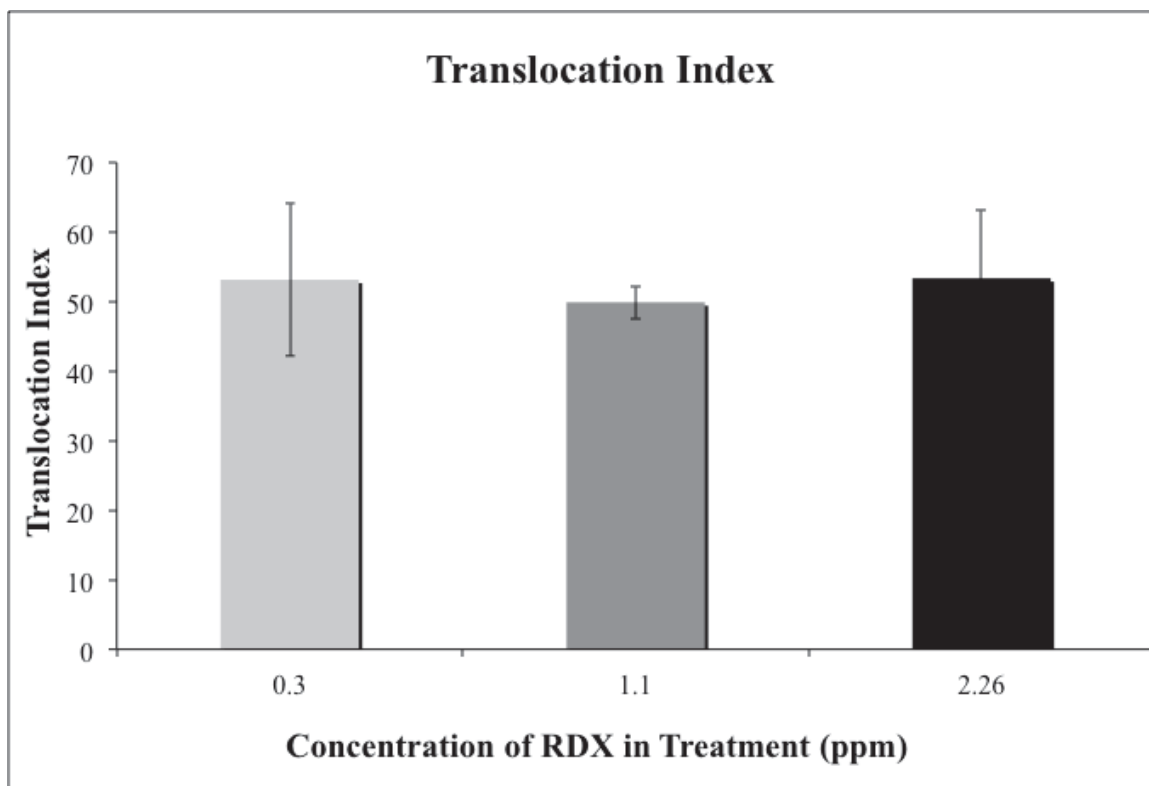


Figure 3.23 Translocation Index of RDX in vetiver. Error bars represent ± 1 standard deviation between triplicate plant samples.

3.3 Partitioning Coefficients of RDX

The log K_{OW} and log K_{OC} values for RDX in vetiver were calculated for the harvested plants for each treatment and are presented in Table 3.7. Values were calculated according to the following (Di Toro 1985; US EPA 1996):

$$K_{oc} = ((\text{mg RDX in plant}) / (\text{kg of Organic Carbon in plant})) / (\text{mg RDX L}^{-1} \text{ media})$$

$$\log K_{OW} = (\log K_{OC} - 0.00028) / 0.983$$

The level of OC in vetiver is approximately 50% of the dry plant matter (Singh 2011)

Table 3.7: Calculated K_{OC} and K_{OW} Values

RDX Treatment (ppm)	Mean log(K_{OC}) \pm stdev	Mean log(K_{OW}) \pm stdev
0.3	0.869 \pm 0.232	1.80 \pm 0.481
1.1	0.896 \pm 0.201	1.85 \pm 0.417
2.26	1.20 \pm 0.363	2.48 \pm 0.752

3.4 Phytotoxicity of RDX

3.4.1 Plant Biomass

Each individual plant was weighed prior to the start of the experiment and at the end of the 30 d experimental period to determine whether or not RDX had any negative or positive effects on the plant growth. The greatest average loss in plant biomass after 30 days was observed in control plants, with an average loss of 13.8g of plant biomass (Figure 3.25). Plants that were harvested at 10, 20, and 30 days were also measured at their harvest point. Similarly, the control plants showed the greatest loss in biomass at 20 and 30 days (Figure 3.24).

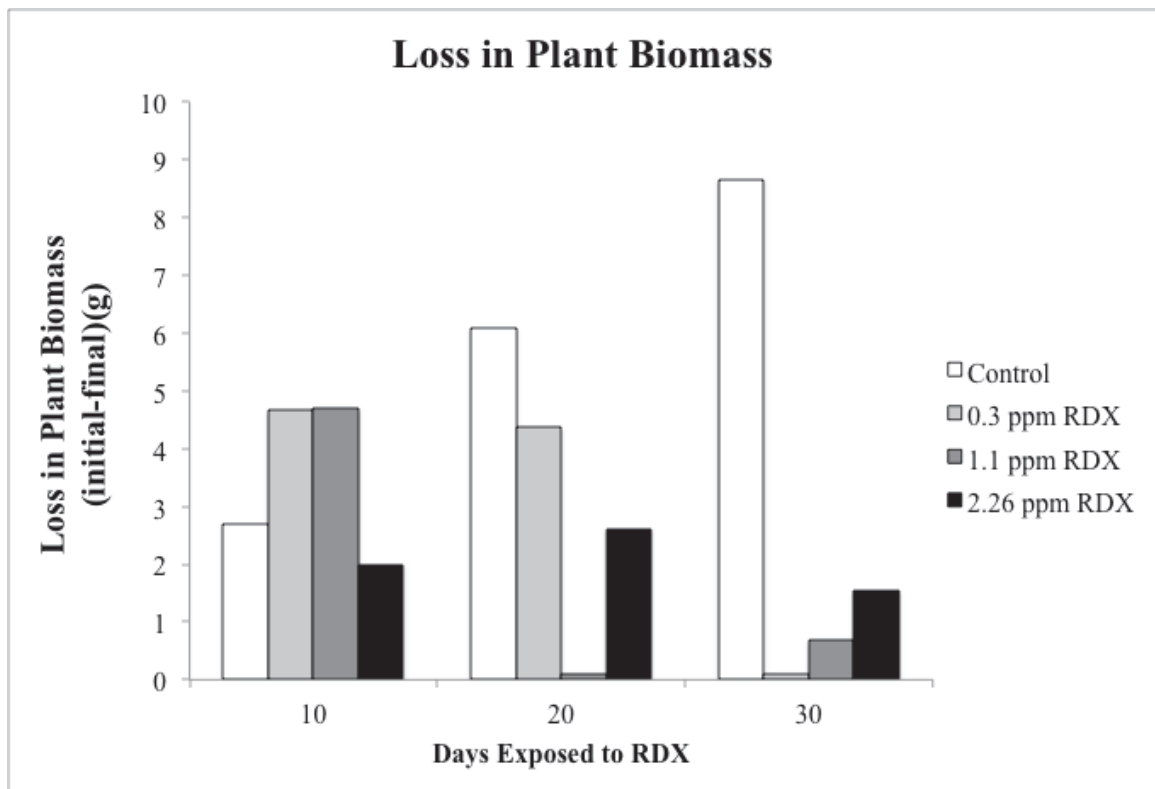


Figure 3.24 Loss in biomass of vetiver exposed to RDX for plants harvested at 10, 20 and 30 days of exposure.

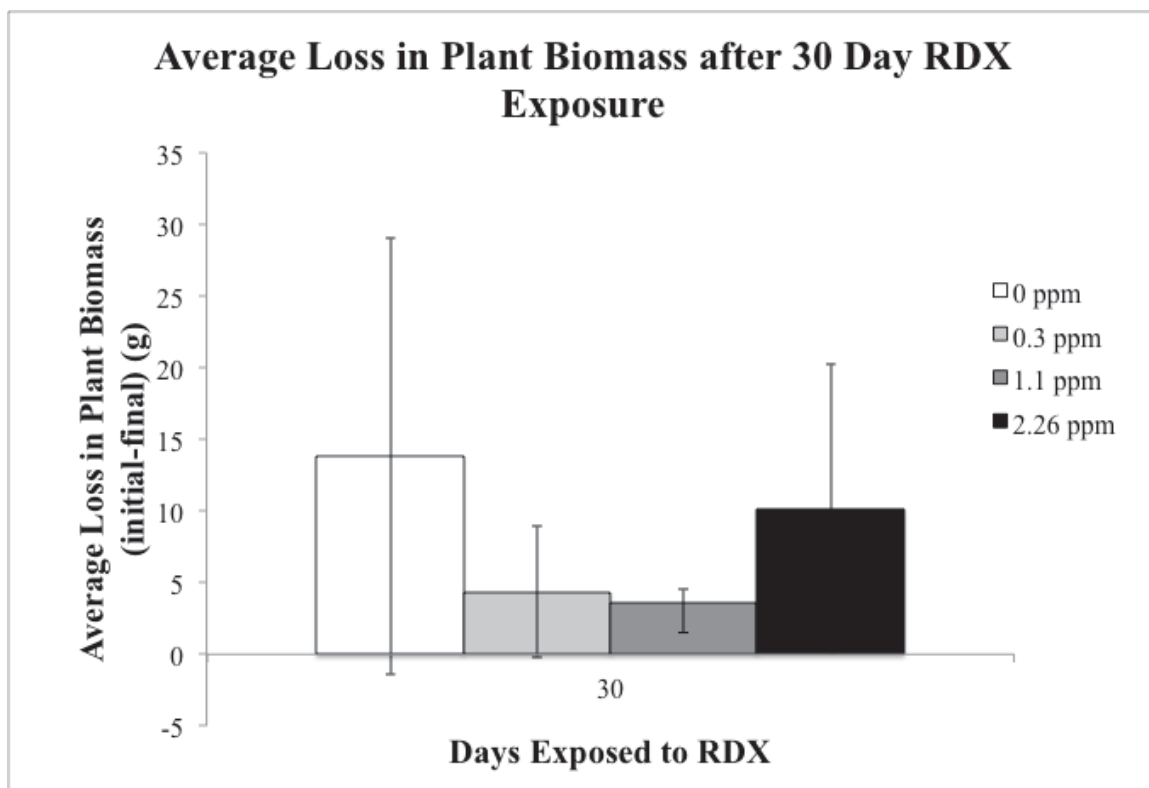


Figure 3.25 Average loss in Plant Biomass after 30 Day Exposure to RDX. Includes all plants harvested at the 30-day endpoint of the experiment.

3.4.2 Chlorophyll Content

Chlorophyll content did not decrease with increasing exposure to RDX and control plants (5.02 ± 4.24) had slightly lower chlorophyll contents than plants exposed to RDX (Figure 3.26). Chlorophyll contents were 7.58 ± 2.12 , 7.25 ± 2.68 , and 6.51 ± 1.44 for the 0.3 ppm, 1.1 ppm, and 2.26 ppm RDX treatments, respectively.

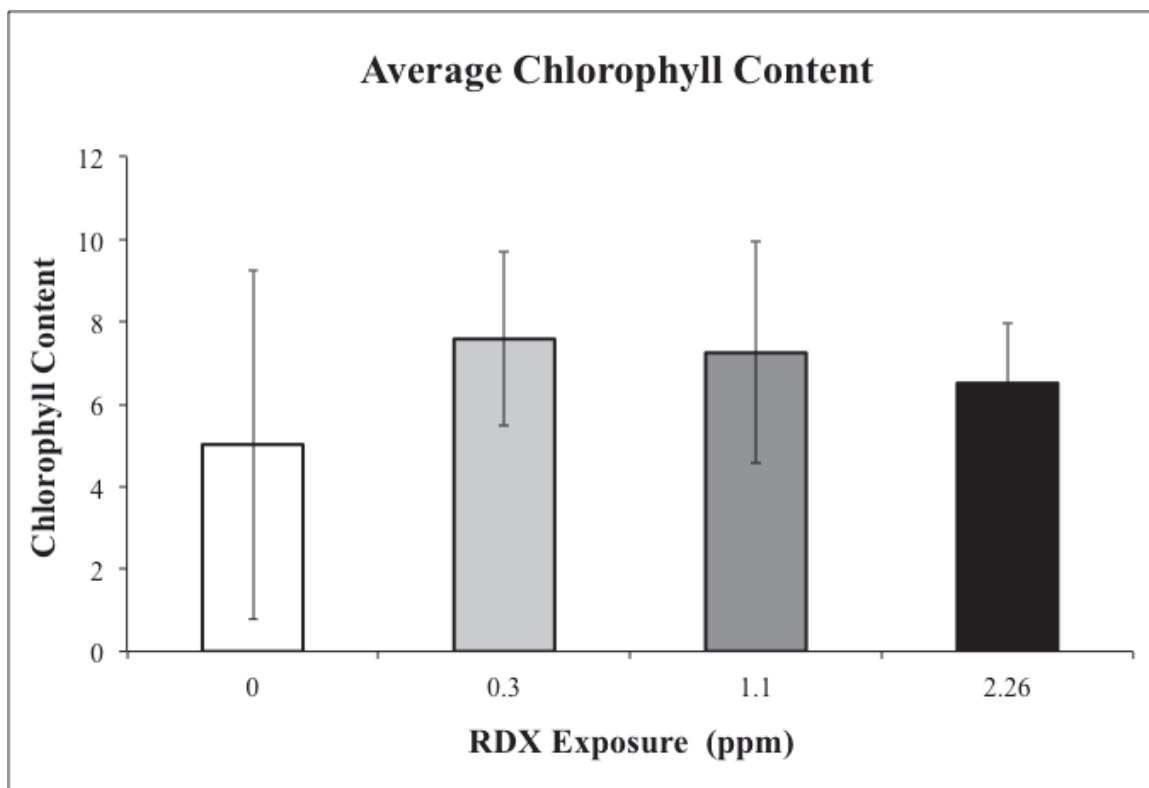


Figure 3.26 Average Chlorophyll Content after 30 day exposure to RDX. Error bars represent ± 1 standard deviation between triplicate plant samples.

Chapter 4

Conclusions

The study represents a preliminary investigation of the uptake of RDX by vetiver grown hydroponically. Measuring uptake of an organic chemical by plants grown hydroponically presents several challenges: (1) organic chemicals are hydrophobic and readily sorb to surfaces, (2) evaporation of hydroponic media is exacerbated by forced aeration and can leave plant roots exposed to air during long exposures, (3) accounting for mass losses of hydroponic media during long exposures is difficult and precludes monitoring plant uptake by measuring chemical losses in the hydroponic media, and (4) replenishing hydroponic media during long exposures might compromise results due to an inability to supply the same level of nutrients to all plant treatments. A rapid and similar decrease in RDX concentration in controls and plant exposures was observed within the first 18 h of the experiment with the greatest loss occurring during the first 6 hours. However, RDX concentrations returned to initial levels in about 30 hr and began to gradually increase throughout the remainder of the experiment. Although RDX is quite hydrophilic, it was apparently sorbed to container walls within the first 18 h and then desorbed from container walls after about 30 h. The gradual increase in RDX concentration throughout the remainder of the experiment is attributed to evaporation of water, which is more volatile than RDX.

A better measure of the uptake of RDX from long exposures in hydroponic media is through quantification of RDX in exposed plants. The mass of RDX in the shoot tissue of vetiver was about equal to the mass of RDX in root tissue of vetiver, with slightly more RDX being found root tissue. The result was contrary to other studies that found RDX more readily translocated to apical parts of plants.

Evaporation of water from the treatment containers precluded a measurement of the loss of RDX from the hydroponic solutions with time. Accurately replenishing the hydroponic solution would be difficult and might also lead to unequal levels of nutrients in the various treatment containers. However, water is more volatile than RDX, and thus,

the theoretical loss in RDX from hydroponic media was calculated using the measured mass of RDX in the plant tissue and measured concentration of RDX in the hydroponic media for different durations of exposure. If replicates were harvested along the way, the level of uncertainty would have been less; however, due to experimental constraints this was not possible.

Measuring the level of RDX in plants and hydroponic media for different durations of exposure provided data to calculate $\log K_{OC}$ and $\log K_{OW}$ for RDX. The $\log K_{OC}$ and $\log K_{OW}$ derived from the experiment were 0.896 ± 0.201 and 1.85 ± 0.417 (derived from 1.1ppm treatment data), respectively, and similar the $\log K_{OC}$ and $\log K_{OW}$ values of 1.80 and 0.87, respectively, reported in the literature (Table 1.1). The result confirms the behavior of RDX in this study to be similar to what is to be expected on the basis of the physical and chemical properties of RDX. Hydrophilic compounds are not likely to pass through the hydrophobic membrane of root tissue and will instead stay in solution. Compounds with $\log K_{ow} < 1.8$ are expected to be too hydrophilic to be taken up by roots (Yoon et al. 2005). The $\log K_{ow}$ of RDX determined here was approximately 2 and is in the range of being too hydrophilic for effective uptake by plant roots. The RDX did accumulate in vetiver tissue: however, the rate of RDX uptake by plants might be much slower than the rate of uptake of more hydrophobic substances.

Similar TLIs were observed for the 3 RDX treatments, with the 1 ppm RDX treatment showing the greatest translocation index. The BCFs were low for all RDX treatments and exposure durations, which might be due to degradation of RDX in plant tissue. Major degradation products of RDX are MNX, DNX, and TNX. The derivatives are denitrated, and thus, are slightly less polar than RDX and would have longer HPLC elution times than RDX. In the HPLC chromatograms of plant extracts in this study, peaks were observed at 4.3 and 5.1 min. On a C18 column, peaks for RDX derivatives follow the RDX peak and elute sequentially, between 4 and 6 minutes (Felt et al. 2003). Mass spectral analysis of the vetiver extracts is required to positively identify the degradation products.

A loss in biomass was observed in plants exposed to all three concentrations of RDX. However, plants grown as controls exhibited a greater loss in biomass indicating a link between the RDX exposure and loss of plant biomass might not exist. Results from chlorophyll analyses did not reveal a trend of increasing phytotoxicity with exposure to increasing concentrations of RDX. Instead, control plants showed less chlorophyll content than those exposed to RDX, which might be related to other environmental factors and greenhouse conditions. For example, containers in different locations on the benchtop experienced different evaporation rates. The placement of plant treatment containers was randomized; however reduction in hydroponic solution and nutrients could cause chlorosis. Phytotoxicity of vetiver to RDX for 0.3, 1.1 and 2.26 ppm treatments was not observed; however higher concentrations might elicit a phytotoxic response.

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