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IMPACTS OF RDX SOIL CONTAMINATION ACROSS AN AGE GRADIENT FOR THE NATIVE SHRUB MORELLA CERIFERA.

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IMPACTS OF RDX SOIL CONTAMINATION ACROSS AN AGE GRADIENT FOR THE NATIVE SHRUB MORELLA CERIFERA.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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B.S., Virginia Polytechnic and State University, 2010

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Abstract

IMPACTS OF RDX SOIL CONTAMINATION ACROSS AN AGE GRADIENT FOR THE NATIVE SHRUB MORELLA CERIFERA.

By Stephen M. Via M.S

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Thesis Director: Dr. Donald R. Young, Department Chair, Department of Biology
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Understanding the impacts of explosive contamination on vegetation is key to understanding explosives behavior in the environment. I quantified shrub growth responses to 1,3,5-trinitroperhydro-1,35-triazine (RDX) soil contamination across three life stages and I hypothesized that RDX would have the greatest impact on seed germination. Morella cerifera seeds were germinated on soils amended with RDX up to 1500 mg RDX kg\(^{-1}\) dry soil. Juvenile and adult individuals were exposed for 6 weeks to soil amended with RDX up to 750 and 1500 mg RDX kg\(^{-1}\) dry soil, respectively. Morphological responses were quantified for juveniles while physiological measurements were quantified for adults. RDX induced a significant response in all age groups and, in accordance with the hypothesis, germination was the most impacted of the
three stages. Impacts varied by concentration in addition to life stage, showing that many variables influence plant response to RDX.
Environmental contamination has been a concern for many years; however the emphasis has been on industrial discharge and oil spills while overlooking many other, potentially more harmful, contaminants. Contaminants may exhibit different behaviors depending on the method of introduction, surrounding environment, and the local flora and fauna. For many soil-based contaminants, microbes and plants are the first organisms that are exposed, frequently leading to contaminant degradation or sequestration. The result of this may be harmful intermediate compounds or biomagnification of the toxin (Pilon-Smits, 2005; Pilon-Smits and Freeman, 2006). Plants also provide the pollutant with a pathway from the soil to the surface (Vila et al., 2006) potentially passing it to other trophic levels (Boyd, 2002; Vickerman and Trumble, 2003). This has spurred numerous studies on the environmental impact of explosives (energetic compounds) contamination over the past 30 years (Best et al., 2006).

Production, storage, and transfer of munitions can lead to environmental contamination. Munitions plants, military bases, and bombing ranges, many of which are located in coastal environments, are common sites of explosives contamination (Robidoux et al., 2002). Additionally, proper as well as improper use can lead to a release of explosive compounds into the environment (Robidoux et al., 2002). Buried across 68 nations are 110 million landmines and incalculable amounts of unexploded ordnances (UXOs) (Unicef, 1995). Persistent landmines and
UXOs pose two environmental threats: explosive potential and the leaching of explosive compounds (Hawari et al., 2000a). Research Demolition Explosive (RDX) (1,3,5-trinitroperhydro-1,3,5-triazine) is one of the most common explosives compounds incorporated into both landmines and bombs, and is one of the most widely distributed organic explosive contaminants (Hawari et al., 2000a; Rylott and Bruce, 2008; Khatishvili et al., 2009). RDX also has industrial (i.e., demolition) and recreational uses (i.e., fireworks), so it may occur in areas far removed from military operations (Hawari et al., 2000a).

RDX is a nitroamine (cyclic nitroaromatic compound) making in structurally dissimilar to many naturally occurring organic compounds. Like other toxic compounds, RDX can be absorbed, moved through the plant vascular system, and distributed throughout the plant. RDX is more mobile than other toxins and is readily absorbed by many plants (Thompson et al., 1999; Pilon-Smits, 2005; Best et al., 2006). RDX typically bioaccumulates in the stems and leaves of the plant and in some cases > 90% of the RDX taken up can be recovered in the parent form (Ahmadi et al., 1980; Thompson et al., 1999; Ampleman et al., 2003; Best et al., 2006; Laurent et al., 2007). Accumulation in aboveground portions of the plant may greatly exceed concentrations in soil (Brannon and Pennington, 2002) causing concern for trophic transfer of the compound. Due to the molecular composition of RDX, many plants, and other organisms, cannot inherently degrade the compound (Hawari et al., 2000b; Best et al., 2006).

RDX is primarily degraded via microbial processes and abiotic reactions. Microbes are capable of anaerobically (Hawari et al., 2000a) and aerobically (Hawari et al., 2000b) degrading RDX to formaldehyde, methanol, nitrous oxide, nitrite, nitrate, and CO2. In plants, once RDX reaches the leaves, it can either be stored in the vacuole or incorporated into the cell wall (Pilon-Smits, 2005). This above-ground accumulation exposes the compound to light, leading to
photodegradation creating potentially harmful byproducts inside and outside of the plant (Coleman et al., 1998; Aken et al., 2004; Just and Schnoor, 2004; Yoon et al., 2005).

The direct impact of the molecule and subsequent degradation in the leaf, leads to a variety of morphological responses (Winnfield et al., 2003; Laurent et al., 2007). RDX can cause leaf necrosis, leaf margin curling, leaf shriveling, leaf bleaching, delayed leaf expansion, stunted root and shoot growth, and thin stems (Winnfield et al., 2003; Laurent et al., 2007). Physiological processes are also impacted by explosives contamination in which metabolic processes may be interrupted and photosynthetic reaction centers damaged (Winnfield et al., 2003; Zinnert et al., 2010). Though other energetic compounds have been studied in this capacity (Zinnert et al., 2010), but there are little data on the physiological impacts of RDX.

Plants in general are capable of tolerating explosives until a toxic threshold is reached (Brannon and Pennington, 2002). The level of toxicity varies by species and at low levels of contamination some plants exhibit increased biomass, which may be due to the nitrogen present in such compounds (Ampleman et al., 2003; Khatisashvili et al., 2009). Contaminant impacts can also vary based on life stage (Ahmadi et al., 1980).

Plants in the early stages of life are generally more sensitive to the effects of stressors (Ahmadi et al., 1980); however, RDX has shown negligible effects on seed germination, unless in substantially high concentrations (Laurent et al., 2007). Studies show that immediately after germination, RDX has significant impacts on plant growth (Ampleman et al., 2003; Winnfield et al., 2003; Laurent et al., 2007). RDX, like many other toxins, may have reduced impacts in older and larger plants (Ahmadi et al, 1980); however the variability across an age gradient has not been studied. Age-related responses are crucial to understanding overall impact of RDX soil contamination.
The purpose of my study was to test the effects of varying concentrations of RDX on three life stages of a common coastal shrub, *Morella cerifera*. I hypothesized that the *M. cerifera* response to RDX will follow convention; the youngest life stage will be most sensitive to RDX and that sensitivity will decrease with age. Prior research has focused on morphological impacts of contaminants on plants such as biomass reduction and leaf deformation (Ampleman et al., 2003; Winnfield et al., 2003; Laurent et al., 2007). My study included physiological responses, such as photosynthetic rate and chlorophyll fluorescence, in addition to the standard morphological responses. Understanding the impact of energetic contaminants on plant physiology and morphology could provide key insights into affected plant processes.
**Materials and Methods**

**Plant material**

Target species that are commonly used for contamination research include grasses, legumes, and transgenic trees (Pilon-Smits, 2005; Thompson et al., 1999). While these species are effective for phytoremediation (decontamination of sites using vegetation), they are not an accurate metric for the response of native plants.

For this study, *Morella cerifera* L. (wax myrtle, Myricaceae) was the target species. Physiology and stress response of *Morella cerifera* has been quantified thoroughly and the species has been used in TNT contamination experiments showing mixed responses (Zinnert et al., 2010). *Morella cerifera* also exhibits traits prized in plants chosen for phytoremediation: rapid growth, a large dense root system, and accumulation a large amount of biomass over its lifetime (Pilon-Smits, 2005). These traits are perfect for such an experiment as they all involve high nutrient/water uptake. *Morella cerifera* naturally colonizes disturbed sites, and is present on bombing ranges and around munitions plants. For this study, *Morella cerifera* was observed at three distinct life stages: seed germination, juvenile (~8 cm), and adult (≥30 cm).

Fruits of *M. cerifera* were collected from Hog Island (37° 40’N; 75° 40’W), a barrier island located on the Eastern Shore of Virginia, U.S.A.. Seeds were kept at 4°C for ~4 months. *Morella cerifera* fruits were run across a mesh screen to break the waxy coating and scarify seeds. For the juvenile individuals, seeds were sown in transparent plastic trays filled with 2.5
cm of Perlite, and watered as necessary. Plants with at least three sets of secondary leaves (~8 cm) were transplanted into 2” plastic pots and grown for at least 3 months prior to experimentation. Adult native-stock plants were purchased from Pinelands Nursery (Pinelands Nursery & Supply, 323 Island Road Columbus, NJ 08022) and pruned to the woody stem. They were allowed to grow out for 3 months prior to experimentation (≥ 30 cm).

Soil contamination

Many recent studies have used hydroponic solutions (Thompson et al., 1999) to test the impacts of explosives contaminants on plants and while this allows more control over the growing environment it introduces a disconnect between lab tests and field applications. Soils naturally contain some amount of organic material which can interact and bind with contaminants (Cunningham et al., 1995). While this may impart some error in the experiment the results are more field applicable.

All plants were grown in a 3:1 mixture of low nutrient topsoil and sand. This mixture was chosen to mimic natural organic, and nutrient, content of field soil. The soil was amended with 200 ml of acetone containing RDX to bring the relative concentration to the proper level (Ali et al., 2006; Zinnert et al., 2010). After amending the soil with acetone, the soil was kept in the dark for 72 hr to allow for the acetone to evaporate and prevent photodegradation of RDX (Ali et al., 2006). A wide concentration range was chosen as the concentration of RDX found in field soils can range from 0.7 to 7500 mg RDX kg⁻¹ soil (Talmage et al., 1999). Age groups considered to be more resilient to RDX (i.e. germination and adults) were given a broader range of contamination while the most sensitive group (i.e. juveniles) was given a reduced range with smaller concentration increments. Germination tests were conducted on soils having 100, 500, 750, or 1500 mg of RDX kg⁻¹ dry soil. Juvenile plants were grown in soil contaminated with 100,
200, 300, 400, 500, or 750 mg RDX kg\(^{-1}\) soil and adults with 100, 750, or 1500 mg RDX kg\(^{-1}\) soil. Reference plants were amended with 200 ml of acetone containing no contaminant.

**Germination**

*Morella cerifera* seeds were placed in Petri dishes containing 20 g of treated soil (n=50). Five dishes for each treatment were used (n=5) and all dishes were placed in plastic bags and stored in a Conviron environmental chamber (CMP 3244, Controlled Environments Limited, Asheville, NC). Seeds were monitored daily until 50% of the control group successfully germinated. Germination was defined as the emergence of the radical from the seed. Once the radical was visible, the seed was removed. Overall percent germination was calculated at the conclusion of the experiment.

**Juvenile**

Individuals were potted two plants per pot in soil taken from a 4.2 kg stock of treated soil. For each concentration, there were 5 plants which were watered daily and kept in a controlled greenhouse for 6 weeks (ambient temperature of 30°C ± 7°C, relative humidity ≈ 60%). Plants were placed in catch dishes to retain water and reduce the impact of RDX leaching from the soil. For this group, root and shoot growth were measured with a ruler. Shoot length was monitored once every week while overall root length was measured upon completion of the experiment. Stem count, leaf drop, necrotic leaf count, curled leaf count, and reduced leaf count were recorded on a weekly basis. Leaves were defined as necrotic if ≥ 30% of the leaf showed necrosis, as curled if the leaf deviated ≥45° from the plane of the mid-vein, and as reduced if at maturation was <50% size of control leaves at maturation.

**Adult**
Soil for adult plants was comprised of 4.2 kg treated soil. For each concentration, there were 5 replicates. Specimens were watered on a daily basis and kept in a controlled greenhouse (ambient temperature of 30°C ± 7°C, relative humidity ≈ 60%) while being monitored for 6 weeks. Catch dishes were placed under each plant to retain water and reduce the impact of leaching. Net photosynthesis ($A_{NET} \mu{mol} m^{-2}s^{-1}$) and stomatal conductance to water vapor diffusion ($g_s \text{ mmol H}_2\text{O} m^{-2}s^{-1}$) were measured at midday (1100-1300.) on a weekly basis using a LI-6400 (LI-COR Biosciences, Inc., Lincoln, NE) portable infrared gas analyzer at 700 µmol m$^{-2}$s$^{-1}$ PAR. Fluorescence measurements of PSII operating efficiency ($\Delta F/F_m^*$) and maximum operating efficiency ($F_v/F_m$) were taken at the end of the experiment using a pulse amplitude modulated fluorometer (Mini-PAM, Waltz, Germany), as previous studies have shown this to be an effective sampling regime (Zinnert et al., 2010). Maximum electron transport rate (ETR) was calculated at the end of the experiment as: $[(F_m^*-F_s)/F_m^*]*$ PAR using 1500 µmol m$^{-2}$s$^{-1}$ as the level of photosynthetically active radiation (PAR). Water potentials ($\Psi_{xylem}$) were taken from leaves biweekly using a Model 600 pressure chamber instrument (PMS Instrument Company, Albany, OR). All physiological measurements were taken from the 2$^{nd}$ or 3$^{rd}$ fully expanded leaves.

**Statistical analysis**

ANOVA for treatment effects were used to determine significant changes in effects of RDX contamination in each life stage at various contaminant concentrations. Dunnett’s multiple comparisons ($\alpha=0.05$) was used to identify difference in treatment plants relative to controls. Linear regression was used to find relationships between parameters using JMP Pro 10 (©SAS Institute Inc.; Zar, 1999).
Results

Germination

All RDX treatment groups exhibited significantly less germination than the control (F=17.67, p<0.0001; Fig. 1). The 100 mg RDX kg\(^{-1}\) soil group was 50% of the control group, the 500 and 750 mg RDX kg\(^{-1}\) soil groups had 24% germination of the control, and the 1500 mg RDX kg\(^{-1}\) soil group had 41% of control germination (Fig. 1). Seedlings that did emerge from the contamination groups showed no obvious deformations of the cotyledons or first set of true leaves.

Juvenile

By the end of the experiment, no significant differences in shoot length for *Morella cerifera* juveniles were present relative to controls (F=3.27, p=0.0116; Fig. 2). Similar to shoot length, *M. cerifera* showed no significant change in root length (F=0.67, p=0.6746; Fig. 2). Stem count was variable among groups (Fig. 3), from 3.0±2.0 stems in control plants to 4.0±2.5 in the 400 mg RDX kg\(^{-1}\) soil group. The 100 mg RDX kg\(^{-1}\) soil group had 3.7±1.7, 200 mg RDX kg\(^{-1}\) soil had 2.0±0.6, 300 mg RDX kg\(^{-1}\) soil had 2.2±0.5, the 500 mg RDX kg\(^{-1}\) soil had 2.0±0, and the 750 mg RDX kg\(^{-1}\) soil had 2.0±0.71 stems. No significant difference in stem count was observed at the end of the experiment (F=1.79, p=0.1309).

Leaf count remained relatively constant through week 2, after which new leaf formation increased in week 4 (F=2.10, p=0.0771, Table 1). Significant differences in leaf count were first
observed in week 6 (F=6.54, p=0.0001) where all contaminant groups above the 100 mg RDX kg\(^{-1}\) soil group were significantly reduced (Table 1). By the end of the experiment, the 500 mg RDX kg\(^{-1}\) soil and 750 mg RDX kg\(^{-1}\) soil groups had 50% less leaves relative to controls plants (Fig. 4). The 200 mg RDX kg\(^{-1}\) soil and 300 mg RDX kg\(^{-1}\) soil groups had 57% and 53%, respectively (Fig. 4). Leaf necrosis appeared first in week 2 where there was a significant increase (F=5.03, p=0.0008) in the 300 mg RDX kg\(^{-1}\) soil group compared to the control (Table 1, Fig. 4). By the end of the experiment, only the 400 mg RDX kg\(^{-1}\) soil and higher concentrations had significantly higher leaf necrosis (F=7.23, p<0.0001) relative to control plants (Fig. 4).

Leaf curling occurred only in the new and middle aged leaves. By week 4, all groups above the 300 mg RDX kg\(^{-1}\) soil concentration had significantly higher leaf curling (F=8.28, p<0.0001; Table 1, Fig. 4). This trend continued until the end of the experiment (F=5.43, p=0.0005; Table 1). Reduced leaf size was observed first in week 4 (F=8.28, p<0.0001) and followed a similar pattern to leaf curling. All groups above 200 mg RDX kg\(^{-1}\) soil had more leaves of reduced size relative to control by the end of the experiment (Fig. 4). The 100 mg RDX kg\(^{-1}\) soil and 200 mg RDX kg\(^{-1}\) soil groups showed no significant amount of leaf reduction compared to the control throughout the experiment (Table 1, Fig. 4).

**Adult**

Midday net photosynthesis was reduced as RDX concentration increased (Fig. 5). By the end of the experiment, 750 mg RDX kg\(^{-1}\) soil group was 47% lower than controls, and 1500 mg RDX kg\(^{-1}\) soil exhibited 68% lower photosynthesis relative to controls (Fig. 5). A drop in photosynthesis occurred in all groups during week 2 and all experimental groups had a photosynthetic value that was significantly lower than the control (F=25.97, p=<0.0001). The
100 mg RDX kg\(^{-1}\) soil group recovered in week 4, and showed no significant difference by the end of the experiment (Fig. 5). In week 4, the 750 mg RDX kg\(^{-1}\) soil and 1500 mg RDX kg\(^{-1}\) soil groups continued to decline to photosynthetic values of 4.3±0.5 and 2.9±0.5 µmol CO\(_2\) m\(^2\)s\(^{-1}\), respectively. By week 6, both the 750 mg RDX kg\(^{-1}\) soil and 1500 mg RDX kg\(^{-1}\) soil groups showed an increase in photosynthetic values to 5.7±0.9 and 3.5±1.2 µmol CO\(_2\) m\(^2\)s\(^{-1}\), respectively (Fig. 5). The overall change in photosynthesis through the 6 weeks showed a significant difference (F=7.644, p=0.0007) between the control and 750 mg RDX kg\(^{-1}\) soil and 1500 mg RDX kg\(^{-1}\) soil groups (Fig. 5).

Stomatal conductance to water vapor diffusion declined in all groups in week 2 although no group differed from the control (F=0.8421, p=0.5148). There were no significant differences observed in the 100 mg RDX kg\(^{-1}\) soil group relative to control for stomatal conductance throughout the experiment (Fig. 6). Stomatal conductance for the 750 and 1500 mg RDX kg\(^{-1}\) soil groups increased the least and were not statistically different (F=2.28, p=0.0963) relative to controls. In week 6, stomatal conductance for the 750 mg RDX kg\(^{-1}\) soil group was 130% greater than that of the control reaching 301±3.0 mmol H\(_2\)O m\(^2\)s\(^{-1}\) and the 1500 mg RDX kg\(^{-1}\) soil was 160% greater reaching 376±9.0 mmol H\(_2\)O m\(^2\)s\(^{-1}\). Only the 1500 group showed significantly higher conductance in week 6 (Fig. 6). With photosynthesis declining as RDX concentration increased and stomatal conductance increasing with concentration there was a negative linear relationship between stomatal conductance and photosynthesis (r\(^2\)=0.60, p<0.0001; Fig. 7).

Chlorophyll fluorescence values followed a similar trend as photosynthesis, declining as RDX concentration increased (Fig. 8). ETR values declined with increased RDX concentration yet there was no significant change, relative to control (F=1.03, p=0.4192; Fig. 8). F\(_{v}/F_{m}\) values were significantly reduced by 18% at the end of the experiment in the 1500 mg RDX kg\(^{-1}\) soil
The ΔF/F’_M values were reduced by 3% in the 100 mg RDX kg\(^{-1}\) soil group, 22% for the 750 mg RDX kg\(^{-1}\) soil group and 29% in the 1500 mg RDX kg\(^{-1}\) soil group. Only the 750 and 1500 mg RDX kg\(^{-1}\) soil groups were significantly (F=12.42, p<0.0001) different compared to the control (Fig. 8). Water potentials showed a significant decline in the 100 and 750 mg of RDX kg\(^{-1}\) of soil in week 2 (F=4.53, p=0.0091), but no significant difference relative to control was present at the end of the experiment (Fig. 9).
Soils contaminated with energetic compounds such as RDX, are a global threat and direct effects on flora and fauna are poorly understood. The objective of my study was to quantify the impacts of varying concentrations of RDX contamination in soils across life stages in the shrub *Morella cerifera*. My hypothesis was that *M. cerifera* response to RDX soil contamination would follow convention; youngest life stage would be most sensitive to RDX and that sensitivity would decrease with age. My results indicated a more complex response. Soil contamination had an impact on both plant morphology and physiological processes. As concentrations increased, the impact was greater for all life stages, while differing concentrations had varied effects across life stages. Seed germination showed a toxicity threshold of at least 100 mg RDX kg$^{-1}$ soil. Based on the juvenile data there was a toxicity threshold between of 300 and 400 mg RDX kg$^{-1}$ soil; morphological deformation occurred. Adults showed a similar threshold between the 100 and 750 mg RDX kg$^{-1}$ soil groups; physiological alterations occurred.

**Germination**

Toxins have negligible effects on seed germination unless in substantially high concentrations (Ampleman et al., 2003; Winnfield et al., 2003; Laurent et al., 2007) and RDX is no different in agronomic plants (Winnfield et al., 2003; Laurent et al., 2007). My findings for RDX in *Morella cerifera* are an exception. Emergence, the least effected part of germination for agronomic plants (Winnfield et al., 2003; Laurent et al., 2007), was significantly reduced for
Morella cerifera even at 100 mg RDX kg\(^{-1}\) dry soil. Percent germination for the 1500 mg RDX kg\(^{-1}\) dry soil group was unexpectedly close to the 100 mg RDX kg\(^{-1}\) dry soil group, perhaps due to an uneven distribution of soil organic material in the Petri dishes. To germinate, seeds must imbibe water from the surroundings (Copeland and McDonald, 1995) and RDX being highly mobile can be easily absorbed by the seeds (Winnfield et al., 2003). Uptake of RDX could lead to the disruption of any number of physiological processes (Copeland and McDonald, 1995), thereby inhibiting germination. The lack of an endosperm in *M. cerifera* seeds may have compounded the impacts of RDX as the emerging plant would require more nutrition from the environment than if an endosperm was present (Young and Young, 1992). Inhibition of germination in *M. cerifera* differs from species in prior studies and this shows that soil contaminated with RDX may differently alter species composition in naturally occurring communities and potentially influence successional trajectory.

**Juvenile**

Plants showed similar morphological responses to RDX as those found in the literature (Winnfield et al., 2003; Laurent et al., 2007; Appendix A). All plants in soils contaminated with 400 mg RDX kg\(^{-1}\) soil or more showed similar morphological responses to RDX. The highest leaf necrosis was seen in the 300 and 400 mg RDX kg\(^{-1}\) soil groups, likely due to increased leaf drop in higher concentrations. The few leaves present were presumably receiving relatively larger amounts of RDX compared to plants at lower concentration. Variable significance in leaf curling, necrosis, and reduction of leaves may also be attributed to variable leaf drop. Leaf curling was only visible in the newly formed and younger leaves while leaf reduction was only observed in newly formed leaves. The reduced size and curled shape could be due to delayed leaf maturation, or other tetrogenic impacts on the meristem (Winfeild et al., 2003). Old leaves
exhibited no leaf curling; therefore, impacts in leaf development are possibly the source of both curling and reduced leaf size. Regardless of age, leaves exhibited necrotic legions suggesting that leaves of all ages may act as a reservoir for RDX. Leaf senescence started in the oldest leaves, after necrosis had set in, and steadily moved to newer leaves possibly due to the older and lower leaves being first to receive RDX moving through the plant and being exposed longer than the upper leaves (personal observations).

The reduced and curled form of leaves was potentially due to delayed leaf emergence and maturation caused by RDX (Winfeild et al., 2003). As RDX photodegrades in the leaves, it is also possible that deformation is a plant response to limit the amount of light incident on the leaves, similar to the mechanism of protection used by sun leaves (Young and Smith, 1980). Necrosis could be induced by intermediate compounds created as RDX degrades (i.e., formaldehyde and oxygen radicals; Bose et al., 1998b; Just and Schnoor, 2004).

While other species have shown reduced root and shoot growth in response to RDX soil contaminations (Winnfield et al., 2003; Laurent et al., 2007), *M. cerifera* did not. Other species also exhibit stem morphology changes that were not seen here. The high stomatal conductance seen in *M. cerifera* adults shows the rapidity of water moving through the plant. This rapid uptake may not allow RDX to reside in the vascular tissue of the plant thereby limiting impacts on roots and stems. This would also explain the significant leaf senescence and deformation.

**Adult**

Adult physiology, similar to juvenile morphology, was significantly impacted across concentrations. Interestingly the reactions of physiology to RDX were different from expected stress responses. The 750 and 1500 mg RDX kg$^{-1}$ dry soil groups exhibited greater impacts to physiology than the 100 mg RDX kg$^{-1}$ dry soil group. This could be due to RDX binding to the
soil (Cunningham et. al., 1995), RDX leaching, sequestration and dilution by plants (Pilon-Smits, 2005), microbial degradation and removal (Yoon et al., 2005), or that the level was insufficient to impact growth. There appeared to be a physiological threshold for adult plants between the 750 mg RDX kg\(^{-1}\) soil and 1500 mg RDX kg\(^{-1}\) soil groups. Photosynthesis at 750 mg RDX kg\(^{-1}\) soil was significantly different from controls however chlorophyll fluorescence characteristics responded differently. The 1500 mg RDX kg\(^{-1}\) soil group showed significant differences in dark adapted maximum operating efficiency of PSII \(F_v/F_m\) and the operating efficiency of PSII \(\Delta F'/F'_M\). \(\Delta F'/F'_M\) was significantly lower in the 750 mg RDX kg\(^{-1}\) soil and 1500 mg RDX kg\(^{-1}\) soil groups relative to control corresponding with the overall decline in photosynthesis in those groups. This decline showed a reduction in the absorption of light by chlorophyll (Baker and Oxborough, 2004) which could be due to RDX or possibly due to the chlorosis and necrosis occurring in the leaves.

Reduced \(F_v/F_m\) is a common indicator of stress in plants (Lichtenthaler and Mehé, 1997). This was observed in the 1500 mg RDX kg\(^{-1}\) soil group which consistently had values under 0.7, suggesting that photoinhibition occurred (Adams et al., 1990). Yet the lack of change in ETR values shows that PSII is operational across concentrations. RDX may be inducing the decline in photosynthesis by altering or impairing the Calvin-Benson cycle, or other process downstream of the dependent portions of the photochemical reaction (Zinnert et al., 2012). The lack of effect on water potential also supports that there is a mechanistic difference between the impacts of explosives contamination and naturally occurring stressors. The morphological changes that were present in juveniles and observed in adult shrubs may also play a role in physiological reduction.
The difference in morphological impacts in between the 100 mg RDX kg$^{-1}$ soil and higher concentrations of juvenile plants helps to explain the difference in physiological function in adult plants. Leaf necrosis limits leaf area for photosynthesis and stomatal conductance, as does the reduced leaf size (Laurent et al., 2007). Defoliation increases stomatal conductance as a compensatory action to balance the root surface area to leaf area ratio (Heichel and Turner, 1983). Plants >100 mg RDX kg$^{-1}$ soil lost older leaves and new leaves were reduced, necrotic, and curled (personal observations). Initial declines in physiological responses can be attributed to transplant stress as this was seen in the control group as well (Rietveld, 1989). Leaf necrosis was observed at the end of the experiment in the 100 mg RDX kg$^{-1}$ soil adult group, however it was only observed on the margins of the oldest leaves (personal observations).

While RDX produced morphological changes there was no reduction in overall stomatal conductance. Interestingly, the 1500 mg RDX kg$^{-1}$ soil groups had significantly higher stomatal conductance compared to the control and other experimental groups, giving stomatal conductance and photosynthesis a negative relationship. This suggests that stomatal conductance was not inhibiting photosynthesis, which is contrary to non-anthropogenic environmental stresses (Sande and Young, 1992, Flexas et al., 1999; Subrahmaniam et al., 2006). Similar results have been reported for ozone contamination of *Populus sp.* trees (Reich and Lassoie, 1984), however most of the literature has shown the reverse to be true (Reiling and Davison, 1995).

Interestingly, although there were significant changes in physiological function in the higher concentrations the values were still well above what are considered “normal” for *M. cerifera* under natural stressed conditions (e.g. drought, salinity, high light; Sande and Young, 1992; Subrahmaniam et al., 2006; Naumann et al. 2007). Therefore despite drastic
morphological impacts RDX had on juvenile *M. cerifera*, adult physiology was still comparatively functional. More research is needed to understand the specific method by which RDX impacts plant physiological processes and morphology.
Conclusions

RDX had significant impacts on both plant morphology and physiology. Across age classes there was a distinct difference in the impact of RDX at varying concentrations. Seed germination was the most influenced by RDX, having significantly reduced germination at the lowest concentration (100 mg RDX kg\(^{-1}\) soil) where the other two age classes did not exhibit significant impacts at that level. Under natural conditions, this threshold difference may alter both succession and seed bank viability at concentrations between 0 and 100 mg RDX kg\(^{-1}\) soil without impacting the established plants. Above 100 mg RDX kg\(^{-1}\) soil however the germination success did not vary while established plant morphology and physiology deteriorated. The morphological impacts were similar across concentrations above 300 mg RDX kg\(^{-1}\) soil while the physiological characteristics declined as concentration increased. This suggests that at low concentrations RDX acts as an ecophysiological filter, preventing seed germination, while at higher concentrations it can act as a biotic filter, influencing the success of established plants.
Literature Cited
Literature Cited


Table 1: Summary of Dunnet’s multiple comparison p-values for leaf morphology impacts of RDX contamination on *Morella cerifera*. All comparisons are relative to the control group for each week. The (-) denote a lack of data due to absence of the morphological characteristic and bold numbers indicate significance at $\alpha=0.05$.

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Figure 1: Effect of RDX soil contamination on overall amount of *Morella cerifera* germination. (*) indicate the presence of a significant difference, relative to control (p<0.05). Error bars represent one standard error.
Figure 2: The effect of RDX soil contamination on the 6-week difference in shoot length and root length for juvenile *Morella cerifera*. There were no significant impacts on shoot or root length found here. Error bars represent one standard error.
Figure 3: Effect of RDX soil contamination on stem count for *Morella cerifera*. No significant differences were observed across concentrations. Error bars represent one standard error.
Figure 4: Effects of RDX soil contamination on leaf morphology in juvenile *Morella cerifera* at week 6. Leaf necrosis, leaf curling, and reduced leaves are shown as a percentage of total leaf count at week 6. (*) show the presence of a significant difference relative to control. Error bars represent one standard error.
Figure 5: Impact of RDX soil contamination on adult *Morella cerifera* photosynthetic values across weeks. (*) indicate the presence of a significant difference relative to control (p<0.05). Error bars represent one standard error.
Figure 6: Effect of RDX soil contamination on average stomatal conductance across weeks on *Morella cerifera*. The 750 and 1500 mg RDX kg\(^{-1}\) soil groups exhibited increased stomatal conductance, surpassing that of the control. Error bars represent one standard error.
Figure 7: The impact of RDX on the relationship between stomatal conductance and photosynthesis in *Morella cerifera*.
Figure 8: Effect of RDX soil contamination levels on chlorophyll fluorescence characteristics of Morella cerifera at the end of the experiment. (*) indicate the presence of a significant difference relative to control (p<0.05). Error bars represent one standard error.
Figure 9: Effect of RDX soil contamination on xylem pressure potential values for *Morella cerifera* at biweekly intervals. Error bars represent one standard error.
Appendix A

Images of test plants: on the right is an average control plant, on the left is an average example of a 500 mg RDX kg$^{-1}$ soil group plant.
Vita

Stephen Michael Via was born in June 5th, 1988 in Newport News, Virginia. He graduated Heritage High School, Newport News, Virginia, in 2006. While in high school he spent his summers working at the Virginia Living Museum, Newport News, Virginia helping with tending to the plants and animals kept there. He received his Bachelor’s of Science in Biology from Virginia Polytechnic and State University, Blacksburg, Virginia, in 2010. As an undergraduate he worked in three labs focusing on water hyacinth impacts on bird populations, nutrient cycling in freshwater streams, and poplar tree genetics.