Shrubs as Sentinels of Ordnance Contamination: Using Plant Physiology and Remote Sensing to Detect TNT in Soils

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SHRUBS AS SENTINELS OF ORDNANCE CONTAMINATION:
USING PLANT PHYSIOLOGY AND REMOTE SENSING TO DETECT TNT IN SOILS

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.A. Virginia Commonwealth University 2004
M.S. Virginia Commonwealth University 2011

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Abstract

SHRUBS AS SENTINELS OF ORDNANCE CONTAMINATION: USING PLANT PHYSIOLOGY AND REMOTE SENSING TO DETECT TNT IN SOILS

By Kathryn Rubis, M.S.

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

Virginia Commonwealth University, 2011

Major Director: Donald R. Young, Department Chair, Biology

Methods for rapid, safe and effective detection of unmapped buried ordnance are vital to the protection of humans and environmental quality throughout the world. This study aimed to investigate the use of phytosensing and to understand the physiological response of woody plants to 2,4,6-trinitrotoluene (TNT) contamination. Baccharis halimifolia were potted in soils containing various concentrations of TNT and physiological responses were observed over a 9-week experimental period. Measurements included the collection of remotely sensed data, such as hyperspectral reflectance and chlorophyll fluorescence, and traditional plant-level physiological data. In accordance with the hypothesis, low levels of TNT improved physiological response in plants due to the slight increase in nitrogen, while high levels of TNT induced stress. Key markers in stress responses were identified, specifically with reflectance indices and derivatives, which may separate TNT-contaminated plants from naturally stressed plants and would allow for accurate detection of buried ordnance at the landscape level.
Introduction

Detection and remediation of ordnance contamination is crucial to national and military security as well as environmental quality. Minefields and abandoned bombing ranges serve as dangerous and oftentimes obscured explosive obstacles to the security of military personnel and civilians across the world. For example, approximately 2 million mines were arbitrarily laid during the war in Croatia (1991-1995) and only a small percentage have been cleared or identified, the majority having no record of geographic location (Radonic et al., 2004). Numerous countries, such as Tajikistan, Afghanistan, and Chechnya, are experiencing similar challenges with indiscriminately laid antipersonnel mines and continuing cases of civilian casualties. In addition to fatalities and severe injuries due to detonation, buried ordnances leak toxic, mutagenic, and carcinogenic compounds into the soil and water supply, further endangering humans and organisms within the ecosystem. It is necessary to construct a method for the safe and remote detection of buried explosives, and secondarily to observe potential techniques for the bioremediation of these ordnance compounds.

By analyzing the physiological response of plants to TNT (2,4,6-trinitrotoluene) contamination, we can potentially detect, with certainty, the presence of explosives in soil. The novel and burgeoning field of phytosensing, defined as the use of plants to remotely detect explosives, has focused mainly on gene expression of genetically modified plants, specifically designed to respond to TNT and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine); however, few advancements have been achieved thus far (Rao et al., 2009). Analysis of existing vegetation in a contaminated landscape may prove useful in the development of detection strategies. Numerous studies indicate that plants store significantly higher amounts of TNT compounds in the root
tissues as opposed to above-ground vegetation (Schoenmuth and Pestemer, 2004) due to strong chemical binding and transformation by the root tissues (Thompson et al., 1998). Though TNT is stored primarily in below-ground tissues, above-ground physiological effects are evident. Biomass is significantly reduced in plants grown in TNT-contaminated soil (Robidoux et al., 2003; Thompson et al., 1998) and seed germination decreases with TNT exposure (Gong et al., 1999b). Due to the transformation and compartmentalization of xenobiotics in plant cellular structures, such as the vacuole or cell wall (Trapp and McFarlane, 1995), photosynthetic rates and pigment content are negatively affected (Naumann et al., 2009). Therefore, remote sensing of plants, via the examination of foliar reflectance or chlorophyll fluorescence, could be useful in the detection of biomass changes and also photosynthetic and pigment content alterations in response to TNT. I argue that it is important to identify phytosensing capabilities of naturally occurring plants presently existing in the contaminated environment. Remote detection of explosives through the analysis of plant canopy responses has potential to be practical and rapid, effective at the landscape level, and economically feasible.

Remote sensing is an efficient tool in the analysis of landscapes due to relatively rapid and easy collection of data over large spatial scales; however, it should be used in conjunction with ground-truthing physiological measurements to validate results. Analysis of canopy-level reflectance can indicate the presence of TNT in the soil. Previous research shows that spectral signatures vary between plants exposed to natural stresses (drought, light, salinity) and anthropogenic stresses (nutrient inputs, heavy metals, explosive compounds) (Naumann et al., 2009). Identification of the sensitive red-edge inflection point through first- and second-derivatives, exploratory statistical analysis of reflectance indices, and the overall examination of the spectral signature in the visible and near-infrared region may reinforce remote sensing
methods when using satellite or airborne hyperspectral imagery at the landscape level. Additionally, the remote detection of chlorophyll fluorescence can indicate heat dissipation mechanisms and photosystem functioning in the leaf as a response to explosive residue contamination. To connect remote sensing observations to plant-level physiological responses, parameters such as photosynthetic gas exchange, water-use efficiency, chlorophyll content, biomass distributions, and dry tissue analysis should be conducted.

The objective of my study was to examine the effects of TNT contamination on the physiological processes and associated stress of the woody shrub, Baccharis halimifolia L. (Asteraceae). It is necessary to understand the physiological responses of a globally distributed woody shrub to TNT. Primarily, I aimed to establish methods to identify TNT-stressed vegetation by analyzing foliar spectral reflectance via hyperspectral imagery and chlorophyll fluorescence, and relating these data to plant-level physiological measurements. I hypothesized that plants contaminated with low levels of TNT would show increased rates of physiological activity due to the slight increased availability of nitrogen, whereas high levels of TNT would induce some stress response. When coupled with remote sensing imagery, results may be used to identify areas of contamination for national security or military missions, and also to recognize areas in need of remediation efforts. Secondarily, I intended to determine if B. halimifolia is tolerant of various levels of TNT, and if so, could perhaps thereby serve as a soil stabilizer at contaminated sites and be recommended for future phytoremediative studies. Because B. halimifolia is generally regarded as a weedy plant that endures a wide range of environmental conditions, I hypothesized that the shrub would be tolerant of high levels of TNT and would potentially be useful in remediation.
Background

Though many chemical explosives have been produced throughout the previous century, TNT has been the most prevalently used, and currently exists in great quantities throughout the environment (Travis et al., 2008). TNT is the primary energetic compound in most buried ordnance. Relatively stable in comparison to earlier explosives, such as dynamite, TNT was first produced in 1863 by German scientist Joseph Wilbrand and became the most widely produced and used explosive by militaries throughout the world (Daun et al., 1998). TNT has been used in ammunition for firearms, shells, tanks, and landmines.

In the United States, numerous production facilities began mass-synthesizing TNT to meet the demands of ammunition for World War I and II, Vietnam and Korean Wars. At production sites, byproducts of TNT include two main hazardous waste water emissions. TNT colored complex (TNTcc), commonly referred to as pink water, is produced from the post-production equipment washdown procedures. The more toxic sellite water, also known as red water, is created during TNT production by using water to purify crude TNT. Laboratory research and field studies have demonstrated that prolonged exposure and/or ingestion of TNT byproducts lead to carcinogenic and mutagenic reactions (Daun et al., 1998) and cause spleen and liver malfunctions, degradation of the immune system, and anemia. In a study examining the effects of TNT on Pimephales promelas (fathead minnow), typical environmental concentrations of pink water showed altered behavioral responses while high concentrations caused mortality within ten minutes of exposure (Smock et al., 1976). Pink water can also form from the leaching of TNT from unexploded ordnances (UXOs) into the groundwater and surface
water (Best et al., 2008). UXOs commonly litter former aerial bombing ranges and, to a lesser extent, are present at artillery and mortar training ranges.

It has been well documented that TNT causes many adverse and potentially lethal reactions among organisms (Gong et al., 1999b). These highly toxic compounds enter delicate ecosystems through wastewater runoff and leachates and can cause chronic diseases and possibly cancer in higher organisms through the process of bioaccumulation (Adamia et al., 2005). Additionally, as residential housing expands onto sites previously used for ammunitions production, edible plants are being cultivated on contaminated soil, posing an immediate threat to public health (Schneider et al., 1996). Due to the toxic affects of TNT to aquatic and terrestrial organisms, as well as the possibility of danger to humans, it is a necessity that these compounds be remediated (Smock et al., 1976; Daun et al., 1998).

Sites containing TNT-contaminated soils are common throughout Virginia due to the prevalence of numerous military sites that have either TNT production facilities, former aerial bombing ranges, or live ammunition artillery and mortar training ranges. A production site of importance in Virginia is Radford Army Ammunition Plant, which has been the leading producer of military-used TNT in the country. Prior to 1986, the facility released 60,000 pounds of red water per day into nearby streams. With the advent of environmental regulations, Radford has eliminated the discharge into streams through the reengineering of the equipment. However, residual contaminants may still be affecting surrounding aquatic environments.

Other production-related sites in Virginia include eight Environmental Protection Agency Superfund Sites. These sites contain buried munitions that are potentially leaking TNT into the soil and the underground water supply. For example, in 1987, Nansemond Ordnance Depot in Suffolk was found to have buried explosives in addition to a several-ton slab of crystalline TNT.
Due to its designation as a Superfund Site, Nansemond Ordnance Depot is currently being remediated; however, present ammunitions may still be affecting water quality.

UXOs at former bombing ranges cause safety, health, and environmental hazards. The former aerial bombing range at Plumtree Island National Wildlife Refuge near Norfolk has been closed to the public due to the extensive distribution of buried UXOs. Though the US Army Corps of Engineers is currently detonating and removing some of the exposed ordnances, numerous other UXOs persist underground. Due to the location of Plumtree Island on the fragile Chesapeake Bay, TNT leaching poses a considerable threat to bay organisms. Another former aerial bombing range that poses a potential risk is located at Duck Pier Field Research Facility in Duck, North Carolina. In response to serious injuries due to scavenging vacationers, cleaning efforts began in 1972 and buried much of the UXOs; however, at present, shifting sands uncover more ordnance on a daily basis. These sites should be a focus for remedial efforts for not only explosive and safety concerns, but also for water contamination issues.

Furthermore, the dissolution of explosive residues at artillery training ranges poses a threat to ground water contamination and introduction into the environment. Previous studies indicate that soil concentrations at military ranges contain a sizable presence of TNT and RDX, and exceptionally high levels of HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) (Jenkins et al., 1997; Thiboutot et al., 1998). In relation to other explosives, TNT has the highest dissolution rate into water, indicating faster mobilization into the environment (Pennington et al., 2001). Therefore, prime objectives of the Department of Defense, in relation to the management of military training ranges, are to minimize the leaching of explosive residues into the groundwater, avoid the uptake of these compounds into edible plants and introduction into the food chain, stabilize soil to discourage erosion, and maintain a sense of vegetative realism on the
battlefield (Best et al., 2008). An effective and pragmatic solution to these objectives is through the use of phytoremediation.

Traditionally, the most common form of TNT remediation has included the physical removal of soil, in addition to thermal processes and the utilization of soil microorganisms to reduce compounds (Hughes et al., 1997; Daun et al., 1998). However, because plants have a tremendous ability to store high concentrations of metals and toxins in their tissues (Salt et al., 1998), many plants are effective in removing toxins from a contaminated site. The use of phytoremediation significantly reduces the cost of cleanup as compared to the physical removal of soil. The cost of TNT-contaminated soil remediation for every cubic meter can cost one hundred to five hundred times more than phytoremediation (Cunningham et al., 1995). Additionally, soil removal is detrimental to ecosystems in that it alters geologic and chemical properties of the area, and that the contaminated soil is disposed of in landfills, compost heaps, or incinerated (Rylott and Bruce, 2008). Certain plant species show great potential for the uptake of TNT from soils, such as wheat and oat (Gong et al., 1999b), specific grasses and forbs (Best et al., 2008), and some aquatic plants (Hughes et al., 1997). Fewer studies have investigated woody plant uptake of TNT (Thompson et al., 1998), yet claim that woody plants possess greater potential than herbaceous plants for the remediation of TNT due to the large root biomass and TNT-degrading potential (Schoenmuth and Pestemer, 2004). The use of plants is effective in not only the remediation of a contaminated site, but also as a means of creating habitat diversity and preventing soil erosion. It is therefore crucial to examine plant species that are tolerant of explosive contaminants or effective in the remediation of a site.

Though environmental contamination due to explosive residues is a significant problem, an urgent solution is required for the detection of unidentified landmines. During the twentieth
century, over a hundred million unmapped landmines were laid by military or rebel forces throughout the world, and have consequently caused mass casualties, loss of property values, restriction of farming, and excessive cost expenditures for landmine detection and removal. The use of modern landmines, initially developed by the Germans and used during the First World War as antitank weapons, spread throughout the world and were refined into smaller and more sensitive antipersonnel mines that would detonate under the weight of a person. In 2009 alone, there were 3,956 reported casualties from landmines in 64 countries, of which over a thousand victims died and the remaining suffered from debilitating disfigurement (Landmine Monitor Report, 2010). Detection methods and clean up efforts, which can cost as much as 300 times that of the initial laying of the landmine, continue to increase in efficacy and safety; however, further research is necessary to develop practical and safe alternatives to the identification of landmines. Today, methods of detection and demining include the use of metal detectors, trained dogs, on-site manual disarming, robots, and armored automotive tillers, with current scientific research focusing on more effective detection methods such as ground-penetrating radar (Takahashi et al., 2011) and the use of odor-detection capabilities of giant African pouched rats (Poling et al., 2011). These methods contrast with the more passive yet potentially more effective approach of using existing vegetation to reveal UXOs.

Much research involving the effects of TNT on naturally occurring plants has focused on seedling development and plant tolerance with the end goal of identifying phytoremediative strategies. Studies using grasses, forbs, and sedges have shown that overall plant development is significantly reduced in response to TNT (>50 mg TNT kg\(^{-1}\) soil) (Best et al., 2008), particularly in the roots (Palazzo and Leggett, 1986). However, herbaceous plants contaminated with low levels of TNT (25 mg TNT kg\(^{-1}\) soil) show an increase in seedling development (Gong et al.,
suggesting that the nitroaromatics enhance nutrient availability. Other studies have focused on gene expression and transcription in plants exposed to TNT to better identify metabolic pathways in remediative plants (Ekman et al., 2003).

Additionally, research has examined plant uptake, fate, and transformation of energetic compounds. Once explosive residues are taken up through the plant roots, the xenobiotics undergo a three-step process involving enzymatic transformation, conjugation, and compartmentalization of the soluble materials being stored in cell walls or vacuoles, and insoluble forms stored in cell walls (Yoon et al., 2005). Tracer studies using $^{14}$C in aquatic plants and poplar trees have shown that bound residues of TNT are highest in root tissues and indicate strong sequestration; however, extractable products of TNT are highest in stems and leaves and are potentially transferable to the environment (Vanderford et al., 1997; Thompson et al., 1998). Due to the soluble nature of the extractable TNT xenobiotics, these compounds are transpired throughout the plant, causing physiological changes in pigment content and photosynthetic activity, which can be examined through the measurement of gas-exchange, chlorophyll fluorescence, foliar reflectance, chlorophyll concentration, and carbon and nitrogen isotopes. Previous research indicates that there are physiological variations in plant responses to TNT, which separate it from natural stress responses, specifically with stomatal conductance and dark-adapted fluorescence (Naumann et al., 2009). It is therefore the aim of this study to further identify markers that would clearly and accurately separate the responses between TNT-contaminated plants and uncontaminated vegetation.

Through the analysis of data obtained using remote sensing technologies, these markers may allow for landscape-level explosive detection. Analysis of canopy reflectance is a useful tool in the understanding of plant chemistry, net primary production, turgidity, and stress from
environmental or anthropogenic pressures and should be used in conjunction with ground-truthing. Results from laboratory analysis using close-range spectrometers can be related to airborne or satellite-derived reflectance data. The near-infrared region of light is indicative of biomass and tissue health, whereas the visible region denotes pigment content and chemistry. Damaged and chlorotic leaves display increased reflectance in the visible region with an overall shift of the red-edge inflection point to shorter wavelengths (Ustin and Curtiss, 1990), which is due to a reduction in chlorophyll (Collins, 1978). Conversely, increases in chlorophyll content induce greater absorption, especially in the green and red wavelengths, and a red-edge shift to longer wavelengths (Collins, 1978).

Another remote sensing technology, passive fluorescence, allows for measurement of photosynthetic fluctuation diurnally and under stress (Evain et al., 2004). Furthermore, as with certain reflectance indices, fluorescence measurements have detected vegetation stress before other physiological changes occurred, especially changes in chlorophyll content (Lichtenthaler, 1996; Evain et al., 2004). Stress causes a direct and rapid decrease in photosynthetic quantum yield and conversely an increase in heat dissipation and chlorophyll fluorescence, as a means to balance the budget of incoming actinic light. In response to stress, partial photoinhibition, or the inactivation of some photosystems within the cell, protects the remaining PSII centers from destruction while maintaining CO₂ assimilation and subsequent carbon gain and plant growth (Lichtenthaler, 1996). Therefore, the measure of chlorophyll fluorescence can immediately quantify the efficiency of the photosynthetic apparatus, where as other physiological parameters (such as gas exchange, water potential, and pigment content) may take longer to react to certain stressors. The measurement of chlorophyll fluorescence induction kinetics is non-invasive and remotely-sensed and has the potential to move towards a larger scale at greater distances from
the plant, as has the analysis of reflectance in hyperspectral imagery. In conclusion, the ability to collect plant physiological data from airborne-obtained imagery suggests that remote sensing has the potential to be more efficient, rapid and safe in comparison to other methods of ground-level explosive detection.
Methods

Plant Materials and Soil Contaminant Preparation

*Baccharis halimifolia* is a wind-dispersed perennial dioecious woody shrub that occupies upland marsh sites and ocean-side shrub thickets, and is common throughout the mid-Atlantic coast. The genus *Baccharis* is widespread throughout the Americas and has become established in Australia, southern Europe, and Africa; therefore, data collected from *B. halimifolia* may potentially be related to the other 400 species within the genus. This weedy species responds well to increased levels of nitrogen and is tolerant of anthropogenically-disturbed sites, allowing for global trends of shrub expansion (Ervin, 2009). *Baccharis halimifolia* contains antiherbivory secondary compounds that make it generally unpalatable to wildlife (Kraft and Denno, 1982); therefore, it may be valuable if used as a TNT remediator or soil stabilizer as it would have a reduced potential for introducing toxins into the food chain. Additionally, *B. halimifolia* was selected due to the extensive physiological data that my laboratory has collected over the previous fifteen years. Physiological data from laboratory experimentation will be analyzed against field data to establish landscape-level applications.

*Baccharis halimifolia* seedlings were collected from an undisturbed barrier island, Hog Island, Virginia (37° 40’N; 75° 40’W). The seedlings were planted in 1.5 L plastic pots filled with low nitrogen soil and allowed to mature in a Conviron environmental chamber (CMP 3244, Controlled Environments Limited, Asheville, NC) set at a photosynthetic photon flux density (PPFD) of approximately 600 mmol m$^{-2}$ s$^{-1}$, 48% relative humidity, a 14 hour photoperiod, and a
day and night temperature of 30°C and 25°C, respectively. After maturing, plants measuring at least 15 cm in height were selected for the experiment.

Low nitrogen topsoil was dried at 80°C for two days in a drying oven to remove excess moisture. A 3:1 ratio of topsoil-to-sand was mixed for each 1.5 L plastic pot (2.59 kg topsoil and 0.86 kg sand) yielding 0.21 ± 0.01 % nitrogen per 3.45 kg of prepared soil. Varying concentrations of TNT were dissolved in 200 mL acetone and mixed with the soil. Treatments included 30, 100, 250, and 500 mg TNT kg⁻¹ soil, n=5 per treatment (Naumann et al., 2009). Reference plants were potted in the topsoil-sand mixture and treated with 200 mL of acetone and 0 mg TNT kg⁻¹ soil. A second experiment was performed at a later date to observe the effects from an extended range of contamination with treatments of 30 mg, 300 mg, 600 mg, and 1200 mg TNT kg⁻¹ soil. Identical methods were employed in this second experiment.

Throughout the experiment, controlled watering was conducted three times a week and was in response to the individual needs of each plant based on turgidity and soil dryness. Water was applied to each plant until a few drops percolated from the drain holes (trays were provided to reduce loss of TNT). As B. halimifolia is sensitive to flooding, overwatering was carefully avoided to minimize additional stress effects. Canopy reflectance, gas exchange, and chlorophyll fluorescence measurements were performed weekly in the mid-morning (starting at 1030 hours) and lasted 9 weeks, starting at week zero. Midday leaf water potential, leaf pigment content, biomass, C:N, and isotopes were measured at the conclusion of the experiment.

Reflectance Measurements

Canopy spectral reflectance measurements between λ 350 nm and 2,500 nm were collected using an ASD FieldSpec Pro reflectance radiometer (Analytical Spectral Devices, Inc.,
Boulder, CO) and a 3,200°K lamp as the source of illumination. Data were compiled and averaged to obtain spectral signatures for each treatment. To determine if differences existed between the inflection points of the stress-sensitive red-edge region between λ 680 and 720 nm, first- and second-derivative analysis was applied to the data using techniques from Milton et al. (1989). To accurately quantify differences in spectral curves and to normalize any noise, an exploratory analysis was conducted by applying 22 established vegetative indices and derivatives to the reflectance data from the primary and secondary experiments. Indices showing potential as statistically significant indicators of TNT contamination were selected and examined for separation responses of the treatments. The relative red-edge position (REP) was calculated based on techniques by Guyot and Baret (Cho and Skidmore, 2006) to later relate to chlorophyll concentrations.

Fluorescence and Gas Exchange Measurements

Light-adapted and dark-adapted chlorophyll fluorescence was quantified with a pulse amplitude modulated fluorometer (MINI-PAM, Photosynthesis Yield Analyzer, Walz, Effeltrich, Germany). Measurements of fluorescence for light-adapted plants included minimal fluorescence (F’s), maximal fluorescence (F’m), and the ratio of ΔF/F’m = (F’m-F’s)/F’m as the effective quantum yield of regulated thermal energy in the xanthophyll cycle. Measurements of fluorescence for dark-adapted plants included minimal fluorescence (F_o), maximal fluorescence (F_m), and the ratio of F_v/F_m = (F_m-F_o)/F_m as the maximum quantum use efficiency of photosystem II (PSII). The physiological reflectance index (PRI), also known as the photochemical reflectance index, was linearly regressed against the effective quantum yield (ΔF/F’_m) of the final weeks to determine whether relationships existed. Leaf net photosynthesis (A_{Net}) and stomatal
conductance to water vapor \( (g_{wv}) \) were measured using a portable infrared gas analyzer at a photosynthetic photon flux density of 800 mmol m\(^{-2}\) s\(^{-1}\), 48\% relative humidity, and 28°C air temperature (LI-6400, LI-COR Biosciences, Inc., Lincoln, NE). Since stomatal aperture may respond within seconds to fluctuations in CO\(_2\) levels, gas exchange measurements were conducted immediately upon removing the plant from the environmental chamber.

Physiological Measurements

At the conclusion of the 56-day experiment, midday leaf water potentials were measured using a Scholander-type pressure chamber (PMS Instrument, Corvallis, Oregon). Leaf pigment concentrations were examined using established chlorophyll extraction protocols determined by Sěsták (1971). The extracted pigment samples were analyzed with a Spectronic 21 spectrophotometer to obtain absorption values, which were used to calculate chlorophyll \( a \), chlorophyll \( b \), and carotenoid concentrations in accordance with the equations of Sěsták (1971).

The plants were extracted from the pots, hand washed to remove soil, and dried at 80° C for 48 hours in a drying oven. Belowground and aboveground biomasses were measured to establish if trends existed among TNT concentrations and carbon sequestration. Dried leaf samples were ground into a fine powder using a mortar and pestle, and analyzed for C:N, %N, \( \delta^{13}C \), and \( \delta^{15}N \) using an elemental analyzer-isotope ratio mass spectrometer at the University of Georgia Stable Isotope and Soil Biology Laboratory (Athens, GA).

Field Measurements

To link laboratory measurements to the landscape level, field work was conducted in late summer at the USACE Duck Pier, FRF in Duck, North Carolina. The 175-acre property was the
site of the Duck Naval Target Facility, used for intense aerial bombing target training between 1941 and 1965 (McDonald, 2009). Unexploded ordnances (UXOs) heavily littered the area with the greatest concentration along the target flight line, which extended northeast from Currituck Sound to the Atlantic Ocean. In 1972, due to trophy scavenging by visitors and subsequent injuries from UXO detonation, 2,287,000 pounds of ordnance were bulldozed into 10 shallow burial pits scattered throughout the property (DPRA, 2001; McDonald, 2009). Using ArcGIS Software, I georeferenced a 1999 survey by Parson’s Engineering Science, Inc. to base map imagery and used the coordinates to identify the exact position of the largest burial pit, #10 (36°10’N, 75°45’W). The site is situated alongside Currituck Sound and is intermixed with small grassy patches and a diverse array of coastal shrubs and vines. Six mature B. halimifolia, measuring 2.5 to 5 meters in height, were growing in this area. Leaf samples from second-order branches were collected for pigment and dry-tissue analysis, using the aforementioned methods involving laboratory plants. Leaf samples were also taken at a reference site at Jockey’s Ridge State Park, North Carolina (35°96’N, 75°64’W), an uncontaminated area located 26.6 km south-southeast of Duck FRF. The reference site was chosen for the presence of B. halimifolia, the absence of excessive anthropogenic contaminants, and for the similarity in environment and proximity to Currituck Sound.

Statistical Analysis

Statistical differences among the treatments for all experimental dependent variables were analyzed using one-factor analysis of variance with a confidence level of 95% (Zar, 1999). Post hoc analysis was performed using Tukey’s HSD. Dependent variable values of chlorophyll a were regressed against percent nitrogen, values of total chlorophyll were regressed against the
relative red-edge position, and PRI was regressed against $\Delta F/F'_m$ to identify statistically
significant predictive relationships. A comparison of means (t-test) was used to analyze site
differences in field measurements with a confidence level of 95%. All statistical analyses were
performed using R™ Statistical Software.
Results

Overall, both control and treatment plants generally declined physiologically throughout the 8-week experiment, markedly so during the last two weeks; however, definitive separation among treatments identified varying responses to TNT concentrations. Visually, all plants appeared healthy having bright green new leaves and continued meristematic growth throughout the entire experiment (Fig. 1). Changes in leaf coloration on middle age and older leaves of higher-contaminated plants were evident by week 5 and became more pronounced through the remainder of the experiment. Plants treated with high concentrations (250 and 500 mg TNT kg\(^{-1}\) soil) produced deep red-purple leaf coloration, whereas plants with low concentrations displayed no reddish coloration and maintained a green leaf color throughout the 8 weeks (Fig. 1).

Canopy Spectral Reflectance

Spectral signatures were generated for each TNT treatment to remotely analyze the potential differences among *Baccharis halimifolia* growing in a range of TNT contaminated soil. There was clear separation of treatments in the visible region (\(\lambda\) 380 nm to 700 nm) and the near-infrared region (\(\lambda\) 700 nm to 1000 nm) (Fig. 2). The visible region, indicative of leaf chemical composition, showed variation in the reflection of green light and less variation in the reflection of red light. The near-infrared region, indicative of biomass and turgidity, showed a general decrease in reflectance for contaminated plants.

To observe shifts in the stress-sensitive red-edge region (\(\lambda\) 680 nm to 720 nm), first- and second-derivative curves were applied to identify the inflection point. All treatments showed
slight shifts to shorter wavelengths (blue shifts) throughout weeks 4, 6, and 8 of the experiment; however, the first and second peaks decreased with higher TNT levels (Fig. 3). Analysis of the second-derivative reflectance curves of week 8 indicated that plants with higher contaminant levels had the greatest blue shifts (Fig. 4).

Due to atmospheric conditions or daily physiological variation in individual plants, it is often necessary with remote sensing to normalize the data by analyzing reflectance indices. Analysis of variance results from an exploratory investigation of reflectance indices indicated similar statistical significance with the primary lower-level contamination experiment and the secondary high-level contamination experiment (Table 1). For further investigation, selected indices were examined to determine statistically significant relationships among treatments for each week (Table 2). Physiological reflectance index (PRI), chlorophyll index (CI), anthocyanin reflectance index (ARI), index of $R_{750} / R_{710}$, derivative of the maximum of $\lambda$ 714 nm, and the derivative of $\lambda$ 715 / $\lambda$ 710 showed statistically significant separation of the treatments throughout the progression of the experiment, markedly so in the final two weeks (Table 3, Fig. 5). Specifically, treatment levels 30 and 100 mg kg$^{-1}$ produced the highest index values, followed by the reference, 250, and 500 mg kg$^{-1}$ plants, respectively.

Chlorophyll fluorescence, gas exchange, and leaf water potential

Clear and predicted separation of the treatments occurred for the dark-adapted chlorophyll fluorescence measurements, indicating that 30 and 100 mg kg$^{-1}$ plants had the highest optimal quantum yield ($F_v/F_m$) (Fig. 6). Statistically significant differences existed among treatment levels at week 4 ($F=3.46, p=0.0266$) and week 5 ($F=5.66, p=0.0033$). Similar separation trends existed for the effective quantum yield of light-adapted plants ($\Delta F/F'_m$) with
significant differences at week 6 ($F=9.47$, $p=0.0002$) and week 7 ($F=2.91$, $p=0.0478$) (Fig. 6). The physiological reflectance index (PRI) was linearly regressed against the effective quantum yield of chlorophyll fluorescence for light-adapted *Baccharis halimifolia* ($\Delta F/F'_m$) in the final weeks of the experiment (Fig. 7). Positive, though weak, trends existed in weeks 7 and 8; however, week 6 produced statistically significant differences ($r^2=0.36$, $p=0.0017$). In week 8 of the experiment, 30 mg kg$^{-1}$ plants had the highest net photosynthetic rate, followed by the reference plants, with a sharp and statistically significant decline for the higher TNT-contaminated plants ($F=13.73$, $p < 0.0001$) (Fig. 8). No significant differences were evident for stomatal conductance (Fig. 8). Gas exchange measurements over the entire 9-week experiment showed independent fluctuations between net photosynthesis and stomatal conductance, and an overall gradual decline in all treatments, including reference plants, indicating soil nutrient depletion (Fig. 9). Midday leaf water potential (measurements recorded at 1130 hours) indicated statistically significant differences between 0 and 30 mg kg$^{-1}$ plants and higher levels ($F=3.57$, $p=0.0237$) (Fig. 10).

Pigment content and dry tissue analysis

Plants contaminated with 30 mg kg$^{-1}$ displayed statistically higher concentrations of chlorophyll $a$ ($F=7.34$, $p=0.0008$) and chlorophyll $b$ ($F=4.07$, $p=0.0142$); carotenoid content varied minimally (Fig. 11, Table 4). Dry tissue analysis indicated that a decrease in percent nitrogen occurred in the 500 mg kg$^{-1}$ plants, though no statistical difference existed (Table 4). A positive, yet statistically insignificant, linear regression trend ($r^2=0.06$, $p=0.2401$) existed between percent nitrogen content in the leaves and chlorophyll $a$ concentrations (mg m$^{-2}$) (Fig. 12, Table 4). Linear regression of total chlorophyll content versus the relative red-edge position...
(indicative of pigment content) signified a positive and statistically significant relationship ($r^2=0.44$, $p=0.0003$), and also indicated a general trend that 30 and 100 mg kg$^{-1}$ plants had both higher total chlorophyll and a red-edge position shifted to longer wavelengths (Fig. 13). Plants contaminated with 250 and 500 mg kg$^{-1}$ had statistically less negative $\delta^{13}$C values ($F=6.64$, $p=0.0014$), whereas $\delta^{15}$N values were greatest for mid-range contamination (Fig. 14). The carbon to nitrogen ratio (C:N) of the 500 mg kg$^{-1}$ plants was statistically greater than for other treatments ($F=2.89$, $p=0.0488$) (Fig. 14).

**Biomass**

Similar to the C:N data, plants with the highest level of contamination sequestered the most carbon and, therefore, displayed the largest overall biomass (Fig. 15). Stems of the 500 mg kg$^{-1}$ plants had statistically greater biomass than the other treatments ($F=4.87$, $p=0.0066$), as were also the leaves ($F=8.89$, $p=0.0003$). This parallels visual observation of changes in above-ground biomass and branching structure in week 8 when above-ground biomass appeared greater in the higher-contaminated plants (Fig. 1). Roots of the 500 mg kg$^{-1}$ plants showed some increase in biomass, but the difference was not statistically significant in comparison to other treatments. The ratio of root to shoot biomass did not produce any trends or significant differences throughout the experiment (Fig. 16).

**Field measurements**

Six mature *Baccharis halimifolia* plants, measuring 2.5 to 5 m in height, were identified directly upon or at the edges of the surveyed burial pit #10 at the USACE Duck Pier Field Research Facility in Duck, North Carolina (Fig. 17). Values of $\delta^{15}$N were statistically more
negative in the potentially contaminated plants at Duck Pier FRF than at the reference site \((p=0.0054)\); however, because the isotopic signature was different compared with laboratory values, it may indicate that other factors influenced the results (Fig. 18). Similar to laboratory results, potentially contaminated plants at Duck Pier FRF displayed the least negative \(\delta^{13}\text{C}\) values, though there was no statistical difference compared to the reference site \((p=0.1924)\) (Fig. 18). Also in accordance with laboratory results were the carbon-to-nitrogen ratios for the potentially contaminated Duck Pier FRF plants, which had significantly greater values \((p=0.0055)\) (Fig. 18). Chlorophyll \(a\) concentrations were statistically greater in the reference plants at Jockey’s Ridge SP \((p=0.0341)\); concentrations of chlorophyll \(b\) and carotenoids also were greater in reference plants \((p=0.0777 \text{ and } p=0.0743, \text{ respectively})\) (Fig. 18). Trends in pigment concentrations were similar to laboratory experiments between higher contamination plants and reference plants (Fig. 11).
Discussion

Methods for rapid, safe and effective detection of unmapped buried ordnance need to be established in order to protect civilians and military personnel throughout the world, and additionally to abate environmental contamination. My study aimed to investigate the use of phytosensing and to broaden the understanding of woody plant physiological responses to explosive contamination. Remotely sensed data, including foliar reflectance and chlorophyll fluorescence, and traditional plant-level physiological data were collected for *Baccharis halimifolia* grown in soils with various levels of TNT during a 9-week experiment. I hypothesized that low levels of TNT would improve physiological response in plants due to the slight increase in nitrogen, while high levels of TNT would induce stress. Also, this study investigated the tolerance of *B. halimifolia* grown in various concentrations of TNT to determine if the species could be recommended for future phytoremediative studies and serve as a soil stabilizer at contaminated sites. Since *B. halimifolia* populations cover a range of environmental conditions, I hypothesized that the shrub would be tolerant of high levels of TNT.

Much research has been conducted to analyze the path of energetic compound uptake in plant tissues and associated effects upon plant development, particularly in herbaceous vegetation. However, very little has been performed on the remote detection of explosive contamination in plants. Best *et al.* (1999) determined in emergent aquatic plants that TNT-derived $^{14}$C was highest in roots and below detection in upper shoots, demonstrating that the maximum uptake by root tissues hinders transport to the aboveground portion of the plant. In review of the limited research performed on the effects of TNT on woody plants, one study
involving *Populus* hybrids found that of the total bound TNT residues sequestered by the plants, up to 75% of the compound was found in the roots and no more than 10% was found in the leaves (Thompson *et al.*, 1998). Similarly, in another experiment, 70 ± 0.1% of total applied TNT was found in the roots of sand-grown *Abies* and *Salix* tree saplings (Schoenmuth and Pestemer, 2004). Therefore, given that the majority of TNT compounds are below ground and that it is inefficient to conduct aboveground tracer studies for soluble and insoluble TNT residue contamination in plants, it is potentially more effective to investigate the physiological response in pigment content and photosynthesis due to the transport of soluble TNT extracts throughout the plant. Information acquired at the plant level may be applied to the landscape level through the analysis of remotely obtained data. It is essential to identify patterns and variations between TNT-induced plant stress, anthropogenic-induced stress, and environmental stress at the cellular, chemical, and physiological level.

Analysis of foliar reflectance signifies biomass and tissue health in the near-infrared region of light, and pigment content and chemistry in the visible region. *Baccharis halimifolia* showed separations in response to TNT concentrations in week 8 of the experiment. Because of the consistent shapes and stacking of the spectral signatures between the visible and infrared regions, the data suggest that as TNT concentrations increase, turgidity increases, but does not seem to reflect physiological stress associated with TNT. Due to noise from external sources, overall analysis of the visible and near-infrared regions suggested conflicting theories and was altogether inconclusive; therefore, further examination using noise-diminishing indices and derivatives was required.

The anthocyanin reflectance index (ARI) provided the first basic link between visible stress at the plant-level and remotely-sensed pigment detection using hyperspectral data. Middle
age and older leaves of plants treated with 250 and 500 mg TNT kg\(^{-1}\) soil produced deep red-purple leaf coloration by week 5 which became more pronounced as the experiment progressed. As indicated by the ARI, highly contaminated plants had significantly elevated anthocyanin content, which is often produced by certain environmental stressors or pollutants (Gitelson \textit{et al.}, 2001). These cytoplasmic red flavonoid pigments protect the photosystems during periods of excess light and during leaf senescence, often signifying plant stress (Schaberg \textit{et al.}, 2008). In accordance with the ARI, the purple-red color observed in the leaves of the higher-contaminated TNT plants was indicative of an increase in anthocyanin pigmentation.

Further investigations into reflectance indices rendered an understanding of pigment content, specifically chlorophyll, within the tissues in response to TNT stress. Using the maximum first derivative spectrum (Milton \textit{et al.}, 1989), the red-edge inflection points were generated for each treatment, signifying a blue shift in the red-edge of plants with increased levels of TNT. Supported by the index \(R_{750}/R_{710}\), which focuses on this stress-sensitive region of reflectance, plants contaminated with 250 and 500 mg kg\(^{-1}\) indicate statistically significant shifts in the red-edge. Shown by Gitelson and Merzlyak (1996) that the red-edge position is directly proportional to the chlorophyll index (CHL), the CHL values of \textit{B. halimifolia} treated with 250 and 500 mg kg\(^{-1}\) were statistically lower, indicating a reduction in chlorophyll. This is supported by laboratory pigment analysis showing that the highly contaminated plants had statistically lower chlorophyll \(a\) and \(b\) concentrations within the leaf tissues. Grouped linear regression of total chlorophyll content versus the red-edge position indicates these statistically significant relations. To note, the mid-range contaminations (30 and 100 mg kg\(^{-1}\)) produced more chlorophyll in relation to controls, as shown through pigment analysis, the indices CHL and \(R_{750}/R_{710}\), and linear regressions of chlorophyll content versus REP. In controls, the low soil
nitrogen content may have hindered the synthesis of chlorophyll molecules. For the 250 and 500 mg kg\(^{-1}\) plants, high levels of xenobiotics may have induced reductions in chlorophyll biosynthesis; this has been attributed to either disturbances in the enzymatic production pathways or the inhibition of proper nutrient metabolism (Zengin and Munzuroglu, 2005). Nitrogen is essential for the production of the chlorophyll molecule, as it comprises the fundamental prophyrin ring that supports the central magnesium ion, and has been shown in numerous studies that nitrogen content and chlorophyll are strongly correlated (Blackmer and Schepers, 1995). Therefore, it is possible that the introduction of TNT to \textit{B. halimifolia} has blocked the metabolism of nitrogen in chlorophyll synthesis, as seen in the weak yet correlated relationship between percent nitrogen and chlorophyll \(a\) concentrations.

Healthy plants under high light conditions display multiple maxima in the red-edge first-derivative, which has been attributed to changes in steady-state fluorescence emission (Zarco-Tejada \textit{et al.}, 2003). In response to long-term stress, the maxima diminish considerably, particularly so for the second peak (Zarco-Tejada \textit{et al.}, 2003). In analyzing the TNT results, the first-derivative of the controls showed some progressive stress throughout the remaining 3 weeks of the experiment, probably due to nutrient removal from the soil; however, plants with 250 and 500 mg kg\(^{-1}\) indicated distinct decreases in the second double peak around \(\lambda\) 715 nm. This reduction in the second peak may serve as a key indicator for TNT-contaminated plants.

Visual analysis of the second-derivative further identified a slight shift to shorter wavelengths of the contaminated plants, though this shift was statistically inconclusive. Additional examination of the red-edge using other techniques, such as the linear four-point interpolation, inverted Gaussian, or linear extrapolation (Cho and Skidmore, 2006), could assist in the reduction of noise within the first- and second-derivatives (Ustin \textit{et al.}, 2009). Studies
have shown that derivatives of the red-edge are more effective, possess less error, and are not obstructed by leaf area index (LAI) values than non-derivative indices in detecting bioindicators (Zarco-Tejada et al., 1999). The red-edge derivatives $D_{715}/D_{705}$ and the inversely proportional $D_{\text{max}}/D_{714}$ were valuable in identifying physiological variations among treatments, further supporting the fact that plants contaminated with 250 and 500 mg kg$^{-1}$ had statistically significant shifts in the red-edge region.

Results indicate that the stress response in the reference and TNT-treated plants was clearly visible by week 5 when analyzing the light-adapted ($\Delta F/F'_m$) values and by week 7 in the dark-adapted ($F_v/F_m$) values. This suggests that all plants, including the controls, where gradually showing signs of stress. However, significant separation of the highest contamination plants with the dark-adapted measurements was evident early at week 4. By week 8, the dark-adapted ratios show that the highest contaminated plants fell below the stress threshold, generally regarded as below 0.75, 0.83 being optimal (Maxwell and Johnson, 2000). As photosynthetic activity diminishes under stress, the excess energy from actinic light must be dissipated and does so through non-photochemical quenching (NPQ). Changes in pH occur within the thylakoid lumen and there is an increase in de-epoxidation of the xanthophyll cycle, where violaxanthin is converted to the photoprotective form zeaxanthin (Maxwell and Johnson, 2000). Lower values of $F_v/F_m$ and $\Delta F/F'_m$ in contaminated plants, specifically 500 mg kg$^{-1}$ plants, indicate an inhibition of the photosystems and an increase in zeaxanthin production due to stress.

The physiological reflectance index (PRI), which is often used to determine photosynthetic light-use efficiency in high light environments, is also used to signify the conversion of violaxanthin to zeaxanthin in response to other stressors and can be used in conjunction with fluorescence measurements (Gamon et al., 1990). Separation of PRI for the
treatments throughout the experiment indicates that photosynthetic activity diminished in the 250 and 500 mg kg\(^{-1}\) plants, and that 30 and 100 mg kg\(^{-1}\) plants were photosynthetically more efficient than controls. Research shows that PRI is positively correlated with \(\Delta F/F'_m\) and NPQ, as these are associated measures with the xanthophyll cycle (Evain et al., 2004; Naumann et al., 2008). Similar trends exist with the TNT-stressed *B. halimifolia*, statistically so in week 6 between PRI and \(\Delta F/F'_m\). Overall, the trend is not considerable enough to warrant this a determinant in the detection of TNT stress and further analysis is needed. Independently, though, PRI and chlorophyll fluorescence show potential as key markers in the identification of TNT stress. As with *B. halimifolia*, PRI and chlorophyll fluorescence have shown statistical significance among treatments in TNT-treated *Morella cerifera*, but not with other treatments such as excess nitrogen (Naumann et al., 2010). This is important to note since it is a long-term goal to tease apart the variations in physiological response between plants exposed to nitrogen and plants contaminated with nitrogen-based TNT.

To further understand the physiological condition of the plants and to relate to or confirm remotely-sensed findings, traditional plant-level analysis was required. It was also important to compare all treatment values to controls, as this experiment showed that control plants were increasingly stressed. Immediately upon recovery from transplanting, control plants exhibited stomatal conductance values typical of *B. halimifolia*. However, midway through the experiment, stress-induced closing of the stomata was evident in the controls probably due to the removal of essential nutrients from the low-nitrogen soil. Other studies examining the physiology of laboratory-grown *B. halimifolia* indicate maintenance of stomatal conductance throughout 40 days; however, this is probably due to the utilization of 10% Hoagland’s solution to deliver sustainable amounts of nutrients (Young et al., 1994, Tolliver et al., 1997). Stomatal
conductance values of TNT-treated plants, however, surpassed the control plants in week 4, possibly due to the added nitrogen from the TNT, and then fell towards the end of the experiment.

As stomatal conductance increases, net photosynthesis and carbon assimilation increases (Farquhar and Sharkey, 1982). Typical of plants undergoing natural stress, photosynthesis and stomatal conductance are tightly paired in the woody shrub *Morella cerifera* when exposed to salinity (Naumann *et al.*, 2008). However, my results indicate that net photosynthesis of *B. halimifolia* functions independently from stomatal conductance with exposure to TNT-contaminated soils. These results are analogous with similar TNT-contaminated shrub studies showing that the two physiological processes operate independently of each other, and that TNT affects metabolic activity but not stomatal control (Naumann *et al.*, 2009). Therefore, the independence between photosynthesis and stomatal conductance is a possible indicator for the presence of TNT-related stress (Naumann *et al.*, 2009).

If stomata are closed for greater periods (lower stomatal conductance), carboxylation depletes the reserve of carbon-12 in the cellular chambers and accepts the heavier isotope, carbon-13, which is discriminated against in photosynthesis under optimal conditions (O’Leary, 1988). Greater (least-negative) δ^{13}C values in the tissues indicate lower stomatal conductance and higher water-use efficiency. Plants treated with 250 and 500 mg kg^{-1} displayed the highest water-use efficiency, inferring that these plants would also show lower stomatal conductance and photosynthetic rates. Though true for photosynthesis, discrepancies existed between stomatal conductance and δ^{13}C values. However, examining biological trends, the 30 mg kg^{-1} treatment displayed the highest stomatal conductance and the lowest δ^{13}C values, possibly indicating a link between the two processes and suggesting that these plants showed the highest rates of
physiological activity possibly due to the increased nitrogen availability from the TNT. As with many physiological activities, stomatal conductance fluctuates greatly throughout a 24-hour cycle, and may slow or stop completely due to the slightest external disturbance. Though water availability and subsequent turgor pressure are the main controlling factors, carbon dioxide concentration, light, and temperature also affect the aperture of the stomata and can induce closure within seconds. Because $\delta^{13}C$ is a measurement of carbon assimilation over the lifetime of those cells, and stomatal conductance is an instantaneous measurement, $\delta^{13}C$ values can arguably be more accurate at assessing carbon efficiency. However, due to the preparation and cost of dry tissue analysis, stomatal conductance is a more efficient method of measurement, and is still relatively dependable with repeated measurements.

In the analytical application of laboratory results to field measurements, TNT-contaminated *B. halimifolia* plants also showed less-negative $\delta^{13}C$ values in comparison to the reference site, indicating higher water-use efficiency and lower metabolic activity, possibly due to TNT contamination. Of note is the lowering of $\delta^{13}C$ values for all field specimens ($< 30$) in contrast to laboratory plants ($> 30$), possibly indicative of the presence of other environmental stressors, such as drought or salinity stress. Therefore, it is important to consider natural impacts upon physiological functions when attempting to identify TNT presence at the landscape level.

Concurrent with my laboratory results, previous research also indicates that plants with the least-negative $\delta^{13}C$ values displayed the greatest biomass (Robinson *et al.*, 2000). *Baccharis halimifolia* grown in highly contaminated soil had statistically higher $\delta^{13}C$ values and biomass than plants grown in lower concentrations. The biomass results of this study, however, are contrary to similar TNT experiments showing that root and shoot biomasses of sedges, grasses, and forbs were negatively affected by TNT concentrations. Sedge root growth was reduced by
95% and shoot growth reduced by 54-74% (Palazzo and Leggett, 1986); additionally, grass root and shoot length was significantly reduced in high levels of TNT contamination (Travis et al., 2008). Gong and colleagues (1999b) showed that slight increases in TNT concentrations (25 mg kg\(^{-1}\)) lead to greater aboveground biomass in selected forbs and grasses, and large increases in TNT (100 to 400 mg kg\(^{-1}\)) cause decreases in biomass, as compared with reference plants. Additionally, studies have found that grasses exposed to 50 to 100 mg kg\(^{-1}\) exhibit decreased root size in relation to above-ground biomass (Best et al., 2008), whereas \textit{B. halimifolia} showed an increase in below-ground growth at 30 and 100 mg kg\(^{-1}\) concentrations, though these differences were statistically insignificant. Results from the grass studies varied widely based on species, however, and may explain the inconclusive results from \textit{B. halimifolia} in that root to shoot ratios may differ greatly between herbaceous and woody plants. My study indicated that no significant variation or trends existed between aboveground and belowground biomass ratios for each treatment of \textit{B. halimifolia}. Though it can be independently inferred from both my study and by research from Gong and colleagues (2009b) that higher photosynthetic rates and greater biomass indicates greater carbon assimilation, the discontinuity between these two physiological processes may suggest differences between shrubs and herbaceous plants. Therefore, when endeavoring to detect TNT contamination in the field, it is important to apply analysis to similar plant types.

In comparison to these previously mentioned studies, it has been shown that total biomass considerably increases with nitrogen availability (Day, 1996). Xenobiotics, such as TNT products, are taken up by the plant roots, undergo enzymatic transformation and conjugation, and are compartmentalized in vacuoles or cell walls as compounds and later used for physiological processes (Yoon et al., 2005). Due to the aromatic arrangement of TNT that contains three nitro-
groups, it is assumed that plants are able to use the added nitrogen from TNT. In accordance with the assumption that increased $\delta^{15}$N values indicate higher available nitrogen (Robinson et al., 2000), nitrogen isotopes were measured in TNT-contaminated $B. \text{halimifolia}$. Results were statistically insignificant and did not show any discernable trends. Evans (2001) stated that $\delta^{15}$N does not indicate nitrogen availability in the soil but rather the synthesis of $\delta^{15}$N from the source, fractionation events, pathways of assimilation, nitrogen recycling, allocation and loss of nitrogen from the plant. For $B. \text{halimifolia}$, it is possible that there is a gradual increase in nitrogen allocation as TNT increases, but that higher levels of TNT cause physiological stress and depletion of transformation pathways in the root tissues. This abnormality may indicate another key marker in physiological responses to TNT stress.

In field studies, $B. \text{halimifolia}$ showed negative $\delta^{15}$N values at both sites, as compared with the positive values from laboratory results. The majority of non-nitrogen fixing plants display positive $\delta^{15}$N values, due to the fact that many soils have higher $\delta^{15}$N than available atmospheric N$^2$ (Vitousek et al., 1989), which explains the positive values for laboratory plants. In several forest and shrubland communities, however, non-nitrogen fixing plants have more negative values, for which reasons are not understood (Vitousek et al., 1989). The negative values of the field-tested plants may be due to nitrogen input from closely surrounding nitrogen-fixing $\text{Morella cerifera}$ and $\text{Morella pensylvanica}$ at both field sites.

Fluctuations in biomass may also be due to stress response anomalies in plants. When faced with a particular stressor, whether natural or anthropogenic, plants may respond counter to decline by producing more fruit or flowers or sequestering more carbon. As an anecdotal example, a common practice among gardeners is to violently beat a shrub in order to produce an impressive flowering, explained by the plant’s production of the hormone traumatin in response
to damage, thereby inducing cellular division in flowers and fruits to disperse genes before death. In regard to contaminant uptake in plants, it has been noted through chlorophyll fluorescence studies that low dosages of xenobiotics or other stressors act as stimulants in cell metabolism and other physiological activities, whereas much higher dosages cause senescence and death (Lichtenthaler, 1996). Additionally, with respect to water stress, the partial root-zone drying of *Vitis*, as compared with conventional drip irrigation, significantly increases leaf area, shoot, and fruit development without affecting gas exchange or water relations (De la Hera *et al.*, 2007). With *Baccharis*, the 500 mg kg\(^{-1}\) plants produced statistically higher biomass, as compared to other treatments, and yet showed little variation in stomatal conductance. Because the 500 mg kg\(^{-1}\) plants were confined to the same size pots as were the other treatments, it can be assumed that despite individually tailored watering, the plants were taking up more water and were perhaps exposed to some water deficit in order to sufficiently hydrate the tissues, thus amplifying vegetative growth. An alternative explanation could be that the soluble TNT xenobiotics interfere with water uptake by altering osmotic balance in the cells and inducing a drought-like effect. Further analysis is needed to understand the mechanisms behind the increase in biomass.

Ratios of carbon to nitrogen indicate nitrogen availability and use efficiency. Concurrent with Robinson and colleagues (2000), Vitousek and Howarth (1991) showed that increased enrichment of nitrogen causes an increase in net primary production (biomass), which therefore leads to a decrease in C:N. However, enrichment of TNT-derived nitrogen caused a statistically significant increase in both biomass and C:N of *B. halimifolia* in the 500 mg kg\(^{-1}\) treatments. Field studies indicated the same trend that C:N was statistically higher in plants growing in TNT-contaminated soils. This incongruity of higher C:N may be an indicator of TNT-derived nitrogen as opposed to other nitrogenous forms.
It is important to note that TNT is more available for plant uptake in sandy substrates rather than clay or highly organic soils (Gong et al., 1999a). It is possible that field plants, both reference and test, were growing in substrates comprised of higher sand content. Laboratory plants were grown in soils containing a 3:1 ratio of topsoil to sand whereas field plants were located upon dunes. Therefore, it can be suggested that lower amounts of TNT in sandy substrates can create the same affects upon plants as those grown in organic soils with higher levels of TNT.

In order to properly detect TNT-contamination within a plant from remotely sensed or plant-level data, it is imperative to determine that the measured stresses are due to ordnance contamination and not to naturally existing variables. Without a priori knowledge of the site, such as soil type, nutrient inputs, abiotic stressors or surrounding vegetation, it may be difficult to definitively conclude ordnance presence. Field measurements in North Carolina replicated this scenario of unknown TNT soil concentrations within the study area. Expected and statistically significant results were found between the contaminated field site and the reference site, though external inputs may have affected the shrubs’ physiological responses. Therefore, to avoid such difficulties and incongruities, the identification of statistically significant or anomalous responses unique to ordnance xenobiotics is essential to distinguishing explosive contamination from natural inputs.

Certain physiological parameters, obtained at the plant level and remotely sensed, showed potential as key indicators of buried ordnance detection. The examination of pigment content, through reflectance and chemical analysis, served to explain nitrogen availability, photosynthetic capacity, and protective methods in response to xenobiotic stress. Remotely sensed data, both reflectance and fluorescence, showed changes in the xanthophyll cycle,
allowing for heat dissipation, and also contributed to the understanding of photosystem functioning. Gas exchange measurements suggested that stomatal control functioned independently from metabolic processes, possibly indicative of TNT contamination. Diminishment of the second peak in the red-edge first derivative also signified fluctuations in fluorescence emissions in response to ordnance stress. Further investigations into dry tissue analysis could allow understanding of carbon allocation, biomass yields, and water use efficiency in TNT-contaminated plants.

Overall, the study showed that it is possible to take laboratory analysis of plant physiology and apply it to the landscape level in order to detect explosive contamination from a remote and safe location. In support of the hypothesis, statistically significant differences existed in many of the physiological parameters between low-level contamination (0 to 100 mg kg\(^{-1}\)) and high-level contamination (250 to 500 mg kg\(^{-1}\)), suggesting that it is possible to accurately detect high concentrations of explosive contaminants using plant physiology. Further research will help delineate between explosive-induced stress and natural stress. In accordance with my hypothesis, plants potted in low TNT concentrations generally responded positively in comparison to reference plants, though differences were statistically insignificant. Therefore, the challenge remains to accurately detect buried ordnance containing low concentrations of explosives, as responses may be variable.

Additionally, my study showed the *B. halimifolia* is tolerant of high levels of TNT contamination and thrives in soils with low concentrations. As it is a goal of the Department of Defense to identify plant species for military training ranges that are tolerant of ammunitions contamination, are good soil stabilizers, are able to sequester some of the explosive residue, and do not introduce contaminants into the food chain, *B. halimifolia* appears to meet many of these
criteria. I suggest that the species be considered for future phytoremediative studies to investigate strength of contaminant removal from the soil.

With additional research, phytosensing shows great potential in the remote detection of unmapped buried ordnance as a safe, rapid and effective method. As all plant species possess unique responses to xenobiotics, I have documented the effects of TNT on *Baccharis halimifolia* through the use of remote sensing technologies and traditional plant-level physiological analysis. Further studies are required to understand exact responses by other plant types, such as grasses, conifers, and nitrogen-fixers. My study has highlighted physiological responses in vegetation that show potential as accurate sentinels of ammunitions contamination. Additionally, this study has identified potential problems encountered with phytosensing at both the cellular and landscape level. Further research and novel methods may overcome these issues in its application to remote sensing detection.
Literature Cited


# Appendix

Table 1. Exploratory analysis of reflectance indices for the primary experiment (concentrations ranging from 0 to 500 mg TNT kg\(^{-1}\) soil) and the secondary experiment (concentrations ranging from 0 to 1200 mg TNT kg\(^{-1}\) soil). Bold numbering indicates statistical significance at \(\alpha = 0.05\).

<table>
<thead>
<tr>
<th>Index</th>
<th>0 to 500 mg kg(^{-1})</th>
<th>0 to 1200 mg kg(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>p-value</td>
<td>F</td>
</tr>
<tr>
<td>AI</td>
<td>6.35</td>
<td>0.0018</td>
</tr>
<tr>
<td>CHL</td>
<td>6.50</td>
<td>0.0016</td>
</tr>
<tr>
<td>CI</td>
<td>4.45</td>
<td>0.0098</td>
</tr>
<tr>
<td>(D_{705} / D_{722})</td>
<td>5.41</td>
<td>0.0040</td>
</tr>
<tr>
<td>(D_{715} / D_{705})</td>
<td>6.00</td>
<td>0.0024</td>
</tr>
<tr>
<td>(D_{730} / D_{706})</td>
<td>2.87</td>
<td>0.0498</td>
</tr>
<tr>
<td>(D_{735} / D_{680})</td>
<td>2.64</td>
<td>0.0641</td>
</tr>
<tr>
<td>Dmax / (D_{705})</td>
<td>3.25</td>
<td>0.0330</td>
</tr>
<tr>
<td>Dmax / (D_{714})</td>
<td>6.73</td>
<td>0.0013</td>
</tr>
<tr>
<td>Dmax / (D_{744})</td>
<td>5.40</td>
<td>0.0041</td>
</tr>
<tr>
<td>Dmax / (D_{745})</td>
<td>2.66</td>
<td>0.0631</td>
</tr>
<tr>
<td>DPI</td>
<td>1.05</td>
<td>0.4057</td>
</tr>
<tr>
<td>Green NDVI</td>
<td>1.26</td>
<td>0.3194</td>
</tr>
<tr>
<td>NDNI</td>
<td>1.76</td>
<td>0.1759</td>
</tr>
<tr>
<td>NDVI</td>
<td>2.85</td>
<td>0.0508</td>
</tr>
<tr>
<td>NDVI2</td>
<td>2.89</td>
<td>0.0489</td>
</tr>
<tr>
<td>PRI</td>
<td>11.97</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PRSI</td>
<td>2.69</td>
<td>0.0605</td>
</tr>
<tr>
<td>(R_{750} / R_{710})</td>
<td>5.94</td>
<td>0.0026</td>
</tr>
<tr>
<td>SIPI</td>
<td>3.56</td>
<td>0.0239</td>
</tr>
<tr>
<td>WBI</td>
<td>0.07</td>
<td>0.9903</td>
</tr>
<tr>
<td>WINDV2</td>
<td>2.22</td>
<td>0.1029</td>
</tr>
</tbody>
</table>
Table 2. Selected reflectance indices and derivatives found to be statistically significant were used in the analysis of TNT-related stress of *Baccharis halimifolia*.

<table>
<thead>
<tr>
<th>Index</th>
<th>Formula</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physiological Reflectance</td>
<td>( \frac{(R_{531} - R_{570})}{(R_{531} + R_{570})} )</td>
<td>(Gamon et al., 1990)</td>
</tr>
<tr>
<td>Index (PRI)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyll Index (CHL)</td>
<td>( \frac{(R_{750} - R_{705})}{(R_{750} + R_{705})} )</td>
<td>(Gitelson and Merzlyak, 1996)</td>
</tr>
<tr>
<td>Anthocyanin Index (ARI)</td>
<td>average ( \frac{(R_{600} : R_{696})}{average (R_{500} : R_{596})} )</td>
<td>(Gitelson et al., 2001)</td>
</tr>
<tr>
<td>( \frac{D_{715}}{D_{705}} )</td>
<td>( \frac{D_{715}}{D_{705}} )</td>
<td>(Zarco-Tejada et al., 1999)</td>
</tr>
<tr>
<td>( \frac{D_{max}}{D_{714}} )</td>
<td>([\text{maximum} (D_{477} : D_{910})]/D_{714} )</td>
<td></td>
</tr>
<tr>
<td>( \frac{R_{750}}{R_{710}} )</td>
<td>( \frac{R_{750}}{R_{710}} )</td>
<td></td>
</tr>
</tbody>
</table>

* where derivative (D) = \[\frac{(R_{\lambda+1} - R_{\lambda})}{1}\]
Table 3. Selected reflectance indices indicating statistical significance in the final four weeks of
the experiment. Bold numbers signify statistical significance at $\alpha = 0.05$.

<table>
<thead>
<tr>
<th>Index</th>
<th>35 days</th>
<th>42 days</th>
<th>49 days</th>
<th>56 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F$</td>
<td>$p$</td>
<td>$F$</td>
<td>$p$</td>
</tr>
<tr>
<td>PRI</td>
<td>8.26</td>
<td>0.0004</td>
<td>3.32</td>
<td>0.0306</td>
</tr>
<tr>
<td>CHL</td>
<td>3.15</td>
<td>0.0368</td>
<td>2.22</td>
<td>0.1039</td>
</tr>
<tr>
<td>ARI</td>
<td>1.01</td>
<td>0.4247</td>
<td>1.75</td>
<td>0.1779</td>
</tr>
<tr>
<td>$D_{715}/D_{705}$</td>
<td>2.50</td>
<td>0.0699</td>
<td>0.71</td>
<td>0.5965</td>
</tr>
<tr>
<td>$D_{max}/D_{714}$</td>
<td>0.69</td>
<td>0.6076</td>
<td>0.91</td>
<td>0.4774</td>
</tr>
<tr>
<td>$R_{750}/R_{710}$</td>
<td>3.10</td>
<td>0.0387</td>
<td>2.03</td>
<td>0.1291</td>
</tr>
</tbody>
</table>
Table 4. Mean ± SE for percent nitrogen and pigment content of treatments (mg TNT kg$^{-1}$ soil). Statistically significant differences existed for chlorophyll $a$ and $b$, and are expressed in superscripts.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% N</th>
<th>chl $a$</th>
<th>chl $b$</th>
<th>carotenoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg kg$^{-1}$</td>
<td>1.30 ± 0.11</td>
<td>157.21 ± 9.25$^{ab}$</td>
<td>130.00 ± 8.65$^{ab}$</td>
<td>36.57 ± 2.99</td>
</tr>
<tr>
<td>30 mg kg$^{-1}$</td>
<td>1.28 ± 0.09</td>
<td>189.79 ± 9.44$^a$</td>
<td>158.74 ± 11.12$^a$</td>
<td>42.37 ± 3.02</td>
</tr>
<tr>
<td>100 mg kg$^{-1}$</td>
<td>1.29 ± 0.14</td>
<td>177.05 ± 9.43$^a$</td>
<td>154.62 ± 10.88$^{ab}$</td>
<td>39.96 ± 2.12</td>
</tr>
<tr>
<td>250 mg kg$^{-1}$</td>
<td>1.31 ± 0.15</td>
<td>143.46 ± 8.43$^b$</td>
<td>122.64 ± 9.49$^{ab}$</td>
<td>34.37 ± 3.53</td>
</tr>
<tr>
<td>500 mg kg$^{-1}$</td>
<td>0.96 ± 0.10</td>
<td>129.63 ± 8.42$^b$</td>
<td>116.37 ± 6.47$^b$</td>
<td>31.17 ± 2.67</td>
</tr>
</tbody>
</table>
Figure 1. Upper images show visual observation of changes in above-ground biomass and branching structure typical for each treatment (mg TNT kg\(^{-1}\) soil) in week 8. Lower image is a detail showing red-purple leaf coloration in week 8 of the experiment for *Baccharis halimifolia* treated with 500 mg kg\(^{-1}\). Plants with small concentrations of TNT displayed no reddish coloration and maintained a green leaf color throughout the experiment.
Fig. 2. Average percent reflectance in week 8 of *Baccharis halimifolia* for each treatment (mg TNT kg\(^{-1}\) soil). The top figure shows the entire spectral curve for the visible and near infrared region; the bottom figure is a detail of the visible color and red-edge region.
Fig. 3. First-derivative reflectance in the red-edge region for each treatment (mg TNT kg\(^{-1}\) soil) throughout the final weeks of the experiment.
Fig. 4. First- and second-derivatives of percent reflectance for each treatment (mg TNT kg$^{-1}$ soil) in week 8.
Fig. 5. Selected reflectance indices (mean ± SE) for each treatment (mg TNT kg\(^{-1}\) soil) over the course of the experiment. Asterisks (*) indicate statistically significant differences among treatments at \(\alpha = 0.05\).
Fig. 6. The change in quantum yield of chlorophyll fluorescence for dark-adapted and light-adapted *Baccharis halimifolia* (mean ± SE) with varying concentrations of TNT over the course of the experiment. Asterisks (*) represents statistically significant differences at $\alpha = 0.05$. 
Fig. 7. Linear regression showing the relation between PRI and the effective quantum yield of chlorophyll fluorescence for light-adapted *Baccharis halimifolia* in response to treatments (mg TNT kg$^{-1}$ soil) throughout the final weeks of the experiment. Week 6 produced statistically significant relations, $r^2=0.36$, $p=0.0017$. 
Fig. 8. Mean ± SE of net photosynthesis (top figure) and stomatal conductance (bottom figure) for each treatment (mg TNT kg⁻¹ soil) in week 8 of the experiment. Post hoc analysis (Tukey’s HSD) rendered statistically significant differences in photosynthesis among treatments, signified by letters a and b, at $\alpha = 0.05$. 
Fig. 9. Mean ± SE of net photosynthesis (top figure) and stomatal conductance (bottom figure) for each treatment (mg TNT kg⁻¹ soil) throughout the experiment. Post hoc analysis (Tukey’s HSD) rendered statistically significant differences in photosynthesis among treatments, signified by asterisks (*), at α = 0.05.
Fig. 10. Midday leaf water potential (mean ± SE) for each treatment (mg TNT kg\(^{-1}\) soil) in week 8 of the experiment. Post hoc analysis (Tukey’s HSD) rendered statistically significant differences among treatments, signified by letters a and b.
Fig. 11. Pigment content (mean ± SE) for each treatment (mg TNT kg\(^{-1}\) soil) at the conclusion of the experiment in week 8. Post hoc analysis (Tukey’s HSD) showed statistically significant differences in chlorophyll concentration among treatments, signified by letters a and b.
Fig. 12. Linear regression showing the positive relationship between total percent nitrogen and chlorophyll $a$ concentration in $B. \ halimifolia$ leaves, $r^2 = 0.06$, $p = 0.2401$. 

![Graph showing the linear regression between total percent nitrogen and chlorophyll a concentration in B. halimifolia leaves.](image)
Fig. 13. Linear regression showing the statistically significant and positive relationship between total chlorophyll content (chl $a$ and $b$) and the relative red-edge position (REP) for each treatment of (mg TNT kg$^{-1}$ soil), $r^2 = 0.44$, $p = 0.0003$. 
Fig. 14. Dry tissue analysis (mean ± SE) of each treatment (mg TNT kg⁻¹ soil) for δ¹³C (top figure), δ¹⁵N (middle figure), and carbon to nitrogen ratio (bottom figure). Letters a and b indicate statistically significant differences from post hoc analysis (Tukey’s HSD) at α = 0.05.
Fig. 15. Biomass in grams (± SE) of stems, leaves, and roots for each treatment (mg TNT kg\(^{-1}\) soil) at the conclusion of the experiment in week 8. Post hoc analysis (Tukey’s HSD) rendered statistically significant differences of stem and leaf biomass among treatments, signified by letters a and b.
Fig. 16. Ratio of root to shoot dry weight biomass (mean ± SE) for each treatment (mg TNT kg$^{-1}$ soil) of *Baccharis halimifolia* at the conclusion of the experiment in week 8.
Fig. 17. Map of the field site at Duck Field Research Facility (FRF), NC, showing the location of six mature *Baccharis halimifolia* used in measurements. An existing survey map was georeferenced to base imagery indicating that the largest UXO burial pit is situated beneath the indicated shrubs. The reference site is located 26.6 kilometers south-southeast at Jockey’s Ridge State Park in a site chosen for its similarity to the protected sound-side environment at Duck FRF.
Fig. 18. Field measurement results (± SE) for *B. halimifolia* in TNT-contaminated areas at Duck FRF, NC (black bars) and at a reference site at Jockey’s Ridge SP, NC (gray bars). Analysis includes δ¹⁵N (top left), δ¹³C (bottom left), C:N (top right), and pigment content (bottom right). Statistical differences at α = 0.05 are designated by asterisks (*).
Vita

Kathryn Tyler Rubis was born in Richmond, Virginia on June 2, 1981. She attended high school at Governor’s School for Government and International Studies in Richmond. She received her Bachelor of Arts degree in 2004 from Virginia Commonwealth University with a focus on Art and Architectural History. Afterward, she worked at the Virginia Department of Historic Resources and enrolled in the Master’s program of Architectural History at the University of Virginia’s Architecture School. While at UVA, she was exposed to the concept of phytoremediation and storm water control in the designed landscape by internationally acclaimed landscape architects. Thereafter, she enrolled in the Master’s program at VCU with a focus on Plant Physiological Ecology and, as a complimentary education, pursued a Professional Certificate in Landscape Design from the University of Richmond, balancing both the scientific and artistic aspects of environmentally sustainable applications in the landscape. While in graduate school at VCU, Kathryn was exposed to courses in aquatic systems (wetlands and streams), plants, urban ecology, and remote sensing and GIS technology. Concurrent with course work and research, and as part of the Graduate Teaching Assistant scholarship, she was fortunate to teach writing- and field-intensive undergraduate courses such as Ecology and Ornithology labs. Additionally, she interned as an ecological monitor and plant consultant at Philip Morris’s Park-500 Natural Treatment System, an international award winning 40-acre wetland and arboretum designed to treat tobacco factory effluent while increasing habitat diversity. During the graduate program, she travelled twice to Panama to study migratory birds in the mangroves, cloud forests and mud flats, and also traveled to Sicily, Italy to study river and marine ecology through the University of Messina. In 2011, she received her Master of Science from VCU.