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Growth changes of eighteen herbaceous angiosperms induced by Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in soil

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ABSTRACT

Study objectives were to describe and quantify growth responses (tolerance as shoot and root biomass accumulation) to soil-applied Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) treatments of eighteen terrestrial, herbaceous, angiospermous species and also; to determine how much of RDX, RDX transformation products, total N and RDX-derived N accumulated in the foliage. RDX altered growth of eighteen plant species or cultivars at levels of 100, 500, and 1,000 mg kg^{-1}dry soil in a 75-d greenhouse study. Sixteen species or cultivars exhibited growth inhibition while two were stimulated in growth by RDX. A maximum amount of foliar RDX in a subset of three plant species was 36.0 mg per plant in Coronilla varia. Foliar concentrations of transformation products of RDX were low relative to RDX in the subset of three species. The proportion of RDX-N with respect to total N was constant, suggesting that foliar RDX transformation did not explain differences in tolerance. There was a δ ^{15}N shift towards that of synthetic RDX in foliage of the three species at a level of 1,000 mg kg^{-1} RDX, proportional in magnitude to uptake of N from RDX and tolerance ranking.Reddened leaf margins for treated Sida spinosa indicate the potential of this species as a biosensor for RDX.

KEYWORDS

phytoremediation; phytoextraction; soil pollutants; royal demolition explosive; RDX

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Introduction

Contamination of military ranges by explosive residues is an international problem (Clausen *et al.* 2004). Extensive closing of military bases across the United States has left approximately 4 million hectares of abandoned military land containing residual explosives and unexploded ordnance (Brentner *et al.* 2010). Furthermore, some energetic compounds have contaminated groundwater (Funk *et al.* 1993). The U.S. department of Defense has identified more than 1000 sites with explosives contamination (Rodgers and Bunce 2001). Hexahydro-1,3,5-trinitro-1,3,5-triazine (Cyclonite or RDX) is an explosive used extensively by military forces throughout the world and military firing range soils are often contaminated by RDX (Pennington *et al.* 2006). This compound also has civilian usages, such as in vehicular air bags (Chakraborty *et al.* 2000).

Military grade RDX generally contains concentrations of Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (HMX) ranging from 3 to 10 percent (U.S. Army 1984, Talmage *et al.* 1999) as an impurity of the manufacturing process. Cyclonite and HMX water solubilities are low (Talmage *et al.* 1999, ATSDR 1997). However, neither RDX nor HMX bind to soil very strongly (Hawari 2000) and both compounds can move rapidly into groundwater (Chen 1993). The U.S. Environmental Protection Agency (EPA online: accessed 2013) suspended military training activities at the Massachusetts Military Reservation in Cape Cod, Massachusetts due to the discovery of RDX in area aquifers and wetlands. Neither RDX nor HMX is typically biodegraded significantly in aerobic soil according to Lynch and others (2001). It is well known that RDX can be taken up from soil by plants (Chen 1993; Best *et al.* 1999).

Review of the literature on RDX contaminants and their phytoremediation

Cyclonite does not accumulate in tissues of fish or in people (ATSDR 2010; U.S. Navy 1972; U.S. Army 1984), but exposure in large amounts can cause seizures in mammals. Cyclonite is toxic to some mammalian species (BAE Systems 2005). Exposure to RDX leads to smaller offspring in rats (U.S. Army 1986). Studies in rats, mice, and rabbits indicate that HMX may also be harmful to the mammalian liver and central nervous systems (ATSDR 1997, 2010). Chronic, local and systemic effects on humans are not known (BAE Systems 2005), but RDX and co-occurring HMX have high potential for toxicity to mammals.

The uptake, accumulation and biodegradation of RDX by plants has been reported (Best *et al.* 2007, Brentner *et al.* 2010, Chen 1993, Just and Schnoor 2004, Van Aken *et al.* 2004a, 2004b). RDX is readily translocated to shoots and leaves of the plants after its uptake, with about 70 % of RDX accumulating in aerial parts of plants (Thompson *et al.* 1999). Photolytic transformation, that occurs in the aerial parts of the plant, is

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the primary mechanism of transformation during the degradation of RDX (Just and Schnoor 2004). Photolysis occurs when water containing organics is taken up by plants and released into the atmosphere through their leaves. Phytophotolysis of RDX in poplar plant tissues was observed by Van Aken and others (2004b).

According to Van Aken and others (2004b) the mineralization of RDX occurs in three steps (1) a light independent reduction of RDX to MNX (Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine) and DNX (Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine) by plant cells, (2) a plant/light mediated breakdown of RDX, MNX, or DNX into formaldehyde and methanol, (3) a light-independent mineralization of metabolites to CO2.

Fournier *et al.* (2002) reported the aerobic biodegradation of RDX in *Rhodococcus* sp. Strain DN22, a soil and water bacterium, to nitrite (NO2-) (30%), nitrous oxide (N2O) (3.2%), ammonia (NH3-) (10%) and formaldehyde (HCHO) which was converted to carbon dioxide.

Hawari and others (2000b), found that non-aromatic, cyclic nitramine explosives, such as RDX, lack electronic stability so that there would be no cyclized breakdown products (Hawari 2000). Instead two nitramine compounds would be produced that spontaneously decompose in water (Lamberton *et al.* 1949a,b; Urbanski 1967; Rocheleau *et al.* 1999).

Studies of explosives as soil contaminants have focused mainly on TNT (reviewed by Rocheleau *et al.* 2005). Military explosives contain both TNT and RDX. Furthermore, the majority of studies of phytoremediation processes in soils contaminated by TNT and RDX have focused on grasses and wetland species (Best *et al.* 2007). We opted to examine less-studied herbaceous, dicotyledonous angiosperms from terrestrial habitats. Studies of RDX and HMX, which is a byproduct of its manufacture, suggest that they are not as toxic to plants as TNT (Schnoor *et al.* 2006). Gong and others (1999) and Winfield and others (2004) suggested that herbaceous monocots (monocotyledonous angiosperms, i.e., grasses) might be more sensitive to TNT and RDX than dicots (broad-leaved angiosperms, i.e., herbs). Others have found contrary evidence and suggest it is not plausible to generalize differences between monocots and dicots with respect to tolerance of TNT and RDX (Gorge *et al.* 1994, Scheidemann *et al.* 1998). Published reports of dicotyledonous plant responses to RDX vary in species tested, plant tissues tested, number of dosage treatments, dosage levels, types of growth substrates, duration of RDX exposure, and co-contaminants (Vila *et al.* 2007; Van Dillewijn *et al.* 2007; Bhadra *et al.* 2001; Thompson *et al.* 1999; Harvey 1991). Measures related to RDX tolerance include short-term uptake (Bhadra *et al.* 2001), seed germination rates (Best *et al.* 2007), presence of a well-developed root system determined by optical scanning (Best *et al.* 2007), uptake of RDX by plants (Best *et al.* 1997), reaction of plants to hydroponic solutions of RDX (Chen *et al.* 2011)), impacts of leachate from RDX-treated soils (Rylott *et al.* 2011), plant root and shoot elongation (Winfiled *et al.* 2004), response of hairy root cultures (Bhadra *et al.* 2001) and growth of plant tissue cultures exposed to RDX (Van Aken *et al.* 2004b). These varied approaches make comparisons of RDX-tolerance assessments problematic. Recent studies have focused on uptake and RDX-transformation processes in genetically modified plants (reviewed by Van Aken 2009). Much research has been conducted using brief exposure durations in the laboratory and in controlled environments. Applying laboratory research results directly to assess potential field performance is not reasonable. Many phytoremediation trials have failed or proved impossible to monitor under field conditions (Gerhardt *et al.* 2009).

This study included hardy, adaptable and resilient herbaceous dicotyledonous plants together with some commonly cultivated plants over a relatively-long, 60-day-RDX-exposure period in a greenhouse under natural daylight supplemented with artificial light to maintain growing-season photoperiod. Plant tolerance and tolerance characteristics of RDX-treated plants was the main focus of this study. The study employed robust, repeatable, quantitative measures of plant biomass changes in response to soil RDX levels found in contaminated soils. The intention was to provide an ecological approximation of field conditions for comparing differences in RDX tolerance among a set of plants adapted to eastern and central North American conditions. In contrast, many studies of plant responses to RDX have focused on RDX uptake and transformation processes in single plant species or smaller assemblages of plants including plants modified with introduced bacterial genes able to reduce RDX.

Objectives

Our main objective was to describe and quantify growth responses (shoot biomass, root biomass, and root:shoot ratios) to soil RDX treatments of eighteen terrestrial, herbaceous, angiospermous species. RDX treatments were at levels found in soils of contaminated military sites in the U.S (Pennington *et al.* (2003, 2004, 2005, 2006). The growth responses of different herbaceous angiospermous species exposed to RDX, relative to controls, allowed plants to be ranked according to RDX tolerance levels. Additional objectives were to determine whether a subset of treated plants had actually taken up RDX from dosed soils and how much RDX, total N and RDX-derived N accumulated in the foliage of treated plants. Using an assay with the ratio of $\delta\ ^{15}N$ of manufactured RDX as a standard, we confirmed uptake of N from RDX (Sharpe & Nichols 2007) and established a possible isotopic method for identification of RDX-tolerant plants. We also determined foliar quantities of RDX, HMX and RDX-transformation products in this subset of plants using high pressure liquid chromatography (HPLC) to assess the possible significance of their ratios and frequencies in foliar tissue.

Methods

RDX Impacts on biomass accumulation

The selected plant species were okra (*Abelmoschus esculentus* (L.) Moench), velvetleaf (*Abutilon theophrasti* Medik), redroot pigweed (*Amaranthus retroflexus* L.), black mustard (*Brassica nigra* (L.) W.D.J.Koch), morning glory (*Ipomoea lacunosa* L.), prickly sida (*Sida spinosa* L.), yarrow (*Achillea millefolium* L.), love lies bleeding (*Amaranthus caudatus* L.), California poppy (*Eschscholzia californica* Cham.), strawberry fields (*Gomphrena haageana* Klotzsch), four o'clock (*Mirabilis* L. sp.), peppermint

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purslane (*Portulaca grandiflora* Hook), nasturtium (*Tropaeolum majus* L.), crown vetch (*Coronilla varia* L.), jimson weed (*Datura stramonium* L.), cotton (USDA, DP 174RF) (*Gossypium hirsutum* L.), common purslane (*Portulaca oleracea* L.), and tomato (*Solanum lycopersicum* L.). The selected plant species and cultivars are a diverse group of hardy, geographically-widespread herbaceous dicots including both native and exotic weeds, forage crops and cultivated plants adapted to conditions in central and eastern North America.

Seeds of these species were sown in multi-cell trays using SBF-500 high-porosity, soil-less plant medium (Sun Gro Horticulture Canada Ltd.) on a mist bench with natural light. Seedlings were transplanted into treated soil after 15 days at the two leaf stage to eliminate any confounding RDX influences of a seed germination bioassay. They were transferred to pots measuring 16.8 cm in diameter by 14 cm in height and containing approximately 2,240 g (= 1,300 cc) of oven-dried, sandy potting soil (10 parts coarse silica quartz sand to 3 parts silt loam, a prairie-derived, silty clay loam mollisol with a bulk density of 1.6). Dry RDX, supplied by BAE System, Ordnance Systems Inc., Holston Army Ammunition Plant, Kingsport, TN, (1% HMX) was ground with a mortar and pestle into a fine powder. At room temperature, RDX and HMX are stable. They burn rather than explode, detonating only with a detonator having a detonation velocity of 8,750 m/s (Cooper 1996), making them safe to grind. RDX was then thoroughly mixed into the steam-sterilized potting soil to prepare three levels of soil treatments of 100, 500, and 1,000 mg RDX kg^{-1} dry soil. We found no differences in plant-growth responses between direct RDX dosing through thorough mixing of finely-powdered RDX into soils and an artificial aging method (Best *et al.* 2007) using methanol to disperse RDX as a solute throughout the medium (data available from authors). The lowest observed effect concentration of RDX in soil is 100 mg kg^{-1} and the maximum *in-situ* contamination levels of RDX in soil found on military training ranges in the U.S. is 1,000 mg kg^{-1} (Pennington *et al.* 2003, 2004, 2005, 2006; Simini *et al.* 1995; Talmage *et al.* 1999). At least seven seedlings were randomly assigned, one per pot, to each of the 3 treatment levels (100, 500 and 1,000 mg kg^{-1}) and the control for a minimum of 28 plants per experiment. After sterilization, RDX treatment and planting, the soil in pots was left open on greenhouse benches under non-sterile conditions. Pots were arranged according to a completely randomized design.

Plants were watered daily as needed and fertilized weekly using the recommended solution of PLANTEX 20-20-20 Water Soluble Fertilizer (Plant Products Company, LTD. Brampton, Ontario, Canada) containing 250 mg kg^{-1} 20% N (as 10% urea $(NH_2)_2CO$, 6% nitrate NO_3 & 4% ammonia NH_3), 20% P (as phosphate P_2O_5), and 20% K (as potash K_2O) plus micronutrients to pot saturation throughout the growing period. Greenhouse bays were maintained at temperatures of 28°C daytime (0600-1800 hours) and 21.1°C nighttime (2200−0400 hours). On cloudy days and to maintain a 16-h photoperiod, natural light was supplemented with 1,000-watt sodium vapor lamps suspended at 2.0 meters over pot surfaces. Trays were placed under individual pots to prevent loss of RDX or nutrients by leaching through screened drainage holes in pot bottoms. Solutions of nutrients and RDX that accumulated in trays remained in treated soil as lowered osmotic potential in drying soils resulted in re-absorption of leachate. At harvest time 60 days after dosing with RDX, the above- and below-ground plant portions were separated and washed in distilled water to remove external soil particles and then blotted dry. Fresh mass of each plant part was recorded. The plant parts were then dried in a forced-air oven for 24 h at 105°C to obtain dry mass values and root:shoot ratios of dry mass for each plant species or cultivar. At harvest time, each plant's dry shoot mass, dry mass of roots, total dry mass, ratio of oven-dry roots to oven-dry shoots, corresponding fresh weights, and water contents were determined.

Data analysis

Responses of dry shoot mass and ratio of root/shoot dry mass to RDX treatments were analyzed using univariate, one-way analysis of variance (ANOVA) in R (R Core Team 2013). Analysis of variance was performed on $\log_{10}$ transformed total dry mass to meet the test assumption of normal distribution. To control experimental-wise error rate, a Bonferroni adjusted analysis ($\alpha = 0.05$) was adopted for transformed total dry weight and root/shoot ratio ANOVAs. The Bonferroni method corrects for multiple comparisons. Errors in inference, including confidence intervals that fail to include their corresponding population parameters are more likely to occur when one considers the set as a whole. This allows significance levels for single and multiple comparisons to be directly compared. This technique is conservative, generally requiring a higher significance threshold for individual comparisons, so as to compensate for the number of inferences being made. Diagnostic plots confirmed that the residuals for both analyses met the assumptions of normality and equal variances. Mean separation for significant factors was conducted using a protected Fisher's Least Significant Difference test ($\alpha = 0.05$). "Protected" means that you only perform the calculations when the overall ANOVA resulted in a P value less than 0.05. This step controls the false positive rate for the entire family of comparisons. Fisher's LSD test computes the pooled standard deviation from all groups. Fisher's LSD does not correct for multiple comparisons, hence the use of the Bonferroni adjusted ANOVAs. Our comparisons were made using the LSD test function provided in the Agricolae package (De Mendiburu 2012).

δ^{15}N shifts of RDX-treated plant tissue

Seeds of *C. varia, T. majus*, and *E. californica* plants were sown to produce seedlings for ^{15}N isotopic analysis using RDX as a standard. Holly (*Ilex vomitoria* Aiton), a woody species not included in our biomass studies, was planted as one-year old seedlings. Plants were cultivated and treated as described previously for the biomass study except that only the control and 1,000 mg kg^{-1} RDX soil treatments were employed. Sixty days after treatment with RDX, the plants were harvested by stripping fully-expanded leaves from the stems to provide foliar bulk samples of at least 1 g dry mass based on pre-determined fresh weight to dry weight conversions. A substantial

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proportions of RDX is translocated from the RDX pool in plant roots, accumulating in leaves (Thompson *et al.* 1999). Leaves are a tissue of primary importance in photosynthesis and growth. There were few if any senesced leaves, making them unrepresentative of the foliar tissue, so they were not sampled. Leaves were rinsed in distilled water and then blotted dry. The leaves were placed in a beaker and sufficient distilled deionized (DDI) water was added to cover the sample.

Samples were homogenized using an Ika T-18 Basic Ultra Turrax, (Wilmington, NC) beginning at a speed of 500 rpm and slowly increasing the speed to 7,500 rpm until the samples were transformed to a frothy paste, avoiding sample temperatures above 40°C from friction. The samples were frozen (−40°C), freeze dried for 2 to 4 days, then ground in using a tissue grinder and stored in UV protective bottles.

The samples were analyzed for stable isotope ratios by continuous flow isotope ratio mass spectrometry (IRMS) (20−20 mass spectrometer, PDZ Europa, Northwick, UK) using the methods outlined in Sharpe & Nichols (2007). Nitrogen isotope values were expressed as $\delta^{15}N$, that is the relative difference in $^{15}N/^{14}N$ ratios between samples and atmospheric nitrogen (Kendall 1998). Duplicates were analyzed to insure consistency of values within 1% of mean.

HPLC analysis of RDX, HMX, and RDX transformation products

Seeds of *C. varia*, T. majus, and *E. californica*, representing high, medium and low RDX tolerances respectively, as well as a woody plant, *Il. Vomitoria*, were selected for analysis. The seeds were cultivated and treated as described previously for the biomass study. Two g dry mass equivalent of fresh plant leaf tissue from bulked samples were placed in a 2.25 cm diameter × 15 cm long test tube, homogenized and freeze dried as previously described. Two ml of deionized, distilled water were added to each tube. Then an aliquot of 10.0 ml of acetonitrile (ACN) was added and mixed for 1 minute using a vortex mixer. After twenty four hours enclosed in a UV-protective container to allow for dissolution of RDX and breakdown products, samples were gravity filtered using Whatman #2 125-mm filter paper. Samples were next filtered twice using a Nalgene 45 μ nylon filter followed by filtration through a Nalgene 20 μ nylon filter and, finally, filtered under pressure (Phenomenex Strata FL-PR syringe filters). The filtrate was vigorously shaken in a vortex mixer for 1 minute. A 0.3 ml aliquot of the filtered sample was analyzed by HPLC using the methods outlined in Jenkins and Grant (1987).

Standards of transformation products of RDX; Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), Hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) and 4-nitro-2,4-diazabutanal (4-NDAB), as well as the RDX-manufacturing by-product octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), were acquired from SRI International (Menlo Park, CA). Samples were analyzed using an HPLC fitted with a C-18 reversed-phase column in a 60% methanol mobile phase and employing a UV detector at 254-nm. Duplicate values for a bulked sample were within 1% of each other.

Results

Biomass study

Treatments resulted in statistically significant differences among biomass accumulation for each species in individual experiments according to one way analysis of variance (Table 1). Biomass generally decreased as RDX concentrations increased (Table 2). In one species tested, *A. retroflexus* growth increased in a dose-dependent manner with RDX concentrations in soil. In the other 17 species or cultivars, there was a significant decrease in biomass compared with controls at the highest level of RDX in treated soil (Table 2). There was no discernable pattern in root:shoot ratio changes with respect to RDX treatments. Root:shoot ratios of controls ranged from 0.24 for *E. californica* to 23.16 for *P. oleracea*. This indicates the need for caution in extrapolating RDX effects on plants based on single treatment concentrations or on shoot or root growth parameters by themselves. Nine species of plants tested showed no significant change in root:shoot ratio with RDX treatment. Three of six species in the most-RDX-tolerant forbs, two of six moderately tolerant and three of the least tolerant species increased significantly in root:shoot ratio, and one species (*A. theophrasti*) decreased significantly in root shoot ratio due to treatment with RDX. Three species changed in a dose-dependent manner by more than 100% in root:shoot ratio with increasing RDX concentration to 1,000 mg kg^{-1} : *E. californica* from 0.24 to 1.00, *Ipomea* sp. from 4.43 to 16.80, and *A. caudatus* from 4.45 to 10.74.

We observed red pigmentation of foliar margins at all RDX-treatment levels for *S. spinosa. Red pigmentation did not occur* in any other species tested. Water contents were consistent with adequate hydration in the greenhouse experimental system. No patterns could be detected in water contents with respect to RDX treatments.

Table 1. One-way Analysis of Variance of mean log_{10} transformed dry mass production after 60 days for each forb species according to four RDX soil treatments (0, 100, 500 and 1,000 ppm). Analysis of variance performed on log_{10}- transformed response variables to meet test assumption of normal distribution. Species are ranked according to tolerance by average percentage of control growth for overall mean (Table 2).

Species	*No. of Plants	DF	Mean Squares	Pr(>F)
Amaranthus retroflexus	34	3	0.90	0.0144
Ipomoea lacunosa	27	3	11.32	0.0001
Coronilla varia	28	3	16.37	0.0002
Portulaca oleracea	22	3	3.01	<0.0001
Achillea millefolium	25	3	34.47	<0.0001
Abelmoschus esculentus	28	3	15.47	0.0044
Abutilon theophrasti	28	3	14.79	<0.0001
Mirabilis sp.	28	3	65.16	<0.0001
Brassica nigra	28	3	53.40	<0.0001
Sida spinosa	27	3	75.53	<0.0001
Gossypium hirsutum	27	3	10.18	<0.0001
Tropaeolum majus	28	3	138.49	<0.0001
Solanum lycopersicum	26	3	6183.01	<0.0001
Gomphrena haageana	28	3	119.09	<0.0001
Datura stramonium	28	3	49.27	<0.0001
Eschscholzia californica	25	3	17.46	<0.0001
Portulaca grandiflora	28	3	3.01	<0.0001
Amaranthus caudatus	51	3	199.14	<0.0001

*The number of plants per analysis is based on a minimum of 7 reps of 4 treatments, or 28 plants, adjusted for missing values due to plant mortality. For *Amaranthus retroflexus* the number of reps was 8 with 2 extra controls and for *Amaranthus caudatus* there were 13 reps with one missing value due to mortality. Standard procedures for dealing with missing data points in the analysis of variance were employed.

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Table 2. Treatment means of total dry mass production (g) per plantand their separation by Least Significant Difference values signified by different letters after 60 days for each angiospermous, herbaceous species or cultivar according to RDX soil treatments of 0, 100, 500, and 1,000 mg kg^{-1}. Species are ranked according to tolerance by average percentage of control growth for overall mean.

Plant Species/Cultivar	n	Control	100	500	1,000
Amaranthus retroflexus	34*	1.12b** (100)	1.24b (110)***	1.48ab (132)	1.86a(166)
Ipomoea lacunosa	27	3.59b (100)	5.24a (146)	2.04c (57)	2.87bc (80)
Coronilla varia	28	8.84a (100)	5.92bc (67)	7.21b (82)	5.41c (61)
Portulaca oleracea	22	3.49a (100)	3.20a (92)	1.77b (50)	1.79b (51)
Achillea millefolium	25	11.42a (100)	7.40b (65)	6.33b (55)	7.64b (67)
Abelmoschus esculentus	28	5.44a (100)	3.24b (60)	3.26b (60)	1.84b (34)
Abutilon theophrasti	28	5.57a (100)	3.31b (59)	2.55bc (46)	2.46c (44)
Mirabilis sp.	28	12.16a (100)	7.04b (58)	5.28c (43)	6.36bc (52)
Brassica nigra	28	10.46a (100)	5.26b (50)	4.55b (43)	5.09b (49)
Sida spinosa	27	10.46a (100)	3.61b (35)	4.20b (40)	3.78b (36)
Gossypium hirsutum	27	3.61a (100)	1.93b (53)	1.43b (40)	0.61c (17)
Tropaeolum majus	28	12.40a (100)	5.20b (42)	3.93b (32)	2.33b (19)
Solanum lycopersicum	26	75.05a (100)	20.63b (27)	26.12b (35)	20.85b (28)
Gomphrena haageana	28	11.58a (100)	3.08b (27)	3.59b (31)	3.35b (29)
Datura stramonium	28	6.74a (100)	1.88b (28)	1.75bc (26)	0.90c (13)
Eschscholzia californica	25	4.05a (100)	1.19b (29)	0.99b (25)	0.23c (6)
Portulaca grandiflora	28	2.13a (100)	0.48b (22)	0.28b (13)	0.49b (23)
Amaranthus caudatus	51	9.25a (100)	1.90b (21)	1.51b (16)	1.46b (16)

*The number of plants per analysis is based on a minimum of 7 reps of 4 treatments, or 28 plants, adjusted for missing values due to plant mortality. For *Amaranthus retroflexus* the number of reps was 8 with 2 extra controls and for *Amaranthus caudatus* there were 13 reps with one missing value due to mortality. Standard procedures for dealing with missing data points in the analysis of variance were employed.

**Means within a row containing different letters are significantly different from others using a protected Fisher's Least Significant Difference Test ($\alpha = 0.05$).

***Values in parentheses are percentages of corresponding control values. for overall mean of 3 RDX treatment levels.

δ^{15}N shifts of RDX-treated plant tissue

Plants of four selected species grown in RDX-treated soil showed a shift in δ ^{15}N values toward that of the manufactured RDX used in the experiment (Figure 1), with the greatest shift occurring in *C. varia*.

RDX, HMX, and RDX transformation products

RDX accumulated in leaf tissue of the three selected herbaceous, angiospermous species to concentrations exceeding those in the soil (Table 3). *E. californica* had the least biomass accumulation and 12 times greater RDX concentration than the soil at a treatment level of 1000 mg kg^{-1} (Table 3). The *C. varia* plants had 5 times the soil concentration of RDX at a treatment level of 1000 mg per kg. *T. majus* plants had about 2.4 times the soil concentration of RDX at a treatment level of 1000 mg kg^{-1} RDX.

C. varia accumulated the greatest amount of RDX per plant, 36 mg at the 1000 mg kg^{-1} treatment level. *E. californica* plants grew the least and accumulated only 2.6 mg per plant at the 1000 mg kg^{-1} RDX-treatment level (Table 3). Total N concentrations of foliar tissue were 2% for *C. varia*, 3.7% *for T. majus*, and 5.8% for *E. californica* with respective percentages of RDX-N as proportions of total N of 10.0, 10.3, and 8.5% (derived from values in Table 3 and Figure 1). One-year-old woody-plant seedlings dosed with RDX in soil were less than or about equal to soil concentrations, and not comparable in RDX accumulation to other plants in the study. Because results were so different than for the herbaceous species, experiments were truncated for *Ilex*.

Concentrations of reductive transformation products of RDX, MNX, DNX, TNX, and 4-NDAB, were small in plant leaf tissue relative to quantities of RDX in the same plants (Table 2). HMX was also present only in small quantities in plant leaf tissue relative to RDX (Table 3), values consistent with its presence as a 1% manufacturing byproduct of the RDX used in this study.

Discussion

Biomass

The general reduction in biomass of selected herbaceous angiosperms indicates that RDX inhibited growth of a set of hardy,

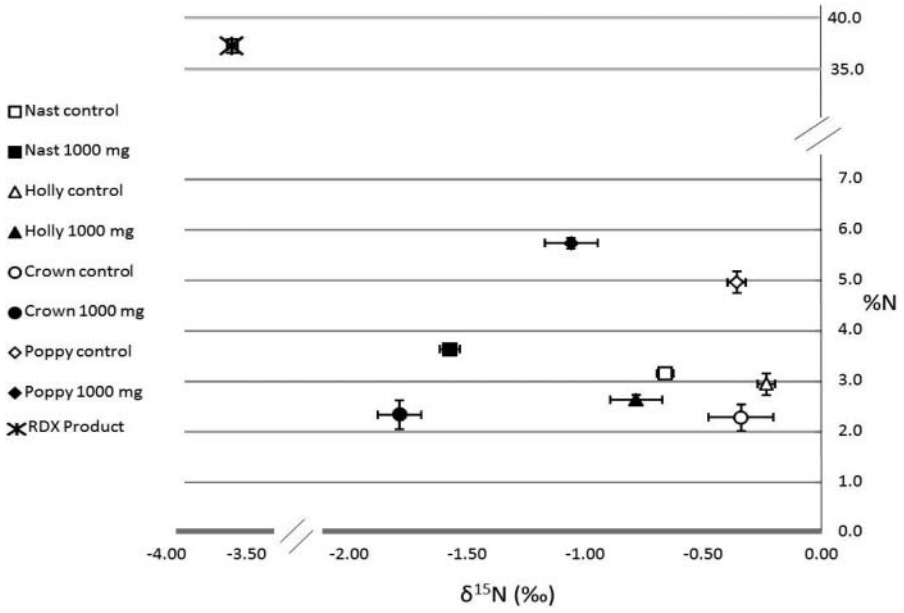

Figure 1. The δ^{15}N in plants treated with 1,000 mg kg^{-1} RDX soil and the RDX used in soil treatments. *Nast = Tropaeolum majus*, *Holly = Ilex vomitoria*, *Crown = Coronilla varia*, *Poppy = Eschscholzia californica*.

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Table 3. RDX soil treatment and RDX in foliage with transformation products of three forbs and one woody plant species (*Ilex vomitoria*). Soil treatment levels are: controls with no RDX, 100, 500, and 1,000 mg RDX per kg soil dry mass. All foliar concentration values are from duplicates of bulked foliage samples having values within 1% of each other. nd = not detected.

Plant Species	Soil RDX Trt.-mg kg^{-1}	mg of leaf RDX/plant	Leaf Concentrations of RDX and RDX Metabolites (mg kg^{-1})					
			RDX	HMX	4-NDAB	TNX	MNX	DNX
Coronilla varia	control	0.00	nd	4	nd	nd	4	nd
	100	0.90	140	7	5	nd	8	nd
	500	4.35	1,600	7	nd	1	20	nd
	1,000	36.00	5,000	52	18	4	8	2
Tropaeolum majus	control	0.00	nd	nd	3	nd	3	nd
	100	0.16	320	4	11	nd	7	nd
	500	6.00	1,500	11	6	nd	10	nd
	1,000	5.60	2,400	13	23	1	14	nd
Eschsholzia californica	control	0.00	80	nd	nd	nd	8	11
	100	0.30	270	10	20	nd	9	9
	500	8.80	960	60	5	8	145	37
	1,000	2.60	11,500	80	140	nd	52	9
Ilex vomitoria	control		nd	nd	1	nd	3	nd
	500		600	nd	1	nd	3	nd
	1,000		300	1	8	nd	3	nd

Minimum detection level was 1 mg kg^{-1}.

angiospermous plants at levels from 100 to 1,000 mg kg^{-1} soil (Table 2). In a similar study, five grasses and five dicotyledonous plant species (dicots) were identified as rapidly colonizing and tolerant of short-term exposure to combined TNT- and RDX-contamination of soils (Best *et al.* 2007). These rankings were based on optical root area determinations and germination rates (Best *et al.* 2007). These species were: the monocots, *Andropogon Gerardii* Vitm., *Bouteloua gracilis* (Willd.ex H.B. K.)Lag. Ex Griffiths, *Elymus canadensis* L., *Eragrostis trichodes (Mutt.)Wood, Sorghastrum nutans* (L.) Nash and the dicots: *A. retroflexus, Asclepias syriaca* L., *I. lacunosa, P. oleracea and S. spinosa*. The results for dicots were in accord with our long-term tolerance rankings based on biomass accumulation of plants after germination. In our study *A. retroflexus, I. lacunosa*, and *P. oleracea* had the greatest tolerance while *S. spinosa* was intermediate in tolerance (Table 2). TNT added as a co-contaminant in the study by Best *et al.* (2007), resulted in tolerance levels that were similar to our RDX-only rankings. The results of other, published RDX uptake and tolerance studies are not easily comparable to our results owing to major differences in experimental methods, including duration of RDX exposure, plant type (herbaceous, woody), photosynthetic system (C3, C4, or Crassulacean Acid Metabolism), environmental conditions of study, soil substrates, tolerance criteria, plant growth systems employed such as seedling, tissue culture and hydroponics, as well as other methodological differences.

In contrast to the observations of Best *et al.* (2007) that RDX and TNT together reduce root growth relative to shoot growth, we found that only one species exposed to soil RDX alone (*A. theophrasti*) decreased significantly in root:shoot ratio. This discrepancy may be due to the fact that RDX is more-readily translocated from roots to shoots than TNT (Harvey 1991). Exposure symptoms in plants resulting from one type of explosive contaminant cannot be generalized to other, similar energetic chemical compounds. Co-contamination studies should employ factorial statistical analyses, including single contaminants combined with combinations, to test for interactions. The remaining seventeen species exhibited either an increase or no change in root:shoot ratios with exposure to RDX. Increases or no change in root:shoot ratios induced by RDX have implications for increased rhizosphere mediation of RDX-degrading bacteria and increased capacity to take up water, nutrients and contaminants from soil in comparison with plants having reductions in root biomass. Root:shoot ratios generally increase in response to limited water and nitrogen availability as well as low light intensities (Larcher 1995).

A. retroflexus and *I. lacunosa* had significant increases in growth with RDX soil treatments (Tables 1 and 2). *A. retroflexus* is a nitrogen accumulator, known to take up nitrogen in shoots at levels that are sufficiently concentrated to be toxic to herbivores (Zadnik *et al.* 2008). *Ipomoea* species such as *I. lacunosa* in this study are commonly known to grow on poor soils and, being in a genus that has Crassulacean Acid Metabolism (CAM), an efficient C4 photosynthetic process under hot and dry conditions. CAM plants typically have high nitrogen use efficiencies (Griffiths 1989, Raven and Spicer 1996). *A. retroflexus* grew in a dose-dependent manner more than its control at all RDX treatment levels to a maximum of 166% of control at 1,000 mg kg^{-1} RDX. Such stimulation in RDX uptake and tolerance is similar to that of bio-engineered plants with introduced bacterial genes able to chemically reduce RDX (Jackson *et al.* 2007; Rylott *et al.* 2011). Similar performance by wild-type plants merits further investigation to determine exact mechanisms of growth/tolerance stimulation by RDX. *A. retroflexus*' superior abilities to accumulate nitrogen, tolerance of RDX, together with possible stimulation of RDX degradation in soils, rhizospheres, endophytic bacteria or plant cytoplasm to forms that can be used in plant metabolism may account for the measured growth stimulation. At the highest treatment level of RDX in soil, the root:shoot ratio of *A retroflexus* increased by 80% over the control value of 8.10.

As RDX treatment levels in soil went up, leaves of sensitive species were observed to be thin and malleable similar to those of plants affected by drought. RDX might have restricted water movement to and retention in leaf cells (Chang *et al.* 2004) or otherwise influenced leaf cell wall growth and structure in a manner similar to drought stress.

δ^{15}N Isotopic shift

The observed isotopic shift in δ^{15}N values towards the value for manufactured RDX for four selected plant species indicates that nitrogen from the supplied RDX was taken up by the plants (Figure 1). The control plants were exposed to the same fertilizer regimen as the RDX-dosed plants, so any shift in δ15N values associated with manufactured nitrogen fertilizer would be reflected equally in control plants.

The shift of foliar δ^{15}N values from control levels towards that of manufactured RDX for a species treated with RDX in soil (Figure 1) was greater in species with higher RDX tolerance and uptake (Table 2). This isotopic method could possibly be used as a method for the assessment of RDX tolerance.

Total N concentrations of foliar tissue were 2% for the more tolerant *C. varia*, 3.7% for intermediately tolerant *T. majus*, and 5.8% for the least tolerant *E. californica* (Figure 1). These

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three species, however, had relatively constant total concentrations of N derived from RDX, 10.0, 10.3, and 8.5% respectively (derived from measurements in Table 3 and Figure 1). Thus, differences in RDX transformation into N available for plant metabolism was not discernable as a differentiating factor in RDX tolerance. The total amount, not the concentration, of accumulated RDX in plant foliage corresponded closely to RDX tolerance.

S. spinosa grown in RDX-contaminated soil exhibited red pigmentation at leaf margins at time of flowering.Anthocyanin is the only red pigment type occurring in plants of this study (Seigler 1998). Brentner *et al.* (2010) found that RDX accumulated in the leaf margins of poplar, the same location at which red pigmentation occurred in prickly sida. Accumulation of RDX in leaf margins at time of flowering may have induced anthocyanin production in leaf cells. Results suggest that prickly sida may be useful as a sentinel plant for RDX contamination of soils (Hagan 2012) and for finding plant biomarkers for RDX contaminants.

RDX, HMX, and RDX transformation compounds in foliage

Pools of nitroso-derivatives were detected in the selected plant species (Table 2). HMX increased proportionally with levels of soil RDX treatment, consistent with its occurrence as a by-product (<1%) of RDX manufacture. Nitroso-derivatives are associated with transformation of RDX (McCormick *et al.* 1981, 1985). Sequentially-produced nitroso-derivatives from RDX reduction were not always detected nor were pools of these compounds large relative to RDX (Table 3). MNX was the most-commonly detected RDX transformation product, detected at all RDX-treatment levels for all species (Table 3), followed in frequency of detection by 4-NDAB, DNX and TNX. DNX, with one exception, was found exclusively in the stunted, RDX-intolerant *E. californica*. Similarly Larson and others (1999) observed small quantities of MNX in extracts from yellow nutsedge plants, which were cultivated in water containing the hexogen. MNX is the first product in the sequential reduction of RDX by anaerobic bacteria, which is further reduced to the dinitroso (DNX) and trinitroso (TNX) derivatives (Halasz *et al.* 2012). MNX, was the most commonly detected RDX transformation product in our foliar samples, being found at all treatment levels in the subset of species assayed (Table 3).

With respect to the model proposed by Van Aken and others (2004b), low levels of the transformation products relative to RDX found in this study could have resulted from limitations on the rate of a light-independent reduction of RDX to MNX and DNX or rapid progression of plant/light mediated breakdown of RDX metabolites and their mineralization, or both, in experimental plants and soils. The occurrence of known microbial transformation products within the plant tissue might also indicate that metabolism of RDX had taken place in anaerobic soil regions or perhaps via microbial endophytes in leaf tissue where oxygen levels vary with cell type and photosynthetic activity (Van Aken *et al.* 2004a,b).

The generally low levels of aerobic biodegradation of RDX in soil include products of *Rhodococcus* sp, such as *Rhodococcus* sp Strain DN22, that produces nitrite, nitrous oxide, ammonia and formaldehyde, which converts to carbon dioxide (Fournier *et al.* 2002). In turn, nitrite and ammonia are converted to nitrate by other bacteria in aerobic soils (Bremner and Keeney 1966). Nitrate and ammonium are the forms of nitrogen taken up from soil by plant roots. These bacterial processes could occur or be enhanced in rhizosphere soils.

We found cyclized RDX metabolites in small quantities in leaf tissue, but did not assess spontaneous decomposition of nitramine compounds in water.

Conclusions

Our results, together with plant species information, might lead to selection of plants with potential for RDX phytoextraction or phytoremediation. For example, in this study, Crown vetch (*C. varia*), a nitrogen-fixing legume, used widely on poor soils to prevent erosion and increase N was identified as an RDX-tolerant species (Table 2). It is a hardy, widespread and widely-cultivated leguminous herb often used in reclamation projects and for soil stabilization (Gucker 2009). Plant species with similar, high RDX tolerance (Table 2) to *C. varia* in this this study (*A. retroflexus, I. lacunose*, and *P. oleracea*) proved to be tolerant of TNT added as a co-contaminant with RDX (Best *et al.* (2007). This indicates that tolerance of TNT, an RDX co-contaminant, by *C. varia* and other herbaceous angiosperms is plausible. It will be necessary to directly ascertain performance of promising plant species in the field under various environmental conditions, cultivation systems, co-contamination with TNT or other compounds, plant resilience and RDX levels. Optimizing sequestration and transformation of RDX in soils by using plant assays for tolerance in carefully-designed greenhouse studies could lead to viable management options for efficient remediation of RDX pollution.

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