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HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE TRANSLOCATION IN POPLAR TREES

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Abstract—This article evaluates the translocation of the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in hybrid poplar trees (*Populus deltoides* × *nigra*, DN34) grown in hydroponic solutions. Mass balances with [U-^{14}C]RDX were used to assess RDX translocation. Up to 60% of the RDX uptaken by the tree accumulated in leaf tissues. Analysis of plant extracts by high-performance liquid chromatography equipped with radiochemical detection indicated that RDX was not significantly transformed during exposure periods of up to 7 d. The bioaccumulation of RDX may be an important concern for phytoremediation efforts.

Keywords—Phytoremediation Translocation Hexahydro-1,3,5-trinitro-1,3,5-triazine

INTRODUCTION

The improper disposal of wastewater laden with hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) from ammunition production lines has led to the contamination of soil and groundwater [1]. Hexahydro-1,3,5-trinitro-1,3,5-triazine (Fig. 1) was first formulated by the German Hans Henning in 1899 [2], and the British coined the acronym, which stands for Research Department explosive [3]. It is not mutagenic, but its acute toxicity toward rodents has led to its use as a rat poison [4]. Exposure to RDX has resulted in epileptic seizures and unconsciousness in humans [5]. As a result, a water-quality limit of 105 μg/L RDX and an estimated acceptable daily RDX intake of 0.21 mg/d for a 70-kg human have been recommended [5].

McCormick et al. [6] reported that the most effective means of RDX transformation is under anaerobic conditions. These researchers found that metabolites included known carcinogens such as dimethylnitrosamine. Spanggord et al. [7] reported that aerobic RDX transformation was highly unlikely, and McCormick et al. [6] claimed that no aerobic microbial transformation processes occur. However, a few strains of RDX-degrading aerobic bacteria have been isolated from contaminated soils [8], although no transformation products were identified.

The white rot fungus (*Phanerochaete chrysosporium*) was able to mineralize up to 67% of the [U-^{14}C]RDX to $^{14}CO_2$ over 30-d periods in liquid cultures and up to 75% in soil [9]. No RDX metabolites other than $^{14}CO_2$ were detected.

Concern over food-chain contamination has resulted in the study of RDX uptake by a number of plants. Harvey et al. [4] used [U-^{14}C]RDX and mass balances to evaluate uptake by bush beans. Their mass balance results averaged 82% and demonstrated that 69% of the uptaken label accumulated in leaf tissues after 7 d. Roughly 18 and 13% of the absorbed label was found in stem and root tissues, respectively. Radiochromatographic analysis of plant extracts revealed that the majority (up to 86%) of the radiolabel remained as RDX. An increasing trend of polar metabolite production with time was observed. After 60 d, plant extracts showed that only 21 to 51% of the radiolabel remained as RDX, 8 to 30% existed as unknown polar compounds, and 20 to 50% was not extractable. Neither mineralization to $^{14}CO_2$ nor production of volatile organic transformation products was observed.

Chen [10] studied the uptake of RDX by four different plant species from hydroponic solutions and soil: maize, soybean, wheat, and sorghum. Those experiments estimated the 30-d hydroponic toxicity of RDX to maize and wheat to be approx. 21 mg/L. No toxicity was observed for sorghum or soybean plants for RDX concentrations up to 21 mg/L. Chen also determined that RDX concentrations in plant tissues increased linearly with the increase in RDX levels in hydroponic solution or soil. Although [U-^{14}C]RDX was not used for these experiments, it was determined that RDX accumulated in leaf tissues.

Schneider et al. [11] detected field evidence at the Iowa Army Ammunition Plant (IAAP) in Middletown, Iowa, USA, that supports research pertaining to the uptake of RDX by plants. These researchers collected a wide variety of abundant plant species and found the accumulation of RDX in the leaf tissues of a large percentage of the species tested. These included the tree species black locust (*Robinia pseudoacacia*) and red cedar (*Juniperus virginiana*) as well as smartweed (*Polygonum capitatum*), bromegrass (*Bromus inermis*), pigweed (*Amaranthus* sp.), reed canary grass (*Phalaris arundinacea*), Canadian goldenrod (*Solidago canadesis*), and ragweed (*Ambrosia artemislilfolia*).

Recent interest in phytoremediation has led to the evaluation of different plant species for hazardous waste cleanup. Hybrid poplar trees are of particular interest because they grow quickly and can use relatively large amounts of water [12]. In addition, poplar trees are being considered for remediating sites contaminated with organic chemicals, as exemplified by field-scale demonstrations initiated by the U.S. Environmental Protection Agency [13]. The objective of this research was to assess the uptake and translocation of RDX by hybrid poplar trees (*Populus deltoides* × *nigra*, DN34).

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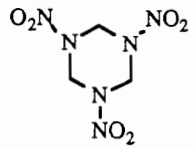

RDX
(1,3,5-trinitro-1,3,5-triazine)

Fig. 1. Chemical structure of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX).

MATERIALS AND METHODS

Analytical-grade (>99% purity) RDX was obtained from SRI International (Menlo Park, CA, USA). Deionized water (Nanopure®) was used, and all other chemicals were of reagent grade or better. Solutions of RDX were prepared by diluting the appropriate volumes from a 40-mg/L stock solution. External standards for RDX were obtained from Accustandard (New Haven, CT, USA).

The measurement of RDX was performed on hydroponic solutions before and after treatment with a Gilson 308 high-performance liquid chromatography (HPLC) isocratic pump, a Spectra 100 ultraviolet light detector set at 254 nm, and an injection loop volume of 100 μl. A guard column was used in series before the 15-cm LC-8 separation column (Supelco, Bellefonte, PA, USA). The mobile phase consisted of a water: 2-propanol (80:20, v/v) mixture fed at a flow rate of 1.5 ml/min. Samples were prepared by centrifuging 1.5-ml aliquots at 12,000 rpm for 10 min followed by dilution (1:1) with methanol.

Experimental design

Genetically identical hybrid poplar cuttings (*P. deltoides* × *nigra,* DN34) were collected in the winter from a nursery at Amana, Iowa, USA. The cuttings were trimmed to 8 inches in length and grown hydroponically in a greenhouse for 30 d. The hydroponic media was a half-strength Hoagland nutrient solution [14] adjusted to pH 7.0 with 1.0 N NaOH.

Prerooted cuttings were transferred to foil-wrapped Erlenmeyer flasks that ranged from 250 to 500 ml in volume and had been acid-washed with 1.0 M HNO_3. Initial hydroponic solution volumes of 150, 200, and 300 ml were used. Evaporation from the flasks was prevented by filling the opening with glass wool. After transpiration was monitored daily for several days, similarly transpiring cuttings were selected for use.

All experiments were conducted in a temperature-controlled room, where plants were randomly positioned under fluorescent lighting having an average light intensity of 300 μmol/$m^2 \cdot s^{-1}$ in the photosynthetically active region (PAR) as measured by a calibrated sensor. The photoperiod was 16 h with an average temperature of 25°C.

Evaluation of RDX uptake

Uptake of RDX was studied with a series of experiments designed to evaluate the loss of RDX from hydroponic solution over time. The four treatments (performed in triplicate) were live trees, heat-inactivated trees, fresh roots only, and a glassware control. Heat-inactivated trees were prepared by drying the trees in an oven at 103°C for 1 h. All plants used in these uptake experiments were maintained at 25°C. These particular experiments were conducted under continuous light. Hydroponic solutions were sampled at intervals ranging from 1 to 8 h.

Determination of [U-^{14}C] translocation

Solutions used for translocation experiments contained 50% Hoagland nutrient media spiked with RDX from a 40-mg/L stock solution and 1 to 6 μCi of [U-^{14}C]RDX (85% purity, Chemsyn, Lenexa, KS, USA) with a specific activity of 8.3 mCi/mmol. The purity of the radiolabel was determined by radiochromatography.

After completion of each experiment, plants were separated into leaves, stems, and roots. The fresh weights of each tissue type were immediately measured. Then, each tissue was split for extraction and dry-weight analysis. Tissues used for dry-weight analysis were dried in an oven at 35°C for 72 h and analyzed for ^{14}C by using an R.J. Harvey bio-oxidizer (BO). Sample blanks were performed using 0.05 g of mannitol or unspiked plant tissues. Standards were prepared by applying approx. 60,000 disintegrations per minute of ring-labeled ^{14}C-RDX to 0.05 g of mannitol, which was then combusted. The combusted RDX standard was compared to the solution in a scintillation vial containing 10 ml of a mixture that had been spiked with the same RDX standard. Bio-oxidizer efficiency was checked in this way at the beginning and end of each run. These combustion efficiency values ranged from 90 to 100%.

Samples no greater than 1.0 g in size were oxidized for 4 min. Carbon dioxide from the BO was trapped in 15 ml of R.J. Harvey ^{14}Carbon Liquid Scintillation mixture. Ten milliliters of the sample mixture was then transferred for analysis by a Beckman LS 6000IC scintillation counter (Fullerton, CA, USA).

Plant tissue extraction

A plant tissue extraction procedure based on the methods of Anderson [15] and Briggs et al. [16] was used. Tissue samples were finely chopped with pruning shears and then homogenized in a high-speed blender for 5 min. The weighed sample was then inserted into a 50-ml polypropylene centrifuge tube, to which 40 ml of pure acetone was added. The samples were allowed to equilibrate for 72 h on a heated shaker table adjusted to 100 rpm and 40°C. The samples were then filtered through 7.0-cm-diameter, 0.2-μm-mesh glass fiber filters (Fisher Scientific, Fair Lawn, NJ, USA) using a ceramic filtration funnel. The filtrate (100 μl) was analyzed by liquid scintillation, and the filter and filter residue were reserved for BO analysis.

The filtrate was mixed with 10 ml of deionized water, and the acetone was evaporated using a Büchi rotary evaporator set to 50°C. After distillation, part of the sample was transferred to two 1.5-ml microfuge tubes, and the remainder was stored in a glass vial at 4°C. No radioactivity distilled over with the acetone. After centrifugation at 12,000 rpm for 10 min, the plant extracts were analyzed using HPLC with radiochemical detection.

Volatilization studies

The transformation of RDX to CO_2 or volatile organic compounds was studied in a plant growth chamber. The chamber had one inlet connected to an air tank with a flow rate of 1.68 L/min and five outlets with combined flow rates of 1.68 L/min. One end of three of the outlets was connected to the glass side arm of a Erlenmeyer flask, and the other end led to the exterior of the chamber, where it was connected to a 27-gauge needle that bubbled through a test tube containing 10 ml of 3 N NaOH. The other two chamber outlets were exits for air

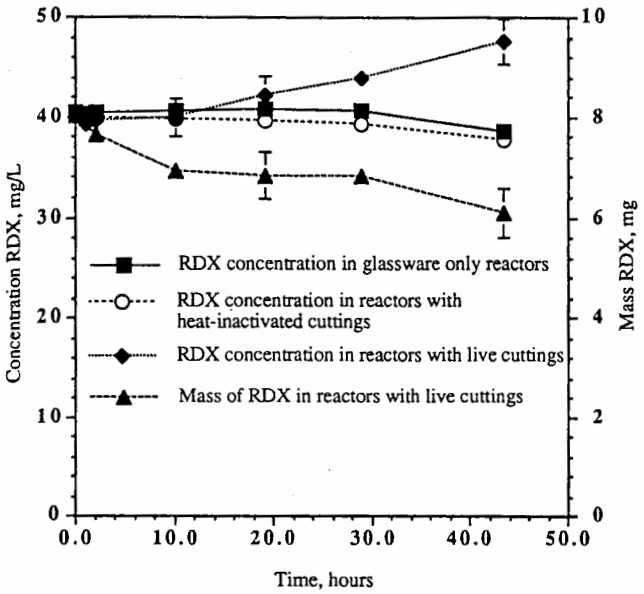

Fig. 2. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) accumulated in batch hydroponic solutions at pH 7.0 and 25°C after a spike input of 40 mg/L RDX. The concentration of RDX remained constant in glassware and dead cutting controls. Error bars indicate 1 SD, and $n = 3$ for each group.

contained within the chamber itself, and they were bubbled through NaOH traps as well. The exhaust from all five NaOH traps was evacuated to the atmosphere with an air pump. Air flow rates were metered with gauges from Gilmont (Barrington, IL, USA). Because evaporation from the NaOH traps could occur, the initial and final mass of each trap was recorded. Volatile organics were trapped in activated carbon-containing traps (Orbo tube 32 large, Supelco) placed inline before the NaOH traps. Transpired water was condensed in the chamber by a copper coil that was continuously cooled with 4°C water from a circulating water bath, maintaining the relative humidity between 50 and 60% at 25°C. Plant lighting was about 200 $\mu mol/m^2 \cdot s^{-1}$ in the PAR, and the photoperiod was 16 h.

RESULTS AND DISCUSSION

Uptake and translocation of RDX

The uptake of RDX by poplar cuttings was studied by monitoring the RDX concentration in each batch reactor for approx. 2 d. As illustrated in Figure 2, the mass of RDX decreased over time, but no new peaks (transformation products) were detected. However, the concentration increased slightly because RDX was concentrated by transpiration.

Plant uptake was further assessed with [U-^{14}C]RDX. The mass balance recoveries for these experiments (Table 1) averaged 79.7 ± 7.1%. These recoveries were similar to those reported by others [4]. Figure 3 summarizes the results of the hydroponic ^{14}C-RDX experiments in terms of the distribution of label between plant tissues. The experiments showed very similar trends in RDX transport, with the majority (60%) of the uptaken label being translocated to the leaves. The amount of [U-^{14}C]RDX partitioning into individual plant tissues (e.g., leaves) was not statistically different ($p < 0.05$) for the three time periods tested.

These experiments showed that RDX is less efficiently uptaken by plants than water. This efficiency was defined by Briggs et al. [16] with the root concentration factor (RCF) and the transpiration stream concentration factor (TSCF). The general forms of these relationships are expressed in Equations 1 and 2, where stem uptake is the mass of compound present in the stems and leaves combined:

Table 1. Mass balance results for [U-^{14}C]RDX uptake experiments from hydroponic solutions[a]

Time (d)	Applied [U-^{14}C] (%) Hydroponic medium	Plant oxidation	Total recovered
2	54.8 ± 12.2A	28.7 ± 12.8B	88.0 ± 0.79C
6	25.1 ± 7.5A	49.7 ± 11.0B	74.8 ± 3.0CD
7	28.5 ± 12.2A	45.9 ± 5.1B	74.4 ± 6.0D

[a] RDX = Hexahydro-1,3,5-trinitro-1,3,5-triazine. $n = 3$ for each group, and values are average ± 1 SD. Values followed by the same letter in each column are not significantly different ($p < 0.05$, analysis of variance).

$$\text{Root Uptake} = \text{RCF (ml/g)} \times \text{Root Mass (g)} \times \text{Water Concentration (mg/ml)} \quad (1)$$

$$\text{Stem Uptake} = \text{TSCF} \times \text{Transpiration (L)} \times \text{Water Concentration (mg/L)} \quad (2)$$

Briggs et al. further characterized the RCF and TSCF with respect to the log K_{ow} of various organic chemicals (Eqns. 3 and 4). The experimentally determined RCF and TSCF values for RDX are presented in Table 2 and are compared to the theoretical values calculated using Equations 3 and 4 and the estimated log K_{ow} of 0.9 for RDX [17]. This overprediction of RDX uptake illustrates the difficulty with using empirical relationships for predicting plant uptake and translocation of organic compounds. Experimentally determined TSCF values can be useful, however, in predicting plant uptake (see below).

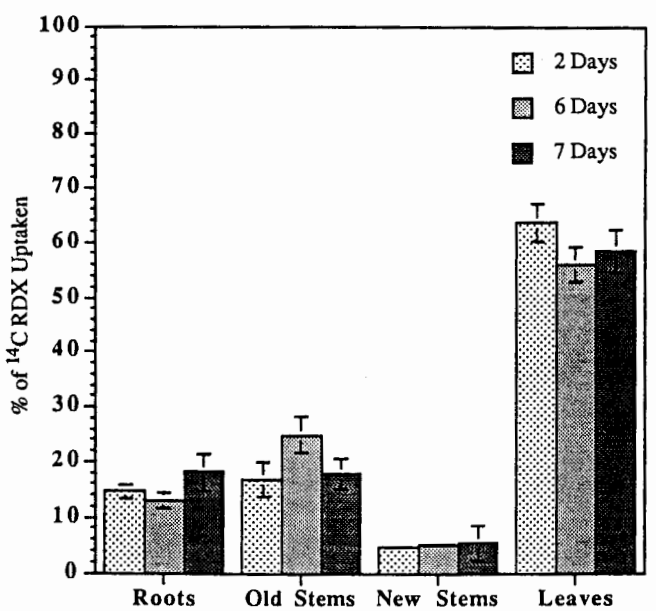

Fig. 3. The majority of the [U-^{14}C]RDX uptaken by hybrid poplars partitioned into the leaves. The amount of partitioning into individual plant tissues (e.g., leaves) was not statistically different ($p < 0.05$) for 2, 6, or 7 d (pH 7.0 and 25°C). $n = 3$ for all samples, and error bars indicate ± 1 SD. RDX = Hexahydro-1,3,5-trinitro-1,3,5-triazine.

Table 2. Extraction efficiency of [U-^{14}C]RDX from poplar cuttings (n = 3) exposed for 7 d[a]

Plant tissue	Average extraction of [U-^{14}C] (%)	SD
Roots	89.0[a]	13.9
Stems	98.4[a]	16.7
Leaves	92.7[a]	7.1

[a] RDX = Hexahydro-1,3,5-trinitro-1,3,5-triazine. Values followed by the same letter in each column are not significantly different ($p < 0.05$, analysis of variance).

$$\log(\mathrm{RCF} - 0.82) = 0.77 \log K_{ow} - 1.52 \quad (3)$$

$$\log \mathrm{TSCF} = 0.784 \times \exp\left[-\frac{(\log K_{ow} - 1.78)^2}{2.44}\right] \quad (4)$$

Transformation of [U-^{14}C]RDX

Plant tissues were extracted and analyzed to determine the extent to which transformation of RDX had occurred. In general, the extraction of RDX was quantitative, with more than 90% being extracted on average (Table 3). Chromatograms (HPLC with radiochemical detection) for tissue extracts of plants exposed to [U-^{14}C]RDX are shown in Figures 4 and 5. They demonstrate the movement of the radiolabel from the roots to the leaves.

Three radioactive chemicals were detected in the hydroponic solution at time 0: RDX and two unknowns at 1.5 and 4.4 min, respectively (Fig. 4, bottom). Even though the [U-^{14}C]RDX in the spiking solution was only 85% pure, the results indicated that the ratio of RDX to the impurities was similar throughout the plant, suggesting a lack of transformation during exposure periods of up to 7 d. Figure 5 (top) illustrates the accumulation of [U-^{14}C]RDX in the leaves of the poplar hybrid. The 15% of radiolabel that was not extractable (the bound fraction) may represent a transformation product of RDX in the plant.

No plant respiration of $^{14}CO_2$ or organic volatiles was measured for exposure periods of 7 d. The microbial production of $^{14}CO_2$ was detected, but the small amounts (<2%) did not exceed the amount of radiolabeled impurities. No signs of phytotoxicity (hindered growth, chlorosis, or decreased transpiration) were evident for concentrations of RDX up to 21 mg/L and exposure periods of 14 d.

The lack of transformation of RDX and its accumulation in leaf tissues resulted in concentrations of RDX ranging from 350 to 720 mg/kg in the leaf tissues (Table 4). (Variation for the second set of experiments may be attributed to the large differences in transpiration and uptake between the plants (100 vs 200ml). These concentrations are comparable to those reported by Chen [10] for agricultural crops.

Table 3. Comparison of calculated and experimental root concentration factors (RCFs) and transpiration stream concentration factors (TSCFs) for RDX[a]

Chemical	Calculated value RCF (ml/g)	Calculated value TSCF	Experimental value RCF (ml/g)	Experimental value TSCF
RDX	0.97	0.57	1.3 ± 0.73	0.16 ± 0.06

[a] RDX = Hexahydro-1,3,5-trinitro-1,3,5-triazine. n = 9, and experimental values are averages ± 1 SD.
[b] [16].

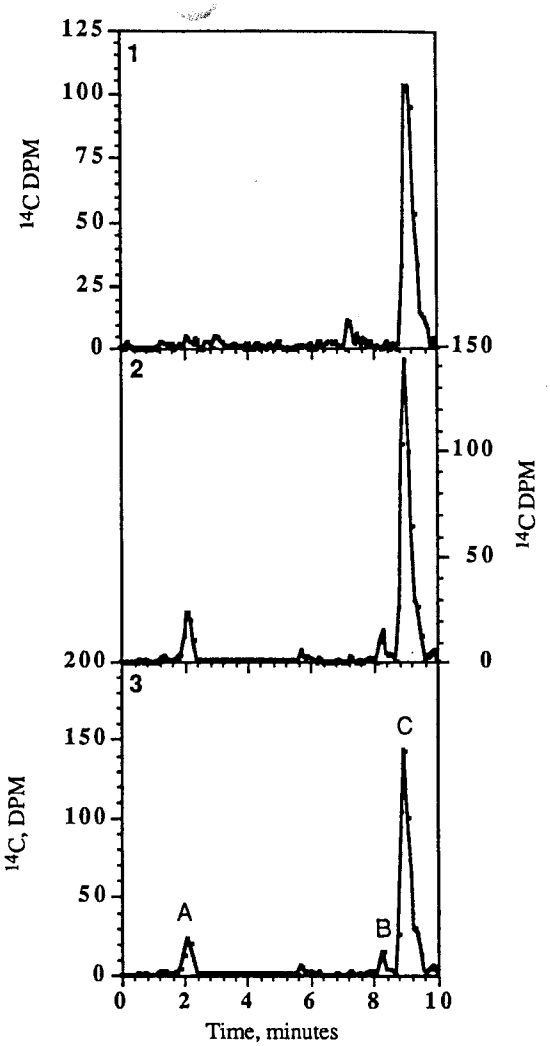

Fig. 4. Radiochromatograms of stem (1) and root (2) extracts after 7 d compared to the feed solution (3), which contains two unknowns (A and B) and [U-^{14}C]RDX (C). RDX = Hexahydro-1,3,5-trinitro-1,3,5-triazine; DPM = disintegrations per minute.

Environmental implications of RDX uptake and fate

Before the results of this research can be applied directly to the field, at least two unknown factors must be considered: time of exposure and scale. First, it is possible that longer exposure times may result in an increase in RDX metabolism, as suggested by Harvey et al. [4]. Furthermore, there is recent evidence that trichloroethylene metabolism is significantly higher for large (20 feet in height) poplar trees under field conditions (natural sunlight, etc.) [18]. With this in mind, the research presented here may be used to develop a worst-case scenario.

By using (1) an experimentally determined TSCF value for RDX, (2) plant biomass and transpiration data from a pilot-scale greenhouse experiment (unpublished data), and (3) an average soil pore-water concentration of 1 mg/L RDX, we estimate that poplar leaves could have concentrations of 20 mg/kg. In comparison, a black locust tree (*R. pseudoacacia*) growing in soil containing 114 mg/kg RDX accumulated 38.6 mg/kg of RDX in its leaves according to the IAAP study

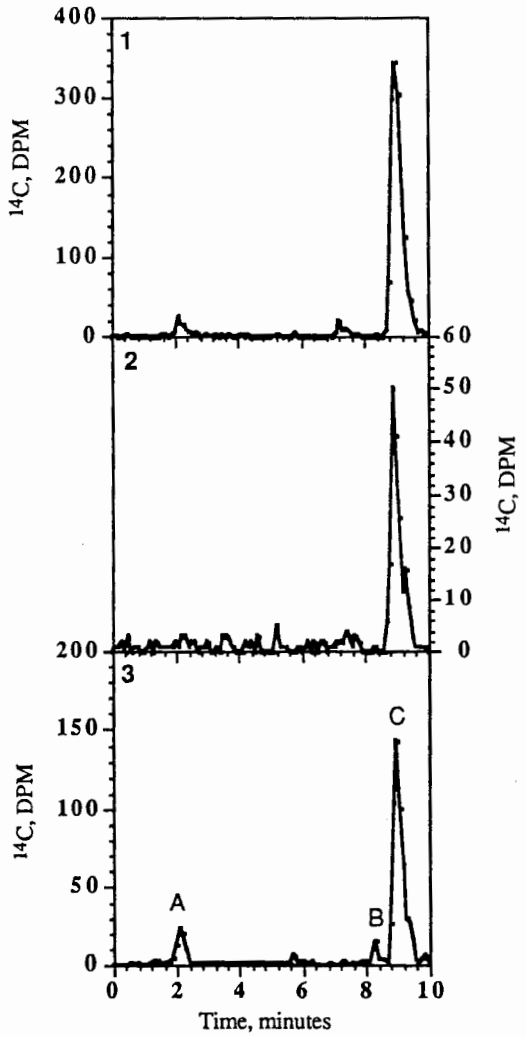

Fig. 5. Radiochromatograms of leaf (1) and new stem growth (2) extracts after 7 d compared to the feed solution (3), which contains two unknowns (A and B) and [U-^{14}C]RDX (C). RDX = Hexahydro-1,3,5-trinitro-1,3,5-triazine; DPM = disintegrations per minute.

conducted by Schneider et al. [11]. They also detected 42 mg/kg RDX in the above-ground portions of a red cedar tree (*J. virginiana*) growing in soil contaminated with 17 mg/kg RDX.

The issue with RDX accumulation in poplar leaves may ultimately be its effect on the large number of deer and other herbivores at the IAAP. With an anticipated 20 mg/kg RDX concentration in leaf tissues, a 70-kg deer would be able to consume only about 10 g of leaves per day given Etnier's [5] estimated acceptable daily RDX intake of 0.21 mg/d for a 70-kg human. This intake limit would have to be increased by more than 100-fold for a deer to consume 2 pounds of leaves per day. If phytoremediation is to be used for RDX contamination, leaves should be monitored for the presence of RDX. Leaf consumption could be prevented by surrounding contaminated areas with high fences.

Table 4. Estimated mass of RDX per kilogram of dry plant tissue[a]

Initial hydroponic concn. of RDX (mg/L)	RDX/Root (mg/kg)	RDX/Stem (mg/kg)	RDX/Leaves (mg/kg)
7.9	694 ± 49A	46 ± 32CD	354 ± 42E
17.4	877 ± 625AB	50 ± 15C	470 ± 296EF
26	1,494 ± 136B	121 ± 17D	723 ± 44F

[a] RDX = Hexahydro-1,3,5-trinitro-1,3,5-triazine. Exposure times for the set of three experiments were 6, 2, and 7 d. $n = 3$ for each group, and values are averages ± 1 SD. Values followed by the same letter in each column are not significantly different ($p < 0.05$, analysis of variance).

CONCLUSION

For field-scale phytoremediation to be effective, the uptake and fate of contaminants must be evaluated. The research presented in this article demonstrates that the explosive RDX was uptaken by a hybrid poplar tree (*P. deltoides* × *nigra*, DN34) to a lesser degree than predicted by empirical methods. The RDX-related radiolabel was rapidly translocated and was distributed throughout the plant in similar proportions in experiments lasting from 2 to 7 d. The largest amounts of label (60% of that uptaken) were found in leaf tissues, with the remainder being equally distributed between root and stem tissues. Radiochromatography analysis demonstrated that no RDX transformation had occurred. Accumulation of RDX in foliage may be a concern for some applications of phytoremediation.

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