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ACCUMULATION OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE BY THE EARTHWORM *EISENIA ANDREI* IN A SANDY LOAM SOIL

Manon Sarrazin,† Sabine G. Dodard,† Kathleen Savard,† Bernard Lachance,† Pierre Y. Robidoux,† Roman G. Kuperman,‡ Jalal Hawari,† Guy Ampleman,§ Sonia Thiboutot,§ and Geoffrey I. Sunahara†

†Biotechnology Research Institute, National Research Council–Canada, 6100 Royalmount Avenue, Montreal, Quebec H4P 2R2, Canada
‡U.S. Army Edgewood Chemical Biological Center, 5183 Blackhawk Road, Aberdeen Proving Ground, Maryland 21010-5424
§Defense Research and Development Canada–Valcartier, 2459 Pie IX Boulevard, Val Bélair, Quebec G3J 1X5, Canada

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Abstract—The heterocyclic polynitramine hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a highly energetic compound found as a soil contaminant at some defense installations. Although RDX is not lethal to soil invertebrates at concentrations up to 10,000 mg/kg, it decreases earthworm cocoon formation and juvenile production at environmentally relevant concentrations found at contaminated sites. Very little is known about the uptake of RDX in earthworms and the potential risks for food-chain transfer of RDX in the environment. Toxicokinetic studies were conducted to quantify the bioaccumulation factors (BAFs) using adult earthworms (*Eisenia andrei*) exposed for up to 14 d to sublethal concentrations of nonlabeled RDX or [^{14}C]RDX in a Sassafras sandy loam soil. High-performance liquid chromatography of acetonitrile extracts of tissue and soil samples indicated that nonlabeled RDX can be accumulated by the earthworm in a concentration- and time-dependent manner. The BAF, expressed as the earthworm tissue to soil concentration ratio, decreased from 6.7 to 0.1 when the nominal soil RDX concentrations were increased from 1 to 10,000 mg/kg. Tissue concentrations were comparable in earthworms exposed to nonlabeled RDX or [^{14}C]RDX. The RDX bioaccumulation also was estimated using the kinetically derived model (BAF_K), based on the ratio of the uptake to elimination rate constants. The established BAF_K of 3.6 for [^{14}C]RDX uptake was consistent with the results for nonlabeled RDX. Radioactivity also was present in the tissue residues of [^{14}C]RDX-exposed earthworms following acetonitrile extraction, suggesting the formation of nonextractable [^{14}C]RDX metabolites associated with tissue macromolecules. These findings demonstrated a net accumulation of RDX in the earthworm and the potential for food-chain transfer of RDX to higher-trophic-level receptors.

Keywords—Earthworm Hexahydro-1,3,5-trinitro-1,3,5-triazine Explosives Bioaccumulation Soil

INTRODUCTION

The polynitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) can adversely affect soil invertebrates at environmentally relevant concentrations found at defense installations [1–4]. Earthworm exposure to RDX adversely affected reproduction, based on the median effective concentrations (EC50s) of 3.7 and 5.0 mg/kg for cocoon and juvenile production, respectively, but did not affect the survival of adult earthworms at concentrations up to and including 756 mg/kg in a 56-d test [5–8]. Sublethal effects of RDX in earthworms included neurotoxicity at 0.21 μg/cm^2 in a 14-d filter-paper study [9].

The toxicity of RDX or its nitroso-metabolites also was demonstrated for different vertebrate species [8,10]. In fact, RDX occasionally has been used as a rat poison [11]. Subchronic toxicity studies using Fischer 344 rats receiving daily doses of 100 mg RDX/kg body weight for 90 d showed that RDX can cause lethality, specific organ toxicities, and neurological effects [12]. Acute oral toxicity studies showed that the RDX metabolite hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) caused 100% lethality at 400 mg/kg in gavaged Sprague–Dawley rats [13]. Convulsions also were observed in MNX-treated animals. Acute toxicity studies showed that oral doses of RDX greater than 630 mg/kg were lethal to Northern Bobwhite [14]. Central nervous system and respiratory distresses also were observed in the RDX-exposed birds.

In humans, RDX is a possible carcinogen, and accidental exposure to RDX has led to central nervous system problems, nausea, and vomiting [11]. These toxicological data for RDX and its metabolites suggest that soil RDX concentrations found at defense installations [1,15] can pose health risks to humans and wildlife through food-chain transfer [10]. Consequently, a better understanding of RDX accumulation in soil invertebrates, including earthworms that are key components of soil trophic webs, requires further study.

Bioaccumulation of environmentally persistent chemicals in animals involves several interacting physiological processes that govern the uptake of a contaminant by an organism following dermal absorption or ingestion, as described elsewhere [16–18]. The accumulation of a chemical in an organism often is conveyed through a bioaccumulation factor (BAF), which can be expressed as the steady state–based distribution coefficient of the tissue to soil concentrations of the test compound [19–21]. Sunahara et al. [22] determined the BAFs for RDX in laboratory microcosm studies with the earthworm *Eisenia andrei* and reported that the BAFs decreased from 13 to 2.9 as nominal RDX concentrations in a Sassafras sandy loam (SSL) soil increased from 10 to 100 mg/kg dry soil. Statistical analysis of these data revealed a log-linear relationship between RDX concentrations in soil and in earthworms [23]. Best et al. [24] determined an average

* To whom correspondence may be addressed (geoffrey.sunahara@cnrc-nrc.gc.ca).
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tissue-to-soil RDX concentration ratio of 1 in a 28-d study with *E. fetida* exposed to a field-collected soil containing RDX and other contaminants, including octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and 1,3-dinitrobenzene. Those authors did not comment on whether the presence of the latter co-contaminants had an effect on RDX accumulation in the earthworm. Discrepancies in the published BAFs for RDX can be attributed to differences in the experimental designs used in the latter studies, including the methods used to assess bioaccumulation, the earthworm species, soil properties, RDX concentrations, duration of exposures, presence of co-contaminants in field-collected soils, and the formation of RDX transformation products in soil or in the organism.

In addition to the steady state–based tissue-to-soil distribution coefficient model described above, the BAF can be estimated using the kinetic approach (BAF_K) [17,25,26]. For example, the BAF_K can be expressed as the ratio of the uptake and elimination rate constants for a test compound using a first-order, single-compartment model. This method has been used to estimate the BAF_K for RDX in aquatic vertebrate and benthic oligochaetes [27,28].

In the present study, the hypothesis that RDX can accumulate in soil invertebrates, such as earthworms, from RDX-amended soil was tested. The objective was to determine and contrast the BAFs for RDX in soil using two approaches: the steady state–based distribution coefficient model (ratio of tissue to soil RDX concentrations) using earthworms exposed to nonlabeled RDX, and the kinetic approach (BAF_K) using earthworms exposed to [^{14}C]RDX. Mass-balance estimation using [^{14}C]RDX was included to determine the fate of RDX and its metabolites in the soil and tissue.

MATERIALS AND METHODS

Chemicals and reagents

Nonlabeled RDX (Chemical Abstracts Service no. 121-82-4; 99.9% purity, with <0.1% MNX) and [^{14}C]RDX (specific activity, 54.4–62.1 μCi/mmol) were obtained from the Defense Research Development Canada–Valcartier. Authentic reference standards, including RDX, MNX, hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) were purchased from Accu-Standard. All other chemicals were of reagent grade and were obtained from commercial suppliers. Acetone and acetonitrile (high-performance liquid chromatography [HPLC] grade) were obtained from Caledon Laboratories. The American Society for Testing and Materials (ASTM) type I water [29] was obtained using a Zenopure Mega-90 water purification system (Zenon Environmental) and was used throughout the present study. Glassware was washed with phosphate-free detergent and rinsed with acetone, nitric acid (10%, v/v), and deionized water.

Culturing and handling of earthworms

Adult earthworms (*E. andrei*) obtained from Carolina Biological Supply were used to establish the initial laboratory cultures. Animals were maintained in earthworm bedding (Magic Products) supplemented with dry cereal (Magic Worm Food; Magic Products). The incubator was kept under a 16:8-h light:dark photoperiod with a light intensity of 800 ± 400 lux (mean ± standard deviation), temperature of 20 ± 1°C, and relative humidity of 70 to 80%. Clitellated earthworms weighing from 425 to 690 mg were used in the present study.

Soil handling and amendments

A natural soil (SSL; fine-loamy, siliceous, mesic semiactive, Typic Hapludult) [30] collected from a grassland field on the property of the U.S. Army Aberdeen Proving Ground (MD, USA) was used in the present study. The physical and chemical characteristics of the SSL soil (11% clay, 1.2% organic carbon, 71% sand, and 18% silt; pH 5.5) were expected to support high relative bioavailability of RDX according to the ecological soil screening level (Eco-SSL) criteria [31] (http://www.epa.gov/superfund/health/exposure/bioavailability/guidance.htm). The SSL soil was air-dried, sieved on a 2-mm mesh screen, and weighed separately to prepare each treatment batch in a glass dish. Soil was spread to a thickness of approximately 2.5 to 4 cm. Nonlabeled RDX was dissolved in acetone and added to each soil batch to prepare target concentrations of 1, 10, 100, 1,000, 3,000, or 10,000 mg/kg. The selection of RDX concentrations in soil was based on published results of the earthworm toxicity tests [6,7]. Acetone solutions of RDX were poured evenly across the soil surface, ensuring that the volume of solution added did not exceed 15% (v/w) of the dry soil mass. The greatest concentration (10,000 mg/kg) was prepared in several steps using a stock solution of 40 g/L, each time not exceeding 15% of soil weight. Acetone was allowed to volatilize for 2 h between the steps [3,32]. The amended soil batches were kept in a darkened chemical hood for a minimum of 48 h to allow acetone evaporation [33]. Each soil treatment batch was transferred into high-density polyethylene containers coated with a Teflon®-like fluorocarbon and covered with aluminum foil to prevent photolysis of RDX. The soil batches were mixed overnight (18 ± 2 h) using a three-dimensional rotary soil mixer. Three replicates from each dry soil batch were hydrated individually to 70 to 75% of the SSL water-holding capacity (21% water based on the dry SSL soil mass) for 3 h before the beginning of the experiment.

Uptake of nonlabeled RDX by earthworms in amended soil

The RDX uptake experiments were performed with SSL soil using *E. andrei* according to the ASTM standard guide for soil bioaccumulation studies [34] with some modifications. Instead of plastic containers (as recommended in the 2004 ASTM guidelines [34]), glass containers were used to avoid adsorption of RDX to the container walls. Earthworms were acclimated for 24 h in nonamended SSL soil before the experiment. One earthworm per 10 g dry weight of soil was placed into each replicate test unit (250-ml glass jar) containing 60 or 100 g dry weight of soil.

Two grams of dry cereal were added to each test unit. Each test unit was then covered by a perforated lid to control soil moisture. Earthworm wet weights were recorded at the start of the experiment. Measurements of RDX in tissue and soil samples were taken by a time-series sampling in which earthworms were sampled destructively after 0.25, 1, 2, 4, 7, and 14 d of exposure. On each sampling day, the earthworms were collected, counted, rinsed with ASTM type I water, and depurated for 24 h on a moistened filter paper. Then, earthworms were rinsed, blotted dry, placed into glass tubes, and immediately frozen at −80°C. Soil samples from each replicate container were homogenized and stored at −20°C until processed for HPLC analysis. Chemical analyses were conducted on triplicate samples of soil or tissue that were collected from each treatment group on the designated sampling days.

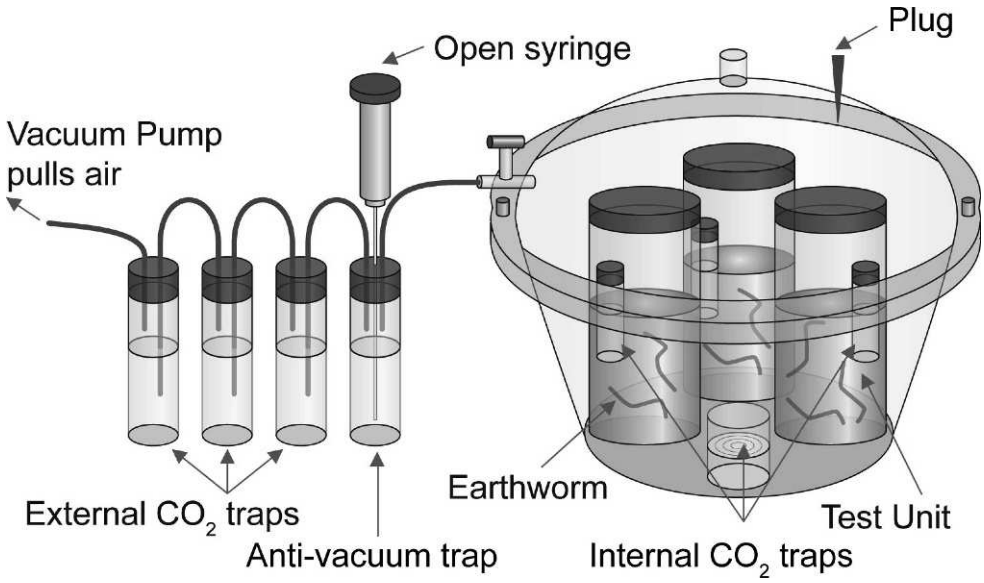

Fig. 1. Earthworm accumulation microcosm (EAM), a closed system. Adult *Eisenia andrei* were exposed to SSL soil amended with [^{14}C]hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Internal and external $^{14}CO_2$ traps contained 0.5 N KOH. The EAM used in the present study was a modification of an earlier design described by Sunahara et al. [22] and by Kuperman et al. (http://www.serdp.org/research/upload/cu-1221-fr-01.pdf).

Chemical analyses of nonlabeled RDX in soil and earthworm samples

A modified U.S. Environmental Protection Agency Method 8330B [35] was used to extract and quantify the concentrations of RDX or its degradation products in the soil samples, using HPLC as described by Dodard et al. [33]. Triplicate soil samples (2 g each) were collected from each test unit, after which 10 ml of acetonitrile were added and the mixture vortexed for 1 min. Samples were sonicated for 18 h and then diluted (1:1, v/v) with 5 g/L of $CaCl_2$. The clear fraction was filtered through a 0.45-µm Millex™ HV cartridge (Millipore) before HPLC analyses. The limit of detection for RDX, MNX, and TNX in liquid samples was approximately 50 µg/L. Precision was 95% or greater (standard deviation, <2%). The laboratory detection limit for RDX, MNX, and TNX in SSL soil was 0.25 mg/kg, based on a signal-to-noise ratio of 10.

Tissue extracts of the earthworms exposed to nonlabeled RDX or of those in the control treatments (no RDX added) were prepared as described by Renoux et al. [5]. For each replicate, all earthworms were lyophilized and crushed using a mortar and pestle to obtain dry material for analysis. Two milliliters of ASTM type I water (4°C) were added to each tissue sample (0.48 ± 0.08 g), followed by vortexing for 10 s. Next, 5 ml of acetonitrile were added to the suspension, which then was vortexed for an additional 60 s. All samples were sonicated (Branson Model 3200) for 18 ± 2 h at 20°C and centrifuged (12,000 *g*) for 10 min at 4°C using a Sorval Super T21 centrifuge (Global Medical Instrumentation). A 3.5-ml aliquot of each supernatant was taken and mixed with 1.5 ml of $CaCl_2$ (16 g/L) and placed at 4°C for 2 h to precipitate the fine particles. The supernatant was filtered through a 0.45-µm Millex™ HV cartridge before HPLC analysis. The detection limit for nonlabeled RDX in the earthworm was 5 mg/kg dry tissue. Nitramine concentrations in earthworm tissue and soil were expressed as mg/kg dry tissue and mg/kg dry soil, respectively.

Uptake and elimination of [^{14}C]RDX by earthworms

The kinetics-based bioaccumulation test consisted of two phases (i.e., a period of uptake, followed by a period of elimination) as described by others [26,36]. The RDX uptake kinetics in earthworms were quantified by a time-series sampling in which earthworms were sampled destructively after 0.25, 1, 2, 3, 7, 9, and 14 d of exposure to 100 mg [^{14}C]RDX/kg dry SSL soil. Earthworms were depurated for 24 h on a moistened filter paper as described for the nonlabeled RDX studies. Based on the nonlabeled RDX exposure studies described above, this exposure concentration was not lethal to earthworms. Preparation of the [^{14}C]RDX-amended SSL soil followed the same procedure as described for the nonlabeled RDX soil studies.

At the start of the experiment, test units were placed into separate earthworm accumulation microcosms (EAMs) (Fig. 1) constructed from clear polycarbonate vacuum desiccators (inner diameter, 23 cm). Each EAM consisted of a maximum of six test units containing the earthworms and the [^{14}C]RDX-amended SSL soil. Control treatments were placed into separate EAMs. The EAM was made air-tight using two metal rings and associated polytetrafluoroethylene-rubber O-rings, fastened by bolted nuts. A 3-mm access port on the top of each EAM allowed sampling of the internal EAM alkali trap for CO_2.

All CO_2 traps contained 0.5 M KOH. Two sets of CO_2 traps were used to collect the evolved $^{14}CO_2$ within the EAM (Fig. 1). The first set of traps consisted of 10-ml glass tubes placed into each separate test unit and a 20-ml beaker placed outside the test units but inside each EAM. The second set of traps consisted of four external, serially connected test tubes. The first external tube from the EAM contained water and acted as the antivacuum trap. The remaining external tubes contained 20 ml of KOH each and trapped the evolved $^{14}CO_2$. The fourth tube also contained an outlet to flush air three times each week using a vacuum pump. Based on the results of preliminary studies, total air flush of the EAM was completed within 4 h.

On the designated sampling day, each external trap was sampled (1 ml) and mixed with ASTM type I water (1 ml) after each air flush. A 1-ml aliquot also was taken from each internal CO_2 trap. Scintillation counting fluid (18 ml) was added to the samples, and radioactivity was determined using a Packard Tri-Carb™ 2100TR (Canberra) liquid scintillation counter (LSC).

To quantify the RDX elimination kinetics, the earthworms exposed to [^{14}C]RDX for 14 d were removed from the soil, rinsed with ASTM type I water, and then transferred (without depuration on filter paper) into test units, with each test unit having 60 g of nonamended SSL soil. Triplicate test units then were placed into separate EAMs for subsequent destructive sampling. The RDX elimination kinetics in earthworms were quantified by a time-series destructive sampling after 0.08, 0.25, 1, 2, 3, 7, and 14 d of exposure in nonamended SSL soil. Earthworms were depurated for 24 h on a moistened filter paper as described for the RDX uptake studies. The concentrations of [^{14}C]RDX and its [^{14}C]metabolites (including MNX or TNX) in the earthworms were determined using the LSC or ^{14}C-HPLC during the uptake and elimination phases of this bioaccumulation test. The total ^{14}C-activity (RDX and its metabolites) in the individual test soil or tissue samples was determined using a PerkinElmer Model 307 sample oxidizer and LSC.

The [^{14}C]RDX uptake studies also were done to quantitate the mass balance using the EAM setup (Fig. 1) and triplicate test units containing earthworms (n = 10 worms/unit and 3 units/EAM) exposed to 100 mg [^{14}C]RDX/kg SSL soil for up to 14 d before destructive sampling and radiochemical analyses. Data are expressed as the percentage recovery of radioactivity in soil or earthworm or the evolved $^{14}CO_2$ relative to the amount of radioactivity added to soil at the start of the present study. As part of the mass-balance studies, the ^{14}C-activity in the acetonitrile-extractable fraction as well as the tissue-residue (or nonextractable) fraction also were examined to follow the fate of [^{14}C]RDX absorbed by the earthworms. The ^{14}C-activity remaining in the tissue pellet was separated from the acetonitrile extract by centrifugation of the solvent–tissue suspension. The radioactivity in these fractions was analyzed using LSC; this value is expressed as disintegrations per minute (dpm). Radioactivity in the acetonitrile-extractable fraction of the earthworm was considered to represent RDX or its unbound degradation products, whereas radioactivity in the nonextractable fraction was considered to represent RDX degradation products that were bound to cellular constituents.

Analysis of ^{14}C-activity in soil and earthworm tissue samples

The ^{14}C-activity in soil or earthworm samples was determined using two methods. Wet combustion was performed using a glass and polytetrafluoroethylene apparatus containing hot acid, as described by others [22,37,38]. This setup consisted of a 100-ml, round-bottom flask with heating mantle, a gas inlet, a water-filled jacket condenser, and an outlet fitted with a separatory funnel used for liquid transfer. The outlet was connected to a KOH trap consisting of five test tubes (17 × 150 mm) attached in series. Each tube was filled with 10 ml of 0.5 M KOH containing a low concentration of thymolphtalein as a pH indicator (for changes in the alkaline range). Soil and earthworm samples were combusted in the following manner: Approximately 1 g of soil or 0.2 g of lyophilized ground earthworm tissue sample was taken. Then, 1.5 g of potassium dichromate ($K_2Cr_2O_7$) was added to each soil sample, and the samples were combusted in 25 ml of hot acid mixture (H_2SO_4:H_3PO_4 [3:2, v/v]) for 20 min. Earthworms were combusted in a 30-ml acid mixture. Four tubes, each containing 10 ml of KOH (0.5 M), were used to trap the evolved $^{14}CO_2$. A 1-ml sample from each of the KOH traps was analyzed using LSC. The detection limit of this method was 10×10^3 dpm/g tissue. The detection limit was improved to 1.3×10^3 dpm/g tissue later in the investigation, when the dry-combustion method became available to this laboratory. This technique involved use of the sample oxidizer that enabled analysis of smaller quantities of earthworm tissue (~0.02 g tissue) compared to the amount required for wet combustion (0.2 g tissue). Samples were prepared according to the manufacturer's instructions and counted using LSC.

BAF calculations

The BAF was determined using two approaches. The first approach involved use of the steady state–based distribution coefficient model, expressed as the ratio of the nonlabeled RDX concentration in the tissue (mg/kg) to the nonlabeled RDX concentration in the soil (mg/kg). The BAF is expressed as kg soil/kg tissue. The second approach was the BAF_K, or the net uptake of RDX, calculated as the ratio of the rate constant for the uptake of RDX (k_1) from soil by the earthworm to the rate constant for elimination of RDX (k_2) from the earthworm, assuming first-order, single-compartment exponential kinetics. The values for the RDX uptake rate constant (k_1), the elimination rate constant (k_2), and the BAF_K were calculated from model equations shown below. The goodness of fit of the models was determined from the coefficients of determination (r^2).

The tissue uptake rate constant (k_1) was derived from a single-compartment exponential uptake model for first-order kinetics as described by Bruns et al. [36]:

$$[RDX_T]_{total} = \frac{k_1}{k_2} \cdot [RDX_S] \cdot \left(1 - e^{-k_2 t}\right) \quad (1)$$

where $[RDX_T]_{total}$ is the total radioactivity (dpm/g dry wt tissue) in the earthworm tissue (^{14}C in extractable plus nonextractable fractions), k_1 is the uptake rate constant (g dry wt soil/g dry wt tissue/d), k_2 is the elimination rate constant (1/d), $[RDX_S]$ is the concentration of radioactivity in dry soil (dpm/g dry wt soil) at the end of RDX exposure (14 d), and t is the duration of uptake (d).

Either [^{14}C]RDX or its unbound metabolites were considered for the calculation of the elimination kinetics. These compounds were present in the acetonitrile-extractable fraction of the earthworm. Consequently, the elimination data of ^{14}C-radioactivity from earthworm extracts was used to estimate the tissue elimination rate constant (k_2) according to the following exponential elimination model:

$$[RDX_T]_{extract} = \left([RDX_T]_{SS} \cdot e^{-k_2 t}\right) + [RDX_T]_R \quad (2)$$

where $[RDX_T]_{extract}$ is the total radioactivity (dpm/g dry wt tissue) in the acetonitrile-extractable fraction of the earthworm, $[RDX_T]_{SS}$ is the extractable radioactivity in the earthworm (dpm/g dry wt tissue) under apparent steady-state conditions (defined as no significant change in tissue RDX concentrations with respect to period of exposure), and $[RDX_T]_R$ is the residual radioactivity (dpm/kg dry wt tissue) at the end of elimination phase.

Under steady-state conditions,

$$[\mathrm{RDX_T}]_{SS} = \frac{k_1}{k_2} \cdot [\mathrm{RDX_S}]_{SS} \quad (3)$$

$$\mathrm{BAF}_K = \frac{k_1}{k_2} \quad (4)$$

where $[\mathrm{RDX_S}]_{SS}$ is the radioactivity in the soil (dpm/g dry wt soil) at apparent steady-state conditions and BAF_K (g dry wt soil/g dry wt tissue) is the ratio of the tissue uptake rate constant (k_1) to the tissue elimination rate constant (k_2).

The biological half-life of RDX in the earthworm—that is, the time required for the organism to eliminate half the radioactivity absorbed under steady-state conditions ($t_{1/2}$, d)—was determined using the equation described by Lotufo and Lydy [28]:

$$t_{1/2} = \frac{\ln(0.50)}{k_2} \cong \frac{0.693}{k_2} \quad (5)$$

Nonlinear regression models were run in SYSTAT™ (Ver 7.01; SPSS) and KaleidaGraph™ (Ver 4.03; Synergy Software) for the iterative curve-fitting procedures. Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Untransformed data for tissue or soil RDX concentration were used in parameter calculations.

Statistical analysis

Analysis-of-variance procedures were used for the uptake and elimination data established over time and among concentrations for different exposure time series. Means separations were done using Fisher's least-significant-difference pairwise comparison tests. Statistical analyses were performed using SYSTAT (Ver 7.01). Student's t test was used for comparisons between treatments using Microsoft® Excel software. A significance level of $p \leq 0.05$ was accepted for all statistical analyses.

RESULTS AND DISCUSSION

Uptake of nonlabeled RDX in earthworms

Preliminary time-course studies were carried out to determine the length of time needed to achieve steady-state conditions for nonlabeled RDX uptake from SSL soil in which adult *E. andrei* were exposed to nominal RDX concentrations of 10, 100, 1000, and 10,000 mg/kg for varying periods of exposure (2–21 d). These studies showed that RDX concentrations in exposed earthworms achieved steady state on or after 7 d (data not shown). All earthworms survived the exposure and showed no signs of adverse effects, which was consistent with the results of earlier studies [4,6,7,22]. Consequently, adult *E. andrei* were exposed for 7 d to nominal RDX concentrations of 0, 1, 10, 100, 1,000, 3,000, and 10,000 mg/kg. The corresponding measured concentrations of RDX in SSL were 0, 0.66 ± 0.09, 10.6 ± 0.03, 102 ± 5, 967 ± 16, 2,850 ± 12, and 9,427 ± 103 mg/kg, respectively. Trace concentrations of MNX (<1% of RDX concentrations) were found in amended soil and earthworm samples from RDX-amended soil. Neither DNX nor TNX was detected in these samples. For ease of reference, the nominal concentrations are reported in the text and in the figures, unless otherwise stated. The concentration of RDX in tissue steadily increased with increasing soil RDX concentration; differences among several treatment groups were statistically significant ($p \leq 0.05$)

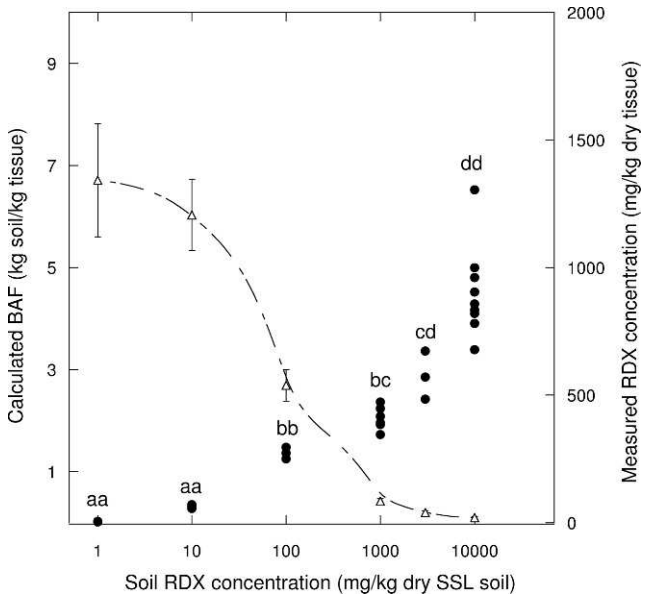

Fig. 2. Uptake of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in earthworms (*Eisenia andrei*) after the 7-d exposure to freshly amended soil. The RDX concentrations in tissue samples (●; right y axis) were determined using the U.S. Environmental Protection Agency Method 8330B [35] at different nominal RDX concentrations in a Sassafras sandy loam (SSL) soil (x axis). The calculated bioaccumulation factor (BAF) of RDX (△; left y axis) is expressed as the mean ± standard deviation (n = 3–8 replicates). The BAF of RDX in earthworms was 2.7 ± 0.3 at 100 mg RDX/kg soil. Common letters between treatment groups indicate no significant difference ($p > 0.05$) using analysis of variance and Fisher's least-significant-difference test.

(Fig. 2). The BAFs, expressed as distribution coefficients, decreased proportionally to the increase in the RDX concentration in soil and were 0, 6.7, 6.0, 2.7, 0.4, 0.2, and 0.1 for nominal soil RDX concentrations of 0, 1, 10, 100, 1,000, 3,000, and 10,000 mg/kg, respectively (Fig. 2). A similar relationship was reported by Sunahara et al. [22]. Based on the BAF distribution coefficients greater than one, the tissue accumulation of RDX occurred at measured RDX concentrations of 0.66, 10.6, and 102 mg/kg soil.

The results presented herein differ from those of Best et al. [24], who reported that the average BAF for RDX in the earthworm was equal to one, although closer examination of their data suggests that BAFs also may have changed as a function of RDX soil concentrations. The results of the latter study, together with the findings presented here, suggest that above a certain concentration of RDX in soil, small quantities of undissolved or crystalline RDX may be present in the exposure matrix and not accessible to the earthworms. This is consistent with the RDX tissue uptake data presented in Figure 2, showing that tissue concentrations of RDX increased 4.3-fold ($p \leq 0.05$) in earthworms exposed to soil RDX concentrations ranging from 10 to 100 mg/kg. In contrast, only a 1.4-fold increase ($p > 0.05$) was observed in tissue RDX concentrations when earthworms were exposed to soil RDX concentrations ranging from 100 to 1,000 mg/kg.

The HPLC analysis of tissue extracts indicated that the parent compound RDX as well as MNX, a reduced product of RDX degradation, were detected in earthworms exposed to different concentrations of RDX in soil (Fig. 3). Concentrations of MNX in earthworm tissues were directly proportional to the RDX concentrations in soil. A transient increase in MNX concentration was found in the tissue after a 1-d

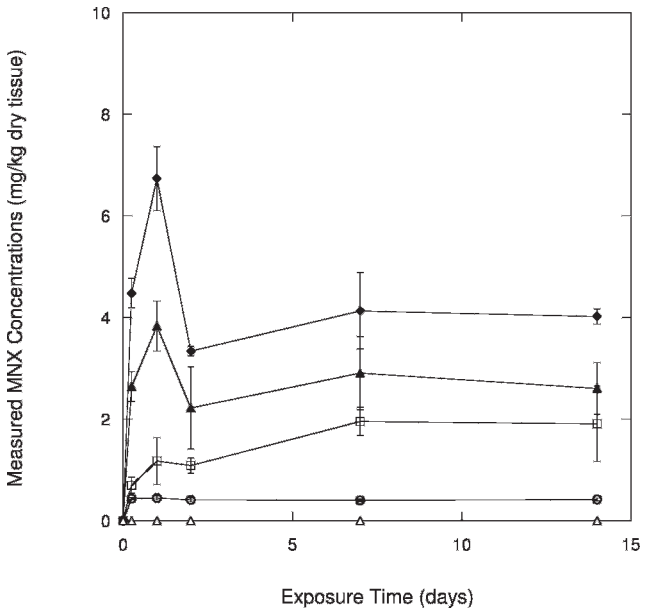

Fig. 3. Presence of hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX; a reduced product of hexahydro-1,3,5-trinitro-1,3,5-triazine [RDX]) in earthworms exposed to different concentrations of RDX in a Sassafras sandy loam soil for up to 14 d. The MNX concentrations in tissue following exposure to soil containing no RDX (△), 10 mg/kg (○), 100 mg/kg (□), 1,000 mg/kg (▲), or 10,000 mg/kg (♦) are shown. Data are expressed as the mean ± standard deviation (n = 3 replicates). If not visible, error bars are smaller than the symbol.

exposure to RDX, after which these concentrations decreased or attained a plateau between 7 and 14 d of the study. The peak of tissue MNX concentrations in the 1-d exposure was most evident in the 10,000 mg/kg treatment. These changes may represent the early phases of MNX equilibration in soils containing earthworms. It is doubtful that these effects are related to RDX degradation in the soil, because control studies indicated that no additional MNX was formed in RDX-amended soil incubated for up to 14 d without earthworms added.

Low levels of MNX and TNX were detected by Best et al. [24] in *E. fetida* exposed to field-collected soils containing a variety of energetic materials, including RDX. It is not clear from the latter study if the reported RDX metabolites were formed in the earthworm or originated in the energetic materials–contaminated soil and were taken up by the organism. In the present study, no TNX was detected in either soil or earthworm tissue using HPLC analyses. It should be noted, however, that after 14 d of exposure to RDX in soil, the tissue MNX concentrations represented less than 0.1% of the RDX concentrations in the earthworm. This amount was consistent with the quantity of MNX as a contaminant in the original RDX product (99.9% purity), and both compounds may have been coabsorbed by the earthworm from the amended soil.

Mass-balance studies using earthworms exposed to [^{14}C]RDX-amended soil

Time-series, mass-balance studies were conducted using the EAM containing *E. andrei* exposed to [^{14}C]RDX-amended SSL soil (100 mg/kg) for up to 14 d. The radioactivity was analyzed from samples of soil and earthworms and the CO_2 traps. Total radioactivity was calculated as the sum of the latter three fractions. Earthworm controls included soils amended with [^{14}C]RDX but with no earthworms added. The recovery of total radioactivity ranged from 90% ± 3% to 100% ± 6% compared to that added at the start of the experiment (Table 1). Most of the radioactivity (88–98%) remained in the soil, whereas from 1.7 to 3.4% was found in earthworms and from 0.1 to 1.8% as evolved $^{14}CO_2$. The recovery of radioactivity was not statistically different ($p > 0.05$) between soil samples with and without earthworms added (data not shown).

Table 1. Mass balance for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) as determined in the microcosm studies using the earthworm *Eisenia andrei* exposed to 100 mg [^{14}C]RDX/kg in a Sassafras sandy loam soil

Exposure duration	Recovery of ^{14}C-activity (%)			
	Soil[a]	Tissue[a]	CO_2 evolved[b]	Total recovery[c]
1 d	88 ± 3	1.7 ± 0.1	0.5	90 ± 3
2 d	98 ± 6	2.5 ± 0.5	0.1 (0; 0.2)	100 ± 6
7 d	90 ± 3	2.5 ± 0.0	0.9	94 ± 3
14 d	92 ± 6	3.4 ± 0.9	1.8 (0.9; 2.7)	97 ± 4

[a] Data are presented as mean percentages ± standard deviation (n = 3–6 replicates) based on the amount of [^{14}C]RDX added at the start of experiment.
[b] Cumulative ^{14}C-activity is the sum of the radioactivity measured in the internal and external CO_2 traps of KOH solution. Triplicate KOH samples were pooled because of the limited amount of radioactivity collected. Values in parenthesis indicate the individual means of two separate experiments.
[c] Total recovery is the sum of individual mass recoveries of [^{14}C] activity from soil, tissue, and evolved CO_2.

Uptake and elimination of [^{14}C]RDX in earthworms

The uptake of [^{14}C]RDX in earthworms was examined using *E. andrei* exposed to 100 mg [^{14}C-RDX]/kg SSL soil for up to 14 d. This RDX exposure concentration provided sufficient amounts of radioactivity for detection in the tissue samples. Figure 4 shows data from a representative study (experiment 3 in Table 2) and summarizes the temporal changes in radioactivity in the acetonitrile-extractable fraction as well as in the nonextractable fraction taken from [^{14}C]RDX-exposed earthworms. The radioactivity in the extracts (representing RDX and some of its metabolites) increased in a curvilinear fashion during the 14-d exposure to [^{14}C]RDX in SSL soil. Radioactivity in the nonextractable fraction was relatively low and increased to 42 × 10^3 dpm/g tissue by the end of the 14-d uptake phase of the experiment. Figure 4 shows the time course of total (sum of extractable and nonextractable fractions) uptake of [^{14}C]RDX in earthworms exposed to [^{14}C]RDX-amended soil. Analysis of the total uptake of [^{14}C]RDX in earthworms obtained from the three experiments revealed that the [^{14}C]RDX uptake rate constant (k_1) was 4.5 ± 2.4 g soil/g tissue/d (Table 2).

Results showed that the uptake of [^{14}C]RDX in earthworms can be described by a single-compartment kinetics model. The elimination of ^{14}C-activity from the earthworm was examined during days 14 to 28 of the experiment (i.e., when ^{14}C-containing earthworms were transferred to the nonamended SSL soil). Figure 4 summarizes the results and shows a rapid decrease of radioactivity in the extractable fraction of earthworms during the elimination phase. The radioactivity in the nonextractable fraction remained relatively stable (i.e., from 42 × 10^3 to 35 × 10^3 dpm/g tissue) during the 14-d elimination period. These results indicate that either the association of [^{14}C]RDX or its metabolites with the cellular

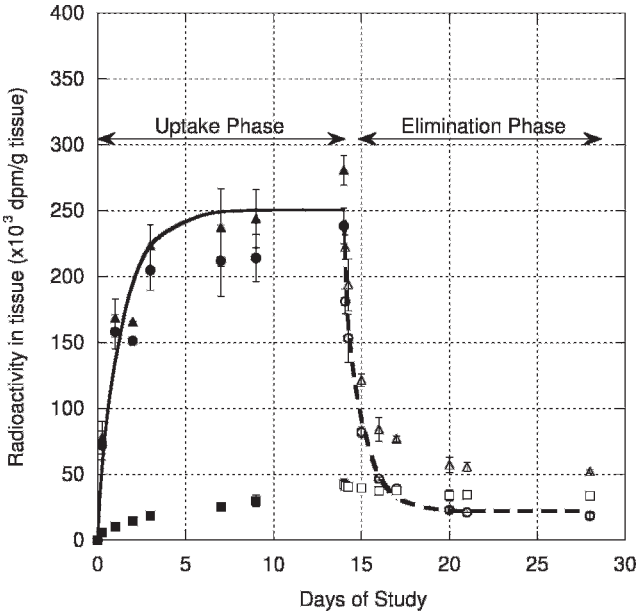

Fig. 4. Uptake and elimination of [^{14}C]hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and its products in earthworm tissues. Shown are radioactivity (dpm) as measured from extracted tissue fractions (●, ○), radioactivity as measured from nonextractable fractions of earthworm tissues (■, □), and total radioactivity (acetonitrile-extractable + nonextractable fractions) from tissue samples (▲, △). Filled and unfilled symbols denote uptake and elimination of radioactivity, respectively. Solid line shows best-fit curve for total uptake of radioactivity (▲—▲); broken line shows best-fit curve for radioactivity in the extractable fraction (○– –○). Data are expressed as the mean ± standard deviation (n = 3 replicates). If not visible, error bars are smaller than the symbol.

constituents of the earthworm was irreversible or that the dissociation of these [^{14}C]RDX metabolites from this ^{14}C-complex was very slow. Further studies will be carried out to identify the [^{14}C]RDX metabolites in the nonextractable fraction. The persistence and toxicological relevance of these RDX metabolites in the earthworm also will require further investigation.

Analysis of the elimination of ^{14}C-activity from the earthworm indicated a rapid efflux (k_2 = 1.2 ± 0.5/d) of radioactivity from an average steady-state concentration (138 × 10^3 dpm/g) at the start of the elimination phase to a residual level (19 × 10^3 dpm/g) that remained in the earthworm until the end of the elimination phase (Table 2). Based on these data, 0.7 ± 0.4 d is required to eliminate half the [^{14}C]RDX absorbed by the earthworms.

The kinetically derived BAF_K of RDX was estimated using the ratio of paired uptake (k_1) to elimination (k_2) rate constants for each experiment. The BAF_K was 3.6 ± 0.5 (n = 3 experiments) and was similar to the BAF (2.7 ± 0.3) obtained when earthworms were exposed to 100 mg/kg nonlabeled RDX in SSL soil (Fig. 2). Recent preliminary studies (K. Savard et al., unpublished data) using earthworms exposed to 10 mg [^{14}C]RDX/kg SSL soil showed that the tissue uptake rate constant (k_1) was 9.1 g soil/g tissue/d and that the tissue elimination rate constant (k_2) was 1.2/d (asymptotic standard error = 0.1). The resulting BAF_K was 7.3 and was consistent with the BAF of 6.0 determined when earthworms were exposed to 10 mg/kg of nonlabeled RDX (Fig. 2). These results indicate that the BAF_K varies according to the soil RDX concentration (i.e., $[RDX_S]$). A similar conclusion was found based on calculation of the steady-state distribution coefficient BAF. Although the bioaccumulation potential or tissue uptake of RDX from soil was determined using two different approaches, they share a common exposure parameter—namely, the measured $[RDX_S]$. The $[RDX_S]$ was used to calculate the BAF (i.e., $[RDX_T]/[RDX_S]$), and the tissue RDX concentration $[RDX_T]_{total}$ parameter (shown in Eqn. 1) that also uses the steady-state RDX concentration in soil (i.e., $[RDX_S]_{SS}$). These soil RDX concentrations were based on acetonitrile extraction of soil samples. It is possible that the $[RDX_S]$ values reported here using the U.S. Environmental Protection Agency Method 8330B [35] may overestimate the actual RDX exposure concentration that is accessible to the earthworm in soil. Therefore, a more accurate exposure parameter, such as the interstitial water fraction of soil, should be considered. Preliminary ongoing studies suggest that this soil fraction becomes saturated with RDX (maximum solubility is 42 mg/L at 20°C) [39]) at soil total RDX concentrations greater than 40 to 50 mg /kg [40]. Whether or not the interstitial water fraction of soil plays a role in affecting RDX accumulation in earthworms, the RDX uptake data described in the present study indicates that earthworms can accumulate RDX from soil. Such accumulation can pose a risk for RDX exposure by higher-trophic-level receptors through the food-chain transfer.

CONCLUSION

The present study has demonstrated that the RDX BAFs, expressed as steady state–based distribution coefficients, ranged from 0.1 to 6.7, depending on the soil RDX concentration. These values were similar to those derived using the kinetic approach. Toxicokinetic studies using [^{14}C]RDX indicated a net accumulation of RDX by the

Table 2. Parameters of uptake and elimination for [^{14}C]hexahydro-1,3,5-trinitro-1,3,5-triazine ([^{14}C]RDX) in the earthworm *Eisenia andrei*[a]

	Body residue[b] (× 10^3 dpm/g tissue)		Rate constants[b]		Coefficient of determination (r^2)			
	$[RDX_T]_{SS}$	$[RDX_T]_R$	k_1 (g soil/g tissue/d)	k_2 (1/d)	Uptake	Elimination	BAF_K	$t_{1/2}$ (d)
Experiment 1	68 (6)	14 (4)	1.9 (0.1)	0.6 (0.1)	0.8	0.9	3.0	1.1
Experiment 2	153 (9)	17 (6)	6.6 (0.3)	1.6 (0.3)	0.8	0.9	4.1	0.4
Experiment 3	192 (7)	26 (4)	4.9 (0.2)	1.3 (0.2)	0.9	1.0	3.6	0.5
Mean ± SD	138 ± 63	19 ± 6	4.5 ± 2.4	1.2 ± 0.5			3.6 ± 0.5	0.7 ± 0.4

[a] $[RDX_T]_{SS}$ is the sum of the radioactivity in the acetonitrile-extractable fraction of the earthworm (dpm/g dry wt tissue) under apparent steady-state conditions, and $[RDX_T]_R$ is the residual radioactivity remaining in the extractable fraction of the earthworm (dpm/g dry wt tissue) at the end of the experiment. Rate constants were determined using the model specified in the text; k_1 is the total ^{14}C-activity uptake rate constant (g dry wt soil/g dry wt tissue/d) and k_2 the elimination rate constant (per day) for extractable ^{14}C-activity. BAF_K is the kinetics-based bioaccumulation factor, defined as k_1/k_2, and $t_{1/2}$ (d) is the eliminated half-life of the extractable ^{14}C-activity in the earthworms. SD is the standard deviation.

[b] Data are expressed as the mean ± standard deviation (n = 3) or with the asymptotic standard error indicated in parentheses.

earthworm *E. andrei*. Unidentified radioactive compounds were detected in the nonextractable fraction of earthworms exposed to [^{14}C]RDX in soil and indicated a tight or irreversible association between RDX metabolites and cellular constituents (proteins or nuclear material) in the organism.

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