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ROLE OF SOIL INTERSTITIAL WATER IN THE ACCUMULATION OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE IN THE EARTHWORM *EISENIA ANDREI*

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Abstract—The uptake of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) from soil by the earthworm *Eisenia andrei* was examined by using the equilibrium partitioning (EqP) theory and a three-compartment model including soil (S), interstitial water (IW), and earthworms (E). The RDX concentrations were measured using U.S. Environmental Protection Agency (U.S. EPA) Method 8330A and high-performance liquid chromatography (HPLC). The S-IW studies were conducted using four natural soils with contrasting physicochemical properties that were hypothesized to affect the bioavailability of RDX. Each soil was amended with nominal RDX concentrations ranging from 1 to 10,000 mg/kg. The HPLC analysis showed that the IW extracted from soil was saturated with RDX at 80 mg/kg or greater soil concentrations. The calculated S-IW coefficient (K_p) values for RDX ranged from 0.4 to 1.8 ml/g soil, depending on the soil type, and were influenced by the organic matter content. In the IW-E studies, earthworms were exposed to nonlethal RDX concentrations in aqueous media. The uptake of RDX by the earthworms correlated well ($r^2 = 0.99$) with the dissolved RDX concentrations. For the E-S studies, earthworms were exposed to RDX-amended soils used in the S-IW studies. The bioconcentration factors (BCF; ratios of E-to-IW RDX concentrations) were relatively constant (~5) up to 80 mg/kg soil RDX concentrations, which encompass the RDX saturation limit in the interstitial water of the tested soils. At this concentration range, the RDX uptake from interstitial water was likely dominated by passive diffusion and could be used as an indicator of bioavailability. Other mechanisms may be involved at greater RDX soil concentrations. Environ. Toxicol. Chem. 2010;29:998–1005. © 2009 SETAC

Keywords—Hexahydro-1,3,5-trinitro-1,3,5-triazine Bioaccumulation Bioavailability Bioconcentration Equilibrium partitioning

INTRODUCTION

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a polynitramine explosive found as a contaminant at sites related to RDX manufacturing, use, and disposal. The RDX concentrations can range from very low levels to 3,500 mg/kg at some military firing and training sites and can reach up to 74,000 mg/kg at open burning/open detonation areas [1–6].

The toxicity of RDX to earthworms has been well documented [6–12]. Although RDX was not lethal to soil invertebrates such as adult enchytraeids up to approximately 20,000 mg/kg [13], adverse sublethal effects at lower concentrations have been reported. For example, exposure to RDX in soil can decrease juvenile production in *Eisenia andrei* [7,8], *Enchytraeus albidus* [14], and *Enchytraeus crypticus* [13], with the lowest-observed effect concentration values ranging from 15 to 3,715 mg/kg, depending on the test species.

Previous experiments demonstrated a limited accumulation potential for RDX in earthworms [1,15–17], as could be expected from its low log K_{OW} of 0.87 [18]. The biota-soil-accumulation factor (BSAF), typically expressed as the ratio of tissue to total soil concentrations [19], is often used to characterize the bioaccumulation potential of a chemical from soil to a soil-dwelling organism, such as the earthworm. Recent studies have shown, however, that the BSAF of RDX in earthworms decreased from 6.7 to 0.10 g soil/g tissue as the RDX concentration in soil increased from 1 to 10,000 mg/kg soil [1,15,16]. A varying BSAF value can increase the uncertainty of estimated food chain transfer potential for RDX during the ecological risk assessment at a contaminated site. Therefore, it is important to examine the approaches used to determine the BSAF value of RDX.

The U.S. Environmental Protection Agency (U.S. EPA) Method 8330A [20] (http://www.epa.gov/waste/hazard/testmethods/sw846/online/8_series.htm) is often used in ecotoxicity studies to estimate the RDX exposure concentration in soil [1,15,16]. This method, which is based on acetonitrile extraction, quantifies the total concentration of RDX that includes the nonsoluble (crystalline plus sorbed) and the water-soluble fractions of RDX. Therefore, U.S. EPA Method 8330A has the potential to overestimate the amount of RDX available to the exposed organism.

The equilibrium partitioning (EqP) theory has been used to assess the uptake of organic compounds by soil organisms [21,22]. This theory stipulates that the bioavailability of an organic compound having a log $K_{OW} < 5$ for uptake by a soil organism is determined by the fraction dissolved in the interstitial water [23]. Moreover, according to the EqP theory, dermal absorption of an organic chemical into the earthworm can be derived from the concentration in the interstitial water using the bioconcentration factor (BCF; Fig. 1) [24].

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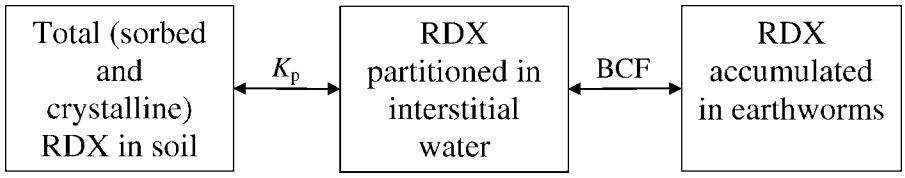

Fig. 1. Equilibrium partitioning (EqP) theory applied to hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). K_p = soil-to-interstitial water partition coefficient; BCF = bioconcentration factor.

To improve our basic understanding of RDX bioaccumulation in soil, uptake of RDX in the earthworm was evaluated by considering independently the uptake of RDX in soil and from interstitial water and the sorption of RDX in soil (Fig. 1). The objectives of the present study were to quantify the RDX uptake in earthworms (*E. andrei*) using either the total RDX concentration in soil (BSAF) or the RDX fraction dissolved in interstitial water (BCF), using earthworm exposures in four natural soils with contrasting physicochemical properties, and to test the hypothesis that soil properties can affect the earthworm uptake of RDX such that the concentration of RDX in interstitial water can be used as an indicator of RDX bioavailability in soil.

MATERIALS AND METHODS

Chemicals and reagents

Hexahydro-1,3,5-trinitro-1,3,5-triazine (Chemical Abstracts Service [CAS] No. 121-82-4; 99.9% purity with <0.1% hexahydro-3,5-dinitro-1-nitroso-1,3,5-triazine; MNX) was supplied by the Defence Research and Development Canada (DRDC), Valcartier. The reference standards included RDX, MNX (CAS No. 5755-27-1), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX; CAS No. 80251-29-2), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX; CAS No. 13980-04-6) from AccuStandard. Reagent-grade calcium chloride was obtained from BDH™, and anhydrous ethyl alcohol was obtained from Commercial Alcohols Inc. High-performance liquid chromatography (HPLC)-grade acetone and acetonitrile were purchased from Caledon Laboratories. The American Society for Testing and Materials (ASTM) type I deionized water [25] was produced using a Super-Q™ water purification system (Millipore) or Zenopure Mega-90. Other reagents were obtained from commercial suppliers. Glassware was washed with phosphate-free detergent followed by rinses with acetone, nitric acid (10%, v/v), and ASTM type I water.

Preparation of RDX-amended aqueous samples

For the aqueous exposure studies, the RDX solutions were prepared using ASTM type I water. A saturated RDX solution (60 mg RDX in 1 L of water) was prepared and stirred overnight in darkness. This mixture was then passed through a 0.22-μm filter (Millipore), and the RDX concentration in the filtrate (stock solution) was confirmed by using HPLC, as described below. The stock solution of RDX was serially diluted to yield nominal concentrations of 1, 3, 5, 10, 20, 25, 30, 35, and 40 mg/L. The ASTM type I water was used as the carrier control.

Preparation of RDX-amended soil samples

Natural soils used in this study included Teller sandy loam (TSL; fine-loamy, mixed, active, thermic Udic Argiustoll soil, obtained from Payne County, Oklahoma, USA), Kirkland loam (KL; fine, mixed, superactive, thermic Udertic Paleustoll soil, also obtained from Payne County, Oklahoma), Webster clay loam (WCL; fine-loamy, mixed, superactive, mesic Typic Endoaquoll soil, obtained from Story County, Iowa, USA), and a sandy soil (DRDC; obtained from a Canadian military training facility in Val-Bélair, Quebec, Canada). Each soil was air dried, sieved on a 2-mm screen, and then stored at room temperature prior to use. Table 1 summarizes the key physicochemical characteristics of the soils used in the present study. The particle size distribution was determined by using the hydrometer method [26], and the organic matter (OM) content was estimated by weight loss following ignition [27]. The pH was measured using a 1:5 (v/v) suspension of soil in water [28].

The TSL, KL, WCL, and DRDC soils were individually amended with RDX using acetone as the carrier to attain nominal RDX concentrations ranging from 1 to 10,000 mg/kg. Individual solutions of RDX in acetone 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% (v/w) of soil weight. Acetone was allowed to volatilize for 2 h between the steps [13,16,29]. All treatment groups including the carrier control (no RDX added) received the same quantity of acetone. All amended soil batches were then kept in a

Table 1. Selected physicochemical characteristics of soils used in the present study

Soil identification[a]	Soil type	Sand (%) 0.08–2 mm	Silt (%) 0.002–0.08 mm	Clay (%) <0.002 mm	OM[b] (%)	WHC[c] (ml/100 g)	pH
TSL	Sandy loam	65	22	13	1.4	16	4.4
KL	Loam	38	42	19	1.5	29	5.7
WCL	Clay loam	33	39	28	5.3	38	5.9
DRDC	Sandy	94	5	1	1.2	23	5.5

[a] TSL = Teller sandy loam soil; KL = Kirkland loam soil; WCL = Webster clay loam soil; DRDC = sandy soil provided by Defence Research and Development Canada, Valcartier.
[b] Organic matter.
[c] Water-holding capacity.

darkened chemical hood for at least 48 h to allow acetone to volatilize [14]. Control treatment groups also included a negative control (no acetone added) for each experiment. Each soil batch was mixed using a three-dimensional rotary soil mixer for 18 h 1 day before the experiment. Nominal concentrations of RDX included 1, 5, 10, 25, 50, 75, 100, 1,000, and 10,000 mg/kg for the soil interstitial water studies; 1, 10, 100, 1,000, and 10,000 mg/kg for studies involving earthworms exposed to RDX in soil; and 5, 10, 25, and 50 mg/kg for earthworms exposed to RDX in interstitial water.

Soil interstitial water collection

Individual samples (200 g dry soil mass) of prepared TSL, KL, WCL, and DRDC soil batches were placed into separate Mason-type 500-ml glass jars, hydrated to 75% of the soil water-holding capacity (WHC), and hand mixed with a spatula. Each jar was covered by a lid perforated with approximately 10 holes (1 mm diameter each), and was kept in an environment-controlled incubator at $20 \pm 1^{\circ}C$ (SD) and 70 to 80% relative humidity, with a 16:8-h light:dark photoperiod cycle with a mean light intensity of 800 ± 400 lux. After 24 h of soil moisture equilibration, the RDX concentration in each treatment group was determined in triplicate using the acetonitrile extraction procedure described below. Triplicate soil samples were collected from each jar to extract the interstitial water according to the coupled filtration–centrifugation method described by Lock and Janssen [30]. Briefly, 10 g of each soil sample was placed into a separate Sera-Separa™ filter (10.8 cm long, 9 ml capacity; Evergreen Scientific). Each filter was inserted into a separate conical polypropylene tube for subsequent centrifugation using a Sorvall Super T21 (Sorvall, Mandel Scientific) set at 1,800 *g* for 45 min at 20°C. The filtrate was collected and passed through a 0.45-μm Millex™-HV cartridge (Millipore) to eliminate the precipitate. A fraction of the filtrate was then mixed with acetonitrile (1:1, v/v) before HPLC analysis.

Earthworm exposures to RDX in aqueous media

Earthworms, *E. andrei,* were obtained from Carolina Biological Supply™ and were cultured at room temperature in earthworm bedding (Magic Products) supplemented weekly with dry food (Magic Worm Food; Magic Products). Adult earthworms with developed clitellum and weighing between 454 and 581 mg (wet wt) were selected for the experiments.

Earthworms were exposed to a solution of ASTM type I water amended with RDX (40 mg/L) to evaluate the RDX uptake in earthworm tissues after different exposure periods. Five depurated earthworms per treatment group were exposed in each replicate ($n = 3$) glass Petri dish containing 5 ml of the RDX solution for 0.13, 0.25, 1, or 2 d. The test medium was not renewed during the exposure. A negative control (water only and earthworms) was included. Petri dishes were placed in darkness in an environment-controlled incubator at $20 \pm 1^{\circ}C$ and 70 to 80% relative humidity. The HPLC analysis of aqueous RDX solutions taken at the beginning and at the end of the exposure confirmed the stability of the RDX concentrations after 1 d. Earthworms were rinsed after each exposure period, blotted, weighed, frozen in a dry ice–ethanol bath, and then kept at $-80^{\circ}C$ until RDX extraction from the tissues and HPLC analysis. In a separate experiment, the procedure described above was applied using earthworms exposed for 1 d to different RDX concentrations ranging from 1 to 40 mg/L.

The uptake of RDX in earthworms was also examined using interstitial water samples prepared from the RDX-amended TSL soil treatments or carrier control batches. Samples of TSL soil from each treatment group were placed into separate plastic containers (1.2 kg soil per container) and then hydrated to 75% of the soil WHC. Each container was covered with a perforated lid and was kept for 24 h in a lighted, environment-controlled incubator (as described above) to attain a steady-state of soil hydration. After this period, the interstitial water was collected using the procedure described above (see *Soil interstitial water collection*). Approximately 15 soil samples from each soil concentration were centrifuged to obtain at least 15 ml interstitial water. Five depurated earthworms were placed in each replicate ($n = 3$) glass Petri dish containing 5 ml of the interstitial water sample representing the specific TSL soil treatment. The test medium was not renewed during the exposure period. After the 1-d exposure in darkness in an environment-controlled incubator (described above), all earthworms were rinsed, blotted, weighed, frozen in a dry ice–ethanol bath, and kept at $-80^{\circ}C$ until RDX extraction from the tissues and HPLC analysis. The RDX concentration in interstitial water was analyzed before and after the 1-d exposure.

Earthworm exposures to RDX-amended soils

Individual samples (60 g dry soil mass) of prepared TSL, KL, WCL, and DRDC soil batches were placed into separate glass jars, using the method described by Sarrazin et al. [16]. Three replicates were used for each treatment. Soils were hydrated to 75% of their WHC for 3 h before the addition of earthworms. Two grams of dry food was added to each test unit, and the soil was hand mixed with a spatula. Six earthworms acclimated in nonamended soils for 1 d before the exposure studies were placed into a separate glass jar containing the amended soil sample, and each jar was covered with a perforated lid. All jars were placed in an illuminated, environment-controlled incubator as described above. Earthworms were removed from the test jars after 0.25, 1, 2, 7, 14, 21, and 28 d of exposure and were depurated on a moistened filter paper for 1 d to ensure the absence of visible soil particles in the intestinal tract. The earthworms were then rinsed, blotted on filter paper, weighed, and frozen in a dry ice–ethanol bath. Also, soil aliquots (20 g) were collected from each test jar at the beginning and at the end of the experiment. Individual earthworms and soil samples were stored at $-80^{\circ}C$ and $-20^{\circ}C$, respectively, before chemical analyses.

Chemical analyses of RDX in soil, tissue, and aqueous media

Concentrations of RDX and its metabolites in soil were determined using the modified U.S. EPA Method 8330A [20] and as described elsewhere [3]. For quantifying RDX in the aqueous media, each RDX solution was mixed with acetonitrile (1:1; v/v) before HPLC analyses. The HPLC detection limits for RDX and its metabolites were 0.25 mg/kg dry soil and 50 μg/L aqueous media.

Concentrations of RDX and its metabolites in the earthworm tissue were determined using a modification of the method

described by Renoux et al. [31]. Briefly, earthworms were lyophilized, ground, rehydrated with distilled water, and sonicated in the dark after addition of acetonitrile. Samples were centrifuged (12,000 *g* for 10 min at 4°C), and 3.5 ml of the supernatant was mixed with 1.5 ml of a 16 g/L calcium chloride solution before filtration and analysis by HPLC. The limit of detection for RDX and its metabolites in the earthworms was 5 mg/kg dry tissue.

Parameter estimations and statistical analyses

The BSAF (expressed as g soil/g tissue), the soil-to-interstitial water partition coefficient (K_p, ml/g soil), and the BCF (ml/g tissue) were calculated using Equations 1 to 3, respectively

$$\text{BSAF} = \frac{[\text{RDX}_T]}{[\text{RDX}_S]} \quad (1)$$

$$K_p = \frac{[\text{RDX}_S]}{[\text{RDX}_{IW}]} \quad (2)$$

$$\text{BCF} = \frac{[\text{RDX}_T]}{[\text{RDX}_W]} \text{ or } \frac{[\text{RDX}_T]}{[\text{RDX}_{IW}]} \quad (3)$$

where $[\text{RDX}_T]$, $[\text{RDX}_S]$, $[\text{RDX}_{IW}]$, and $[\text{RDX}_W]$ are RDX concentrations in the tissue (expressed as μg/g tissue), the soil (mg/kg soil), the soil interstitial water (mg/L), and water (mg/L), respectively. The RDX concentrations in soil, interstitial water, or tissue measured at the end of exposure were used for all calculations.

Soil-property data were log-transformed to normalize distribution. The analysis of variance and Student's *t* test for pairwise means separation was used to detect significant differences among treatments. Pearson's analysis and uncorrected probabilities were used to identify significant correlations among the selected soil parameters (clay content, OM content, and pH) and the K_p values. The WHC was not included in these analyses because of its dependence on OM and clay content. A significance level of $p \leq 0.05$ was accepted for statistical tests. All statistical analyses were performed using measured chemical concentrations and SYSTAT™ 11.0 for Windows (SPSS) and JMP IN™ version 4.0 software (SAS).

RESULTS AND DISCUSSION

Partitioning of RDX in amended soils

Concentrations of RDX were determined in the interstitial water of the four natural soils amended with nominal RDX concentrations ranging from 1 to 10,000 mg/kg. Preliminary studies showed that the RDX concentration in the soil interstitial water increased over time and reached a plateau after 1 d of soil hydration (data not shown). The interstitial water samples from TSL, KL, WCL, or DRDC soils were therefore collected at that time using the coupled filtration–centrifugation method. Concentrations of RDX in the soil interstitial water samples increased with increasing RDX concentrations in the amended soils (Fig. 2), approximately up to the aqueous solubility limit of RDX (42 mg/L at 20°C) [18]. Based on the data shown in Figure 2, the K_p values for RDX in each soil type were calculated as the ratio of the RDX concentration in a given soil sample to the concentration of RDX in the nonsaturated interstitial water sample (Eqn. 2). For this analysis, only data ranging from 0 to the limit of RDX saturation in each soil (20 to 80 mg/kg, depending on the soil type) were used. Statistical analyses revealed that the K_p values were significantly different (Student's *t* test; $p \leq 0.05$) among the four soils tested. The K_p value (ml/g) for RDX was greatest in WCL (1.8), followed by KL (0.9), TSL (0.6), and DRDC (0.4) soils, and indicated that the WCL soil had the lowest bioavailability for RDX compared with the other soils tested.

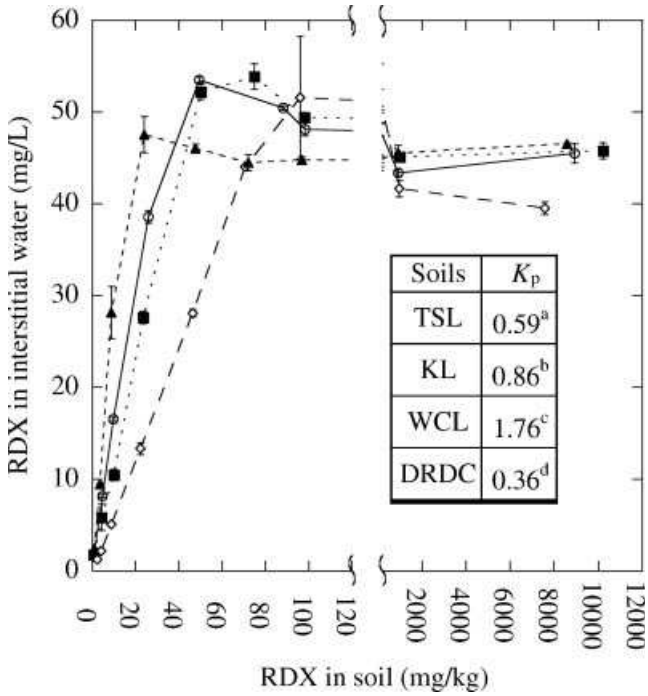

Fig. 2. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) partitioned to soil interstitial water at different soil RDX concentrations (mg/kg) and soil types (circles, Teller sandy loam [TSL]; squares, Kirkland loam [KL]; lozenges, Webster clay loam [WCL]; triangles, sandy soil [DRDC]) after 1 d of soil hydration. The K_p (soil-to-interstitial water partition coefficient, ml/g) is the concentration of RDX in soil divided by the concentration of RDX in interstitial water. Different letters show significant differences between soil types (Student's *t* test, $p \leq 0.05$). Data are expressed as mean ± standard deviation ($n = 3$ replicates). If not visible, error bars are smaller than the symbol.

The OM, clay, and soil pH are known to play important roles in the sorption of organic compounds in soils [32,33]. Correlation analyses revealed that the K_p value was correlated strongly and significantly ($r = 0.978$; $p = 0.022$) with the OM content. The effect of clay content on RDX sorption was strong but not statistically significant ($r = 0.729$, $p = 0.271$; Table 2). This contrasted with earlier reports that suggested

Table 2. Pearson correlations and corresponding uncorrected probabilities for hexahydro-1,3,5-trinitro-1,3,5-triazine-amended soil-to-water partition coefficients (K_p) with clay, organic matter content, or pH of the four natural test soils

Soil properties	Correlation coefficients (*r*)	Uncorrected probability (*p*)
Clay	0.729	0.271
Organic matter	0.978	0.022
pH	0.546	0.454

a predominating role of clays in the sorption of RDX in soil [33–36]. The relationship between soil pH and the K_p value was weak and nonsignificant ($r = 0.546$, $p = 0.454$). Based on a limited data set, these findings indicated that the bioavailability of RDX in the soil interstitial water was influenced by the RDX sorption to the OM in the four soils tested.

Uptake of RDX by earthworms exposed in aqueous media

The BCF values for RDX were determined using RDX-amended water samples and interstitial water extracted from RDX-amended soil. A time course study was done using destructive sampling of earthworms exposed to RDX dissolved in water at concentrations approaching maximal aqueous solubility (40 mg/L) for up to 2 d. Concentrations of RDX in water attained an apparent steady state after a 1-d exposure (Fig. 3A). Therefore, this exposure period was chosen to determine RDX uptake by the earthworms in interstitial water that was extracted from TSL soil amended with different concentrations of RDX. The initial measured RDX concentrations in the interstitial water samples were 9.1 ± 0.01, 18 ± 0.1, 53 ± 0.1, and 50 ± 0.2 mg/L, corresponding to nominal soil RDX concentrations of 5, 10, 25, and 50 mg/kg, respectively. The corresponding RDX concentrations at the end of the study were 4.7 ± 0.23, 8.5 ± 0.22, 26 ± 1.3, and 24 ± 0.3 mg/L, respectively. In a separate study, earthworms were exposed for 1 d to different RDX concentrations in amended deionized water. Concentrations of RDX in tissue, in interstitial water, and in deionized water at the end of the exposure period are shown in Figure 3B. These data showed that uptake of RDX by the earthworms correlated strongly with the dissolved RDX concentrations in the interstitial water or the ASTM type I water ($r = 0.96$, $p = 0.0001$).

The calculated BCF (Eqn. 3) for the earthworms exposed separately in interstitial water from RDX-amended soil and in amended water was 13 ± 1 ml/g dry tissue or 2 ± 0.1 ml/g wet biomass. These results are similar to the BCF values for RDX (ml/g wet tissue) of 2.1 and 2.4 established in studies with channel catfish (*Ictalurus punctatus*) and aquatic oligochaetes (*Lumbriculus variegatus*), respectively [37].

Uptake of RDX by the earthworms exposed in soil

The RDX accumulation in earthworms from each soil type was evaluated on the basis of the BSAF determined under steady-state conditions (Eqn. 1). To determine the duration necessary to achieve steady-state conditions, earthworms were exposed to RDX in soils for up to 28 d. The uptake of RDX by the earthworms approached a steady state (indicated by the leveling off in tissue RDX concentrations) between 2 and 7 d from the start of exposure in WCL soil amended with RDX concentrations of 10, 100, or 1,000 mg/kg (Fig. 4A). Similar results were obtained in studies with the other three soils (data not shown). Therefore, a 7-d exposure period was chosen for quantifying the RDX bioaccumulation in all soils tested. For each soil, the RDX uptake in tissue increased from nondetectable concentrations to 1,514 mg/kg dry tissue, as the nominal RDX concentrations in soil increased from 1 to 10,000 mg/kg dry soil (Fig. 4B). The HPLC analyses showed that neither DNX nor TNX was found in the soil or the earthworm tissue samples; however, MNX was detected to a maximum concentration of 11 mg/kg when the earthworms were exposed to soil RDX concentration of 10,000 mg/kg. Similar results have been obtained by other authors [1,16]. Control studies showed that there was no MNX formed in RDX-amended soil incubated for up to 14 d without earthworms. However, MNX was present as a contaminant in the original RDX product (99.9% purity), so both compounds could be taken up by the earthworms from the amended soils.

The BSAF values for RDX were calculated for each treatment group (Table 3). If the bioavailable and total (acetonitrile-extractable) soil RDX concentrations were directly related, then increases in soil RDX concentrations would be associated with increases in tissue RDX concentrations and thus would result in a constant BSAF (according to Eqn. 1). The results presented here showed that the BSAF values were not constant and decreased from 13 to 0.05 g soil/g tissue as the RDX concentrations in soil increased from 1 to 10,000 mg/kg. These results are consistent with findings of earlier studies [1,16] and indicate that the total soil RDX concentration, as measured using acetonitrile extraction, does not specifically represent the

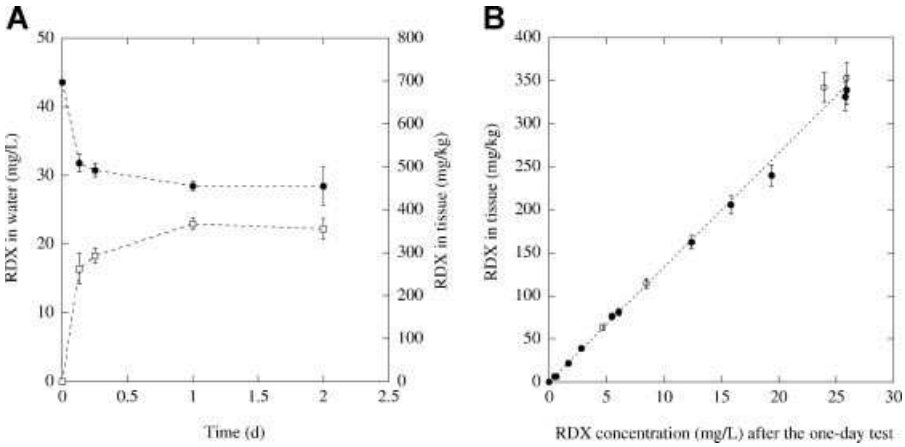

Fig. 3. Uptake of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by earthworms exposed in aqueous media. (**A**) A time-series change in RDX concentrations in water (circles, left *y*-axis mg/L) and the earthworms (squares, right *y*-axis; mg/kg) exposed to single RDX concentration of 40 mg/L. (**B**) Linear relationship showing RDX uptake = 13.3 [RDX concentration in aqueous media] − 0.1 ($r^2 = 0.99$) by earthworms after a 1-d exposure in American Society for Testing and Materials type I water (solid circles) or in interstitial water extracted from RDX-amended soil (open circles). Data are expressed as mean ± standard deviation ($n = 3$ replicates). If not visible, error bars are smaller than the symbol.

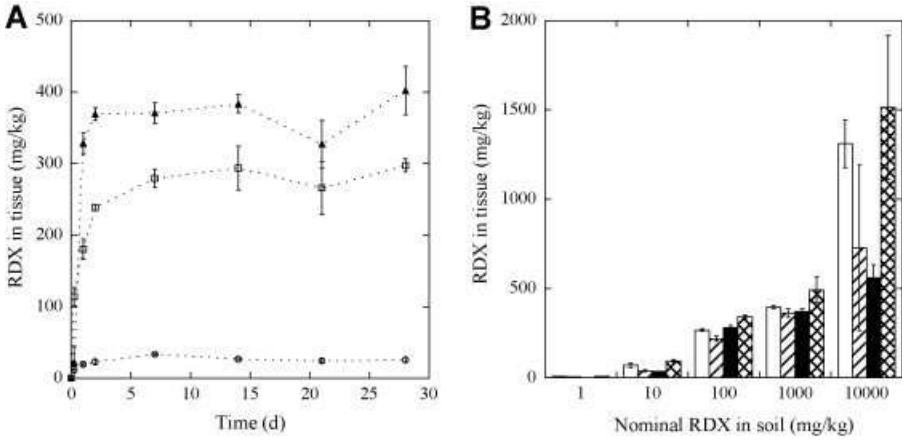

Fig. 4. Uptake of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by earthworms exposed in soil. (**A**) A time-series RDX uptake by earthworms (expressed as mg/kg) exposed to RDX concentrations 10 (circles), 100 (squares), and 1,000 (triangles) mg/kg in Webster clay loam soil. (**B**) RDX uptake (expressed as mg/kg) by earthworms exposed in natural soils (open columns, Teller sandy loam; diagonally hatched columns, Kirkland loam; solid columns, Webster clay loam; cross-hatched columns, sandy soil for 7 d. Data are expressed as mean ± standard deviation ($n = 3$ replicates).

bioavailable form of RDX. It is conceivable that the total soil RDX concentration represents two fractions of RDX in soil, a bioavailable fraction and another that is nonaccessible (e.g., undissolved or adsorbed RDX). In such a case, the increase in total RDX concentrations in soil relative to the constant concentrations of RDX in the tissue would lead to decreases in the BSAF values, as calculated with Equation 1.

Variations in the BSAF values were soil specific and decreased in the order DRDC > TSL > KL > WCL at RDX concentrations of 10 mg/kg (Table 3). The smallest BSAF was determined for WCL soil, which had the greatest OM content and the lowest bioavailability of RDX among soils tested in these studies. A similar inverse relationship between BSAF and OM was found in other studies [38–42]. At 100 mg/kg or greater RDX concentrations in soil, the trend was less clear (Table 3).

Concentrations of RDX dissolved in interstitial water of amended TSL, KL, WCL, and DRDC soils (shown in Fig. 2) were used to determine the RDX uptake in the earthworms exposed to RDX in soil. Interstitial water RDX concentrations correlated strongly and significantly with tissue RDX concentrations in nominal soil treatments of 1 and 10 mg/kg ($n = 15$, $r = 0.96$, $p = 0.0001$). In contrast, there were no significant correlations between RDX concentrations in interstitial water and in earthworms for nominal soil treatments of 100, 1000, and 10,000 mg/kg. These results indicate that the RDX partitioning in the soil interstitial water plays a determining role in RDX uptake by the earthworms from soil, up to the limit of RDX saturation in the interstitial water (i.e., below 100 mg/kg).

The BCFs were determined for each soil type and for each soil exposure concentration (Table 4). For nominal soil RDX concentrations of 1 and 10 mg/kg, the statistical analyses showed no significant differences in BCFs among soils tested ($n = 21$, analysis of variance and Student's t test, $p > 0.05$). The BCF values for all tested soils increased with increasing nominal RDX concentrations of 100, 1,000, and 10,000 mg/kg. Differences among many of those treatments were statistically significant (Table 4). These data and those shown in Figure 2 indicate that passive diffusion across the earthworm integument may be the predominant mechanism of RDX uptake from interstitial water at 80 mg/kg or lower soil RDX concentrations. The possible contribution of other xenobiotic uptake mechanisms (e.g., absorption and diffusion in the gut following soil ingestion) [23,24,43] could contribute at higher soil RDX concentrations. Further studies would be required to elucidate these mechanisms.

Table 3. Biota-soil-accumulation factor (BSAF) values for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) determined in studies with the earthworm *Eisenia andrei* exposed in four natural soils

	Nominal RDX concentrations in soil (mg/kg)				
Soil types[a]	1	10	100	1,000	10,000
TSL	9.0 ± 1.0A,W	8.0 ± 1.0A,W	2.8 ± 0.1A,X	0.43 ± 0.03A,Y	0.15 ± 0.02A,Y
KL	4.7 ± 0.3B,W	5.0 ± 0.7B,W	2.4 ± 0.2B,X	0.39 ± 0.03A,Y	0.08 ± 0.05AB,Y
WCL	ND[b]	3.2 ± 0.1B,W	3.1 ± 0.1A,X	0.39 ± 0.03A,Y	0.05 ± 0.01B,Z
DRDC	13 ± 1C,W	15 ± 2C, X	3.9 ± 0.3C,Y	0.58 ± 0.09B,Z	0.19 ± 0.07A,Z

BSAF (g soil/g tissue) is the RDX concentration in tissue (μg/g tissue) divided by the RDX concentration measured in soil (mg/kg soil). Data are expressed as the mean ± standard deviation ($n = 3$). Different capital letters (A, B, C) indicate significant differences between soil types having a same soil RDX concentration (analysis of variance [ANOVA] followed by a Student's t test, $p \leq 0.05$). Different capital letters (W, X, Y, Z) indicate significant differences between different soil RDX concentrations within the same soil type (ANOVA followed by a Student's t test, $p \leq 0.05$).

[a] TSL = Teller sandy loam soil; KL = Kirkland loam soil; WCL = Webster clay loam soil; DRDC = sandy soil provided by Defence Research and Development Canada, Valcartier.

[b] RDX was not detected (ND) in earthworm tissue. Limit of detection in tissue = 5 μg/g dry tissue.

Table 4. Bioconcentration factor (BCF) values for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) determined in studies with the earthworm *Eisenia andrei* exposed in four natural soils

	Nominal RDX concentrations in soil (mg/kg)				
Soil types[a]	1	10	100	1,000	10,000
TSL	5.5 ± 0.1A,X	5.0 ± 0.9A,X	5.5 ± 0.1A,X	9.1 ± 0.2A,Y	29 ± 3AB,Z
KL	4.0 ± 0.1A,X	4.3 ± 0.6A,X	4.4 ± 0.3B,X	8.0 ± 0.5A,XY	16 ± 10BC,Y
WCL	ND[b]	5.6 ± 0.1A,X	5.4 ± 0.3A,X	8.9 ± 0.3A,Y	14 ± 2C,Z
DRDC	4.6 ± 0.2A,X	5.5 ± 0.5A,X	7.6 ± 0.2C,X	11 ± 2B,X	33 ± 9A,Y

BCF (ml/g tissue) is the RDX concentration in tissue (μg/g tissue) divided by the RDX concentration in interstitial water (mg/L). Data are expressed as the mean ± standard deviation ($n = 3$). Different capital letters (A, B, C) indicate significant differences between soil types having a same soil RDX concentration (ANOVA followed by a Student's t test, $p \leq 0.05$). Different capital letters (X, Y, Z) indicate significant differences between different soil RDX concentrations within the same soil type (ANOVA followed by a Student's t test, $p \leq 0.05$).

[a] TSL = Teller sandy loam soil; KL = Kirkland loam soil; WCL = Webster clay loam soil; DRDC = sandy soil provided by Defence Research and Development Canada, Valcartier.

[b] RDX was not detected (ND) in earthworm tissue. Limit of detection in tissue = 5 μg/g dry tissue.

CONCLUSIONS

The uptake of RDX in earthworms was evaluated by considering independently the RDX partitioning in three compartments, including soil—interstitial water, soil—earthworm, and interstitial water—earthworm. The RDX partitioning coefficients (K_p) determined in four natural soils with contrasting physicochemical properties and various RDX concentrations correlated strongly and significantly with the soil OM content. The results showed that the RDX concentration in the interstitial water played a determining role in RDX uptake by the earthworms exposed to 80 mg/kg or lower soil RDX concentrations, which is consistent with EqP theory. At this concentration range, the RDX uptake from interstitial water was likely dominated by passive diffusion and could be used as an indicator of bioavailability. Other mechanisms may be involved at greater RDX soil concentrations.

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