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Accumulation of ^{14}C-trinitrotoluene and related nonextractable (bound) residues in *Eisenia fetida*

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The majority of trinitrotoluene accumulation in earthworms occurs as nonextractable residues that have a significantly longer half life in the worm as compared to TNT and its solvent-extractable deaminated metabolites.

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ABSTRACT

To determine if trinitrotoluene (TNT) forms nonextractable residues in earthworms and to measure the relative degree of accumulation as compared to TNT and its deaminated metabolites, *Eisenia fetida* was exposed to ^{14}C-TNT using dermal contact to filter paper or exposure to soil. Nonextractable residues made up 32–68% of total body burden depending on exposure media and depuration time. Parent TNT accounted for less than 3% of radioactivity, while ADNTs accounted for 7–38%. Elimination half-lives were 61–120 h for TNT, ADNTs, and DANTs, which was significantly lower than the half-lives found for nonextractable residues, 201–240 h. However, over 80% of the nonextractable residue was solubilized using weak acid (pH 2). Based on our findings that TNT accumulation occurs primarily as nonextractable residues, which have a longer half-life, and that nonextractable residues can be solubilized, we propose that nonextractable residues could be used as a selective biomarker for assessing TNT contamination.

1. Introduction

The nitroaromatic compound 2,4,6-trinitrotoluene (TNT) is manufactured exclusively as an explosive and has been used extensively by the military since the early 1900s (Hathaway, 1977). Synthesis, manufacture, use, and disposal of TNT and associated by-products have resulted in contamination of soil in the environment, sometimes at a large-scale (Talmage et al., 1999; Jenkins et al., 2006). Despite reduction of TNT production and usage over the past two decades, the presence of unexploded ordinance in the environment has continued to allow the release of TNT as the ordinance corrodes and breaches (Lewis et al., 2009). Exposure to TNT can cause acute and chronic effects to soil organisms as summarized in Kuperman et al. (2009). For example, TNT and its major transformation products promoted decreased survival of earthworms as well as decreased reproduction and growth (Phillips et al., 1993; Robidoux et al., 2000, 2002; Lachance et al., 2004; Hund-Rinke and Simon, 2005; Gong et al., 2008).

Explosives such as TNT and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) accumulate to a relatively low extent in aquatic invertebrates, fish, and terrestrial invertebrates due to a low degree of lipophilicity ($\log K_{ow} < 3$) and in the case of TNT, rapid biotransformation. Thus, reduced TNT biotransformation products including 2,6-diamino 4-nitrotoluene (2,6DANT), 2,4-diamino 6-nitrotoluene (2,4DANT), 4-amino 2,6-dinitrotoluene (4ADNT), and 2-amino, 4,6-dinitrotoluene (2ADNT) are typically found at higher concentrations within tissue than parent TNT with ADNTs occurring most frequently (Renoux et al., 2000; Conder et al., 2004; Dodard et al., 2004; Lachance et al., 2004; Belden et al., 2005a,b; Ownby et al., 2005; Lotufo et al., 2009). Several recent studies in aquatic organisms have reported that bound or unextracted residues bioaccumulate to a larger extent than TNT, ADNTs, or DANTs. In these studies, bioaccumulation of the explosives was measured using radiolabeled version (^{14}C) of TNT and RDX. The formation of bound residues was evidenced by the inability of solvents such as methanol and acetonitrile to remove the radiolabeled metabolites. Those solvents efficiently extract standard analytes such as TNT, ADNTs, and DANTs and are commonly used in US Environmental Protection Agency methods (8330, USEPA, 2008) and most published studies (Renoux et al., 2000; Conder et al., 2004; Belden et al., 2005a; Ownby et al., 2005; Sarrazin et al., 2009). To best

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imply the unknown nature of this material and match the current terminology used for bound residues in soil, the term non-extractable residue (Barriuso et al., 2008) is used herein. It should be noted that the term does not imply that extraction is impossible, only that solvents that readily extract the parent compound do not extract this type of metabolite. Within this study, nonextractable is defined as residue that was not extracted by acetonitrile.

Although previous studies demonstrated the presence of non-extractable residues and suggested that nonextractable residues have longer half-lives (Conder et al., 2004; Belden et al., 2005a), they did not fully investigate the chemical nature and the toxicokinetics of the nonextractable residues. If nonextractable residues accumulate to a greater extent and have longer half-lives as compared to TNT, ADNTs, and DANTs, insight into the mechanism of TNT toxicity may be gained and potentially the nonextractable residues could be useful as a biomarker of exposure for TNT. Furthermore, no studies have evaluated if nonextractable TNT-related residues form within earthworms, which are ecologically relevant and widespread in terrestrial systems.

Thus, the objectives of this study were to first determine if TNT-related nonextractable residues are formed in *Eisenia fetida* and the relative degree of accumulation as compared to TNT and standard measured metabolites (ADNTs and DANTs); second, to determine if the half-lives of nonextractable residues within the worms are different as compared to TNT and standard measured metabolites (ADNTs and DANTs), and third, to determine if nonextractable residues can be solubilized using aqueous extraction techniques. Our approach was to start with a simplified exposure regime using dermal contact to filter paper for exposure to avoid transformation of TNT in soil prior to uptake. Secondary experiments were then conducted in soil to verify formation of nonextractable residues under a realistic exposure scenario.

2. Materials and methods

2.1. Chemicals

Trinitrotoluene (ring-^{14}C; specific activity 40 mci/mmol) was purchased from Perkin Elmer Life Sciences, Boston, MA. Purity was determined to be >96% based on HPLC as described later. The remaining 4% was unidentified. For use as analytical standards, unlabelled TNT was obtained from Chem Service (WestChester, PA, USA) and TNT metabolite standards, including: 2,6-diamino 4-nitrotoluene (2,6DANT), 2,4-diamino 6-nitrotoluene (2,4DANT), 4-amino 2,6-dinitrotoluene (4ADNT), and 2-amino, 4,6-dinitrotoluene (2ADNT), were purchased from Ultra Scientific (>97% purity, N. Kingstown, RI, USA). All solvents were high-performance liquid chromatography (HPLC) grade or purer. Liquid scintillation counting (LSC) was performed using Scintisafe Plus 50% (Fisher Scientific, Pittsburg, PA, USA).

2.2. Test organisms

E. fetida were maintained in continuous on-site culture at the US Army Engineer Research and Development Center (Vicksburg, Mississippi) from stocks obtained from Carolina Biological Supply Company (Burlington, NC, USA). Worms were kept at 22–25 °C in moistened sphagnum peat with calcium carbonate added to adjust the pH to 7.0 ± 0.5. Moisture content was adjusted to 50% and they were fed *ad libitum* on a diet of Magic Worm Food (Carolina Biological Supply). Fully clitellate adults weighing 0.3–0.6 g live weight were selected for all experiments.

2.3. Design of toxicokinetic studies

Two sets of toxicokinetic studies were conducted. Both studies used a single uptake point followed by a complete depuration study since the objectives of the study were focused towards measuring the presence and biological half-life of nonextractable residues. The primary study utilized exposure of the earthworms to ^{14}C-TNT from moist filter paper to ensure that biotransformation of the TNT was not occurring prior to uptake and reducing the amount of radioactive material required for experiments. In the second smaller study, earthworms were exposed to ^{14}C-TNT using spiked soil to determine if a comparable pattern of uptake and elimination would occur under more realistic conditions.

2.4. Filter paper exposure studies

Filter paper exposures were performed in 500 mL glass jars lined with an 8-cm diameter qualitative-grade filter paper. ^{14}C-TNT (200,000 dpm) was applied to the bottom using methanol as a carrier to saturate the filter paper and distribute the TNT. The methanol was allowed to evaporate completely, more than 2 h, and then the filter paper was moistened to remain damp but with limited standing water (1.2 mL). An individual worm was added to each experimental unit. Initially, 25 mg of cerophyll was added and aluminum foil with small air holes was used to seal the container and help control moisture. Daily, 25 mg of cerophyll was added and moisture balance retained. Although some uptake from food may have occurred, discerning sources of uptake was irrelevant for this study as this phase of the experiment was aimed at loading the organism and not calculating uptake rates. No avoidance behavior or toxicity was observed. At 72 h of exposure, worms were removed, weighed, and either directly frozen (5 worms) or placed into a depuration chamber (32 worms). Depuration chambers consisted of a jar as before, but with 200 g soil added. Collection, processing and characterization of the soil are described in Inouye et al. (2006). The soil is silty-loam of the Grenada–Loring soil series (3% sand, 72% silt, and 26% clay, pH 6.7, 0.7%; total organic carbon). Animals were fed 20 mg cerophyll and moisture was maintained daily. At 0.5, 1, 4, 12, 24, 96, 192, 384 h, four worms were removed, rinsed quickly with water, blotted dry, weighed, and frozen for later analysis.

2.5. Soil exposure studies

Soil, as described in the previous section, was spiked with ^{14}C-labeled TNT and cold TNT to obtain a TNT concentration of 20 mg/kg and a radioactivity concentration of 100×10^6 dpm/kg. This TNT concentration was not lethal to *E. fetida* in preliminary experiments or to the related species *E. andrei* in a previous investigation (Lachance et al., 2004). To spike the soil, a 5-mL acetone spiking solution containing the appropriate amounts of non-radiolabeled and radiolabeled TNT was poured evenly across the surface of 200 g of air-dried soil. Soil and spiking solution were mixed thoroughly, and the acetone was allowed to volatilize for 2 h. Spiked soil was thoroughly mixed with 800 g of non-spiked air-dried soil while water was added stepwise to a total of 300 mL to restore the soil to its original moisture content (78% solids content) and create a homogenous exposure media. To verify spiking levels, samples were collected for radioactivity and chemical analysis following spiking and mixing. Greater than 90% of the radioactivity in the soil was extractable and 98% of the extractable residue was TNT following initial spiking. Hydrated soils were allowed to age at room temperature (20–22 °C) in the dark for 7 d before use. Following aging, soil was placed in a 1-L beaker with 12 worms weighing 0.4–0.5 g that had been allowed to depurate their gut content overnight. Glass containers were used to minimize adsorption of TNT to container walls. Within 2–3 h all worms buried in the soil. Beakers were covered with Nitex screen and placed under 24-h fluorescent light to minimize burrowing avoidance. Worms were exposed to TNT-spiked sediment for a total of 14 days, during which proper moisture was maintained and no burrowing avoidance behavior or toxicity was observed.

Following uptake, worms were removed from beakers and counted. Three worms were rinsed with deionized water, weighed, and each added to a scintillation vial that was frozen and stored at −80 °C until chemical analysis. Worms were not allowed to depurate their gut content to prevent loss of TNT and its transformation products taken up during exposure.

To quantify the TNT elimination kinetics, the remaining 9 earthworms were rinsed with deionized water and transferred into mason jars that contained 250 g of unspiked soil. Three worms were added to each jar. After 24, 48, and 168 h, three worms, from different jars, were removed and individually prepared for analysis and stored as described for the filter-paper exposure.

2.6. Chemical analysis

Individual frozen worms were thawed and homogenized in 15 mL acetonitrile using a T10 Ultra-Turrax (Ika Works Inc., Wilmington, NC, USA) homogenizer, followed by two 10 mL ACN rinses for a total volume of 40 mL. Samples were centrifuged for 10 min in a bench top centrifuge (4100 rpm, Eppendorf Centrifuge Model 5702, Hauppauge, NY, USA), and the supernatant forced through a 0.45-μm syringe filter. The extract volume was reduced to 5 mL using a TurboVap (Zymark Corporation, Hopkinton, MA, USA) and to a final volume of 1 mL under with a slow stream of nitrogen. A 0.1-mL aliquot of extract was placed in 10 mL of ScintiSafe 50% scintillation cocktail (Fisher Scientific, Fair Lawn, NJ, USA) for radioactivity measurements of the total extract. Extracts were then amended with 0.9 mL HPLC grade water and frozen to precipitate lipids that would otherwise precipitate on the HPLC column. Less than 3% of the extractable radioactivity in the extract was lost in this step. A 0.1-mL aliquot of the extract was used for HPLC fractionation. Total radioactivity measurements were done on a LSC (LS 6000SC, Beckman Coulter, Brea, CA, USA). Radioactivity directly measured in the extract was defined as “extractable”. The pellet remaining after extraction was dried under nitrogen and weighed. A 15-mg subsample was then added to 1 mL of Scintigest (Fisher Scientific), allowed to digest overnight, and combined with 10 mL of scintillation cocktail. Syringe filters for each sample from the initial extraction were also combined with 1 mL Scintigest