ABSTRACT

Accumulation of Trifluralin and Trinitrotoluene (TNT) in Two Aquatic Invertebrates: Formation and Persistence of Unextractable Biotransformation Products

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Exposure to nitroaromatic compounds has been reported to result in unextractable residues, or residues not readily extracted by traditional techniques. However, limited information is available about the formation rate and biological half-life of unextractable residues in aquatic organisms. In this study, two aquatic invertebrates, *Lumbriculus variegatus* and *Hyalella azteca*, were exposed in aqueous media to ^{14}C-labeled trinitrotoluene and trifluralin. Unextractable residues were formed in both organisms by both compounds. TNT formed a greater percentage of unextractable residues than trifluralin. *L. variegatus* accumulated more TNT-related unextractable residues compared to *H. azteca,* while the reverse was true for trifluralin. *H. azteca* eliminated unextractable residues more efficiently than *L. variegatus*. Additionally, the biological half-life of unextractable residues was longer than that of the extractable residues. The presence and biological persistence of unextractable residues indicates that these compounds, and especially TNT, could cause subtle and chronic biological effects and serve as indicators of exposure.

Accumulation of Trifluralin and Trinitrotoluene (TNT) in Two Aquatic Invertebrates: Formation and Persistence of Unextractable Biotransformation Products

by

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A Thesis

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CHAPTER ONE

Introduction

Nitroarenes are ubiquitous environmental contaminants originating from military application (e.g. trinitrotoluene (TNT)), agricultural use (e.g. dinitroanaline herbicides), and additives found in personal care products (e.g. nitro musks and nitro ketones). The two compounds of interest in this thesis are TNT and trifluralin. Both contaminants are commonly found in aquatic systems and have been documented to accumulate in biota.

Trinitrotoluene is an explosive used in bombs, grenades shells, demolition explosives, and propellant compositions (Talmage et al. 1999). It is also used to a lesser extent as a chemical intermediate in the production of dyestuffs and photographic chemicals (Talmage et al. 1999). By the time of the First World War, the use of TNT was common. This was because of its low manufacturing cost and the fact that it was relatively safe compared to other explosives of the time (Akhavan 2004, Liu et al. 1995). Contamination of TNT can occur during several stages of munitions production, storage, transport, usage, and demilitarization. Currently, the most common way contamination occurs is through seepage from unexploded ordinances (UXO). TNT is readily transformed in the environment by both biotic and abiotic factors. Its four most common metabolites are 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2, 4-diamino,6-nitrotoluene (2,4-DANT), and 2,6-diamino, 4-nitrotoluene (2,6-DANT).

Trifluralin is a dinitroanaline herbicide, extensively used in the United States to control broad-leaf weeds and grasses. It is traded under the name, Treflan, and is used

heavily on soybean and cotton. Contamination occurs primarily due to agricultural runoff as it is incorporated into or applied directly onto sediment. Trifluralin is described by the US EPA (1999) as bioaccumulative, persistent, and toxic; and therefore presents itself as a contaminant of concern.

Accumulation of Nitroarenes into Organisms

TNT and all of its major metabolites have been found in a number of organisms, including aquatic oligochates, chironomids, earthworms, and vertebrates (Conder et al. 2004, Belden et al. 2002, Renoux et al. 2000, and Ownby et al. 2005). There is much less data available concerning the bioaccumulation of trifluralin than for TNT, although a study by Guerrero et al. (2002) indicated that unextractable residues occur in *Sphaerium corneum*, *Chironomous riparus*, and *Lumbriculus variegatus*. Also, trifluralin residues are often present in a fruits and vegetables, including carrots and melons that have been grown in treated soil (Tiryaki et al. 1997, Golab et al. 1967).

In some accumulation studies, the contaminant is tracked by using a radiolabeled analog of the chemical.. For example, TNT could be synthesized using a process that incorporates ^{14}C into the aromatic ring. By tracing the radioactivity, two pools of residue are realized. The first residue pool is readily extracted by the selected solvent. The second is not. It remains in the tissue homogenate after the extraction step. This pool of unextractable residue, rendered by exposure to nitroaromatic compounds, has been noted in a variety of organisms.

Nature of Unextractable Residues

Unextractable residues have been noted in numerous studies evaluating a variety of contaminants and matrices. Measurement of unextractable residues within an experiment could be explained several ways. First, it is possible that the unextractable portion of the radiolabeled compound is an artifact of poor extraction efficiency. However, in most cases, extraction efficiency has been determined to be acceptable (>95%) and other extraction solvents have yielded only negligible increases in efficiency (Belden et al. 2005). Most studies have used solvent systems that readily extract lipids indicating that the unextractable residues are associated with the remaining macromolecules, especially protein.

A second explanation of unextractable residues is that the residue is physically trapped within the matrix. This has been proposed for soil, but is less likely to occur in animal tissue due its macromolecular structure. It has been confirmed that certain biotransformation products of TNT are able to covalently bind and become physically sequestered to soil (Achnitch 1999). One study reports up to 95.5% of total radioactive compound was found bound in soils under aerobic conditions, even after confirming that extraction efficiency was adequate (Achnitch et al. 2002). Thompson et al. (1998) reported that 85% of TNT was absorbed from soils into hybrid poplar trees, the majority of which was unable to be extracted. Similarly, Hughes et al. (1997) noted a large percentage of unknown TNT transformation products that were associated with a mixture of exposed aquatic plants.

A third suggested cause of unextractable residues was discovered by Leung et al. (1995). He reported covalent binding of radiolabeled TNT to microsomal proteins in rat

liver cells. Also, the formation of these adducts increased with time. This lead Leung and colleagues to believe that the adducts were being formed by a biotransformation product resulting from TNT reduction and not TNT itself. Athough direct experimental evidence is not available; the third mechanism provides the most likely explanation for animal tissue.

Unextractable Residues of Nitroarenes in Animal Tissue

The accumulation of unextractable, nitroaromatic residues in aquatic organisms is of particular interest to this study. The most widely studied example present in the literature is the accumulation of unextractable nitro musk residues in vertebrate tissue, called adducts in that body of literature. The accumulation of nitro musks and their amine metabolites has been documented to form adducts with hemoglobin in marine and fresh water biota, and human adipose tissue, hemoglobin, and breast milk (Gatermann et. al., 1995; Rimkus, 1999; Rimkus and Wolf, 1995). These identified adducts have served as useful biomarkers of exposure (Mottaleb 2005, Riedel 1999, Liu et al. 1995). Except in the case of the nitro musks, limited research has been pursued concerning unextractable residues. The main focus has generally been on the accumulation and persistence of parent compound and major metabolites in the exposed organism.

However, a few studies have documented that unextractable residues of TNT and trifluralin occur and may have different toxicokinetic properties. For example, a study by Conder et al. (2004) described the accumulation of ^{14}C-TNT and its major metabolites in an aquatic oligochaete, *Tubifex tubifex* exposed in water. Of extractable ^{14}C-labeled TNT equivalents, only 15-47% was identified as parent TNT, 4-ADNT, or 2-ADNT. Of the

total ^{14}C-TNT equivalents, 14-23% was unextractable; moreover, unidentified ^{14}C equivalents were not eliminated during the 54 hour depuration period. However, Conder et al., did not investigate the unextractable equivalents in more detail.

Guerrero et al. (2002) described similar results using trifluralin. Trifluralin accumulated in all model species (*Sphaerium corneum*, *Chironomous riparus*, and *Lumbriculus variegatus*). In *S. corneum* and *L. variegatus*, the majority of the extractable residue was identified as parent trifluralin. However, very little of ^{14}C-equivalents were recovered as parent in *C. riparus*. As much as 12.5% and 56.1% of the total radioactivity was described as unextractable, in *L. variegatus* and *C. riparus*, respectively. The purpose of this research was to determine whether exposure chemicals remained as parent compound after treatment and not to describe the presence of unextractable residues. No depuration studies were performed and unextractable residues were described as possible metabolites that may require a different extraction system.

Ownby et al. (2005) exposed channel catfish (*Ictalurus punctatus*) to ^{14}C-labeled TNT in water. Uptake rates, bioconcentration factors, and body distributions of TNT were described. Uptake rates were relatively fast for parent TNT. TNT and metabolites reached a steady state within hours, while unextractable residues increased throughout the study without reaching a steady state. Bioconcentration factors were low for parent compound due to its rapid biotransformation once absorbed. In a sister study by Belden et al. (2005), unextractable residues are described in more detail using channel catfish and two invertebrates, *Chironomus tentans* and *Lumbriculus variegatus*. Organisms were exposed in moderately hard water fortified with ^{14}C-labeled TNT. Within hours of exposure, the largest radioactive portion in *C. tentans* was the unextractable fraction

(89.1%), which was still increasing up to the 24-hour uptake time point. Of the extractable radioactivity, ~35% were recovered as unidentified biotransformation products, indicating extensive biotransformation of parent compound. In *L. variegatus*, the unextractable portion dominated the recoverable radioactivity (82.0%) and only ~36% of the extractable portion was accounted for by identifiable TNT or known metabolites. The majority of the extractable portion was composed of unidentified polar biotransformation products.

In the same study, channel catfish were exposed manually with pellets dosed with ^{14}C-labeled TNT. After 10 days of exposure, almost 50% of ^{14}C-TNT equivalents were unextractable biotransformation products. Approximately 28% of the total extractable radioactivity was that of unidentifiable, polar metabolites, while 51% was attributed to TNT and major biotransformation products. When catfish were dosed using exposed *L. variegatus* and *C. tentans.* However, accumulation was minimal; suggesting trophic transfer is not a huge consideration in regard to body burden. It has been suggested by Belden et al. (2005) that the unextractable portions were likely covalently bound products associated with proteins (Leung et al. 1995). Although this study described full uptake kinetics of TNT in all three selected organisms, the persistence of the unextractable residues during depuration was not considered.

While the above studies have suggested the presence or formation of unextractable residues, this notion has been more of an artifact or side note to the principal goals of the studies. Except in the case of the nitro musks, these residues are generally attributed to poor extraction efficiency. Also, the majority of the work with nitro musks has been done using vertebrates which tend to form a smaller percentage of

unextractable residues than invertebrates (Belden et al. 2005). Therefore, organisms that have a tendency to be more affected by exposure to nitroarenes have not been adequately investigated. Furthermore, no full toxicokinetics studies have been attempted in order to describe the presence or persistence of unextractable residues.

Study Objectives

The present study aimed to describe the toxicokinetics of unextractable residues in *Lumbriculus variegatus* and *Hyalella azteca* using trinitrotoluene (TNT) and trifluralin as model nitroarenes. The organisms were selected based on the previously referenced research by Belden et al. (2005) that showed that the model invertebrates bioaccumulated significantly more unextractable residues than vertebrates. The nitroarenes were selected based on environmental importance as previously detailed. The first objective of the study was to determine if TNT and trifluralin exposure yields unextractable, or bound, residues in *Lumbriculus variegatus* and *Hyalella azteca*. Although previous work has indicated unextractable residues may occur for both compounds in *L. variegatus*, no work has been conducted in regard to *H. azteca*. The second objective was to determine the biological half lives for both extractable and unextractable residues in the selected species. This work expands on previous work, as previous studies suggest unextractable residues may persist even after long depuration periods; however full toxicokinetic studies that allow calculation of depuration rates and biological half-lives of the bound residue have not been performed.

CHAPTER TWO

Methods

Materials and Purity of Compounds

The radiolabeled trifluralin (ring-^{14}C) was purchased from Sigma-Aldrich (specific activity16.8 mCi/mmol, St. Louis, MO, USA). Purity of ^{14}C-trifluralin was determined using silica gel 60 f_{254} thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany), using acetone:hexane (18:82, v/v) mobile phase. After development, the plates were cut into measured sections and placed into scintillation vials with 8 ml of scintillation cocktail and analyzed for ^{14}C on a Beckman LS6500 scintillation counter (Fullerton, CA, USA). The purity of radiolabeled trifluralin was determined to be >96%. The number of replicates was three. Purity was also confirmed by high performance liquid chromatography (HPLC). Refer to the *Trifluralin HPLC Method* below for a detailed description of the elution method used.

The trinitrotoluene (ring-^{14}C) was received as a donation from the Army Corps of Engineers (specific activity 40 mci/mmol), originally purchased from Perkin Elmer Life Sciences, Boston, MA. TNT purity was determined using a toluene:methanol (99:1, v/v) mobile phase on silica gel 60 f_{254} thin layer chromatography plates. After development, the plates were cut into measured sections and placed into scintillation vials with 8 ml of scintillation cocktail and analyzed for ^{14}C on a Beckman LS6500 scintillation counter (Fullerton, CA, USA). The purity of the three replicate samples was determined to be

>96%. This was also confirmed using (HPLC). See the *TNT HPLC Method* below for a detailed description of the elution method used.

Test Organisms

The two organisms that were chosen for the project were *Lumbriculus variegatus*, an aquatic oligochaete and *Hyalella azteca*, an amphipod. *L. variegatus* is an aquatic oligochaete prevalent in waters throughout the northern hemisphere (Maenpaa et al. 2003). Its wide geographical distribution renders *L. variegatus* ecologically relevant. It has been used as a test organism for many years and is able to incorporate a number of exposure routes, via water and sediment, further promoting it as a possible indicator organism (Maenpaa et al. 2003). Similarly, *H. azteca* is also a suitable model organism as it is a commonly used test organism, is relatively easy to culture, and is capable of incorporating the same exposure routes as *L. variegatus.*

A starter culture of *Lumbriculus variegatus* and *Hyalella azteca* were originally obtained from Aquatic Research Organisms (Hampton, NH, USA) and the Department of Zoology, Southern Illinois University (Carbondale, IL, USA), respectively. Continuous cultures were maintained at Baylor University as a source for test organisms. Briefly, the culture of *L. variegatus* was maintained using unbleached paper towels as substrate. Tank water was continually aerated and renewed weekly to ensure that dissolved oxygen content did not drop and that waste products were removed. Organisms were fed ground TetraMin ® daily. *H. azteca* were maintained similarly. Tanks were on a flow-through system and were continually aerated. Leached maple leaves were used as substrate and as a food source in this culture.

Lipid Analysis

Lipid analysis was performed to evaluate the health of the organisms and provide a reference for future users of the bioaccumulation data. Percent lipid in both organisms was determined by a vanillin-phosphoric acid method as described by Van Handel (1985). The vanillin-phosphoric acid reagent was prepared by dissolving 600 mg of vanillin in 100 ml of hot deionized water. Four-hundred ml of 85% phosphoric acid was added to the vanillin solution. The wet mass of the organism was measured. In a 16 × 100 mm test tube, one whole organism was crushed with a glass rod in 0.5 ml of chloroform:methanol (1:1, v:v) solution. The test tube was then placed on a heating block. Once the solvent evaporated, 0.2 ml of sulfuric acid (95%, VWR, West Chester, PA, USA) was added and heating continued for ten minutes. After ten minutes, the sulfuric acid/organism mixture was allowed cool. The vanillin-phosphoric acid reagent was brought to a final volume of five ml. The mixture was allowed to develop for at least five minutes and absorbance was measured at 525nm against a reagent blank using a spectronic 20D+ spectrophotometer (Milton Roy, Rochester, NY, USA). A calibration line was created in duplicate using 10, 25, 50, 100, and 200 µL of the lipid standard (canola oil) in 5ml of vanillin. Each standard was prepared and measured exactly like the samples. Lipid analysis for both organisms was done in triplicate. Absorbance values of samples were compared to the calibration curve to determine percent lipid.

Exposure Design

The investigation involved aqueous exposure of organisms to each selected parent compound. Dissolved oxygen and chemical concentration were monitored throughout experiments with less than 20% loss in all combinations. Organisms were exposed in 175 ml reconstituted hard water fortified with a radiolabeled compound (~30 mg total mass; 3 replicate exposures per time point per treatment). Volumes and masses were based on preliminary studies performed to optimize the system for stability of parent compound in water (< 20% loss), maintaining dissolved oxygen (>4 mg/L), and using a minimal amount of radioactivity. For trifluralin, the initial water concentration for both *H. azteca* and *L. variegatus* was 14.39 µg/L (1600 dpm/ml). This concentration is well below recorded LC50 values for invertebrates (>2000 µg/L) (USEPA, 1996). The initial water concentration for *H. azteca* chambers spiked with TNT was 12.48 µg/L (4000 dpm/ml) and will be 6.24 µg/L (2000 dpm/ml). Both of these concentrations are well below the acute toxicity concentrations for aquatic invertebrates (>4000µg/L) (Talmage et al. 1999). Immediately following the radioactive spike, each chamber was allowed to equilibrate for approximately 5 minutes, then a 300µl water sample was taken and analyzed by liquid scintillation counting to ensure that the spike was accurate.

During uptake studies, organisms were collected from exposure chambers and evaluated over a 72-h period for uptake including at least 5 time points with a minimum of three replicates per time point. During depuration studies, organisms from each experimental unit were removed from exposure chambers after 72-h and transferred to a chamber containing unspiked reconstituted hard water. At timed intervals, experimental units were evaluated over a 96-h period. The depuration study also included a minimum

of 5 time points with 3 replicates per time point. During depuration, renewals and feeding were performed every 24 hours.

At each time point, the concentration of ^{14}C was monitored in the test water. For uptake time points, 300 µl aliquots were directly analyzed for ^{14}C to check for stability of the compound, and for depuration time points, a 1 ml aliquot was directly analyzed to ensure that reuptake was not occurring. Water samples from depuration chambers were also analyzed by high performance liquid chromatography and scintillation counting. These samples were prepared for analysis by diluting a 500 µl aliquot with an equal volume of acetonitrile. These samples were also spiked with either non-radiolabeled TNT or Trifluralin, as appropriate, to ensure fractions were being collected properly. Chromatographic analysis of depuration water showed that virtually no radioactivity was in the water, (<100 dpm/sample).

Extraction Technique

Protocols used for the extraction technique were adapted from a Belden et al. 2005 study. The organisms from each replicate were blotted dry, weighed, and then homogenized in 8 ml acetonitrile using a Fisher Scientific Sonic Dismembrator (Model 500) with a pre-programmed method set to sonicate for two seconds, then rest for two seconds for a total of 40 seconds. The homogenate was then filtered through a 0.45-µm glass fiber filter (25 mm diameter) (Whatman International Ltd., Maidstone, England). The filtrate was evaporated and diluted to a final volume of 1ml under N_2 gas and a 50 µl aliquot was directly analyzed by scintillation counting. The rest of the filtrate was immediately frozen and retained at -4 °C until HPLC analysis. The tissue remaining on the filter was dissolved with 1 ml of Fisher Scientific Tissue Solubilizer, then directly

analyzed by scintillation counting. The amount of ^{14}C determined in this portion is experimentally defined as unextractable, or bound, residue (Figure 1). All samples that were analyzed by scintillation counting were allowed to sit in the dark for at least 24 hours before analysis to maximize counting efficiency.

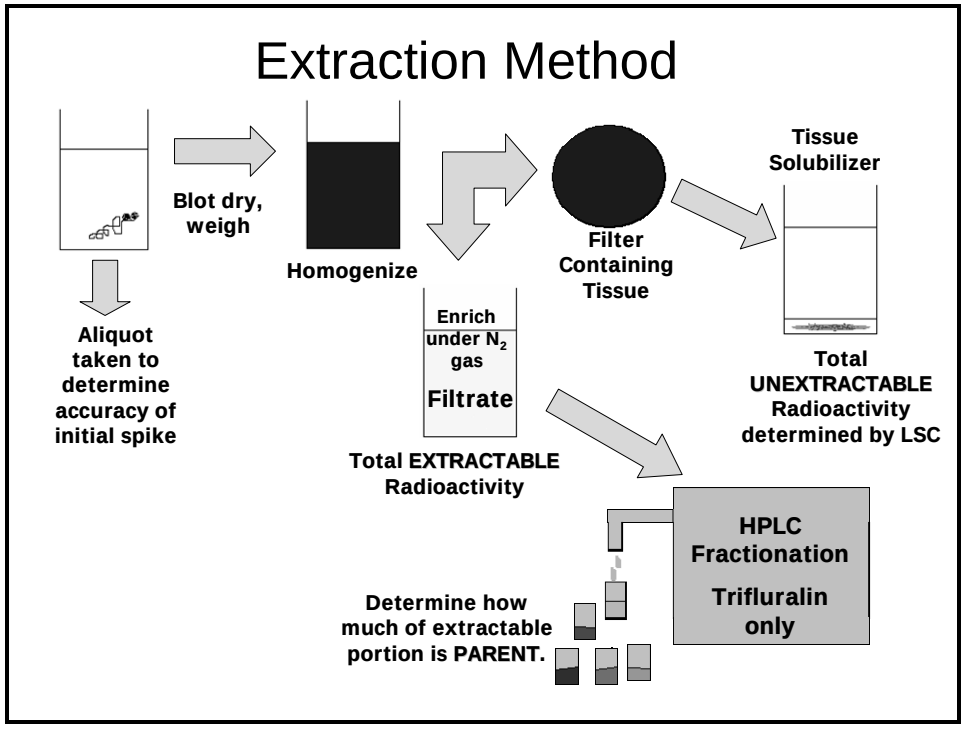

Figure 1. Flow chart describing extraction and separation approach. Following exposure, whole organisms were homogenized, extracted with acetonitrile, and the extract was filtered. The volume of the filtered extract was then reduced under N_2 gas to enrich the sample, and an aliquot was directly analyzed by liquid scintillation counting (LSC) to measure "extractable" radioactivity. A second aliquot was separated into fractions corresponding to parent and biotransformed products using HPLC and each fraction was measured using LSC. The residual tissue on the filter was dissolved in 1ml of tissue solubilizer and directly analyzed by LSC to measure the "unextractable" portion.

Separation/Chromatography for Trifluralin

Parent compound was separated from any biotransformation products that may have been present in the extracts of the trifluralin-exposed organisms by high performance liquid chromatography (Varian, Chromspher C-18 (150 * 4.6 mm) Lake Forest, CA, USA). All samples were prepared for HPLC analysis by creating a sample:deionized water (1:1) solution, spiked with the non-radiolabeled compound to ensure proper fraction collection following HPLC separation. A 100 µl sub-sample of the HPLC-prepared solution was analyzed and fractions were collected. Each fraction was then analyzed for ^{14}C by scintillation counting on a liquid scintillation counter (Beckman LS6500 Scintillation System, Fullerton, CA, USA). This analysis determined how much of the extract was still parent and how much had been biotransformed. This analysis was not done for TNT samples.

All HPLC analyses were carried out using a Varian ProStar-9000 series liquid chromatograph (Varian, Walnut Creek, CA, USA). The HPLC system consisted of a ProStar 410 autosampler, PrepStar 218 solvent delivery module, and a ProStar 335 photodiode array detector. Chromatographic separation was achieved using a 150 × 4.6 mm C-18 column (Varian, Chromspher, Lake Forest, CA, USA). Primary wavelengths used for detection were 280 nm and 220 nm for trifluralin and TNT samples, respectively. Gradient separations were carried out using a water-methanol (85:15) solution and acetonitrile for trifluralin samples. For TNT samples, gradient separations were carried out using nanopure and acetonitrile. Additional parameters employed in both HPLC analyses were as follows: injection volume, 50µl; flow rate, 0.8ml/min. The

nonlinear gradient elution profile employed to achieve chromatographic separation for each analyte is given in Tables 1 and 2.

Table 1. HPLC gradient elution profile for trifluralin

Time (min)	Mobile phase composition (%)	
	Water:Methanol (85:15, v:v)	Acetonitrile
0	95	5
2	95	5
3	3	97
10	3	97
11	95	5
13	95	5

Table 2. HPLC gradient elution profile for TNT

Time (min)	Mobile phase composition (%)	
	Nanopure water	Acetonitrile
0	90	10
8	50	50
12	50	50
17	30	70
18	0	100
22	0	100
24	90	10
25	90	10

Solvent Extraction Efficiency

To ensure that the acetonitrile solvent was efficient at extracting both trifluralin and trinitrotoluene residues out of both organisms, extraction efficiencies were

determined. This was accomplished by spiking a known amount of radioactive compound into a scintillation vial containing acetonitrile and organisms (10 organisms for *H. azteca*, 4 for *L. variegatus*). Immediately following the radioactive spike, the organisms and solvent were homogenized on the sonic dismembrator for 40 seconds (homogenize for 2 seconds, pause for 2 seconds). After homogenation, test replicates were split into one of two groups: the first group was immediately filtered as described in the extraction/filtration method above and the second group was placed on a shaker for 30 minutes to allow time for any possible partitioning into tissue, and then filtered as described in the extraction/filtration method above. The number of replicates per treatment was three. Each sample was independently analyzed for radioactivity. The extraction efficiency of both groups was determined using the following equation:

$$\frac{\text{Radioactivity in filtrate (extractable)}}{\text{Radioactivity in filtrate (extractable) + radioactivity in tissue (unextractable)}} * (100)$$

Statistical Analysis

Kinetics parameters were determined assuming a first-order toxicokinetic model and steady-state concentration during the uptake phase. Uptake rate (k_u) was calculated for extractable portions of each compound using initial rate estimates. A plot of concentration in organism (y-axis) verses time (x-axis) was constructed. The initial slope of the line prior to loss of linearity was used to calculate k_u. The depuration rate (k_d) was also determined graphically based on a plot of the natural log of concentration of contaminant within the organisms (y-axis) versus time (x-axis). The slope of the line indicated k_d. The bioconcentration factor, or the accumulation of chemical in an exposed

organism, was determined for both extractable and unextractable portions of each compound. This was accomplished by using the following formula(s):

Formula 1. $$BCF = \frac{[\text{residue in organism}]}{[\text{water}]} \quad \text{(if at steady state)}$$

Formula 2. $$BCF = \frac{k_u}{k_d}$$

The half-life ($t_{1/2}$) of extractable and unextractable residues was determined using the following formula:

Formula 3 $$t_{1/2} = \frac{\ln 0.5}{k_d} = \frac{0.693}{k_d}$$

The statistical program, JMP ®, was used to determine if percentages of unextractable residues for each compound were significantly different between organisms. A pooled t-test was used to determine significance ($\alpha = 0.05$). In each table and graph for all values means and standard deviations are shown.

CHAPTER THREE

Results

Lipid Analysis

The results of the lipid analysis showed that *L.variegatus* was 10.5% (±1.5) lipid. This value is within the range of lipid percentages found in the literature, 8-12.2% (Liebig et al., 2005, Landrum et al. 2004a). Percent lipid for *H. azteca* was determined to be 14.4% (±3.8). Percent lipid for *H. azteca* ranged in the literature from about 7-10% based on dry weight (Lotufo et al. 2002, Morris et al. 2003, Landrum et al. 2004b, and Driscoll et al. 1997)

Extraction Efficiency

For both compounds in both organisms extraction efficiencies were all >90%. Therefore the extraction efficiency is acceptable for parent compound and we are confident that the unextractable residues accumulated are not a result of the choice of a poor extraction solvent, but because they are bound within the organism in some way. Results of the extraction efficiency experiments also showed negligible decrease in the amount of recovered radioactivity between samples that were immediately filtered and those that were placed on a shaker for thirty minutes (to allow for possible partitioning).

Specifically, for *L.variegatus* exposed to trifluralin the extraction efficiencies were 99.1% ±0.2% and 99.4% ±0.7% for immediately filtered samples and samples placed on the shaker for thirty minutes, respectively. The extraction efficiencies for trifluralin in *H. azteca* were 95.9% ±1.0% and 95.8% ±1.2%, respectively for immediately-filtered samples versus samples that were placed on the shaker for thirty

minutes. Extraction efficiencies for TNT exposures for *L. variegatus* were 95.6% ±1.3% and 96.6% ±1.5%, respectively and for *H. azteca* were 95.6% ±1.8% and 93.7% ±0.8%, respectively.

Trinitrotoluene in Lumbriculus variegatus

Over the 24 hour uptake, extractable residues reached an approximate steady state with an average concentration of 310 ng/g ±30 ng/g and a bioconcentration factor (BCF) of 60 ±10 (Table 1). The initial rate of uptake for extractable residues into L. variegatus was 12 ng/g/h (Table 1). The concentration of unextractable residues exceeded extractable residues, 830 ng/g ±80 ng/g and therefore contributed more to overall body burden of TNT in *L. variegatus* with a bioconcentration factor of 160 ± 30 (Figure 2, Table 3). Because the unextractable residues did not reach a steady state, the given BCF may be underestimated. During depuration, unextractable TNT residues were eliminated more slowly than extractable ones. The biological half-lives of the two residues were 124 and 39 hours, respectively (Figure 3). The k_d values for extractable and unextractable residues were 0.018 and 0.0056, respectively, indicating that unextractable residues were more slowly eliminated (Table 3).

Trinitrotoluene in Hyalella azteca

Uptake of TNT into *H. azteca* demonstrated similar trends to those found in *L. variegatus*. Unextractable residues increased without reaching a steady state at the 24 hour uptake time-point. The initial uptake rate for extractable TNT residue was 4 ng/g/h (Table 3). The concentration of unextractable residues exceeded that of extractable residues, 180 ng/g ±6 ng/g and 110ng/g ±6 ng/g, respectively (Figure 4). Again,

unextractable residues contributed more to overall body burden, with a bioconcentration factor of 20 ±1, than extractable residues did, BCF of 10 ±1 (Table 3). During depuration, unextractable residues were eliminated more slowly than extractable ones, with biological half-lives of 64 and 47 hours, respectively (Figure 5). The k_d values also showed unextractable residues were eliminated more slowly, 0.015 and 0.011 for extractable and unextractable fractions, respectively (Table 3).

Table 3. Bioconcentration factors and depuration and uptake rates for residues in *L. variegatus* and *H. azteca*. * indicates that steady state condition had not been reached. The BCF for unextractable residue was significantly different in all cases (α=0.05).

Organisms/Compound	Bioconcentration Factor		K_d		K_u
	Extractable	Unextractable	Extractable	Unextractabl	Extractable
L. variegatus/ TNT	60±10	160±30*	0.018	0.0056	12
H.azteca/ TNT	10±1	20±1*	0.015	0.011	4
L.variegatus/Trifluralin	580±70	30±5*	0.044	0.0030	225
H. azteca/ Trifluralin	1200±200	350±140*	0.022	0.013	180

Trifluralin in *Lumbriculus variegatus*

Extractable and unextractable residues reached an apparent steady state by the 72 hour uptake time point, leveling off at concentrations of approximately 7400 ng/g ±760 ng/g and 330 ng/g ± 60 ng/g, respectively. The initial uptake rate for extractable trifluralin was 225 ng/g/h (Table 3). At 72 hours, greater than 90% of the extractable portion was still parent trifluralin. During uptake, the concentration of extractable residues was never exceeded by that of the unextractable residues (Figure 6). As a result, the extractable residues contributed more to overall body burden as compared to unextractable ones, with mean bioconcentration factors of 580 ±70 and 30 ± 5, respectively (Table 3).

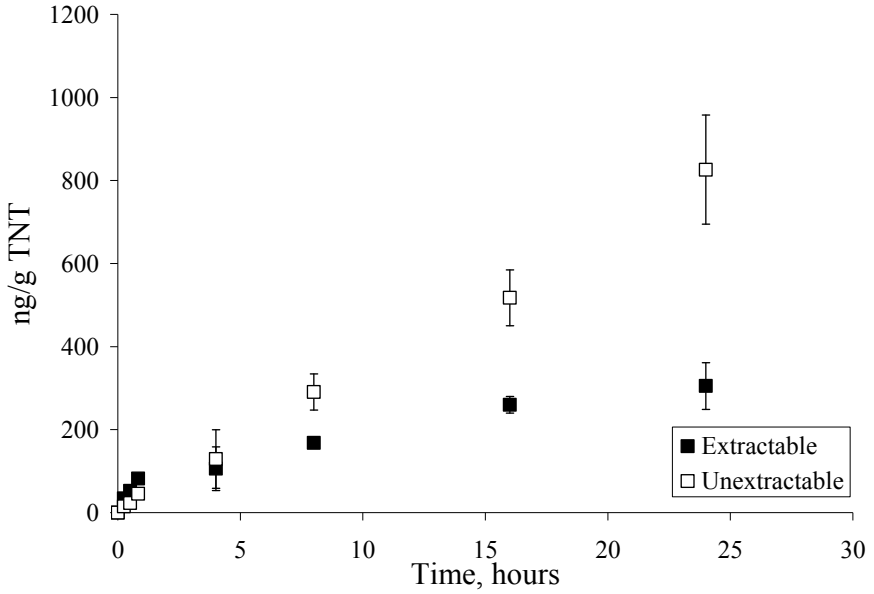

Figure 2. Uptake of TNT into *L. variegatus* (ng/g) over 24 hours. Neither residue reached a steady state by the 24 hour uptake time point. Error bars represent standard deviation (n=3).

During depuration, unextractable residues took longer to be eliminated than extractable ones. The biological half-life of extractable residue was 16 hours. The percent of the extractable residue that remained parent decreased from greater than 90%, at time zero (72 hour uptake time point), to less than 70% at the 96 hour depuration time-point. The concentration of the unextractable residue did not decrease by half during the depuration period; therefore, a specific value for the biological half-life could not be assigned, but was defined as greater than 96 hours (Figure 7).

The elimination rates also indicated that unextractable residues were eliminated more slowly than extractable ones, 0.0030 and 0.044, respectively (Table 3). The k_d estimate for unextractable is only an estimate as the slope of the line was approaching

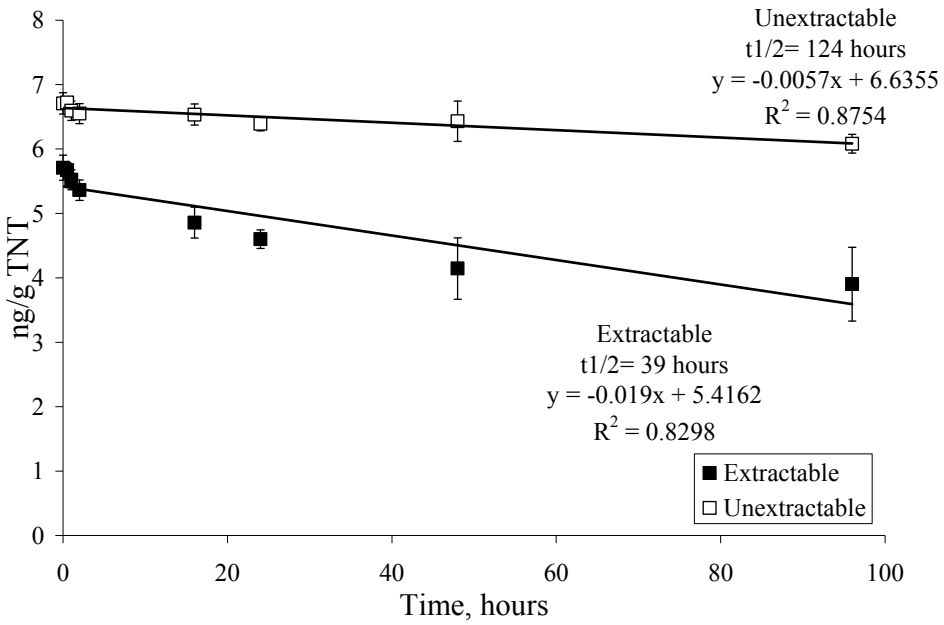

Figure 3. Depuration of TNT from *L. variegatus*. Unextractable residues were eliminated more slowly than extractable ones; biological half-lives were 124 hours and 39 hours, respectively. Error bars represent standard deviation (n=3).

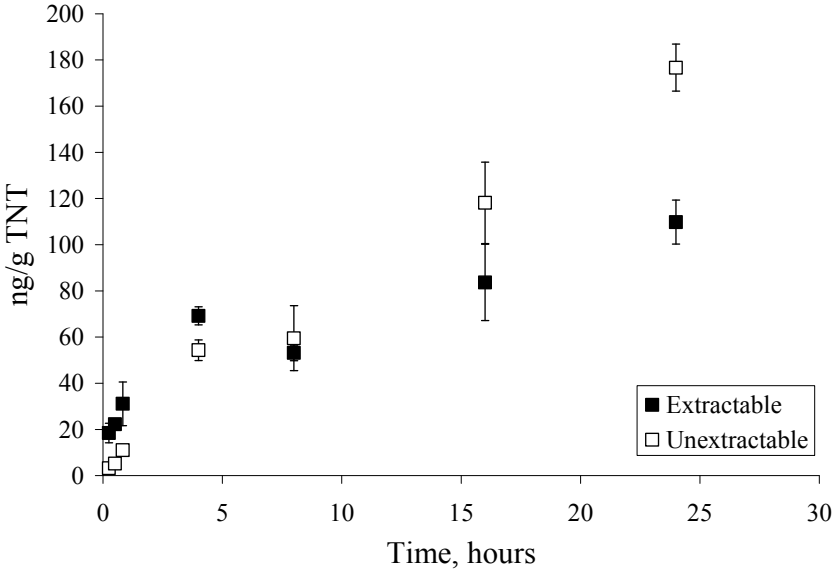

Figure 4. Uptake of TNT into *H. azteca* (ng/g). Neither residue reached a steady state by the 24 hour uptake time point. Error bars represent standard deviation (n=3).

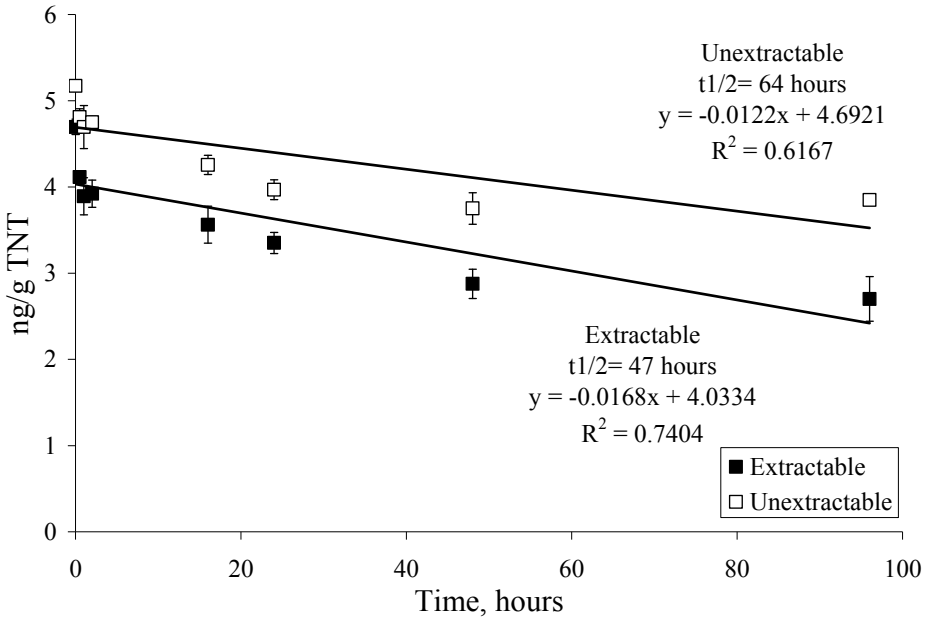

Figure 5. Depuration of TNT from *H. azteca*. Unextractable residues were eliminated more slowly than extractable ones, biological half-lives were 64 and 47, respectively. Error bars represent standard deviation (n=3).

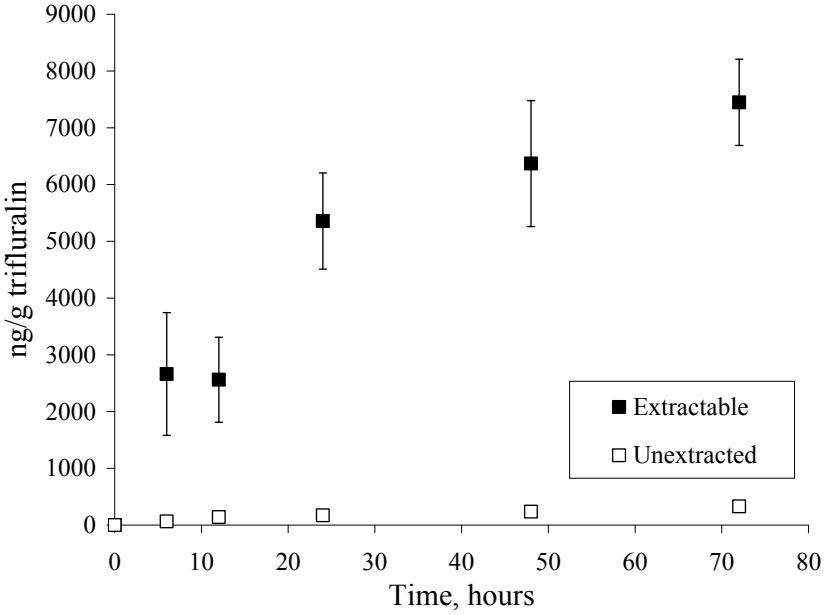

Figure 6. Uptake of trifluralin into *L. variegatus*. Error bars represent standard deviation, (n=3).

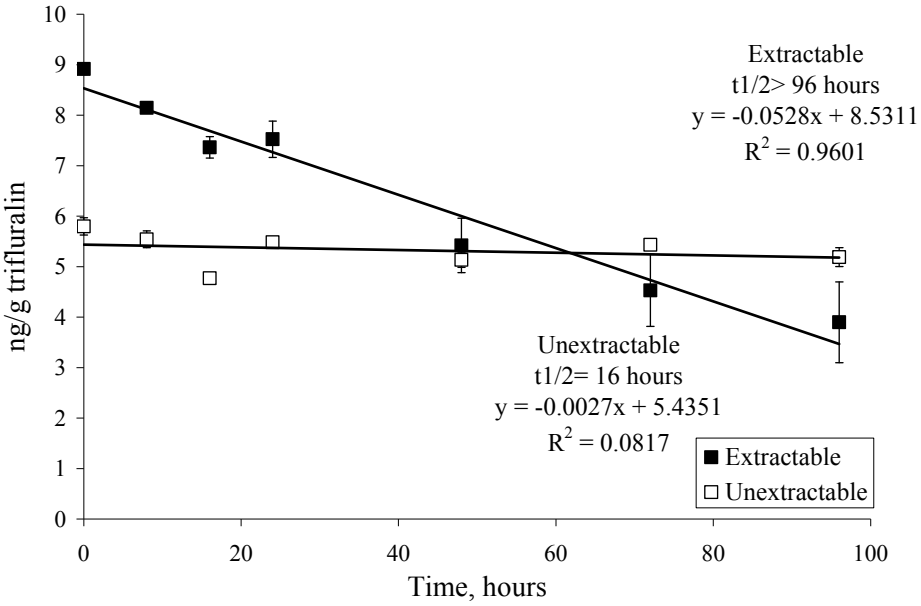

Figure 7. Depuration of trifluralin residues from *L. variegates.* Error bars represent standard deviation (n=3).

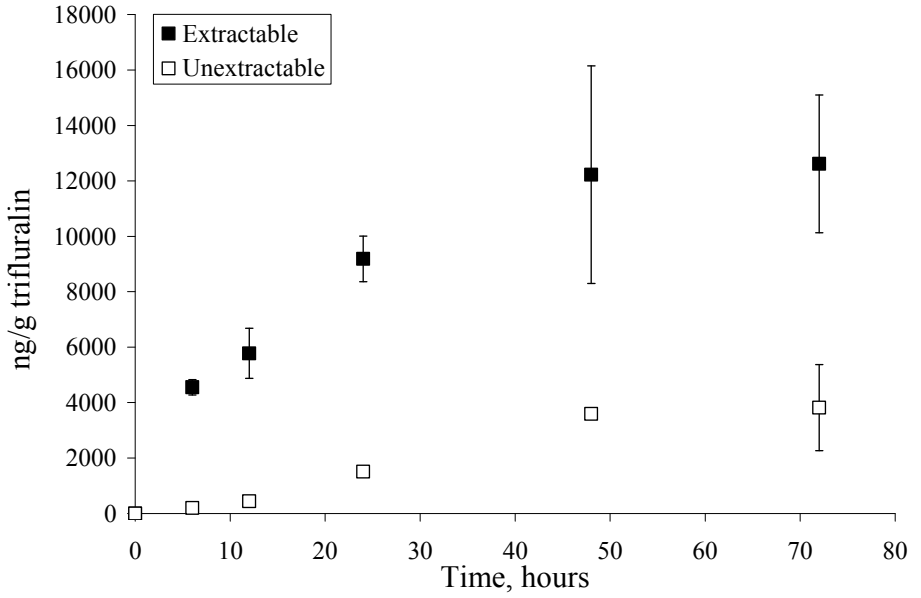

Figure 8. Uptake of trifluralin into *H. azteca.* Error bars represent standard deviation (n=3).

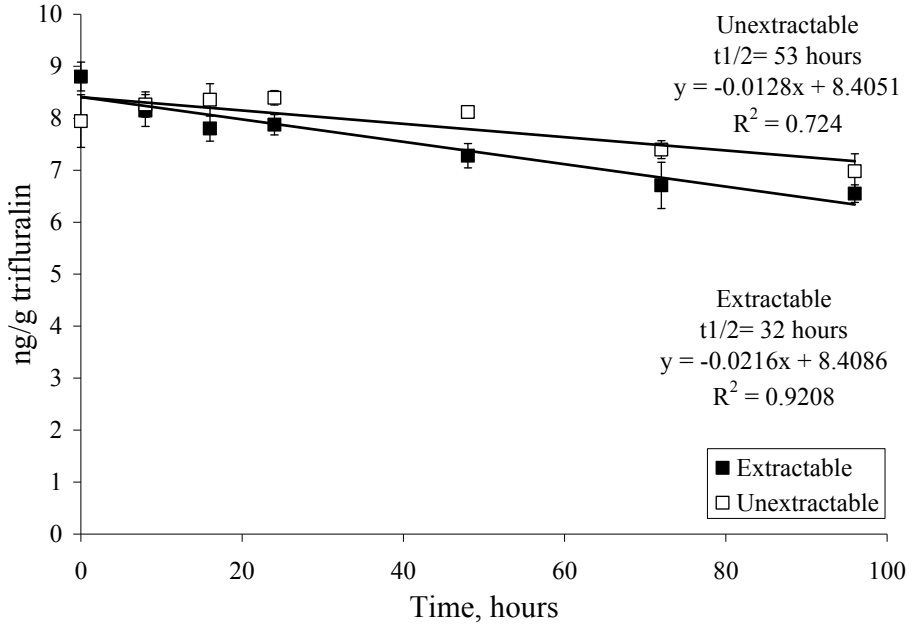

Figure 9. Depuration of trifluralin from *H. azteca*. Error bars represent standard deviation (n=3).

zero and the biological half life of the compound was not exceeded during the depuration period.

Trifluralin in Hyalella azteca

Both extractable and unextractable trifluralin residues reached a steady state by the 72 hour uptake time-point, with concentrations of 13000ng/g (±1000 ng/g) and 3800 ng/g (±900 ng/g), respectively (Figure 8). The initial uptake rate for extractable trifluralin residue was 180 ng/g/h (Table 3). At 72 hours, approximately 85% of extractable residues were still parent trifluralin. Extractable residues contributed more to overall body burden than unextractable residues, BCF, 1200 ±200 and 350±140, respectively. During depuration, the percent of extractable residue that remained parent decreased from approximately 85% at time zero, to less than 30% at the 96 hour depuration time-point. Depuration of trifluralin residues from *H. azteca,* again showed

the trend that unextractable residues had longer biological half-lives than extractable ones, 53 and 32 hours, respectively (Figure 9). Elimination rates also showed that unextractable residues were eliminated more slowly than extractable ones, 0.013 and 0.022, respectively.

CHAPTER FOUR

Discussion

Formation of Unextractable Residues

Unextractable residues were formed in both organisms when exposed to each nitroarene. At the 24 hour uptake time-point for TNT exposure, unextractable residues contributed more to overall body burden than extractable ones, 73% ±1% of all TNT residues in *L. variegatus* and 62% ±1% of all TNT residues in *H. azteca* (Table 2). Moreover, these percentages may be underestimates as the unextractable TNT residues had not reached a steady state by the 24 hour uptake time-point (Figure 2, Figure 4). The findings for *L. variegatus* are close to the percentages found in a similar study by Belden et al (2005) that reported 80% of all TNT residues in *L. variegatus* as unextractable after 24 hours of uptake.

For trifluralin exposures, *H. azteca* accumulated more unextractable residues than *L. variegatus*. At the 72 hour uptake time-point, unextractable residues represented only 4.0% ±0.5% and 23% ±5% of all trifluralin residues in *L. variegatus* and *H. azteca*, respectively (Table 4). The percentage of unextractable residue found in *L. variegatus* in our study was slightly different than what was reported in a study by Guerrero et al. (2002), who reported ~12.5% of all trifluralin residues in *L. variegatus* as unextractable. This difference in the percentage of unextractable trifluralin residue in *L. variegatus* between the two studies may be attributed to the different extraction solvents that were

used: *n*-hexane-diethyl ether (70:30) in Guerrero et al. (2002) versus acetonitrile in our study.

Table 4. Percent of compound that was yielded extractable and unextractable at longest uptake time point (24h for TNT, 72h for trifluralin). The percentages of unextractable residue for both compounds was significantly different between organisms (n=3, +- standard deviation, α= 0.05).

	Trinitrotoluene		Trifluralin	
Organism	Percent Extractable	Percent Unextractable	Percent Extractable	Unextractable
L. variegatus	27 $\pm$1	73 $\pm$1	96 $\pm$1	4 $\pm$1
H. azteca	38 $\pm$1	62 $\pm$1	77 $\pm$5	23 $\pm$5

Bioaccumulation Factors

In our study, total BCF of TNT in *L. variegatus* was approximately 212. This value was close agreement with previously reported measurements. Total TNT BCF values of 216 and 202 were reported by Belden et al. (2005) and Liu et al. (1983), respectively. Furthermore, the proportion of residues that made up the total was similar between our study and the Belden et al. (2005) study. The BCF values for extractable and unextractable residues reported by Belden et al. (2005) were 42 and 174, respectively compared to 60 $\pm$10 and 160 $\pm$30 for *L. variegatus* in our study.

The BCF values of the extractable and unextractable TNT residues in *H. azteca* were also similar to what was found in the literature. In our study the BCF for total TNT residues was 30, of which approximately 62% (BCF of 20 $\pm$1) and 38% (BCF of 10 $\pm$1) was contributed by unextractable residues and extractable residues, respectively (Table 4). A study by Sims et al. (2007) reported a total BCF of 87 in *H. azteca*. This BCF

value was higher than what we found in our study; however, the BCF in the Sims et al. (2007) paper was determined at a 96 hour uptake time point while ours was found at a 24 hour uptake time point.

When comparing extractable triflurlain and TNT residues, trifluralin extractable residues were higher. This however, would be expected based on the compounds' differing log K_{ow} values. The log K_{ow} for trifluralin is 5.34 (MSDS, Dow AgroChemical 2004). For TNT, the log K_{ow} ranges in the literature from 1.60-2.7. TNT's relatively low log K_{ow} indicates that it may partition to biota lesser extent (Talmage et al, 1999, Lotufo et al, 2001, Green et al, 1999, Conder et al, 2004). Therefore, it is not a surprise that trifluralin accumulated more total residues in both organisms than TNT.

Differences Between Nitroarene Compounds

The majority of trifluralin residues were accumulated as extractable in both organisms. In fact, this is the major difference between trifluralin and TNT accumulation: for TNT, unextractable residues contributed more to overall body burden, while for trifluralin, extractable residues contributed more to overall body burden.

This sort of variance in accumulation of different nitroaromatic compounds is documented in the literature. In *L.variegatus,* 82% of accumulated TNT residues were yielded unextractable after aqueous exposure, while only 12.5% of trifluralin residues were rendered unextractable (Belden et al. 2005, Guerrero et al. 2002). In *Chironomous tentans*, 89.1% of TNT residues were unextractable, while 56.1% of trifluralin residues were unextractable in *Chironomous riparius* (Belden et al. 2005, Guerrero et al. 2002).

The chemical structures of these compounds may offer a clue as to why this is so. Trifluralin has two large alkyl-groups that may cause steric hindrance, disabling the

organisms' enzymatic capability to break it down (Figure 10). Therefore, no groups are cleaved, thus no reactive intermediate is formed that is capable of forming unextractable residues, or covalent bonds within the organism. TNT, however, has three nitro-groups that are not hindered by large constituents and are therefore more amenable to metabolic transformations resulting in unextractable residues within the organism (Figure 10).

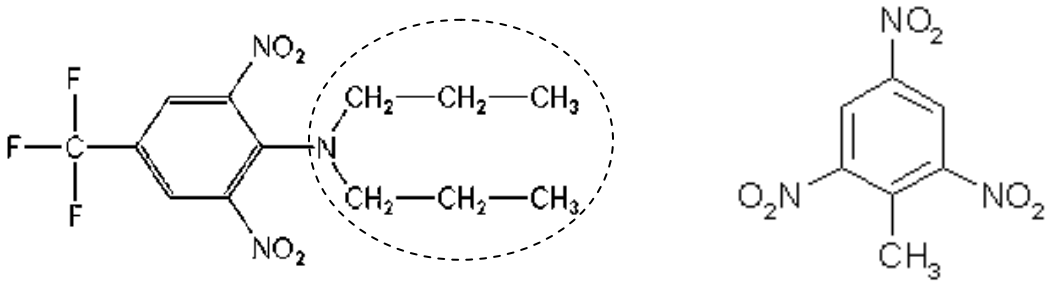

Figure 10. Chemical structure of trifluralin (left) and trinitrotoluene (TNT) (right). The dashed circle indicates the two alkyl groups which may be the cause of steric hindrance, disabling the nitro-groups from being cleaved. Notice there are no large constituents in the TNT structure that may cause similar hindrance.

Covalent binding of a reactive TNT intermediate, was described by Leung et al. (1995). In their study, they found that when rat liver microsomes were incubated in the presence of ^{14}C-TNT and NADPH, covalent protein adducts increased with time. Because adduct formation increased with time, the researchers believed that the adducts were not formed by TNT directly, but by an intermediate species. They suggested that TNT was bioactivated by the reduction of the nitroso-group on the fourth position, to form 4-hydroxylamino-2,6-dinitrotoluene, (4-HA). Furthermore, when they performed the same experiment but exposed the liver microsomes to the reactive 4-HA instead of parent TNT, the level of covalent adduct formation increased. The levels were even further increased with higher oxygen concentrations. It is probable that a similar

mechanism was operable in our study, which allowed the TNT to form more unextractable residues in both organisms.

Differences Between Organisms in Accumulation of Unextractable Residues

L. variegatus accumulated more overall TNT residues than *H. azteca***.** Based on a comparison of mean concentrations determined at the longest uptake time point, extractable residues accumulated approximately five times more and unextractable residues accumulated approximately nine times more in *L. variegatus* than *H. azteca.* Unextractable TNT residues contributed to more than 60% of overall body burden in both organisms (Table 4**).**

H. azteca accumulated more overall residues when exposed to trifluralin. Approximately 1.7 times more extractable residues and approximately 11.6 times more unextractable residues were formed in *H. azteca* than in *L. variegatus.* Unextractable trifluralin residues contributed to less than 23% of overall body burden in both organisms (Table 4).

Interspecies variance in accumulation of unextractable nitroaromatic residues have been noted between other species as well. Belden et al. (2005) reported that *L. variegatus* accumulated anywhere from 7.5 to 20 times more unextractable TNT residues than *I. punctatus*. Also, *Chironomous riparus* accumulated approximately 4.5 times and almost 7.5 times more unextractable trifluralin residues than *L. variegatus* and *S. corneum*, respectively (Guerrero et al. 2002).

The aforementioned study by Sims et al. (2007) reported that after the initial reduction of TNT to either 2-ADNT or 4-ADNT, cytochrome P450 is involved in the N-hydroxylation of the newly formed amine group, which can lead to the formation of 4-

hydroxylamino-2,6-dinitrotoluene (4-HA), the highly reactive intermediate species that formed adducts in the Leung et al. (1995) study. Interspecies differences in basal enzymatic activity and differences in the sensitivity to induction of enzymes may lead to differences in the rate that the reactive intermediate is formed which may ultimately explain the differences in the accumulation of unextractable residues in each organism (Di Giulio et al., 1995).

Toxicokinetics of Residues: Biological Half-lives

The biological half-life of unextractable residues was always longer than that of the extractable residues in both organisms. *H. azteca* eliminated unextractable residues of both compounds more efficiently than *L. variegatus*. The unextractable TNT residues in *L. variegatus* had a biological half-life approximately 3.2 times longer than the extractable residue, (124 hours and 39 hours, respectively). *H. azteca* eliminated unextractable TNT residues approximately 1.5 times slower than extractable residues, (64 hours versus 47 hours). The biological half lives for both extractable and unextractable TNT residues was shorter in *H. azteca* than *L. variegatus*.

The biological half-life of unextractable trifluralin residue was about 6 times longer than that of the extractable residue in *L. variegatus* (>96 hours versus 16 hours). However, the half life for the unextractable residue was only an estimate as its biological half-life was not reached by the end of the depuration period. In *H. azteca,* the biological half-life of the unextractable trifluralin residue was approximately 1.6 times longer than that of the extractable residue, (53 and 32 hours, respectively).

The difference noted in toxicokinetics parameter between the extractable and unextractable residues indicate that the residues behave and are metabolized differently

within both organisms. This provides support for the idea that the unextractable residues are something other than the parent compound and not simply left in the organism due to poor extraction efficiency.

Conclusions

In summary, the results of the study have established that unextractable residues form in both *Lumbriculus variegatus* and *Hyalella azteca* when independently exposed to TNT and trifluralin. Additionally, the unextractable residues are always eliminated more slowly than the extractable ones, as evident by their longer biological half-lives. The presence and biological persistence of unextractable residues indicates that these compounds, and especially TNT, could cause subtle and chronic biological effects and serve as indicators of exposure. However, further research must be pursued to determine the exact chemical nature of the bound residue and if the residues result in a biologically significant effect.

Future Research

There are a couple different directions that could be taken in future research. One interesting path to take for new research may be to look closer at what causes each species to accumulate and eliminate these compounds both at different rates and proportions. This could be accomplished by looking at different protein and enzyme levels within each organism and comparing them to accumulation and elimination rates.

It is unknown whether there are physiological, reproductive, or other effects that may be caused by the accumulation of unextractable residues. Therefore another area of research may investigate these possible effects. A series of bioassays may provide

insight not only into effects on the organism, but also a comparison of those effects among organisms.

Finally, a third avenue to pursue would be to take a closer look at the bound residue itself. If the suspected bound residues, or adducts, can be isolated and cleaved, the species of the compound that is forming the unextractable residue may be identifiable using analytical techniques. For example, if the TNT or trifluralin adduct is cleaved by an acid digestion, the resulting extract could be analyzed using HPLC to determine if the parent compound, a known metabolite, or an unknown biotransformation product were the adduct-forming species. Furthermore, if the acid digestion works, it is possible that ecotoxicological effects may ensue which could completely reshape the ecological risk assessments for these compounds.

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