Dietary Exposure of Fathead Minnows to the Explosives TNT and RDX and to the Pesticide DDT using Contaminated Invertebrates

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Abstract: Explosive compounds have been released into the environment during manufacturing, handling, and usage procedures. These compounds have been found to persist in the environment and potentially promote detrimental biological effects. The lack of research on bioaccumulation and bioconcentration and especially dietary transfer on aquatic life has resulted in challenges in assessing ecological risks. The objective of this study was to investigate the potential trophic transfer of the explosive compounds 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) using a realistic freshwater prey/predator model and using dichlorodiphenyltrichloroethane (DDT), a highly bioaccumulative compound, to establish relative dietary uptake potential. The oligochaete worm *Lumbriculus variegatus* was exposed to ^{14}C-labeled TNT, RDX or DDT for 5 hours in water, frozen in meal-size packages and subsequently fed to individual juvenile fathead minnows (*Pimephales promelas*). Fish were sampled for body residue determination on days 1, 2, 3, 4, 7, and 14 following an 8-hour gut purging period. Extensive metabolism of the parent compound in worms occurred for TNT but not for RDX and DDT. Fish body residue remained relatively unchanged over time for TNT and RDX, but did not approach steady-state concentration for DDT during the exposure period. The bioaccumulation factor (concentration in fish relative to concentration in worms) was 0.018, 0.010, and 0.422 g/g for TNT, RDX and DDT, respectively, confirming the expected relatively low bioaccumulative potential for TNT and RDX through the dietary route. The experimental design was deemed successful in determining the potential for trophic transfer of organic contaminants via a realistic predator/prey exposure scenario.

Key words: TNT, RDX, DDT, trophic transfer, bioaccumulation

Introduction

Explosive compounds were released to the environment during the manufacturing, handling, use, and disposal of munitions at military sites in the United States and throughout the world. The result was contamination of ground and superficial waters, and soils and sediments, sometimes at exceedingly high concentrations (e.g., 34 mg/L for TNT in superficial water) [1-2]. Explosives such as 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and their degradation products typically degrade slowly in many environmental matrices, therefore yielding long-term contamination at the military sites where they were released [3]. The nitroaromatic compound TNT was the most abundantly produced explosive in the world and was released to surface and groundwater mainly from runoff and leaching from storage and disposal areas and from receiving lagoons at munitions production and

processing plants [1]. The cyclonitramine compound RDX is one of the most commonly used and most powerful munitions and was released in waste streams generated during manufacturing and processing activities, leaching from storage lagoons and burial areas, and from demilitarization operations [1]. RDX has a lower sorption coefficient in topsoil and is more commonly found in groundwater compared to TNT [4].

Challenges in establishing ecological risks and remediation goals at contaminated military sites typically exist because of inadequate knowledge of the environmental fate and effects of explosives in aquatic ecosystems. Explosives and several related compounds are known to cause a variety of adverse effects in animals. Organism-level effects have been reported in a relatively small number of aquatic species (see review [1] and also [5-9]. The fate of explosives and related compounds in fish and aquatic invertebrates is poorly understood. Explosives and related compounds have

low potential to bioaccumulate in animals as expected due to their low hydrophobicity [10-16]. Moreover, recent investigations revealed that TNT entering animals undergo extensive chemical transformations and the bioaccumulation of breakdown products typically exceeds the bioaccumulation of the parent compound in animal tissue [13-17]. All investigations of the bioaccumulation of explosive compounds in aquatic organisms used spiked water as the exposure medium.

Fish bioaccumulate xenobiotic compounds through direct absorption (mostly through the gills) from contaminated water and through the ingestion of contaminated food [18] and water. Dietary exposure to organic contaminants results in significant bioaccumulation for a variety of compounds [19-20] and sometimes results in detrimental biochemical and physiological effects [21, 22]. While the dietary uptake of explosives in aquatic species has never been investigated, significant bioaccumulation of TNT metabolites in a species of salamander through exposure to contaminated prey has been reported [23]. Therefore, there is potential for dietary uptake of TNT and other explosives in fish.

This study investigates the potential for dietary uptake of the explosives, TNT and RDX, to the fathead minnow (*Pimephales promelas*). The organochlorine dichlorodiphenyltrichloroethane (DDT) was used as a comparative compound because it is substantially more hydrophobic, and hence bioaccumulative, than explosives. Methods were designed to provide realistic exposure estimates for the trophic transfer of explosive compounds in aquatic systems. The source of dietary uptake was the freshwater oligochaete, *Lumbriculus variegatus,* pre-exposed to contaminants. Previous investigations of dietary uptake of xenobiotics typically used spiked food pellets.

Materials and Methods

Chemicals

Radiolabeled trinitrotoluene (^{14}C-TNT, 40 Ci/mol) was purchased from Chem Service (Westchester, PA). Non-labeled TNT was purchased from Sigma Chemical (St. Louis, MO). Radiolabeled hexahydro-1,3,5-trinitro-1,3,5-triazine (^{14}C-RDX) was purchased from New England Nuclear Research Products (Boston, MA). Non-labeled RDX (>98 percent pure) was obtained from the Naval Surface Warfare Center (Indian Head, MD). Radiolabeled dichlorodiphenyltrichloroethane (^{14}C-DDT) was purchased from Sigma Chemical Co. (St. Louis, MO). Manufacturer reported radiochemical purity and chemical purity were >98 percent for all compounds.

Experimental Organisms

Oligochaete worms, *Lumbriculus variegatus,* were obtained from a commercial vendor (Aquatic Bio Systems Inc., Fort Collins, CO) and maintained under flow-through culture conditions according to standard procedures [24] before use in the experiments. Typical individual worm mass was 6 mg. Laboratory-cultured juvenile fathead minnows (*Pimephales promelas*)

approximately 6 weeks old were purchased from Aquatic Bio Systems Inc. (Fort Collins, CO). Fish were maintained in dechlorinated tap water prior to use in the experiments. Typical fish biomass was 100 mg.

Aqueous Exposure of Prey Worms

Exposure solutions were created by spiking 1 L of water with 2 ml of an acetone solution consisting of radiolabeled and non-radiolabeled compounds (TNT and RDX) or radiolabeled compound only (DDT). The specific activity (disintegrations per minute [dpm]/μmol) of the TNT and RDX exposure water was determined by measuring radioactivity (dpm/ml) via liquid scintillation counting (LSC) and explosive concentrations (μmol/ml) via high performance liquid chromatography (HPLC). Target water concentrations were 5, 8, and 0.02 mg/L for the TNT, RDX and DDT exposures, respectively. Target radioactivity in water was 33, 66, and 3dpm/μL for the TNT, RDX, and DDT exposures, respectively.

Approximately 650 worms were exposed to ^{14}C-TNT, ^{14}C-RDX or ^{14}C-DDT for 5 hours in separate 1-L glass beakers. Such short exposure period was selected to maximize the fraction of the total radioactivity in the tissue corresponding to parent compound, as the relative contribution of the breakdown products of TNT increase with exposure duration for *L. variegatus* [14]. Transfer factors were determined as the ratio between radioactivity in the worm (dpm/mg) and in the exposure water (dpm/μL). Water samples were taken for chemical analysis at the beginning and end of the exposure period. At termination of the exposure period, a subset of worms was sampled for chemical analysis and a subset was processed for use as prey items in the dietary exposure experiment. Groups of two worms were wrapped in aluminum foil packages and frozen (-20°C) until fed to the fathead minnows.

Dietary Exposure of Predator Fish

Individual fish were placed in 600-ml glass beakers with 500 ml dechlorinated tap water. Fish were fed twice a day at 8:00 AM and 5:00 PM with a meal consisting two frozen worms. Three replicate fish were sampled on days 1, 2, 3, 4, 7, and 14. Fish sampling took place 8 hours after the morning feeding to allow purging of food from the gut.

Chemical Analysis

Water Samples

Water (1 ml) from worm and fish exposure beakers were mixed in 12 ml of xylene-based scintillation cocktail (3a70b, Research Product International, Mt. Prospect, IL) and analyzed for radioactivity on a Tri-Carb Liquid Scintillation Analyzer (Model 2500 TR, Packard Instrument, Meridien, CT, USA). Water was also analyzed for TNT, RDX, and the TNT breakdown products aminodinitrotoluenes (ADNTs) and diaminonitrotolunes (DANTs) using the U.S. Environmental Protection Agency method 8330 [25].

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Worm Samples

For radioactivity analysis, two worms in six replicates were placed in scintillation cocktail and analyzed as described above. For TNT and RDX, 20 worms in triplicates were transferred to polypropylene bead-beater vials. Each vial received 100 mg of 1-mm glass beads and 0.5 ml of HPLC-grade acetonitrile. Samples were homogenized using a mini bead-beater (Biospec, Barttlesville, OK) for 100 sec at 4200 oscillations/min and sonicated for 1 hour at 18°C in a water bath. Samples were centrifuged for 10 min at 7500 *g* at 4°C. A fraction of the acetonitrile supernatant (0.05 ml) was assayed for radioactivity as described above and another fraction received 0.5 ml of 1% $CaCl_2$, and was filtered through a PTFE 0.45-µm syringe filter into amber sample vials for HPLC analysis as described above. Laboratory reporting limits for tissue samples were approximately 1.2 mg/kg (6µmol/kg) for all analytes. For DDT, 20 worms in triplicates were transferred to scintillation vials. Each vial received 5 ml of HPLC grade acetonitrile. Samples were homogenized using probe sonication and centrifuged for 10 min at 7500-x g at 4°C. A fraction of the acetonitrile supernatant (1 ml) was analyzed for radioactivity as described above and another fraction (1 ml) was used for separation of DDT parent compound and breakdown products using the thin layer chromatography analysis as previously described [26].

Fish samples

Fish sampled at different time points of the dietary exposure were individually transferred to scintillation vials. Each vial received 1.0 ml of tissue solubilizer (T2, Research Product International, Mt. Prospect, IL). Following complete tissue solubilization (overnight), each vial received 1.0 ml of 1.2 N hydrochloric acid and was assayed for radioactivity as described above.

Data Analysis

For fish bioaccumulation data, completely randomized one-way analysis of variance (ANOVA) on ranks (Kruskal-Wallis) was used to determine differences between means of the various exposure periods at a 0.05 level of significance. Pairwise comparisons (Fisher LSD method) were used to determine significant differences between fish burden at different exposure periods.

Results and Discussion

Radioactivity was used as a surrogate for expressing the sum concentration of parent compound and all the degradation products for TNT, RDX and DDT in water and whole animal samples. Parent compound and all its degradation products will be collectively referred to as TNT*, RDX*, and DDT*.

Bioaccumulation in Prey

Aqueous exposures of *L. variegatus* were conducted to produce prey material for use in the dietary transfer experiments. Mean measured compound concentration in the exposure solution was 4.8 mg/L for TNT and 8.1 mg/L for RDX. The concentration of DDT in the water was not measured using analytical chemistry. Breakdown of parent compound during the 5-h aqueous exposure, determined using HPLC analysis, was minimal (<3%) for TNT and non-detectable for RDX. The relative accumulation of compounds in prey tissue was expressed as water-to-prey transfer factors calculated as the ratio between radioactivity in the worm (dpm/mg) and in the exposure water (dpm/µL). The 5-hour transfer factor for DDT* in *L. variegatus* was much greater than those of TNT* or RDX*, and the relative bioaccumulation of TNT* was greater than that of RDX*, based on concentration determinations using total radioactivity (Table 1). Using measured tissue concentrations of parent compounds in worms determined using HPLC analysis (7.48 and 2.14 µg/g for TNT and RDX, respectively), 5-h bioconcentration factors (BCFs) were much lower (1.5 and 0.27 µL/g for TNT and RDX, respectively) than the transfer factors determined using radioactivity presented in Table 1. Higher relative bioaccumulation of DDT* in *L. variegatus* was expected based on the major differences in K_{ow} among the compounds used in this study (log K_{ow} = 6.2, 1.60 and 0.87 for DDT [27], TNT [28], and RDX [29], respectively) and the positive relationship between log BCF and log K_{ow} [30].

Table 1: Radioactivity in water, prey (*Lumbriculus variegatus*) , and fish (*Pimephales promelas*) expressed as mean (± 1 standard deviation) disintegrations per minute (dpm) per unit of volume or mass representing the total concentration of the parent compounds (TNT, RDX or DDT) and all their degradation products. Transfer factors represent the ratio between prey body residue (dpm/mg) and water concentration (dpm/L) and between fish body burden (dpm/mg) and prey body burden (dpm/mg).

	Concentration (dpm/mg)			Transfer factor	
	Water (dpm/µL)	*Prey (dpm/mg)*	*Fish (dpm/mg)*	*Water to prey (µL/mg)*	*Prey to fish* [*] *(mg/mg)*
TNT	38.3 ± 3.7	1,245.6 ± 453.5	22.7 ± 10.5	32.5	0.018
RDX	135.1 ± 4.0	280.0 ± 19.0	2.8 ± 0.7	2.1	0.010
DDT	2.0 ± 1.0	423.7 0 ± 158.5	178.8 ± 28.4	214.5	0.422

[*] Bioaccumulation factor

Table 2: Percent of total sum-molar concentrations in worm tissues corresponding to unextractable or extractable parent, known or unknown compounds. Numbers (1-4) represent mean ± 1 standard deviation. Unextractable is defined as compounds that are resistent to solvent extraction from tissue. Extractable compounds includes the parent compound, known, or identified, compounds and unknown compounds, which are more polar than the parent compound and were not identified.

Exposure	Unextractable	Extractable		
		Parent	Known	Unknown
TNT	11.9 ± 4.7	4.6 ± 0.9	13.8 ± 4.0	73.7 ± 8.1
RDX	36.0 ± 9.5	12.9 ± 5.7	0	51.1 ± 3.8
DDT	2.6 ± 0.6	92.5 ± 0.5	1.5 ± 0.3	4.0 ± 0.7

The relative contribution of parent compound to the overall bioaccumulation of radioactive compounds in *L. variegatus* varied substantially among exposures (Table 2). While for DDT the parent compound was dominant in worm tissues, TNT and RDX parent compound was present at much lower concentrations compared to its breakdown products. The fraction of total radioactivity corresponding to non-solvent-extractable metabolites also varied greatly and was highest for RDX and lowest for DDT. Extractable breakdown products were identified as 2- and 4-ADNT for the TNT exposure and dichlorodiphenyldichloroethane (DDD) for the DDT exposure (Table 2). Peaks of potential RDX breakdown products were not discernible in HPLC chromatograms, indicating the polar nature of non-identified extractable breakdown products of RDX. The bioaccumulation profile reported for *L. variegatus* in an aqueous exposure to TNT by Belden et al. [17] was similar to that determined in this study, indicating that TNT is efficiently biotransformed in *L. variegatus*, as reported for other aquatic invertebrates [31, 13-14], terrestrial invertebrates [17] and fish [15]. Different from the nitroaromatic explosive TNT, the cyclonitramine explosive RDX was less efficiently biotransformed in *L. variegatus* as all extractable radioactivity corresponded to the parent compound. Biotransformation of RDX has been reported for microorganisms [32] and plants [33] but not for invertebrates or fish. The breakdown of DDT in the tissues of *L. variegatus* was minimal, as expected based on the reported inefficiency of that invertebrate to metabolize hydrophobic organic compounds [34]. Unextractable radioactivity detected in animal tissue corresponded likely to covalently bound products associated with organic molecules. Such strong binding has been previously reported for TNT in invertebrates [14,16], cell cultures [35] and plants [36]. RDX was found as either parent compound or bound residues in the intracellular compartment of plant roots [37].

Dietary Bioaccumulation in Predator Fish

Detectable concentrations of TNT*, RDX* and DDT* in fish fed contaminant-laden prey worms were determined over the 14-day exposure period (Figure. 1) using radioactivity as a surrogate for the parent compounds and their breakdown products. Tissue concentrations of TNT* and RDX* did not vary significantly over time (Figure. 1). However, an overall

trend for increasing DDT* body burden was observed (Figure. 1) and concentrations measured at experiment termination were significantly higher than those determined during the first seven exposure periods. Therefore, the concentration of DDT in fish would likely have increased beyond levels detected at day 14 if the experiment had continued for longer exposure periods. Based on results from preliminary experiments, the whole-body concentrations of TNT and its major breakdown products and RDX in fish fed contaminant-laden worms were too low for detection and quantification using acetonitrile extraction and HPLC analysis. Therefore, the identity of the compounds accumulated in exposed fish from this experiment is unknown.
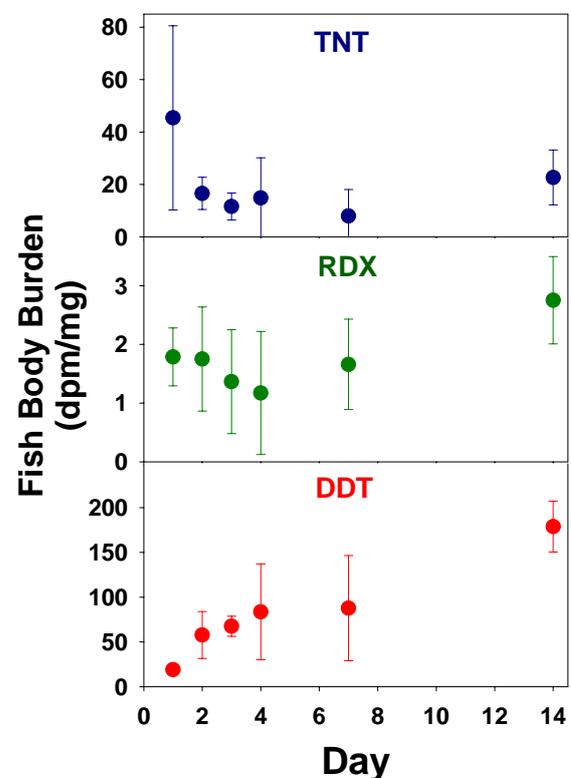
Figure 1: Body burden expressed as radioactivity (disintegrations per minute) per milligram of mass representing the total concentration (as parent compound equivalents) of the parent compound (TNT, RDX or DDT) and all its degradation products at different time points during the 14-day dietary exposure period.

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The experimental design employed in this study proved successful for investigating the potential for dietary uptake of organic contaminants from invertebrates to fish. Juvenile fathead minnows consumed contaminant-laden frozen worms quickly and completely. Hansen et al. [38] also successfully used *L. variegatus* as the contaminant source in an investigation of metal dietary uptake in juvenile rainbow trout.

Prey-to-predator transfer factors or bioaccumulation factors (BAFs) were determined as the ratio between radioactivity in the fish (dpm/mg) and in the prey (dpm/mg). The BAF for DDT* (0.422g/g) was substantially higher compared to that for TNT* (0.018g/g) and RDX* (0.010g/g) (Table 2). Comparison of those ratios indicates that while the concentration of DDT* in the fish was approaching the concentration of DDT* in its food source, the concentration of TNT* and RDX* in fish represented only a very small fraction of the concentration in the prey (>2 percent). The higher potential for trophic transfer of DDT and hydrophobic organochlorine compounds relative to less hydrophobic and more readily metabolized compounds has been well documented [39]. Johnson et al. [23] used earthworms exposed to TNT or PCBs (Arochor 1260) as prey and salamanders as predator to investigate the dietary uptake of those compounds in terrestrial systems. In that investigation, dietary bioaccumulation was much greater for the highly hydrophobic PCBs than for TNT and its metabolites, therefore corroborating our finding of much greater trophic transfer of DDT compared to TNT in the aquatic prey-predator system. The bioaccumulation factor for DDT obtained in this study (0.422 g/g) was lower than similar factors (0.89 – 2.80 g/g) reported for hydrophobic organochlorine compounds in rainbow trout [40], likely due to the short exposure duration used in the present study.

Fish were held for 10-hours in clean water to allow for the digestion of their last meal and also the egestion of undigested prey from their guts. However, this period may not have been sufficient for complete gut clearance. Therefore, radioactivity associated with undigested prey in the fish gut may have accounted for at least a fraction of the radioactivity determined in whole fish. For TNT*, the radioactivity associated with a meal (two frozen worms), approximately 13,000 dpm, far exceeded the mean radioactivity in fish at exposure termination (3,100 dpm). For RDX, the radioactivity associated with a meal, approximately 3,000 dpm, also far exceeded the mean radioactivity in fish at exposure termination (370 dpm). Therefore, for both explosives, even a small amount of undigested prey could have accounted for the whole body burden measured in the fish. For DDT*, however, the mean radioactivity associated with a meal (4,400 dpm) was substantially lower than the mean concentration in the fish at exposure termination (24,200 dpm), indicating that most of the body burden in fish corresponded to radioactivity present in fish tissues rather than associated with undigested prey.

Conclusions

The experimental design employing frozen aquatic worms as prey and juvenile fish as predator proved successful for investigating the potential for dietary uptake of organic contaminants in aquatic systems. This study demonstrated that dietary transfer from invertebrates to fish was negligible for TNT and RDX but relatively high for DDT, a compound substantially more hydrophobic than TNT and RDX.

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