2008

EFFECTS, UPTAKE, AND FATE OF 2,4,6-TRINITROTOLUENE AGED IN SOIL IN PLANTS AND WORMS

Elly PH Best
U.S. Army Engineer Research and Development Center, Environmental Laboratory;, elly.p.best@erdc.usace.army.mil

Henry E. Tatem
U.S. Army Engineer Research and Development Center, Environmental Laboratory

Kaaren N. Geter
Analytical Services

Melissa L. Wells
U.S. Army Engineer Research and Development Center, Environmental Laboratory

Brian K. Lane
Analytical Services

SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 27, No. 12, pp. 2539–2547, 2008

Printed in the USA
0730-7268/08 $12.00 + .00

EFFECTS, UPTAKE, AND FATE OF 2,4,6-TRINITROTOLUENE AGED IN SOIL IN PLANTS AND WORMS

ELLY P.H. BEST,*† HENRY E. TATEM,† KAAREN N. GETER,‡ MELISSA L. WELLS,† and BRIAN K. LANE‡
†U.S. Army Engineer Research and Development Center, Environmental Laboratory, 3909 Halls Ferry Road, Vicksburg, Mississippi 39180, USA
‡Analytical Services, 3532 Manor Drive, Suite 3, Vicksburg, Mississippi 39180, USA

(*Received* 9 *January* 2008; *Accepted* 17 *June* 2008)

Abstract—The present study was aimed at providing data to be used at predicting exposure-based effects of 2,4,6-trinitrotoluene (TNT) aged in soil on endpoint organisms representing two trophic levels. These data can be used to define criteria or reference values for environmental management and conducting specific risk assessment. Long-term exposure tests were conducted to evaluate sublethal toxicity and uptake of aged soil-based explosives, with TNT as the main contaminant. In these tests, plants were exposed for 55 d, and biomass and explosives residues were determined. Worms were exposed for 28 and 42 d, and biomass, number, and tissue residues were determined. Biomass of *Lolium perenne* significantly decreased with soil–TNT concentration, and an effective concentration causing a 20% decrease in biomass (EC20) for TNT metabolites of 3.75 mg/kg was calculated. The concentrations of TNT metabolites in shoots and roots were significantly related to concentrations in soil, as were concentrations of hexahydro-1,3,5-trinitro-1,3,5 triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7- tetrazocine (HMX). The mean bioconcentration factors, indicating the potential of a chemical to accumulate in an organism, were 0.9 for TNT metabolites, 71.8 for RDX, and 12.2 for HMX in *L. perenne* shoots. Biomass of *Eisenia fetida* adults significantly decreased with soil–TNT concentration, and an EC20 for TNT of 3.70 mg/kg was calculated. The TNT, RDX, and HMX levels in *E. fetida* were below detection.

Keywords—2,4,6-Trinitrotoluene Aged Bioavailability Plants Worms

INTRODUCTION

Explosives, including 2,4,6-trinitroluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), and associated byproducts and degradation compounds, have been released into the environment from munitions production and processing facilities. 2,4,6-Trinitroluene has been identified at 19 National Priority List for Superfund Cleanup sites across the United States [1]. These sites include U.S. Army Ammunition Plants and load, assemble, and pack processing sites. 2,4,6-Trinitroluene is also present in the environment as a result of decommissioning activities and through field usage and disposal activities such as open burning. It has been found in environmental media only in the vicinity of such sites. Explosive concentrations in contaminated soil are extremely heterogeneous, ranging from 0.08 to 87,000 mg/kg for TNT, from 0.7 to 74,000 mg/kg for RDX, and from 0.7 to 5,700 mg/kg dry weight for HMX [2].

The use of toxicity data is an important tool in predicting the effects of contaminants on populations, defining criteria or reference values for environmental management, and conducting site-specific risk assessment [3]. The U.S. Environmental Protection Agency is developing ecological screening level (Eco-SSL) values for some energetic materials, including TNT and RDX, on the basis of terrestrial plant and soil invertebrate data [4]. To determine if concentrations at a site might be harmful to the indigenous species, the maximum measured media-specific concentration can be compared with a criterion or screening benchmark. The criterion or benchmark is a concentration that should not result in adverse ecological effects to the populations of indigenous species. Both terrestrial plants and invertebrates are important, because they contribute to the functional aspects of the soil and because of their role in the food chain. For plants and soil invertebrates, lowest- or no-observed effect concentrations (LOECs or NOECs) have to be determined as a basis for these screening benchmarks. In plants effective concentrations causing a 20% decrease in biomass (EC20) are often used as a measure for a LOEC. In animals, concentrations causing 50% mortality (LC50s) have traditionally been used as a measure for toxicity. The preference for derivation of screening benchmarks from the toxicity data established from concentration–response relationships (EC estimates) compared with data from the analysis of variance (ANOVA)-type studies (LOEC and NOEC values) has been mentioned in many publications and in the Eco-SSL Guidance [4]. To date, effects-based ecotoxicological criteria for explosives-contaminated soil are extremely scarce. These would be required for safe management of the future use of decommissioned military training sites. Moreover, among the existing test data relating to TNT, concentration-dependent effects in plants in hydroponics do not compare well with effects in plants on soil, because the characteristics of the matrix differ. Also, effects on plants and worms of TNT spiked onto soil and of TNT from aged soil are difficult to compare. Spiking can cause solvent effects. The time allowed for evaporation of the solvent prior to incubation can permit a decrease in the concentration of the parent compound and the formation of undefined degradation compounds in the test. In aged soil, other contaminants, such as nitroaromatics and metals, may be present that may confound the TNT effects.

The published screening benchmark for TNT in soil for terrestrial plants is 30 mg/kg [2]. This value is based on the LOEC of 30 mg TNT/kg for aged soil in bush bean (*Phaseolus*

* To whom correspondence may be addressed (elly.p.best@erdc.usace.army.mil).
Published on the Web 7/11/2008.

Table 1. Characteristics of the soils used to create the test mixtures and controls. All values are mean ± standard deviation ($n = 5$ for explosives, $n = 3$ for other characteristics)[a]

Characteristic	Soil				
	Nebraska ordnance plant load and pack line, high-TNT	Nebraska ordnance plant reference[b]	Sharkey-clay[b]	Plant control	Worm control[b]
Explosives (mg/kg dry wt)					
TNT	170.85 ± 43.20	<1.684	ND	ND	ND
TNB	23.35 ± 5.54	<1.000	ND	ND	ND
RDX	1,512.50 ± 246.95	<3.122	ND	ND	ND
HMX	150.30 ± 29.68	<1.913	ND	ND	ND
Nutrients (mg/kg dry wt)					
Nitrate-N	ND	117.93 ± 15.71		122.6 ± 26.1	3.93
Infinite-sink P	76.2 ± 18.8	0.98 ± 0.24		14.3 ± 9.8	0.6 ± 0.05
Total K	ND	1,339		ND	5.3 ± 1.2
Other					
pH_{water}	5.44 ± 0.07	6.51 ± 0.04	5.62 ± 0.06	5.79	7.06 ± 0.08
Organic matter (% dry wt)	3.37 ± 0.31	5.22 ± 0.06	5.83 ± 3.01	76.29	1.33 ± 0.16
Dry weight (% fresh wt)	89.88 ± 0.08	90.55 ± 0.15	86.91 ± 0.07	41.8	99.6 ± 0.24
Bulk density (g dry wt/ml)	2.52 ± 0.20	2.17 ± 0.22	0.95 ± 0.06	9.3	2.39 ± 0.25

[a] TNT = 2,4,6-trinitrotoluene; TNB = trinitrobenzoic acid; RDX = hexahydro-1,3,5-trinitro-1,3,5 triazine; HMX = octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine; ND = not determined.
[b] These soils were not subjected to analyses of explosives and organics since no prior history of exposure existed.

vulgaris) [5]. The published screening benchmarks for TNT in soil for soil invertebrates are 140 mg/kg for earthworms, 37 mg/kg for potworms [6], and 200 mg/kg for other invertebrates [2]. The benchmark for earthworms is based on the LOEC of 140 mg TNT/kg for spiked artificial soil [7]. The benchmark for soil nematodes and arthropods is based on a 7-d LOEC of 200 mg TNT/kg for spiked forest soil [8].

The response of plants to TNT exposure depends on the way TNT is administered (spiked or aged), matrix (hydroponics or soil), and plant species. In this paper, the term ''spiked'' is used for an uncontaminated soil amended with a chemical for the experiment, and the term ''aged'' for a contaminated soil originating from a field site and used directly or after mixing with other soil types for the experiment. No indication of a difference in TNT sensitivity [9] or TNT uptake and transformation [10] between dicotyledonous and monocotyledonous plants has been identified. Uptake of TNT by plants takes place through roots, and translocation to other plant organs is limited. Metabolism of TNT by the plant usually occurs by reduction to 2-amino-4,6-dinitrotoluene (2ADNT), 4-amino-4,6-dinitrotoluene (4ADNT) [11,12], 2,4-dinitrotoluene (2,4DNT), and 2,6-dinitrotoluene [12,13]. Residues of TNT are rarely recovered, but have been identified in shoots and belowground organs exposed to concentrated aqueous TNT solutions (20 mg/L) [11] and in roots exposed to elevated soil–TNT concentrations (471–1,920 mg/kg soil dry wt) [14]. The response of worms to TNT exposure also depends on the way TNT is administered (spiked or aged), matrix (artificial or forest soil), and worm species. Worms are less sensitive to other explosive parent compounds that often co-occur with TNT, such as RDX and HMX [7]. Earthworms reduce TNT [15], and residues that have been identified are 2ADNT and 4ADNT [16].

Our present study is aimed at providing data that can be used to predict the exposure-based effects of aged TNT in soil on multiple endpoint organisms representing two trophic levels of a terrestrial food chain. These data can be utilized for defining criteria or reference values for environmental management and conducting specific risk assessment. Dose–response experiments formed the basis for the evaluation of toxic effects and uptake of contaminants from soil into two trophic levels, taking bioavailability modifying soil characteristics into account.

MATERIALS AND METHODS

Experimental

A sublethal toxicity test was carried out to evaluate the effects and uptake of TNT contamination aged in soil on the test organisms. Dose–response curves for TNT concentrations between 0 and 18 mg/kg dry weight were constructed for both plant and animal tests. The concentration of 18 mg TNT/kg dry weight was considered high enough to cause a decrease in biomass in both plants and worms, since some plant and worm species died at TNT concentrations of approximately 19 mg/kg in tests published by others [17], while our test organisms survived short-term exposures. Moreover, a concentration of 17.2 mg TNT/kg soil dry weight was the remedial cleanup goal identified for soil at the Nebraska Ordnance Plant, Nebraska, USA, based on risk assessment data [18]. Three soils were mixed for the tests. The TNT-contaminated and reference soils both originated from the Nebraska Ordnance Plant and Sharkey-clay from Vicksburg, Mississippi, USA. The Nebraska Ordnance Plant soil was of the Sharpsburg–Fillmore association, comprised of mostly Sharpsburg silty loam on well-drained locations. Control potting soil served as a test to verify plant performance, and control soil according to Organization for Economic Cooperation and Development guidelines [19] to verify worm performance (Table 1). The clay amendment was used to evaluate a tentative bioavailability decreasing effect of clay on the explosives' toxicity and uptake in the test organisms. The availability of the aged TNT-contaminated soil was limited, and, therefore, only two clay amendments were done. All treatments were replicated five times and followed a randomized block design. Plant and worm studies each included a total of 70 test units: One reference

soil, times two species, times two clay amendments, times five replicates (20 units); three TNT treatments times two species times five replicates (30 units); two TNT and clay treatments times two species times five replicates (20 units). The following responses were measured: in plants, toxicity as plant biomass formed in 55 d (measured as g dry wt per pot, and reported as g dry wt/m^2 to facilitate use of the data on a field-scale) and accumulation as plant tissue concentrations of explosives accumulated in 55 d (mg/kg dry wt); in worms, toxicity in earthworms as biomass (measured as g dry wt per 100-g-soil, and reported as g dry wt) and number of adults after 28 d, toxicity in potworms as number of juveniles after 42 d, and accumulation as earthworm tissue concentrations of explosives accumulated in 28 d (mg/kg dry wt). The three soils were spread and dried in the greenhouse to reach a moisture content of 5 to 10%. The explosives-contaminated soil was crushed and thoroughly mixed. The reference and clay soils were ground to pass a 2-mm sieve.

Test organisms

Two plant species were selected for the tests based on their worldwide use and general acceptance in standard test procedures [20–22]. The monocotyledonous *Lolium perenne* (*L. perenne*, perennial ryegrass) and dicotyledonous *Medicago sativa* (*M. sativa*, alfalfa); both have a wide geographical distribution, rapid growth, and profuse generative reproduction. In addition, their seeds germinate simultaneously within several days and the species can be cultivated in the testing environment. Both species are relatively insensitive towards organic contaminants and widely used as a response and bioaccumulating indicator for organics contamination of soils [23]. Both plant species are considered as moderately ecologically relevant test species and *L. perenne* as highly standardized [24].

Two worm species were selected, also based on their worldwide use in standard test procedures, facilitating comparison with bioaccumulation and toxicity data of other sites, and ease of culture under laboratory conditions. The earthworm *Eisenia fetida* (*E. fetida*) and the enchytraeid potworm *Enchytraeus crypticus* (*E. crypticus*). Both worm species are suitable bioaccumulation and response indicators for metals as well as organics and are relatively insensitive [25]. Earthworms are of low ecological relevance and are highly standardized; potworms are highly ecologically relevant and are moderately standardized test species [25].

Plant exposure

Seeds of *L. perenne* var. Linn and *M. sativa* var. Ladack were purchased from the Granite Seed Company (Lehi, Utah, USA). For each *L. perenne* unit, 0.230 g of seeds (200) was weighed and placed on top of 1 L (1,580 g fresh wt) of the appropriate soil mixture contained in 2-L plastic pots. For each *M. sativa* unit, 0.201 g of seeds was seeded, without inoculation with nitrogen-fixing bacteria prior to sowing. Plants were cultivated in a greenhouse at the Waterways Experiment Station, Vicksburg, grounds. Irradiance and temperature followed ambient conditions during the period June 28 to August 22, 2002. The pots were watered daily with reverse osmosis (RO) water to maintain the soil at a moisture level of 36% (field capacity was 38%). A moisture level at field capacity allows maximum specific mass transport of contaminants with soil solution. Plants were amended with slow-release Osmocote® fertilizer 10 d (Scotts-Sierra Horticultural Products, Marysville, OH, USA) after onset of the experiment to attain target levels of 352 kg N/ha, 59.2 kg P/ha, and 331.9 kg/K ha, commonly used for pastures. They were harvested after 55 d of cultivation. Seeds germinated synchronously, as was verified before the onset of the tests.

Worm exposure

Adult earthworm specimens were taken from the Engineer Research and Development Center, Environmental Laboratory culture, originally purchased from Carolina Biological Supply Company (Burlington, NC, USA). Adult potworm specimens were taken from an Engineer Research and Development Center laboratory culture reared from a mass culture obtained from R. Kuperman (U.S. Army Aberdeen Proving Ground, MD, USA) in 2001. Food for both worm species was supplied regularly as needed. It was composed of rolled oats, purchased locally, and powdered earthworm food purchased from Magic Products (Amherst Junction, WI, USA). All units were moistened regularly with RO water. For earthworms, 10 specimens were placed on top of 100 g fresh weight of the appropriate soil mixture contained in a 250-ml glass Mason jar. Animals were cultivated under continuous fluorescent illumination at 20°C [25]. After 28 d adults were harvested. For potworms, 10 specimens were placed on top of 2 g fresh weight of the appropriate soil mixture contained in a 5-ml petri dish. The relatively low worm density, compared to the density recommended [26,27], was selected because results of prior range-finding experiments indicated that higher worm densities exceeded our counting ability. Animals were cultivated in darkness at 16°C. After 14 d adults were removed, and after 42 d the juveniles were harvested.

Sample processing

After incubation, plants and worms were harvested and freed from dust and soil particles by rinsing with RO water. Worms were counted, and earthworms were purged. After collecting, weighing, washing, purging, and reweighing were completed, plant and worm tissues were placed in plastic Ziploc® bags (SC Johnson, Racine, WI, USA) and frozen at −80°C. Subsamples were used to determine dry weight. Dry weight in plants was determined in subsamples by drying the fresh material in a forced-air oven to constant weight (105°C). Earthworm samples were freeze-dried.

Analyses

Explosives in plant and worm tissues and in soil were quantified using modifications of U.S. Environmental Protection Agency method 8330B for soils [28]. Plant extracts were prepared from fresh materials, worm extracts from freeze-dried materials, and soil extracts from fresh material. Three replicate samples of each treatment were extracted. Plants were clipped into small pieces and mixed. Subsamples for extraction were homogenized by grinding them in liquid nitrogen. Two-gram fresh weight portions were spiked with 4-nitrotoluene (4NT) as an internal standard for recovery (50 μl of a 1 mg/ml solution), heated at 100°C to remove water, and extracted in 5-ml acetonitrile by an 18-h sonication in a water-cooled bath at 15°C. Freeze-dried worm tissue aliquots, equivalent to 0.7 g fresh weight, were amended with 0.8 ml of acetonitrile in bead-beater vials, and extracted by two successive cycles of 1-min bead-beating at room temperature (22–24°C) and sonication for 1 h at 15°C. All extracts were freed from particles by centrifugation for 10 min at 2,000 *g*. Supernatants were

diluted 1:1 with Millipore-filtered RO water (Milli-Q, Bedford, MA, USA), recentrifuged, and cleaned up. Plant extracts were cleaned up over a 0.5-g Florisil® column (U.S. Silica, Berkeley Springs, WV, USA); worm extracts, over a 0.45-μm polytetrafluroethylene disk. Each soil mixture was analyzed for explosives and other chemical and physical characteristics just before incubation. Explosives in soil were determined by extracting a 2-g fresh weight aliquot in 10 ml of acetonitrile by 18-h sonication at 15°C, cleanup over a Florisil column, and 10× concentration. The plant, worm, and soil extracts were analyzed for explosives using high-performance liquid chromatography (HPLC) analysis. The HPLC separations were performed on an Agilent 1100 Series HPLC (Palo Alto, CA, USA) equipped with a quaternary pump, autosampler with a 200-μl loop injector, diode array ultraviolet absorbance detector, and a column oven. A Hypersil ODS reverse-phase C-18 HPLC column (100 by 4.6 mm; 5-μm particle size; Fisher Scientific, Waltham, MA, USA) was used as the primary column, along with the Hypersil ODS C-18 guard column (20 by 4 mm; 5-μm particle size). The column compartment was operated at 39°C and the flow rate of the mobile phase was 1.5 ml/min. The composition of the mobile phase was 68% 20 mM NH_4Cl, 31.4% methanol, and 0.6% *n*-butanol. The energetics were measured at 254 nm. The calibration curves of the standard compounds were linear between 0.1 and 50 mg/L for 1:1 (v/v) solutions of solvent and water. The extracts of the samples with the highest levels of energetics were first screened for the presence of all compounds listed by U.S. Environmental Protection Agency method 8330B. After identifying the energetics' parent compounds and metabolites in these extracts, only the relevant compounds were determined in all other extracts. The latter compounds were usually TNT, 2ADNT, 4ADNT, RDX, and HMX, and 4NT as internal standard. The method detection level in milligrams per kilogram dry weight for several target compounds, spiked on plants, worms, and soil directly before extraction, varied with compound. In freshly ground plant tissues, the detection levels were as follows: TNT, 0.081; 2ADNT, 0.103; 4ADNT, 0.161; 4NT, 0.314; RDX, 0.142; and HMX, 0.110 mg/kg dry weight. In freeze-dried worm tissue, the levels were TNT, 1.174; 2ADNT, 1.750; 4ADNT, 1.773; RDX, 2.176; and HMX, 1.645 mg/kg dry weight. In freshly ground soil, the levels were TNT, 1.684; 2ADNT, 3.043; 4ADNT, 1.225; RDX, 3.122; and HMX, 1.913 mg/kg dry weight. Recovery of 4NT ranged from 65 to 95% [29].

Data analysis

Statistical analyses were conducted with the software STATGRAPHICS Plus for Windows Version 32S package (Manugistics, Rockville, MD, USA). Normal distribution of the data was tested using Shapiro–Wilk's test. Analysis of variance was expanded with a multiple range test using Fisher's least significant difference procedure. The p value in the ANOVA is a measure of the significance of the analysis; it was set at a 95% confidence level (p value of ≤ 0.05). Regression models were used to estimate EC values as well as bioaccumulation. Linear and nonlinear equations were fitted with the regression module using the least squares method. For these analyses zero values for tissue levels of explosives were replaced by half of the detection levels. The p value in the regression model was set at a 95% confidence level (p value of ≤ 0.05) unless stated otherwise. The R^2 value of the regression model indicates the proportion of the variance explained by the model. Regression models explaining at least 50% of the variability in the data set, $R^2 > 0.50$, were considered as good fits. In ANOVAs and regression analyses the sum of plant tissue 2ADNT and 4ADNT concentrations was included as TNT-derived metabolites (recalculated on a molar basis).

RESULTS AND DISCUSSION

The concentrations of explosives in the seven soil mixtures ranged from nondetectable to 18 mg TNT/kg dry weight, 154 mg RDX/kg dry weight, and 17 mg HMX/kg dry weight at the beginning of the incubations (Table 2). The TNT, RDX, and HMX concentrations were significantly correlated, with correlation coefficients between TNT, RDX, and HMX of ≤97%. After a 55-d incubation with plants, the TNT levels were below the detection level of 1.684 mg/kg (nonextractable).

Toxicity and bioaccumulation in plants

Plant biomass of *L. perenne* was significantly reduced by soil-TNT concentration in all TNT treatments relative to control, including 18 mg/kg dry weight (ANOVA; $p < 0.001$). Although plant biomass was greater on the nonamended soils than on the clay-amended soils, the effect of clay amendment was not statistically significant (ANOVA; $p = 0.119$), and, therefore, data pertaining to plants exposed to nonamended and amended soils were included in the subsequent regression analysis. Regression models were fitted to the plant data to relate them to soil-TNT concentration. The linear regression showed a significant fit ($p < 0.001$) and explained 40% of the variability in the data set ($R^2 = 0.40$; Fig. 1). The linear regressions between the plant data and soil-RDX and soil-HMX were also significant but explained relatively less of the variability in the dataset. Plant biomass on the reference soil was similar to that on control soil, confirming that the test soil without contamination supported adequate plant growth (shoot and root weights on control soil were 43.92 ± 5.87 g dry wt/m^2 and 19.60 ± 2.26 g dry wt/m^2, respectively). According to the fitted linear regression equation, for soil-TNT concentrations up to 18 mg/kg, $y = 34.79 - 1.86x$, in which y = plant biomass (g dry wt/m^2) and x = soil-TNT concentration (mg TNT/kg soil-dry wt), a 20% reduction in plant biomass may occur at a soil-TNT concentration of 3.75 mg TNT/kg soil dry weight in *L. perenne* (EC20; 95% CI, 0.5–7.0). Plant biomass of *M. sativa* was substantial on the reference soil without and with clay amendment (2.14 to 1.83 g dry wt/m^2), but reduced to zero by soil-TNT concentrations ≥5.4 mg/kg dry weight. Since no concentration–response relationship was established within the TNT concentration range tested in this study, no EC value could be estimated for *M. sativa*. This plant material was not further analyzed. Because the relationships between the soil-TNT concentrations and plant and shoot biomass of *L. perenne*, established via regression models, explained larger proportions of the data sets (plants, $R^2 = 0.40$) than those between soil-RDX and soil-HMX concentrations (plants, RDX $R^2 = 0.23$; HMX $R^2 = 0.27$), the toxicity of the soil was attributed mainly to the contamination by TNT. However, the other explosives in the soil may have also contributed.

The parent compound TNT was not recovered from the *L. perenne* plant material. However, concentrations of the TNT metabolites 2ADNT and 4ADNT up to 19 mg/kg dry weight were found in the shoots of plants exposed to the 10-mg TNT and 18-mg TNT soil mixtures. Concentrations of TNT metab-

Table 2. Characteristics of the soil mixtures prior to incubation. Target TNT concentrations, in mg/kg dry weight. All values are mean ± standard deviation (n = 5 for explosives, n = 3 for other characteristics)[a]

Characteristic	Soil mixtures						
	Reference,[b] 0	Reference,[b] 0 + clay[c]	TNT, 5 mg	TNT,[b] 5 mg + clay[c]	TNT, 10 mg	TNT, 10 mg + clay[c]	TNT, 18 mg
Explosives (mg/kg dry wt)							
TNT	<1.684	<1.684	5.40 ± 2.76	4.33 ± 3.36	10.30 ± 33.40	8.90 ± 4.16	18.02 ± 8.06
2ADNT	<3.043	<3.043	0.95 ± 0.93	0.96 ± 0.16	2.70 ± 1.86	1.12 ± 1.05	3.69 ± 1.43
4ADNT	<1.225	<1.225	<1.225	<1.225	0.26 ± 0.59	<1.225	0.37 ± 0.34
TNB	<1.000	<1.000	<1.000	0.67 ± 0.77	6.86 ± 9.99	0.75 ± 1.02	2.04 ± 1.08
RDX	<3.122	<3.122	8.57 ± 0.73	11.45 ± 3.04	59.05 ± 11.21	59.34 ± 12.31	153.86 ± 4.22
HMX	<1.913	<1.913	1.54 ± 0.12	1.72 ± 0.40	6.51 ± 0.13	7.48 ± 1.45	17.15 ± 0.56
Nutrients (mg/kg dry wt)							
Nitrate-N	117.93 ± 15.71	94.91 ± 1.66	86.30 ± 0.75	88.00 ± 11.45	68.86 ± 11.30	74.21 ± 0.76	79.35 ± 1.60
Infinite-sink P	0.98 ± 0.24	1.89 ± 0.31	1.35 ± 0.40	1.80 ± 0.14	1.46 ± 0.40	4.67 ± 1.45	7.63 ± 2.08
Other							
pH_{water}	6.51 ± 0.04	6.53 ± 0.02	6.61 ± 0.01	6.56 ± 0.02	6.57 ± 0.01	6.55 ± 0.02	6.15 ± 0.13
Organic matter (% dry wt)	5.22 ± 0.06	5.25 ± 1.12	5.01 ± 0.22	4.63 ± 0.64	4.51 ± 0.25	3.78 ± 0.08	3.76 ± 0.11
Dry weight (% fresh wt)	90.55 ± 0.15	90.48 ± 0.14	91.95 ± 0.07	90.46 ± 0.48	91.59 ± 0.56	89.82 ± 0.37	89.91 ± 0.65
Bulk density (g dry wt/ml)	2.17 ± 0.22	2.47 ± 0.12	2.40 ± 0.24	1.92 ± 0.16	1.99 ± 0.14	2.10 ± 0.27	1.96 ± 0.04

[a] TNT = 2,4,6-trinitrotoluene; ADNT = amino-dinitrotoluene; TNB = trinitrobenzoic acid; RDX = hexahydro-1,3,5-trinitro-1,3,5 triazine; HMX = octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine.
[b] Clean Nebraska Ordnance Plant soil (NE, USA).
[c] Clean clay, used in amendments of 30% w/w.

olites in roots usually exceeded those in shoots, and were detectable in all plants exposed to TNT-contaminated soils (Fig. 2). Of the other explosives and metabolites initially present in the soil mixtures, both RDX and HMX were recovered from the plant material, but TNB was not. RDX and HMX accumulated in the plants to an extent that greatly exceeded the concentrations in the soil mixtures: RDX mainly in shoots, and HMX in shoots and roots (Fig. 2). The TNT metabolite concentrations in shoots increased significantly with the soil-TNT concentrations (R^2 = 0.53; Fig. 2). The TNT metabolite concentrations in roots also increased significantly with soil-TNT, but the variability in the dataset was greater (R^2 = 0.46; Fig. 2). The following regression equations were fitted to the data and can be used to predict tissue explosive levels in plants upon exposure to soils contaminated with explosives, in which y = the tissue concentration of explosives (in mg/kg dry wt), and x = the concentration of explosives in the soil (in mg/kg dry wt). In *L. perenne* shoot: for soil-TNT concentrations up to a level of 18 mg/kg, $y = -3.61 + 0.24x$, with y being the shoot-TNT metabolite concentration; for soil-RDX concentrations up to a level of 154 mg/kg, $y = 938.43 + 26.47x$; for soil-HMX concentrations up to a level of 17 mg/kg, $y = 18.09 + 4.98x$. In *L. perenne* roots: for soil-TNT, $y = -0.86 + 6.20x$, with y being the root-TNT metabolite concentration; for soil-RDX, $y = 299.88 + 16.01x$; for soil-HMX, $y = 9.06 + 4.92x$.

Toxicity and bioaccumulation in worms

Survival of *E. fetida* after 28-d exposure was usually 60 to 100%, except upon exposure to 18 mg TNT/kg soil dry weight when all worms died. There was a significant (ANOVA; $p < 0.001$) weight and number loss in earthworms exposed to soil-TNT concentrations >5 mg/kg (Fig. 1). The effect of clay amendment was significant ($p < 0.001$), and, therefore, only data pertaining to worms exposed to nonamended soils were included in the subsequent regression analysis. Weight loss was related to increasing soil-TNT concentration, and the linear regression model fitted to the biomass values explained 91% of the variability in the dataset, whereas the fit for clay-amended soil was not significant (for soil-TNT, $p = 0.298$; for soil-RDX, $p = 0.338$; for soil-HMX, $p = 0.269$). According to the fitted regression equation for nonamended soil-TNT, $y = 0.83 - 0.05x$, in which y = earthworm biomass (g dry wt per jar) and x = soil-TNT concentration (mg TNT/kg soil dry wt), the EC20 for earthworm is 3.70 mg TNT/kg soil dry weight (95% CI, 2.60–4.80). Earthworm number also significantly decreased with increasing soil-TNT concentration, and the linear regression model for the worm number was $y = 10.90 - 0.57x$ ($p < 0.001$, $R^2 = 0.78$), in which y = worm number (n per jar), with the fit for clay-amended soil being nonsignificant. Earthworm biomass on reference soil was similar to that on control soil, confirming that the test soil supported adequate worm growth (worm wt on control soil was 0.791 ± 0.075 g dry wt). *Enchytraeus crypticus* survived in the control and reference treatments, but numbers were reduced to zero after 42-d exposure at a concentration ≥4.3 mg TNT/kg soil dry weight. Since no concentration–response relationship was established within the TNT concentration range tested in this study, no EC value could be estimated for potworm. None of the explosives and metabolites identified in the soil mixtures prior to incubation, were detected in earthworms while potworms were not further analyzed by lack of mass available for analysis. Although the relationships between the

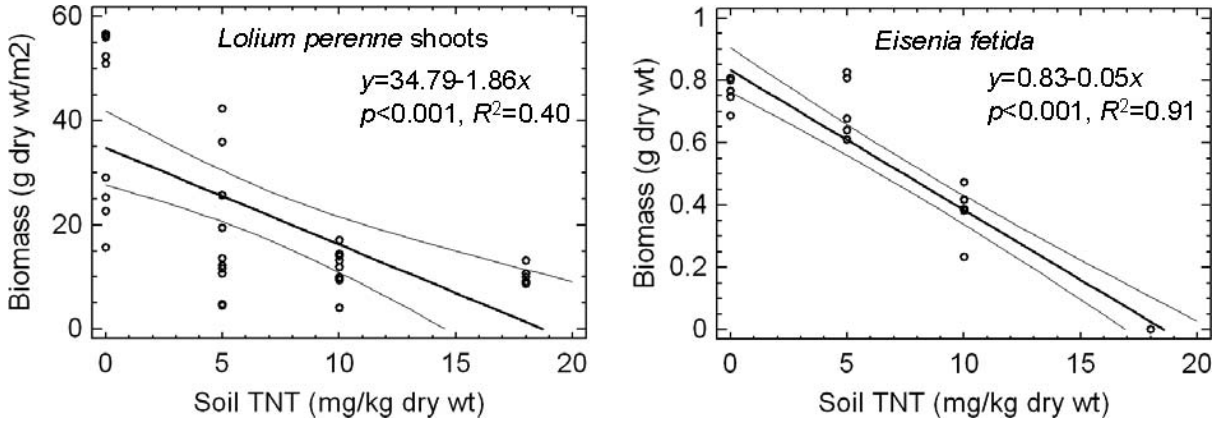

Fig. 1. Relationships between concentrations of aged explosives in the soil, and biomass in *Lolium perenne* and *Eisenia fetida*. Lines represent regression lines and 95% confidence levels. Regression equation, significance (p), and proportion of variance explained (R^2) by the fitted model are indicated. TNT = 2,4,6-trinitrotoluene.

soil–TNT concentrations and earthworm biomass, established via regression, explained similar proportions of the data sets as those between soil–RDX and soil–HMX concentrations (TNT $R^2 = 0.91$; RDX $R^2 = 0.94$; HMX $R^2 = 0.94$), the toxicity of the soil was attributed to TNT contamination because the worms died upon exposure to 18 mg/kg soil dry weight in the present experiment where concomitant levels of RDX and HMX were 154 mg/kg and 17 mg/kg soil dry weight; whereas worms proved to tolerate RDX levels up to 1,540 mg/kg and HMX-levels up to 41 mg/kg soil dry weight in a previous study [30].

Toxicity screening benchmarks

The LOECs for TNT based on the presently found EC20 values for plants and worms are considerably lower than the published screening benchmarks [2]. For plants, the presently found EC20 value of 3.75 mg/kg soil dry weight for *L. perenne* was less than the screening benchmark of 30 mg/kg for bush bean exposed to TNT-contaminated, aged soil. It is also lower than recently published 16- to 19-d EC20s for *L. perenne* and *M. sativa* of 43 to 61 mg/kg soil dry weight, in which soils were TNT-amended [31]. However, the presently found EC20 for *L. perenne* is higher than the LOEC of 0.3 mg/kg soil dry weight reported for *M. sativa* exposed to TNT weathered and aged in sandy loam soil for an even longer exposure period of 13 weeks [31]. The noted difference may be explained by differences in species-specific tolerance towards TNT, and possibly other explosives usually co-occurring with TNT in explosives-contaminated, aged soils, soil amendment method, and exposure duration. Since *L. perenne* and *M. sativa* proved to be relatively insensitive to RDX-contaminated, aged soil up to a concentration of 1,540 mg/kg soil [26], the only other explosive that might have contributed to the soil toxicity to these plants is HMX.

For worms, the presently found EC20 value of 3.70 mg/kg soil dry weight for *E. fetida*, is low compared to the screening benchmarks for soil invertebrates, which range from 37 to 200 mg/kg in earthworms, nematodes, and arthropods exposed to soil-TNT. It is also less than the LOEC of 7 mg/kg TNT in aged soil containing several other explosives besides TNT, reported for earthworms [17]. The latter difference may be explained by the spiked soils and short exposure periods employed in the tests on which the published screening benchmark for earthworm is based. However, part of the observed toxicity may be attributed to the effect of RDX. Concentrations of RDX in the present tests ranged from 8.6 to 155 mg/kg soil, which exceeded the EC20-values of 1.2 and 1.6 mg/kg for cocoon and juvenile production, respectively, by *E. fetida* in freshly amended sandy loam soil [32].

Bioconcentration and biotransfer of explosives

To predict the effects of contaminants on populations and in food chains, it is important to evaluate the biotransfer of the contaminant as a basis for potential trophic transfer in the ecological groups constituting the food chain. Empirical data on the biotransfer of explosives in lower trophic levels such as plants and worms are extremely scarce, and in the absence of these data ecological risk approaches are based on linear regression equations derived from residues of organics other than explosives in vegetation and beef [33]. The latter approach assumes that the potential of a chemical to accumulate in an organism, the bioconcentration factor, is defined as a chemical's concentration in an organism or tissue divided by its concentration in food. The RDX concentrations in plants increased upon exposure to RDX-contaminated soil up to a concentration of 856 mg/kg and decreased at higher soil concentrations. Bioconcentration factors in the current study were on average 0.9 (range, 0–1.7) for TNT metabolites, 71.8 (range, 19.2–108.3) for RDX, and 12.2 (range, 3.6–18.6) for HMX in plant shoots (Table 3). Bioconcentration factors were far higher in plants exposed to low explosive concentrations than in plants exposed to high concentrations of explosives. Bioconcentration factors in worms could not be calculated because levels of explosives in worms were below detection.

However, the biotransfer factor is more useful in risk assessment, since exposure of the organism to the chemical may occur through both food and water pathways. To relate tissue concentrations in the aboveground biomass of vegetation to soil concentrations of a contaminant, the following equation was used [33]: $\log B_v = 1.588 - 0.578 \log K_{OW}$, in which B_v is the biotransfer factor in herbaceous plant shoots and K_{OW} is the octanol–water partitioning coefficient of that particular contaminant. Similarly, to relate tissue concentrations in beef to soil concentrations of a contaminant, the following equation is used [33]: $\log B_b = -7.6 + \log K_{OW}$, in which B_b is the biotransfer factor in beef of that particular contaminant. Using these biotransfer factor equations, the following explosive concentrations would be expected: in plants, for TNT metabolites,

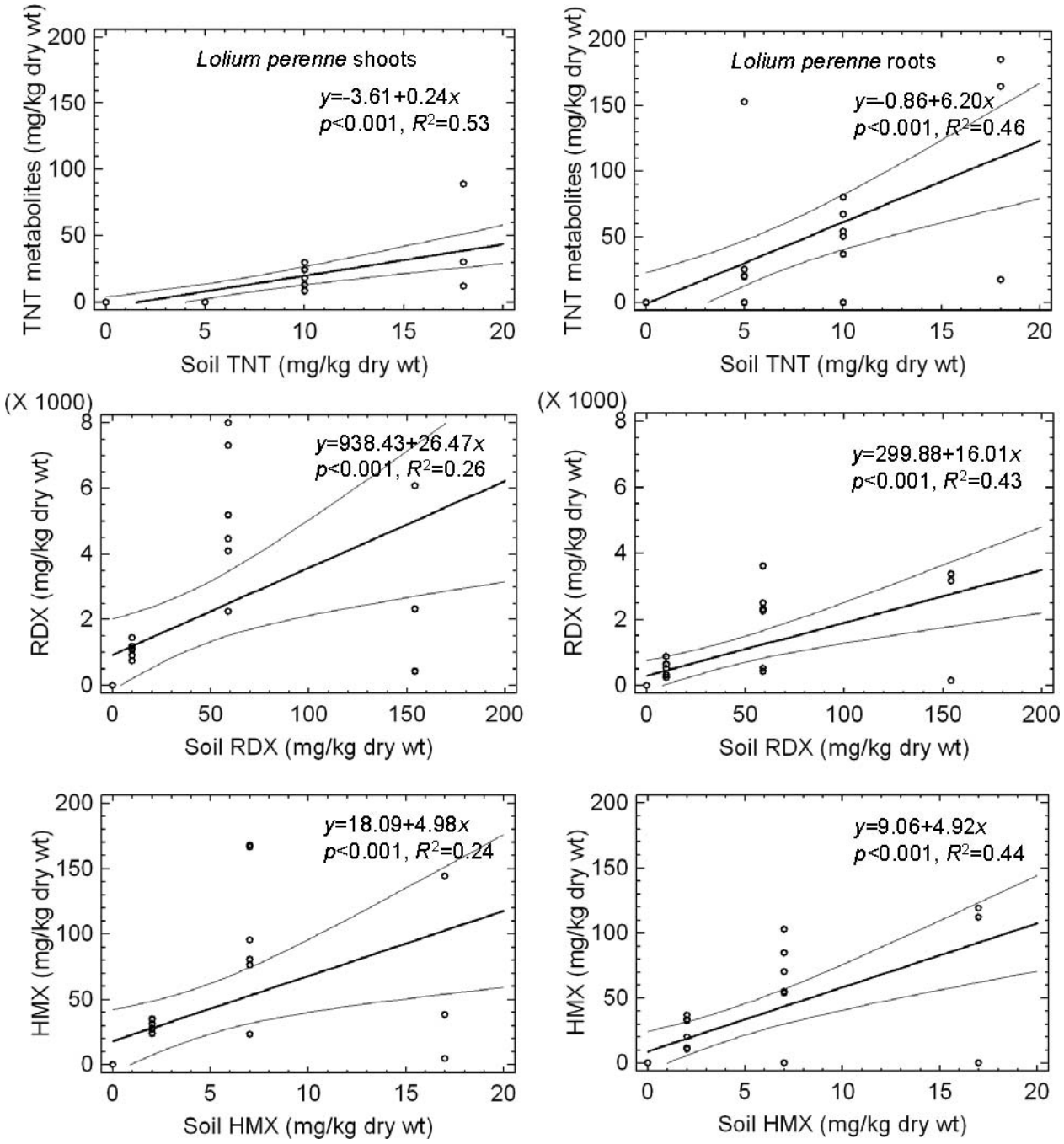

Fig. 2. Relationships between concentrations of aged explosives in the soil, and explosives and metabolite concentrations in *Lolium perenne*. Lines represent regression lines and 95% confidence levels. Regression equation, significance (p), and proportion of variance explained (R^2) by the fitted model are indicated. TNT = 2,4,6-trinitrotoluene; RDX = hexahydro-1,3,5-trinitro-1,3,5 triazine; HMX = octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine.

Table 3. Tissue concentrations of explosives TNT metabolites, RDX, and HMX, in mg/kg dry weight and bioconcentration factors (BCFs) in *Lolium perenne* shoots[a]

	Soil explosive (mg/kg dry wt)			Tissue explosive concentrations and BCF in *L. perenne* shoots		
Soil-mixture	TNT	RDX	HMX	TNT[a] metabolites	RDX	HMX
5-mg TNT	4.9	10.0	1.6	0 (0)	1,083 (108.3)	29.8 (18.6)
10-mg TNT	9.6	59.2	7.0	17.1 (1.7)	5,217 (88.1)	101.7 (14.5)
18-mg TNT	18.0	153.9	17.2	19.1 (1.1)	2,948 (19.2)	62.3 (3.6)
Overall mean	10.8	74.6	8.6	12.1 (0.9)	3,083 (71.8)	64.6 (12.2)

[a] TNT = 2,4,6-trinitrotoluene; RDX = hexahydro-1,3,5-trinitro-1,3,5 triazine; HMX = octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine.

19.1 to 82.9 mg/kg; for RDX, 1876.4 mg/kg; and for HMX, 470.3 to 607.1 mg/kg plant dry weight; in worms, for TNT, RDX, and HMX in the range of 10^{-5} to 10^{-3} mg/kg dry weight. The concentrations of explosives recovered from the plant shoots in the current study were in the calculated ranges for TNT metabolites and for RDX, but they were lower than calculated for HMX. The nondetectable explosive residue levels in the worms of the current study were in agreement with the calculated range, which is lower than the lowest explosive (metabolite) detection level attained by us (>1.1 mg/kg).

CONCLUSIONS

The present study provides data that can be used in predicting exposure-based effects of TNT in aged soil on two plant and two worm species. These data contribute to the full data set used for defining criteria or reference values for environmental management. An EC20 of 3.75 TNT mg/kg soil dry weight was found for *L. perenne*, but no concentration–response relationship was found for *M. sativa* because the latter plants died at TNT concentrations ≥5.4 mg/kg soil. An EC20 of 3.70 mg TNT/kg was found for *E. fetida* but no concentration–response relationship was found for *E. crypticus* because the potworms died at TNT concentrations ≥4.3 mg/kg soil. The TNT metabolite concentrations in plant shoots were significantly related to concentrations in soil after 55 d exposure. The mean bioconcentration factors were 0.9 for TNT (metabolites), 71.8 for RDX, and 12.2 for HMX in plant shoots. Explosive parent compounds and metabolites were below detection in the worms. The toxicity of the soil was attributed mainly to the contamination by TNT.

Acknowledgement—This research was funded by the U.S. Army Corps of Engineers Environmental Quality Technology Program. The present work was part of the Army Environmental Research and Development Center's Project Bioavailability, Uptake, Toxicity, and Transfer Across Trophic Levels of TNT and RDX in Aged Soil. Margaret Richmond (Analytical Services) provided partial technical assistance.

REFERENCES

1. Agency for Toxic Substances and Disease Registry. 1995. Toxicological profile for 2,4,6-trinitrotoluene. Public Health Service, U.S. Department of Health and Human Services, Washington, DC.
2. Talmage SS, Opresko DM, Maxwell CJ, Welsh CJE, Cretella FM, Reno PH, Daniel FB. 1999. Nitroaromatic munition compounds: Environmental effects and screening values. *Rev Environ Contam Toxicol* 161:1–156.
3. Renoux AY, Caumartin J, Thiboutot S, Ampleman G, Sunahara GI. 2001. Derivation of environmental soil quality guidelines for 2,4,6-trinitrotoluene in soil using the CCME approach. *Human and Ecological Risk Assessment* 7:1715–1735.
4. U.S. Environmental Protection Agency. 2005. Guidance for developing ecological soil screening levels. Office of Solid Waste and Emergency Response, Washington, DC.
5. Cataldo DA, Harvey SD, Fellows RJ, Bean RM, McVeety BD. 1989. An evaluation of the environmental fate and behavior of munitions material (TNT, RDX) in soil and plant systems. Technical Report PNL-7370, AD-A223 546. U.S. Army Medical Research and Development Command, Fort Detrick, MD.
6. Kuperman RG, Checkai RT, Simini M, Phillips CT, Anthony JS, Kurnas CW. 2005. Weathering and aging of TNT in soil increases toxicity to potworm *Enchytraeus crypticus*. *Environ Toxicol Chem* 24:2509–2518.
7. Phillips CT, Checkai RT, Wentsel RS. 1993. Toxicity of selected munitions and munition-contaminated soil on the earthworm (*Eisenia foetida*). Technical Report AD-A264408. Edgewood Research DEC, Aberdeen Proving Ground, MD, USA.
8. Parmelee RW, Wentsel RS, Phillips CT, Simini M, Checkai RT. 1993. Soil microcosm for testing the effects of chemical pollutants on soil fauna communities and trophic structure. *Environ Toxicol Chem* 12:1477–1486.
9. Gong P, Wilke B-M, Fleischmann S. 1999. Soil-based phytotoxicity of 2,4,6-trinitrotoluene (TNT) to terrestrial higher plants. *Arch Environ Contam Toxicol* 36:152–157.
10. Scheidemann P, Klunk A, Sens C, Werner D. 1998. Species dependent uptake and tolerance of nitroaromatic compounds by higher plants. *J Plant Physiol* 152:242–247.
11. Palazzo AJ, Legett DC. 1986. Effects and disposition of TNT in a terrestrial plant. *J Environ Qual* 15:49–52.
12. Sens C, Scheidemann P, Werner D. 1998. Distribution of 14C-TNT and derivatives in different biochemical compartments of *Phaseolus vulgaris*. *Environ Sci Pollut Res* 5:202–208.
13. Best EPH, Sprecher SL, Larson S, Fredrickson HL, Bader DF. 1999. Environmental behavior of explosives in groundwater from the Milan Army Ammunition Plant in aquatic and wetland plant treatments. Uptake and fate of TNT and RDX in plants. *Chemosphere* 39:2057–2072.
14. Thorne PG. 1999. Fate of explosives in plant tissues contaminated during phytoremediation. Special Report 99-19. U.S. Army Corps of Engineers Waterways Cold Regions Research and Engineering Laboratory, Hanover, NH.
15. Renoux AY, Sarrazin M, Hawari J, Sunahara GI. 2000. Transformation of 2,4,6-trinitrotoluene (TNT) in soil in the presence of the earthworm *Eisenia andrei*. *Environ Toxicol Chem* 19: 1473–1480.
16. Robidoux PY, Svendsen C, Sarrazin M, Hawari J, Thiboutot S, Ampleman G, Weeks JM, Sunahara GI. 2002. Evaluation of tissue and cellular biomarkers to assess 2,4,6-trinitrotoluene (TNT) exposure in earthworms: Effects-based assessment in laboratory studies using *Eisenia andrei*. *Biomarkers* 7:306–321.
17. Simini M, Wentsel RS, Checkai RT, Phillips CT, Chester NA, Major MA, Amos JC. 1995. Evaluation of soil toxicity at Joliet Army Ammunition Plant. *Environ Toxicol Chem* 14:623–630.
18. Price RA, Pennington JC, Larson SL, Neumann D, Hayes CA. 2002. Uptake of RDX and TNT by agronomic plants. *Soil Sediment Contam* 11:307–326.
19. Organization for Economic Co-operation and Development. 1984. Terrestrial plants, growth test. Guideline 208. Paris, France.
20. U.S. Environmental Protection Agency. 1996. Ecological effects test guidelines. OPPTS 850.4800 Plant Uptake and Translocation Test. EPA 712-C-96-159. Washington, DC.
21. U.S. Environmental Protection Agency. 1999. Data collection for the hazardous waste identification rule. Section 14.0 Ecological Benchmarks. Office of Solid Waste, Washington, DC.
22. American Society for Testing and Materials. 1999. Standard guide for conducting terrestrial plant toxicity tests. E1963-98. In *Annual Book of Standards,* Vol 11.05. West Conshohocken, PA, pp 1028–1046.
23. Gorsuch JW, Kringle RO, Robillard KA. 1990. Chemical effects on the germination and early growth of terrestrial plants. In Wang W, Gorsuch JW, Lower WR, eds, *Plants for Toxicity Assessment.* American Society for Testing and Materials, Philadelphia, PA, pp 49–58.
24. Markwiese JT, Ryti RT, Hooten MM, Michael DI, Hlohowskyj I. 2000. Toxicity bioassays for ecological risk assessment in arid and semiarid ecosystems. *Rev Environ Contam Toxicol* 168:43–98.
25. American Society for Testing and Materials. 1998. Standard guide for conducting laboratory soil toxicity or bioaccumulation tests with the lumbricid earthworm *Eisenia fetida*. E1676-97. In *Annual Book of ASTM Standards,* Vol 11.05. Philadelphia, PA, pp 1040–1058.
26. American Society for Testing and Materials. 2004. Standard guide for conducting laboratory soil toxicity or bioaccumulation tests with the Lumbricid earthworm *Eisenia fetida* and the enchytraeid potworm *Enchytraeus albidus*. E 1676-04. West Conshoshocken, PA.
27. International Organization for Standardization. 2005. Soil quality: Effects of pollutants on Enchytraeidae (*Enchytraeus* sp)—Determination of effects on reproduction and survival. ISO 16387: 2005, Geneva, Switzerland.
28. U.S. Environmental Protection Agency. 2006. Nitroaromatics, nitramines, and nitrate esters by high performance liquid chromatography (HPLC) Method 8330B. In *Test Methods for Evaluating*

Solid Waste, Physical/Chemical Methods, Office of Solid Waste and Emergency Response. SW-846. Washington, DC.
29. Best EPH, Tatem HE, Geter KN, Wells ML, Lane BK. 2004. Toxicity and metabolites of 2,4,6-trinitrotoluene (TNT) in plants and worms from exposure to aged soil. Technical Report ERDC/EL TR-04-18. U.S. Army Engineer Research and Development Center, Vicksburg, MS.
30. Best EPH, Geter KN, Tatem HE, Wells ML, Lane BK. 2006. Effects, transfer, and fate of RDX from aged soil in plants and worms. *Chemosphere* 62:616–625.
31. Rocheleau S, Kuperman RG, Martel M, Paquet L, Bardai G, Wong S, Sarrazin M, Dodard S, Gong P, Hawari J, Checkai RT, Sunahara GI. 2006. Phytotoxicity of nitroaromatic energetic compounds freshly amended or weathered and aged in sandy loam soil. *Chemosphere* 62:545–558.
32. Simini M, Checkai RT, Kuperman RG, Phillips CT, Kolakowski JE, Kurnas CW, Sunahara GI. 2003. Reproduction and survival of *Eisenia fetida* in a sandy loam soil amended with the nitroheterocyclic explosives RDX and HMX. *Pedobiologia* 47:657–662.
33. Travis CC, Arms AD. 1988. Bioconcentration of organics in beef, milk, and vegetation. *Environ Sci Technol* 22:217–274.