DEVELOPMENT OF ECOLOGICAL TOXICITY AND BIOMAGNIFICATION DATA FOR EXPLOSIVES CONTAMINANTS IN SOIL

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1. PROJECT BACKGROUND

Soil contamination was identified at more than 21,000 sites among Department of Defense (DoD) installations (Bridges and Whaley, 1997). By 2001, the number of known waste sites on current and former DoD installations in the United States exceeded 28,000. Many of these sites are associated with military operations that involve munition manufacturing, disposal, testing, and training contain elevated levels of explosives and related materials in soil. Concentrations of explosives in soil have been reported to exceed 87,000 mg kg^{-1} for TNT and 3,000 mg kg^{-1} for RDX or HMX (Simini *et al*., 1995). Although the energetic materials (EM) RDX and HMX are persistent and highly mobile in the environment, their effects on soil biota have not been sufficiently investigated. This presented a challenge for site managers who have to distinguish those sites that pose significant environmental risks from those that do not, prioritize contaminated sites by the level of risk posed, quantify the risks at each site, and develop appropriate remedial actions and cleanup goals. Recognizing a need for quantifying ecotoxicological benchmarks that can be used for development of scientifically based Ecological Soil Screening Levels (Eco-SSLs), the Strategic Environmental Research and Development Program (SERDP) has supported this research to extend the knowledge of the toxicity of explosives-related soil contaminants to ecological receptors, and to assess the potential for EM bioaccumulation in soil organisms that may affect higher level receptors through trophic chain transfer. Eco-SSL concentrations can be used in a Screening Level Ecological Risk Assessment (ERA) to identify those contaminants in soil that warrant additional evaluation in a Baseline ERA, and to eliminate those that do not. Eco-SSLs are derived using published data generated from laboratory toxicity tests with different test species relevant to soil ecosystems. The Eco-SSL workgroup, after an extensive literature review (United States Environmental Protection Agency, USEPA, 2000), determined that there was insufficient information for EMs to generate Eco-SSLs for terrestrial plants and soil invertebrates, which necessitated these studies to fill the knowledge gap.

2. OBJECTIVES

The goal of this investigation was to obtain direct experimental data on toxicity and biomagnification potential of nitroamine and nitroaromatic compounds hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), and 1,3,5-trinitrobenzene (TNB) for terrestrial plants and soil invertebrates in soil with parameters (i.e., pH, organic matter, clay content, etc.) promoting a relatively high bioavailability of the energetic materials (EM). To further understand the environmental impacts of exposure to EM soil contaminants, phytogenotoxicity of dinitrotoluenes was assessed using the *Tradescantia* Micronucleus (Trad-MCN) bioassay. In order that Eco-SSLs are appropriately effects-based, receptor responses must be coupled with appropriate measures of chemical exposure that integrate chemical bioavailability. This project aimed at determining which chemical measure of exposure better correlates with toxicity by measuring EM concentrations as acetonitrile-extractable (total) and as the labile water-extractable (which was hypothesized to be more immediately bioavailable) chemical concentrations. Special consideration in assessing chemical toxicity for

ecotoxicological benchmark development was given to examining the effects of weathering and aging of contaminant EMs in soil on exposure effects for soil organisms. Simulated weathering/aging of amended soils was incorporated into experimental design of toxicity testing to produce a soil microenvironment more similar to field conditions. The ultimate goal of this project was to develop draft Eco-SSL values for the five EMs from the toxicity benchmark values generated for terrestrial plants and soil invertebrates. The goals of this research were achieved by addressing the following technical objectives:

- Quantifying the toxicity of RDX, HMX, 2,4-DNT, 2,6-DNT and TNB to terrestrial plants and soil invertebrates using soil with parameters promoting a relatively high bioavailability of the EMs
- Evaluating soil extraction methods to determine which chemical measure of exposure better correlates with toxicity
- Examining the effect of a simulated weathering/aging process on EM toxicity
- Assessing EM bioaccumulation potential in terrestrial plants and soil invertebrates
- Assessing phytogenotoxicity potential of dinitrotoluenes 2,4-DNT and 2,6-DNT using the *Tradescantia* Micronucleus (Trad-MCN) bioassay
- Developing draft Eco-SSLs for RDX, HMX, 2,4-DNT, 2,6-DNT and TNB for terrestrial plants and soil invertebrates, based upon concentration-response relationships established during these studies

3. TECHNICAL APPROACH

The USEPA in conjunction with stakeholders is developing Eco-SSLs for contaminants frequently found at Superfund sites. Eco-SSLs are defined as concentrations of chemicals in soil that, when not exceeded, will be protective of terrestrial ecosystems from unacceptable harmful effects. This study was designed to produce benchmark data for the development of Eco-SSLs for RDX, HMX, 2,4-DNT, 2,6-DNT and TNB for terrestrial plants and soil invertebrates, and meet specific criteria (USEPA, 2000), including: (1) tests were conducted in soil having physico-chemical characteristics that support relatively high bioavailability of chemicals; (2) experimental designs for laboratory studies were documented and appropriate; (3) both nominal and analytically determined concentrations of chemicals of interest were reported; (4) tests included both negative and positive controls; (5) tests that included growth measurement endpoint were used; (6) appropriate chemical dosing procedures were reported; (7) concentration-response relationships were reported; (8) statistical tests used to calculate the benchmark and level of significance were described; and (9) the origin of test species were specified and appropriate.

The project consisted of five interrelated parts, including (1) phytotoxicity assessments, (2) soil invertebrate toxicity assessments, (3) analytical determinations of EM concentrations in test media, (4) determination of bioaccumulation in terrestrial plants and earthworms, and (5) phytogenotoxicity assessments. The detailed methodology is described in respective appendices and is summarized in sections addressing: i) test soil, ii) test energetic materials, iii) preparation of soils, iv) chemical extractions and analyses, v) toxicity bioassays, vi)

bioaccumulation assays, vii) data analysis, and viii) phytogenotoxicity assessments. An overview of the technical approach to investigations is shown in Figure 1.

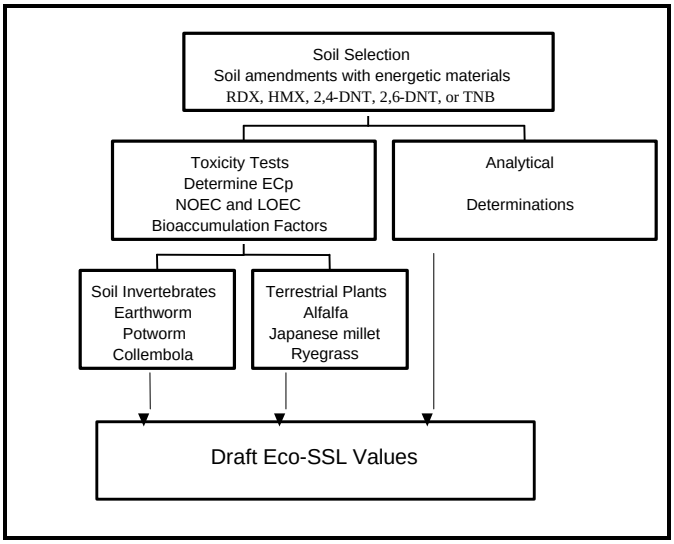

Figure 1. Overview of the technical approach to toxicity assessments and development of draft Ecological Soils Screening Levels for terrestrial plants and soil invertebrates.

3.1 Test Soil.

A natural soil, Sassafras sandy loam [Fine-loamy, siliceous, mesic Typic Hapludult] (SSL) was used in this study to assess the EM toxicity for the test species used. This soil was selected for developing ecotoxicological values protective of soil biota because it has physical and chemical characteristics supporting relatively high bioavailability of the test chemicals (low organic matter and clay contents). The SSL soil was collected from an open grassland field on the property of the U.S. Army Aberdeen Proving Ground (APG; Edgewood, MD). Vegetation and the organic horizon were removed to just below the root zone and the top six inches of the A horizon were then collected. The soil was sieved through a 5-mm^2 mesh screen, air-dried for at least 72 hours and mixed periodically to ensure uniform drying, passed through a 2-mm sieve for soil invertebrate testing, then stored at room temperature before use in testing. Soil was analyzed for physical and chemical characteristics by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD. Results of these analyses are presented in Table 1. Changes in SSL soil characteristics, including pH, redox potential, and cation exchange capacity (CEC) resulted from testing procedures are described in appendices for individual reports.

Table 1. Physical and chemical characteristics of Sassafras sandy loam soil analyzed by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD.

Soil Parameter	Sassafras Sandy Loam
Sand %	69
Silt %	13
Clay %	17
Texture	Sandy loam
CEC cmol kg^{-1}	5.5
Organic matter %	1.2
pH	5.2

3.2 Test Energetic Materials.

Energetic materials used in this investigation included nitroamines hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX; CAS: 121-82-4; Purity: 99%) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX; CAS: 2691-41-0; Purity: 99%), and nitroaromatic chemical intermediates of TNT production 2,4-dinitrotoluene (2,4-DNT; CAS: 121-14-2; Purity: 97%), 2,6-dinitrotoluene (2,6-DNT; CAS: 606-20-2; Purity: 98%), and 1,3,5-trinitrobenzene (TNB; CAS: 99-35-4; Purity: 99.7%). All EMs tested were obtained from the Defense Research Establishment Valcartier of the Canadian Ministry of National Defense (Val Bélair, QC, Canada).

3.3 Preparation of Soil.

Sassafras sandy loam (SSL) soil was individually amended with RDX, HMX, 2,4-DNT, 2,6-DNT or TNB. Prepared SSL soil was weighed separately for each treatment in a glass dish. For each treatment, soil was spread to a thickness of approximately 2.5 - 4 cm. Each concentration of EM was prepared separately in glass volumetric flasks and dissolved in acetone. This was necessary to dissolve the nonpolar chemicals, giving a more homogeneous mixture than the addition of solid chemical crystals to soil. The EM/acetone solution was quantitatively transferred to the soil evenly across the soil surface, ensuring that the volume of solution added at any one time did not exceed 15% (v m^{-1}) of the dry mass soil. After addition of the EM solution, the volumetric flask was rinsed twice with a known volume of acetone and this was also applied to the soil surface. If the total volume of solution needed to amend the soil exceeded 15% (v m^{-1}), the solution was added in successive stages, allowing the acetone to evaporate under a chemical hood for a minimum of 2 h. The same total EM/acetone solution volume was added to every treatment, equaling the volume required to dissolve the EM at the highest concentration tested. The amended soil was then air-dried overnight (minimum of 18 h) in a darkened chemical hood. Each amended soil sample was transferred into a high-density polyethylene container coated with fluoropolymer (Teflon-like chemical) and covered with aluminum foil, to prevent photolysis of the EM. The sample was mixed overnight (18 h) using a three-dimensional mixer. Soil was then

ready for the phytotoxicity assays. For the soil invertebrate toxicity assays, soil was hydrated with ASTM type I water to a moisture level required for individual test species and was allowed to equilibrate for 24 h before exposing test organisms.

Standardized methods for weathering/aging of EMs in soil are not available. We have developed approaches that simulate, at least partially, the weathering and aging process of chemicals in soil and more closely approximate the exposure effects on soil biota in the field. This included exposing both treated and control soils (prepared in the same manner as the freshly amended soil) hydrated to 60-75 percent of the water holding capacity (WHC) to alternating wetting and drying cycles for a period of 13 weeks in open chemically inert containers in the green house. All soil treatments were periodically weighed and readjusted to their initial mass by adding ASTM type I water. Hydration frequency varied from one to two times each week depending on the rate of soil drying. All soil treatments were brought to the appropriate soil moisture level required for individual tests 24 h prior to initiation of bioassays. The effect of weathering and aging on EM ecotoxicity was determined by comparing test results in weathered/aged and freshly amended soils.

3.4 Chemical Extractions and Analyses.

Acetonitrile extractions of soils were performed according to USEPA Method 8330A using freshly amended or weathered/aged amended soils, respectively. Samples for chemical analysis were taken after the 24-h hydration. For each treatment, 2.0 g soil was weighed in triplicate into 50-mL centrifuge tubes, 10 mL acetonitrile was added and the samples vortexed for 1 min, then sonicated in the dark for 18 h at 20°C. Five mL of sonicated sample were transferred to a glass tube, to which 5 mL of $CaCl_2$ solution (5 g L^{-1}) was added. Supernatant was filtered through 0.45 µm syringe cartridges. Soil extracts were analyzed and quantified using an HPLC. In this report, acetonitrile soil extraction is reported as the concentration in dry soil.

In addition to acetonitrile extraction, soil samples were extracted using an Adapted Toxicity Characteristic Leaching Procedure (ATCLP; Haley *et al*., 1993) at the beginning of each definitive test with freshly amended or weathered/aged amended soils. The ATCLP is based on modification of the Toxicity Characteristic Leaching Procedure (TCLP; 40 CFR Part 268.41, Hazardous Waste Management, Method 1311). The modification involved substitution of CO_2-saturated ASTM type I water for acetic acid, better simulating field soil-water conditions due to respiration by soil biota. Prior to ATCLP extraction, soil samples were equilibrated in the dark for 24 h at room temperature, after addition of ASTM type I water. All analytical measurements were done in triplicate at the beginning of each test. For each treatment concentration, 4 g of soil were transferred into 20 mL vials. Sixteen mL of CO_2-saturated water at pH 4.0 was added to the vials, and vials were rapidly sealed tight. Soil samples were vortexed 45 sec, then mixed in the dark for 18 h using a rotary mixer (30 rpm) at room temperature. Soil solids were allowed to settle and supernatants were filtered through 0.45 µm syringe cartridges. An equivalent volume of acetonitrile was added to filtered soil extract prior to HPLC analysis. In this report, ATCLP soil extraction is referred to as the water-soluble fraction of EM.

The soil and plant extracts were analyzed by HPLC using a modified EPA Method 8330A (USEPA, 1998). The method was modified in two ways. First, the final solvent for the energetic compounds was a mixture of 60 parts water and 40 parts acetonitrile rather than a 50:50 ratio. Secondly, the flow rate of the methanol:water mobile phase was 1.0 mL min^{-1} rather than 1.5 mL min^{-1}. A 25 cm x 4.6 mm x 5 micron particle size C-18 column was used for all determinations since only one energetic compound was analyzed at a time. In studies with soil invertebrates, the instrument used was a Beckman *System Gold*, consisting of a model 126 programmable solvent module, model 168 diode array detector and a model 507 automatic sampler. In studies with plants, a Thermo Separation Products chromatographic system composed of model P4000 pump, a model AS1000 injector, including temperature control for the column, and a model UV6000LP photodiode-array detector was used. Other differences in analytical procedures used by the U.S. Army ECBC and BRI groups are described in respective appendices. Calibration curves were generated before each HPLC run by dissolving certified standards (AccuStandard, Inc., New Haven, CT) in a range of concentrations appropriate for each run. The method detection limit was 50 ppb for each chemical. Blanks and standards were placed intermittently between unknown samples to maintain quality assurance of the samples. All reagents used in extraction of chemicals from soils were either reagent or trace metal grade, and ASTM Type I water was used throughout the analytical studies. Glassware was washed with phosphate-free detergent followed by rinses with tap water, ASTM type II water, nitric acid 1% (v/v) and, again with ASTM type I water.

3.5 Toxicity Assessments.

Toxicity assays were conducted to determine the effects of RDX, HMX, 2,4-DNT, 2,6-DNT and TNB on terrestrial plants and soil invertebrates. All assays included range-finding tests to bracket EM concentration range for each test species, and definitive tests to determine ecotoxicological benchmarks required for development of draft Eco-SSL values. Definitive toxicity tests for either terrestrial plants or soil invertebrates were conducted with three test species to comply with the Eco-SSL requirement of using multiple species for Eco-SSL development (USEPA, 2000). Each toxicity test was appropriately replicated and included negative (no chemicals added), positive (reference chemical), and carrier (acetone) controls. The complete study reports for terrestrial plant and soil invertebrate species are presented in appendices A-D.

3.5.1 Plant Toxicity Assays.

The plant toxicity assays were performed according to protocols of American Society for Testing and Materials (ASTM) standard guide for conducting terrestrial plant toxicity tests (ASTM, 1998a) and USEPA early seedling growth test (USEPA, 1982). Range-finding tests were performed using corn (*Zea mays*), lettuce (*Lactuva sativa*), alfalfa (*Medicago sativa*), perennial ryegrass (*Lolium perenne*) and Japanese millet (*Echinochloa crusgalli*). Twenty seeds of each plant species were sown per 10-cm pot containing 200 g dry soil, except for corn where 7 seeds were sown. The bottom of each plant pot was previously covered with a piece of cheesecloth to prevent soil loss during testing. Alfalfa seeds were inoculated with nitrogen-fixing bacteria prior to sowing. Thirty mL of ASTM type I water was added to obtain 75% of WHC.

Plant pots were placed in 1-L polyethylene bags closed with an elastic band to minimize loss of soil water due to evapotranspiration. Plant toxicity tests were performed in a temperature and light controlled growth chamber. Plants were incubated in the dark for the first two days and then exposed to a normal diurnal cycle afterwards. The growth chamber conditions were set as follows: light intensity at 5000 ± 500 lux, day time at 25°C for 16 h, night time at 20°C for 8 h. Luminosity level was measured weekly using a photometer and the light intensity was adjusted when needed. Based on the results of range-finding tests, definitive tests were performed using the three most sensitive plant species, including alfalfa, perennial ryegrass, and Japanese millet using six to nine treatment concentrations.

The numbers of emerged seedlings were counted after 5 days for alfalfa, Japanese millet and corn, and after 7 days for lettuce and ryegrass. Shoot number, shoot fresh mass, and shoot dry mass were measured after 16 days for alfalfa, Japanese millet and corn, and after 19 days for lettuce and ryegrass. Shoot dry mass was obtained after drying at 70°C for 24 ± 2 h. Reference toxicant, boric acid, was used as the positive control (ASTM, 1998a). Definitive toxicity tests were repeated when the percentage of germination in the controls were lower than 85% for ryegrass or Japanese millet, or lower than 70% for alfalfa, and when boric acid EC_{50} values were outside the quality control limit equivalent to EC_{50} average value ± 2 times standard deviation.

3.5.2 Soil Invertebrate Toxicity Assays.

The chronic 56-day earthworm reproduction assay was used assess the effects of EMs on earthworm *Eisenia fetida*. The test is an adaptation of an International Standardization Organization (ISO) bioassay, ISO/11268-2:1998 *Soil Quality – Effects of Pollutants on Earthworms (Eisenia fetida) – Part 2: Determination of Effects on Reproduction* (ISO, 1998a) that is based on test developed by Van Gestel *et al*. (1989). Guidelines for this assay was originally developed for use with Artificial Soil (USEPA Standard Artificial Soil), however research in our laboratory has shown that this assay could also be successfully conducted using natural soils (Kuperman *et al*., 1999; 2003). The measurement endpoints of this test included number of juveniles produced, number of cocoons produced, and adult survival.

Adult *E. fetida* were exposed to a range of concentrations of EM amended SSL soil. The test consisted of two steps. In a range-finding test (21 days), adult survival and cocoon production were assessed using five treatment concentrations and two replicates. Adult survival and cocoon production data from the range-finding test were used to determine the range of EMs concentrations for use in the definitive tests. In a definitive test (56 days), adult survival, adult live and dry weights, cocoon production, and juvenile production were assessed using a greater number of concentrations and replicates. In the definitive tests, surviving adult were counted and removed from the soil after 28 days. Soil with cocoons was incubated for additional 28 days. After 56 days from the start of the assay, cocoons and juveniles were harvested and counted. Ecotoxicological parameters were derived from regression analysis and Analysis of Variance. These parameters included the bounded No Observed Effect Concentration (NOEC), the Lowest Observed Effect Concentration (LOEC) values, and the effective concentration that caused a p percent reduction in adults, i.e. ECp (e.g., EC_{20}, and EC_{50}).

The Enchytraeid Reproduction Test (ERT) was used to assess the effects of EMs on the reproduction of the potworm *Enchytraeus crypticus*. The test is an adaptation of an ISO bioassay ISO/16387 *Soil quality — Effects of pollutants on Enchytraeidae (Enchytraeus sp.) — Determination of effects on reproduction and survival* (ISO, 2001). The ERT is a chronic assay. The ISO Guideline for this assay was originally developed for use with Artificial Soil (USEPA Standard Artificial Soil), however our research showed that this test could also be conducted using natural soils (Kuperman *et al*., 1999; 2003). The ISO ERT was initially developed using the enchytraeid worm species *Enchytraeus albidus*. Results of our previous studies using *E. albidus* showed that this species requires soils containing high organic matter content with a soil pH 6 (± 0.5) for optimal test conditions. This species performed poorly in natural soils with physical and chemical characteristics that support a higher level of EM bioavailability (Kuperman *et al*., 1999). The species of Enchytraeidae, *E. crypticus*, listed in the ISO protocol as an acceptable alternative to *E. albidus*, was selected for toxicity testing.

Adult *E. crypticus* were exposed to a range of EM concentrations added to SSL soil. The test consisted of two steps. They were a range-finding test in which adult survival and total number of juveniles produced were assessed using few treatment concentrations (five) and reduced number of replicates (two), and a definitive test in which the same endpoints were assessed using greater number of concentrations and replicates. The duration of each test was four weeks. After the first two weeks, the adult worms were removed, counted, and any morphological changes were recorded. After an additional two-week exposure, the number of juveniles produced was counted. The number of adults and juveniles in treatment concentrations were compared to those in the control treatments to quantify ecotoxicological parameters. These parameters included the bounded No Observed Effect Concentration (NOEC), the bounded Lowest Observed Effect Concentration (LOEC) and the effective concentration that caused a p percent reduction in juvenile numbers, ECp (e.g., EC_{20}, and EC_{50}).

The Folsomia Reproduction Test (FRT) was used to assess the effects of EMs on the reproduction of the collembolan *Folsomia candida*. The test is an adaptation of an ISO bioassay ISO/11267 *Soil quality —Inhibition of Reproduction of Collembola* (*Folsomia candida*) *by Soil Pollutants* (ISO, 1998b). The FRT is a chronic assay. The ISO Guideline for this assay was originally developed for use with Artificial Soil (USEPA Standard Artificial Soil). Research in our laboratory has shown that this test can also be conducted using natural soils (Phillips *et al*., 2002; Kuperman *et al*., 2003). The measurement endpoints for the test included adult survival and juvenile production.

Similar to earthworm and potworm assays, collembola were exposed to a range of EM concentrations added to SSL soil. The total number of juveniles produced and the survival of adult collembola were assessed. The duration of assay was 28 days. After 28 days both the number of adults and the number of juveniles were counted. The reproduction and survival of adults exposed to the test EMs were compared to that of the control treatments to quantify ecotoxicological parameters. These parameters included the bounded No Observed Effect Concentration (NOEC), the bounded Lowest Observed Effect Concentration (LOEC) and the effective concentration that caused a p percent reduction in juvenile numbers, i.e. ECp (e.g., EC_{20}, and EC_{50}).

3.6 Data Analysis.

Measurement endpoint data were analyzed using nonlinear regression models described in Stephenson *et al*. (2000) and Kuperman *et al*. (2003). The EC_{20} and EC_{50} values for seedling emergence and growth measurement endpoints in the phytotoxicity assays, and cocoon/juvenile production in the soil invertebrate reproduction assays were determined using SYSTAT software, version 7.0 (SPSS Inc., 1997). Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to decide whether to weight the data, and to select potential models. The following nonlinear regression models were used:

Exponential model: $Y = a \times e^{(([\log(1-p)] / \mathrm{ECp}) \times C)} + \mathrm{b}$

Logistic Gompertz model: $Y = a \times e^{([\log(1-p)] \times [C/\mathrm{ECp}]^{b})}$

Logistic Hormetic model: $Y = (t \times [1 + hC] / \{1 + [(p + h\,\mathrm{ECp}) / (1 - p)] \times [C/\mathrm{ECp}]^{b}\}$

where Y is the number for a measurement endpoint (e.g., number of juveniles, of emerged seedlings or the shoot mass), a is the control response, t is the control response in the hormetic model, e is the base of the natural logarithm, p is the percent inhibition/100 (e.g., 0.50 for EC_{50}), C is the exposure concentration in test soil, ECp is the estimate of effect concentration for a specified percent effect, h is the hormetic effect parameter, and b is the scale parameter. The ECp parameters used in this study included the EM concentration producing a 20% (EC_{20}) or 50% (EC_{50}) reduction in the measurement endpoint. The EC_{20} parameter based on a growth (for plants) or reproduction (for soil invertebrates) endpoint is the preferred parameter for deriving Eco-SSL values. The EC_{50}, a commonly reported value, was included to enable comparisons of the results produced in this study with results reported by other researchers. The asymptotic standard error (a.s.e.) and 95% confidence intervals (CI) associated with the point estimates were determined.

Analysis of Variance (ANOVA) was used to determine the bounded No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values. Mean separations were done using Fisher's Least Significant Difference (LSD) pairwise comparison tests. When NOAEC (no observed adverse effect concentration) or LOAEC (lowest observed adverse effect concentration) values were determined, which usually happened in tests with hormetic response at low exposure concentrations of chemicals, the same statistical methods were used. A significance level of $p < 0.05$ was accepted for determining the NOEC and LOEC values. Student's t-Test (two-tailed) with significance level set at $p < 0.05$ was used in the limit tests with plants and potworms exposed to RDX or HMX using EXCEL software (Microsoft Corporation, 1997). All analyses were done using measured EM concentrations.

3.7 Bioaccumulation.

Bioaccumulation potential of nitramine EM in terrestrial plants and in earthworm (*Eisenia andrei*) was assessed with [^{14}C]-RDX or [^{14}C]-HMX freshly amended SSL soil using a microcosm system designed for mass-balance studies. The use of radiolabeled compounds had an advantage of employing a mass-balance approach that allowed us to account for the portion of EMs undetectable by the USEPA Method 8330A due to mineralization, production of volatile metabolites, or fixation within the soil. The use of ^{14}C-labeled molecules also eliminated analytical problems associated with interference from other organic compounds during determination of RDX or HMX in soil or tissue samples. The radiolabeled [^{14}C]-RDX (specific activity = 54.4 µCi mmole^{-1}) and [^{14}C]-HMX (specific activity = 101.4 µCi mmole^{-1}) were provided by Dr. Guy Ampleman (Defense Research Establishment Valcartier, Val Bélair, QC, Canada).

A modified clear polycarbonate vacuum desiccator (Nalgene Part No. 5311-0250) was used to construct an enclosed system, a microcosm that can house the earthworms or plants (Figure 2). The microcosm was made pressure tight by using a metal rod and associated PTFE / rubber O-rings and nuts that joined the top and bottom sections of the modified desiccator. An access hole (3 mm) was drilled in the top to allow watering of plants, and to allow filling and emptying KOH traps. One of the ports was used to pump air while the second was connected as an outlet to a series of 3 tubes containing 10.0 mL of 0.25 M KOH to trap CO_2. A catalytic conversion unit made of potassium permanganate mixed with activated charcoal was used to convert putative volatile organic compounds into CO_2.

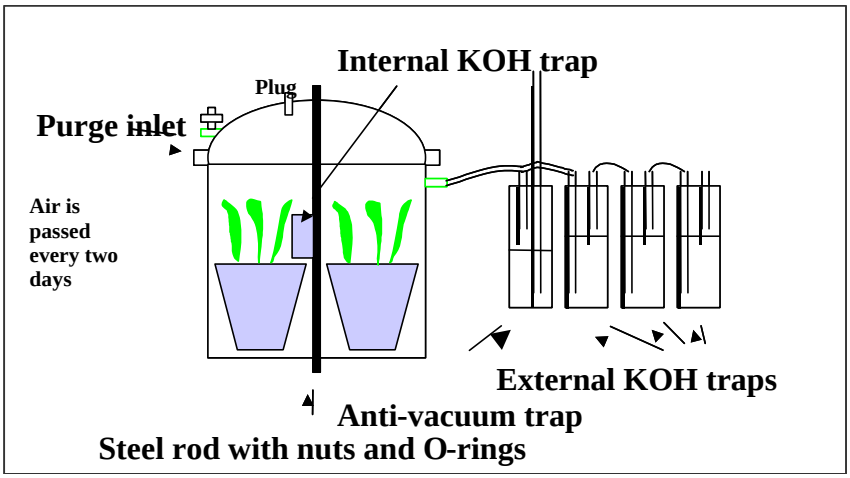

Figure 2. Microcosm design for assessing bioaccumulation of [^{14}C]-RDX or [^{14}C]-HMX in plants and earthworms.

In addition to using radiolabeled RDX and HMX, bioaccumulation potential of nitramine and nitroaromatic EM tested was determined using unlabeled materials and the USEPA Method 8330A. This approach allowed us to compare two methods to estimate RDX or

HMX bioaccumulation potentials, and provided bioaccumulation data for 2,4-DNT, 2,6-DNT, and TNB. Studies of bioaccumulation potential of nitramine and nitroaromatic EMs were conducted with freshly amended and weathered/aged EM amended SSL soil. This allowed us to examine the effect of weathering and aging of EM amended soils on the bioaccumulation potential of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB in plants. A draft of the report on the bioaccumulation studies is presented in Appendix E.

3.7.1 Bioaccumulation in Plants.

The plant bioaccumulation tests were performed according to modified protocols of ASTM standard guide for conducting terrestrial plant toxicity tests (ASTM, 1998a), and USEPA early seedling growth test (USEPA, 1982). Assays with non-radiolabeled compounds were performed using alfalfa (*Medicago sativa*), Japanese millet (*Echinochloa crusgalli*) and perennial ryegrass (*Lolium perenne*) selected from the five plant species tested in the range-finding toxicity tests as described in section 3.5.1. Assays with radiolabeled compounds were performed with corn, alfalfa, Japanese millet, lettuce and ryegrass. Bioaccumulation measurements were done using selected treatment concentrations of the five EMs tested. Two concentrations of each EM (except for RDX and HMX) were chosen, at which shoot mass was sufficient for extraction, and an onset of growth inhibition was evident from data generated in definitive toxicity assays.

The measured concentrations of TNB used in freshly amended soils for alfalfa were 2.58 ± 0.08 and 67.01 ± 2.04 mg kg^{-1}, for Japanese millet they were 2.58 ± 0.08 and 67.02 ± 1.09 mg kg^{-1} and for ryegrass they were 4.97 ± 0.18 and 112.18 ± 2.32 mg kg^{-1}. Concentrations of 2,4-DNT in freshly amended soils for alfalfa were 4.72 ± 0.11 and 46.52 ± 0.51 mg kg^{-1}, for Japanese millet they were 4.72 ± 0.11 and 21.48 ± 0.58 mg kg^{-1} and for ryegrass they were 1.03 ± 0.02 and 9.06 ± 0.22 mg kg^{-1}. Concentrations of 2,6-DNT used in freshly amended soils for alfalfa were 8.04 ± 0.26 and 13.89 ± 0.63 mg kg^{-1}, for Japanese millet they were 4.13 ± 0.05 and 13.89 ± 0.63 mg kg^{-1} and for ryegrass they were 4.13 ± 0.05 and 29.74 ± 1.42 mg kg^{-1}. A single concentration of 9740 ± 150 mg kg^{-1} RDX and of 10410 ± 810 mg kg^{-1} HMX was used for freshly amended soils for alfalfa, Japanese millet and ryegrass.

For weathered / aged soils, the measured concentrations were as follows: for TNB in alfalfa 22.10 ± 0.44 mg kg^{-1}, in Japanese millet 5.17 ± 0.17 mg kg^{-1}. It was found that after weathering/aging TNB amended at a nominal soil concentration of 5.0 mg kg^{-1} was no longer detectable in acetonitrile extracts of the soil. Measured concentrations of 0.32 ± .01 and 80.70 ± 1.89 mg kg^{-1} were used for ryegrass in weathered / aged soils. Concentrations of 2,4-DNT used for weathered / aged soils for alfalfa were 3.70 ± 0.23 and 14.86 ± 0.33 mg kg^{-1}, for Japanese millet and for ryegrass they were 3.70 ± 0.23 and 7.75 ± 0.11 mg kg^{-1}. Concentrations of 2,6-DNT used in weathered / aged soils for alfalfa were 0.60 ± 0.04 and 5.35 ± 0.12 mg kg^{-1}, for Japanese millet they were 1.16 ± 0.01 and 5.35 ± 0.12 mg kg^{-1} and for ryegrass they were 1.16 ± 0.01 and 14.93 ± 0.13 mg kg^{-1}. Finally, a single concentration of 9540 ± 210 mg kg^{-1} RDX and of 9340 ± 800 mg kg^{-1} HMX was used for weathered / aged soils for alfalfa, Japanese millet and ryegrass.

Acetonitrile extraction of EM amended soil was performed according to a modified USEPA Method # 8330A (USEPA, 1998) described in section 3.4. Plant tissue samples from four replicates of the two EM concentration treatments and from the negative controls were lyophilized individually in the dark for 24 h. Dried shoots were kept in a desiccator at a room temperature before weighing. At least 0.02 g of finely ground shoot was transferred to a glass conical tube, to which a volume of internal standard/acetonitrile mixture equating to 25 times dry biomass was added. For plants exposed to TNB, 2,4-DNT, or 2,6-DNT, 100 µL of 0.5 mg L^{-1} 1,3-DNB internal standard solution in acetonitrile was added. For plants exposed to RDX or HMX, the internal standard was 0.5 mg 2,4-DNT L^{-1}. Plant tissue extracts were sonicated in the dark at 20°C for 18 ± 2 h and then centrifuged at 1500 rpm (360 x g) for one hour. Supernatants were transferred into glass vials, to which an equal volume of ASTM type I water was added and kept at 4°C for 24 h. Supernatants were filtered using 0.45 µm cartridges and analyzed by HPLC.

For bioaccumulation studies with radiolabeled RDX and HMX, plant pots were placed inside microcosms and were incubated in the dark for 2 d at room temperature, and then transferred to the greenhouse. The light intensity was 4000 ± 500 lux. The microcosms were placed away from direct sunlight in the definitive tests to prevent large variations in temperature (observed inside the microcosms when they were placed near the greenhouse windows during range-finding tests). In bioaccumulation tests with radiolabeled RDX and HMX, samples were processed using essentially the same methods as for non-radiolabeled samples, except for the additional precautions related to work with radioactive materials (dedicated radionuclide-approved hood and analytical balance). Plant shoots were washed in distilled water and then placed on filter paper before being transferred to glass vials. Plants were then subjected to lyophilization. All calculations were expressed on the plant dry mass basis.

3.7.2 Bioaccumulation in Earthworms.

The earthworm bioaccumulation tests were performed according to a modified ASTM protocol for conducting laboratory soil toxicity or bioaccumulation tests with the lumbricid earthworm *Eisenia fetida* (ASTM, 1998b). Modifications included reducing the exposure from the recommended 28 d to 14 d to eliminate the need for feeding the earthworms, and using glass instead of plastic pots to avoid adsorption of EMs.

Earthworms *Eisenia andrei* used in this study were obtained from Carolina Biological Supply (Burlington, NC). They were maintained in earthworm bedding (Magic Products, Amherst, Jct, WI) at 20 ± 1°C, 70-80% (w/w) moisture and a 16 h : 8 h (light:dark) cycle, and fed with dry cereal (Magic Worm Food, Magic Products). Adult *E. andrei* used for the bioaccumulation tests had a well-developed clitellum and a wet mass ranging from 300 mg to 600 mg. Earthworms were acclimated for 24 h in non-amended SSL soil prior to the experiment. Ten earthworms were placed into each test unit (1-L glass jar), filled with 200 g of test soil (dry weight). Test units were prepared in triplicate for each concentration of chemical. Nominal concentrations for the definitive assays were 10 and 100 mg kg^{-1}. Measured concentrations were 11.2 ± 1.0 and 99.0 ± 6.9 mg kg^{-1} for RDX; and 8.5 ± 0.9 mg kg^{-1} and 83.0 ± 3.2 mg kg^{-1} for HMX. Each test unit was covered by perforated caps and a chemically inert porous geotextile (Landscape Fabric, Select) and placed in the microcosm. After a 14-d exposure, surviving

earthworms were counted, rinsed with ASTM type I water, and allowed to depurate for 24 h on moistened (5 mL of ASTM type I water) filter paper. Depurated earthworms were rinsed, blotted-dry, placed into Teflon tubes and were immediately frozen at –80°C. Soil was mixed and stored at –20°C until extracted with acetonitrile for HPLC analyses.

3.7.3 Chemical Analysis of Non-radiolabeled Samples.

Soil and plant extracts were analyzed using a Thermo Separation Products chromatographic system composed of model P4000 pump, a model AS1000 injector, including the temperature control for the column, and a model UV6000LP photodiode-array detector. For TNB, 2,4-DNT and 2,6-DNT analyses, a Supelcosil C8 column (25 cm x 4.6 mm ID, 5 μm particles) and an 18% 2-propanol / 82% water mobile phase were used. The flow rate was 1 mL min^{-1} and the run time was 40 min. For RDX and HMX analyses, the column used was a Supelcosil LC-CN (25 cm x 4.6 mm ID, 5 μm), which was held at 35°C. The initial solvent composition was 30% methanol / 70% water, which was held for 8 min, then a linear gradient was run from 30 to 65% methanol over 12 min. This solvent ratio was then changed to initial conditions (30% methanol) over 5 min. These initial conditions were then held for an additional 5 min. The injection volume was 50 μL. The detector was set to scan from 200 to 350 nm and chromatograms were extracted at 254 nm. The detection limit of the instrument was 50 μg L^{-1} for all chemicals. Precision was > 95% (SD < 2%, S/N = 10). The detection limit of the radiodetector was approximately 100 dpm.

3.7.4 Wet Combustion of Radiolabeled Samples.

Plant sample preparation prior to total combustion included an additional washing step in ASTM type I water. Leaves were left for 5-10 min on absorbent paper before their wet weight was measured. Preparation of roots involved separation of soil by soaking and repeated washing in ASTM type I water. Soil and plant samples were lyophilized before analysis. Soil samples were combusted after lyophilization. Plant samples were extracted using acetonitrile extraction-sonication method prior to combustion, and the extracts were analyzed by HPLC. Earthworm samples were combusted directly without lyophilization.

The combustion method was adapted from Allison (1960) as described by Nelson and Sommers (1982). A glass and PTFE apparatus was assembled (Figure 3), consisting of a 100 mL round bottom flask (A) with heating mantle, a gas inlet (B), a water filled jacket condenser (C) and an outlet (D) fitted with a separatory funnel (E) used for liquid transfer. The outlet was connected to a CO_2 trap consisting of 5 bubbling tubes (17 x 150 mm) attached in series. Each tube was filled with 10 mL of 0.25 M KOH containing a low concentration of thymolphtalein as pH indicator (for changes in the alkaline range).

The separatory funnel was filled with 10 mL of a mixture of 60% concentrated sulfuric acid and 40% concentrated phosphoric acid. A sample of dried material was weighed to the nearest 0.1 mg and transferred to a 100 mL flask. Sample weight was between 0.5 -1.2 g for soil, between 0.02 - 0.05 g for plants and approximately 0.2 g for earthworms (two lyophilized earthworms), respectively. One gram (± 5%) of potassium dichromate was then added, the flask

was attached to the condenser, and fitted with the heating mantle. After the dropwise addition of 10 mL of the concentrated sulfuric and phosphoric acids, the mixture was slowly heated. Nitrogen flow was turned on at a flow rate of approximately 2 bubbles per second. Heating was stopped after 10 min and nitrogen was passed at an increased flow rate (10-15 bubbles per second) for another 10 min. The contents of the KOH traps were mixed (traps 1 and 2, separately from 3, 4, and 5) and duplicate 2 mL aliquots of each were counted in 18 mL of scintillation counting fluid (ACS, Amersham, Oakville, Canada) in a Packard Tri-Carb 2100 TR scintillation counter.

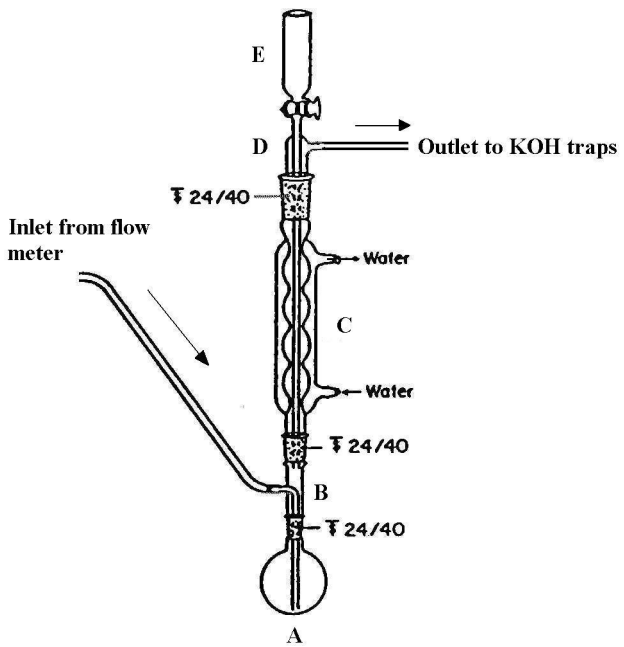

Figure 3. Apparatus used for measurement of [^{14}C]-RDX or [^{14}C]-HMX in plants, soil, and earthworms by wet combustion (modified from Nelson and Sommers, 1982).

3.7.5 Chemical Analysis of Radiolabeled Samples.

Determinations of radiolabeled RDX or HMX concentrations in earthworm tissue were performed using the method described in Renoux *et al.* (2000). Whole frozen earthworms were thawed in acetonitrile at room temperature for 15 min, then homogenized for 1 min (Kinematica Model CH-6010 with 10 mm probe, Kriens-Lu, Switzerland) at 4°C and vortexed for 1 min. Samples were then sonicated for 18 h and centrifuged (12000 x g) for 10 min at 4°C. An aliquot of the supernatant was combined with an equivalent volume of $CaCl_2$ (10 g L^{-1}) to precipitate fine particles. Samples were then left for 2 h at 4°C prior to filtration through a 0.45-µm cartridge (Millipore) for subsequent HPLC analysis.

Extracts were analyzed by HPLC using a chromatographic system composed of a Beckman System Gold Model 128 pump, a Beckman Model 166 UV detector, a Waters Model

717 Plus sample injector and a Waters temperature control module. A radiodetector (Model β-RAM, IN/US Systems Inc., Tampa, FL) was used with IN-Flow BD scintillation cocktail and Win-Flow software. A Supelcosil C8 column (25 cm × 4.6 mm i.d., 5 μm particles) was used for separation at 35°C. The mobile phase composition was 82% water and 18% 2-propanol (v/v), and the flow rate was 1 mL/min. The sample volume injected was 50 μL with a run time of 40 min. Analytes were separated by HPLC and detected simultaneously using a radiodetector and a UV detector set at 254 nm. The detection limit of the instrument (UV detector) was 50 μg L^{-1} for RDX or HMX. Chromatograms were examined for the presence of metabolites, which were identified on the basis of their retention time.

3.8 Phytogenotoxicity.

The *Tradescantia* micronucleus (Trad-MCN) assay was included in this project as a component of assessment of EM toxicity to terrestrial plants. The Trad-MCN bioassay measures the mutagenicity of environmental agents or radiation to living organisms. It uses the meiotic pollen mother cells of *Tradescantia* as the target cells and the chromosome breakage revealed in the form of micronuclei in the tetrads as the measurement endpoint. This assay is used for environmental genotoxicity detection and has been intensively reviewed under the Gene-Tox Program initiated by the USEPA's Office of Toxic Substances in 1980, and validated and standardized initially by the Collaborative Study on Plant Systems as part of the International Program on Chemical Safety (IPCS) from 1985 to 1992, and then by the International Program on Plant Bioassays (IPPB) that began in 1993 (Grant, 1999). Two nitraromatic EMs, including 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) were tested in water solutions using the Trad-MCN assay, and 2,4-DNT amended soil was also tested using a modified procedure of the Trad-MCN assay.

The Trad-MCN assay was conducted as described by Ma *et al*. (1994) with slight modifications. *Tradescantia* (clone 4430) cuttings (12-15) bearing young inflorescence were exposed to 200 mL of EM solutions or stored tap water for 6 h, and were then rinsed with ASTM Type I water and recovered in 200 mL of stored tap water for 24 h. The nominal concentration of test solutions were 0, 1.9, 3.8, 7.5, 15, 30, 60, 100, 150, 200 mg L^{-1} 2,4-DNT, and 0, 7.5, 15, 30, 60, 90, 120, 180 mg L^{-1} 2,6-DNT. During the entire course of exposure and recovery (30 h), aeration was provided to avoid possible oxygen depletion in solution. After recovery the inflorescences were fixed for 24 h in acetic acid:ethanol (1:3) solution and then transferred to 70% ethanol for storage. Cadmium chloride was used as positive control. Micronuclei (MCN) in the tetrad-stage pollen mother cells were determined. The assessment endpoint was the number of MCN per 100 tetrads. At least 5 slides containing early tetrads were made for each treatment and 300 tetrads were scored on each slide. Every experiment was repeated three or four times. Test solutions (prior to and right after exposure) as well as some of the post recovery tap water (recovery solution) were sampled for DNT determination.

Soils amended with 2,4-DNT were also tested for phytogenotoxicity. A known quantity of 2,4-DNT was dissolved in acetonitrile and applied to 50 g of dry SSL soil. Acetonitrile was allowed to volatilize, and 100 mL of de-chlorinated water was added to the soil. The slurry was mixed using a shaker at 250 rpm for 24 h to allow for the aqueous and solid

phases in soil slurries reached a steady state. Plant cuttings (12-15) were then immersed in the soil slurry and exposed for 6 h. Constant aeration was provided to supply oxygen and to keep the solution well mixed by the bubbling action. The test treatments included background control (fixed inflorescence right after cutting), negative control (SSL soil slurry with no added chemicals added), solvent control (SSL soil slurry treated with acetonitrile), 25, 250, 500, 1000, and 2000 mg kg^{-1} 2,4-DNT nominal concentrations. Chemical analysis confirmed the 2,4-DNT concentrations in soil prior to the test.

Chemical analyses used methods described in section 3.7.3. Statistical analyses included ANOVA and Dunnett's test to determine the bounded NOEC and LOEC values for induction of micronuclei (MCN), using ToxCalc™ Version 5.0 (Tidepool Scientific Software, McKinleyville, CA, USA). A draft of the report on the phytogenotoxicity studies is presented in Appendix F.

4. PROJECT ACCOMPLISHMENTS

4.1 Plant Toxicity Assays.

We assessed the toxicity of two explosives RDX and HMX, and three TNT by-products 2,4-DNT, 2,6-DNT and TNB to alfalfa, corn, lettuce, Japanese millet and ryegrass in a natural soil, Sassafras sandy loam. Preliminary range-finding tests identified the three plant species most sensitive to energetic materials tested and with performance parameters in SSL soil required by the validity criteria of standardized toxicity tests. These species included a dicotyledonous symbiotic species alfalfa, and two monocotyledonous species Japanese millet and ryegrass.

Nitro-heterocyclic explosives RDX or HMX did not adversely affect alfalfa, Japanese millet or ryegrass seedling emergence or growth at nominal concentrations of 10,000 mg kg^{-1} in the definitive limit tests with either freshly amended or weathered/aged amended SSL soil. Significant growth stimulation was observed in studies with Japanese millet and ryegrass exposed to these concentrations of RDX or HMX. Relatively low exposure concentrations of these energetic materials in pore waters of amended soil, resulting from their low solubility levels in water, may in part be responsible for these results. The solubility levels in water at 20°C of RDX and HMX are 42 and 6.6 mg L^{-1}, respectively (Sikka *et al.*, 1980; McLellan *et al.*, 1992).

Dinitrotoluenes (DNTs) and trinitrobenzene (TNB) adversely affected alfalfa, Japanese millet and ryegrass in the definitive toxicity tests at concentration ranges selected from the range-finding tests. Plant growth was a more sensitive endpoint compared with seedling emergence in both freshly amended and weathered/aged amended soils. Fresh shoot mass was a more sensitive measurement endpoint compared with dry shoot mass, as evidenced by lower EC_{20} and EC_{50} values for TNB, 2,4-DNT and 2,6-DNT in most tests. These results support strongly the USEPA decision of giving a higher priority to ecotoxicological benchmarks based on growth over other assessment endpoints (e.g., seedling emergence and root elongation) for developing Eco-SSLs for terrestrial plants (USEPA, 2000).

Definitive toxicity tests with both freshly amended and weathered/aged amended soils showed that EM toxicity order based on EC_{20} values for plant growth (fresh or dry shoot mass) in tests with alfalfa was 2,6-DNT > 2,4-DNT > TNB (Table 2). Toxicity order for these endpoints in tests with ryegrass was 2,4-DNT > 2,6-DNT > TNB. Toxicity order varied for Japanese millet depended on exposure type and measurement endpoint used. In freshly amended soil, toxicity order was 2,6-DNT > 2,4-DNT > TNB, based on dry mass, and 2,4-DNT > 2,6-DNT > TNB, based on fresh mass. In weathered/aged amended soils, toxicity order based on fresh or dry mass was TNB > 2,4-DNT ≥ 2,6-DNT. These results show that toxicity of these nitroaromatic energetic materials varied among the three test species and that the USEPA requirement of using multiple species for Eco-SSLs development is well justified.

Table 2. Summary of the plant growth NOEC, LOEC and EC_{20} values (mg kg^{-1}) for freshly amended (F/A) and weathered/aged (W/A) TNB, 2,4-DNT or 2,6-DNT amended Sassafras sandy loam soil.

Exposure type	Fresh shoot growth (n=4)			Dry shoot growth (n=4)		
	NOEC	LOEC	EC_{20} (95% C.I.)	NOEC	LOEC	EC_{20} (95% C.I.)
F/A 2,4-DNT						
Alfalfa	<5	5**	11 (0-24)	<5	5**	34 (10-59)
Japanese millet	1	5	4 (2-5)	5	9	25 (18-33)
Ryegrass	2***	4****	11 (10-12)	9***	17****	11 (10-12)
W/A 2,4-DNT						
Alfalfa	6	10	7 (2-11)	6***	10****	15 (9-21)
Japanese millet	1*	4	3.5 (2.3-4.6)	4	8	6 (5-8)
Ryegrass	4*	8	5 (4-7)	4***	8****	2 (0-4)
F/A 2,6-DNT						
Alfalfa	1.4	4.1	1.3 (0-2.9)	1.4	4.1	3 (0-6)
Japanese millet	<4	4**	13 (12-14)	8***	14****	11 (9-13)
Ryegrass	30***	89****	18 (4-32)	14***	30****	26 (21-32)
W/A 2,6-DNT						
Alfalfa	3.3	5.4	1.6 (0.1-3.2)	3.3	5.4	0.4 (0-1.4)
Japanese millet	1	3	5 (4-6)	3	5	6 (3-8)
Ryegrass	8*	20	24 (21-27)	8*	20	21 (18-23)
F/A TNB						
Alfalfa	5*	39	38 (10-66)	39	88	62 (28-96)
Japanese millet	8***	22****	16 (12-21)	22	64	43 (27-59)
Ryegrass	39***	125****	45 (35-56)	39***	125****	56 (43-67)
W/A TNB						
Alfalfa	22	114	20 (0-49)	22	114	46 (2-89)
Japanese millet	<0.3	0.3**	0.3 (0.1-0.4)	<0.3	0.3**	0.7 (0.4-0.9)
Ryegrass	<0.3	<0.3**	26 (13-78)	2	81	51 (30-72)

* Unbounded NOEC ** Unbounded LOEC *** NOAEC **** LOAEC

Hormesis, a stimulatory effect caused by low levels of potentially toxic chemicals followed by inhibitory effects at higher concentrations, was observed in all plant species exposed to TNB, 2,4-DNT and 2,6-DNT. Weathering/aging of EM amended soils did not reduce the toxicity for terrestrial plant species tested. In fact, weathering/aging of 2,4-DNT, 2,6-DNT, or TNB amended soils significantly increased toxicity for Japanese millet, which was the most sensitive species among plant species tested. Weathering/aging of amended soils also significantly increased the toxicity of 2,4-DNT for ryegrass. Overall results of our study showed that special consideration given to the effects of weathering and aging of energetic contaminants in soil for assessing phytotoxicity was well justified. Benchmark values generated in these investigations and summarized in Table 2, will contribute to development of Eco-SSLs that better represent the exposure conditions of terrestrial plants at contaminated sites.

4.2 Soil Invertebrate Toxicity Assays.

4.2.1 Earthworm Toxicity Assays.

Definitive studies using the Earthworm Reproduction Tests were conducted to assess the effects of RDX, HMX, 2,4-DNT, 2,6-DNT, or TNB on the reproduction of the earthworm *E. fetida*. Adult *E. fetida* were exposed to a range of concentrations of each EM in SSL soil in independent investigations. Measurement endpoints were assessed using treatment concentrations determined from the results of the range-finding studies and included number of surviving adults after 28 days, and number of cocoons and juveniles after 56 days. All ecotoxicological parameters were estimated using measured chemical concentrations for each treatment level.

Definitive toxicity tests conducted with freshly amended soils showed that the order of EM toxicity, based on EC_{20} values for juvenile production with *E. fetida* was HMX >RDX > 2,6-DNT> TNB > 2,4-DNT. The EM toxicity order in tests with weathered/aged amended soils was RDX > 2,6-DNT > TNB > 2,4-DNT > HMX. Reproduction measurement endpoints in all tests were more sensitive compared with adult survival.

Adult *E. fetida* survival was not affected in all RDX or HMX concentrations tested in definitive tests, producing unbounded NOEC values for RDX and HMX in freshly amended soils of 148 and of 141 mg kg^{-1}, respectively. The unbounded NOEC values for RDX and HMX in weathered/aged-amended soils were 527 and 562 mg kg^{-1}, respectively. RDX or HMX did not affect adult *E. fetida* survival even at concentrations as high as 5,000 mg kg^{-1} in range-finding tests.

Both cocoon and juvenile production were reduced at relatively low levels of RDX or HMX in freshly amended soils (Table 3). Juvenile production was affected by RDX with EC_{20} estimates of 1.6 and 4.8 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively. However, some cocoons were still found at 148 mg kg^{-1} in the definitive tests and at 5,000 mg kg^{-1} in the range-finding tests. HMX in freshly amended SSL soil was the most toxic to *E. fetida* reproduction of the five EM compounds tested in this study with EC_{20} values for cocoon and juvenile production of 2.7 and 0.4 mg kg^{-1}, respectively. Weathering and aging

of RDX amended soil did not significantly affect its toxicity to *E. fetida*. HMX toxicity was greatly reduced after weathering and aging of amended SSL soil, and no ecotoxicological benchmarks could be determined. However, most of the HMX (mean = 75%) was still present in the acetonitrile fraction after the 3-months weathering/aging processes.

Table 3. Summary of reproduction ecotoxicological parameters (mg kg^{-1}) for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using earthworm reproduction test with *Eisenia fetida*.

Exposure type	Cocoon Production				Juvenile production			
	NOEC	LOEC	EC_{20}	EC_{50}	NOEC	LOEC	EC_{20}	EC_{50}
RDX								
Freshly amended	8.6	18.2	1.2	3.7	7.5	8.6	1.6	5
P or 95% C.I.	0.06	0.001	0.4-2.0	1.2-6.2	0.31	0.001	0.4-2.7	1.4-8.5
Aged/weathered	56.6	61.5	19.2	59.6	8.4	15.7	4.8	14.9
P or 95% C.I.	0.45	0.01	0-39	0-120	0.06	0.02	0.2-9	0.66-29
HMX								
Freshly amended	15.6	36.0	2.7	8.5	6.5	11.2	0.4	1.2
P or 95% C.I.	0.16	0.007	0-7	0-22	0.1	0.02	0-0.9	0.5-2.8
Aged/weathered	>561.7	ND	ND	ND	>561.7	ND	ND	ND
P or 95% C.I.	0.46				0.59			
2,4-DNT								
Freshly amended	20.3	40.9	31	43	55	64.7	44	52
P or 95% C.I.	0.91	0.003	17-44	34-52	0.066	0.021	11-76	33-70
Aged/weathered	21.5	31.0	25	40	37.3	71.7	29	36
P or 95% C.I.	0.1	0.01	16-34	32-50	0.12	0.002	17-41	30-41
2,6-DNT								
Freshly amended	9.4	12.9	14	25	20	40.2	9	27
P or 95% C.I.	0.545	0.035	7-22	17-32	0.56	0.001	0-30	0-92
Aged/weathered	18.1	37.4	16	19	13.9	18.1	8.3	11
P or 95% C.I.	0.58	0.002	10-22	13-25	0.09	0.03	1.6-15.1	7-16
TNB								
Freshly amended	13.6	45.0	27	59	13.6	45.0	21	33
P or 95% C.I.	0.09	0.0001	6-48	37-81	0.23	0.04	0-55	9-57
Aged/weathered	19.9	78.7	18	57	19.9	78.7	13	41
P or 95% C.I.	0.13	0.0001	11-26	33-80	0.52	0.0001	7-19	23-60

Table note: ND, Not Determined. ECp values could not be determined because cocoon and juvenile numbers were not significantly different in all treatment concentrations compared with carrier control.

Adult survival, cocoon production, and juvenile production by *E. fetida* were affected by exposure to 2,4-DNT amended SSL. For adult survival in freshly amended soil, the bounded NOEC and LOEC values for 2,4-DNT were 55 and 65 mg kg^{-1}, respectively. For adult survival in weathered/aged amended soil, the bounded NOEC and LOEC values were 37 and

71.7 mg kg^{-1}, respectively. No adults survived in the 179 mg kg^{-1} treatment. EC_{20} values for juvenile production were 44 and 29 mg kg^{-1} in freshly amended and weathered/aged soils, respectively (Table 3). EC_{20} values for cocoon production were 31 and 25 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively. The differences between freshly amended and weathered/aged amended soils in cocoon and juvenile productions were not statistically significant based on 95% CI, indicating that the 3-month weathering/aging process did not appreciably affect the toxicity of 2,4-DNT to *E. fetida*.

Among the nitroaromatic compounds evaluated in our study, 2,6-DNT was most toxic (Table 3). For adult survival, the bounded NOEC and LOEC values for 2,6-DNT were 20 and 40 mg kg^{-1}, respectively in freshly amended soil, and 14 and 18 mg kg^{-1}, respectively in weathered/aged amended soil. EC_{20} values for juvenile production were 9 and 8.3 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively. EC_{20} values for cocoon production were 14 and 16 mg kg^{-1} in freshly amended and in weathered/aged amended soil, respectively (Table 3). The differences in cocoon and juvenile productions between freshly amended and weathered/aged amended soils were not statistically significant based on 95% CI, indicating that the 3-month weathering/aging process did not appreciably affect the toxicity of 2,6-DNT to *E. fetida*.

Toxicity testing with TNB amended SSL soil showed that bounded NOEC and LOEC values for adult survival in freshly amended soil were 45 and 107 mg kg^{-1}, respectively. The bounded NOEC and LOEC values in weathered/aged amended soil were 79 and 191 mg kg^{-1}, respectively. No adults survived in the 302 mg kg^{-1} treatment. EC_{20} values for juvenile production, based on total extraction of TNB, were 21 and 13 mg kg^{-1} in freshly amended and weathered/aged soils, respectively (Table 3). EC_{20} values for cocoon production were 27 and 19 mg kg^{-1} in freshly amended soil and in weathered/aged amended soil, respectively (Table 3). The differences in cocoon and juvenile productions between freshly amended and weathered/aged amended soils were not statistically significant based on 95% CI, indicating that the 3-month weathering/aging process did not appreciably affect the toxicity of TNB to *E. fetida*.

This study generated ecotoxicological benchmarks for *E. fetida* reproduction measurement endpoints that were used for derivation of the draft Ecological Soil Screening Level for soil invertebrates for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB.

4.2.2 Potworm Toxicity Assays.

Definitive toxicity tests conducted with both freshly amended and weathered/aged amended soils showed that EM toxicity order based on EC_{20} values for juvenile production in tests with *E. crypticus* was TNB > 2,4-DNT > 2,6-DNT > RDX > HMX. Reproduction measurement endpoint in all tests was more sensitive compared with adult survival. This supported the Eco-SSL requirement of the use of reproduction endpoints for benchmark development (USEPA, 2000). Nitro-heterocyclic explosives RDX and HMX did not affect adult *E. crypticus* survival even at concentrations as high as 21,383 and 21,750 mg kg^{-1}, respectively (Table 4). Juvenile production was affected by RDX but the toxicity was relatively low, with EC_{20} estimates of 3,715 and 8,797 mg kg^{-1} in freshly amended and weathered/aged amended

soils, respectively. Weathering and aging of RDX amended soil did not significantly affect its toxicity to *E. crypticus*.

Table 4. Ecotoxicological benchmarks (mg kg^{-1}) for nitramine energetic materials RDX and HMX determined in freshly amended (F) and weathered/aged (W/A) amended Sassafras sandy loam soil using Enchytraeid Reproduction Test with *Enchytraeus crypticus*.

Exposure type	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
RDX (F)	21,383	>21,383	1,194	2,203	3,715	51,413
p or 95% C.I.			0.055	0.001	0-8,100	6,336-96,491
RDX (W/A)	18,347	>18,347	2,379	3,985	8,797	142,356
p or 95% C.I.			0.056	0.001	761-16,834	0-373,753
HMX (F)	21,750	>21,750	21,750	>21,750	ND	ND
HMX (W/A)	17,498	>17,498	17,498	>17,498	LT	LT

Table notes:
ND, Not Determined. ECp values could not be determined due to stimulation of juvenile production in all treatment concentrations.
LT, Limit Test is based on data comparison between carrier control and one treatment concentration of 17,498 mg kg^{-1}.

Exposure of *E. crypticus* to HMX in freshly amended SSL soil produced a significant stimulating effect on juvenile production (11-56% increase), which disappeared in weathered and aged soil. A hormetic response in freshly amended SSL soil, which also disappeared in weathered/aged amended soil, was observed in our toxicity test with TNB amended soil. To date, no studies investigated the mechanisms responsible for stimulating effects of these EMs at specific concentrations. Stevens *et al*. (2002) suggested that these mechanisms could include the direct effect on test organisms through the release of metabolic products of explosives that may have a specific effect on growth and reproduction, and indirect effects through increased supply of nitrogen for bacteria, fungi, or algae (an important food source for higher trophic levels) from mineralization of explosives.

The relatively low toxicity of RDX and the absence of HMX toxicity to *E. crypticus* in SSL soil at concentrations tested in our study can be in part attributed to the relatively low immediate bioavailability of these energetic materials in soil pore water, as evidenced by low ATCLP-based extractions of both compounds. Considering *E. crypticus* exposure to RDX and HMX in soil pore water in relation to ATCLP results provides explanation, at least partially, on the basis of solubility for the observed effects of these nitro-heterocyclic explosives. Additional research would be required to better understand the reasons for low toxicity of RDX to *E. crypticus*, and elucidation of mechanisms of a stimulating response to HMX exposure.

Table 5. Ecotoxicological parameters (mg kg^{-1}) for nitroaromatic energetic materials 2,4-DNT, 2,6-DNT, and TNB determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using Enchytraeid Reproduction Test with *Enchytraeus crypticus*.

Exposure type	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
2,4-DNT						
Freshly amended soil	4.9	55.0	9.9	20.3	19	36
p or 95% C.I.	0.659	0.013	0.271	0.037	13 - 26	30 - 41
Weathered/aged soil	37.3	71.7	5.2	11.5	14	27
p or 95% C.I.	0.711	0.015	0.318	<0.0001	10 - 18	24 - 31
2,6-DNT						
Freshly amended soil	64	>64	20.0	40	37	57
p or 95% C.I.	0.369		0.136	0.019	28 - 47	51 - 63
Weathered/aged soil	37	108	18	37	18	29
p or 95% C.I.	1.000	<0.0001	0.055	<0.0001	13 - 23	25 - 34
TNB						
Freshly amended soil	45	107	2.6*	3.9*	5	11
p or 95% C.I.	1.000	<0.0001	0.010	0.012	3 - 7	7 - 16
Weathered/aged soil	75.8	176	1.3	8.8	9	22
p or 95% C.I.	1.000	<0.0001	0.722	0.009	4 - 14	13 – 32

Table note:
*Values are No Observed Adverse Effect Concentration, NOAEC and Lowest Observed Adverse Effect Concentration, LOAEC due to a significant (p = 0.01) increase in juvenile production in 2.6 mg kg^{-1} treatment.

Toxicity to *E. crypticus* juvenile production of nitroaromatic EMs tested was considerably greater (more than two orders of magnitude) compared with RDX and even greater compared with HMX (Table 5). EC_{20} estimates for juvenile production ranged from 5 to 37 mg kg^{-1} in freshly amended soils, and from 9 to 20 mg kg^{-1} in weathered/aged amended soils. Weathering and aging of amended soils significantly increased the toxicity of 2,6-DNT to *E. crypticus*, while toxicity of 2,4-DNT and TNB was unaffected.

This study generated ecotoxicological benchmarks for reproduction measurement endpoint that were used for derivation of the draft Ecological Soil Screening Level for soil invertebrates for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB.

4.2.3 Collembola Toxicity Assays.

Definitive toxicity assays using the Folsomia Reproduction Test were conducted to assess the effects of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB on the reproduction of the Collembolan *F. candida*. Juvenile collembolans were exposed in SSL soil to a range of concentrations for each EM, in independent investigations. Measurement endpoints were assessed using 6-10 treatment concentrations determined from the range-finding studies, and

included the number of surviving adults and the number of juveniles produced after 28 days. All ecotoxicological parameters were estimated using measured chemical concentrations for each treatment level.

Definitive toxicity tests conducted with freshly amended soil showed that EM toxicity order based on EC_{20} values for juvenile production was TNB > 2,6-DNT > 2,4-DNT > RDX > HMX. Definitive toxicity tests conducted with weathered/aged amended soil showed that EM toxicity order based on EC_{20} values was 2,6-DNT > 2,4-DNT > TNB > RDX > HMX. The juvenile production measurement endpoint based on EC_{20} values was more sensitive or similar compared with adult survival in all tests.

Adult survival and juvenile production by *F. candida* were affected by exposure to RDX in freshly amended SSL soil. Juvenile production was affected by exposure to RDX in both freshly amended and weathered/aged amended soil. For adult survival in freshly amended soil, the bounded NOEC and LOEC values for RDX were 44.4 and 138.7 mg kg^{-1}, respectively. Adult survival in weathered/aged amended soil was not affected by exposure to RDX, producing an unbounded NOEC value of 527 mg kg^{-1}. EC_{20} values for juvenile production were 28 and 113 mg kg^{-1} in freshly amended and weathered/aged soils, respectively (Table 6). The difference in EC_{20} values for juvenile production between freshly amended and weathered/aged amended soils was not statistically significant based on 95% CI, indicating that the 3-month weathering/aging process did not appreciably affect the toxicity of RDX to *F. candida*.

Adult survival and juvenile production by *F. candida* were affected by exposure to HMX amended SSL. For adult survival in freshly amended soil, the bounded NOEC and LOEC values were 642 and 1,491 mg kg^{-1}, respectively. For adult survival in weathered/aged-amended soil, the bounded NOEC and LOEC values were 2,491 and 4,784 mg kg^{-1}, respectively. EC_{20} values for juvenile production were 235 and 1,046 mg kg^{-1} in freshly amended and weathered/aged soils, respectively (Table 6). The difference in EC_{20} values for juvenile production between freshly amended and weathered/aged amended soils was not statistically significant based on 95% CI, indicating that the 3-month weathering/aging process did not appreciably affect the toxicity of HMX to *F. candida*.

Exposure to 2,4-DNT amended SSL affected adult survival and juvenile production by *F. candida* in freshly amended and in weathered/aged amended SSL soil. For adult survival in freshly amended soil, the bounded NOEC and LOEC values for 2,4-DNT were 3.1 and 5.4 mg kg^{-1}, respectively. Bounded NOEC and LOEC values for adult survival in weathered/aged amended soil were 5.2 and 11.5 mg kg^{-1}, respectively. LOEC values for adult survival were lower compared with EC_{20} values for juvenile production in freshly amended and in weathered/aged amended SSL soil. However, when nonlinear regression analysis-based benchmarks for adult survival and juvenile production were compared, the differences between these benchmarks in both exposure types were not statistically significant based on 95% CI. These benchmark values were: EC_{20} for adult survival and juvenile production 7.5 (4 – 11, 95% CI) and 9.9 (5.6 – 14, 95% CI), respectively in freshly amended soil; and 12 (4 – 20, 95% CI) and 14.9 (11 – 19, 95% CI), respectively in weathered/aged amended soil. The difference in EC_{20} values for juvenile production between freshly amended and weathered/aged amended soils was

not statistically significant based on 95% CI, indicating that the 3-month weathering/aging process did not appreciably affect the toxicity of 2,4-DNT to *F. candida*.

Table 6. Ecotoxicological parameters (mg kg^{-1}) for energetic materials RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using Folsomia Reproduction Test with *Folsomia candida*

Exposure type	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
RDX						
Freshly amended soil	44.4	138.7	20.4	44.37	28	86.5
p or 95% C.I.	1.000	<0.0001	0.535	0.005	14 – 41	45 – 128
Weathered/aged soil	527	>527	56.6	61.5	113	771
p or 95% C.I.	0.264	0.264	0.079	0.012	29 – 197	444 – 1097
HMX						
Freshly amended soil	642	1,491	642	1,491	235	8,799
p or 95% C.I.	0.075	0.006	0.054	0.001	0 - 730	0-22,648
Weathered/aged soil	2,491	4,784	129	280	1,046	10,370
p or 95% C.I.	0.744	<0.0001	0.069	0.019	58-2,033	3,156-17,583
2,4-DNT						
Freshly amended soil	3.1	5.4	3.1	5.4	10	21
p or 95% C.I.	1.000	<0.0001	0.239	0.004	6 – 14	16 – 25
Weathered/aged soil	5.2	11.5	3.0	5.2	15	23
p or 95% C.I.	0.325	<0.0001	0.143	0.004	11 – 19	20 – 25
2,6-DNT						
Freshly amended soil	7.6	9.4	7.6	9	6	11
p or 95% C.I.	0.809	0.007	0.073	0.002	2 – 10	7 – 15
Weathered/aged soil	1.6	3.7	1.6	3.7	0.96	3.6
p or 95% C.I.	0.285	0.001	0.167	<0.0001	0 - 2.1	1.4 - 5.9
TNB						
Freshly amended soil	45	107	3.9	13.6	4.4	24.7
p or 95% C.I.	0.279	<0.0001	0.481	0.002	0 - 12	2.7 - 46.7
Weathered/aged soil	76	176	8.8	75.8	48	87.5
p or 95% C.I.	0.608	0.001	0.676	<0.0001	27 - 68	70 - 105

Adult survival and juvenile production by *F. candida* were affected by exposure to 2,6-DNT amended SSL. For adult survival, the bounded NOEC and LOEC values for 2,6-DNT were 7.6 and 9 mg kg^{-1}, respectively in freshly amended soil; and 1.6 and 3.7 mg kg^{-1}, respectively, in weathered/aged amended soil. EC_{20} values for juvenile production were 5.9 and 0.96 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively. The 3-month weathering and aging of SSL soil amended with 2,6-DNT significantly increased the toxicity of 2,6-DNT to *F. candida* based on 95% CI.

Toxicity testing with TNB amended SSL soil showed that bounded NOEC and LOEC values for adult survival in freshly amended soil were 45 and 107 mg kg^{-1}, respectively. The bounded NOEC and LOEC values in weathered/aged amended soil were 76 and 176 mg kg^{-1}, respectively. EC_{20} values for juvenile production, based on total acetonitrile extraction, were 4.4 and 48 mg kg^{-1} in freshly amended and weathered/aged soils, respectively (Table 6). The difference in juvenile production between freshly amended and weathered/aged amended soils was statistically significant based on 95% CI, indicating that the 3-month weathering/aging process decreased the toxicity of TNB to *F. candida*.

This study generated ecotoxicological benchmarks for *F. candida* reproduction measurement endpoint that were used for derivation of the draft Ecological Soil Screening Level for soil invertebrates for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB.

4.3 Weathering and Aging Effect on Toxicity of Energetic Materials for Terrestrial Plant and Soil Invertebrates.

Special consideration in assessing chemical toxicity for Eco-SSL development was given to the effects of weathering and aging of contaminant explosives in soil on the exposure of terrestrial receptors. Assessment of the EM toxicity for Eco-SSL development included studies with weathered and aged EM-amended soils to more closely simulate the exposure effects in the field, and because Eco-SSL development by USEPA was specifically undertaken for use at Superfund sites (locations where contaminants have been long-present). Weathering/aging of chemicals in soil may reduce exposure of terrestrial plants and soil invertebrates to EMs due to photodecomposition, hydrolysis, reaction with organic matter, sorption/fixation, precipitation, immobilization, occlusion, microbial transformation and other fate processes that commonly occur at contaminated sites. The 3-month weathering and aging of amended SSL soil significantly reduced toxicity of TNB to collembolan *F. candida* (Table 7, Figure 4). Based on EC_{20} estimates for juvenile production, TNB toxicity decreased by an order magnitude from 4.4 mg kg^{-1} in freshly amended soil to 48 mg kg^{-1} in weathered/aged TNB amended soil. This was the only instance of a decrease in EM toxicity in weathered/aged EM amended soils among all EMs and species tested in this investigation.

Certain fate processes, including microbial transformation of EMs can produce chemicals that are more bioavailable or more toxic to soil organisms than parent EM compounds freshly introduced into soil. Identification of breakdown products of EMs was not included in the scope of current investigation. However inclusion of weathering and aging of amended soil in the experimental design of toxicity assessments presented the research team with the opportunity to initially investigate the issue of the presence of EM breakdown products in weathered/aged EM-amended soils. In additional research efforts undertaken by the CU-1221 research team (Appendix A), transformation products of nitroaromatic EMs 2,4-DNT and TNB were detected in weathered/aged amended soils. These results strongly suggest that the EMs 2,4-DNT and TNB were transformed due to exposure to sunlight and/or soil drying/wetting cycles, as occurs normally in nature. The transformation products detected included 3,5-dinitroaniline (3,5-DNA), 2-amino-4-nitrotoluene (2-A-4 NT), and 4-amino-2-nitrotoluene (4-A-2 NT). The transformation product 3,5-DNA was detected in all concentrations of TNB amended weathered/aged soil, but in

greater amount at concentrations 40, 60 and 80 mg kg^{-1}. Measurable amounts of 2-A-4 NT and 4-A-2 NT were detected in weathered/aged soil amended with 2,4-DNT at concentrations of 25, 50 and 200 mg kg^{-1}.

Table 7. Ecotoxicological parameters (mg kg^{-1}) for TNB determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using Folsomia Reproduction Test with *Folsomia candida*.

Exposure type	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Freshly amended soil	45	107	3.9	13.6	4.4	24.7
p or 95% C.I.	0.279	<0.0001	0.481	0.002	0 - 12	2.7 - 46.7
Weathered/aged amended soil	76	176	8.8	75.8	48	87.5
p or 95% C.I.	0.608	0.001	0.676	<0.0001	27 - 68	70 - 105

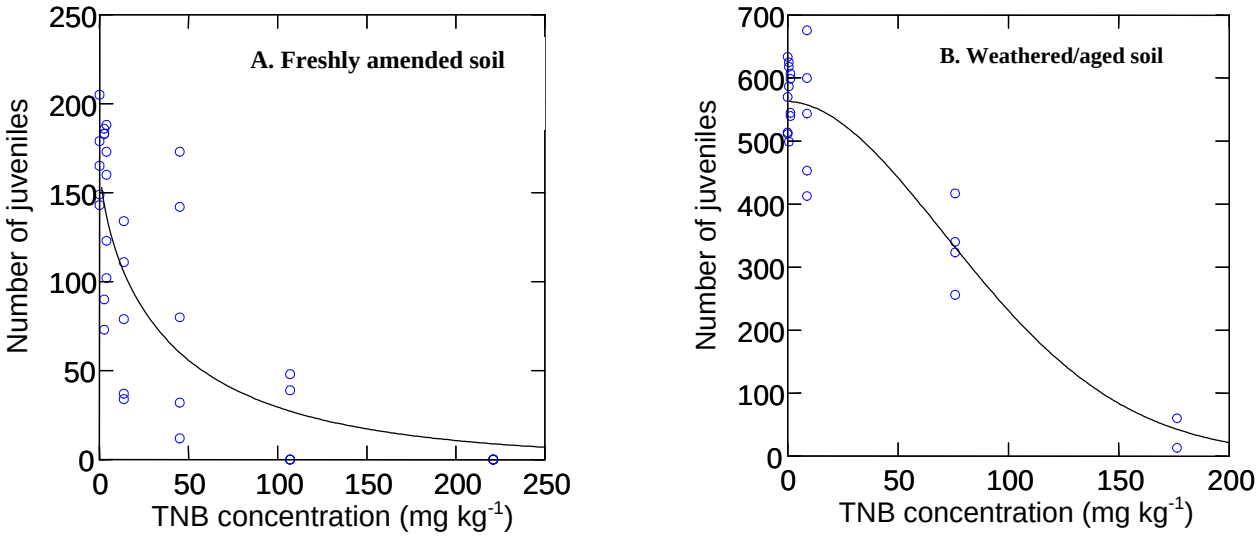

Figure 4. Effects of TNB on juvenile production in freshly amended and weathered/aged TNB amended Sassafras sandy loam soils determined in toxicity testing using Folsomia Reproduction Test with *Folsomia candida*.

Results of toxicity tests with weathered/aged 2,4-DNT, 2,6-DNT, or TNB amended soils showed significantly increased toxicity to Japanese millet (Table 8, Figures 5-10), and significantly increased toxicity to ryegrass in weathered/aged 2,4-DNT amended soils (Table 9, Figures 11 and 12). Toxicity was also significantly increased to potworm *Enchytraeus crypticus* (Table 10, Figure 13) and collembola *Folsomia candida* (Table 11, Figure 14) in 2,6-DNT weathered/aged amended soil. These increases in toxicities to terrestrial plants and soil invertebrates exposed in weathered/aged 2,4-DNT, 2,6-DNT, or TNB amended soils strongly

indicate that the soil chemical environment was altered during the 3-month weathering and aging period, similar to changes that can occur in vadose zone soil environments in the field.

Specific mechanisms of changes in the toxicity of EMs in weathered/aged amended soil are unknown. Degradation products produced during the weathering and aging process may be more toxic to soil organisms compared with the parent material, and may be one of the factors contributing to the increased toxicity in weathered/aged amended soil. Dodard *et al*. (1999) investigated the toxicity of 2,4-DNT and 2,6-DNT, and their respective metabolites in aquatic ecosystems using the 15-min Microtox (*Vibrio fischeri*) and 96-h freshwater green alga (*S. capricornutum*) growth inhibition tests. The authors reported that the reduced metabolites of 2,6-DNT tested were less toxic compared to the toxicity of parent compound. However, partially reduced metabolites of 2,4-DNT (4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene), which were also detected in our study, were more toxic compared with the parent compound. These results may not be directly compared to our study because the biotic reductive degradation pathway for 2,4-DNT and 2,6-DNT in aquatic environment contrasts with the aerobic metabolic processes in the vadose zone, simulated in our investigations. However reducing microenvironments can exist in waterlogged soil microsites within the soil vadose zone, and this may contribute to the presence of the more toxic metabolites of dinitrotoluenes degradation even within the vadose zone of otherwise aerobic soils.

Overall, our findings of increased toxicity for several terrestrial species in weathered/aged amended soil clearly show that additional studies are required to investigate the toxicity of the EM degradation products. Analogously, investigation of the more toxic transformation compounds that arise within soils amended with 2,4-DNT, 2,6-DNT, or TNB should also have a weathering/aging component, so that the level of persistence and long-term impact of the ecotoxicity of these toxic transformation products may also be assessed. Such studies should be designed to generate benchmark data for EM breakdown/transformation products so results may be used for deriving draft Eco-SSLs for these chemicals, while providing more complete information on ecotoxicological effects of energetic contaminants in soil for risk assessors and site managers.

Table 8. Effect of weathering/aging (W/A) of amended soil on toxicity of nitroaromatic energetic materials (mg kg^{-1}) for Japanese millet.

Measurement endpoint	Fresh TNB	W/A TNB	Fresh 2,4-DNT	W/A 2,4-DNT	Fresh 2,6-DNT	W/A 2,6-DNT
Growth - Fresh mass						
EC_{20}	16	0.3	5	4	13	5
Confidence interval	12-21	0.1-0.5	1.6-5.4	2.3-4.6	12-14	4-6
Significant difference	**yes**		no		**yes**	
EC_{50}	36	0.9	10	7	16	9
Confidence interval	27-45	0.4-1.4	7.6-13.1	5.4-7.5	15-18	8-10
Significant difference	**yes**		**yes**		**yes**	
Growth - Dry mass						
EC_{20}	43	0.7	25	6	11	6
Confidence interval	27-59	0.4-0.9	18-33	5-8	9.4-13.4	3.1-8.5
Significant difference	**yes**		**yes**		**yes**	
EC_{50}	89	2.0	34	10	18	11
Confidence interval	73-104	1-3	28-40	9-12	16-20	8-13
Significant difference	**yes**		**yes**		**yes**	

Table 9. Effect of weathering/aging of 2,4-DNT (mg kg^{-1}) amended soil on toxicity for ryegrass.

Measurement endpoint	Freshly amended soil	Weathered/aged amended soil
Growth - Fresh mass		
EC_{20}	11	5
Confidence interval	10-12	4-7
Significant difference	**yes**	
EC_{50}	13	7
Confidence interval	12-15	6-8
Significant difference	**yes**	
Growth - Dry mass		
EC_{20}	11	2
Confidence interval	10-12	0-4.5
Significant difference	**yes**	
EC_{50}	13	7.6
Confidence interval	12-15	---
Significant difference		

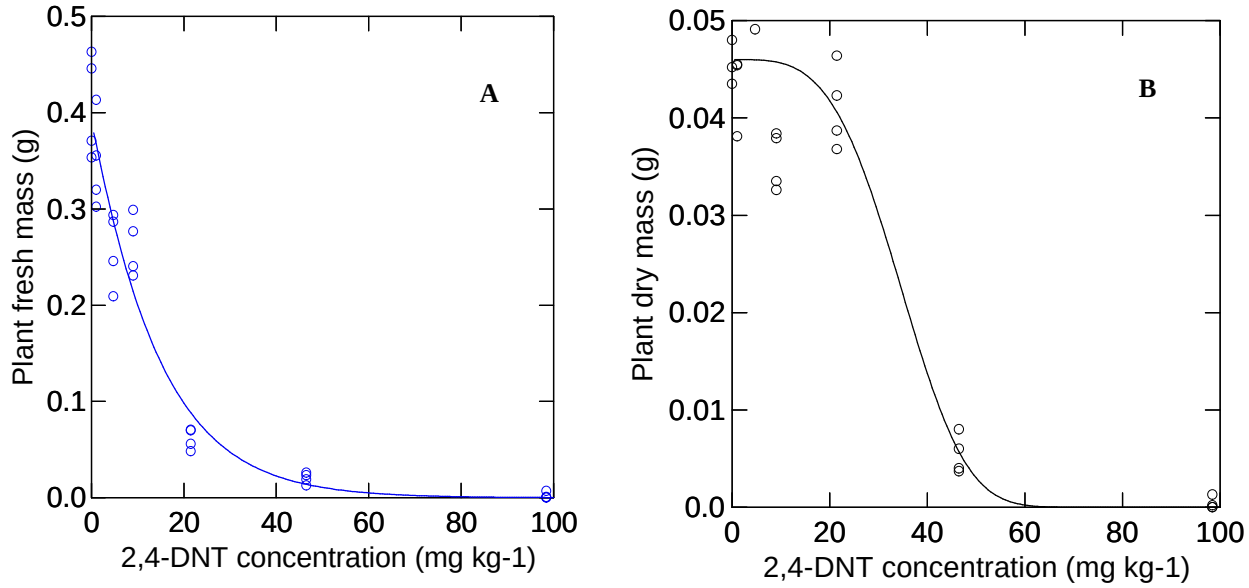

Figure 5. Effect of 2,4-DNT in freshly amended SSL soil on Japanese millet shoot growth (fresh [A] and dry [B] mass).

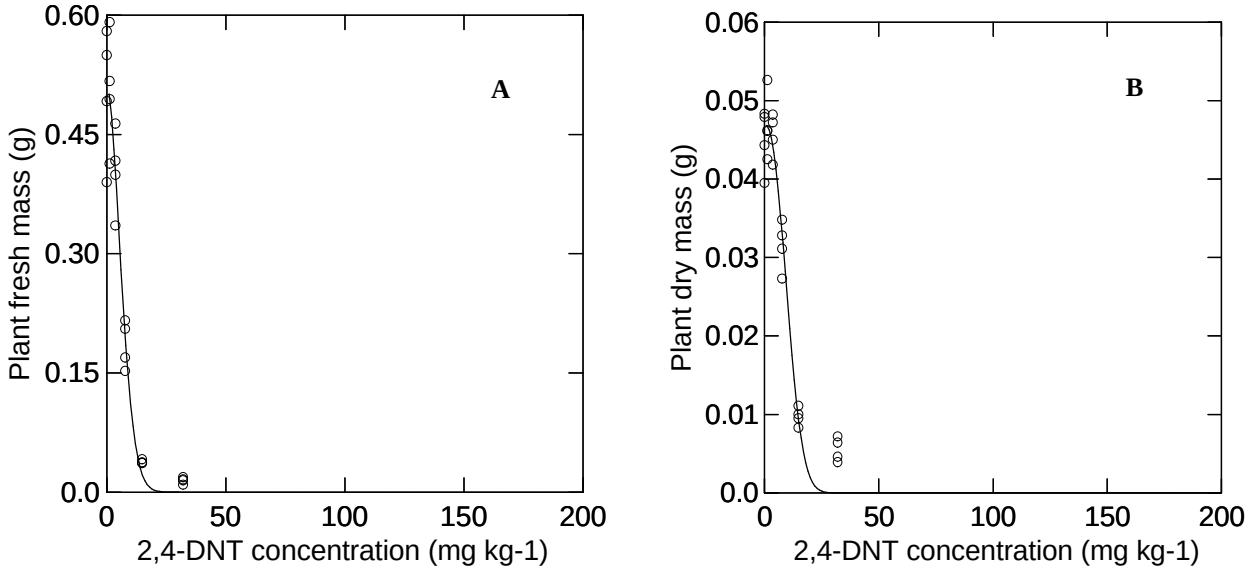

Figure 6. Effect of weathered/aged 2,4-DNT amended SSL soil on Japanese millet shoot growth (fresh [A] and dry [B] mass).

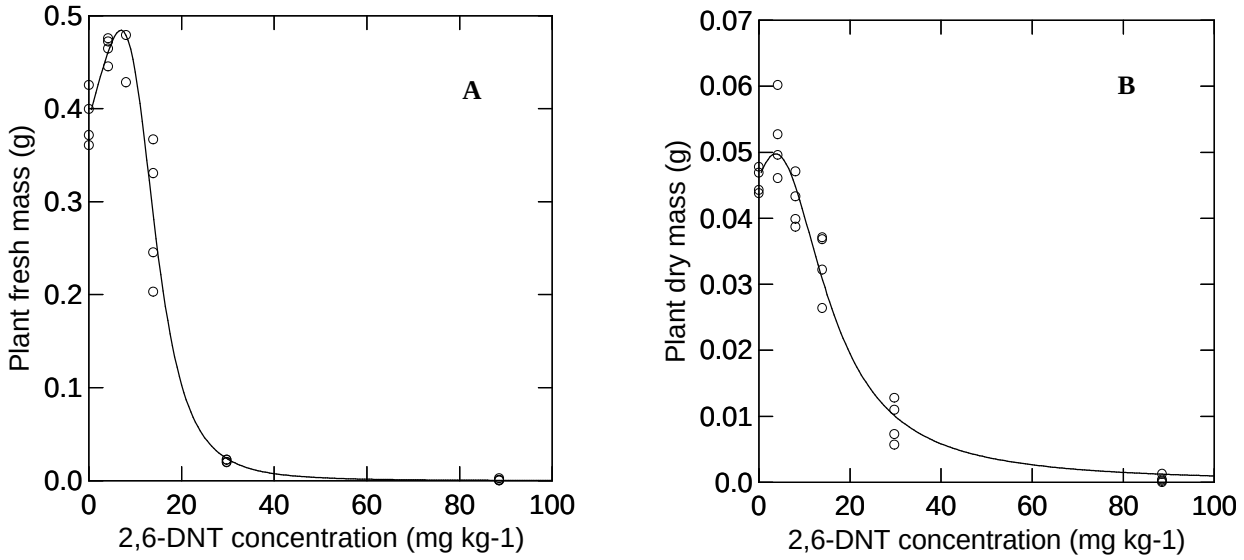

Figure 7. Effect of 2,6-DNT in freshly amended SSL soil on Japanese millet shoot growth (fresh [A] and dry [B] mass).

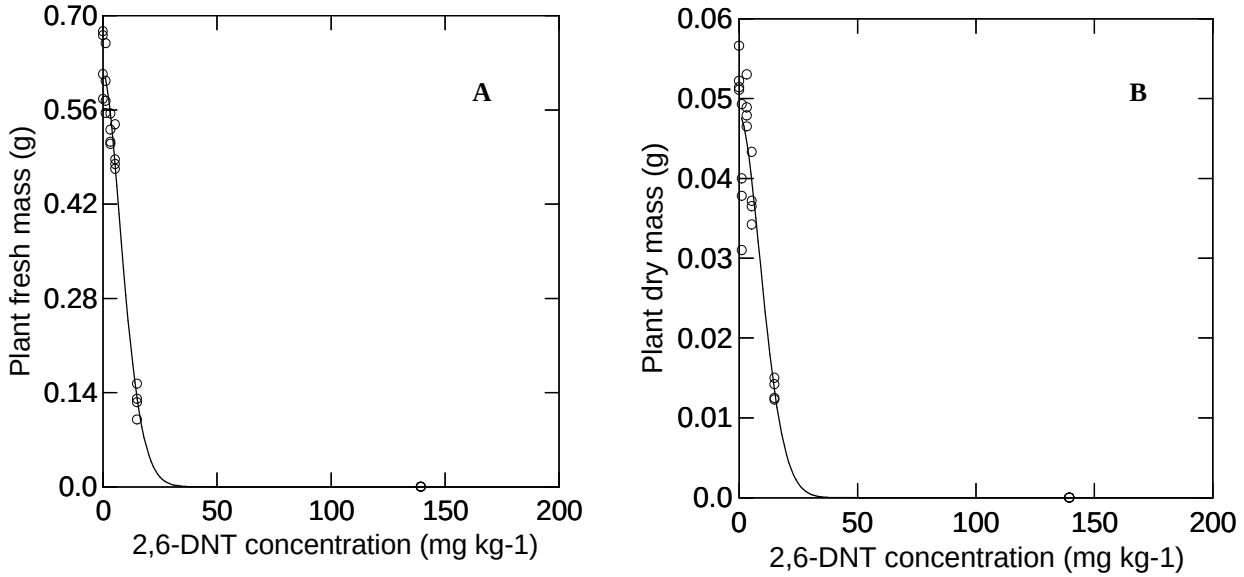

Figure 8. Effect of weathered/aged 2,6-DNT amended SSL soil on Japanese millet shoot growth (fresh [A] and dry [B] mass).

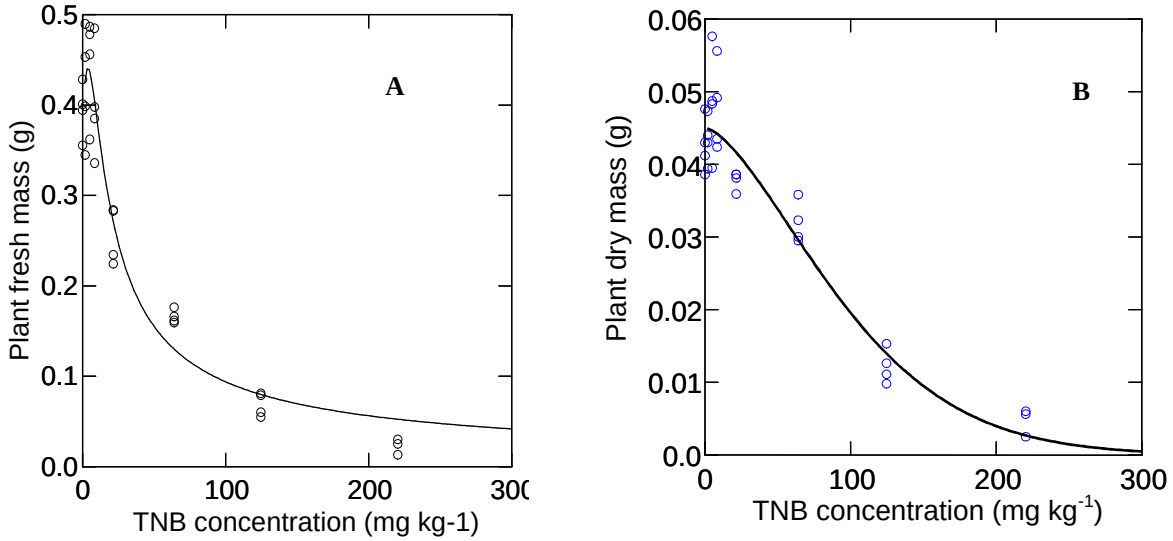

Figure 9. Effect of TNB in freshly amended SSL soil on Japanese millet shoot growth (fresh [A] and dry [B] mass).

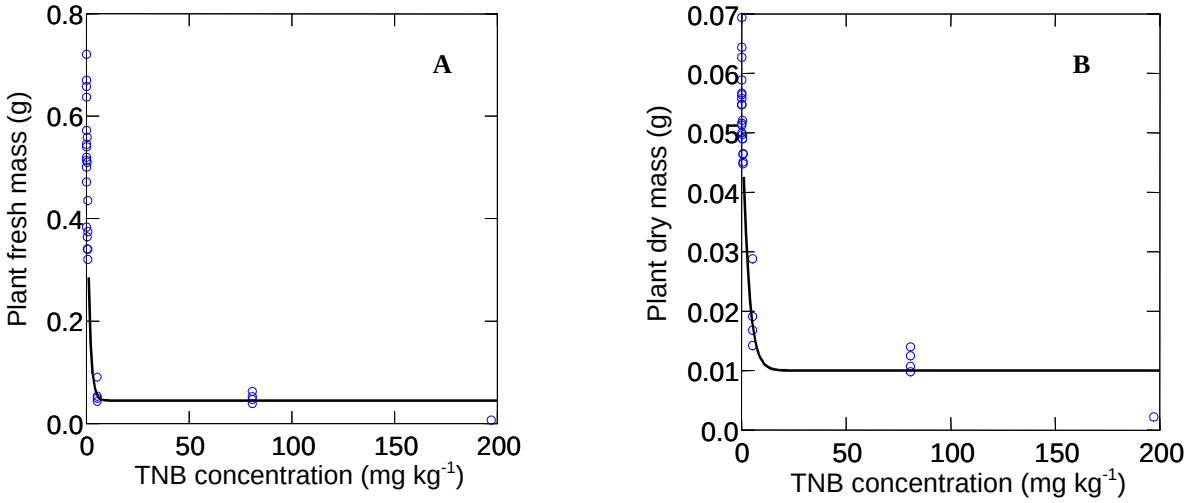

Figure 10. Effect of weathered/aged TNB amended SSL soil on Japanese millet shoot growth (fresh [A] and dry [B] mass).

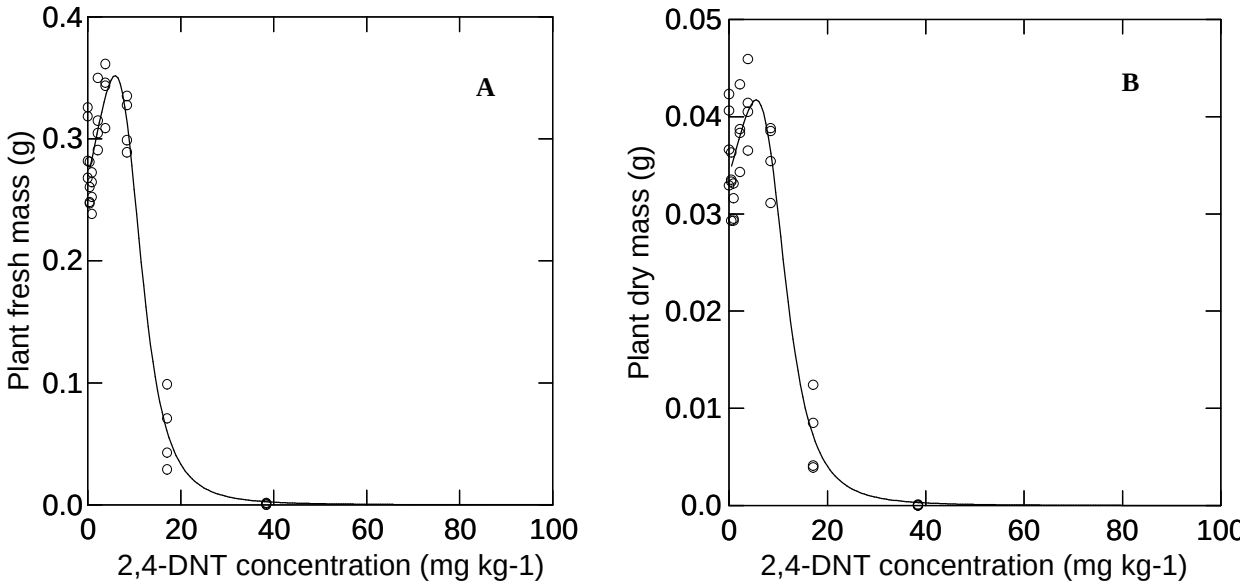

Figure 11. Effect of 2,4-DNT in freshly amended SSL soil on ryegrass shoot growth (fresh [A] and dry [B] mass).

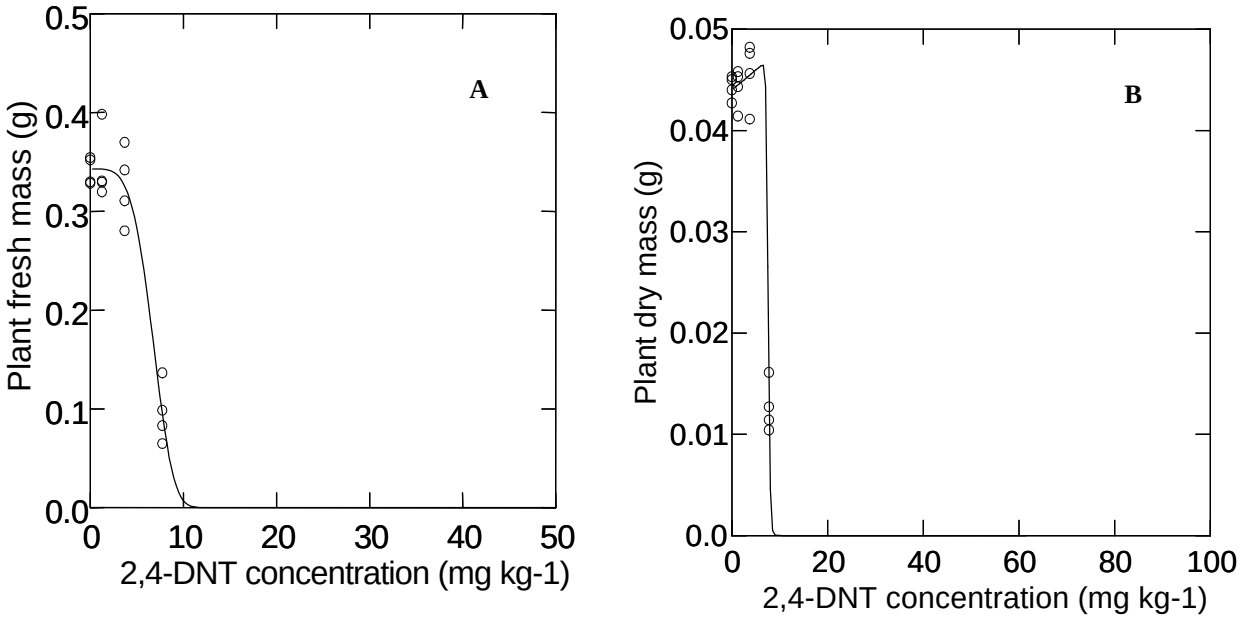

Figure 12. Effect of weathered/aged 2,4-DNT amended SSL soil on ryegrass shoot growth (fresh [A] and dry [B] mass).

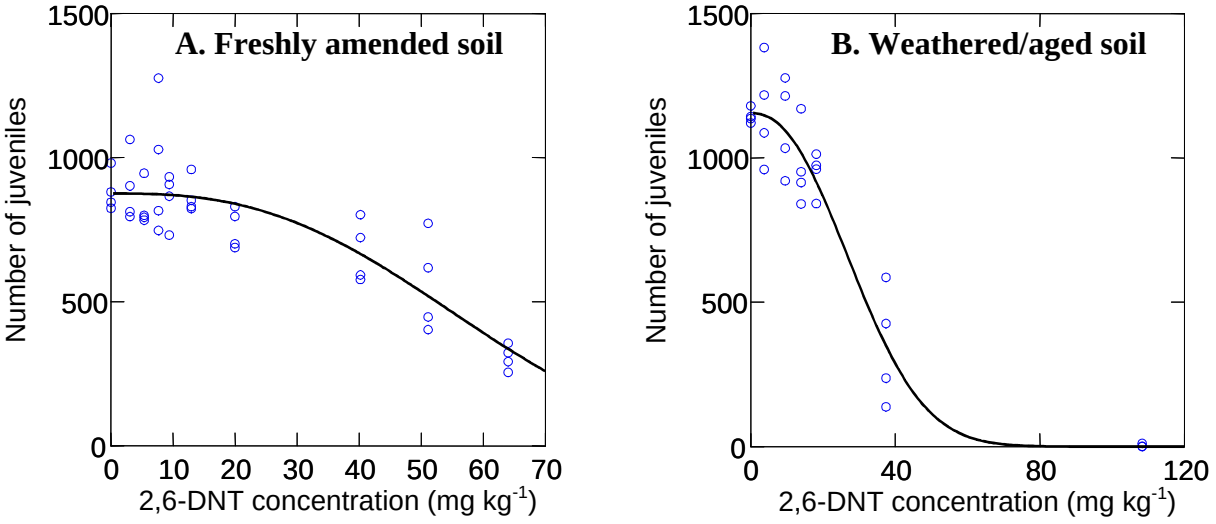

Figure 13. Effects of 2,6-DNT on juvenile production in freshly amended and weathered/aged 2,6-DNT amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*.

Table 10. Ecotoxicological parameters (mg kg^{-1}) for 2,6-DNT determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using Enchytraeid Reproduction Test with *Enchytraeus crypticus*.

Exposure type	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Freshly amended soil	64	>64	20	40.2	37	57
p or 95% C.I.	0.37		0.14	0.019	28 - 47	51 - 63
Weathered/aged amended soil	37.4	108.3	18	37.4	18	29
p or 95% C.I.	1.0	<0.0001	0.055	<0.0001	13 - 23	25 - 34

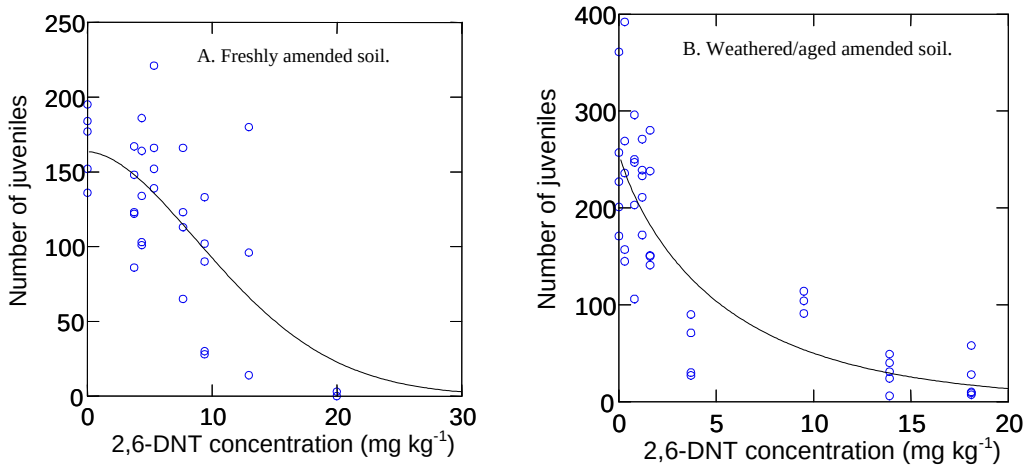

Figure 14. Effects of 2,6-DNT on juvenile production in freshly amended and weathered/aged 2,6-DNT amended Sassafras sandy loam soils determined in toxicity testing using Folsomia Reproduction Test with *Folsomia candida*.

Table 11. Ecotoxicological parameters (mg kg^{-1}) for 2,6-DNT determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using Folsomia Reproduction Test with *Folsomia candida*.

Exposure type	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Freshly amended soil	7.6	9.4	7.65	9.4	5.9	11.1
p or 95% C.I.	0.8	0.007	0.07	0.002	1.8 - 10	7.4 - 14.9
Weathered/aged amended soil	1.6	3.7	1.6	3.7	0.96	3.6
p or 95% C.I.	0.285	0.001	0.167	<0.0001	0 - 2.1	1.4 - 5.9

4.4 Chemical Measures of Exposure.

The commonly used "total" chemical measurements represent a simplified measure of the environmentally available portion of a soil contaminant. Such measurements do not take into account soil factors that may modify bioavailability. The bioavailability of nonpolar organic chemicals in soil was hypothesized to be determined primarily by soil organic matter content (Belfroid *et al*., 1996). These authors also suggested that bioaccumulation and toxicity are well correlated with the concentration of chemical in the soil solution or pore water, rather than total chemical levels. Although it has been shown that total chemical levels are often not well correlated with bioaccumulation or toxicity (Linz and Nakles, 1997), until recently, few alternatives to "total" bulk chemical measures were available. For this reason, identifying measures of exposure that may better represent the bioavailable fractions of the contaminants was included in this investigation. A better measure of the contaminant concentration immediately available to terrestrial plants or soil invertebrates as well as the concentration rigorously extractable from the soil may provide more relevant estimates of actual exposure. To this end, we employed two extraction methods for measuring the exposure concentrations in EM amended soils. These methods included acetonitrile extraction performed according to USEPA Method 8330A (USEPA, 1998), and an Adapted Toxicity Characteristic Leaching Procedure (ATCLP; Haley *et al*., 1993) based on modification of the Toxicity Characteristic Leaching Procedure (TCLP). The modification involved substitution of CO_2-saturated ASTM type I water for acetic acid, better simulating field soil-water conditions due to respiration by soil biota. Both extraction methods were used for freshly amended and weathered/aged EM amended soils.

Coefficients of determinations (R^2) for acetonitrile and ATCLP based extractions determined in nonlinear regression analyses of the plant germination and growth data (Table 12), and soil invertebrate reproduction data (Table 13) from studies with freshly amended and weathered/aged EM amended soils, were compared to determine which chemical measure of exposure better correlated with toxicity. These comparisons showed that neither extraction method had an advantage for characterizing bioavailability of EMs to the three terrestrial plant or soil invertebrate species tested in this study. This was true for both freshly amended and weathered/aged amended soils, indicating that. This result supported our decision for developing draft Eco-SSLs for EM contaminants in soil on the basis of acetonitrile extraction of test compounds. The acetonitrile extraction-based Eco-SSL values will be especially useful for Ecological Risk Assessment at contaminated sites because EM concentrations determined during site characterization are usually based on acetonitrile extraction by the USEPA Method 8330A.

Table 12. Summary of coefficients of determination (R^2) for acetonitrile and ATCLP extractable measures of exposure determined by nonlinear regressions for plant measurement endpoints (EC_{20} levels) in definitive toxicity tests of energetic materials in freshly amended and weathered/aged amended SSL soil.

Compound	**Seedling emergence**		**Shoot fresh mass**		**Shoot dry mass**	
Plant species	Acetonitrile	ATCLP	Acetonitrile	ATCLP	Acetonitrile	ATCLP
Freshly amended TNB						
Alfalfa	0.967	0.899	0.971	0.971	0.972	0.972
Japanese millet	0.988	0.987	0.984	0.983	0.985	0.976
Ryegrass	0.958	0.985	0.981	0.970	0.980	0.979
Weathered/aged TNB						
Alfalfa	0.989	0.989	0.930	0.929	0.966	0.966
Japanese millet	0.992	0.992	0.972	0.948	0.990	0.983
Ryegrass	0.992	0.992	0.969	0.971	0.989	0.989
Freshly amended 2,4-DNT						
Alfalfa	ND	0.975	0.923	0.923	0.902	0.901
Japanese millet	0.994	0.994	0.975	0.977	0.978	0.978
Ryegrass	0.995	0.995	0.991	0.991	0.987	0.987
Weathered/aged 2,4-DNT						
Alfalfa	0.989	0.981	0.976	0.977	0.979	0.980
Japanese millet	0.994	ND	0.982	0.982	0.989	0.989
Ryegrass	ND	0.995	0.992	0.992	0.990	ND
Freshly amended 2,6-DNT						
Alfalfa	0.956	0.953	0.919	0.922	0.935	0.939
Japanese millet	0.992	0.992	0.991	0.991	0.989	0.990
Ryegrass	0.992	0.991	0.944	0.955	0.984	0.983
Weathered/aged 2,6-DNT						
Alfalfa	0.971	0.972	0.962	0.966	0.911	0.929
Japanese millet	ND	0.935	0.995	0.994	0.979	0.980
Ryegrass	0.995	0.995	0.994	0.993	0.995	0.995

ND: not determined

Table 13. Summary of coefficients of determination (R^2) for acetonitrile extractable (ANE) and ATCLP extractable measures of exposure determined by nonlinear regressions for soil invertebrate reproduction measurement endpoints (EC_{20} levels) in definitive toxicity tests of energetic materials in freshly amended (F) and weathered/aged (W/A) amended SSL soil.

Endpoint/	2,4-DNT		2,6-DNT		TNB		RDX		HMX	
Exposure	ANE	ATCLP	ANE	ATCLP	ANE	ATCLP	ANE	ATCLP	ANE	ATCLP
Potworm										
F	0.98	0.97	0.98	0.98	0.98	0.98	0.99	ND	ND	ND
W/A	0.99	0.98	0.98	0.98	0.99	0.99	0.99	ND	ND	ND
Earthworm cocoons										
F	0.94	0.86	0.92	0.93	0.94	0.95	0.94	0.86	0.82	0.81
W/A	0.91	0.95	0.91	0.85	0.96	0.97	0.95	0.95	ND	ND
Earthworm juveniles										
F	0.84	0.83	0.71	0.73	0.92	0.81	0.84	0.83	0.74	0.73
W/A	0.80	0.82	0.71	0.67	0.95	0.96	0.80	0.82	ND	ND
Collembola										
F	0.97	0.97	0.91	0.91	0.88	0.88	0.98	0.97	0.98	0.97
W/A	0.98	0.98	0.90	0.90	0.98	0.98	0.99	0.99	0.99	0.98

ND: not determined

4.5 Derivation of Draft Eco-SSLs for Terrestrial Plant and Soil Invertebrates.

The main objective of this project was to generate toxicity benchmark values for terrestrial plants and soil invertebrates that could be used for developing draft Eco-SSLs for RDX, HMX, 2,4-DNT, 2,6-DNT and TNB. Ecotoxicological testing was specifically designed to meet the criteria for Eco-SSL derivation outlined in the Eco-SSL Draft Guideline (USEPA, 2000). General concepts of this Draft Guideline are summarized in this section of report to assist users in reviewing and interpreting its findings. USEPA may revise some concepts and definitions in the final Eco-SSL document due for release in 2003 and the user of this report is encouraged to review the final Eco-SSL document upon its release.

The Eco-SSLs are screening values that can be used to identify contaminants of potential concern (COPCs) in soils that require further evaluation in a Baseline Ecological Risk Assessment (ERA), and to eliminate those that do not. Eco-SSLs are concentrations of contaminants in soils that are protective of ecological receptors that commonly come into contact with soil or ingest biota that live in or on soil. Eco-SSLs are derived separately for two groups of ecological receptors, plants and soil invertebrates. As such, these values are expected to provide adequate protection of terrestrial ecosystems.

The draft Eco-SSLs developed for five EMs in this project, if accepted by USEPA, should be used during Step 2 of the Superfund ERA process, the screening-level risk

calculation. It is expected that the Eco-SSLs will be used to screen the site soil data to identify those EM contaminants that are not of potential ecological concern, and do not need to be considered in the subsequent Baseline ERA. The draft Eco-SSLs are intentionally conservative in order to provide confidence that contaminants that potentially present an unacceptable risk are not screened out early in the ERA process. This conservative nature of Eco-SSLs is achieved by using a natural soil type that has properties maximizing EM bioavailability to ecologically relevant test species, by using growth (for terrestrial plants) or reproduction (for soil invertebrates) measurement endpoints for benchmark derivation, and by relying on a low, EC_{20} (20 percent reduction) level of the effect on a measurement endpoint for Eco-SSL development.

The draft Eco-SSLs may apply only to sites where terrestrial receptors may be exposed directly or indirectly to EM contaminated soil. They were derived for two groups of ecological receptors: terrestrial plants and soil invertebrates. The Eco-SSLs for terrestrial plants consider direct contact of EMs in soils, and for soil invertebrates they consider ingestion of soil as well as direct contact exposures. Both exposures were considered under conditions of relatively high EM bioavailability in SSL soil. By deriving conservative soil screening values protective of these receptor groups, it is assumed that the terrestrial ecosystem will be protected from possible adverse effects associated with soil contamination when used in conjunction with Eco-SSLs developed for avian wildlife and mammalian wildlife (if available).

Soil physical and chemical properties affect the exposure of organisms, including terrestrial plants and soil invertebrates to contaminants in soils (Alexander, 1995; Loehr and Webster, 1996; Linz and Nakles, 1997; Allen *et al*., 1999). Eco-SSLs are applicable to all sites where key soil parameters fall within a certain range of chemical and physical parameters (USEPA, 2000). They apply to upland aerobic soils where: the pH is greater than or equal to 4.0 and less than or equal to 8.5 and the organic matter content is less than or equal to 10%. The majority of soil toxicity tests that were reported in literature utilized standard artificial soil with high organic matter content (10%), which limited their usefulness for Eco-SSL derivation. In contrast, our toxicity studies designed to specifically fill the knowledge gap regarding ecotoxicity of energetic material contaminants in soil, used a natural soil that meet the criteria for Eco-SSL development, in large part because it has characteristics supporting relatively high bioavailability of EMs. This was necessary to ensure that draft Eco-SSLs for terrestrial plants and soil invertebrates developed in this project are adequately conservative for a broad range of soils within the specified boundary conditions (USEPA, 2000).

Derivation of Eco-SSL values prioritizes ecotoxicological benchmarks that are based on measured soil concentration of a chemical over those based on nominal concentrations (USEPA, 2000). In this project, the exposure concentrations of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB in soil were analytically determined in all definitive toxicity tests. Chemical analysis utilized the USEPA Method 8330A (USEPA, 1998) for extraction of EMs from soil and for measuring acetonitrile-extractable chemical concentrations. Comparison of results obtained based on acetonitrile extraction of freshly amended soils showed good agreement between nominal and measured concentrations for the five energetic materials. This confirmed that the soil amendment procedure used in toxicity tests developing ecotoxicological benchmarks for

draft Eco-SSL derivation was appropriate, and that the USEPA Method 8330A was efficient for quantifying the amount of energetic materials in soil.

Among the important aspects for a draft Eco-SSL development are selections of test methods and test species for toxicity testing to generate ecotoxicological benchmarks. The USEPA preference for using standardized toxicity assays for generating benchmarks, and ecological relevance of test species used to soil ecosystems was emphasized in the draft guideline (USEPA, 2000). A limited number of terrestrial toxicity tests have been developed, or improved by standardization by different agencies and organizations since the early 1990s. Leading among them are: the International Standardization Organization (ISO), the American Society for Testing and Materials (ASTM), Environment Canada (EC), Organization for European Co-operation and Development (OECD), the United States Environmental Protection Agency (USEPA), and the European initiative SECOFASE (Development, Improvement, and Standardization of Test Systems for Assessing **S**ublethal **E**ffects of **C**hemicals **o**n **F**auna in the **S**oil **E**cosystem) with its mandate to develop test systems for the early detection and evaluation of sublethal effects of chemicals on organisms in soil ecosystems. The new or improved test methods developed under the auspices of SECOFASE have been summarized in a handbook of soil invertebrate toxicity tests by Løkke and van Gestel (1998).

After an extensive review of existing standardized test methods, and based on the experience accumulated in the participating laboratories, we selected ASTM standard guide for conducting terrestrial plant toxicity tests (ASTM, 1998a), and USEPA early seedling growth test (USEPA, 1982) for assessing EM effects on terrestrial plants. Preliminary range-finding tests identified the three plant species most sensitive to EMs tested and with performance parameters in SSL soil required by the validity criteria of standardized definitive toxicity tests. These species included a dicotyledonous symbiotic species alfalfa, and monocotyledonous species Japanese millet and ryegrass.

ISO assays were selected for toxicity testing with soil invertebrates. These assays included ISO/11268-2:1998 *Soil Quality – Effects of Pollutants on Earthworms (Eisenia fetida) – Part 2: Determination of Effects on Reproduction* (ISO, 1998a); ISO/16387 *Soil quality — Effects of pollutants on Enchytraeidae (Enchytraeus sp.) — Determination of effects on reproduction and survival* (ISO, 2001); and ISO/11267 *Soil quality —Inhibition of Reproduction of Collembola* (*Folsomia candida*) *by Soil Pollutants* (ISO, 1998b). Guidelines for these ISO assays were originally developed for use with Artificial Soil (OECD/USEPA Standard Artificial Soil), however research in our laboratory has shown that they could be successfully adapted for use with natural soils (Kuperman *et al*., 1999; 2003), which was necessary for draft Eco-SSLs development. Further, the ISO/16387 assay was initially developed using the enchytraeid worm species *Enchytraeus albidus*, which requires soil containing high organic matter content with a soil pH 6 (± 0.5) for optimal test conditions. This species performed poorly in natural soils with physical and chemical characteristics that support a higher level of EM bioavailability (Kuperman *et al*., 1999). The species of Enchytraeidae, *E. crypticus*, listed in the ISO protocol as an acceptable alternative to *E. albidus*, was selected for toxicity testing.

Energetic materials can affect populations of terrestrial plants and soil invertebrates in different ways. These include (1) direct acute toxicity, (2) chronic toxicity such as effects on growth and/or reproduction, (3) indirect toxicity by altering soil structure or fertility, (4) indirect toxicity by adversely affecting nutrient and food supplies, or (5) by affecting predators and parasites. In addition, soil organisms may alter their environment changing the overall bioavailability of chemicals within the soil. No single test can address these types of effects, and a battery of tests is required to reasonably do so. Inclusion of species from different taxonomic groups representing a range of sensitivities, which often correlate with physiologically-determined modes of action and can vary among taxa, was an important consideration for selecting the test battery for Eco-SSL development. The selected species were expected to represent the spectrum of diverse ecological functions that are attributed to organisms comprising soil communities: primary producers, and different functional groups of soil invertebrates. Test species selected for our studies are representative surrogates of species that normally inhabit a wide range of site soils and geographical areas (i.e., ecologically relevant). Test invertebrate species used in this investigation actively move through soil, thus ensuring contact with contaminants. Both terrestrial plant and soil invertebrate species tested are sensitive to a wide range of contaminants, and reflect different routes of exposure (e.g., ingestion, inhalation, dermal absorption for soil invertebrates, and uptake from soil solution for plants). It was important for Eco-SSL development, that selected test invertebrate species were amenable to life cycle tests to identify the most vulnerable developmental stage of the test organisms (e.g., adult survival, or cocoon and/or juvenile production). Finally, selected terrestrial toxicity tests with representative test species, have been standardized and generate reproducible, statistically-valid results, which imparts a greater confidence in the data and generates less uncertainty associated with the decisions and recommendations that are based on the test data, especially for draft Eco-SSL development.

A draft Eco-SSL for an EM-receptor pairing (e.g., RDX-invertebrates) was calculated as the geometric mean of EC_{20} toxicity values determined from the individual studies. Three toxicity data values generated under specified conditions were the minimum required to calculate an Eco-SSL (USEPA, 2000). The draft Eco-SSL derivation process was completed separately for terrestrial plants and soil invertebrates for each energetic material. Separate draft Eco-SSL values were derived for freshly amended and for weathered/aged amended SSL soil. Growth measurement endpoints in all tests with terrestrial plants, and reproduction measurement endpoints in tests with soil invertebrates, were more sensitive compared with seedling emergence or adult survival, respectively. This supported the Eco-SSL requirement of the use of growth or reproduction endpoints for benchmark development (USEPA, 2000). Consequently, growth measurement endpoints, including fresh and dry shoot mass for all species tested were used for derivation of draft Eco-SSLs for terrestrial plants. Reproduction measurement endpoints were used for derivation of draft Eco-SSLs for soil invertebrates. These endpoints included cocoon production and juvenile production for earthworms, and juvenile production for potworms and collembola.

Draft Eco-SSLs for terrestrial plants were developed for nitroaromatic EMs 2,4-DNT, 2,6-DNT, and TNB for both freshly amended and weathered/aged amended soil. Nitramine EMs RDX and HMX were not phytotoxic up to 10,000 mg kg^{-1} (nominal), the highest

concentration tested in the limit test with the three plant species. Consequently, no draft Eco-SSLs for terrestrial plants could be developed for RDX and HMX. Draft Eco-SSLs for soil invertebrates were developed for the five EMs RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB freshly amended in SSL soil and for RDX, 2,4-DNT, 2,6-DNT, and TNB in weathered/aged amended soil. No Eco-SSL for soil invertebrates could be developed for HMX in weathered/aged amended soil because EC_{20} value was estimated only for one test species, *F. candida*. HMX did not adversely affect reproduction of either earthworms or potworms in weathered/aged amended soil at concentrations tested. The calculated draft Eco-SSLs for each EM- receptor are presented in Tables 14-29, listed here for sake of comparison and increased understanding of different outcomes based on different experimental conditions. These Eco-SSL values are unofficial, since USEPA must review experimental designs of studies, the data produced, and its applicability, before accepting benchmarks or deriving Eco-SSL values.

Table 14. Derivation of Draft Eco-SSL values for 2,4-DNT in freshly amended Sassafras sandy loam soil using growth benchmarks for terrestrial plants alfalfa (*Medicago sativa*), Japanese millet (*Echinochloa crusgalli*), and perennial ryegrass (*Lolium perenne*). All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Alfalfa			
Fresh shoot	11	0-24	
Dry shoot	34	10-59	
Japanese millet			
Fresh shoot	4	2-5	12.6
Dry shoot	25	18-33	
Ryegrass			
Fresh shoot	11	10-12	
Dry shoot	11	10-12	

Table 15. Derivation of Draft Eco-SSL values for 2,4-DNT in weathered/aged amended Sassafras sandy loam soil using growth benchmarks for terrestrial plants alfalfa (*Medicago sativa*), Japanese millet (*Echinochloa crusgalli*), and perennial ryegrass (*Lolium perenne*). All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Alfalfa			
Fresh shoot	7	2-11	
Dry shoot	15	9-21	
Japanese millet			
Fresh shoot	4	2-5	5.3
Dry shoot	6	5-8	
Ryegrass			
Fresh shoot	5	4-7	
Dry shoot	2	0-4	

Table 16. Derivation of Draft Eco-SSL values for 2,6-DNT in freshly amended Sassafras sandy loam soil using growth benchmarks for terrestrial plants alfalfa (*Medicago sativa*), Japanese millet (*Echinochloa crusgalli*), and perennial ryegrass (*Lolium perenne*). All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Alfalfa			
Fresh shoot	1.3	0-3	
Dry shoot	3	0-6	
Japanese millet			
Fresh shoot	13	12-14	8.0
Dry shoot	11	9-13	
Ryegrass			
Fresh shoot	18	4-32	
Dry shoot	26	21-32	

Table 17. Derivation of Draft Eco-SSL values for 2,6-DNT in weathered/aged amended Sassafras sandy loam soil using growth benchmarks for terrestrial plants alfalfa (*Medicago sativa*), Japanese millet (*Echinochloa crusgalli*), and perennial ryegrass (*Lolium perenne*). All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Alfalfa			
Fresh shoot	1.6	0.1-3.2	
Dry shoot	0.4	0-1.4	
Japanese millet			
Fresh shoot	5	4-6	4.5
Dry shoot	6	3-9	
Ryegrass			
Fresh shoot	24	21-27	
Dry shoot	21	18-23	

Table 18. Derivation of Draft Eco-SSL values for TNB in freshly amended Sassafras sandy loam soil using growth benchmarks for terrestrial plants alfalfa (*Medicago sativa*), Japanese millet (*Echinochloa crusgalli*), and perennial ryegrass (*Lolium perenne*). All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Alfalfa			
Fresh shoot	38	10-66	
Dry shoot	62	28-96	
Japanese millet			
Fresh shoot	16	12-21	40.0
Dry shoot	43	27-59	
Ryegrass			
Fresh shoot	45	35-56	
Dry shoot	56	43-67	

Table 19. Derivation of Draft Eco-SSL values for TNB in weathered/aged amended Sassafras sandy loam soil using growth benchmarks for terrestrial plants alfalfa (*Medicago sativa*), Japanese millet (*Echinochloa crusgalli*), and perennial ryegrass (*Lolium perenne*). All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Alfalfa			
Fresh shoot	20	0-49	
Dry shoot	46	2-89	
Japanese millet			
Fresh shoot	0.3	0.1-0.4	8.6
Dry shoot	0.7	0.4-0.9	
Ryegrass			
Fresh shoot	46	13-78	
Dry shoot	51	30-72	

Table 20. Derivation of Draft Eco-SSL values for RDX in freshly amended Sassafras sandy loam soil using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus crypticus* and collembolan *Folsomia candida*. All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Earthworm			
Cocoon Production	1.2	0.4-2.0	
Juvenile Production	1.6	0.4-2.7	
Potworm			21.1
Juvenile Production	3,715	0-8,100	
Collembola			
Juvenile Production	27.8	14.8-41.2	

Table 21. Derivation of Draft Eco-SSL values for RDX in weathered/aged amended Sassafras sandy loam soil using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus crypticus* and collembolan *Folsomia candida*. All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Earthworm			
Cocoon Production	19.2	0-39.0	
Juvenile Production	5.0	0.2-9.0	
Potworm			98.6
Juvenile Production	8,797	761-16,834	
Collembola			
Juvenile Production	113.0	28.6-197.5	

Table 22. Derivation of Draft Eco-SSL values for HMX in freshly amended Sassafras sandy loam soil using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus crypticus* and collembolan *Folsomia candida*. All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Earthworm			
Cocoon Production	2.7	0-7	
Juvenile Production	0.4	0-0.9	
Potworm			6.3
Juvenile Production	No adverse effect on juvenile production		
Collembola			
Juvenile Production	234.8	0-729.6	

Table 23. Derivation of Draft Eco-SSL values for HMX in weathered/aged amended Sassafras sandy loam soil using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus crypticus* and collembolan *Folsomia candida*. All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Earthworm			
Cocoon Production	No adverse effect on cocoon production		Insufficient information to derive Draft Eco-SSL
Juvenile Production	No adverse effect on juvenile production		
Potworm			
Juvenile Production	No adverse effect on juvenile production		
Collembola			
Juvenile Production	1,046	58.4-2,033	

Table 24. Derivation of Draft Eco-SSL values for 2,4-DNT in freshly amended Sassafras sandy loam soil using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus crypticus* and collembolan *Folsomia candida*. All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Earthworm			
Cocoon Production	31	17-44	22.6
Juvenile Production	44	9-77	
Potworm			
Juvenile Production	19	13-26	
Collembola			
Juvenile Production	10	6-14	

Table 25. Derivation of Draft Eco-SSL values for 2,4-DNT in weathered/aged amended Sassafras sandy loam soil using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus crypticus* and collembolan *Folsomia candida*. All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Earthworm			
Cocoon Production	25	16-35	
Juvenile Production	29	17-42	
Potworm			19.8
Juvenile Production	14	10-18	
Collembola			
Juvenile Production	15	11-19	

Table 26. Derivation of Draft Eco-SSL values for 2,6-DNT in freshly amended Sassafras sandy loam soil using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus crypticus* and collembolan *Folsomia candida*. All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Earthworm			
Cocoon Production	14	7-77	
Juvenile Production	9	0-30	
Potworm			12.9
Juvenile Production	37	28-47	
Collembola			
Juvenile Production	6	2-10	

Table 27. Derivation of Draft Eco-SSL values for 2,6-DNT in weathered/aged amended Sassafras sandy loam soil using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus crypticus* and collembolan *Folsomia candida*. All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Earthworm			
Cocoon Production	16	10-22	
Juvenile Production	8	2-15	
Potworm			6.9
Juvenile Production	18	13-23	
Collembola			
Juvenile Production	0.96	0-2.1	

Table 28. Derivation of Draft Eco-SSL values for TNB in freshly amended Sassafras sandy loam soil using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus crypticus* and collembolan *Folsomia candida*. All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Earthworm			
Cocoon Production	27	6.5-48	
Juvenile Production	21	0-55	
Potworm			10.3
Juvenile Production	5	3-7	
Collembola			
Juvenile Production	4	0-12	

Table 29. Derivation of Draft Eco-SSL values for TNB in weathered/aged amended Sassafras sandy loam soil using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus crypticus* and collembolan *Folsomia candida*. All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Receptor Group	EC_{20} (mg kg^{-1})	95% Confidence Internals (mg kg^{-1})	Draft Eco-SSL (mg kg^{-1})
Earthworm			
Cocoon Production	19	10-27	
Juvenile Production	13	7-19	
Potworm			18.1
Juvenile Production	9	4-14	
Collembola			
Juvenile Production	48	27-68	

Review of ecotoxicological benchmark values, herein used for developing draft Eco-SSLs under different stipulations, shows that although majority of values were fairly uniform, there were instances of high variability among EC_{20} estimates determined in toxicity assays. Greatest variability among terrestrial plant benchmark values was found for alfalfa and ryegrass growth estimates for 2,6-DNT, which ranged from 1.3 mg kg^{-1} for alfalfa fresh shoot mass to 26 mg kg^{-1} for ryegrass dry shoot mass in studies with freshly amended soil, and from 0.4 mg kg^{-1} for alfalfa dry shoot mass to 24 mg kg^{-1} for ryegrass fresh shoot mass in studies with weathered/aged amended soil. Even greater contrast was found for alfalfa and Japanese millet growth estimates for TNB, which ranged from 46 mg kg^{-1} for alfalfa dry shoot mass to 0.3 mg kg^{-1} for Japanese millet fresh shoot mass in studies with weathered/aged amended soil. Similar variability was found in toxicity assessments with soil invertebrates. Greatest differences were found for earthworm and potworm juvenile production benchmark estimates for RDX, which ranged from 1.6 mg kg^{-1} for earthworm to 3,715 mg kg^{-1} for potworm in studies with freshly amended soil, and from 5 mg kg^{-1} to 8,797 mg kg^{-1}, respectively in studies with weathered/aged amended soil. Large difference was also found for benchmark estimates for earthworm and collembola juvenile production for HMX, which ranged from 0.4 mg kg^{-1} for earthworm to 234.8 mg kg^{-1} for collembola in studies with freshly amended soil, while no adverse effect was evident for potworm juvenile production up to 21,750 mg kg^{-1}, the highest HMX concentration tested. These examples of species-specific variability in toxicity endpoint values provide clear evidence in support of the USEPA requirement for use of multiple species for generating ecotoxicological benchmarks for Eco-SSL development, and for having selection rules for determining which data are most appropriate for developing Eco-SSLs.

The draft Eco-SSL values were derived using the EC_{20} level of the EM effects on plant growth or soil invertebrate reproduction measurement endpoints. The preference for growth/reproduction benchmarks and for low effect level was justified to ensure that Eco-SSL values would be protective of majority of ecological receptors in soil, and provide confidence that EM concentrations posing an unacceptable risk are not screened out early in the ERA process. Review of the ecotoxicological benchmarks generated in our studies shows that both requirements, including the use of growth/reproduction effects and the use of EC_{20} response level were well justified. Growth measurement endpoints were more sensitive indicators of EM effects on terrestrial plants compared with germination, while reproduction measurement endpoints were more sensitive (or not statistically different based on 95% CI) compared with adult survival in all soil invertebrate tests. The EC_{20} level for growth/reproduction benchmarks generally approximated the ANOVA-based no effect (NOEC) levels for EMs tested in most studies.

Project design was evaluated using the Literature Evaluation Criteria accepted by the Eco-SSL Workgroup and summarized in Table 30. This was done to ensure that draft Eco-SSLs developed by our studies comply with all criteria and would obtain the highest score in each category. Such review would also expedite the transition of the results of our investigations to Eco-SSL Workgroup, who will also apply rules of selection to determine the most appropriate benchmarks for establishing the respective Eco-SSL values.

Table 30. Summary of Literature Evaluation Process for Plant and Soil Invertebrate Eco-SSLs (modified from USEPA, 2000).

Criteria	Rationale
1: Testing was Done Under Conditions of High Bioavailability.	Bioavailability of metals and polar organic compounds is influenced by pH and soil organic matter, cationic exchange capacity, and clay content. The scoring is intended to favor relatively high bioavailability.
2A: (Laboratory) and **2B:** (field): Experimental Designs for Studies are Documented and Appropriate.	Experimental design can significantly influence the quality of a study. Higher quality studies will use an experimental design sufficiently robust to allow analysis of the test variables and discriminate non-treatment effects.
3: Concentration of Test Substance in Soil is Reported.	The concentration of the contaminant tested must be reported unambiguously.
4: Control Responses are Acceptable**.**	Negative controls are critical to distinguish treatment effects from non-treatment effects.
5: Chronic or Life Cycle Test was Used**.**	Chronic toxicity tests assessing long-term adverse sub-lethal impacts on the life-cycle phases of an organism are considered superior to acute toxicity tests.
6: Contaminant Dosing Procedure is Reported and Appropriate for Contaminant and Test.	Contaminant dosing procedure may affect the outcome of a test. Dosing procedure should include: (A) The form of the contaminant; (B) The carrier or vehicle (e.g., solvent, water, etc.); (C) How the carrier was dealt with following dosing (i.e., allowed to volatilize, controls, etc.); (D) procedure for mixing of soil with contaminant (homogeneity).
7: A Dose-Response Relationship is Reported or can be Established from Reported Data.	Two methodologies that can be used to identify this benchmark concentration. The first method generates a no observed effect concentration (NOEC) and a lowest observed effect concentration (LOEC). The second method uses a statistical model to calculate a dose response curve and estimate an effect concentration for some percentage of the population (EC_x), usually between an EC_5 and an EC_{50}.
8: The Statistical Tests used to Calculate the Benchmark and the Level of Significance were Described.	Statistical tests and results reported in the study should be sufficient to determine the significance of the results.
9: The Origin of the Test Organisms is Described.	The results of a toxicity test can be influenced by the condition of the test organisms. Culture conditions should be maintained such that the organisms are healthy and have had no exposure above background to contamination prior to testing (invertebrates) or detailed information is provided about the seed stock (plants).

Information relevant for each criterion of the evaluation processes is summarized below.

1. A natural soil, Sassafras sandy loam [Fine-loamy, siliceous, mesic Typic Hapludult] (SSL) was used in this study to assess the EM toxicity for the test species used. This soil was selected for developing ecotoxicological values protective of soil biota because it has physical and chemical characteristics supporting relatively high bioavailability of the test chemicals (low organic matter and clay contents).

2. Toxicity assays were conducted to determine the effects of RDX, HMX, 2,4-DNT, 2,6-DNT and TNB on terrestrial plants and soil invertebrates. Testing was designed to specifically meet the requirements for Eco-SSL development. All methods used are documented in relevant sections of this report and in appendices presenting the detailed account of individual studies. All assays included range-finding tests to bracket EM concentration range for each test species, and definitive tests to determine ecotoxicological benchmarks required for development of draft Eco-SSL values.

3. Nominal concentrations were analytically verified in all definitive test treatments. All ecotoxicological parameters were estimated using measured chemical concentrations for each treatment level.

4. Each toxicity test was appropriately replicated and included negative (no chemicals added), positive (reference chemical), and carrier (acetone) controls. Test validity criteria were used in all definitive assays. Validity criteria in definitive toxicity tests with terrestrial plants specified minimal percent germination in negative controls for each species tested, and the quality control limit for EC_{50} values in positive control (boric acid). Validity criteria for negative controls in the definitive toxicity tests with soil invertebrates specified minimal percent adult survival, minimal number of juveniles produced, boundaries for coefficient of variation for reproduction, and percent reduction in positive control (beryllium sulfate) from negative control, determined for reproduction measurement endpoint based on the baseline established for the laboratory cultures of earthworms, potworms, and collembola.

5. All toxicity tests were based on the assessments of EM effects on growth (for plants) and reproduction (for soil invertebrates) in addition to acute endpoints germination and adult survival, respectively.

6. Soil amendment procedures were documented and included the form of EMs used, analytical purity of each EM, procedures for preparation of treatment concentrations using acetone carrier, time allowed to volatilize acetone in chemical hood, and duration of 3-dimentional mixing to ensure the homogeneity of EM incorporation in test soil.

7. Measurement endpoint data were analyzed using nonlinear regression models to establish concentration-response relationships for each EM-test species measurement endpoint pairing. The EC_{20} and EC_{50} values for seedling emergence and growth measurement endpoints in the phytotoxicity assays, and for cocoon/juvenile production in the soil

invertebrate assays were determined using SYSTAT software, version 7.0 (SPSS Inc., 1997). The EC_{20} parameter is preferred for deriving Eco-SSL values. The EC_{50}, a commonly reported value, was included to enable comparisons of the results produced in this study with results reported by other researchers.

8. Statistical tests included nonlinear regression analyses and Analysis of Variance (ANOVA). Nonlinear regression analyses were performed using SYSTAT software, version 7.0 (SPSS Inc., 1997). Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to decide whether or not to weight the data, and to select potential models. The asymptotic standard error (a.s.e.) and 95% confidence intervals (CI) associated with the point estimates were determined. ANOVA was used to determine the bounded No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values. Mean separations were done using Fisher's Least Significant Difference (LSD) pairwise comparison tests. When NOAEC (no observed adverse effect concentration) or LOAEC (lowest observed adverse effect concentration) values were determined, the same statistical methods were used. A significance level of $p < 0.05$ was accepted for determining the NOEC and LOEC values. Student's *t*-Test (two-tailed) with significance level set at $p < 0.05$ was used in the limit tests with plants and potworms exposed to RDX or HMX using EXCEL software (Microsoft Corporation, 1997).

9. Sources of seed stocks and soil invertebrates included:
   - Alfalfa (variety Canada no. 1; Cat. # 550, Lot packed and tested 2000). Supplier: Williams Dam Seeds Ltd., Box 8400, Dundas Ontario, Canada, L9H 6M1.
   - Nitrogen-fixing bacteria for alfalfa (Nitragin Gold; Cat. # 309-9, Lot #NGA33). Supplier: Labon Inc. 1350 Newton, Boucherville, Quebec, Canada, J4B 5H2.
   - Japanese millet (variety Common no. 1; Cat. # 300-380, Lot # 9-6). Supplier: Labon Inc. 1350 Newton, Boucherville, Quebec, Canada, J4B 5H2.
   - Perennial ryegrass (variety Express; Cat. # 1269). Supplier: Pickseed Canada Inc., St-Hyacinthe, Quebec, Canada.
   - Corn (variety Kandy corn Canada no. 1; Cat # 199, Lot packed and tested 2001). Supplier: Williams Dam Seeds Ltd., Box 8400, Dundas Ontario, Canada, L9H 6M1.
   - Lettuce (variety Buttercrunch; Cat. # 172, Lot packed and tested Jan. 2001). Supplier: Stokes Seeds Ltd, 296 Collier Road, Box 10, Thorold, Ontario, Canada, L2V 5E9
   - All soil invertebrate test species used in toxicity assays came from cultures maintained by the Environmental Toxicology laboratory, U.S. Army Edgewood Chemical Biological Center, APG, MD, USA.
   - Bioaccumulation tests were performed at the Biotechnology Research Institute (BRI), National Research Council Canada, Montreal, Quebec, Canada. Earthworm *Eisenia andrei* cultures were maintained at BRI and were purchased from Carolina Biological Supply Company, 2700 York Road, Burlington, NC, USA, 27215-3398.

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Review of the information provided for each criterion shows that experimental design of ecotoxicological investigations complied with all screening criteria used by the Eco-SSL Workgroup during literature evaluation processes for selecting terrestrial plant and soil invertebrate benchmarks for Eco-SSL development. The Draft Eco-SSL values developed in this project will be provided to the Ecological Soil Screening Level (Eco-SSL) Workgroup for review. Results will undergo quality control review by the Eco-SSL task group and rules of selection before determining which benchmarks may be included in the Eco-SSL database, and before acceptance as Ecological Soil Screening Levels (Eco-SSLs) for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB.

4.6 Bioaccumulation Potential of Energetic Materials.

This study was designed to obtain direct experimental data on bioaccumulation potential of nitramine and nitroaromatic energetic materials in terrestrial plants and to determine whether these EMs pose a potential risk for toxic effects on higher trophic levels. Bioaccumulation potential of nitramine EMs (RDX and HMX) in earthworms was also included in this investigation to assess the potential risk of contaminant transfer in a food chain that contains a soil invertebrate consumer. Experiments were based on exposure of selected plant species to sublethal concentrations of RDX, HMX, 2,4-DNT, 2,6-DNT or TNB, and on exposure of the earthworm *E. andrei* to sublethal concentrations of RDX or HMX.

4.6.1 Bioaccumulation of Nitramine EMs in Plants.

Bioaccumulation of nitramine EMs in plants was evaluated using exposures in the microcosms and the growth chamber studies. Calculated BCFs for RDX and HMX in plants decreased inversely with soil concentration. In freshly amended soil, BCF values of RDX in alfalfa were 79, 6.8, and 0.27 at nominal concentrations of 100, 1000, and 10000 mg kg^{-1}, respectively. RDX accumulation in plant tissue was significantly greater in weathered/aged amended SSL soil compared with freshly amended soil for alfalfa, Japanese millet and ryegrass. For HMX, BCF values determined in alfalfa using freshly amended soil were 2.2, 0.22, and 0.03 at nominal concentrations of 100, 1000, and 10000 mg kg^{-1}, respectively. BCF values 23-27 for HMX were determined following exposure to 21 mg kg^{-1} in a preliminary test. Weathering/aging did not significantly affect HMX accumulation in plant species tested. These results show that BCFs decrease proportionally with the increase in soil EM concentrations. The same type of proportional decrease in BCF with increasing soil concentrations was apparent for Japanese millet and ryegrass (Table 31).

Table 31. Summary of bioconcentration factors determined in preliminary and definitive plant tests for nitramine EMs using freshly amended (F) and weathered/aged (W/A) EM amended SSL soil.

Measured concentration (mg kg^{-1})	Alfalfa	Japanese millet	Ryegrass
RDX (F) 87	79 (42 d)	70 (42 d)	45 (42 d)
RDX (F) 985	6.8 (42 d)	5.1 (42 d)	3.5 (42 d)
RDX (F) 9,740	0.27 (16 d)	0.17 (16 d)	0.14 (16 d)
RDX (W/A) 9,537	0.66 (16 d)	0.53 (16 d)	0.39 (16 d)
HMX (F) 20.3	23 – 27 (42 d)		26 – 29 (42 d)
HMX (F) 101	2.2 (28 d)	2.2 (28 d)	1.4 (28 d)
HMX (F) 1,125	0.22 (28 d)	0.36 (28 d)	0.13 (28 d)
HMX (F) 10,411	0.03 (16 d)	0.01 (16 d)	0.02 (16 d)
HMX (W/A) 9,341	0.04 (16 d)	0.03 (16 d)	0.02 (16 d)

The inverse relationship between BCF values and soil concentrations may be controlled by an EM accumulation limit in plant tissue, referred to as a saturation level. This relationship may not apply to all plants and may be typical only of exposure to high concentrations in soil. A study at the BRI laboratory (Dr. Pierre Yves Robidoux, personal communication) supported the hypothesis of a saturation level in plants exposed to high soil concentrations of HMX. That study showed that HMX tissue concentrations reached a plateau at 146 mg kg^{-1} in lettuce exposed to soil HMX concentrations 1110 and 2250 mg kg^{-1} for 14 days.

4.6.2 Bioaccumulation of Nitroaromatic EMs in Plants.

Accumulation of 2,4-DNT in plants was detected only in weathered/aged amended SSL soil. The average BCF of 2,4-DNT for alfalfa and ryegrass was 0.3 for weathered/aged amended soil. Accumulation of 2,4-DNT was observed only following weathering/aging process, but given the low values of the BCFs, bioaccumulation of 2,4-DNT remains negligible. All plant species tested accumulated measurable amounts of 2,6-DNT. The BCF values for 2,6-DNT ranged 0.54 – 1.7 for alfalfa, 0.3 - 1.8 for Japanese millet and 0.26 – 0.74 for ryegrass in both freshly amended and in weathered/aged amended soils. Accumulation of 2,6-DNT was not affected by weathering/aging of amended soil. Only alfalfa had measurable tissue TNB concentration following exposure to 67 mg kg^{-1} freshly amended soil, producing a BCF value of 0.3. Bioavailability of TNB was reduced in weathered/aged soil, as was soil TNB concentration. Plants exposed in weathered/aged TNB amended soil had no detectable EM in the tissue.

Variations in BCF values among plant species suggest the species-specific EM biotransformation dynamics, although accumulation in plants was very limited in these tests. Accumulation in plants is usually dependent on the persistence of a compound in soil and on its

uptake/degradation rate in plants. The negligible accumulation of TNB, 2,4-DNT, and 2,6-DNT by plants could result from a low affinity for uptake by plants, or smaller pool of these EMs in soil where they are easily transformed. EMs taken-up by plants could also undergo further degradation/transformation, which would explain their low concentrations in plant tissue. The combination of these factors would decrease the capacity of these EMs to bioaccumulate in their unchanged form. Additional studies using radiolabeled chemicals would be required to verify if biotransformation is occurring in plants.

4.6.3 Bioaccumulation of Nitramine EMs in Earthworms.

The results of our investigations show that nitramines can accumulate in the earthworm *E. andrei*. RDX was moderately accumulated in the earthworm tissues (BCF $\leq$ 13) following exposure in freshly amended SSL soil (Table 32). RDX tissue concentration 125 mg kg^{-1} was determined following exposure to 10 mg kg^{-1} RDX soil. The exposure of *E. andrei* to a soil RDX concentration 99 mg kg^{-1} (a 9-fold increase) increased tissue RDX concentration only by a factor of 2 (283 mg kg^{-1}). This disproportional RDX accumulation by *E. andrei* suggests the possibility of an accumulation saturation mechanism. The differing RDX accumulation by earthworms determined from exposures to different soil RDX concentrations produced contrasting BCF values.

Table 32. Summary of bioconcentration factors (BCF) for nitramine EMs determined for earthworm *Eisenia andrei* in freshly amended soil.

Exposure concentration (mg kg^{-1})	BCF
RDX 10	13 ± 1.0
RDX 99	2.9 ± 0.2
HMX 9	1.0 ± 0.24
HMX 83	0.32 ± 0.02

HMX accumulated in earthworm tissue in considerably lower quantity producing a BCF value $\leq$ 1.0 for freshly amended soil. The tissue concentration determined following exposure to 9 mg kg^{-1} soil was 9 mg kg^{-1} dry weight. The highest HMX tissue concentration was 26 mg kg^{-1} following *E. andrei* exposure to soil HMX concentration of 83 mg kg^{-1} (measured). Exposure of earthworms at the two different soil HMX concentrations resulted in significantly different tissue HMX concentrations. Calculated BCFs were close to or less than 1, indicating that earthworms do not accumulate HMX above the ambient soil concentrations (Table 32).

Acetonitrile extracts from earthworm tissues contained no detectable levels of RDX or HMX demonstrating a clear advantage of using radiolabeled EMs for bioaccumulation studies. The BCF value for HMX was approximately 4 - 5% of the BCF for RDX and

approximated the ratio (11%) of HMX and RDX solubility in water. This difference in water solubility can partially explain the difference in bioaccumulation.

4.6.4 BCF Relevance for Ecological Risk Assessment.

BCF values can be grouped into three categories of bioaccumulation potential for the purposes of ecological risk assessment. BCF values less than 10 would indicative low accumulation potential, values between 10 and 50 would be indicative of a moderate potential, and between 50 and 100 would suggest a relatively high bioaccumulation potential. Based on our results, bioaccumulation potential in plants is moderate for HMX at lower soil concentrations (25 –29 for HMX at 21 mg kg^{-1} soil); relatively high for RDX at lower soil concentrations (45 – 79 for RDX at 87 mg kg^{-1} soil), and low for nitroaromatic EMs (< 0.3 for TNB at 67 mg kg^{-1} soil; $\leq$ 0.44 for 2,4-DNT at 8 – 15 mg kg^{-1} soil; $\leq$ 2 for 2,6-DNT at 4 - 30 mg kg^{-1} soil). However, consideration of a single value BCF may not be sufficient to evaluate the risk of transfer of EMs from soil to various species of plants, invertebrates, or animals.

The concept of BCF developed for exposures in aquatic environment is not easily applied to the exposures in soil because of a large disparity between the soil solution concentration and the bulk soil concentration. This problem can be further accentuated when compounds with low water solubility are considered, as was demonstrated in our studies with acetonitrile-based extraction and ATCLP-based extraction methods for RDX or HMX amended soil. The nonlinear relationship described previously for plants, between soil concentrations and tissue concentrations, shows obvious limitations of using a BCF-based approach for ERA. Due to the BCF calculation procedure (ratio of tissue to soil concentration), its value for compounds with low water solubility will generally be low in soils with relatively high chemical concentrations. This may result in an erroneous conclusion that risks of food chain transfer are decreasing with increasing soil contamination level. Van Gestel *et al*. (2002) discussed similar drawbacks of using BCF values (or BSAF; biota to soil accumulation factor) for metal-contaminated soils, where the BSAF values might increase with decreasing soil concentrations and provide inadequate indication of potential risk. While the use of background levels was suggested for metal contaminated soils, there is no simple solution available for EM contaminated soils. Potentially promising approaches may include determinations of critical body residue (CBR), or internal effect concentrations. This approach is currently being investigated as part of our SERDP CU-1210 project. As an alternative, a model accounting for variations in BCF in relation to soil concentration, or soil quantity/intensity relationships could be used.

4.6.5 Mass Balance Studies with Plants and Earthworms Using Radiolabeled RDX and HMX.

In studies with alfalfa and ryegrass, mineralization of RDX in soil was stimulated in the presence of plants by a factor of 2. Mineralization was less pronounced at nominal soil concentration of 1000 mg kg^{-1}, but increases of 36% – 68% were still observed. Plants may affect RDX mineralization directly, or indirectly through stimulation of microbial activity in the rhizosphere. Additional studies would be required to definitively confirm the capacity of plant tissue to degrade RDX. Supporting evidence for such mineralization was reported in literature (Harvey *et al*., 1991).

There was no obvious difference between HMX mineralization in soil with and without plants at the two concentrations tested. This confirmed the principal role of soil microorganisms in the HMX mineralization, and the limited capacity of plant species used in this study to transform HMX. This limited capacity of plants to metabolize nitramine EMs was further confirmed by recovery of EMs from plant tissue, which ranged from 80% to 95% for RDX, and from 85% to 100% for HMX. Mass balance data showed that plants accumulated less than 3% of amended RDX, or less that 0.1% of amended HMX. The acetonitrile-extractable radioactivity determined by HPLC accounted for 21% to 100% of RDX, and 49% to 100% of HMX. This indicates that a high percentage of the plant radioactivity could be bound to the plant residue after acetonitrile extraction. A consistently high percentage, 92% – 94% (100% in one case), of the radioactivity present in plant extract could be identified as authentic RDX or HMX by HPLC. Only a small fraction of the soluble radioactivity was either CO_2 assimilation products or RDX/HMX derivatives.

Analysis of plant tissue by HPLC consistently produced lower values compared to radioactivity counting, in part due to the radioactivity associated with insoluble/bound material (Table 33). Since this non-extractable radioactivity is likely not readily available for transfer to other trophic levels, it was not accounted for in the calculations of BCFs.

Table 33. Summary of bioconcentration factors determined using radiolabeled and non-labeled RDX or HMX in definitive phytotoxicity tests using freshly amended (F) and weathered/aged (W/A) amended SSL soil.

Exposure type	Alfalfa	Japanese millet	Perennial ryegrass
Labeled EMs (mg kg^{-1})			
RDX (F) 87	79	70	45
RDX (F) 985	6.8	5.1	3.5
HMX (F) 101	2.2	2.2	1.4
HMX (F) 1,125	0.22	0.36	0.13
Non-Labeled EMs (mg kg^{-1})			
RDX (F) 9,740	0.27	0.17	0.14
RDX (W/A) 9,537	0.66	0.53	0.39
HMX (F) 10,411	0.03	0.01	0.02
HMX (W/A) 9,341	0.04	0.03	0.02

Mineralization data from studies with earthworms show that biotransformation of RDX in soil decreased proportionally as the soil concentration increased, similar to the results observed in studies with plants. The presence of earthworms resulted in a 100% increase in RDX mineralization at 10 mg kg^{-1} RDX soil concentration. No increase in RDX mineralization was evident in the 99 mg kg^{-1} RDX soil concentration. Mass balance calculations determined that the earthworms in the 10 mg kg^{-1} RDX treatment accumulated approximately 5% of the added radiolabeled compound, and 1% was taken up in the 99 mg kg^{-1} RDX treatment. Recovery was similar at all soil concentrations. The portion of unaccounted RDX amount ranged from 4% to 17% and could consist of volatile products.

Similar to the results of studies with plants, HMX mineralization was decreased by 2-fold when soil HMX concentration increased from 9 to 83 mg kg^{-1}. The presence of earthworms had no effect on HMX mineralization. Mass balance calculations determined that earthworms accumulated approximately 0.34% of the added radiolabeled HMX in the 9 mg kg^{-1} treatment, and 0.10% was taken up by the earthworms exposed to the 83 mg kg^{-1} HMX treatment. Based on measured soil concentrations, recovery was over 100% at both soil concentrations, indicating a lower degradation rate for this compound compared to RDX.

4.7 Phytogenotoxicity of Dinitrotoluenes.

Chemical analyses of exposure solutions prior to Trad-MCN assays confirmed the test nominal concentrations. Analysis of the recovery solutions showed a linear relationship between the nominal and measured EM concentrations (Fig. 15). EM concentrations were reduced following plant exposure. A portion of EM was retained in the recovery solution, while measurable amounts of EMs were adsorbed or absorbed by the plant cuttings.

Exposure of *Tradescantia* plant cuttings to 2,4-DNT produced a linear dose-dependent response at concentrations between 0 and 30 mg L^{-1} (Fig. 16a). Increasing concentrations up to its solubility limit, did not induce further significant ($p = 0.65$) increase in micronuclei frequencies (Fig. 16b). 2,6-DNT had no significant effect up to 85 mg L^{-1} (Fig. 17). Exposure of *Tradescantia* plant cuttings to 135 and 188 mg L^{-1} of 2,6-DNT induced significantly ($p < 0.05$) higher MCN frequencies compared with the control exposure.

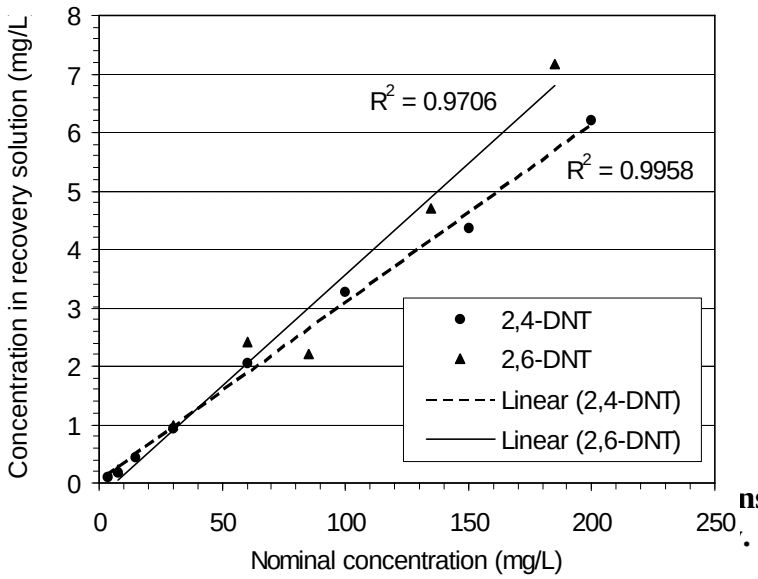

Figure 15. R ns of 2,4-DNT and 2,6-DNT in the e .

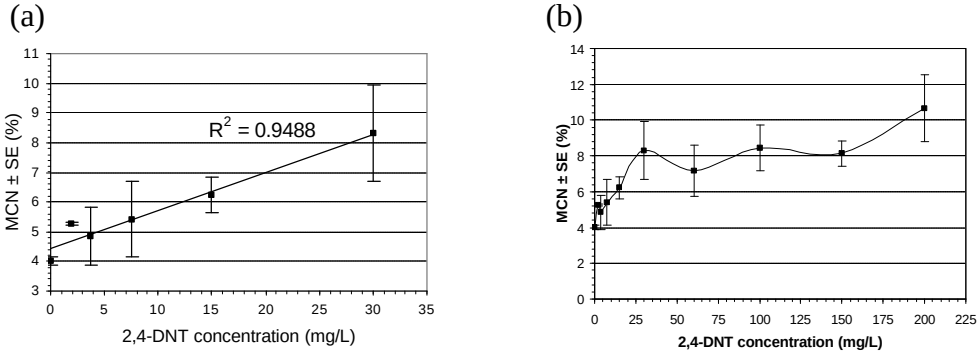

Figure 16. Induction of micronuclei by 2,4-DNT in the tetrad-stage pollen mother cells of *Tradescantia* (exposure time = 6 hr, n = 3 or 4).

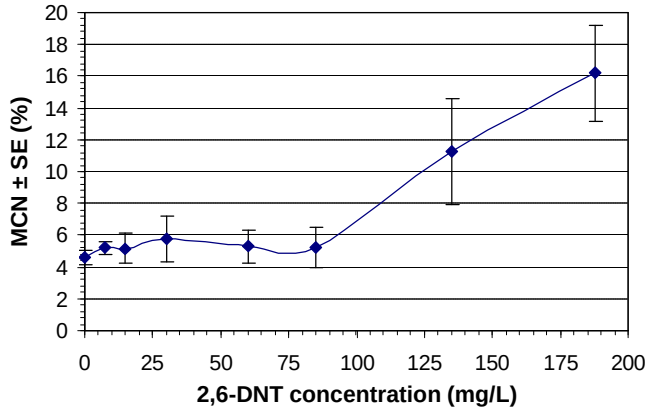

Figure 17. Induction of micronuclei by 2,6-DNT in the tetrad-stage pollen mother cells of *Tradescantia* (exposure time = 6 hr, n = 3 or 4)

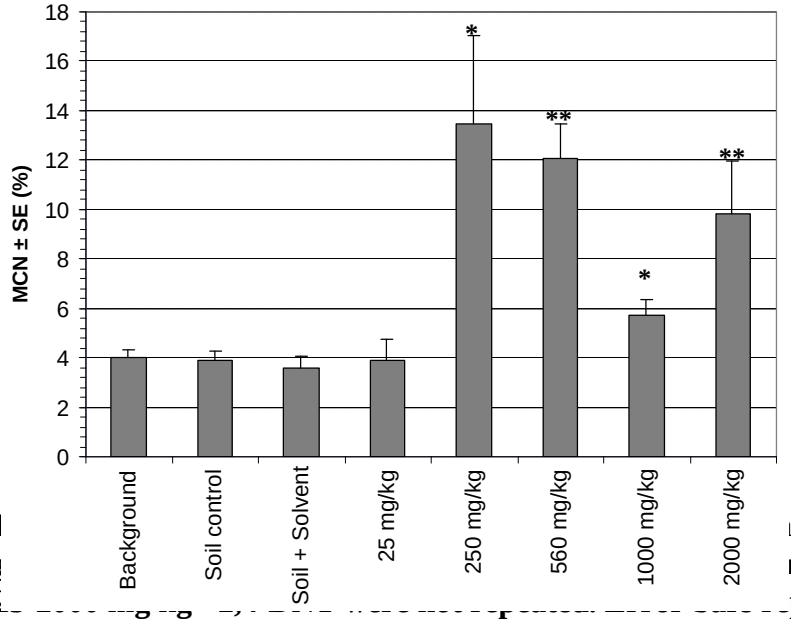

Figure 18. E icronuclei frequency in tetrad-sta n = 3 or 4). Treatments present standard errors from results of 5 to 7 slides scored. (*significant at 95%; **significant at 99%).

Based on the results of Trad-MCN assay conducted with exposure solutions, the NOEC and LOEC values for micronuclei induction were 15 and 30 mg L^{-1}, respectively for 2,4-DNT, and 85 and 135 mg L^{-1}, respectively for 2,6-DNT. Potential for phytogenotoxicity of 2,4-DNT was also investigated in amended SSL soil. Soil was amended with various concentrations of 2,4-DNT to prepare the exposure slurries composed of 100 mL of dechlorinated tap water and

50 g of SSL soil. All exposure treatments above 25 mg kg^{-1} 2,4-DNT produced significantly higher micronuclei frequency compared with the controls (Fig. 18). The significantly higher MCN frequencies in *Tradescantia* pollen mother cells (tetrads) in *Tradescantia* plant cuttings exposed to 2,4-DNT or 2,6-DNT in solution and 2,4-DNT in SSL soil slurries suggest that that these EMs may be phytogenotoxic.

5. SUMMARY AND CONCLUSIONS

This investigation produced experimental data on toxicity and biomagnification potential of nitroamine and nitroaromatic compounds hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), and 1,3,5-trinitrobenzene (TNB) for terrestrial plants and soil invertebrates. Ecotoxicological testing was specifically designed to meet the criteria for Eco-SSL derivation. A natural soil, Sassafras sandy loam was used in all toxicity tests. Sassafras sandy loam had low organic matter and clay contents, which fulfilled the USEPA requirement of using soil with characteristics that support relatively high contaminant bioavailability for developing conservative Eco-SSL values. All ecotoxicological parameters were determined using measured chemical concentrations. This complied with USEPA preference for derivation of Eco-SSL values on the basis of measured soil concentration of a chemical over those based on nominal concentrations.

Growth measurement endpoints in all tests with terrestrial plants and reproduction measurement endpoints in tests with soil invertebrates were more sensitive compared with seedling emergence or adult survival, respectively. Consequently, growth measurement endpoints, including fresh and dry shoot mass for all species tested were used for derivation of draft Eco-SSLs for terrestrial plants. In the derivation of Eco-SSL values, only one of these two endpoints may be accepted per study. Reproduction measurement endpoints were used for derivation of draft Eco-SSLs for soil invertebrates. These endpoints included cocoon production and juvenile production for earthworms, and juvenile production for potworms and collembola. In the derivation of Eco-SSL values, only one of the two soil invertebrate reproduction endpoints may be accepted per study.

Special consideration in assessing chemical toxicity for Eco-SSL development was given to the effects of weathering and aging of contaminant explosives in soil on the exposure of terrestrial receptors. Assessment of the EM toxicity for Eco-SSL development included studies with weathered and aged EM-amended soils to more closely simulate the exposure effects in the field, and because Eco-SSL development by USEPA was specifically undertaken for use at Superfund sites (locations where contaminants have been long-present). Transformation products of nitroaromatic EMs 2,4-DNT and TNB were detected in weathered/aged amended soils. These results strongly suggest that the EMs 2,4-DNT and TNB were transformed due to exposure to sunlight or soil drying/wetting cycles, as occurs normally in nature. Results of toxicity tests with weathered/aged 2,4-DNT, 2,6-DNT, or TNB amended soils showed significantly increased toxicity for Japanese millet, and significantly increased toxicity in weathered/aged 2,4-DNT amended soils for ryegrass. Toxicity was also significantly increased

for potworm *Enchytraeus crypticus* and collembola *Folsomia candida* in 2,6-DNT weathered/aged amended soil. These increases in toxicity for terrestrial plants and soil invertebrates exposed in weathered/aged 2,4-DNT, 2,6-DNT, or TNB amended soils strongly indicate that the soil chemical environment was altered during the 3-month weathering and aging period, similar to changes that can occur in vadose zone soil environments in the field.

In order that Eco-SSLs are appropriately effects-based, receptor responses were coupled with appropriate measures of chemical exposure. This project aimed at determining which chemical measure of exposure better correlated with toxicity by measuring EM concentrations as acetonitrile-extractable and as the labile water-extractable chemical concentrations, which was perceived to measure the immediately bioavailable fraction of chemicals in soil pore water. Two extraction methods included acetonitrile extraction performed according to USEPA Method 8330A, and an Adapted Toxicity Characteristic Leaching Procedure (ATCLP) based on modification of the Toxicity Characteristic Leaching Procedure (TCLP). Coefficients of determinations (R^2) for acetonitrile and ATCLP based extractions determined in nonlinear regression analyses of the plant germination and growth data, and soil invertebrate reproduction data from studies with freshly amended and weathered/aged EM amended soils were compared to determine which chemical measure of exposure better correlated with toxicity. These comparisons showed that coefficients of determinations were generally similar or higher when acetonitrile extractable concentrations were used compared with ATCLP extractable concentrations. This was true for both freshly amended and weathered/aged amended soils indicating that neither extraction method had an advantage for characterizing bioavailability of EMs to the three terrestrial plant or soil invertebrate species tested in this study. This result supported our decision for developing draft Eco-SSLs for explosives contaminants in soil on the basis of acetonitrile extraction of test compounds.

The ultimate goal of this project was to develop toxicity benchmark values for RDX, HMX, 2,4-DNT, 2,6-DNT and TNB from which draft Eco-SSL values may be derived for terrestrial plants and soil invertebrates. Draft Eco-SSL values were derived using the EC_{20} level of the EM effects on plant growth or soil invertebrate reproduction measurement endpoints determined from standardized toxicity tests. The preference for growth/reproduction benchmarks and for low effect level was justified to ensure that Eco-SSL values would be protective of majority of ecological receptors in soil and provide confidence that EM concentrations posing an unacceptable risk, are not screened out early in the ERA process.

Draft Eco-SSL values were developed using ecotoxicological benchmarks based on analytically determined soil concentration of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB in soil in all definitive toxicity tests. A draft Eco-SSL for an EM-receptor pairing was calculated as the geometric mean of EC_{20} toxicity values determined from the individual studies with terrestrial plants and soil invertebrates for each energetic material. Separate draft Eco-SSL values were derived for freshly amended and for weathered/aged amended SSL soil.

Draft Eco-SSLs for terrestrial plants were developed for nitroaromatic EMs 2,4-DNT, 2,6-DNT, and TNB for both freshly amended and weathered/aged amended soil. Nitramine EMs RDX and HMX were not phytotoxic up to 10,000 mg kg^{-1}, the highest concentration tested

in the limit test with the three plant species. Consequently, no draft Eco-SSLs for terrestrial plants were developed for RDX and HMX. Draft Eco-SSLs for soil invertebrates were developed for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB freshly amended in SSL soil and for RDX, 2,4-DNT, 2,6-DNT, and TNB in weathered/aged amended soil. No Eco-SSL for soil invertebrates were developed for HMX in weathered/aged amended soil because EC_{20} value was estimated only for one test species, *F. candida*. HMX did not adversely affect reproduction of either earthworms or potworms in weathered/aged amended soil at concentrations tested.

Experimental design of ecotoxicological investigations complied with all requirements for draft Eco-SSL development for terrestrial plant and soil invertebrates. The toxicity benchmark values and reports detailing these studies will be provided to the Ecological Soil Screening Level Workgroup for review. Results will undergo quality control review by the Eco-SSL task group before inclusion in the Eco-SSL database, and before acceptance for derivation of Ecological Soil Screening Levels (Eco-SSLs) for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB.

Bioaccumulation and mass-balance characteristics of two nitro-heterocyclic energetic materials RDX and HMX were investigated using alfalfa, Japanese millet, ryegrass, lettuce and corn, and the earthworm *Eisenia andrei*. Bioaccumulation of TNT by-products 2,4-DNT, 2,6-DNT, and TNB was investigated using alfalfa, Japanese millet, and ryegrass. Tests were conducted in Sassafras sandy loam soil and included assessments of effect of simulated weathering/aging of amended soil on the bioaccumulation potential of these EMs.

Results showed that [C^{14}]-RDX and [C^{14}]-HMX were accumulated in the earthworm and plant tissues. Little or no accumulation of TNB or of the DNT's was observed in plants. This study showed that the nitroamine EM bioaccumulation potential is relatively high for plants and moderate for earthworms. The bioaccumulation potential for nitroaromatic EMs for plants is low.

Bioconcentration factors (BCFs) for plants exposed to 87 mg kg^{-1} RDX in freshly amended in SSL soil ranged from 45 to 79. BCFs decreased at higher soil concentrations, ranging 3.5 - 6.8 in 985 mg kg^{-1} RDX exposure, and 0.14 - 0.27 in 9740 mg kg^{-1} RDX exposure. For HMX these values ranged 23 – 29, 1.4 – 2.2, 0.13 – 0.36, and 0.01 - 0.03 at the exposure concentrations 20, 101, 1125 and 10411 mg kg^{-1}, respectively. Weathering/aging of amended soil increased average accumulation values for plants by 64% for RDX, and 17% for HMX in the highest exposure concentrations tested. The nitroaromatic TNB and 2,4-DNT did not accumulate in plants and 2,6-DNT accumulation was very low in freshly amended soil. The effect of weathering/aging of nitroaromatic EM amended soil on bioaccumulation was negligible. Bioaccumulation in earthworm in freshly amended soil was moderate for RDX (BCF of 13 and 3 at concentrations of 10 and 99 mg kg^{-1} soil) and low for HMX (BCF of 1 and 0.3 at concentrations of 9 and 83 mg kg^{-1} soil, respectively).

The nonlinear relationship between soil and tissue concentrations, showed obvious limitations of using a traditional BCF-based approach for ERA. Due to the BCF calculation procedure, its value for compounds with low water solubility was lower in soils with

higher chemical concentrations. This may result in an erroneous conclusion that risks of food chain transfer are decreasing with increasing soil contamination level. Potentially promising approach can be determination of critical body residue (CBR). Alternatively, a model accounting for variations in BCF in relation to soil concentration could be developed, such as soil quantity/intensity relationships.

BCF values can be grouped into three categories of bioaccumulation potential for the purposes of ecological risk assessment. BCF values less than 10 would indicative low accumulation potential, values between 10 and 50 would be indicative of a moderate potential, and between 50 and 100 would suggest a relatively high bioaccumulation potential. Based on our results, bioaccumulation potential in plants is moderate for HMX at lower soil concentrations (25 – 29 at 21 mg kg^{-1} soil HMX); relatively high for RDX at lower soil concentrations (45 – 79 at 87 mg kg^{-1} soil RDX), and low for nitroaromatic EMs (< 1 for TNB and 2,4-DNT, and $\leq$ 2 for 2,6-DNT).

To further understand the environmental impacts of exposure to EM soil contaminants, phytogenotoxicity of dinitrotoluenes was assessed using the *Tradescantia* Micronucleus (Trad-MCN) bioassay. Based on the results of this assay, the NOEC and LOEC values for micronuclei induction were 15 and 30 mg L^{-1}, respectively for 2,4-DNT, and 85 and 135 mg L^{-1}, respectively for 2,6-DNT. The significantly higher MCN frequencies in *Tradescantia* pollen mother cells (tetrads) in *Tradescantia* plant cuttings exposed to 2,4-DNT and 2,6-DNT suggest that that these EMs may be phytogenotoxic.

6. TRANSITION PLAN

The toxicity benchmark values and reports detailing these studies will be provided to the Ecological Soil Screening Level Workgroup for review. Results will undergo quality control review by the Eco-SSL task group before inclusion in the Eco-SSL database, and before acceptance for derivation of Ecological Soil Screening Levels (Eco-SSLs) for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB.

Dr. Checkai, Co-PI of this project, is a Co-Chair of the Eco-SSL Task Group and a member of the Eco-SSL Workgroup Steering Committee. Dr. Kuperman, PI of this project, is a member of the Eco-SSL Task Group. Both Dr. Checkai and Dr. Kuperman will provide a direct conduit for the transitioning data generated during this research to USEPA for the development of Eco-SSLs.

The draft Eco-SSLs developed for five EMs in this project, if the toxicity benchmark values and resulting Eco-SSLs are accepted by USEPA, should be used during Step 2 of the Superfund Ecological Risk Assessment process, the screening-level risk calculation. It is expected that the Eco-SSLs will be used to screen the site soil data to identify those EM contaminants that are not of potential ecological concern and do not need to be considered in the subsequent Baseline ERA, resulting in significant cost saving during sites assessments and remedial investigations.

A portion of results of our studies have been published or accepted for publication in peer reviewed journals and several manuscripts are in preparation. These publications will further aid in transition of our findings.

7. RECOMMENDATIONS

Our findings of increased toxicity for several terrestrial species in weathered/aged amended soil clearly show that additional studies are required to investigate the toxicity of the EM degradation products. Analogously, investigation of the more toxic transformation compounds that arise within soils amended with 2,4-DNT, 2,6-DNT, TNB or TNT should also have a weathering/aging component, so that the level of persistence and long-term impact of the ecotoxicity of these toxic transformation products may also be assessed. Such studies should be designed to generate benchmark data for EM breakdown/transformation products so results may be used for deriving draft Eco-SSLs for these chemicals, while providing more complete information on ecotoxicological effects of energetic contaminants in soil for risk assessors and site managers.

Additional studies will be required to address the limitations of BCF approach. The bioaccumulation studies for nitramine and nitroaromatic EMs in plants and soil invertebrates should include studies using lower soil EM concentrations, to confirm the non-linear relationship between soil concentrations and receptor tissue levels. Studies will be required to definitively establish whether a tissue saturation level exists for plants and soil invertebrates exposed to nitramine EMs in freshly amended and weathered/aged soils. Such studies should be conducted with a variety of soil types to determine the effects of soil properties on EM bioavailability and bioaccumulation.

Studies using radiolabeled EMs are needed to evaluate the distribution and degradation of EM compounds in soil and plants, and to determine if a significant portion of the tissue bound radioactivity (EM related products) can be transferred to higher trophic level receptors. The utility of critical body residue approach for evaluating the EM transfers in the food chain should be included in the future investigations.

8. LITERATURE CITED

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APPENDIX A

TOXICITY OF NITRO-HETEROCYCLIC AND NITROAROMATIC ENERGETIC MATERIALS TO TERRESTRIAL PLANTS IN A NATURAL SANDY LOAM SOIL

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USEPA is developing Ecological Soil Screening Level (Eco-SSL) values for ecological risk assessment of contaminants at Superfund sites. Insufficient information for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB to generate Eco-SSLs for terrestrial plants necessitated toxicity testing to fill the data gaps. We used standardized toxicity tests selected on the bases of their ability to measure chemical toxicity to ecologically relevant test species, and their inclusion of growth component among the measurement endpoints. Tests were conducted in Sassafras sandy loam soil, which supports relatively high bioavailability of the energetic materials. Weathering/aging of amended treatment soil was incorporated in the study to better reflect the exposure conditions in the field soils. Definitive toxicity tests conducted with both freshly amended and weathered/aged amended soils generated ecotoxicological benchmarks, including EC_{20} values for growth that can be used for Eco-SSL development. These study results will be provided to the Ecological Soil Screening Level (Eco-SSL) workgroup for review and for developing Ecological Soil Screening Levels (Eco-SSLs) for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB.

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PREFACE

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TOXICITY OF NITRO-HETEROCYCLIC AND NITROAROMATIC ENERGETIC MATERIALS TO TERRESTRIAL PLANTS IN A NATURAL SANDY LOAM SOIL

1. INTRODUCTION

The Strategic Environmental Research and Development Program (SERDP) has identified a research need under the FY00 Broad Agency Announcement (BAA) CUSON-SP-00-04 entitled "Development of Ecological Toxicity and Biomagnification Data for Explosives Contaminants in Soil" to extend the knowledge of the toxicity of explosives-related soil contaminants to ecological receptors. Ecological receptors of interest included terrestrial plants and soil invertebrates. The focus of this investigation was to obtain direct experimental data on toxicity of nitro-heterocyclic and nitroaromatic compounds to terrestrial plants in soil with parameters (i.e., pH, organic matter, clay content, etc.) promoting a relatively high bioavailability of the energetic materials (EM).

Many scientists have investigated the toxicity of TNT to plants, but few have investigated the phytotoxicity of RDX, HMX, TNB or dinitrotoluenes. Phytotoxicity of TNT has been evaluated using single aquatic species, such as Eurasian Watermilfoil and duckweed, and terrestrial plant species, including yellow nutsedge, poplar, lettuce and tall fescue (Schott and Worthley, 1974; Palazzo and Leggett, 1986; Cataldo *et al.*, 1989; Peterson *et al.*, 1996; Pavlostathis *et al.*, 1998; Thompson *et al.*, 1998; Sunahara *et al.*, 2001). Toussaint *et al.* (1995) reported an EC_{50} value of 1 μM (0.2 mg L^{-1}) for the effect of TNT on lettuce root elongation. Robidoux *et al.* (2003) estimated IC_{20} values of 204 and 3113 mg kg^{-1} TNT for lettuce seedling emergence in forest soil and artificial soil (silica), respectively. Exposure of barley seeds to TNT in forest soil produced IC_{20} values of 398, 139, 272 and less than 91 mg kg^{-1} TNT for barley seedling emergence, fresh shoot mass, dry shoot mass, and root mass, whereas these values were 8133, 8133, 133, 1199 and less than 56 mg kg^{-1} TNT in artificial soil (silica) (Robidoux *et al.*, 2003).

Other studies compared the toxicity of different plant species to TNT, RDX and TNB, individually. Gong *et al.* (1999) compared the toxicity of TNT to cress, turnip, oat and wheat and determined a lowest observable adverse effect concentration (LOAEC) of 50 mg kg^{-1} TNT in soil, and stimulation of seedling growth at lower concentrations of TNT (5 to 50 mg kg-^{1}). Scheidemann *et al.* (1998) showed that alfalfa could not grow in soil contaminated with 100 mg kg^{-1} TNT, whereas wheat and bush bean could develop at 500 mg kg^{-1} TNT in soil. Winfield *et al.* (1999) found that sunflower and sanfroin were the most sensitive species among ten species exposed to RDX at soil concentrations up to 4000 mg kg^{-1}. Reddy *et al.* (1994) assessed the toxicity of TNB in sand using lettuce and oat. The authors reported seed germination EC_{50} values of 19 mg kg^{-1} for lettuce and greater than 375 mg kg^{-1} for oat.

Few studies investigated the toxicity of energetic material (EM) mixtures to terrestrial plants. In a study of collected field soils, Simini *et al.* (1995) compared the toxicity of

soils contaminated with TNT, TNB, RDX, HMX and heavy metals to cucumber and radish. They determined that toxicity was mostly related to TNT and TNB, with a LOEC of 7 to 19 mg kg^{-1} TNT in soil. In another field study (Price *et al.*, 1997; Pennington and Brannon, 2002), corn stover was more tolerant compared with tomato vine, nutsedge, corn ears, tomato fruit, and lettuce. In that study, corn, tomato and lettuce died when exposed to 580 mg kg^{-1} RDX and 1720 mg kg^{-1} TNT. All these studies demonstrated that phytotoxicity of explosives was species dependent, but no generalization for sensitivity between monocotyledonous and dicotyledonous plants could be drawn.

Soil type also influences the chemical bioavailability and toxicity of a contaminant. In a comparative study of TNT or HMX toxicities to lettuce and barley using artificial and forest soils, Robidoux *et al.* (2003) determined that TNT was more toxic to barley in organic forest soil than in mineral artificial (silica) soil, while HMX was not toxic to lettuce and barley up to 1866 mg kg^{-1} HMX in artificial soil, and up to 3320 mg kg^{-1} HMX in forest soil. A 45-d exposure to 80 mg kg^{-1} TNT in Tunica silt or Sharkey clay soils, did not affect yellow nutsedge growth compared with controls (Pennington, 1988; Talmage *et al.*, 1999).

Review of the literature showed that, except for TNT, few studies have sufficiently investigated the effects of EMs on terrestrial plants although these contaminants are persistent and some are highly mobile in the environment. As a result, no screening values, which could be used in the Ecological Risk Assessment (ERA), are available for these EM soil contaminants. Scientifically based ecological soil screening levels (Eco-SSLs) are needed to identify EM concentrations in soil that present an acceptable ecological risk. Eco-SSLs are defined as concentrations of chemicals in soil that, when not exceeded, will be protective of terrestrial ecosystems from unacceptable harmful effects. These Eco-SSL concentrations can be used in a Screening Level ERA to identify those contaminants in soil that warrant additional evaluation in a Baseline ERA, and to eliminate those that do not. The insufficient information for EMs required to generate Eco-SSLs for terrestrial plants necessitated our study to fill this knowledge gap.

This study was designed to produce benchmark data for the development of Eco-SSLs for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), and 1,3,5-trinitrobenzene (TNB) for terrestrial plants, and meet specific criteria (United States Environmental Protection Agency, 2000), including: (1) tests were conducted in soil having physico-chemical characteristics that support relatively high bioavailability of chemicals; (2) experimental designs for laboratory studies were documented and appropriate; (3) both nominal and analytically determined concentrations of chemicals of interest were reported; (4) tests included both negative and positive controls; (5) tests that included growth measurement endpoint were used; (6) appropriate chemical dosing procedures were reported; (7) concentration-response relationships were reported; (8) statistical tests used to calculate the benchmark and level of significance were described; and (9) the origin of test species were specified and appropriate. The specific objectives of this study included the assessment of EM toxicity by determining the bounded NOEC and LOEC values, and EC_{20} and EC_{50} values for plant germination and growth measurement endpoints based on concentration-response

relationships; evaluation of soil extraction methods to determine which chemical measure of exposure better correlates with toxicity; and examination of the potential effects of weathering and aging of amended soil on EM toxicity to terrestrial plants.

2. MATERIAL AND METHODS

2.1 Sassafras sandy loam Soil.

A natural soil, Sassafras sandy loam [Fine-loamy, siliceous, mesic Typic Hapludult] (SSL) was used in this study to assess the toxicity of test chemicals to plants. This soil was selected for developing ecotoxicological values protective of soil biota because it has physical and chemical characteristics supporting relatively high bioavailability of the test chemicals (low organic matter and clay contents). The SSL soil was collected from an open grassland field on the property of the U.S. Army Aberdeen Proving Ground (APG; Edgewood, MD). Vegetation and the organic horizon were removed to just below the root zone, and the top six inches of the A horizon were then collected. The soil was sieved through a 5 mm^2 mesh screen, air-dried for at least 72h and mixed periodically to ensure uniform drying, then stored at room temperature before use in testing. Soil was analyzed for physical and chemical characteristics by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD. Results of these analyses are presented in Table 1.

Table 1. Physical and chemical characteristics of Sassafras sandy loam soil analyzed by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD.

Soil Parameter	Sassafras Sandy Loam
Sand (%)	71
Silt (%)	18
Clay (%)	11
Texture	Sandy loam
CEC (cmol $kg^{-1)}$	4.27
Organic matter (%)	1.3
pH	5.0

2.2. Chemicals.

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX; CAS: 121-82-4; Purity: 99%), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX; CAS: 2691-41-0; Purity: 99%), and 1,3,5-trinitrobenzene (TNB; CAS: 99-35-4; Purity: 99.7%) were obtained from the Defense Research Establishment Valcartier of the Canadian Ministry of National Defense (Val Bélair, QC, Canada). 2,4-dinitrotoluene (2,4-DNT; CAS: 121-14-2; Purity: 97%), 2,6-dinitrotoluene (2,6-DNT; CAS: 606-20-2; Purity: 98%) were obtained from Sigma-Aldrich Canada (Oakville, ON, Canada). Boric acid (H_3BO_3; CAS: 10043-35-3; Purity: 99.9%) was used as the positive control. Acetone (CAS: 67-64-1; HPLC Grade) was used for preparing EM solutions during soil amendments. Acetonitrile (CAS: 75-05-8; HPLC Grade), calcium chloride ($CaCl_2 \cdot 2H_2O$; analytical grade) and sodium bisulfate ($NaHSO_4 \cdot H_2O$; certified grade) were used for extractions for chemical analyses and 1,3-dinitrobenzene (1,3-DNB) was used as the internal standard.

Glassware was washed with phosphate-free detergent followed by rinses with acetone, nitric acid, and ASTM type I water (American Society of Testing and Materials, http://www.astm.org). ASTM type I water was obtained using Millipore® Super Q water purification system (Millipore®, Nepean, ON, Canada) and was used throughout the study.

2.3. Soil amendment procedure.

Sassafras sandy loam (SSL) soil was individually amended with RDX, HMX, 2,4-DNT, 2,6-DNT or TNB. Prepared SSL soil was weighed separately for each treatment in a glass dish. For each treatment, soil was spread to a thickness of approximately 2.5 - 4 cm. Each concentration of EM was prepared separately in glass volumetric flasks and dissolved in acetone. The EM/acetone solution was quantitatively transferred to the soil evenly across the soil surface, ensuring that the volume of solution added at any one time did not exceed 15% (volume $mass^{-1}$) of the dry mass soil. After addition of the EM solution, the volumetric flask was rinsed twice with a known volume of acetone and this was also applied to the soil surface. If the total volume of solution needed to amend the soil exceeded 15% (v m^{-1}), the solution was added in successive stages, allowing the acetone to evaporate for a minimum of 2 h under a chemical hood. The same total EM /acetone solution volume was added to every treatment, equaling the volume required to dissolve the EM at the highest concentration tested. The amended soil was then air-dried overnight (minimum of 18 h) in a darkened chemical hood. Each amended soil sample was transferred into a high-density polyethylene container coated with fluoropolymer (Teflon-like chemical) and covered with aluminum foil, to prevent photolysis of the EM. The sample was mixed overnight (18 h ± 2h) using a three-dimensional mixer. Soil was then ready for the phytotoxicity assays.

Weathered/aged amended soil was prepared in the same manner as the freshly amended soil. ASTM type I water was added to adjust the soil moisture to a level equivalent to 75 % of the water holding capacity (WHC). Hydrated soil was exposed to wetting and drying cycles and sunlight in a greenhouse for a period of 13 weeks. Each week, ASTM type I water was added to adjust the soil moisture to initial level (75% of WHC), and was allowed to dry until the next addition of water. The week before the initiation of plant toxicity test using weathered/aged amended soil, each air-dry soil treatment was mixed overnight using a three-dimensional mixer one day prior to the initiation of the test.

2.4. Water holding capacity of soil.

Water holding capacity of the soil was measured accordingly to the procedure provided by Dr. Ronald Checkai (U.S. Army ECBC). Briefly, SSL soil was transferred into10 cm plastic pots in triplicate so that the soil surface was 2 cm below the rim of the pot. Pots were placed on 2 mm mesh sieves to allow free water drainage. A volume of ASTM Type I water equal to the soil volume was slowly added onto the settled soil. Water was allowed to dry for 24 hours. A first aliquot of soil was sampled below the soil surface (below 1-3 cm). Moist soil was immediately weighed and recorded as wet mass ($Mass_{moist\ soil}$). Similar aliquots were taken from the two other replicates. Sub-samples of the moist soil were dried in a 105 °C oven for 18 h and transferred in a desiccator at room temperature for 30 min prior to weighing the dry mass (Mass

$_{dry\ soil}$). This procedure was repeated after 48 h and 72 h, to ensure that a steady state for WHC had been achieved. Water holding capacity (WHC) was calculated according to the following formula:

$$WHC\ \% = [(Mass_{moist\ soil} - Mass_{dry\ soil}) / Mass_{dry\ soil})] * 100$$

2.5. Measurement of soil pH.

Soil pH was measured in each treatment concentration at the beginning of each range-finding tests using freshly amended soil, and in each treatment concentration at the beginning and end of each definitive tests using freshly amended and weathered/aged amended soils. The soil pH was measured according to ISO 10390 method (International Standardization Organization, 1994). Briefly, approximately 5 mL volume of soil was placed in a 50-mL tube, to which 25 mL of ASTM type I water was added. Sample was vortexed for 20 sec and rotated for 5 min at 90 rpm. Soil slurry was let stand at 21 ± 3 °C for 3 h prior to measurement. The pH reading was taken after 1 min, which was sufficient to have a stable reading.

2.6. Measurement of soil redox potential.

The oxidation-reduction (redox) potential was measured in each treatment concentration at the beginning (1 reading per concentration) and at the end of each definitive tests using freshly amended and weathered/aged amended soils (3 replicate per concentration and per plant species). The redox potential of soil was measured according to supplier's instruction (Accumet; Patrick *et al.*, 1996). Prior to redox measurement, soil samples were equilibrated with ASTM type I water (75% of the WHC) during 24 h, in the dark at room temperature. Redox readings were taken after 5 min, which was sufficient to obtain a stable reading.

2.7. Cation exchange capacity of soil.

Soil cation exchange capacity was measured in duplicate for each treatment concentration at the beginning of each definitive tests using freshly amended and weathered/aged amended soils. The effective cation exchange capacity (CEC) was measured according to Hendershot *et al.* (1993) and was performed by Hélène Lalande at McGill University, Montreal, Quebec, Canada. An aliquot of 0.5 - 3.0 g of air-dried soil (< 2 mm) was weighed in a 50-mL centrifuge tube, in duplicate. To each tube, 30.0 mL of 0.1 M of $BaCl_2$ was added and shaken slowly on an end-over-end shaker (15 rpm) for 2 h. Each tube was centrifuged (15 min, 700 x g) and the supernatant was filtered with Whatman No. 41 filter paper. The cations Ca, Mg, K, Na, Al, Fe and Mn were analyzed with an atomic absorption spectrophotometer. Effective CEC was calculated using the following equation:

$$\text{Effective CEC cmol (+) kg}^{-1} = \Sigma\ M^{+}\ \text{cmol (+) kg}^{-1}$$

2.8. Soil acetonitrile extraction.

Acetonitrile extractions of soil samples were performed at the beginning of each range-finding tests using freshly amended soil, and at the beginning and end of each definitive tests using freshly amended and weathered/aged amended soils. Acetonitrile extraction procedure is a modification of USEPA Method # 8330A (United States Environmental Protection Agency, USEPA, 1998). At the beginning of each toxicity test, soil samples were equilibrated in the dark for 24 h at room temperature, after addition of ASTM type I water (75% of WHC). Aliquots of 2.0 g were sampled in triplicate from each treatment concentration. At the end of each definitive toxicity test, aliquots of 2.0 g were taken from each treatment replicate. To each soil aliquot placed in individual glass tubes, 100 µL of 50 mg 1,3-dinitrobenzene (1,3-DNB) L^{-1} internal standard solution and 10 mL of acetonitrile were added. Glass tubes were vortexed for 1 min and then sonicated in the dark for 18h ± 2 h at 20 °C. Five mL of sonicated sample was transferred to a new tube, to which 5 mL of 5 g L^{-1} $CaCl_2$ solution was added. For soil samples amended with TNB, a solution of 5 g L^{-1} $CaCl_2$ + 0.2 g L^{-1} $NaHSO_4$ was added to prevent TNB degradation. Supernatant was filtered through 0.45 µm Millex-HV cartridges. Soil extracts were analyzed and quantified using an HPLC. Extraction was repeated if 1,3-DNB internal standard recovery was lower than 90%.

2.9. Soil ATCLP extraction.

In addition to acetonitrile extraction, soil samples were extracted using an Adapted Toxicity Characteristic Leaching Procedure (ATCLP; Haley *et al.*, 1993) at the beginning of each definitive tests using freshly amended and weathered/aged amended soils. The ATCLP is based on modification of the Toxicity Characteristic Leaching Procedure (TCLP; 40 CFR Part 268.41, Hazardous Waste Management, Method 1311). The modification involved substitution of CO_2-saturated ASTM type I water for acetic acid, better simulating field soil-water conditions due to respiration by soil biota. Prior to ATCLP extraction, soil samples were equilibrated in the dark for 24 h at room temperature, after addition of ASTM type I water (75% of WHC). For each treatment concentration, aliquots of 4.0 g soil were transferred in triplicate into 20-mL scintillation vials. Sixteen mL of CO_2 saturated water at pH 4.5 added and vials were rapidly sealed tight. Soil samples were vortexed 45 sec and were mixed in the dark for 18 h ± 2 h using a rotary mixer (30 rpm) at room temperature. Soil was allowed to settle and supernatants were filtered through .45 µm Millex-HV cartridges. An equivalent volume of acetonitrile was added to filtered soil extract prior to HPLC analysis. For TNB soil extracts, an equivalent volume of acetonitrile: 0.2 g L^{-1} $NaHSO_4$ solution 1:1 was added. In the present report, ATCLP soil extraction is referred to as the water-soluble fraction of EM, which was perceived to measure the portion of EM bioavailable to plants.

2.10. Chemical analysis.

Soil and plant extracts were analyzed using a Thermo Separation Products chromatographic system composed of model P4000 pump, a model AS1000 injector, including the temperature control for the column, and a model UV6000LP photodiode-array detector. For TNB, 2,4-DNT and 2,6-DNT analyses, a Supelcosil C8 column (25 cm x 4.6 mm ID, 5 µm particles) and an 18% 2-propanol / 82% water mobile phase were used. The flow rate was 1 mL min^{-1} and the run time was 40 min. For RDX and HMX analyses, the column used was a

Supelcosil LC-CN (25 cm x 4.6 mm ID, 5 μm), held at 35 °C. The initial solvent composition was 30% methanol / 70% water, which was held for 8 min, then a linear gradient was run from 30 to 65% methanol over 12 min. This solvent ratio was then changed to initial conditions (30% methanol) over 5 min. These initial conditions were then held for an additional 5 min. The injection volume was 50 μL. The detector was set to scan from 200 to 350 nm and chromatograms were extracted at 254 nm. The limit of quantification was 50 ppb for each chemical.

2.11. Plant toxicity tests.

The plant toxicity tests were performed according to protocols of ASTM standard guide for conducting terrestrial plant toxicity tests (American Society for Testing and Materials, 1998) and USEPA early seedling growth test (United States Environmental Protection Agency, USEPA, 1982)

Range-finding tests were performed using Kandy corn Canada no. 1, *Zea mays* (Williams Dam Seeds Ltd., Dundas, Ontario, Canada), lettuce variety Buttercrunch, *Lactuva sativa* (Stokes Seeds Ltd, Thorold, Ontario, Canada), alfalfa variety Canada no. 1, *Medicago sativa* (Williams Dam Seeds Ltd., Dundas, Ontario, Canada), perennial ryegrass variety Express, *Lolium perenne* (Pickseed Canada Inc., St- Hyacinthe, Quebec, Canada) and Japanese millet variety Common no. 1, *Echinochloa crusgalli* (Labon Inc. Boucherville, Quebec, Canada). Five nominal concentrations 1, 10, 100, 1000, and 10000 mg kg^{-1} as well as negative control (ASTM type I water) and a carrier control (acetone) were tested in triplicate. The soil was amended as described in section 2.3. Twenty seeds of each plant species were sown per 10-cm pot containing 200 g dry soil, except for corn where 7 seeds were sown. The bottom of each plant pot was previously covered with a piece of cheesecloth to prevent soil loss during testing. Alfalfa seeds were inoculated with nitrogen-fixing bacteria prior to sowing. Thirty mL of ASTM type I water was added to obtain 75% of WHC. Plant pots were placed in 1-L polyethylene bags closed with an elastic band to minimize loss of soil water due to evapo-transpiration. Plant toxicity tests were performed in a temperature and light controlled growth chamber. Plants were incubated in the dark for the first two days and then exposed to a normal diurnal cycle afterwards. The growth chamber conditions were set as follows: light intensity at 5000 ± 500 lux, day time at 25°C for 16 h, night time at 20°C for 8 h. Luminosity level was measured weekly using a photometer and the light intensity was adjusted when needed.

Based on the results of range-finding tests, definitive tests were performed using the three most sensitive plant species, with four replicates per treatment. The most sensitive plant species tested were alfalfa (*Medicago sativa*), perennial ryegrass (*Lolium perenne*) and Japanese millet (*Echinochloa crusgalli*). Six to nine nominal concentrations as well as negative control (ASTM type I water) and a carrier control (acetone) were used.

The numbers of emerged seedlings were counted after 5 days for alfalfa, Japanese millet and corn, and after 7 days for lettuce and ryegrass. Shoot number, shoot fresh mass, and shoot dry mass were measured after 16 days for alfalfa, Japanese millet and corn, and after 19 days for lettuce and ryegrass. Shoot dry mass was obtained after drying at 70°C for 24 ± 2 h.

Reference toxicant, boric acid, was used as the positive control (ASTM, 1998). Definitive toxicity tests were repeated when the percentage of germination in the controls were lower than 85% for ryegrass or Japanese millet, or lower than 70% for alfalfa, and when boric acid EC_{50} values were outside the quality control limit equivalent to EC_{50} average value ± 2 times standard deviation.

2.12. Statistical methods.

The EC_{20} and EC_{50} values for seedling emergence, shoot fresh mass and shoot dry mass measurement endpoints were calculated using SYSTAT software, version 7.0 (SPSS Inc., 1997). Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to decide whether or not to weight the data, and to select potential models. The following nonlinear regression models were used:

Logistic Gompertz model: $Y = a \times e([\log(1\text{-}p)] \times [C/\mathrm{ECp}]^{b})$

Exponential model: $Y = a \times e(([\log(1\text{-}p)] / \mathrm{ECp}) \times C) + \mathrm{b}$

Logistic Hormetic model: $Y = (t \times [1 + hC] / \{1 + [(p + h\,\mathrm{ECp}) / (1 - p)] \times [C/\mathrm{ECp}]^{b}\}$

where Y is the number of emerged seedlings or the shoot mass, a is the control response, t is the control response in the hormetic model, e is the base of the natural logarithm, p is the percent inhibition/100 (e.g., 0.5 for EC_{50}), C is the exposure concentration in test soil, ECp is the estimate of effect concentration for a specified percent effect, h is the hormetic effect parameter, and b is the scale parameter. The ECp parameters used in this study included the EM concentration producing a 20% (EC_{20}) or 50% (EC_{50}) reduction in the measurement endpoint. The EC_{20} parameter based on a growth endpoint is the preferred parameter for deriving terrestrial plant Eco-SSL benchmarks. The EC_{50}, a commonly reported value, was included to enable comparisons of the results produced in this study with results reported by other researchers. The asymptotic standard error (a.s.e.) and 95% confidence intervals (CI) associated with the point estimates were determined. The raw R-squared values, which reflect the variation of the measurement endpoints (dependent variable) that is explained by the chemical concentration (independent variable), were reported.

Analysis of Variance (ANOVA) was used to determine the bounded No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values for germination or growth data. Mean separations were done using Fisher's Least Significant Difference (LSD) pairwise comparison tests. When NOAEC (no observed adverse effect concentration) or LOAEC (lowest observed adverse effect concentration) values were determined, which usually happened in tests with hormetic response at low exposure concentrations of chemicals, the same statistical methods were used. A significance level of $p < 0.05$ was accepted for determining the NOEC and LOEC values. Student's t-Test (two-tailed) with significance level set at $p < 0.05$ was used in the limit tests with plants exposed to RDX or

HMX using EXCEL software (Microsoft Corporation, 1997). All analyses were done using measured EM concentrations.

3. RESULTS

3.1. EM concentrations in range-finding toxicity tests.

Analytical determinations of EM soil concentrations using acetonitrile extractions of freshly amended soils in the range-finding tests showed relatively good concordance between nominal and measured concentrations (Table 2). Measured/nominal ratio ranged from 0.80 to 1.17. Higher discrepancy determined for the 10 mg kg^{-1} RDX amended soil may be due to the presence of residual ethanol, the solvent in which RDX was stored. Variation within each concentration was lower then 10%, indicating a relatively low variation among the three replicates.

Table 2. Acetonitrile soil extraction of range-finding tests (n = 3).

Chemical	Nominal value (mg kg^{-1} soil)	Measured value (mg kg^{-1} soil)	Standard deviation	Deviation (%)	Measured/nominal ratio
HMX	10	10.5	0.4	4.3	1.05
	100	97.7	1.4	1.4	0.98
	1000	1025	58	5.7	1.02
	10000	9930	830	8.3	0.99
RDX	10	15.3	9.5	61.8	1.53
	100	90.9	1.4	1.6	0.91
	1000	800	38	4.7	0.80
	10000	8550	155	1.8	0.85
TNB	10	11.7	0.3	2.9	1.17
	100	115.8	5.5	4.9	1.16
	1000	1083	33	3.0	1.08
	10000	10620	315	2.9	1.06
2,4-DNT	10	10.2	0.6	5.6	1.02
	100	94.7	4.4	4.7	0.95
	1000	967	99	10.2	0.97
	5000	4900	320	6.6	0.98
2,6-DNT	10	9.6	0.6	6.3	0.96
	100	100.0	5.4	5.4	1.00
	1000	970	38	3.9	0.97
	5000	4900	530	10.9	0.98

3.2. Physico-chemical characterization of Sassafras Sandy Loam soil.

Soil pH, redox potential, and CEC were measured at the beginning of each definitive test. Results are presented in Tables 3 to 10. Initial soil pH values ranged from 5.8 to 6.2 in the negative controls, from 5.9 to 6.2 in the carrier controls, from 5,5 to 6.2 in the soil freshly amended with the five EMs, and from 5.7 to 6.3 in the weathered/aged amended soil. No

significant difference was observed among controls and soil exposed to the different EMs, and no correlation was observed between pH values and concentrations of EMs.

Initial redox potentials ranged from 281 to 463 in the negative controls, from 295 to 473 in the carrier controls, from 316 to 481 in soil freshly amended with energetic compounds, and from 241 to 347 the weathered/aged amended soil exposed to energetic compounds. Although the redox variation within each definitive test was broad, no significant difference was observed among controls and soil amended with different energetic compounds, and no correlation was observed between redox values and concentrations of energetic compounds.

Initial CEC values ranged from 2.9 to 3.5 in the negative controls, from 3.1 to 3.4 in the carrier controls, from 2.8 to 3.9 in the freshly amended soil, and from 2.6 to 3.4 in the weathered/aged amended soil. No significant difference was observed among controls and soil amended with the different energetic compounds and no correlation was observed between CEC values and concentrations of EMs.

At the end of test with TNB freshly amended soil, the soil pH was measured in triplicate for each concentration and each plant species (Table 11). Since pH variation was low among replicates, soil pH was measured in one sample per concentration and per species in the remaining definitive tests. Redox potential was measured using 3 replicates per concentration and per plant species. Results are presented in Tables 12 to 18.

Significant differences ($p < 0.05$) between pH values measured at the beginning and end of phytotoxicity tests were observed in most soil exposed to the different EMs, except for ryegrass (Table 19). For ryegrass, the pH difference was significant only in 2,4-DNT or 2,6-DNT freshly amended soils.

Significant differences ($p < 0.05$) between redox values measured at the beginning and end of phytotoxicity tests were also observed in most amended soils (Table 19). However, there was no significant difference in redox values in TNB freshly amended soil for all three plant species, no difference in weathered/aged TNB amended soil as well as in 2,4-DNT freshly amended soil for Japanese millet and ryegrass, respectively, and no difference in weathered/aged 2,4-DNT amended soil for alfalfa and Japanese millet.

Table 3. Initial soil pH, redox potential, and CEC in SSL soil used for freshly amended TNB definitive plant toxicity test.

TNB concentration (mg kg^{-1})	pH	Redox potential (mV)	Cation-exchange capacity (cmol kg^{-1})
Control (negative)	5.88	463.4	3.161
Control (carrier)	5.93	473.3	3.139
Average of controls	**5.91**	**468.4**	**3.150**
2	5.83	445.8	2.817
5	6.04	466.8	3.190
10	5.72	471.9	3.132
20	5.77	461.8	3.035
40	5.97	462.9	2.859
60	6.01	464.9	2.849
80	6.06	469.6	3.123
120	6.04	476.8	2.941
160	6.11	471.8	3.035
250	5.75	480.7	2.974
320	6.2	472.8	2.861
600	5.93	482.2	2.897
800	5.52	478.9	3.189
Average of TNB soil	**5.92 ± 0.05[a]**	**469.8 ± 2.7[a]**	**2.992 ± 0.037[a]**

a: values are mean ± standard error.

Table 4. Initial soil pH, redox potential, and CEC in SSL soil used for weathered/aged TNB definitive plant toxicity test.

TNB concentration (mg kg^{-1})	pH	Redox potential (mV)	Cation-exchange capacity (cmol kg_{-1})
Control (negative)	5.96	332.8	3.23
Control (carrier)	5.95	352.5	3.13
Average of controls	**5.96**	**342.7**	**3.18**
2	5.93	338.0	3.20
5	5.93	323.5	3.22
10	6.00	347.3	3.26
20	6.00	329.4	3.05
40	5.93	305.4	3.11
60	5.95	292.6	3.12
80	5.95	296.2	3.25
120	5.82	317.5	3.27
160	5.77	305.5	3.20
250	5.84	312.6	3.22
320	5.86	309.9	3.25
600	5.79	310.0	3.13
800	5.77	303.1	2.98
1200	5.91	314.9	3.18
1600	5.86	318.1	3.19
Average of TNB soil	**5.89 ± 0.02[a]**	**314.9 ± 4.0[a]**	**3.18 ± 0.02[a]**

a: values are mean ± standard error.

Table 5. Initial soil pH, redox potential, and CEC in SSL soil used for freshly amended 2,4-DNT definitive plant toxicity test.

2,4-DNT concentration (mg kg^{-1})	pH	Redox potential (mV)	Cation-exchange capacity (cmol kg^{-1})
Control (negative)	5.94	322.5	2.85
Control (carrier)	5.87	318.2	3.12
Average of controls	**5.91**	**320.4**	**2.99**
0.5 - ryegrass repeat	5.84	387.3	
1	5.81	362.8	3.47
1 - ryegrass repeat	5.87	391.0	
2	5.90	316.0	3.55
2.5 - ryegrass repeat	5.87	387.1	
5	5.85	330.8	3.64
5 - ryegrass repeat	5.87	385.5	
10	5.83	335.0	3.44
10 - ryegrass repeat	5.95	368.1	
25	5.88	348.2	3.43
40 - ryegrass repeat	5.98	378.6	
50	5.88	361.7	3.53
100	5.88	373.3	3.53
300	5.87	369.2	3.27
600	5.90	361.2	3.18
Average of 2,4-DNT soil	**5.88 ± 0.01[a]**	**363.7 ± 5.8[a]**	**3.45 ± 0.05[a]**

Table 6. Initial soil pH, redox potential, and CEC in SSL soil used for weathered/aged 2,4-DNT definitive plant toxicity test.

2,4-DNT concentration (mg kg^{-1})	pH	Redox potential (mV)	Cation-exchange capacity (cmol kg^{-1})
Control (negative)	5.82	308.0	2.958
Control (carrier)	6.00	332.7	3.056
Average of controls	**5.91**	**320.4**	**3.007**
5	6.11	341.9	2.842
10	6.09	344.6	2.898
25	6.15	312.2	2.984
50	6.11	287.2	2.783
100	6.15	298.9	2.593
200	6.25	293.8	2.772
300	6.22	303.1	3.000
600	6.36	304.9	3.290
1200	6.33	313.4	3.370
Average of 2,4-DNT soil	**6.20 ± 0.03[a]**	**311.1 ± 6.7[a]**	**2.948 ± 0.083[a]**

a: values are mean ± standard error.

Table 7. Initial soil pH, redox potential, and CEC in SSL soil used for freshly amended 2,6-DNT definitive plant toxicity test.

2,6-DNT concentration (mg kg^{-1})	pH	Redox potential (mV)	Cation-exchange capacity (cmol kg^{-1})
Control (negative)	6.17	436.6	3.540
Control (carrier)	6.19	370.3	3.378
Average of controls	**6.18**	**403.5**	**3.459**
1	6.12	425.7	3.777
2	6.17	425.3	3.898
5	6.16	403.3	3.719
10	6.1	404.1	3.622
20	6.08	418.5	3.755
40	6.08	375.2	3.619
100	6.12	405.5	3.602
500	5.98	392.9	3.638
Average of 2,6-DNT soil	**6.10 ± 0.02[a]**	**406.3 ± 6.0[a]**	**3.704 ± 0.036[a]**

a: values are mean ± standard error.

Table 8. Initial soil pH, redox potential, and CEC in SSL soil used for weathered/aged 2,6-DNT definitive plant toxicity test.

2,6-DNT concentration (mg kg^{-1})	pH	Redox potential (mV)	Cation-exchange capacity (cmol kg^{-1})
Control (negative)	5.92	344.7	2.958
Control (carrier)	5.99	294.7	3.122
Average of controls	**5.96**	**319.7**	**3.040**
2	6.08	265.8	3.131
5	6.06	302.7	3.037
10	6.11	306.3	2.901
20	6.06	310.1	3.001
40	6.18	287	3.063
50 - ryegrass repeat	5.72	244.4	
100	6.09	314.5	2.938
100 - ryegrass repeat	5.68	240.7	
150 - ryegrass repeat	5.68	263.2	
200	6.02	280.5	3.274
200 - ryegrass repeat	5.69	280	
250 - ryegrass repeat	5.71	283.9	
300 - ryegrass repeat	5.76	268.5	
500	5.99	304.8	3.109
1000	5.93	298.3	3.102
Average of 2,6-DNT soil	**5.92 ± 0.05[a]**	**283.4 ± 6.0[a]**	**3.062 ± 0.037[a]**

a: values are mean ± standard error.

Table 9. Initial soil pH, redox potential, and CEC in definitive plant toxicity test in SSL soil used for RDX or HMX freshly amended definitive toxicity tests.

Nominal concentration (mg kg^{-1})	pH	Redox potential (mV)	Cation-exchange capacity (cmol kg^{-1})
Control (negative)	6.19	318	3.031
Control (carrier)	5.98	313	3.086
Average of controls	**6.09**	**315.5**	**3.059**
RDX 10000	6.01	324	3.202
HMX 10000	6.05	336	3.178

a: values are mean ± standard error.

Table 10. Initial soil pH, redox potential, and CEC in definitive plant toxicity test in SSL soil used for RDX or HMX weathered/aged definitive toxicity tests.

Nominal concentration (mg kg^{-1})	pH	Redox potential (mV)	Cation-exchange capacity (cmol kg^{-1})
Control (negative)	5.91	281.3	2.719
Control (carrier)	5.96	308.2	2.879
Average of controls	**5.94**	**294.8**	**2.799**
RDX 10000	5.95	312.9	2.941
HMX 10000	5.96	307.4	3.032

Table 11. Measurement of soil pH (n = 3) and redox potential (n = 3) at the end of freshly amended TNB defi toxicity test.

TNB concentration (mg kg^{-1})	pH - Alfalfa	pH - Japanese millet	pH - Ryegrass	Redox potential – Alfalfa	Redox po Japanes
Control (negative)	5.46 ± 0.08	5.82 ± 0.08	5.86 ± 0.03	435.1 ± 0.9	489.2 ±
Control (carrier)	5.48 ± 0.05	5.87 ± 0.05	5.83 ± 0.05	467.3 ± 0.8	463.4 ±
Average of controls	**5.47 ± 0.01[a]**	**5.84 ± 0.02[a]**	**5.84 ± 0.01[a]**	**451.2 ± 16.1[a]**	**476.3 ±**
2		5.93 ± 0.03	5.87 ± 0.02		479.9 ±
5	5.66 ± 0.02	5.82 ± 0.02		465.1 ± 0.7	479.9 ±
10		5.90 ± 0.02	5.96 ± 0.03		467.9 ±
20		5.86 ± 0.04	5.95 ± 0.02		472.4 ±
40	5.74 ± 0.04		6.05 ± 0.02	462.7 ± 3.9	
60		5.89 ± 0.02			460.8 ±
80	5.85 ± 0.04			457.2 ± 1.1	
120		5.72 ± 0.02	5.72 ± 0.04		450.3 ±
160	5.85 ± 0.02			461.0 ± 5.1	
250		5.57 ± 0.01	5.69 ± 0.04		470.9 ±
320	5.51 ± 0.06			467.8 ± 1.3	
600	5.64 ± 0.03	5.59 ± 0.04	5.60 ± 0.01	473.8 ± 0.9	468.7 ±
800	5.64 ± 0.02			472.6 ± 0.3	
Average of TNB soil	**5.70 ± 0.05[a]**	**5.78 ± 0.05[a]**	**5.83 ± 0.06[a]**	**465.7 ± 2.3[a]**	**468.8 ±**

a: values are mean ± standard error.

Table 12. Measurement of soil pH (n = 1) and redox potential (n = 3) at the end of weathered/aged TNB defin toxicity test.

TNB concentration (mg kg^{-1})	pH - alfalfa	pH - Japanese millet	pH - ryegrass	Redox potential - alfalfa	Redox potential - Japanese millet	I
Control (negative)	6.14	6.61	5.98	318.7 ± 8.6	334.4 ± 9.7	
Control (carrier)	6.30	6.62	5.96	354.2 ± 6.8	357.4 ± 9.1	
Average of controls	**6.22**	**6.62**	**5.97**	**336.5 ± 17.7[a]**	**345.9 ± 11.5[a]**	
2		6.61	5.98		356.7 ± 10.2	
5	6.34	6.55		363.2 ± 8.5	326.8 ± 29.5	
10		6.45	5.93		332.4 ± 31.7	
20		6.50	5.87		319.1 ± 56.5	
40	6.45		6.02	314.2 ± 6.2		
60		6.55			290.2 ± 19.2	
80	6.46			287.4 ± 1.1		
120		6.45	5.95		329.4 ± 10.8	
160	6.46			282.9 ± 9.5		
250		6.04	5.75		292.5 ± 7.5	
320	6.29			286.8 ± 23.4		
600	6.32	6.14	5.73	305.8 ± 2.4	299.0 ± 7.8	
800	6.29			291.1 ± 9.6		
1200		5.93	5.73		296.2 ± 3.1	
1600	6.20			294.1 ± 8.0		
Average of TNB soil	**6.35 ± 0.03[a]**	**6.36 ± 0.08[a]**	**5.87 ± 0.04[a]**	**303.2 ± 9.3[a]**	**315.8 ± 7.6[a]**	

a: values are mean ± standard error.

Table 13. Measurement of soil pH (n = 1) and redox potential (n = 3) at the end of freshly amended 2,4-DNT toxicity test.

2,4-DNT nominal concentration (mg kg^{-1})	pH - alfalfa	pH - Japanese millet	pH - ryegrass	Redox potential - alfalfa	Redox poten Japanese m
Control (negative)	5.92	6.23	6.31	324.3 ± 17.9	421.6 ± 6.5
Control (carrier)	6.07	6.26	6.41	330.2 ± 12.5	346.6 ± 18
Average of controls	**6.00**	**6.25**	**6.36**	**327.2 ± 3.0[a]**	**384.1 ± 37**
1		6.41	6.37		395.0 ± 14
2			6.56		
5	6.12	6.34	6.53	339.5 ± 6.0	392.2 ± 8.2
10	6.23	6.41	6.54	304.5 ± 28.7	398.4 ± 4.0
25	6.19	6.43	6.46	336.2 ± 11.3	376.8 ± 11
50	6.12	6.26		347.6 ± 2.9	339.7 ± 35
100	6.35	6.34	6.39	312.5 ± 6.4	324.7 ± 8.3
300	6.17			297.9 ± 6.4	
600	6.17			301.0 ± 6.4	
Average of 2,4-DNT soil	**6.19 ± 0.03[a]**	**6.37 ± 0.03[a]**	**6.48 ± 0.03[a]**	**319.89 ± 7.80[a]**	**371.12 ± 12**

a: values are mean ± standard error.

Table 14. Measurement of soil pH (n = 1) and redox potential (n = 3) at the end of weathered/aged 2,4-DNT d toxicity test.

2,4-DNT nominal concentration (mg kg^{-1})	pH - alfalfa	pH - Japanese millet	pH - ryegrass	Redox potential - alfalfa	Redox potential Japanese mille
Control (negative)	6.38	6.37	6.07	305.6 ± 10.0	286.2 ± 30.5
Control (carrier)	6.45	6.4	6.09	334.2 ± 11.4	303.6 ± 27.5
Average of controls	**6.42**	**6.39**	**6.08**	**319.9 ± 14.3[a]**	**294.9 ± 8.7[a]**
5		6.30	6.20		366.8 ± 7.3
10	6.39		6.31	341.4 ± 6.1	
25	6.37	6.28	6.26	320.3 ± 9.0	345.9 ± 9.6
50	6.35	6.29	6.30	328.8 ± 4.2	328.6 ± 5.5
100	6.42	6.27	6.36	302.0 ± 10.2	313.5 ± 9.1
200		6.23	6.24		289.2 ± 4.7
300	6.40			300.3 ± 13.7	
600	6.38			279.8 ± 25.8	
1200	6.44			289.3 ± 8.2	
Average of 2,4-DNT soil	**6.39 ± 0.01[a]**	**6.27 ± 0.01[a]**	**6.28 ± 0.02[a]**	**308.8 ± 8.4[a]**	**328.8 ± 13.3[a]**

a: values are mean ± standard error.

Table 15. Measurement of soil pH (n = 1) and redox potential (n = 3) at the end of freshly amended 2,6-DNT toxicity test.

2,6-DNT nominal concentration (mg kg^{-1})	pH - alfalfa	pH - Japanese millet	pH - ryegrass	Redox potential - alfalfa	Redox potential - Japanese millet
Control (negative)	5.9	5.9	5.6	358.4 ± 6.3	400.1 ± 4.9
Control (carrier)	5.9	5.9	5.7	374.3 ± 11.1	420.0 ± 6.5
Average of controls	**5.9**	**5.9**	**5.6**	**366.4 ± 7.9**[a]	**410.1 ± 10.0**[a]
1	5.8			354.9 ± 5.9	
2	6.0			357.4 ± 16.8	
5	6.1	6.1	5.8	352.6 ± 7.1	419.0 ± 3.7
10	6.0	6.0	5.8	353.1 ± 2.1	406.3 ± 6.5
20	6.0	6.0	5.7	345.9 ± 7.5	381.4 ± 22.2
40	6.1	5.9	5.8	368.3 ± 3.0	349.5 ± 4.5
100	5.8	6.0	5.9	349.3 ± 2.3	358.4 ± 15.9
500		5.9	5.8		350.9 ± 16.6
Average of 2,6-DNT soil	**5.98 ± 0.04**[a]	**5.98 ± 0.03**[a]	**5.81 ± 0.02**[a]	**354.50 ± 2.70**[a]	**377.58 ± 12.14**[a]

a: values are mean ± standard error.

Table 16. Measurement of soil pH (n = 1) and redox potential (n = 3) at the end of weathered/aged 2,6-DNT d toxicity test.

2,6-DNT nominal concentration (mg kg^{-1})	pH - alfalfa	pH - Japanese millet	pH - ryegrass	Redox potential - alfalfa	Redox potenti Japanese mil
Control (negative)	6.11	6.26	6.03	301.8 ± 8.8	368.9 ± 3.5
Control (carrier)	6.19	6.30	6.11	357.0 ± 14.9	312.5 ± 16.1
Average of controls	**6.15**	**6.28**	**6.07**	**329.4 ± 27.6**[a]	**340.7 ± 28.2**
2	6.12			367.4 ± 2.6	
5	6.13			366.1 ± 12.8	
10	6.17	6.31	6.15	361.1 ± 8.4	361.6 ± 3.2
20	6.08	6.31	6.10	367.3 ± 3.7	390.7 ± 1.9
40	6.15	6.30	5.88	363.3 ± 8.9	346.7 ± 16.3
50					
100	6.22	6.26	5.95	350.4 ± 8.5	340.5 ± 4.9
150					
200	6.21			325.6 ± 3.6	
250					
300					
500		6.11	5.93		304.3 ± 3.7
1000		6.09	5.98		300.3 ± 7.3
Average of 2,6-DNT soil	**6.15 ± 0.02**[a]	**6.23 ± 0.04**[a]	**6.00 ± 0.04**[a]	**357.3 ± 5.7**[a]	**340.7 14.1**[a]

a: values are mean ± standard error.

Table 17. Measurement of soil pH (n = 1) and redox potential (n = 3) at the end of freshly amended RDX or H plant toxicity test.

Nominal concentration (mg kg^{-1})	pH - alfalfa	pH - Japanese millet	pH - ryegrass	Redox potential - alfalfa	Redox potential - Japanese millet
Control (negative)	5.76	5.82	5.54	369.0 ± 10.3	427.5 ± 5.6
Control (carrier)	5.63	5.67	5.60	431.5 ± 9.3	416.5 ± 5.5
Average of controls	**5.70**	**5.75**	**5.57**	**400.3 ± 31.3**[a]	**422.0 ± 5.5**[a]
RDX 10000	5.72	5.70	5.62	401.8 ± 7.7	382.9 ± 13.4
HMX 10000	5.73	5.69	5.57	400.0 ± 3.4	382.7 ± 5.6

Table 18. Measurement of soil pH (n = 1) and redox potential (n = 3) at the end of weathered/aged RDX or H plant toxicity test.

Nominal concentration (mg kg^{-1})	pH - alfalfa	pH - Japanese millet	pH - ryegrass	Redox potential - alfalfa	Redox potential - Japanese millet
Control (negative)	6.09	6.40	6.25	291.7 ± 11.8	288.1 ± 29.0
Control (carrier)	6.18	6.49	6.22	301.3 ± 10.0	297.2 ± 13.0
Average of controls	**6.14**	**6.45**	**6.24**	**296.5 ± 4.8**[a]	**292.7 ± 4.6**[a]
RDX 10000	6.18	6.51	6.25	299.3 ± 10.6	333.2 ± 10.9
HMX 10000	6.31	6.49	6.36	316.9 ± 30.6	336.8 ± 10.3

a: values are mean ± standard error.

Table 19. Comparisons of the initial and final soil pH and redox values determined in definitive phytotoxicity tests.

Compound Plant species	**pH**	**redox**
Freshly amended TNB		
Alfalfa	Yes-	No
Japanese millet	Yes-	No
Ryegrass	No	No
Weathered/aged TNB		
Alfalfa	Yes+	Yes-
Japanese millet	Yes+	No
Ryegrass	No	No
Freshly amended 2,4-DNT		
Alfalfa	Yes+	Yes-
Japanese millet	Yes+	No
Ryegrass	Yes+	No
Weathered/aged 2,4-DNT		
Alfalfa	Yes+	No
Japanese millet	No	No
Ryegrass	No	Yes+
Freshly amended 2,6-DNT		
Alfalfa	Yes-	Yes-
Japanese millet	Yes-	Yes-
Ryegrass	Yes-	Yes-
Weathered/aged 2,6-DNT		
Alfalfa	Yes+	Yes+
Japanese millet	Yes+	Yes+
Ryegrass	No	Yes+
Freshly amended RDX and HMX		
Alfalfa	Yes-	Yes+
Japanese millet	Yes-	Yes+
Ryegrass	Yes-	Yes+
Weathered/aged RDX and HMX		
Alfalfa	Yes+	No
Japanese millet	Yes+	Yes+
Ryegrass	Yes+	Yes+

Yes+: Significant increase of pH or redox potential at the end of phytotoxicity test ($p<0.05$).
Yes-: Significant decrease of pH or redox potential at the end of phytotoxicity test ($p<0.05$).

3.3. EM concentrations in freshly amended SSL soil.

Concentrations of EMs in freshly amended soils were determined at the beginning (initial, T_0) and at the end (final, T_f) of each definitive toxicity test using both acetonitrile and ATCLP extractions. Results of these analyses for each plant species test are presented in Tables 20-29. The initial percent recovery in freshly amended soils ranged from 84 to 110% for TNB, from 86 to 103% for 2,4-DNT, from 68 to 119% for 2,6-DNT, and from 97 to 104% for RDX and HMX. Lower recovery values of 68 and 70% were observed for 2,6-DNT at concentrations of 2 and 20 mg kg^{-1}, respectively (Tables 26, 27, 28).

ATCLP extractable TNB, 2,4-DNT or 2,6-DNT concentrations increased proportionally with their nominal/acetonitrile concentrations (Tables 20-28). At concentrations below 100 mg kg^{-1}, ATCLP-based recovery ranged from 6 to 61% for TNB, from 0 to 70% for 2,4-DNT and from 45 to 75% for 2,6-DNT. Higher ATCLP extractable values were determined in higher concentrations, ranging from 57 to 96% for TNB, from 81 to 84% for 2,4-DNT, and 86% for 2,6-DNT. ATCLP extractable concentrations of TNB, 2,4-DNT and 2,6-DNT were below their water solubility level, which are 340, 280 and 206 mg L^{-1}, respectively (Hawari *et al.*, 2002). Both RDX and HMX had low ATCLP-based recovery (Table 29). Only 2 and 0.2 % of RDX and HMX, respectively, were ATCLP extractable in soils freshly amended with 10 000 mg kg^{-1} RDX or HMX. These low ATCLP-based recoveries reflect the lower water solubility of both compounds, which were reported for RDX at 42 mg L^{-1} at 20°C (Sikka *et al.*, 1980) and at 60 mg L^{-1} at 25°C (Banerjee *et al.*, 1980). The water solubility of HMX was reported between 5 and 6.6 mg L^{-1} at 20°C and 25°C, respectively (Glover and Hoffsommer, 1973; McLellan *et al.*, 1992).

The presence of 3,5-dinitroaniline (3,5-DNA), a transformation product of TNB, was detected in every TNB treatment concentration in freshly amended SSL soil (data not shown). This suggests that some TNB was likely transformed at the beginning of these phytotoxicity tests. No transformation products or metabolites were detected at the beginning of the phytotoxicity tests with 2,4-DNT, 2,6-DNT, RDX or HMX in freshly amended soil.

The percent decrease of EMs extracted by acetonitrile at the end (T_f) of each definitive test was calculated using the formula:

$$EM_{acetonitrile} \text{ decrease } (\%) = 100 - (\text{Concentration at } T_f \,/\, \text{Concentration at } T_0 \times 100)$$

In freshly amended soil, percent decrease of TNB, 2,4-DNT or 2,6-DNT was inversely related to their concentrations in acetonitrile extracts. At soil concentrations below 100 mg kg^{-1}, decrease in concentrations of TNB, 2,4-DNT or 2,6-DNT ranged from 78 to 100%, from 43 to 100% and from 39 to 100%, respectively. At concentrations above 100 mg kg^{-1}, decrease in concentrations of TNB, 2,4-DNT or 2,6-DNT ranged from 0 to 52%, from 19 to 24% and from 21 to 24%, respectively. There was no significant decrease in acetonitrile extracted RDX in freshly amended soil in the 10000 mg kg^{-1} treatment, except for ryegrass where a 4% decrease was observed. In the 10000 mg kg^{-1} HMX treatment, acetonitrile extractable concentrations of HMX was decreased by 10, 17, and 13% in tests with alfalfa, millet and ryegrass, respectively.

Table 20. Nominal and measured TNB concentrations (mean ± S.E.; n = 3) in freshly amended SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with alfalfa.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
5	5.1 ± 0.1	101	0.1 ± 0.1	99	1	12
40	39.2 ± 1.9	98	2.5 ± 0.2	94	18.2 ± 0.2	47
80	87.8 ± 0.9	110	19.6 ± 0.3	78	53.4 ± 1.8	61
160	170.9 ± 3.7	107	111.1 ± 6.3	35	122.8 ± 9.4	72
320	343 ± 13	107	286.7 ±2.4	16	301.7 ± 5.0	88
600	648 ± 14	108	587 ± 14	9	622.9 ± 6.5	96

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

Table 21. Nominal and measured TNB concentrations (mean ± S.E.; n = 3) in freshly amended SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with Japanese millet.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
2	1.8 ± 0.0	91	0.0 ± 0.0	100	0.1 ± 0.0	6
5	5.1 ± 0.1	101	0.1 ± 0.1	98	0.6 ± 0.0	12
10	8.4 ± 0.1	84	0.36 ± 0.02	96	1.5 ± 0.1	18
20	21.5 ± 0.5	107	0.70 ±0.03	97	5.7 ± 0.2	27
60	64.1 ± 1.3	107	7.0 ± 0.6	89	33.8 ± 2.6	53
120	124.7 ± 4.4	104	59.5 ± 3.9	52	71.3 ± 1.3	57
250	220 ± 27	88	236 ± 11	0	191 ± 13	87
600	648 ± 14	108	587 ± 11	9	622.9 ± 6.5	96

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

Table 22. Nominal and measured TNB concentrations (mean ± S.E.; n = 3) in freshly amended SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with ryegrass.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
2	1.8 ± 0.0	91	BDL	100	0.1 ± 0.0	6
10	8.4 ± 0.1	84	0.2 ± 0.1	98	1.5 ± 0.1	18
20	21.5 ± 0.5	107	0.53 ±0.04	98	5.7 ± 0.2	27
40	39.2 ± 1.9	98	2.3 ± 0.3	94	33.8 ± 2.6	47
120	124.7 ± 4.4	104	61.1 ± 8.0	51	71.3 ± 1.3	57
250	220 ± 27	88	231.6 ± 9.1	0	191.0 ±12.5	87
600	648 ± 14	108	605 ± 13	7	623 ± 7	96

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

Table 23. Nominal and measured 2,4-DNT concentrations (mean ± S.E.; n = 3) in freshly amended SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with alfalfa.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
5	4.7 ± 0.1	95	0.37 ± 0.02	92	1.8 ± 0.1	39
10	9.1 ± 0.2	91	0.93 ± 0.03	90	3.8 ± 0.3	42
25	21.5 ± 0.2	86	4.9 ± 0.2	77	11.8 ± 0.8	55
50	46.5 ± 0.5	93	19.8 ± 1.0	57	11.8 ± 0.2	58
100	98.5 ± 1.3	99	53.8 ± 1.9	45	68.6 ± 2.0	70
300	278 ± 14	93	211.0 ± 7.0	24	225.9 ± 6.3	81
600	613 ± 43	102	496.1 ± 4.4	19	516.8 ± 3.2	84

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

Table 24. Nominal and measured 2,4-DNT concentrations (mean ± S.E.; n = 3) in freshly amended SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with Japanese millet.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
1	1.0 ± 0.0	100	BDL	100	0.3 ± 0.0	25
5	4.7 ± 0.1	95	0.5 ± 0.02	90	1.8 ± 0.1	39
10	9.1 ± 0.2	91	1.3 ± 0.1	86	3.8 ± 0.3	42
25	21.5 ± 0.6	86	5.8 ± 0.7	73	11.8 ± 0.8	55
50	46.5 ± 0.5	93	21.4 ± 0.6	54	26.8 ± 0.2	58
100	98.5 ± 1.3	99	56.0 ± 3.3	43	68.6 ± 2.0	70

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

Table 25. Nominal and measured 2,4-DNT concentrations (mean ± S.E.; n = 3) in freshly amended SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with ryegrass.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
1	1.0 ± 0.0	100	BDL	100	0.3 ± 0.0	30
2	2.1 ± 0.1	105	0.1 ± 0.0	94	0.7 ± 0.0	33
5	4.7 ± 0.1	94	0.5 ± 0.0	89	1.8 ± 0.1	38
10	9.1 ± 0.2	91	1.5 ± 0.04	84	3.8 ± 0.3	42
25	21.5 ± 0.6	86	8.3 ± 1.6	61	11.8 ± 0.8	55
50	46.5 ± 0.5	93	22.8 ± 1.4	51	26.8 ± 0.2	58
100	98.4 ± 1.3	98	51.6 ± 2.8	48	68.6 ± 1.9	70
Concentrations used for EC_{50}, EC_{20}, LOEC and NOEC final calculations						
0.5	0.5 ± 0.0	100	ND		BDL	BDL
1	0.9 ± 0.0	90	ND		0.3 ± 0.0	32
2.5	2.2 ± 0.1	88	ND		0.8 ± 0.0	36
5	3.8 ± 0.1	77	ND		1.4 ± 0.1	37
10	8.5 ± 0.1	85	ND		3.6 ± 0.1	43
20	17.1 ± 0.2	86	ND		8.8 ± 0.0	51
40	38.4 ± 0.6	96	ND		19.3 ± 0.7	50

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.
ND: Not determined.

Table 26. Nominal and measured 2,6-DNT concentrations (mean ± S.E.; n = 3) in freshly amended SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with alfalfa.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
1	1.2 ± 0.0	119	BDL	100	0.5 ± 0.0	46
2	1.4 ± 0.0	68	BDL	100	0.7 ± 0.0	52
5	4.1 ± 0.1	83	BDL	100	2.5 ± 0.2	60
10	8.0 ± 0.3	80	0.4 ± 0.3	95	4.3 ± 0.3	54
20	13.9 ± 0.6	70	4.6 ± 0.1	67	7.6 ± 0.2	55
40	29.7 ± 1.4	74	9.7 ± 0.2	67	22.0 ± 0.5	74
100	88.5 ± 1.7	89	53.8 ± 1.6	39	66.0 ± 2.0	75

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

Table 27. Nominal and measured 2,6-DNT concentrations (mean ± S.E.; n = 3) in freshly amended SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with Japanese millet.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
5	4.1 ± 0.1	83	0.5 ± 0.1	89	2.5 ± 0.2	60
10	8.0 ± 0.3	80	1.2 ± 0.1	85	4.3 ± 0.3	54
20	13.9 ± 0.6	70	2.7 ± 0.1	81	7.6 ± 0.2	55
40	29.7 ± 1.4	74	16.9 ± 0.6	43	22.0 ± 0.5	74
100	88.5 ± 1.7	89	32.5 ± 2.1	63	66.0 ± 2.0	75
600	644.5 ± 6.8	107	489 ± 33	24	555.1 ± 4.8	86

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

Table 28. Nominal and measured 2,6-DNT concentrations (mean ± S.E.; n = 3) in freshly amended SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with ryegrass.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
5	4.1 ± 0.1	83	0.1 ± 0.1	99	2.5 ± 0.2	60
10	8.0 ± 0.3	80	1.0 ± 0.1	88	4.3 ± 0.3	54
20	13.9 ± 0.6	70	4.0 ± 0.5	71	7.6 ± 0.2	55
40	29.7 ± 1.4	74	13.0 ± 0.9	56	22.0 ± 0.5	74
100	88.5 ± 1.7	89	40.5 ± 4.1	54	66.0 ± 2.0	75
600	644.5 ± 6.8	107	508 ± 61	21	555.1 ± 4.8	86

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

Table 29. Nominal and measured RDX or HMX concentrations (mean ± S.E.; n = 3) in freshly amended SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with alfalfa, Japanese millet and ryegrass.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL				BDL	
Control (carrier)	BDL				BDL	
RDX 10000 - alfalfa	9740 ± 150	97	10300 ± 180	0	197.8 ± 1.4	2
RDX 10000 – Japanese millet	9740 ± 150	97	10240 ± 110	0	197.8 ± 1.4	2
RDX 10000 - ryegrass	9740 ± 150	97	9370 ± 200	4	197.8 ± 1.4	2
HMX 10000 - alfalfa	10411 ± 810	104	9430 ± 380	10	18 ± 0.2	0.2
HMX 10000 – Japanese millet	10411 ± 810	104	8600 ± 210	17	18 ± 0.2	0.2
HMX 10000 - ryegrass	10411 ± 810	104	9060 ± 310	13	18 ± 0.2	0.2

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

3.4. EM concentration in weathered/aged SSL soil.

Weathering/aging of amended soils reduced concentrations of TNB, 2,4-DNT and 2,6-DNT (Tables 30-38). Acetonitrile extraction-based recovery at concentrations below 100 mg kg^{-1} ranged from 0 to 28% for TNB, from 25 to 37% for 2,4-DNT, and from 12 to 20% for 2,6-DNT. At concentrations above 100 mg kg^{-1}, recovery ranged from 67 to 98% for TNB, from 44 to 73% for 2,4-DNT and from 21 to 45% for 2,6-DNT, respectively. These data indicate that TNB was either strongly sorbed onto soil or was transformed at low concentrations (from 2 to 80 mg kg^{-1} nominal concentrations) and that TNB was more resistant to transformation or that the transformation process proceeded at relatively low rates at higher concentrations (from 120 to 1600 mg kg^{-1} nominal concentrations). Similarly, 2,4-DNT was strongly sorbed onto soil or was transformed at low concentrations (from 5 to 300 mg kg^{-1} nominal concentrations) and was more resistant to transformation or the transformation process proceeded at relatively low rates at nominal concentrations of 600-1200 mg kg^{-1}. Recovery of 2,6-DNT was below 45 percent at all tested concentrations, indicating that a portion was likely sorbed onto soil. Concentrations of RDX or HMX remained stable in the 10000 mg kg^{-1} treatments with 95 and 93% recoveries respectively, following the 3-month weathering/aging period (Table 39).

Similar to the results in freshly amended soils, ATCLP extractable portions of TNB, 2,4-DNT and 2,6-DNT in weathered/aged amended soils increased proportionally with EM concentrations. At nominal concentrations below 100 mg kg^{-1}, ATCLP-based recovery ranged from 0 to 31% for TNB, from 27 to 53% for 2,4-DNT, and from 0 to 64% for 2,6-DNT. At nominal concentrations above 100 mg kg^{-1}, ATCLP-based recovery ranged from 48 to 93% for TNB, from 55 to 81% for 2,4-DNT, and from 68 to 89% for 2,6-DNT. In contrast with 2,4-DNT and 2,6-DNT, which could be extracted using ATCLP method at concentrations as low as 5 mg kg^{-1}, TNB could not be extracted using this method at concentrations ranging from 2 to 20 mg kg^{-1}. Overall, ATCLP-based EM recoveries were significantly (Student's *t* test $p < 0.05$) lower in weathered/aged amended soil compared with freshly amended soil. RDX and HMX were not appreciably transformed during weathering/aging procedure and their ATCLP-based recoveries remained at 2 and 0.2% respectively, in weathered/aged amended soils (Table 39).

Transformation products detected in weathered/aged TNB and 2,4-DNT amended soils, suggest that these two EMs were in part transformed following exposure to sunlight and soil drying/wetting cycles. These transformation products included 3,5-dinitroaniline (3,5-DNA), 2-amino-4-nitrotoluene (2-A-4 NT), and 4-amino-2-nitrotoluene (4-A-2 NT). 3,5-DNA was detected in all concentrations of weathered/aged TNB soil, but in greater amount at concentrations 40, 60 and 80 mg kg^{-1}. Measurable amounts of 2-A-4 NT and 4-A-2 NT were detected in weathered/aged soil amended with 2,4-DNT at concentrations of 25, 50 and 200 mg kg^{-1}. No metabolites were detected at the beginning of the phytotoxicity tests performed with 2,6-DNT, RDX and HMX weathered/aged amended soils.

The decrease of TNB extracted by acetonitrile at the end of phytotoxicity tests (T_f) with weathered/aged amended soils exceeded 52% in treatments with nominal concentrations below 160 mg kg^{-1}, but was small (< 10%) at concentrations above than 250 mg kg^{-1} for all three

plant species (Tables 30, 31, 32). The decrease of 2,4-DNT extracted by acetonitrile at T_f ranged from 0 to 34%, and was not related to its nominal concentration (Tables 33, 34, 35). The decrease of 2,6-DNT extracted by acetonitrile at T_f was inversely proportional to nominal soil concentrations. At concentrations below 100 mg kg^{-1}, the decrease in extractability by acetonitrile ranged from 44 to 84% for alfalfa, from 41 to 100% for millet, and was 52% for ryegrass. At concentrations above 100 mg kg^{-1}, the decrease in extractability by acetonitrile was 39% for alfalfa, ranged from 19 to 26% for millet, and from 40 to 51% for ryegrass (Tables 36, 37, 38).

Photolysis, microbial degradation, adsorption or fixation at binding sites within the soil, and plant uptake are among possible mechanisms contributing to the decrease in concentrations of TNB, 2,4-DNT and 2,6-DNT in weathered/aged amended soils. TNB was transformed in both freshly amended soil and in weathered/aged amended soil at concentrations below 100 mg kg^{-1}. At the higher concentrations, however, TNB was barely transformed in weathered/aged soil. Greater amounts of 3,5-DNA detected at TNB concentrations between 40 and 250 mg kg^{-1} support the TNB transformation hypothesis, although soil sorption at low concentrations cannot be discounted. 2,4-DNT was more readily transformed in freshly amended soil than in weathered/aged 2,4-DNT amended soil. Some 2-A-4 NT and 4-A-2 NT were detected in 2,4-DNT weathered/aged amended soil at all concentrations but none of these transformation products were detected at the concentration of 1200 mg kg^{-1}. At concentrations below 100 mg kg-^{1}, the decrease in extractability of 2,6-DNT by acetonitrile was similar in freshly amended and weathered/aged amended soils and was inversely proportional to nominal concentrations. No transformation products were detected in 2,6-DNT amended soils, but soil sorption cannot be discounted.

There was no decrease in extractability of RDX by acetonitrile in weathered/aged amended soil in the 10000 mg kg^{-1} treatment, except for the test with Japanese millet where a 4% decrease in extractability by acetonitrile occurred. In the 10000 mg kg^{-1} HMX treatment, decrease in extractability by acetonitrile of 3 and 2% of HMX occurred in tests with Japanese millet and ryegrass, respectively. No metabolites were detected in RDX and HMX amended soils. Analytical results show that RDX was not significantly transformed during the course of the phytotoxicity assays and that the decrease in extractability by acetonitrile of HMX during the assays was greater in freshly amended soils compared with weathered/aged amended soils.

Table 30. Nominal and measured TNB concentrations (mean ± S.E.; n = 3) in weathered/aged SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with alfalfa.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
5	BDL	0	BDL		BDL	0
40	2.1 ± 0.0	5	1 ± 0.1	65	0.5 ± 0.0	22
80	22.1 ± 0.4	28	11 ± 0.2	52	6.8 ± 0.7	31
160	114 ± 4	71	54 ± 2	53	66 ± 5	59
320	280 ± 10	88	271 ± 5	4	168 ± 1	59
600	580 ± 40	96	570 ± 15	2	430 ± 10	75
800	720 ± 10	90	710 ± 20	1	600 ± 20	83
1600	1560 ± 30	98	1530 ± 10	2	1460 ± 15	93

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

Table 31. Nominal and measured TNB concentrations (mean ± S.E.; n = 3) in weathered/aged SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with Japanese millet.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
2	BDL	0	BDL		BDL	0
5	BDL	0	0.1 ± 0.1		BDL	0
10	0.3 ± 0.0	3	0.1 ± 0.1	79	BDL	0
20	0.6 ± 0.0	3	0.1 ± 0.1	89	BDL	0
60	5 ± 0.2	9	1.3 ± 0.1	74	1.4 ± 0.1	27
120	81 ± 2	67	27 ± 1	66	39.1 ± 1.4	49
250	197 ± 7	79	187 ± 5	5	126 ± 1	64
600	575 ± 40	96	560 ± 20	3	430 ± 10	75
1200	984 ± 1	82	1160 ± 50	0	790 ± 3	80

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

Table 32. Nominal and measured TNB concentrations (mean ± S.E.; n = 3) in weathered/aged SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with ryegrass.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
2	BDL	0	BDL		BDL	0
10	0.3 ± 0.0	3	BDL	100	BDL	0
20	0.6 ± 0.0	3	BDL	100	BDL	0
40	2 ± 0.0	5	0.7 ± 0.0	65	0.5 ± 0.0	22
120	81 ± 2	67	30 ± 3	63	39 ± 1	49
250	197 ± 7	79	181 ± 8	8	126 ± 1	64
600	575 ± 40	96	520 ± 25	10	430 ± 10	75
1200	984 ± 1	82	1280 ± 90	0	790 ± 3	80

Table 33. Nominal and measured 2,4-DNT concentrations (mean ± S.E.; n = 3) in weathered/aged SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with alfalfa.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
10	3.7 ± 0.2	37	2.8 ± 0.1	23	1.3 ± 0.1	35
25	7.8 ± 0.1	31	5.1 ± 0.1	34	2.8 ± 0.0	36
50	14.9 ± 0.3	30	15.8 ± 0.8	0	6.2 ± 0.3	42
100	32.1 ± 0.7	32	24.4 ± 0.6	24	16.9 ± 0.2	53
300	132 ± 3	44	128 ± 3	3	102 ± 3	78
600	353 ± 2	59	342 ± 9	3	270 ± 20	77
1200	880 ± 10	73	880 ± 30	0	710 ± 4	81
Concentrations used for EC_{50}, EC_{20}, LOEC and NOEC final calculations						
5	3.2 ± 0.1	64	ND		1.1 ± 0.1	34
10	6.2 ± 0.2	62	ND		2.2 ± 0.0	35
25	10.3 ± 0.5	41	ND		4.1 ± 0.1	40
50	25.2 ± 0.6	50	ND		11.5 ± 0.7	46
100	55.6 ± 2.3	56	ND		27.7 ± 0.3	50
150	89.2 ± 2.1	59	ND		47.6 ± 1.2	53
200	120.6 ± 4.4	60	ND		70.9 ± 0.5	59
250	153.4 ± 4.9	61	ND		104.6 ± 3.5	68

BDL: Below detection limit. HPLC detection limit = 0.5 mg kg^{-1} soil. ND: Not determined.

Table 34. Nominal and measured 2,4-DNT concentrations (mean ± S.E.; n = 3) in weathered/aged SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with Japanese millet.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
5	1.3 ± 0.0	25	1.1 ± 0.0	15	0.3 ± 0.0	27
10	3.7 ± 0.2	37	2.9 ± 0.1	23	1.3 ± 0.1	35
25	7.8 ± 0.1	31	5.7 ± 0.1	27	2.8 ± 0.0	36
50	14.9 ± 0.3	30	10.2 ± 0.3	32	6.2 ± 0.3	42
100	32.1 ± 0.7	32	24.9 ± 0.7	22	16.9 ± 0.2	53
200	90 ± 7	45	68 ± 1	25	50 ± 6	55

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

Table 35. Nominal and measured 2,4-DNT concentrations (mean ± S.E.; n = 3) in weathered/aged SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with ryegrass.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
5	1.3 ± 0.0	25	1.1 ± 0.1	15	0.3 ± 0.0	27
10	3.7 ± 0.2	37	3.0 ± 0.1	20	1.3 ± 0.1	35
25	7.8 ± 0.1	31	5.6 ± 0.2	27	2.8 ± 0.03	36
50	14.9 ± 0.3	30	12 ± 2	19	6.2 ± 0.3	42
100	32.1 ± 0.7	32	25.4 ± 0.1	21	16.9 ± 0.2	53
200	90 ± 7	45	73.9 ± 0.7	18	50 ± 6	55

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

Table 36. Nominal and measured 2,6-DNT concentrations (mean ± S.E.; n = 3) in weathered/aged SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with alfalfa.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
2	0.4 ± 0.1	19	0.1 ± 0.1	84	0.0 ± 0.0	0
5	0.6 ± 0.0	12	0.3 ± 0.0	52	0.2 ± 0.0	37
10	1.2 ± 0.0	12	0.8 ± 0.3	33	0.6 ± 0.1	48
20	3.3 ± 0.3	17	1.3 ± 0.1	61	1.5 ± 0.0	44
40	5.4 ± 0.1	13	2.7 ± 0.1	50	3.2 ± 0.1	59
100	14.9 ± 0.1	15	8.4 ± 0.4	44	9.5 ± 0.3	64
200	41.1 ± 0.8	21	25.0 ± 0.5	39	27.8 ± 0.2	68

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

Table 37. Nominal and measured 2,6-DNT concentrations (mean ± S.E.; n = 3) in weathered/aged SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with Japanese millet.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL	BDL	BDL	
Control (carrier)	BDL		BDL	BDL	BDL	
10	1.2 ± 0.0	12	BDL	100	0.6 ± 0.1	48
20	3.3 ± 0.3	17	1.6 ± 0.0	53	1.5 ± 0.0	44
40	5.4 ± 0.1	13	3.4 ± 0.1	38	3.2 ± 0.1	59
100	14.9 ± 0.1	15	8.9 ± 0.3	41	9.5 ± 0.3	64
500	139.5 ± 4.5	28	103.3 ± 3.2	26	104 ± 7	74
1000	447.3 ± 16.3	45	362.8 ± 14.5	19	397 ± 7	89

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

Table 38. Nominal and measured 2,6-DNT concentrations (mean ± S.E.; n = 3) in weathered/aged SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with ryegrass.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
50	7.5 ± 0.4	15	3.6 ± 0.1	52	3.6 ± 0.2	48
100	19.7 ± 0.6	20	9.4 ± 0.2	52	11.7 ± 0.8	59
150	37 ± 2	25	18.1 ± 0.6	51	23 ± 1	61
200	60 ± 2	30	33.3 ± 0.8	44	38 ± 1	63
250	75 ± 2	30	45 ± 2	40	54 ± 3	72
300	118 ± 4	39	69 ± 5	41	81 ± 3	69

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

Table 39. Nominal and measured RDX or HMX concentrations (mean ± S.E.; n = 3) in weathered/aged SSL soil determined at the beginning (T_0) and at the end (T_f) of definitive test with alfalfa, Japanese millet and ryegrass.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction at T_0 (mg kg^{-1})	Recovery at T_0 (%)	Acetonitrile extraction at T_f (mg kg^{-1})	Decrease at T_f (%)	ATCLP extraction at T_0 (mg kg^{-1})	ATCLP/ Acetonitrile (%)
Control (negative)	BDL		BDL		BDL	
Control (carrier)	BDL		BDL		BDL	
RDX 10000 - alfalfa	9500 ± 200	95	9500 ± 100	0	192 ± 1	2
RDX 10000 – Japanese millet	9500 ± 200	95	9100 ± 200	4	192 ± 1	2
RDX 10000 - ryegrass	9500 ± 200	95	9500 ± 200	0.1	192 ± 1	2
HMX 10000 - alfalfa	9300 ± 800	93	9800 ± 600	0	16 ± 0.1	0.2
HMX 10000 – Japanese millet	9300 ± 800	93	9000 ± 300	3	16 ± 0.1	0.2
HMX 10000 - ryegrass	9300 ± 800	93	9200 ± 400	2	16 ± 0.1	0.2

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

3.5. Range-finding plant toxicity tests.

Different varieties of alfalfa, corn, Japanese millet, lettuce and ryegrass were compared to choose the best performing in the Sassafras sandy loam soil. The soil moisture condition was optimized and a level equivalent to 75% water holding capacity was chosen. This hydration produced better germination rates for most seeds. Kandy corn Canada no. 1 and Japanese millet seeds gave satisfactory germination, with seedling emergence of 100% and 85%, respectively. Two varieties of perennial ryegrass (Cutter and Express) were compared. The Express variety gave slightly better results in the Sassafras soil, with germination rate of 97% compared to 95% for the Cutter variety.

Alfalfa seeds were tested with and without inoculation of nitrogen-fixing bacteria. Germination rate of alfalfa was lower compared with the other species, as we obtained 55% with bacterial inoculation and 62% without bacterial inoculation. When lyophilized nitrogen-fixing bacteria were moisten and incubated at room temperature for 1 h prior to inoculation onto alfalfa seeds, germination rate improved to 70%.

Despite germinating and growing well in silica and OECD artificial soil, the lettuce Buttercrunch seeds germinated poorly in the Sassafras sandy loam soil when sown at a depth of few mm in the soil. Germination of six varieties of lettuce, including Buttercrunch, Grand Rapids, Boston Genecorp, Parris Island, Simpson Elite and Green salad bowl, were tested. After 5 days of incubation, the lettuce Buttercrunch had the highest germination rate of 92%, compared to 48% for Grand Rapids, 82% for Boston Genecorp, 87% for Parris Island, 73% for Simpson Elite and 5% for Green salad bowl variety.

Based on these results, Kandy Corn Canada no. 1, Japanese millet, Express perennial ryegrass, alfalfa inoculated with nitrogen-fixing bacteria pre-incubated at room temperature, and Buttercrunch lettuce seeds were used for the range-finding tests to determine the three most sensitive species. Plants were exposed in triplicate to RDX, HMX, TNB, 2,4-DNT and 2,6-DNT at concentrations of 1, 10, 100, 1000 and 10000 mg kg^{-1}.

During the range-finding tests, no toxic effects were observed for RDX and HMX at concentrations of up to 10000 mg kg^{-1} for all five plants tested, based on seedling emergence and shoot growth measurement endpoints (Table 43). The NOEC values were 9363 and 10373 mg kg^{-1} for RDX and HMX respectively, as derived from ANOVA.

TNB, 2,4-DNT and 2,6-DNT range-finding tests showed that these three energetic compounds affected the five plant species within test concentration ranges selected. For TNB range-finding tests, the three most sensitive species were Japanese millet, ryegrass and lettuce, based on preliminary seedling emergence and growth EC_{20} values (Table 40). For 2,4-DNT range-finding tests, the three most sensitive species were alfalfa, Japanese millet and ryegrass, based on seedling emergence and growth preliminary EC_{20} values (Table 41). For 2,6-DNT range-finding tests, corn and ryegrass showed very similar EC_{20} values, therefore the four most sensitive species were alfalfa, corn, Japanese millet and ryegrass, based on preliminary seedling emergence and growth EC_{20} values (Table 42).

On the basis of these range-finding tests, it appeared that all plant species tested were sensitive to 2,6-DNT, except for lettuce, which also showed high resistance to 2,4-DNT. The second most resistant species was corn, based on its response to TNB and 2,4-DNT. In addition, lettuce showed a poor germination rate in the carrier control, compared with the water control. Therefore, the three most sensitive species selected for use in the definitive tests were alfalfa, a dicotyledonous species, Japanese millet, and ryegrass, both monocotyledonous species.

Neither RDX nor HMX were toxic to the five plant species in the range-finding tests (Table 43). Limit tests were performed with these two compounds. Limit tests included 8 replicates of 10 000 mg kg^{-1} HMX or RDX, 8 replicates of control, 8 replicates of carrier control, using both freshly amended and weathered/aged amended SSL soil.

Boric acid positive controls were tested in triplicate. Concentrations were 175, 200, 230, 260, 300 mg kg^{-1} for alfalfa, 65, 110, 175, 260, 345, 460 mg kg^{-1} for Japanese millet and 50, 80, 110, 150, 200 mg kg^{-1} for ryegrass.

Table 40. Summary of ecotoxicological parameters determined from the range-finding assays with TNB.

Ecotoxicological parameters	Alfalfa (mg kg^{-1})	Corn (mg kg^{-1})	Japanese millet (mg kg^{-1})	Ryegrass (mg kg^{-1})	Lettuce (mg kg^{-1})
EC_{50}-seedling emergence-T $_{5\text{-}7d}$	345	696	438	101	5854
EC_{20}-seedling emergence-T $_{5\text{-}7d}$	49	309	89	21	88
LOEC	116	1083	116	12	12
NOEC	12	116	12	<12	<12
EC_{50}-Fresh growth	>116	114	53	99	61
EC_{20}-Fresh growth	58	48	8	46	11
LOEC	116	116	12	116	12
NOEC	12	12	<12	12	<12
EC_{50}-Dry growth	>116	428	94	116	82
EC_{20}-Dry growth	69	59	42	53	22
LOEC	116	116	116	116	116
NOEC	12	12	12	12	12

Table 41. Summary of ecotoxicological parameters determined from the range-finding assays with 2,4-DNT.

Ecotoxicological parameters	Alfalfa (mg kg^{-1})	Corn (mg kg^{-1})	Japanese millet (mg kg^{-1})	Ryegrass (mg kg^{-1})	Lettuce (mg kg^{-1})
EC_{50}-seedling emergence-T $_{5\text{-}7d}$	443	84	52	52	3128
EC_{20}-seedling emergence-T $_{5\text{-}7d}$	130	40	24	27	304
LOEC	967	95	94	10	4897
NOEC	95	10	10	<10	967
EC_{50}-Fresh growth	91	72	46	>10	55
EC_{20}-Fresh growth	42	35	14	>10	16
LOEC	>95	95	10	10	10
NOEC	95	10	<10	<10	<10
EC_{50}-Dry growth	>95	76	56	>10	71
EC_{20}-Dry growth	67	36	22	>10	35
LOEC	>95	95	10	10	10
NOEC	95	10	<10	<10	<10

Table 42. Summary of ecotoxicological parameters determined from the range-finding assays with 2,6-DNT.

Ecotoxicological parameters	Alfalfa (mg kg^{-1})	Corn (mg kg^{-1})	Japanese millet (mg kg^{-1})	Ryegrass (mg kg^{-1})	Lettuce (mg kg^{-1})
EC_{50}-seedling emergence-T $_{5\text{-}7d}$	65	78	279	249	1954
EC_{20}-seedling emergence-T $_{5\text{-}7d}$	32	37	58	39	401
LOEC	100	100	100	100	>4905
NOEC	10	10	10	10	4905
EC_{50}-Fresh growth	34	68	54	83	64
EC_{20}-Fresh growth	5	33	22	36	31
LOEC	100	100	100	100	10
NOEC	10	10	10	10	<10
EC_{50}-Dry growth	44	71	56	83	69
EC_{20}-Dry growth	7	34	26	39	25
LOEC	100	100	100	100	10
NOEC	10	10	10	10	<10

Table 43. Summary of ecotoxicological parameters determined from the range-finding assays with RDX and HMX.

Ecotoxicological parameters	Alfalfa (mg kg^{-1})	Corn (mg kg^{-1})	Japanese millet (mg kg^{-1})	Ryegrass (mg kg^{-1})	Lettuce (mg kg^{-1})
RDX					
LOEC-seedling emergence-T $_{5\text{-}7d}$	>9363	>9363	>9363	>9363	>9363
NOEC-seedling emergence-T $_{5\text{-}7d}$	9363	9363	9363	9363	9363
LOEC-Fresh growth	>9363	>9363	>9363	>9363	>9363
NOEC-Fresh growth	9363	9363	9363	9363	9363
LOEC-Dry growth	>9363	>9363	>9363	>9363	>9363
NOEC-Dry growth	9363	9363	9363	9363	9363
HMX					
LOEC-seedling emergence-T $_{5\text{-}7d}$	>10373	>10373	>10373	>10373	>10373
NOEC-seedling emergence-T $_{5\text{-}7d}$	10373	10373	10373	10373	10373
LOEC-Fresh growth	>10373	>10373	>10373	>10373	>10373
NOEC-Fresh growth	10373	10373	10373	10373	10373
LOEC-Dry growth	>10373	>10373	>10373	>10373	>10373
NOEC- Dry growth	10373	10373	10373	10373	10373

3.6. Definitive plant toxicity tests.

Definitive plant toxicity tests were conducted to assess the effects of RDX, HMX, 2,4-DNT, 2,6-DNT and TNB on the terrestrial plant species alfalfa, Japanese millet and ryegrass in freshly amended and weathered/aged amended SSL soil. Measurement endpoints included germination (measured as the number of emerged seedlings), and growth (measured as both fresh and dry shoot mass). These endpoints were assessed using 6 to 9 treatment concentrations determined from the range-finding studies (Tables 44 to 50). All ecotoxicological parameters were determined using measured chemical concentrations.

Germination in the negative and carrier (acetone) controls complied in all cases with quality control requirements. These were 70% for alfalfa and 85% for Japanese millet and ryegrass. Alfalfa germination in negative controls ranged from 68% to 76% and from 69% to 82% in carrier controls. Japanese millet germination in negative controls ranged from 83% to 96% and from 79% to 98% in carrier controls. Ryegrass germination in negative controls ranged from 85% to 94%, and from 75% to 95% in carrier controls.

Alfalfa fresh shoot mass ranged from 0.12 g to 0.38 g in negative controls, and from 0.27 g to 0.45 g in carrier controls. Japanese millet had higher biomass, with fresh shoot mass ranging from 0.44 to 0.62 g in negative controls, and from 0.39 to 0.63 g in carrier controls. Ryegrass fresh shoot mass was similar to alfalfa biomass, with fresh shoot mass ranging from 0.15 to 0.30 g in negative controls, and from 0.13 to 0.34 g in carrier controls. Dry shoot mass was usually ten times lower than fresh shoot mass, due to approximately 90% water content in plant shoot tissue.

The ecotoxicological parameters determined in this study included bounded NOEC/NOAEC and LOEC/LOAEC, and EC_{20} and EC_{50} values. These parameters were determined for seedling emergence; shoot fresh and dry mass measurement endpoints. Measured concentrations from acetonitrile and ATCLP extractions were used in statistical analyses and parameter estimations. Coefficients of determinations (R^2) and ECp values were determined by nonlinear regression analyses using logistic (Gompertz), logistic hormetic and exponential models (Tables 51 to 62). The effect of weathering/aging of amended soils on EM toxicity for terrestrial plant species tested was determined by examining coefficients of determination from regression analyses performed to estimate shoot growth EC_{20} and EC_{50} values and their respective 95% confidence intervals. Data presented in Tables 64 to 69 identify EMs with a significant effect of the weathering/aging of amended soils for toxicity measurement endpoints used in the study.

3.6.1. Phytotoxicity of RDX and HMX.

Results of the limit tests performed with freshly amended and weathered/aged RDX or HMX amended soils (Table 50) confirmed that these two EMs were not toxic to alfalfa, Japanese millet and ryegrass. RDX was not toxic at concentrations of 9740 and 9537 mg kg^{-1}

respectively for freshly amended and weathered/aged RDX amended soils, and HMX was not toxic at concentrations of 10410 and 9340 mg kg^{-1} respectively for freshly amended and weathered/aged HMX amended soils (unbounded NOEC). Furthermore, significant ($p < 0.0001$) growth stimulation was observed in Japanese millet and ryegrass exposed to these high concentrations of RDX or HMX (Tables 55 to 62).

3.6.2. Phytotoxicity of TNB.

3.6.2.1. Freshly amended soils.

TNB affected germination of all plant species tested within the concentration ranges selected for definitive test (Table 44). The bounded NOEC and LOEC values for Japanese millet were 39 and 125 mg kg^{-1} respectively (Table 55), based on acetonitrile extractable concentrations of TNB. Since hormetic responses were measured at low TNB concentrations for alfalfa and ryegrass germination, the bounded NOAEC and LOAEC values based on acetonitrile extractable concentrations were 88 and 171, 64 and 125, 39 and 125 mg kg^{-1}, respectively (Tables 51 and 59). The bounded NOEC and LOEC values based on ATCLP extractable concentrations were 34 and 71 for Japanese millet (Table 56). The bounded NOAEC and LOAEC values based on ATCLP extractable concentrations were 53 and 123 for alfalfa, and 18 and 71 mg kg^{-1} for ryegrass (Tables 52 and 60). The EC_{50} and EC_{20} values of TNB for germination for alfalfa, Japanese millet and ryegrass based on acetonitrile extractable concentrations were 172 and 145, 204 and 109, and 95 and 28 mg kg^{-1}, respectively, and 123 and 30, 168 and 63, and 49 and 32 mg kg^{-1}, respectively using ATCLP extractable concentrations.

For the growth endpoint using fresh shoot mass, the NOEC/NOAEC and LOEC/LOAEC values based on acetonitrile extractable concentrations of TNB for alfalfa (unbounded NOEC), Japanese millet (bounded NOAEC) and ryegrass (bounded NOAEC) were 5 and 39, 8 and 22, and 39 and 125 mg kg^{-1}, respectively, and 0.6 and 18, 1.5 and 6, and 18 and 71 mg kg^{-1}, respectively using ATCLP extractable concentrations. Using dry shoot mass, the bounded NOEC/NOAEC and LOEC/LOAEC values based on acetonitrile extractable concentrations of TNB for alfalfa (bounded NOEC), Japanese millet (bounded NOAEC) and ryegrass (bounded NOEC) were 39 and 88, 22 and 64, and 39 and 125 (bounded NOAEC) mg kg^{-1}, respectively, and 18 and 53, 6 and 34, and 18 and 71 mg kg^{-1}, respectively using ATCLP extractable concentrations.

Concentration-response relationships for plant growth determined by nonlinear regressions are shown in Figures 1, 3 and 5. Logistic Gompertz model had the best fit for data in tests with alfalfa. Logistic hormetic model had the best fit for Japanese millet and ryegrass data. The EC_{50} and EC_{20} values of TNB for growth using fresh shoot mass based on acetonitrile extractable concentrations of TNB were 107 and 38, 36 and 16, and 75 and 45 mg kg^{-1}, respectively for alfalfa, Japanese millet and ryegrass; and 68 and 18, 11 and 3, and 46 and 20 mg kg^{-1}, respectively using ATCLP extractable concentrations. For dry shoot mass, the acetonitrile extractable concentration of TNB based EC_{50} and EC_{20} values for these species were 129 and 62,

89 and 43, and 89 and 56 mg kg^{-1}, respectively, and 86 and 34, 49 and 10, and 49 and 27 mg kg^{-1}, respectively using ATCLP extractable concentrations.

3.6.2.2. Weathered/aged amended soils.

Weathering/aging of amended soils increased the TNB toxicity to Japanese millet based on 95% confidence intervals. For germination, the bounded NOEC and LOEC values based on acetonitrile extractable concentrations of TNB for Japanese millet and ryegrass were 81 and 197mg kg^{-1} for both plant species. Since hormetic response was measured at TNB low concentrations for alfalfa germination, the bounded NOAEC and LOAEC values based on acetonitrile extractable concentrations were 22 and 114 mg kg^{-1}. The bounded NOEC/NOAEC and LOEC/LOAEC values based on ATCLP extractable concentrations for alfalfa (bounded NOAEC), Japanese millet (bounded NOEC) and ryegrass (bounded NOEC) were 7 and 67, 39 and 126, and 0.5 and 39 mg kg^{-1}, respectively. The EC_{50} and EC_{20} values of TNB for germination for alfalfa, Japanese millet and ryegrass based on acetonitrile extractable concentrations of TNB were 114 and 109, 163 and 139, and 150 and 107 mg kg^{-1}, respectively, and 67 and 64, 98 and 80, and 88 and 57 mg kg^{-1}, respectively using ATCLP extractable concentrations.

For growth using fresh shoot mass, the bounded NOEC and LOEC values based on acetonitrile extractable concentrations of TNB for alfalfa, Japanese millet (unbounded LOEC) and ryegrass (unbounded LOEC) were 22 and 114, <0.3 and 0.3, and <0.3 and 0.3 mg kg^{-1}, respectively, and 7 and 67, <1.4 and 1.4, and 0.5 and 39 (NOAEC/LOAEC) mg kg^{-1}, respectively using ATCLP extractable concentrations. Using dry shoot mass, the bounded NOEC and LOEC values based on acetonitrile extractable concentrations of TNB for alfalfa, Japanese millet (unbounded LOEC) and ryegrass were 22 and 114, <0.3 and 0.3, and 2 and 81 mg kg^{-1}, respectively, and 7 and 67, <1.4 and 1.4, and 0.5 and 39 mg kg^{-1}, respectively using ATCLP extractable concentrations.

Concentration-response relationships for plant growth determined by nonlinear regressions are shown in Figures 2, 4 and 6. The nonlinear regression model selection based on the fit for the data was species and endpoint specific in the weathered/aged TNB amended soils. Logistic Gompertz model had the best fit for data in tests with alfalfa and ryegrass. Exponential model had the best fit for Japanese millet data. The EC_{50} and EC_{20} values for alfalfa, Japanese millet and ryegrass growth using fresh shoot mass based on acetonitrile extractable concentrations of TNB were 63 and 20, 0.9 and 0.3, and 83 and 46 mg kg^{-1}, respectively, and 29 and 7, 0.3 and 0.1, and 40 and 33 mg kg^{-1}, respectively using ATCLP extractable concentrations. For dry shoot mass, the acetonitrile extractable concentration based EC_{50} and EC_{20} values of TNB for these species were 92 and 46, 2 and 0.7, and 86 and 51 mg kg^{-1}, respectively, and 51 and 22, 0.7 and 0.2, and 43 and 21 mg kg^{-1}, respectively using ATCLP extractable concentrations.

3.6.3. Phytotoxicity of 2,4-DNT.

3.6.3.1. Freshly amended soils.

2,4-DNT affected germination of all plant species tested within the concentration ranges of 2,4-DNT selected for definitive test (Table 46). The bounded NOEC and LOEC values based on acetonitrile extractable concentrations of 2,4-DNT for alfalfa and Japanese millet were 47 and 99, and 9 and 22, respectively (Tables 51 and 55). Since hormetic response was measured at low 2,4-DNT concentrations for ryegrass germination, the bounded NOAEC and LOAEC LOEC values based on acetonitrile extractable concentrations of 2,4-DNT were 9 and 17 mg kg^{-1}, respectively (Table 59). The NOEC/LOAEC and LOEC/LOAEC values based on ATCLP extractable concentrations were 27 and 69, 4 and 12, and 4 and 9 (NOAEC/LOAEC) mg kg^{-1}, respectively (Tables 52, 56, 60). The EC_{50} and EC_{20} values for germination for alfalfa, Japanese millet and ryegrass based on acetonitrile extractable concentrations of 2,4-DNT were >47 and >47, 70 and 55, and 16 and 12 mg kg^{-1}, respectively, and >27 and 39, 45 and 33, and 8 and 3 mg kg^{-1}, respectively using ATCLP extractable concentrations.

Hormetic response was measured for ryegrass growth at low concentrations of freshly amended 2,4-DNT. For growth using fresh shoot mass, the NOEC/NOAEC and LOEC/LOAEC values based on acetonitrile extractable concentrations of 2,4-DNT for alfalfa (unbounded LOEC), Japanese millet (bounded NOEC) and ryegrass (bounded NOAEC) were <4.7 and 4.7, 1.0 and 4.7, and 2 and 4 mg kg^{-1}, respectively (Tables 51, 55, 59). The NOEC and LOEC values based on ATCLP extractable concentrations for alfalfa (unbounded LOEC), Japanese millet (unbounded LOEC) and ryegrass (unbounded NOEC) were <1.8 and 1.8, <0.3 and 0.3, and 0.3 and 0.8 mg kg^{-1}, respectively (Tables 52, 56, 60). Using dry shoot mass, the NOEC/NOAEC and LOEC/LOAEC values based on acetonitrile extractable concentrations of 2,4-DNT for alfalfa (unbounded LOEC), Japanese millet (bounded NOEC) and ryegrass (bounded NOAEC) were <5 and 5, 5 and 9, and 9 and 17 mg kg^{-1}, respectively, and <1.8 and 1.8, 1.8 and 4, and 4 and 9 mg kg^{-1}, respectively using ATCLP extractable concentrations.

Concentration-response relationships for plant growth determined by nonlinear regressions are shown in Figures 7, 9, 11. Logistic Gompertz model had the best fit for data in tests with alfalfa and Japanese millet. Logistic hormetic model had the best fit for ryegrass data. The EC_{50} and EC_{20} values of 2,4-DNT for growth using fresh shoot mass based on acetonitrile extractable concentrations were 38 and 11, 10 and 4, and 13 and 11 mg kg^{-1}, respectively, and 27 and 10, 5 and 1, and 6 and 5 mg kg^{-1}, respectively using ATCLP extractable concentrations. For dry shoot mass, the acetonitrile extractable concentration based EC_{50} and EC_{20} values of 2,4-DNT for these species were 56 and 34, 34 and 25, and 13 and 11 mg kg^{-1}, respectively, and 34 and 19, 20 and 14, and 6 and 5 mg kg^{-1}, respectively using ATCLP extractable concentrations.

3.6.3.2. Weathered/aged amended soils.

Weathering/aging of amended soils increased the 2,4-DNT toxicity to Japanese millet and ryegrass based on 95% confidence intervals (Tables 66-69). Hormetic responses were measured at low concentrations of 2,4-DNT for alfalfa and Japanese millet germination. The bounded NOEC/NOAEC and LOEC/LOAEC values based on acetonitrile extractable

concentrations of 2,4-DNT for alfalfa (NOAEC/LOAEC), Japanese millet (NOAEC/LOAEC) and ryegrass (NOEC/LOEC) were 89 and 121, 32 and 90, and 4 and 8 mg kg^{-1}, respectively. The bounded NOEC/NOAEC and LOEC/LOAEC values based on ATCLP extractable concentrations were 48 and 71 (NOAEC/LOAEC), 17 and 50 (NOAEC/LOAEC), and 1 and 3 (NOEC/LOEC) mg kg^{-1}, respectively. The EC_{50} and EC_{20} values of 2,4-DNT for germination for alfalfa, Japanese millet and ryegrass based on acetonitrile extractable concentrations were 115 and 104, >32 and 86, and >8 and >8 mg kg^{-1}, respectively, and 66 and 36, >17 and >17, and >3 and 3 mg kg^{-1}, respectively using ATCLP extractable concentrations.

For growth using fresh shoot mass, the bounded NOEC and LOEC values based on acetonitrile extractable concentrations of 2,4-DNT for alfalfa, Japanese millet (unbounded NOEC) and ryegrass were 6 and 10, 1 and 4, and 4 and 8 mg kg^{-1}, respectively, and 2 and 4, 0.3 and 1.3, and 1 and 3 mg kg^{-1}, respectively using ATCLP extractable concentrations. Hormetic responses were measured for alfalfa and ryegrass dry shoot mass. The bounded NOEC/NOAEC and LOEC/LOAEC values based on acetonitrile extractable concentrations of 2,4-DNT for alfalfa (NOAEC/LOAEC), Japanese millet (NOEC/LOEC) and ryegrass (NOAEC/LOAEC) were 6 and 10, 4 and 8, and 4 and 8 mg kg^{-1}, respectively, and 2 and 4, 1 and 3, and 1 and 3 mg kg^{-1}, respectively using ATCLP extractable concentrations.

Concentration-response relationships for plant growth determined by nonlinear regressions are shown in Figures 8, 10 and 12. The nonlinear regression model selection based on the fit for the data was species and endpoint specific in weathered/aged 2,4-DNT amended soils. Gompertz model had the best fit for fresh shoot mass data in tests with alfalfa, Japanese millet and ryegrass. Logistic hormetic model had the best fit for dry shoot mass data in tests with alfalfa and ryegrass. The EC_{50} and EC_{20} values of 2,4-DNT for alfalfa, Japanese millet and ryegrass growth using fresh shoot mass based on acetonitrile extractable concentrations of 2,4-DNT were 30 and 7, 7 and 4, and 7 and 5 mg kg^{-1}, respectively, and 14 and 2, 2.3 and 1.2, and 2.4 and 1.8 mg kg^{-1}, respectively using ATCLP extractable concentrations. For dry shoot mass, the acetonitrile extractable concentration based EC_{50} and EC_{20} values of 2,4-DNT for these species were 42 and 15, 10 and 6, and 8 and 2 mg kg^{-1}, respectively, and 20 and 6, 4 and 2, and >1.3 and >1.3 mg kg^{-1}, respectively using ATCLP extractable concentrations.

3.6.4. Phytotoxicity of 2,6-DNT.

3.6.4.1. Freshly amended soils.

Germination of all plant species tested was affected by exposure to 2,6-DNT within the concentration ranges of 2,6-DNT selected for definitive tests (Table 48). The bounded NOEC and LOEC values based on acetonitrile extractable concentrations of 2,6-DNT for alfalfa, Japanese millet and ryegrass (unbounded LOEC) were 8 and 14, 30 and 89, and <4 and 4 mg kg^{-1}, respectively (Tables 51, 55, 59). The bounded NOEC and LOEC values based on ATCLP extractable concentrations were 4 and 8, 22 and 66, and <3 and 3 mg kg^{-1}, respectively (Tables 52, 56, 60). The EC_{50} and EC_{20} values of 2,6-DNT for germination for alfalfa, Japanese millet and ryegrass based on acetonitrile extractable concentrations were 19 and 11, 57 and 40, and 38

and 29 mg kg^{-1}, respectively, and 12 and 6, 43 and 30, and 28 and 21 mg kg^{-1}, respectively using ATCLP extractable concentrations.

Hormetic responses were measured at low concentrations of freshly amended 2,6-DNT for Japanese millet and ryegrass growth. For growth using fresh shoot mass, the bounded NOEC/NOAEC and LOEC/LOAEC values based on acetonitrile extractable concentrations of 2,6-DNT for alfalfa (bounded NOEC), Japanese millet (unbounded NOEC) and ryegrass (bounded NOAEC) were 1 and 4, <4 and 4, and 30 and 89 mg kg^{-1}, respectively (Tables 51, 55, 59), and 1 and 3, <3 and 3, and 22 and 66 mg kg^{-1}, respectively using ATCLP extractable concentrations (Tables 52, 56, 60). Using dry shoot mass, the bounded NOEC/NOAEC and LOEC/LOAEC values based on acetonitrile extractable concentrations of 2,6-DNT for alfalfa (bounded NOEC), Japanese millet (bounded NOAEC) and ryegrass (bounded NOAEC) were 1 and 4, 8 and 14, and 14 and 30 mg kg^{-1}, respectively, and 1 and 3, 4 and 8, and 8 and 22 mg kg^{-1}, respectively using ATCLP extractable concentrations.

Concentration-response relationships for plant growth determined by nonlinear regressions are shown in Figures 13 and 15. Logistic Gompertz model had the best fit for data in tests with alfalfa. Logistic hormetic model had the best fit for Japanese millet and ryegrass data. The EC_{50} and EC_{20} values of 2,6-DNT for alfalfa, Japanese millet, and ryegrass growth using fresh shoot mass based on acetonitrile extractable concentrations were 5 and 1, 16 and 13, and 39 and 18 mg kg^{-1}, respectively, and 3 and 1, 9 and 7, and 29 and 20 mg kg^{-1}, respectively using ATCLP extractable concentrations. For dry shoot mass, the acetonitrile extractable concentration based EC_{50} and EC_{20} values of 2,6-DNT for these species were 10 and 3, 18 and 11, and 39 and 26 mg kg^{-1}, respectively, and 5 and 1, 11 and 6, and 28 and 20 mg kg^{-1}, respectively using ATCLP extractable concentrations.

3.6.4.2. Weathered/aged amended soils.

Weathering/aging of amended soils increased the 2,6-DNT toxicity to Japanese millet based on 95% confidence intervals (Tables 66 and 67). Hormetic responses were measured at low concentrations of 2,6-DNT for alfalfa and Japanese millet germination. The bounded NOEC/NOAEC and LOEC/LOAEC values based on acetonitrile extractable concentrations of 2,6-DNT for alfalfa (NOAEC/LOAEC), Japanese millet (NOAEC/LOAEC) and ryegrass (NOEC/LOEC) were 5 and 15, 15 and 140, and 20 and 37 mg kg^{-1}, respectively. The bounded NOEC/NOAEC and LOEC/LOAEC values based on ATCLP extractable concentrations of 2,6-DNT were 3 and 10, 10 and 104, and 12 and 23 mg kg^{-1}, respectively. The EC_{50} and EC_{20} values of 2,6-DNT for germination for alfalfa, Japanese millet and ryegrass based on acetonitrile extractable concentrations were 55 and 26, >15 and >15, and 54 and 42 mg kg^{-1}, respectively, and 41 and 4, 53 and 3, and 34 and 25 mg kg^{-1}, respectively using ATCLP extractable concentrations.

For growth using fresh shoot mass, the bounded NOEC and LOEC values based on acetonitrile extractable concentrations of 2,6-DNT for alfalfa, Japanese millet (unbounded NOEC for ATCLP) and ryegrass (unbounded NOEC for acetonitrile and ATCLP) were 3 and 5,

1 and 3, and 8 and 20 mg kg^{-1}, respectively, and 2 and 3, 0.6 and 1.5, and 4 and 12 mg kg^{-1}, respectively using ATCLP extractable concentrations. Using dry shoot mass, the bounded NOEC and LOEC values based on acetonitrile extractable concentrations of 2,6-DNT for alfalfa, Japanese millet and ryegrass (unbounded NOEC) were 3 and 5, 3 and 5, and 8 and 20 mg kg^{-1}, respectively, and 1 and 3, 1 and 3, and 4 and 12 mg kg^{-1}, respectively using ATCLP extractable concentrations.

Concentration-response relationships for plant growth determined by nonlinear regressions are shown in Figures 14 and 16. The nonlinear regression model selection based on the fit for the data was and endpoint specific in weathered/aged 2,6-DNT amended soils. Logistic hormetic model had the best fit for germination data and Gompertz model had the best fit for growth endpoints. The EC_{50} and EC_{20} values of 2,6-DNT for alfalfa, Japanese millet and ryegrass growth using fresh shoot mass based on acetonitrile extractable concentrations were 7 and 2, 9 and 5, and 39 and 24 mg kg^{-1}, respectively, and 4 and 1, 6 and 3, and 24 and 14 mg kg^{-1}, respectively using ATCLP extractable concentrations. For dry shoot mass, the acetonitrile extractable concentration based EC_{50} and EC_{20} values of 2,6-DNT for these species were 5 and 0.4, 11 and 6, and 34 and 21 mg kg^{-1}, respectively, and 2 and 0.1, 6 and 3, and 21 and 12 mg kg^{-1}, respectively using ATCLP extractable concentrations.

3.6.5. Relationship between chemical extraction method and phytotoxicity.

Coefficients of determinations (R^2) for acetonitrile and ATCLP based extractions determined in nonlinear regression analyses of the plant germination and growth data from studies with fresh and weathered/aged amended soils were compared to determine which chemical measure of exposure better correlated with toxicity (Table 63). These comparisons of coefficients of determinations showed that neither extraction method had an advantage for characterizing bioavailability of EMs to the three terrestrial plant species tested in this study. This was true for both freshly amended and weathered/aged amended soils.

Table 44. Average (n = 4) seedling counts, fresh shoot mass and dry shoot mass of alfalfa, Japanese millet and ryegrass exposed to freshly amended TNB in Sassafras sandy loam soils. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330A. Twenty (20) seeds were sown in each replicate.

Concentration in freshly amended soil (mg kg^{-1})	Seedling counts	Standard error	Fresh shoot mass (g)	Standard error	Dry shoot mass (g)	Standard error
Alfalfa	After 5 d		After 16 d		After 16 d	
Control (negative)	14.0 (70%)	1.6	0.2432	0.0408	0.0245	0.0064
Control (carrier)	13.8 (69%)	1.2	0.3092	0.0280	0.0308	0.0021
5.1	13.8	1.1	0.2955	0.0352	0.0310	0.0034
39.2	14.5	0.7	0.2332	0.0122	0.0288	0.0017
87.8	14.8	0.8	0.1838	0.0071	0.0204	0.0011
170.9	7.0	2.5	0.0941	0.0256	0.0110	0.0035
343.4	0	0	0.0030	0	0.0003	0
647.8	0	0				
Japanese millet	After 5 d		After 16 d		After 16 d	
Control (negative)	18.5 (93%)	0.3	0.5316	0.0115	0.0526	0.0008
Control (carrier)	16.8 (84%)	1.1	0.3946	0.0150	0.0426	0.0019
1.8	17.3	0.7	0.4214	0.0317	0.0434	0.0017
5.1	16.3	0.9	0.4455	0.0287	0.0485	0.0037
8.4	17.8	1.5	0.4007	0.0311	0.0477	0.0031
21.5	17.5	0.3	0.2563	0.0157	0.0378	0.0007
64.1	17.5	0.7	0.1657	0.0038	0.0319	0.0015
124.7	10.5	1.2	0.0688	0.0066	0.0122	0.0012
220.3	4.5	1.7	0.0227	0.0044	0.0047	0.0020
647.8	1	0	0.0016	0	0.0002	0
Ryegrass	After 7 d		After 19 d		After 19 d	
Control (negative)	18.8 (94%)	0.5	0.3012	0.0131	0.0514	0.0018
Control (carrier)	15.0 (75%)	1.6	0.1947	0.0214	0.0301	0.0026
1.8	15.3	0.9	0.2137	0.0140	0.0278	0.0019
8.4	17.0	0.7	0.2505	0.0114	0.0373	0.0019
21.5	17.5	0.7	0.2080	0.0088	0.0336	0.0021
39.2	16.8	0.8	0.1902	0.0061	0.0298	0.0009
124.7	5.0	1.2	0.0663	0.0091	0.0127	0.0014
220.3	0	0	0.0058	0.0016	0.0008	0.0002
647.8	0	0	0.0031	0.0010	0.0004	0.0003

Table 45. Average (n = 4) seedling counts, fresh shoot mass and dry shoot mass of alfalfa, Japanese millet and ryegrass exposed to weathered/aged TNB in Sassafras sandy loam soils. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330A. Twenty (20) seeds were sown in each replicate.

Concentration in weathered/aged soil (mg kg^{-1})	Seedling counts	Standard error	Fresh shoot mass (g)	Standard error	Dry shoot mass (g)	Standard error
Alfalfa	After 5 d		After 16 d		After 16 d	
Control (negative)	15.3 (76%)	1.6	0.3551	0.0757	0.0397	0.0088
Control (carrier)	15.6 (78%)	0.9	0.3489	0.0749	0.0346	0.0043
BDL	14.3	0.5	0.3402	0.0513	0.0355	0.0043
2.1	15.0	0.8	0.3563	0.0318	0.0315	0.0023
22.1	16.8	0.5	0.2643	0.0190	0.0318	0.0020
113.5	8.3	1.3	0.1104	0.0145	0.0128	0.0017
282.0	0	0	0.0024	0	0.0004	0
575.2	0	0				
722.0	0	0				
1564.1	0	0				
Japanese millet	After 5 d		After 16 d		After 16 d	
Control (negative)	18.5 (93%)	0.9	0.5997	0.0393	0.0625	0.0035
Control (carrier)	19.5 (98%)	0.5	0.6029	0.0752	0.0604	0.0020
BDL	19.0	0.4	0.5724	0.0305	0.0549	0.0012
BDL	18.8	0.8	0.5077	0.0153	0.0564	0.0045
0.3	19.0	0.4	0.4435	0.0536	0.0500	0.0008
0.6	18.0	0.9	0.3676	0.0252	0.0457	0.0005
5.2	18.0	0.4	0.0593	0.0107	0.0197	0.0032
80.7	19.0	0	0.0503	0.0050	0.0118	0.0010
197.1	0	0				
575.2	0	0				
984.3	0	0				
Ryegrass	After 7 d		After 19 d		After 19 d	
Control (negative)	18.8 (94%)	1.0	0.1734	0.0197	0.0364	0.0014
Control (carrier)	19.0 (95%)	0.7	0.2024	0.0290	0.0362	0.0016
BDL	18.5	0.9	0.1984	0.0073	0.0320	0.0014
0.3	20	0	0.2450	0.0166	0.0399	0.0007
0.6	19.3	0.5	0.2564	0.0221	0.0379	0.0017
2.1	17.0	0.4	0.2543	0.0139	0.0359	0.0018
80.7	14.8	2.1	0.1192	0.0067	0.0200	0.0023
197.1	0.5	0.5	0.0071	0.0024	0.0006	0.0004
575.2	0	0				
984.3	0	0				

BDL: Below detection limit. HPLC detection limit = 0.05 mg L^{-1} = 0.5 mg kg^{-1} soil.

Table 46. Average (n = 4) seedling counts, fresh shoot mass and dry shoot mass of alfalfa, Japanese millet and ryegrass exposed to freshly amended 2,4-DNT in Sassafras sandy loam soils. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330A. Twenty (20) seeds were sown in each replicate.

Concentration in freshly amended soil (mg kg^{-1})	Seedling counts	Standard error	Fresh shoot mass (g)	Standard error	Dry shoot mass (g)	Standard error
Alfalfa	After 5 d		After 16 d		After 16 d	
Control (negative)	14.3 (71%)	0.8	0.1213	0.579	0.0152	0.0056
Control (carrier)	15.2 (76%)	0.4	0.2705	0.041	0.0282	0.0029
4.7	11.5	0.5	0.1739	0.012	0.0148	0.0018
9.1	13.5	1.1	0.1935	0.032	0.0181	0.0022
21.5	14.8	1.8	0.1833	0.015	0.0202	0.0007
46.5	14.5	0.7	0.1353	0.013	0.0139	0.0043
98.5	1.3	1.0	0.0099	0.002	0.0013	0.0004
278.1	0	0				
612.7	0	0				
Japanese millet	After 5 d		After 16 d		After 16 d	
Control (negative)	18.0 (90%)	0.7	0.4636	0.0348	0.0418	0.0043
Control (carrier)	18.5 (93%)	0.3	0.4083	0.0271	0.0478	0.0024
1.0	19.0	0.4	0.3476	0.0245	0.0451	0.0027
4.7	17.3	0.9	0.2588	0.0197	0.0525	0.0018
9.1	17.3	0.7	0.2617	0.0159	0.0356	0.0015
21.5	16.8	0.5	0.0612	0.0054	0.0411	0.0021
46.5	16.0	0.4	0.0203	0.0030	0.0054	0.0010
98.5	0.8	0.8	0.0028	0.0019	0.0005	0.0004
Ryegrass	After 7 d		After 19 d		After 19 d	
Control (negative)	18.5 (93%)	0.3	0.2680	0.0066	0.0362	0.0014
Control (carrier)	19.0 (95%)	0.4	0.2985	0.0140	0.0381	0.0021
0.5	19.0	0.7	0.2592	0.0079	0.0331	0.0015
0.9	19.0	0.4	0.2569	0.0074	0.0309	0.0009
2.2	19.0	0.4	0.3151	0.0126	0.0387	0.0019
3.8	19.5	0.3	0.3399	0.0112	0.0411	0.0020
8.5	18.8	0.8	0.3127	0.0111	0.0360	0.0018
17.1	8.3	1.5	0.0602	0.0155	0.0072	0.0021
38.4	0.3	0.3	0.0006	0.0004	0	0

Table 47. Average (n = 4) seedling counts, fresh shoot mass and dry shoot mass of alfalfa, Japanese millet and ryegrass exposed to weathered/aged 2,4-DNT in Sassafras sandy loam soils. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330A. Twenty (20) seeds were sown in each replicate.

Concentration in weathered/aged soil (mg kg^{-1})	Seedling counts	Standard error	Fresh shoot mass (g)	Standard error	Dry shoot mass (g)	Standard error
Alfalfa	After 5 d		After 16 d		After 16 d	
Control (negative)	17.5 (88%)	1.9	0.4792	0.0446	0.0389	0.0021
Control (carrier)	16.2 (81%)	1.8	0.4843	0.0481	0.0370	0.0026
3.2	17.0	2.2	0.4479	0.0774	0.0383	0.0040
6.2	17.3	0.5	0.4394	0.0123	0.0401	0.0017
10.3	15.3	1.3	0.3235	0.0916	0.0313	0.0057
25.2	17.0	2.2	0.2437	0.0306	0.0227	0.0017
55.6	17.0	2.2	0.1795	0.0373	0.0191	0.0034
89.2	16.5	1.0	0.1435	0.0173	0.0169	0.0011
120.6	5.8	2.2	0.0403	0.0147	0.0042	0.0020
153.4	0.3	0.5	0.0030	0.0061	0.0002	0.0005
Japanese millet	After 5 d		After 16 d		After 16 d	
Control (negative)	16.5 (83%)	0.3	0.4915	0.0169	0.0438	0.0013
Control (carrier)	15.8 (79%)	0.7	0.5030	0.0419	0.0450	0.0021
1.3	16.3	0.5	0.5040	0.0366	0.0469	0.0021
3.7	15.0	0.8	0.4039	0.0266	0.0456	0.0014
7.8	15.5	0.3	0.1858	0.0150	0.0315	0.0016
14.9	16.3	0.9	0.0380	0.0011	0.0097	0.0006
32.1	16.3	0.9	0.0146	0.0020	0.0055	0.0008
90.4	0	0				
Ryegrass	After 7 d		After 19 d		After 19 d	
Control (negative)	17.0 (85%)	1.1	0.2964	0.0148	0.0401	0.0010
Control (carrier)	18.3 (90%)	0.5	0.3412	0.0070	0.0443	0.0006
1.3	18.0	0.7	0.3445	0.0185	0.0442	0.0010
3.7	18.8	0.5	0.3257	0.0194	0.0456	0.0016
7.8	15.8	1.1	0.0957	0.0153	0.0126	0.0013
14.9	0	0				
32.1	0	0				
90.4	0	0				

Table 48. Average (n = 4) seedling counts, fresh shoot mass and dry shoot mass of alfalfa, Japanese millet and ryegrass exposed to freshly amended 2,6-DNT in Sassafras sandy loam soils. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330A. Twenty (20) seeds were sown in each replicate.

Concentration in freshly amended soil (mg kg^{-1})	Seedling counts	Standard error	Fresh shoot mass (g)	Standard error	Dry shoot mass (g)	Standard error
Alfalfa	After 5 d		After 16 d		After 16 d	
Control (negative)	13.5 (68%)	0.5	0.2079	0.0501	0.0308	0.0078
Control (carrier)	15.4 (77%)	1.6	0.3684	0.0806	0.0359	0.0058
1.2	15.0	1.0	0.3604	0.0019	0.0359	0.0003
1.4	15.3	1.2	0.3486	0.0356	0.0351	0.0024
4.1	15.0	1.2	0.1729	0.0606	0.0207	0.0041
8.0	17.0	0.4	0.2455	0.0561	0.0292	0.0052
13.9	11.0	1.4	0.0414	0.0129	0.0144	0.0044
29.7	4.5	1.0	0.0089	0.0030	0.0017	0.0004
88.5	0.3	0.3				
Japanese millet	After 5 d		After 16 d		After 16 d	
Control (negative)	19.3 (96%)	0.3	0.4455	0.0103	0.0408	0.0008
Control (carrier)	18.3 (91%)	1.1	0.3893	0.0146	0.0457	0.0010
4.1	18.3	0.3	0.4645	0.0068	0.0522	0.0030
8.0	18.5	0.5	0.4773	0.0171	0.0423	0.0019
13.9	17.5	0.9	0.2865	0.0377	0.0331	0.0025
29.7	16.8	1.1	0.0209	0.0008	0.0092	0.0017
88.5	1.0	0.7	0.0012	0.0006	0.0006	0.0003
644.5	0	0				
Ryegrass	After 7 d		After 19 d		After 19 d	
Control (negative)	18.3 (91%)	0.9	0.2011	0.0054	0.0342	0.0019
Control (carrier)	13.8 (94%)	0.3	0.1337	0.0072	0.0221	0.0008
4.1	18.5	0.3	0.1951	0.0075	0.0296	0.0023
8.0	18.0	0.6	0.1519	0.0145	0.0262	0.0007
13.9	17.8	0.5	0.1400	0.0233	0.0252	0.0014
29.7	11.8	0.8	0.1145	0.0089	0.0179	0.0006
88.5	0	0	0.0053	0.0027	0.0010	0.0006
644.5	0	0				

Table 49. Average (n = 4) seedling counts, fresh shoot mass and dry shoot mass of alfalfa, Japanese millet and ryegrass exposed to weathered/aged 2,6-DNT in Sassafras sandy loam soils. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330A. Twenty (20) seeds were sown in each replicate.

Concentration in weathered/aged soil (mg kg^{-1})	Seedling counts	Standard error	Fresh shoot mass (g)	Standard error	Dry shoot mass (g)	Standard error
Alfalfa	After 5 d		After 16 d		After 16 d	
Control (negative)	13.5 (68%)	1.0	0.3853	0.0271	0.0071	0.0019
Control (carrier)	16.4 (82%)	1.1	0.4497	0.0279	0.0385	0.0025
0.4	16.3	1.5	0.4632	0.0426	0.0408	0.0023
0.6	15.8	0.8	0.4156	0.0123	0.0229	0.0032
1.2	11.8	1.4	0.2991	0.0455	0.0287	0.0035
3.3	14.5	1.5	0.3021	0.0862	0.0276	0.0062
5.4	13.5	1.2	0.2560	0.0212	0.0025	0.0031
14.9	12.3	1.1	0.1157	0.0348	0.0134	0.0029
41.1	9.0	1.1	0.0436	0.0066	0.0094	0.0015
Japanese millet						
Control (negative)	19.0 (95%)	0.6	0.6171	0.0257	0.0345	0.0018
Control (carrier)	18.5 (93%)	0.4	0.6342	0.0241	0.0528	0.0013
1.2	17.0	0.4	0.5976	0.0227	0.0395	0.0038
3.3	17.8	1.0	0.5268	0.0104	0.0491	0.0014
5.4	17.8	0.3	0.4941	0.0151	0.0378	0.0020
14.9	18.0	0.9	0.1274	0.109	0.0135	0.0007
139.5	0	0				
447.3	0	0				
Ryegrass						
Control (negative)	17.8 (88%)	0.9	0.2897	0.0162	0.0397	0.0017
Control (carrier)	19.3 (94%)	0.5	0.3054	0.0121	0.0419	0.0022
7.5	19.5	0.3	0.3021	0.0035	0.0409	0.0005
19.7	18.8	0.3	0.2732	0.0136	0.0349	0.0005
37.2	16.8	1.1	0.1620	0.0079	0.0178	0.0008
59.7	6.8	0.8	0.0480	0.0084	0.0037	0.0011
75.3	1.0	0.6	0.0141	0.0054	0.0004	0.0003
117.7						

Table 50. Average (n = 4) seedling counts, fresh shoot mass and dry shoot mass of alfalfa, Japanese millet and ryegrass exposed to freshly amended and weathered/aged RDX and HMX in Sassafras sandy loam soils. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330A. Twenty (20) seeds were sown in each replicate.

Concentration in freshly amended soil (mg kg^{-1})	Seedling counts	Standard error	Fresh shoot mass (g)	Standard error	Dry shoot mass (g)	Standard error
Alfalfa	After 5 d		After 16 d		After 16 d	
Control (negative)	14.3 (71%)	0.9	0.1743	0.0697	0.0208	0.0056
Control (carrier)	14.8 (74%)	1.1	0.3618	0.0293	0.0309	0.0031
RDX - 9740	14.9	0.7	0.3522	0.0520	0.0347	0.0039
HMX - 10411	15.1	0.7	0.3002	0.0444	0.0312	0.0036
Japanese millet	After 5 d		After 16 d		After 16 d	
Control (negative)	18.5 (93%)	0.5	0.4809	0.0291	0.0489	0.0013
Control (carrier)	18.1 (91%)	0.5	0.3041	0.0216	0.0312	0.0014
RDX - 9740	17.6	0.4	0.4751	0.0260	0.0476	0.0026
HMX - 10411	17.6	0.2	0.4765	0.0198	0.0457	0.0015
Ryegrass	After 7 d		After 19 d		After 19 d	
Control (negative)	18.7 (94%)	0.5	0.2600	0.0073	0.0340	0.0005
Control (carrier)	18.1 (91%)	0.3	0.2119	0.0042	0.0279	0.0006
RDX - 9740	18.8	0.3	0.2106	0.0041	0.0279	0.0004
HMX - 10411	19.0	0.4	0.2351	0.0066	0.0311	0.0008
Concentration in weathered/aged soil (mg kg^{-1})	**Seedling counts**	**Standard error**	**Fresh shoot mass (g)**	**Standard error**	**Dry shoot mass (g)**	**Standard error**
Alfalfa	After 5 d		After 16 d		After 16 d	
Control (negative)	14.5 (73%)	1.0	0.2630	0.0725	0.0285	0.0030
Control (carrier)	12.8 (64%)	1.0	0.2431	0.0424	0.0255	0.0028
RDX - 9537	14.0	0.9	0.2587	0.0290	0.0274	0.0023
HMX - 9341	13.3	0.9	0.1569	0.0299	0.0247	0.0028
Japanese millet	After 5 d		After 16 d		After 16 d	
Control (negative)	17.0 (85%)	0.7	0.1770	0.0239	0.0392	0.0025
Control (carrier)	17.9 (89%)	0.2	0.1698	0.0045	0.0364	0.0013
RDX - 9537	17.1	0.3	0.2284	0.0327	0.0395	0.0019
HMX - 9341	16.3	0.5	0.2658	0.0139	0.0378	0.0009
Ryegrass	After 7 d		After 19 d		After 19 d	
Control (negative)	18.0 (90%)	0.0	0.1500	0.0195	0.0304	0.0013
Control (carrier)	18.1 (91%)	0.4	0.1886	0.0179	0.0283	0.0018
RDX - 9537	17.9	0.8	0.2157	0.0093	0.0264	0.0023
HMX - 9341	18.3	0.5	0.3178	0.0178	0.0361	0.0012

Table 51. Effect of freshly amended energetic materials on alfalfa in Sassafras sandy loam soil (acetonitrile extraction; n = 3).

Ecotoxicological parameters (n = 4)	RDX (mg kg^{-1})	HMX (mg kg^{-1})	TNB (mg kg^{-1})	2,4-DNT (mg kg^{-1})	2,6-DNT (mg kg^{-1})
Germination - T 5d					
NOEC or NOAEC	9740*	10411*	87.8***	46.5	8.0
p			0.553	0.601	0.364
LOEC or LOAEC	>9740	>10411	170.9****	98.5	13.9
p			<0.0001	<0.0001	0.001
EC_{20}			145.1	>46.5	10.9
Confidence interval			69.3-220.9		6.0-15.9
EC_{50}			171.6	>46.5	18.9
Confidence interval			155.5-187.6		14.1-23.6
Model used (EC_{20})			H	H & G	G
R^2 (EC_{20})			0.967		0.956
Growth - Fresh mass					
NOEC	9740*	10411*	5.1*	<4.7	1.37
p			0.671		0.148
LOEC	>9740	>10411	39.2	4.7**	4.13
p or P(T<=t) two-tail	0.875	0.269	0.028	0.004	<0.0001
EC_{20}			37.5	11.3	1.3
Confidence interval			9.6-65.5	0-24.2	0-2.9
EC_{50}			106.6	37.6	5.0
Confidence interval			72.2-141.0	17.0-58.3	2.0-8.0
Model used (EC_{20})			G	G	G
R^2 (EC_{20})			0.971	0.923	0.919
Growth - Dry mass					
NOEC	9740*	10411*	39.2	<4.7	1.37
p			0.556		0.351
LOEC	>9740	>10411	87.8	4.7**	4.13
p or P(T<=t) two-tail	0.468	0.953	0.007	0.001	0.001
EC_{20}			61.9	34.4	2.8
Confidence interval			27.8-96.0	9.7-59.1	0-6.1
EC_{50}			128.9	56.2	9.5
Confidence interval			96.5-161.4	32.9-79.4	4.3-14.6
Model used (EC_{20})			G	G	G
R^2 (EC_{20})			0.972	0.902	0.935

G: Gompertz model * Unbounded NOEC *** NOAEC
H: Hormetic model ** Unbounded LOEC **** LOAEC

Table 52. Effect of freshly amended energetic materials on alfalfa in Sassafras sandy loam soil (ATCLP extraction; n = 3).

Ecotoxicological parameters (n = 4)	RDX (mg kg^{-1})	HMX (mg kg^{-1})	TNB (mg kg^{-1})	2,4-DNT (mg kg^{-1})	2,6-DNT (mg kg^{-1})
Germination - T 5d					
NOEC or NOAEC	9740*	10411*	53.4***	26.8	4.3
p			0.553	0.601	0.364
LOEC or LOAEC	>9740	>10411	122.8****	68.6	7.6
p			<0.0001	<0.0001	0.001
EC_{20}			30.4	39.3	5.9
Confidence interval			15.7-45.2	9.9-68.8	2.3-9.6
EC_{50}			123.3	>26.8	12.2
Confidence interval			107.8-138.7		7.9-16.5
Model used (EC_{20})			H	H	G
R^2 (EC_{20})			0.899	0.975	0.953
Growth - Fresh mass					
NOEC	9740	10411	0.6*	<1.8	0.7
p			0.671		0.148
LOEC	>9740	>10411	18.2	1.8**	2.5
p or P(T<=t) two-tail	0.875	0.269	0.028	0.008	<0.0001
EC_{20}			18.1	10.4	0.7
Confidence interval			1.1-35.1	0-22.1	0-1.6
EC_{50}			68.3	27.4	2.8
Confidence interval			40.5-96.1	12.2-42.6	1.1-4.4
Model used (EC_{20})			G	G	G
R^2 (EC_{20})			0.971	0.923	0.922
Growth - Dry mass					
NOEC	9740	10411	18.2	<1.8	0.7
p			0.556		0.351
LOEC	>9740	>10411	53.4	1.8**	2.5
p or P(T<=t) two-tail	0.468	0.953	0.007	0.001	0.001
EC_{20}			34.0	18.6	1.4
Confidence interval			10.3-57.6	2.6-34.6	0-3.06
EC_{50}			86.3	34.1	5.3
Confidence interval			58.9-113.6	16.7-51.5	2.3-8.2
Model used (EC_{20})			G	G	G
R^2 (EC_{20})			0.972	0.901	0.939

G: Gompertz model * Unbounded NOEC *** NOAEC
H: Hormetic model ** Unbounded LOEC **** LOAEC

Table 53. Effect of weathered/aged energetic materials on alfalfa in Sassafras sandy loam soil (acetonitrile extraction; n = 3).

Ecotoxicological parameters (n = 4)	RDX (mg kg^{-1})	HMX (mg kg^{-1})	TNB (mg kg^{-1})	2,4-DNT (mg kg^{-1})	2,6-DNT (mg kg^{-1})
Germination - T 5d					
NOEC or NOAEC	9537*	9341*	22.1***	89.2***	5.4***
p			0.059	0.802	0.089
LOEC or LOAEC	>9537	>9341	113.5****	120.6****	14.9****
p			<0.0001	0.0006	0.018
EC_{20}			109.3	103.8	26.4
Confidence interval			107.1-111.6	91.0-116.6	0-127.5
EC_{50}			114.4	114.9	54.5
Confidence interval			---	108.7-121.0	8.8-100.2
Model used (EC_{20})			H	H	H
R^2 (EC_{20})			0.989	0.989	0.971
Growth - Fresh mass					
NOEC or NOAEC	9537*	9341*	22.1	6.2	3.3
p			0.125	0.188	0.154
LOEC or LOAEC	>9537	>9341	113.5	10.3	5.4
p or P(T<=t) two-tail	0.766	0.120	<0.0001	<0.0001	<0.0001
EC_{20}			20.4	6.5	1.6
Confidence interval			0-48.9	2.0-11.1	0.1-3.2
EC_{50}			63.4	30.2	7.2
Confidence interval			19.3-107.4	20.1-40.3	3.7-10.6
Model used (EC_{20})			G	G	G
R^2 (EC_{20})			0.930	0.976	0.962
Growth - Dry mass					
NOEC	9537*	9341*	22.1	6.2***	3.3
p			0.361	0.153	0.207
LOEC	>9537	>9341	113.5	10.3****	5.4
p or P(T<=t) two-tail	0.614	0.852	<0.0001	0.011	<0.0001
EC_{20}			45.7	15.1	0.4
Confidence interval			2.4-89.0	8.8-21.4	0-1.4
EC_{50}			91.9	42.2	5.2
Confidence interval			58.8-125.0	28.5-55.9	0-10.6
Model used (EC_{20})			G	H	G
R^2 (EC_{20})			0.966	0.979	0.911

G: Gompertz model * Unbounded NOEC *** NOAEC
H: Hormetic model **** LOAEC

Table 54. Effect of weathered/aged energetic materials on alfalfa in Sassafras sandy loam soil (ATCLP extraction; n = 3).

Ecotoxicological parameters (n = 4)	RDX (mg kg^{-1})	HMX (mg kg^{-1})	TNB (mg kg^{-1})	2,4-DNT (mg kg^{-1})	2,6-DNT (mg kg^{-1})
Germination - T 5d					
NOEC or NOAEC	9537*	9341*	6.8***	47.6***	3.1***
p			0.059	0.802	0.060
LOEC or LOAEC	>9537	>9341	66.5****	70.9****	9.5****
p			<0.0001	0.0006	0.009
EC_{20}			64.4	36.0	4.3
Confidence interval			63.3-65.5	17.1-54.9	0-13.3
EC_{50}			67.0	66.4	40.7
Confidence interval			---	61.7-71.1	5.3-76.0
Model used (EC_{20})			H	H	H
R^2 (EC_{20})			0.989	0.981	0.972
Growth - Fresh mass					
NOEC	9537*	9341*	6.8	2.2	1.5
p			0.125	0.188	0.086
LOEC	>9537	>9341	66.5	4.1	3.1
p or P(T<=t) two-tail	0.766	0.120	<0.0001	<0.0001	<0.0001
EC_{20}			6.7	2.4	0.7
Confidence interval			0-19.3	0.5-4.3	0-1.3
EC_{50}			29.0	14.0	3.9
Confidence interval			1.4-56.6	8.7-19.3	2.0-5.9
Model used (EC_{20})			G	G	G
R^2 (EC_{20})			0.929	0.977	0.966
Growth - Dry mass					
NOEC or NOAEC	9537*	9341*	6.8	2.2***	1.4
p			0.361	0.153	0.095
LOEC or LOAEC	>9537	>9341	66.5	4.1****	3.1
p or P(T<=t) two-tail	0.614	0.852	<0.0001	0.011	<0.0001
EC_{20}			22.3	6.2	0.06
Confidence interval			0-49.2	3.4-9.1	0-0.2
EC_{50}			51.2	20.2	2.1
Confidence interval			27.0-75.4	12.7-27.8	0-4.5
Model used (EC_{20})			G	H	G
R^2 (EC_{20})			0.966	0.980	0.929

G: Gompertz model * Unbounded NOEC *** NOAEC
H: Hormetic model **** LOAEC

Table 55. Effect of freshly amended energetic materials on Japanese millet in Sassafras sandy loam soil (acetonitrile extraction; n = 3).

Ecotoxicological parameters (n = 4)	RDX (mg kg^{-1})	HMX (mg kg^{-1})	TNB (mg kg^{-1})	2,4-DNT (mg kg^{-1})	2,6-DNT (mg kg^{-1})
Germination - T 5d					
NOEC	9740*	10411*	64.1	9.1	29.7
p			0.721	0.141	0.211
LOEC	>9740	>10411	124.7	21.5	88.5
p			0.001	0.044	<0.0001
EC_{20}			109.1	54.5	39.9
Confidence interval			74.0-144.2	46.4-62.7	28.3-51.6
EC_{50}			203.5	70.3	56.8
Confidence interval			167.8-239.2	62.7-77.9	46.0-67.6
Model used (EC_{20})			G	G	G
R^2 (EC_{20})			0.988	0.994	0.992
Growth - Fresh mass					
NOEC or NOAEC	9740*	10411*	8.4***	1.0	<4.1
p			0.837	0.019	
LOEC or LOAEC	>9740	>10411	21.5****	4.7	4.1**
p or P(T<=t) two-tail	0.0002	0.00004	<0.0001	<0.0001	0.009
EC_{20}	stimulation	stimulation	16.3	3.5	13.3
Confidence interval			11.5-21.1	1.6-5.4	12.3-14.3
EC_{50}			35.7	10.4	16.3
Confidence interval			26.7-44.6	7.6-13.1	14.8-17.8
Model used (EC_{20})			H	G	H
R^2 (EC_{20})			0.984	0.975	0.991
Growth - Dry mass					
NOEC	9740*	10411*	21.5	4.7	8.0***
p			0.118	0.083	0.225
LOEC	>9740	>10411	64.1	9.1	13.9****
p or P(T<=t) two-tail	0.0002	0.000006	0.001	<0.0001	<0.0001
EC_{20}	stimulation	stimulation	42.6	25.2	11.4
Confidence interval			26.5-58.7	17.6-32.7	9.4-13.4
EC_{50}			88.8	34.4	17.8
Confidence interval			73.3-104.3	28.4-40.3	15.5-20.1
Model used (EC_{20})			G	G	H
R^2 (EC_{20})			0.985	0.978	0.989

G: Gompertz model * Unbounded NOEC *** NOAEC
H: Hormetic model **Unbounded LOEC **** LOAEC

Table 56. Effect of freshly amended energetic materials on Japanese millet in Sassafras sandy loam soil (ATCLP extraction; n = 3).

Ecotoxicological parameters (n = 4)	RDX (mg kg^{-1})	HMX (mg kg^{-1})	TNB (mg kg^{-1})	2,4-DNT (mg kg^{-1})	2,6-DNT (mg kg^{-1})
Germination - T 5d					
NOEC	9740*	10411*	33.8	3.8	22.0
p			0.721	0.141	0.211
LOEC	>9740	>10411	71.3	11.8	66.0
p			0.001	0.044	<0.0001
EC_{20}			63.4	32.5	29.9
Confidence interval			31.7-95.1	26.5-38.5	21.0-38.8
EC_{50}			168.1	44.8	42.5
Confidence interval			119.6-216.5	38.8-50.8	34.3-50.6
Model used (EC_{20})			G	G	G
R^2 (EC_{20})			0.987	0.994	0.992
Growth - Fresh mass					
NOEC or NOAEC	9740*	10411*	1.5***	<0.3	<2.5
p			0.837		
LOEC or LOAEC	>9740	>10411	5.7****	0.3**	2.5**
p or P(T<=t) two-tail	0.0002	0.00004	<0.0001	0.019	0.009
EC_{20}	stimulation	stimulation	3.4	1.3	7.3
Confidence interval			2.0-4.7	0.5-2.1	6.6-7.9
EC_{50}			10.8	4.7	9.2
Confidence interval			6.5-15.1	3.3-6.1	8.0-10.3
Model used (EC_{20})			H	G	H
R^2 (EC_{20})			0.983	0.977	0.991
Growth - Dry mass					
NOEC or NOAEC	9740*	10411*	5.7***	1.8	4.4***
p			0.118	0.083	0.225
LOEC or LOAEC	>9740	>10411	33.8****	3.8	7.6****
p or P(T<=t) two-tail	0.0002	0.000006	0.001	<0.0001	<0.0001
EC_{20}	stimulation	stimulation	9.9	14.4	6.2
Confidence interval			4.4-15.5	9.7-19.1	5.1-7.4
EC_{50}			49.0	19.7	10.5
Confidence interval			40.5-57.5	16.0-23.4	8.8-12.2
Model used (EC_{20})			H	G	H
R^2 (EC_{20})			0.976	0.978	0.99

G: Gompertz model * Unbounded NOEC *** NOAEC
H: Hormetic model ** Unbounded LOEC **** LOAEC

Table 57. Effect of weathered/aged energetic materials on Japanese millet in Sassafras sandy loam soil (acetonitrile extraction; n = 3).

Ecotoxicological parameters (n = 4)	RDX (mg kg^{-1})	HMX (mg kg^{-1})	TNB (mg kg^{-1})	2,4-DNT (mg kg^{-1})	2,6-DNT (mg kg^{-1})
Germination - T 5d					
NOEC or NOAEC	9537*	9341*	80.7	32.1***	15.0***
p			0.374	0.584	0.581
LOEC or LOAEC	>9537	>9341	197.1	90.4****	139.5****
p			<0.0001	<0.0001	<0.0001
EC_{20}			139.1	86.2	>15.0
Confidence interval			0-294.3	---	
EC_{50}			163.1	>32.1	>15.0
Confidence interval			63.7-262.4		
Model used (EC_{20})			G	H	H & G
R^2 (EC_{20})			0.992	0.994	
Growth - Fresh mass					
NOEC	9537*	9341*	<0.32	1.3*	1.2
p				0.977	0.125
LOEC	>9537	>9341	0.32**	3.7	3.3
p or P(T<=t) two-tail	0.119	0.0002	0.017	0.015	<0.0001
EC_{20}		stimulation	0.29	3.5	4.8
Confidence interval			0.14-0.44	2.3-4.6	3.9-5.8
EC_{50}			0.91	6.5	9.4
Confidence interval			0.4-1.4	5.4-7.5	8.4-10.4
Model used (EC_{20})			E	G	G
R^2 (EC_{20})			0.972	0.982	0.995
Growth - Dry mass					
NOEC	9537*	9341*	<0.32	3.7	3.0
p				0.802	0.184
LOEC	>9537	>9341	0.32**	7.8	5.4
p or P(T<=t) two-tail	0.200	0.393	0.013	<0.0001	<0.0001
EC_{20}			0.65	6.3	5.8
Confidence interval			0.39-0.91	4.9-7.7	3.1-8.5
EC_{50}			2.0	10.3	10.7
Confidence interval			1.2-2.8	9.1-11.5	8.3-13.2
Model used (EC_{20})			E	G	G
R^2 (EC_{20})			0.990	0.989	0.979

G: Gompertz model * Unbounded NOEC *** NOAEC
H: Hormetic model ** Unbounded LOEC **** LOAEC
E: Exponential model

Table 58. Effect of weathered/aged energetic materials on Japanese millet in Sassafras sandy loam soil (ATCLP extraction; n = 3).

Ecotoxicological parameters (n = 4)	RDX (mg kg^{-1})	HMX (mg kg^{-1})	TNB (mg kg^{-1})	2,4-DNT (mg kg^{-1})	2,6-DNT (mg kg^{-1})
Germination - T 5d					
NOEC	9537*	9341*	39.1	16.9	9.5***
p			0.622	0.584	0.581
LOEC	>9537	>9341	125.5	50.0	103.5****
p			<0.0001	<0.0001	<0.0001
EC_{20}			79.7	>16.9	3.2
Confidence interval			0-196		2.8-3.6
EC_{50}			98.0	>16.9	53.1
Confidence interval			20-176		---
Model used (EC_{20})			G	G	H
R^2 (EC_{20})			0.992		0.935
Growth - Fresh mass					
NOEC	9537*	9341*	<1.4	0.3*	0.6*
p				0.977	0.125
LOEC	>9537	>9341	1.4**	1.3	1.5
p or P(T<=t) two-tail	0.119	0.0002	<0.0001	0.015	<0.0001
EC_{20}		stimulation	0.1	1.2	2.8
Confidence interval			0-0.33	0.7-1.6	2.1-3.6
EC_{50}			0.3	2.3	5.8
Confidence interval			0-1.03	1.9-2.7	5.0-6.5
Model used (EC_{20})			E	G	G
R^2 (EC_{20})			0.948	0.982	0.994
Growth - Dry mass					
NOEC	9537*	9341*	<1.4	1.3	1.4
p				0.802	0.184
LOEC	>9537	>9341	1.4**	2.8	3.1
p or P(T<=t) two-tail	0.200	0.393	<0.0001	<0.0001	<0.0001
EC_{20}			0.21	2.2	3.1
Confidence interval			0.1-0.33	1.6-2.8	1.5-4.8
EC_{50}			0.66	4.0	6.4
Confidence interval			0.3-1.0	3.5-4.6	4.8-8.0
Model used (EC_{20})			E	G	G
R^2 (EC_{20})			0.983	0.989	0.980

G: Gompertz model * Unbounded NOEC *** NOAEC
H: Hormetic model ** Unbounded LOEC **** LOAEC
E: Exponential model

Table 59. Effect of freshly amended energetic materials on ryegrass in Sassafras sandy loam soil (acetonitrile extraction; n = 3).

Ecotoxicological parameters (n = 4)	RDX (mg kg^{-1})	HMX (mg kg^{-1})	TNB (mg kg^{-1})	2,4-DNT (mg kg^{-1})	2,6-DNT (mg kg^{-1})
Germination - T 5d					
NOEC or NOAEC	9740*	10411*	39.2***	8.5***	<4.1
p			0.192	0.803	
LOEC or LOAEC	>9740	>10411	124.7****	17.1****	4.1**
p			<0.0001	<0.0001	<0.0001
EC_{20}			28.4	8.1	28.6
Confidence interval			1.4-55.4	6.9-9.2	25.5-31.7
EC_{50}			95.4	16.2	38.4
Confidence interval			76.6-114.1	15.2-17.3	33.0-43.8
Model used (EC_{20})			H	H	H
R^2 (EC_{20})			0.958	0.995	0.992
Growth - Fresh mass					
NOEC or NOAEC	9740*	10411*	39.2***	2.2***	29.7***
p			0.783	0.295	0.294
LOEC or LOAEC	>9740	>10411	124.7****	3.8****	88.5****
p or P(T<=t) two-tail	0.0005	<0.0001	<0.0001	0.013	<0.0001
EC_{20}	stimulation	stimulation	45.4	11.1	18.0
Confidence interval			35.1-55.7	10.2-12.0	4.4-31.7
EC_{50}			75.2	13.4	39.1
Confidence interval			59.2-91.1	12.2-14.6	19.1-59.1
Model used (EC_{20})			H	H	H
R^2 (EC_{20})			0.981	0.991	0.944
Growth - Dry mass					
NOEC or NOAEC	9740*	10411*	39.2***	8.5***	13.9***
p			0.892	0.366	0.085
LOEC or LOAEC	>9740	>10411	124.7****	17.1****	29.7****
p or P(T<=t) two-tail	<0.0001	<0.0001	<0.0001	<0.0001	0.026
EC_{20}	stimulation	stimulation	56.0	10.6	26.4
Confidence interval			42.9-67.3	9.5-11.7	21.0-31.8
EC_{50}			89.1	13.3	38.5
Confidence interval			69.5-108.7	11.6-14.5	30.8-46.2
Model used (EC_{20})			H	H	H
R^2 (EC_{20})			0.980	0.987	0.984

G: Gompertz model * Unbounded NOEC *** NOAEC
H: Hormetic model ** Unbounded LOEC **** LOAEC

Table 60. Effect of freshly amended energetic materials on ryegrass in Sassafras sandy loam soil (ATCLP extraction; n = 3).

Ecotoxicological parameters (n = 4)	RDX (mg kg^{-1})	HMX (mg kg^{-1})	TNB (mg kg^{-1})	2,4-DNT (mg kg^{-1})	2,6-DNT (mg kg^{-1})
Germination - T 5d					
NOEC or NOAEC	9740*	10411*	18.2***	3.6***	<2.5
p			0.192	0.769	
LOEC or LOAEC	>9740	>10411	71.3****	8.8****	2.5**
p			<0.0001	<0.0001	<0.0001
EC_{20}			31.6	3.1	21.3
Confidence interval			23.3-39.9	2.6-4.2	19.0-23.5
EC_{50}			49.1	8.2	27.7
Confidence interval			38.1-60.1	7.6-8.9	23.2-32.1
Model used (EC_{20})			H	H	H
R^2 (EC_{20})			0.985	0.995	0.991
Growth - Fresh mass					
NOEC or NOAEC	9740*	10411*	18.2***	0.3*	22.0***
p			0.783	0.151	0.294
LOEC or LOAEC	>9740	>10411	71.3****	0.8	66.0****
p or P(T<=t) two-tail	0.0005	<0.0001	<0.0001	0.022	<0.0001
EC_{20}	stimulation	stimulation	19.8	5.0	19.6
Confidence interval			9.1-30.8	4.5-5.5	11.3-28.0
EC_{50}			46.0	6.3	28.6
Confidence interval			30.4-61.5	5.6-7.1	8.1-49.0
Model used (EC_{20})			H	H	H
R^2 (EC_{20})			0.970	0.991	0.955
Growth - Dry mass					
NOEC or NOAEC	9740*	10411*	18.2***	3.6***	7.6***
p			0.892	0.873	0.085
LOEC or LOAEC	>9740	>10411	71.3****	8.8****	22.0****
p or P(T<=t) two-tail	<0.0001	<0.0001	<0.0001	<0.0001	0.026
EC_{20}	stimulation	stimulation	26.9	4.7	19.7
Confidence interval			19.0-34.8	4.1-5.3	15.2-24.2
EC_{50}			48.8	6.1	28.2
Confidence interval			35.6-62.0	5.3-7.0	22.4-34.0
Model used (EC_{20})			H	H	H
R^2 (EC_{20})			0.979	0.987	0.983

G: Gompertz model * Unbounded NOEC *** NOAEC
H: Hormetic model ** Unbounded LOEC **** LOAEC

Table 61. Effect of weathered/aged energetic materials on ryegrass in Sassafras sandy loam soil (acetonitrile extraction; n = 3).

Ecotoxicological parameters (n = 4)	RDX (mg kg^{-1})	HMX (mg kg^{-1})	TNB (mg kg^{-1})	2,4-DNT (mg kg^{-1})	2,6-DNT (mg kg^{-1})
Germination - T 5d					
NOEC	9537*	9341*	80.7	3.7	19.7
p			0.172	0.586	0.547
LOEC	>9537	>9341	197.1	7.8	37.2
p			<0.0001	0.014	0.006
EC_{20}			107.2	>7.8	41.6
Confidence interval			81.1-133.4		38.0-45.3
EC_{50}			149.6	>7.8	54.2
Confidence interval			131-168		51.9-56.4
Model used (EC_{20})			G	H & G	G
R^2 (EC_{20})			0.992		0.995
Growth - Fresh mass					
NOEC	9537*	9341*	<0.3	3.7	7.5*
p			0.034	0.498	0.631
LOEC	>9537	>9341	0.3**	7.8	19.7
p or P(T<=t) two-tail	0.201	0.0002	<0.0001	<0.0001	0.022
EC_{20}		stimulation	45.5	5.2	23.8
Confidence interval			12.6-78.4	3.6-6.8	20.5-27.0
EC_{50}			82.8	6.7	38.7
Confidence interval			61.1-104.4	5.9-7.5	36.0-41.3
Model used (EC_{20})			G	G	G
R^2 (EC_{20})			0.969	0.992	0.994
Growth - Dry mass					
NOEC or NOAEC	9537*	9341*	2.08	3.7***	7.5*
p			0.366	0.421	0.974
LOEC or LOAEC	>9537	>9341	80.7	7.8****	19.7
p or P(T<=t) two-tail	0.541	0.003	<0.0001	<0.0001	<0.0001
EC_{20}		stimulation	50.9	1.9	20.8
Confidence interval			29.6-72.2	0-4.0	18.2-23.3
EC_{50}			86.3	7.6	34.0
Confidence interval			73.6-99.0	---	31.9-36.0
Model used (EC_{20})			G	H	G
R^2 (EC_{20})			0.989	0.990	0.995

G: Gompertz model * Unbounded NOEC *** NOAEC
H: Hormetic model ** Unbounded LOEC **** LOAEC

Table 62. Effect of weathered/aged energetic materials on ryegrass in Sassafras sandy loam soil (ATCLP extraction; n = 3).

Ecotoxicological parameters (n = 4)	RDX (mg kg^{-1})	HMX (mg kg^{-1})	TNB (mg kg^{-1})	2,4-DNT (mg kg^{-1})	2,6-DNT (mg kg^{-1})
Germination - T 5d					
NOEC	9537*	9341*	0.5	1.3	11.7
p			0.148	0.586	0.547
LOEC	>9537	>9341	39.1	2.8	22.7
p			0.05	0.014	0.006
EC_{20}			56.7	2.9	24.8
Confidence interval			38.6-74.8	2.8-2.9	22.2-27.4
EC_{50}			87.6	>2.9	33.9
Confidence interval			73.2-102		32.3-35.5
Model used (EC_{20})			G	G	G
R^2 (EC_{20})			0.992	0.995	0.995
Growth - Fresh mass					
NOEC or NOAEC	9537*	9341*	0.5***	1.3	3.6*
p			0.179	0.498	0.631
LOEC or LOAEC	>9537	>9341	39.1****	2.8	11.7
p or P(T<=t) two-tail	0.201	0.0002	<0.0001	<0.0001	0.022
EC_{20}		stimulation	32.6	1.8	14.0
Confidence interval			20.0-45.2	1.3-2.4	11.9-16.2
EC_{50}			40.1	2.4	23.8
Confidence interval			34.1-46.0	2.1-2.7	22.0-25.5
Model used (EC_{20})			H	G	G
R^2 (EC_{20})			0.971	0.992	0.993
Growth - Dry mass					
NOEC	9537*	9341*	0.5	1.3***	3.6*
p			0.785	0.421	0.974
LOEC	>9537	>9341	39.1	2.8****	11.7
p or P(T<=t) two-tail	0.541	0.003	<0.0001	<0.0001	<0.0001
EC_{20}			21.4	>1.3	12.3
Confidence interval			9.7-33.1		10.7-13.9
EC_{50}			42.7	>1.3	20.7
Confidence interval			34.5-51.0		19.4-22.0
Model used (EC_{20})			G	G & H	G
R^2 (EC_{20})			0.989		0.995

G: Gompertz model * Unbounded NOEC *** NOAEC

H: Hormetic model ****** LOAEC**

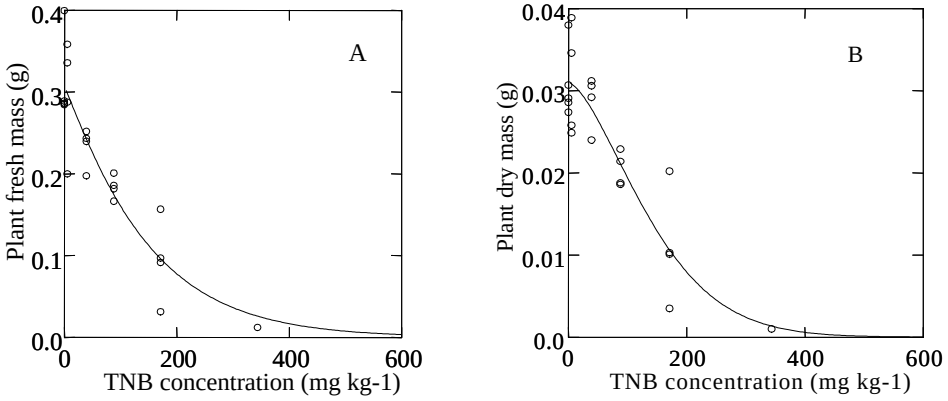

Figure 1. Effect of freshly amended TNB (acetonitrile extraction) on alfalfa shoot growth (fresh [A] and dry [B] mass)

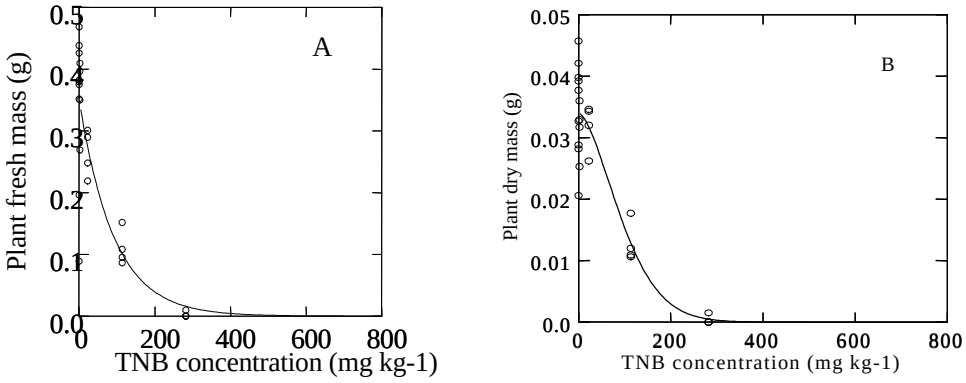

Figure 2. Effect of weathered/aged TNB (acetonitrile extraction) on alfalfa shoot growth (fresh [A] and dry [B] mass)

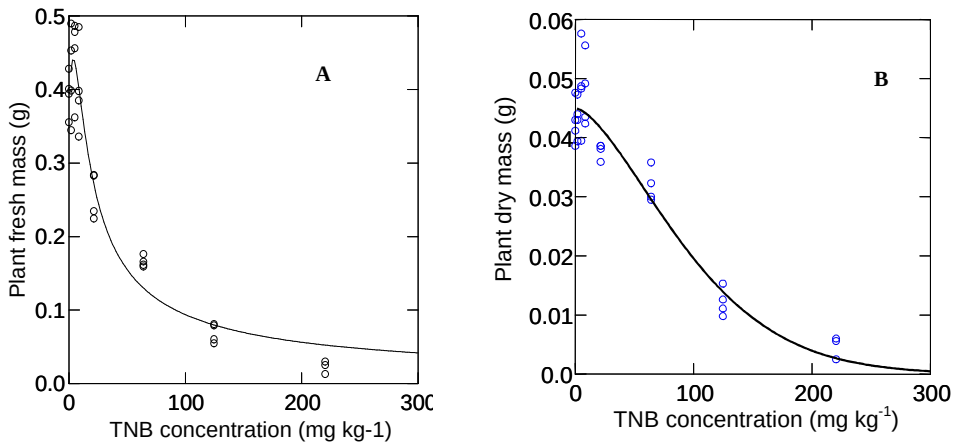

Figure 3. Effect of freshly amended TNB (acetonitrile extraction) on Japanese millet shoot growth (fresh [A] and dry [B] mass)

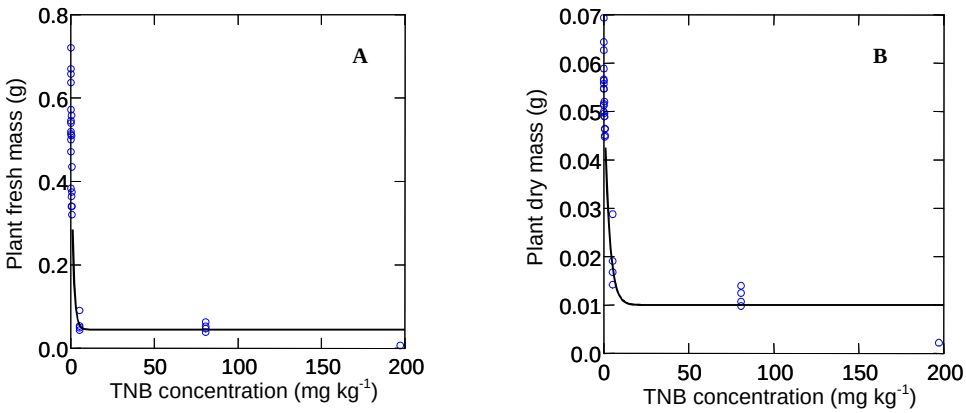

Figure 4. Effect of weathered/aged TNB (acetonitrile extraction) on Japanese millet shoot growth (fresh [A] and dry [B] mass)

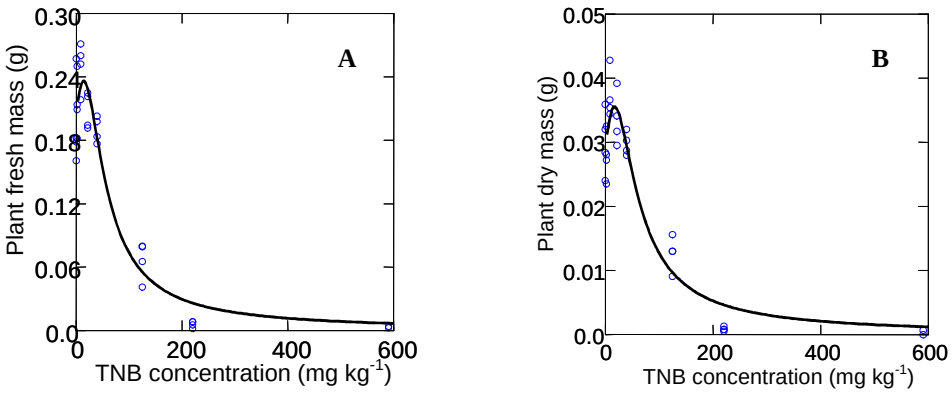

Figure 5. Effect of freshly amended TNB (acetonitrile extraction) on ryegrass shoot growth (fresh [A] and dry [B] mass)

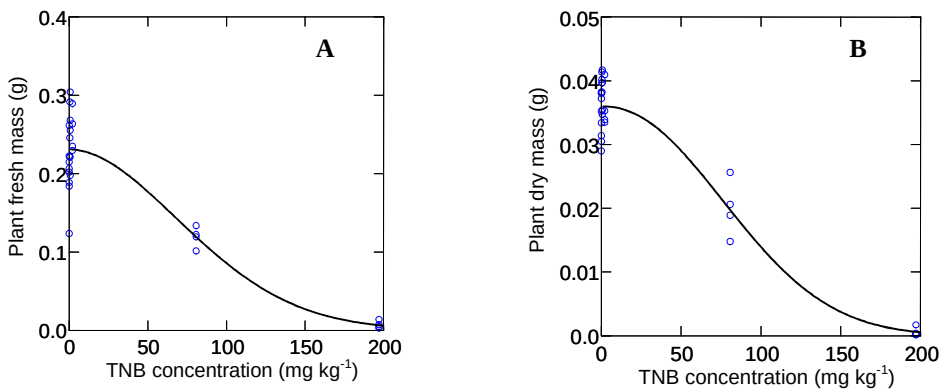

Figure 6. Effect of weathered/aged TNB (acetonitrile extraction) on ryegrass shoot growth (fresh [A] and dry [B] mass)

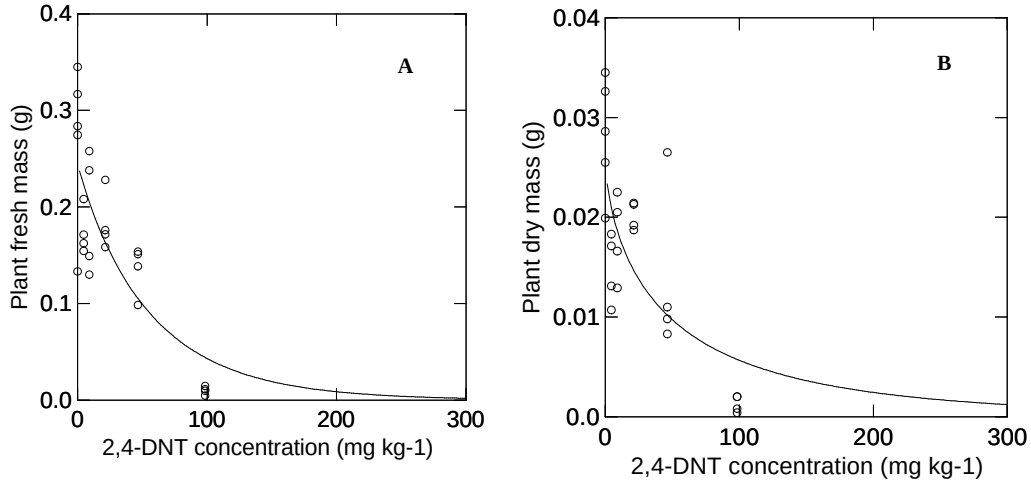

Figure 7. Effect of freshly amended 2,4-DNT (acetonitrile extraction) on alfalfa shoot growth (fresh [A] and dry [B] mass)

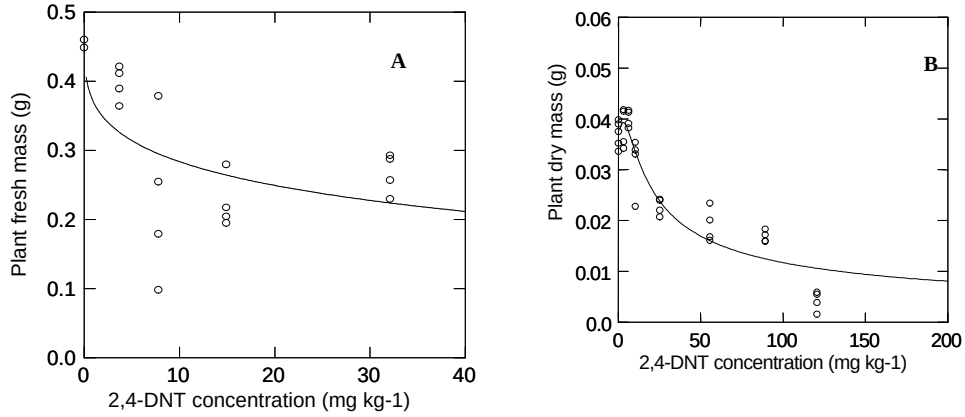

Figure 8. Effect of weathered/aged 2,4-DNT (acetonitrile extraction) on alfalfa shoot growth (fresh [A] and dry [B] mass)

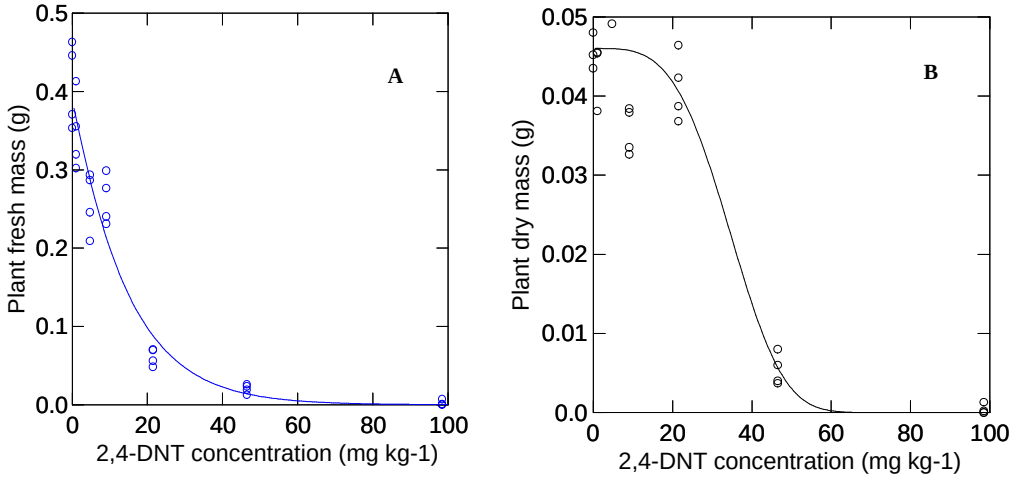

Figure 9. Effect of freshly amended 2,4-DNT (acetonitrile extraction) on Japanese millet shoot growth (fresh [A] and dry [B] mass)

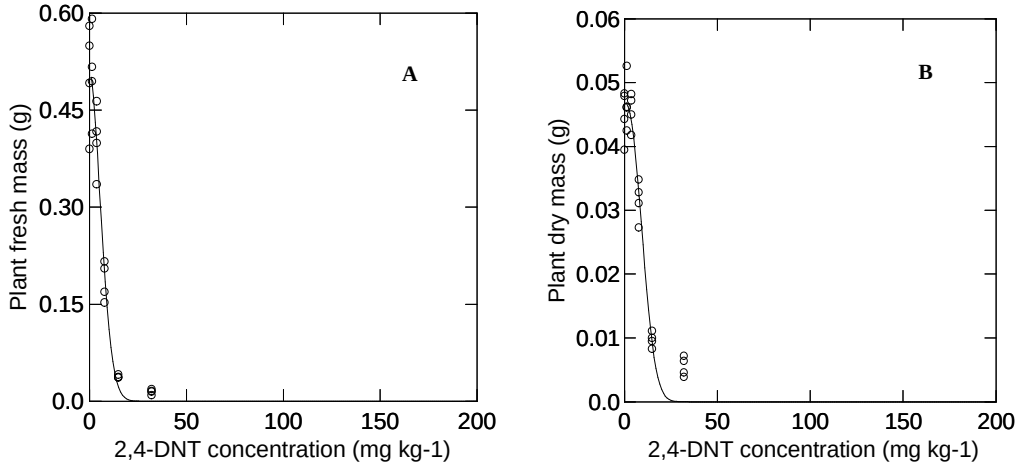

Figure 10. Effect of weathered/aged 2,4-DNT (acetonitrile extraction) on Japanese millet shoot growth (fresh [A] and dry [B] mass)

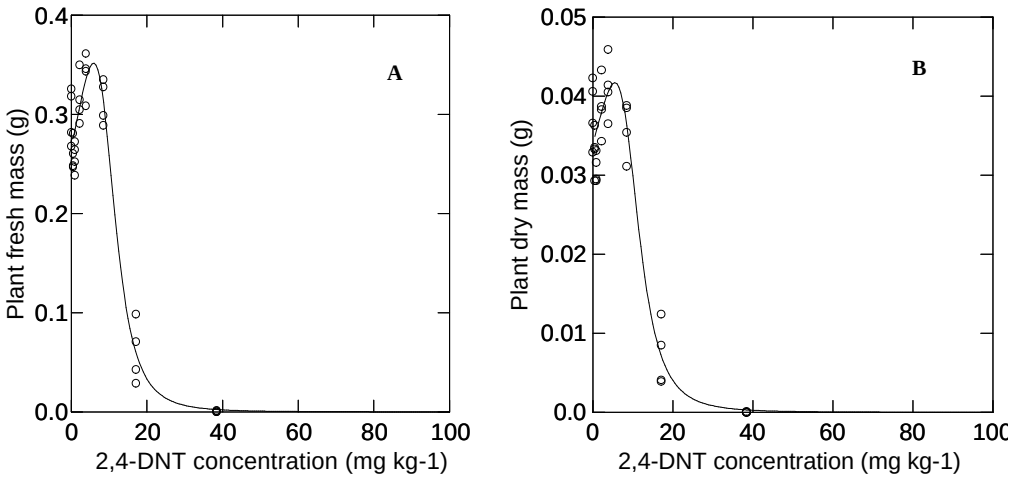

Figure 11. Effect of freshly amended 2,4-DNT (acetonitrile extraction) on ryegrass shoot growth (fresh [A] and dry [B] mass)

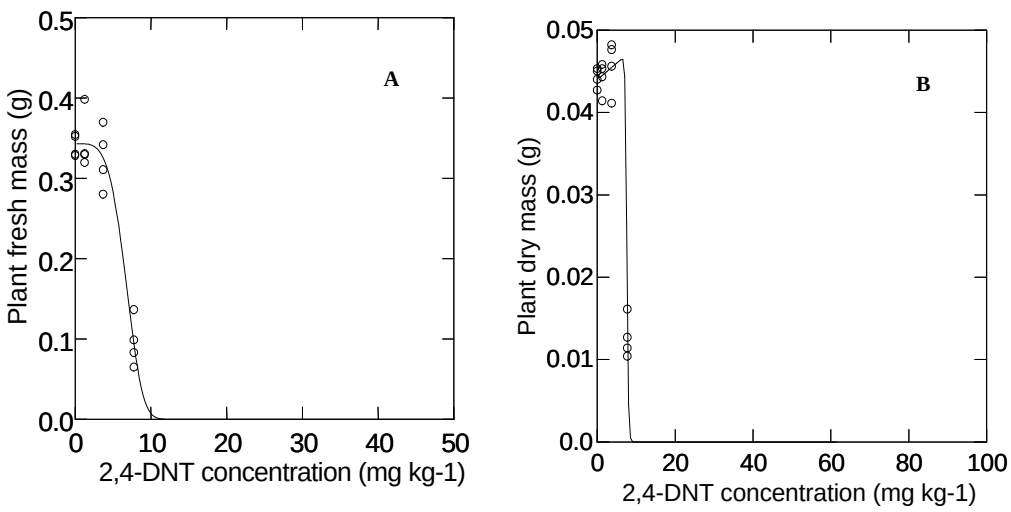

Figure 12. Effect of weathered/aged 2,4-DNT (acetonitrile extraction) on ryegrass shoot growth (fresh [A] and dry [B] mass)

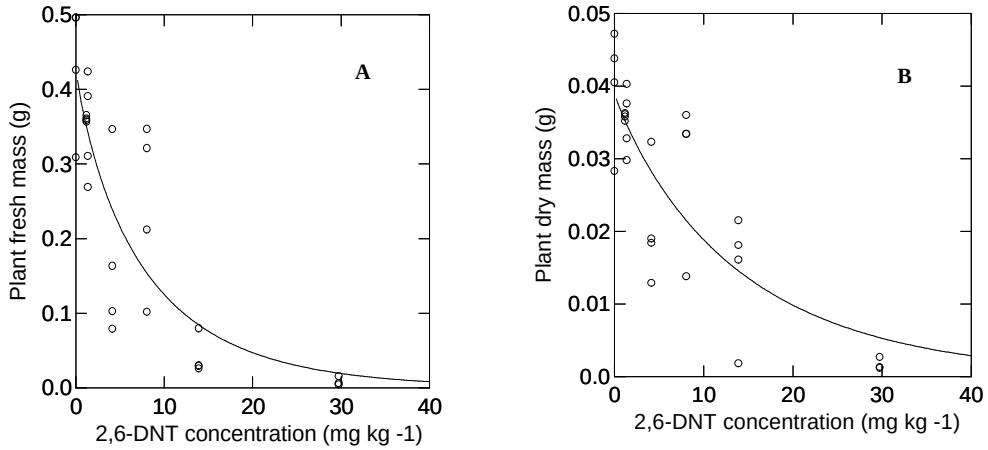

Figure 13. Effect of freshly amended 2,6-DNT (acetonitrile extraction) on alfalfa shoot growth (fresh [A] and dry [B] mass)

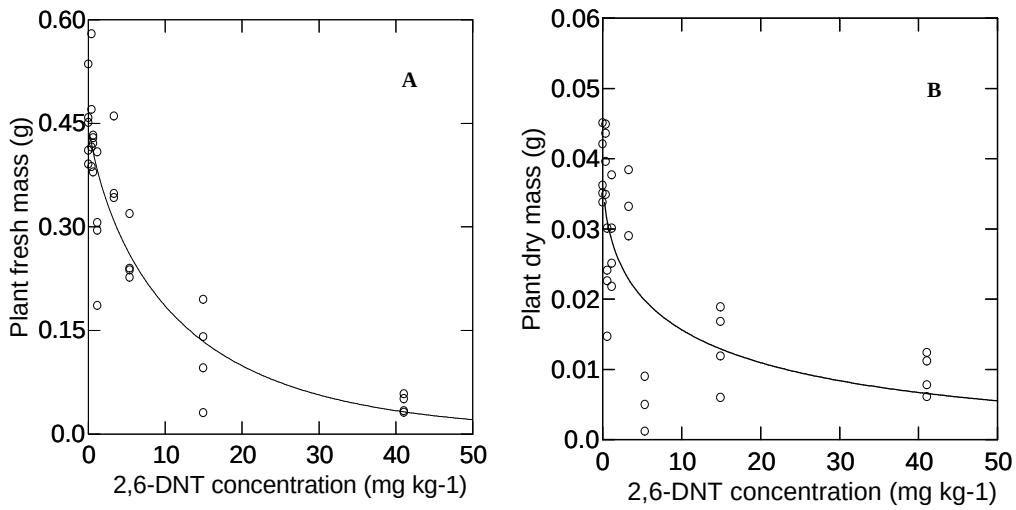

Figure 14. Effect of weathered/aged 2,6-DNT (acetonitrile extraction) on alfalfa shoot growth (fresh [A] and dry [B] mass)

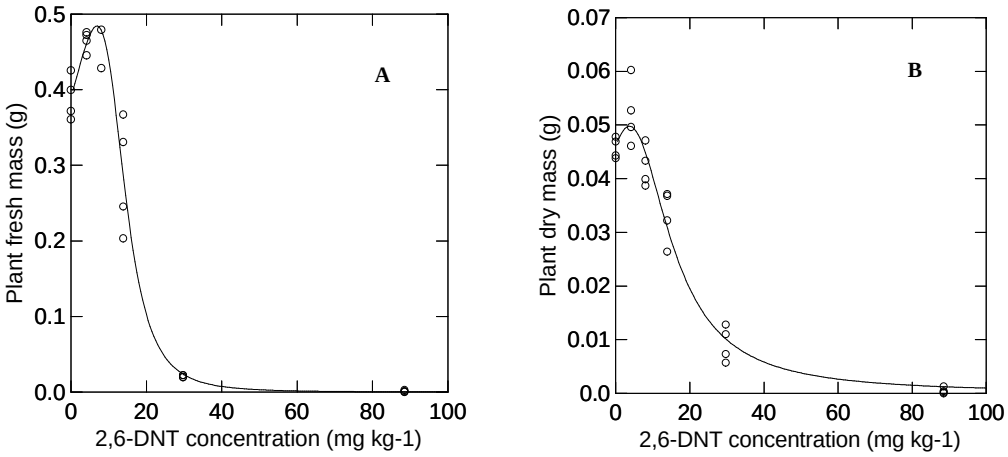

Figure 15. Effect of freshly amended 2,6-DNT (acetonitrile extraction) on Japanese millet shoot growth (fresh [A] and dry [B] mass)

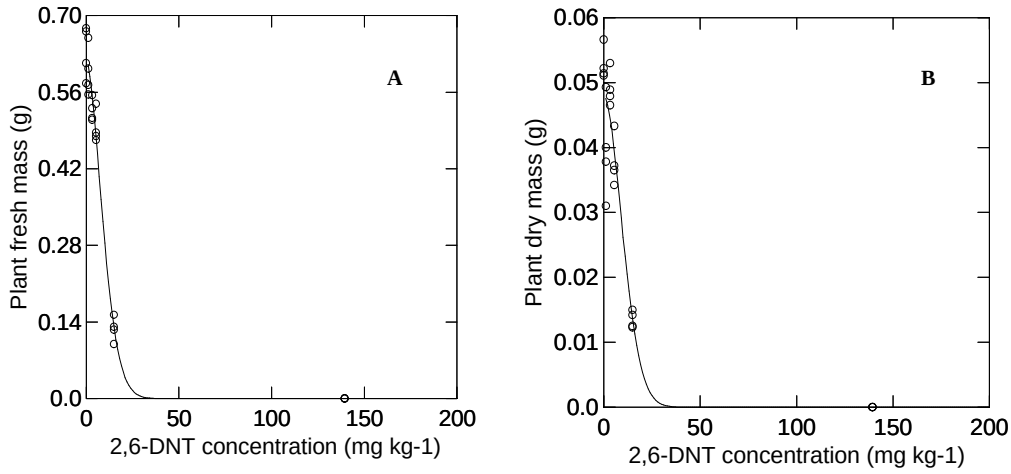

Figure 16. Effect of weathered/aged 2,6-DNT (acetonitrile extraction) on Japanese millet shoot growth (fresh [A] and dry [B] mass)

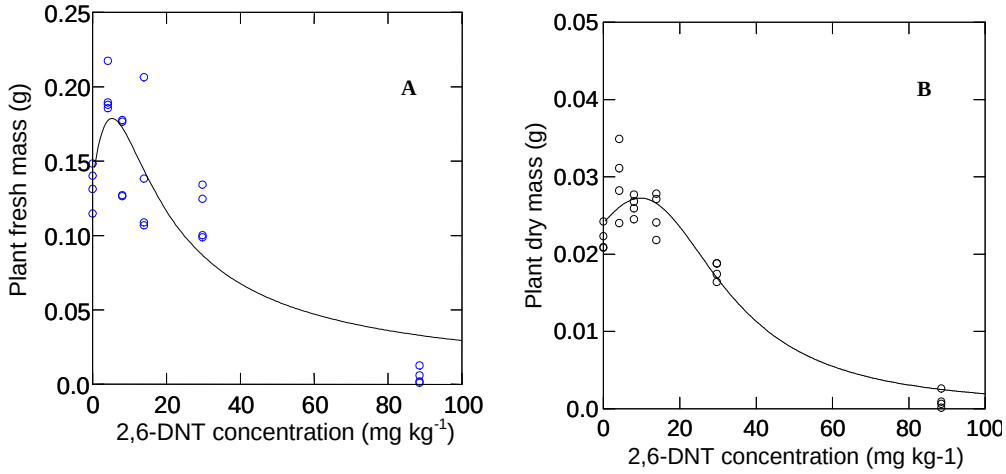

Figure 17. Effect of freshly amended 2,6-DNT (acetonitrile extraction) on ryegrass shoot growth (fresh [A] and dry [B] mass)

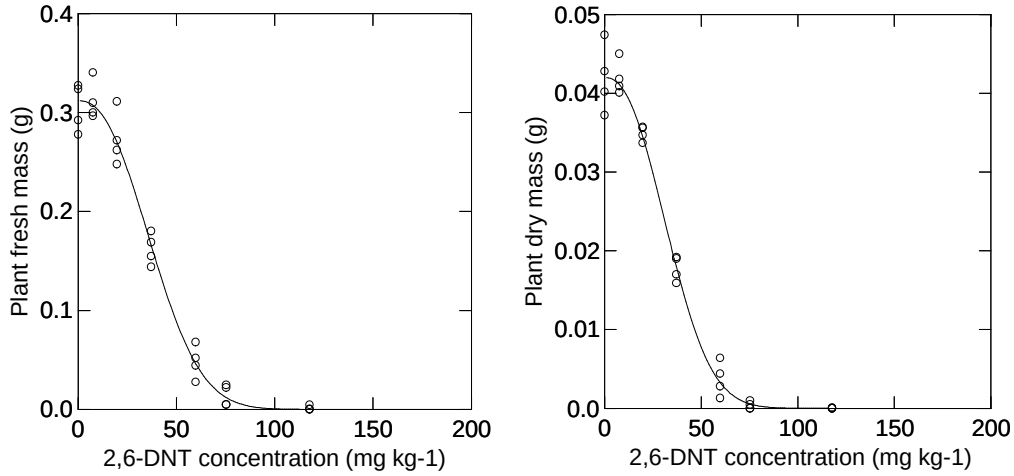

Figure 18. Effect of weathered/aged 2,6-DNT (acetonitrile extraction) on ryegrass shoot growth (fresh [A] and dry [B] mass)

Table 63. Summary of coefficients of determination (R^2) for acetonitrile and ATCLP extractable measures of exposure determined by nonlinear regressions for plant measurement endpoints (EC_{20} levels) in definitive toxicity tests of energetic materials in freshly amended and weathered/aged amended SSL soil.

Compound	**Seedling emergence**		**Shoot fresh mass**		**Shoot dry mass**	
Plant species	Acetonitrile	ATCLP	Acetonitrile	ATCLP	Acetonitrile	ATCLP
Freshly amended TNB						
Alfalfa	0.967	0.899	0.971	0.971	0.972	0.972
Japanese millet	0.988	0.987	0.984	0.983	0.985	0.976
Ryegrass	0.958	0.985	0.981	0.970	0.980	0.979
Weathered/aged TNB						
Alfalfa	0.989	0.989	0.930	0.929	0.966	0.966
Japanese millet	0.992	0.992	0.972	0.948	0.990	0.983
Ryegrass	0.992	0.992	0.969	0.971	0.989	0.989
Freshly amended 2,4-DNT						
Alfalfa	ND	0.975	0.923	0.923	0.902	0.901
Japanese millet	0.994	0.994	0.975	0.977	0.978	0.978
Ryegrass	0.995	0.995	0.991	0.991	0.987	0.987
Weathered/aged 2,4-DNT						
Alfalfa	0.989	0.981	0.976	0.977	0.979	0.980
Japanese millet	0.994	ND	0.982	0.982	0.989	0.989
Ryegrass	ND	0.995	0.992	0.992	0.990	ND
Freshly amended 2,6-DNT						
Alfalfa	0.956	0.953	0.919	0.922	0.935	0.939
Japanese millet	0.992	0.992	0.991	0.991	0.989	0.990
Ryegrass	0.992	0.991	0.944	0.955	0.984	0.983
Weathered/aged 2,6-DNT						
Alfalfa	0.971	0.972	0.962	0.966	0.911	0.929
Japanese millet	ND	0.935	0.995	0.994	0.979	0.980
Ryegrass	0.995	0.995	0.994	0.993	0.995	0.995

ND: not determined

Table 64. Effect of weathering/aging (W/A) of amended soil on toxicity of nitroaromatic energetic materials for alfalfa using acetonitrile extraction.

	Fresh TNB	W/A TNB	Fresh 2,4-DNT	W/A 2,4-DNT	Fresh 2,6-DNT	W/A 2,6-DNT
Growth - Fresh mass						
EC_{20}	38	20	11	7	1.3	2
Confidence interval	10-66	0-49	0-24	2-11	0-3	0.1-3
Significant difference	no		no		no	
EC_{50}	107	63	38	30	5	7
Confidence interval	72-141	19-107	17-58	20-40	2-8	4-11
Significant difference	no		no		no	
Growth - Dry mass						
EC_{20}	62	46	34	15	3	0.4
Confidence interval	28-96	2-89	10-59	9-21	0-6	0-1
Significant difference	no		no		no	
EC_{50}	129	92	56	42	10	5
Confidence interval	97-161	59-125	33-79	29-56	4-15	0-11
Significant difference	no		no		no	

Table 65. Effect of weathering/aging (W/A) of amended soil on toxicity of nitroaromatic energetic materials for alfalfa using ATCLP extraction.

	Fresh TNB	W/A TNB	Fresh 2,4-DNT	W/A 2,4-DNT	Fresh 2,6-DNT	W/A 2,6-DNT
Growth - Fresh mass						
EC_{20}	18	7	10	2	0.7	0.7
Confidence interval	1-35	0-19	0-22	0.5-4.3	0-1.6	0-1.3
Significant difference	no		no		no	
EC_{50}	68	29	27	14	2.8	4
Confidence interval	41-96	1-57	12-43	9-19	1-4	2-6
Significant difference	no		no		no	
Growth - Dry mass						
EC_{20}	34	22	19	6	0	0.1
Confidence interval	10-58	0-49	3-35	3-9	0-3	0-0.2
Significant difference	no		no		no	
EC_{50}	86	51	34	20	5	2
Confidence interval	59-114	27-75	17-52	13-28	2-8	0-5
Significant difference	no		no		no	

Table 66. Effect of weathering/aging (W/A) of amended soil on toxicity of nitroaromatic energetic materials for Japanese millet using acetonitrile extraction.

	Fresh TNB	W/A TNB	Fresh 2,4-DNT	W/A 2,4-DNT	Fresh 2,6-DNT	W/A 2,6-DNT
Growth - Fresh mass						
EC_{20}	16	0.3	4	4	13	5
Confidence interval	12-21	0.1-0.5	1.6-5.4	2.3-4.6	12-14	4-6
Significant difference	**yes**		no		**yes**	
EC_{50}	36	0.9	10	7	16	9
Confidence interval	27-45	0.4-1.4	7.6-13.1	5.4-7.5	15-18	8-10
Significant difference	**yes**		**yes**		**yes**	
Growth - Dry mass						
EC_{20}	43	0.7	25	6	11	6
Confidence interval	27-59	0.4-0.9	18-33	5-8	9.4-13.4	3.1-8.5
Significant difference	**yes**		**yes**		**yes**	
EC_{50}	89	2	34	10	18	11
Confidence interval	73-104	1-3	28-40	9-12	16-20	8-13
Significant difference	**yes**		**yes**		**yes**	

yes: weathering/aging process significantly increased toxicity.

Table 67. Effect of weathering/aging (W/A) of amended soil on toxicity of nitroaromatic energetic materials for Japanese millet using ATCLP extraction.

	Fresh TNB	W/A TNB	Fresh 2,4-DNT	W/A 2,4-DNT	Fresh 2,6-DNT	W/A 2,6-DNT
Growth - Fresh mass						
EC_{20}	3	0.1	1.3	1.2	7	3
Confidence interval	2-5	0-0.33	0.5-2.1	0.7-1.6	7-8	2-4
Significant difference	no		no		**yes**	
EC_{50}	11	0.3	5	2.3	9	6
Confidence interval	7-15	0-1	3-6	2-3	8-10	5-7
Significant difference	**yes**		**yes**		**yes**	
Growth - Dry mass						
EC_{20}	10	0.2	14	2	6	3
Confidence interval	4-16	0.1-0.3	10-19	1-3	5.1-7.4	1.5-4.8
Significant difference	**yes**		**yes**		**yes**	
EC_{50}	49	0.7	20	4	11	7
Confidence interval	41-58	0.3-1	16-23	3.5-4.6	9-12	5-8
Significant difference	**yes**		**yes**		**yes**	

yes: weathering/aging process significantly increased toxicity.

Table 68. Effect of weathering/aging (W/A) of amended soil on toxicity of nitroaromatic energetic materials for ryegrass using acetonitrile extraction.

	Fresh TNB	W/A TNB	Fresh 2,4-DNT	W/A 2,4-DNT	Fresh 2,6-DNT	W/A 2,6-DNT
Growth - Fresh mass						
EC_{20}	45	46	11	5	18	24
Confidence interval	35-56	13-78	10-12	4-7	4-32	21-27
Significant difference	no		**yes**		no	
EC_{50}	75	83	13	7	39	39
Confidence interval	59.2-91.1	61-104	12-15	6-8	19-59	36-41
Significant difference	no		**yes**		no	
Growth - Dry mass						
EC_{20}	56	51	11	2	26	21
Confidence interval	43-67	30-72	10-12	0-4.5	21-32	18-23
Significant difference	no		**yes**		no	
EC_{50}	89	86	13	7.6	39	34
Confidence interval	70-109	74-99	12-15	---	31-46	32-36
Significant difference	no				no	

yes: weathering/aging process significantly increased toxicity.

Table 69. Effect of weathering/aging (W/A) of amended soil on toxicity of nitroaromatic energetic materials for ryegrass using ATCLP extraction.

	Fresh TNB	W/A TNB	Fresh 2,4-DNT	W/A 2,4-DNT	Fresh 2,6-DNT	W/A 2,6-DNT
Growth - Fresh mass						
EC_{20}	20	33	5	2	20	14
Confidence interval	9-31	20-45	4.5-5.5	1.3-2.4	11-28	12-16
Significant difference	no		**yes**		no	
EC_{50}	46	40	6.3	2.4	29	24
Confidence interval	30-62	34-46	5.6-7.1	2.1-2.7	8-49	22-26
Significant difference	no		**yes**		no	
Growth - Dry mass						
EC_{20}	27	21	4.7	>1.3	20	12
Confidence interval	19-35	10-33	4.1-5.3		15-24	11-14
Significant difference	no				**yes**	
EC_{50}	49	43	6.1	>1.3	28	21
Confidence interval	36-62	35-51	5-7		22-34	19-22
Significant difference	no				no	

yes: weathering/aging process significantly increased toxicity.

4. DISCUSSION

Development of ecotoxicological benchmarks for energetic soil contaminants has become a critical need in recent years. These benchmarks are required for derivation of ecological soil screening levels (Eco-SSLs) for use in Ecological Risk Assessment (ERA) of contaminated sites (United States Environmental Protection Agency, 2000). Eco-SSLs represent concentrations of chemicals in soil that, when not exceeded, will be theoretically protective of terrestrial ecosystems within specific soil boundary conditions from unacceptable harmful effects. An extensive review of literature determined that there was insufficient information for energetic material contaminants in soil to generate Eco-SSL values for terrestrial plants (United States Environmental Protection Agency, 2000). The majority of soil toxicity tests that were reported in literature utilized standard artificial soil with high organic matter content (10%). In contrast, our toxicity studies designed to specifically fill this knowledge gap, used a natural soil that meet the criteria for Eco-SSL development, in large part because it has characteristics supporting relatively high bioavailability of EMs. In addition, our weathering/aging procedure applied to soils loaded with range of EM concentrations allowed us to more realistically assess the toxicity under conditions more closely resembling the potential toxic effects of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB to terrestrial plants in the field.

4.1 Determination of energetic materials in soil by chemical analysis.

Derivation of Eco-SSL values prioritizes ecotoxicological benchmarks that are based on measured soil concentration of a chemical over those based on nominal concentrations (USEPA, 2000). In this study, the exposure concentrations of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB in soil were analytically determined in all definitive toxicity tests. Chemical analysis utilized the USEPA Method 8330A based on acetonitrile extraction of EMs from soil. Results from acetonitrile extraction of freshly amended soils showed good correlation between nominal and measured concentrations for the five energetic materials, confirming that the soil amendment procedure used in toxicity tests was appropriate and that the USEPA Method 8330A was efficient for quantifying the amount of energetic materials in soil.

An additional procedure that measures the water extractable portion of each EM in amended soil was performed using the Adapted Toxicity Characteristic Leaching Procedure (ATCLP). This water extractable portion of each EM was perceived to measure the bioavailable fraction of chemicals in soil pore water that is potentially better correlated with toxicity as compared to acetonitrile extracted chemical measure. ATCLP extractable concentrations of 2,4-DNT, 2,6-DNT, and TNB freshly amended in SSL soil increased proportionally with their respective concentrations. In contrast, only 2 and 0.2 % of RDX and HMX concentrations, respectively, were ATCLP extractable in soils freshly amended with 10000 mg kg^{-1} RDX or HMX. These low ATCLP-based recoveries reflected the low water solubility of both compounds, which were reported for RDX as 42 mg L^{-1} at 20°C (Sikka *et al.*, 1980) and as 60 mg L^{-1} at 25°C (Banerjee *et al.*, 1980). The water solubility of HMX was reported between 5 and 6.6 mg L^{-1} at 25°C and 20°C, respectively (Glover and Hoffsommer, 1973; McLellan *et al.*, 1992).

Assessment of the EM toxicities to terrestrial plants for Eco-SSL development included studies with weathered and aged EM amended soils to simulate more closely the exposure effects in the field. Weathering/aging of chemicals in soil may reduce exposure of plants to EMs due to photodecomposition, hydrolysis, reaction with organic matter, sorption, fixation, precipitation, immobilization, occlusion, microbial transformation and other fate processes that commonly occur at contaminated sites. These fate processes can reduce the amount of chemical that is bioavailable, compared to tests conducted with freshly amended soils, or may reveal increased toxicity due to the presence of more toxic transformation products.

Acetonitrile extractable concentrations of TNB, 2,4-DNT and 2,6-DNT were significantly reduced in weathered/aged amended soils. Transformation of TNB was evident in soil amended with low concentrations ranging from 2 to 80 mg kg^{-1}, and was not proportional to the amount of EM in soil at higher concentrations ranging from 120 to 1600 mg kg^{-1}. A transformation product of TNB, 3,5-DNA was detected in weathered/aged amended soil, suggesting that TNB was undergoing microbial and/or photolytic degradation. Two metabolites of 2,4-DNT, including 2-A-4 NT and 4-A-2 NT 2,4-DNT were detected in weathered/aged soil amended with low concentrations of 2,4-DNT, confirming that this EM was also undergoing transformation. Bacteria able to mineralize 2,4-DNT, such as *Pseudomonas sp*. strain, have been isolated from a variety of contaminated soils (Spain, 1995). 2,4-DNT and 2,6-DNT are readily biotransformed by *Pseudomonas sp.* and eventually eliminated as nitrite (Spanggord *et al.*, 1991; Kaplan, 1992; Haidor and Ramos, 1996). In our study, transformation of 2,4-DNT was less pronounced at higher concentrations of 600 and 1200 mg kg^{-1}. 2,6-DNT was transformed in all concentrations tested although no measurable quantities of transformation products were detected in the weathered/aged amended soils. Data analysis of ATCLP/acetonitrile ratios confirmed that the water extractable portions of TNB and DNTs in weathered/aged amended soils were significantly lower compared with freshly amended soils, presumably a result of fate processes in the amended soils undergoing weathering and aging. In contrast to nitroaromatic EMs, there were no appreciable reductions in RDX or HMX acetonitrile extractable concentrations after the 3-month weathering/aging period and their water extractable fractions remained low in weathered/aged soil. Under aerobic conditions, RDX and HMX transformation is limited (Rosenblatt *et al.*, 1991; Hawari and Halasz, 2002). Soil contaminated with RDX and bioaugmented with *Rhodococcus* bacterial strain showed a limited 10% mineralization (Jones *et al.*, 1995). Increasing the concentration of RDX gradually decreased mineralization to undetectable levels at concentrations above 3000 mg kg^{-1}, which is below the 10000 mg kg^{-1} tested in the present study. Overall, chemical analyses demonstrated that EM exposure conditions of terrestrial plants in weathered/aged amended soils differed from those of freshly amended soils. The inclusion of weathering/aging component in the EM toxicity assessments allowed us to incorporate potential alterations in EM bioavailability at contaminated sites in the ecotoxicological benchmarks development for terrestrial plants.

The fate of EMs in soil can modify the exposure concentrations of plant species tested and affect the accuracy of ecotoxicological benchmarks determined from concentration-response relationships based on the initial chemical concentrations. Assessment of the change in chemical concentration during the exposure period is particularly important for organic compounds with high transformation rates and/or sorption ability when weathering/aging of the

EMs is not carried out prior to toxicity testing. Furthermore, it has been demonstrated that TNT, a nitroaromatic compound, has a stronger sorption ability that HMX and RDX, both nitro-heterocyclic compounds (Monteil-Rivera *et al.*, 2003). For that reason, we measured concentrations of all EMs tested at the end of each toxicity assay in addition to analytical determinations at the beginning of the assay. Results showed that concentrations of nitroaromatic EMs were considerably decreased in soil freshly amended with low treatment concentrations and that the decrease in acetonitrile extractability of TNB, 2,4-DNT or 2,6-DNT was inversely related to the initial (T_0) acetonitrile extractable concentrations of these EMs. At concentrations below 100 mg kg^{-1}, decrease in concentrations of TNB, 2,4-DNT or 2,6-DNT ranged from 43 to 100 percent while above that treatment concentration, decrease in concentrations ranged from 0 to 63 percent. There was almost no RDX concentration decrease (4%) in the single 10,000 mg kg^{-1} treatment, and HMX concentration decrease (in the single 10,000 mg kg^{-1} treatment) ranged from 10 to 17 percent of the initial acetonitrile extractable concentration in freshly amended soil to 0 to 4 percent in weathered/aged amended soil in tests with three plant species. Decrease in concentrations of TNB and 2,6-DNT during the toxicity tests in weathered/aged amended soil were similar to decreases in freshly amended soil, while 2,4-DNT concentration decrease in weathered/aged soil was less than that in freshly amended soil.

Decrease in concentrations of freshly amended test compounds during toxicity testing posed the challenge of selecting the appropriate concentrations to use for estimating the concentration-response relationship. We used the initial chemical concentrations in nonlinear regression analyses to estimate EC_{50} and EC_{20} values since it was impossible to determine what level of constantly decreasing exposure concentration could account for toxic response (or a portion of such response). This choice was based on the assumption that for weathered/aged amended soil, the initial concentration was the best representation of the exposure condition of test species and was most appropriate for Eco-SSL derivation. In future investigations, alternative approaches may include measuring concentration over duration of test and expressing “dose” as area under the curve, or using a geometric mean of chemical concentrations determined during the test. An alternative to soil analytical determination approach can be use of organism chemical residue as measure of exposure.

The persistent concentration decrease of nitroaromatic EMs even in weathered/aged amended soil shows clearly the important role terrestrial plants play in the fate of these compounds in soil. Although substantial portions of these EMs were degraded/transformed during the three-month weathering and aging period, presence of plants further accelerated the degradation/transformation of TNB, 2,4-DNT or 2,6-DNT from amended soil during a short period of toxicity testing. Both plant uptake and stimulation of rhizosphere processes could contribute to the decrease of test compounds and additional studies are required to elucidate the specific mechanisms. It has been reported that RDX and HMX are bioaccumulated by some plants (French *et al.*, 2001; Pennington and Brannon, 2002). The present study results showed that capacity of plants to facilitate the degradation of nitroaromatic compounds beyond the microbially and/or abiotically mediated degradation pathways of plant-free soil was concentration dependent. At concentrations below 100 mg kg^{-1}, plants contributed to degradation of up to 70% of TNB, 30% of 2,4-DNT, and 100% of 2,6-DNT while the compound concentration decrease was not as important at higher soil concentrations. This clearly shows the

importance of developing Eco-SSL values that are protective of the terrestrial plant communities potentially capable of contributing to degradation, detoxification, and ultimately, to the remediation of energetic nitroaromatic contaminated sites.

Coefficients of determinations (R^2) for acetonitrile and ATCLP based extractions determined in nonlinear regression analyses of the plant germination and growth data from studies with fresh and weathered/aged amended soils were compared to determine which chemical measure of exposure better correlated with toxicity. These comparisons of coefficients of determinations showed that neither extraction method had an advantage for characterizing bioavailability of EMs to the three terrestrial plant species tested in this study. This was true for both freshly amended and weathered/aged amended soils. This result supports our decision of developing Eco-SSLs for explosives contaminants in soil on the basis of acetonitrile extraction of test compounds. The acetonitrile extraction-based Eco-SSL values will be especially useful for Ecological Risk Assessment at contaminated sites because EM concentrations determined during site characterization are usually based on acetonitrile extraction by the USEPA Method 8330A.

4.2 Plant toxicity tests in Sassafras sandy loam soil

We assessed the toxicity of two explosives RDX and HMX, and three TNT by-products 2,4-DNT, 2,6-DNT and TNB to alfalfa, corn, lettuce, Japanese millet and perennial ryegrass in a natural soil, Sassafras sandy loam. SSL soil has low organic matter and clay contents, low cation exchange capacity, and high sand content. Such characteristics support relatively high bioavailability of energetic contaminants in soil, preferred for Eco-SSLs development. Preliminary range-finding tests identified the three plant species most sensitive to energetic materials tested and with performance parameters in SSL soil required by the validity criteria of standardized toxicity tests. These species included a dicotyledonous symbiotic species alfalfa, and two monocotyledonous species Japanese millet and ryegrass.

Nitro-heterocyclic explosives RDX or HMX did not adversely affect alfalfa, Japanese millet or ryegrass seedling emergence or growth at concentrations of 9740 and 10411 mg kg^{-1}, respectively, in the definitive limit tests with either freshly amended or weathered/aged amended SSL soil. Significant growth stimulation was observed in studies with Japanese millet and ryegrass exposed to these concentrations of RDX or HMX. Relatively low exposure concentrations of these EMs in pore water of amended soil, resulting from their low solubility levels in water, could contribute to these results. The solubility levels in water at 20°C of RDX and HMX are 42 and 6.6 mg L^{-1}, respectively (Sikka *et al.*, 1980; McLellan *et al.*, 1992).

Dinitrotoluenes (DNTs) and trinitrobenzene (TNB) adversely affected alfalfa, Japanese millet and ryegrass in the definitive toxicity tests at concentration ranges selected from the range-finding tests. Plant growth was a more sensitive endpoint compared with seedling emergence in both freshly amended and weathered/aged amended soils. Sunahara *et al.* (2001) suggested that lower sensitivity of seedling emergence based toxicity endpoint could be related to the use of energy reserves by cotyledons plants for germination. Fresh shoot mass was a more sensitive measurement endpoint compared with dry shoot mass, as evidenced by lower EC_{20} and

EC_{50} values for TNB, 2,4-DNT and 2,6-DNT in most tests. These results support strongly the USEPA decision of giving a higher priority to ecotoxicological benchmarks based on growth over other assessment endpoints (e.g. seedling emergence, root elongation) for developing Eco-SSLs for terrestrial plants (United States Environmental Protection Agency, 2000).

Definitive toxicity tests with both freshly amended and weathered/aged amended soils showed that EM toxicity order based on EC_{20} values for plant growth (fresh or dry shoot mass) in tests with alfalfa was 2,6-DNT > 2,4-DNT > TNB. Toxicity order for these endpoints in tests with ryegrass was 2,4-DNT > 2,6-DNT > TNB. Toxicity order varied for Japanese millet depended on exposure type and measurement endpoint used. In freshly amended soil, toxicity order was 2,6-DNT > 2,4-DNT > TNB, based on dry mass, and 2,4-DNT > 2,6-DNT > TNB, based on fresh mass. In weathered/aged amended soils, toxicity order based on fresh or dry mass was TNB > 2,4-DNT ≥ 2,6-DNT. These results show that toxicity of these nitroaromatic energetic materials varied among the three test species and that the USEPA requirement of using multiple species for Eco-SSLs development (United States Environmental Protection Agency, 2000) is well justified.

Because this study was designed to produce benchmark data for development of Eco-SSLs for explosives contaminants in soil, the results of this study may not directly compare to those of other studies in the literature, since none of them were designed to specifically quantify EM toxicity to terrestrial plants under Eco-SSL conditions of testing. Studies on soil-based phytotoxicity of explosives to higher plants are scant (Sunahara *et al.*, 2001). Simini *et al.* (1995) reported statistically significant reductions in cucumber and radish height and survival in soils with mixture of energetic contaminants containing up to 3574 mg kg^{-1} RDX, 3000 mg kg^{-1} HMX, 2,655 mg kg^{-1} TNT, and up to 180 mg kg^{-1} of byproducts of TNT manufacturing and/or degradation. However, these results cannot be directly compared with our studies due to compounding effects of contaminant mixtures in these studies. Robidoux *et al.* (2003) estimated IC_{20} values of 204 and 3113 mg kg^{-1} TNT for lettuce seedling emergence in forest soil and artificial soil (silica), respectively. Exposure of barley seeds to TNT in forest soil or silica produced IC_{20} values of 398, 139, 272 and less than 91 mg kg^{-1} TNT for barley seedling emergence, fresh shoot mass, dry shoot mass, and root mass in forest soil, whereas these values were 8133, 8133, 133, 1199 and less than 56 mg kg^{-1} TNT in artificial soil, respectively (Robidoux *et al.*, 2003). Winfield *et al.* (1999) found that exposure to RDX (up to 4000 mg kg^{-1} soil) during early life stage resulted in adverse responses in sensitive terrestrial plants such as sunflower and sanfroin. Bean, wheat, and blando brome plants were grown in soil amended with 10 mg kg^{-1} RDX (Cataldo *et al.*, 1989), and bush bean (*Phaseolus vulgaris*) was also hydroponically exposed to 10 mg L^{-1} RDX for 1 or 7 d (Harvey *et al.*, 1991), but effects on growth were not reported. Although a screening benchmark of 100 mg kg^{-1} RDX soil was determined by Talmage *et al.* (1999), confidence in the benchmark is low because the available data were insufficient. Results of our studies showing no adverse effects of RDX or HMX at 10,000 mg kg^{-1} on the terrestrial plants tested are in disagreement with these reported results.

Hormesis, a stimulatory effect caused by low levels of potentially toxic chemicals followed by inhibitory effects at higher concentrations (Stebbing, 1982; Calabrese *et al.*, 1987), was observed in all plant species exposed to TNB, 2,4-DNT and 2,6-DNT. Hormesis has been

reported in plants exposed to heavy metals and aromatic hydrocarbons (Stebbing, 1982; Calabrese *et al.*, 1987). Hormetic responses were reported in EM exposure studies for microbial nitrogen fixation activity at TNT concentrations in soil of 200 and 400 mg kg^{-1} (Gong *et al.*, 1999). Hormetic responses have also been shown in aquatic investigations, including offspring production by *Daphnia magna* exposed to 0.08 mg L^{-1} TNT (Bailey *et al.*, 1985), egg production per female fathead minnow exposed to 6.3 mg L^{-1} RDX (Bentley *et al.*, 1977), and density of *Selanastrum capricornutum* cells, based on total chlorophyll measures following HMX exposure ranging 36-572 mg L^{-1} (Bentley *et al.*, 1984). To date, no studies investigated the mechanisms responsible for stimulating effects of these explosives at specific concentrations. Stevens *et al.* (2002) suggested that these mechanisms could include the direct effect on test organisms through the release of metabolic products of explosives that may have a specific effect on growth and reproduction, and indirect effects through increased supply of nitrogen from mineralization of explosives.

Weathering/aging of EM amended soils did not reduce the toxicity for terrestrial plant species tested. In fact, weathering/aging of 2,4-DNT, 2,6-DNT, or TNB amended soils significantly increased toxicity for Japanese millet, which was the most sensitive species among the plant species tested. Weathering/aging of amended soils also significantly increased the toxicity of 2,4-DNT for ryegrass. Specific mechanisms of changes in the toxicity of EMs in weathered/aged amended soil are unknown. Transformation products produced during the weathering and aging process may be more toxic to soil organisms compared with the parent material, and can contribute to the increased toxicity in weathered/aged amended soil. Dodard *et al.* (1999) investigated the toxic effects of 2,4-DNT and 2,6-DNT, and their respective metabolites using the 15-min Microtox (*Vibrio fischeri*; marine bacteria) and 96-h freshwater green alga (*S. capricornutum*) growth inhibition tests. The toxicities of DNTs were species-dependent: 2,4-DNT was more toxic than 2,6-DNT to *S. capricornutum*, while the reverse was true in the test with *Vibrio fischeri*. The authors reported that the reduced metabolites of 2,6-DNT tested were less toxic compared to the toxicity of parent compound. However, certain partially reduced metabolites of 2,4-DNT (4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene) were more toxic than the parent compound. Although these results cannot be directly compared to our study because the biotic reductive degradation pathway for 2,4-DNT and 2,6-DNT in aquatic environment contrasts with metabolic processes in the aerobic conditions of vadose zone simulated in our investigations, the reducing environment can exist in intermittently water-logged soil microsites, where more toxic metabolites of dinitrotoluenes transformation can be present. The higher toxicity of these metabolites may in part explain the increased toxicity of nitroaromatic energetic materials in weathered/aged amended SSL soil observed in our study. Overall results of our study showed that special consideration given to the effects of weathering and aging of energetic contaminants in soil for assessing phytotoxicity was well justified. Benchmark values generated in these investigations will contribute to development of Eco-SSLs that better represent the exposure conditions of terrestrial plants at contaminated sites. Table 70 summaries the EC_{20} values that will be submitted to the Ecological Soil Screening Level (Eco-SSLs) workgroup for quality control review by the Eco-SSL task group before inclusion in the Eco-SSL database, and before being used for developing Eco-SSLs for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB for terrestrial plants..

Table 70. Summary of the plant growth EC_{20} values (mg kg^{-1}) for freshly amended and weathered/aged TNB, 2,4-DNT or 2,6-DNT amended Sassafras sandy loam soil.

EC_{20} for fresh shoot growth (n = 4)	Freshly amended TNB	Fresh amended 2,4-DNT	Fresh amended 2,6-DNT
Alfalfa	38	11	1.3
Japanese millet	16	4	13
Ryegrass	45	11	18
EC_{20} for fresh shoot growth (n = 4)	Weathered/aged TNB	Weathered/aged 2,4-DNT	Weathered/aged 2,6-DNT
Alfalfa	20	7	1.6
Japanese millet	0.3	4	5
Ryegrass	46	5	24
EC_{20} for dry shoot growth (n = 4)	Freshly amended TNB	Fresh amended 2,4-DNT	Fresh amended 2,6-DNT
Alfalfa	62	34	3
Japanese millet	43	25	11
Ryegrass	56	11	26
EC_{20} for dry shoot growth (n = 4)	Weathered/aged TNB	Weathered/aged 2,4-DNT	Weathered/aged 2,6-DNT
Alfalfa	46	15	0.4
Japanese millet	0.7	6	6
Ryegrass	51	1.9	21

5. CONCLUSIONS

This study has produced ecotoxicological data for terrestrial plants alfalfa, Japanese millet and ryegrass for energetic materials RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB. All ecotoxicological parameters were determined using measured chemical concentrations. This complies with USEPA preference for derivation of Eco-SSL values on the basis of measured soil concentration of a chemical over those based on nominal concentrations (USEPA, 2000). Chemical analyses of freshly amended soils using the USEPA Method 8330A showed good correlation between nominal and measured acetonitrile extracted concentrations for the five energetic materials confirming that the soil amendment procedure used in toxicity tests was appropriate and that this method was efficient for quantifying the amounts of energetic materials in soil. The water extractable portion of each EM, which was perceived to measure the immediately bioavailable fraction of chemicals in soil pore water, was determined using the Adapted Toxicity Characteristic Leaching Procedure (ATCLP). Comparisons of the results of nonlinear regression analyses of the toxicity tests data showed that neither extraction method had a statistical advantage for characterizing bioavailability and toxicity of EMs to the three terrestrial plant species tested. This result supports our decision to recommend developing Eco-SSLs for explosives contaminants in soil on the basis of acetonitrile extractable concentrations of test compounds.

A natural soil, Sassafras sandy loam was used in all toxicity tests. Sassafras sandy loam had low organic matter and clay contents, which fulfilled the USEPA requirement of using soil with characteristics that support relatively high contaminant bioavailability for developing conservative Eco-SSL values (USEPA, 2000). Weathering and aging of amended soils were incorporated into experimental design of toxicity testing to produce a soil microenvironment more similar to field conditions. Results of chemical analyses showed that exposure conditions of terrestrial plants to EMs tested in weathered/aged amended soils differed from those of freshly amended soils due to significant transformation of TNB, 2,4-DNT, and 2,6-DNT, and the formation of transformation products, including 3,5-DNA, 2-A-4 NT, and 4-A-2 NT. The inclusion of weathering/aging component in the EM toxicity assessments allowed us to assess the potential alterations in EM bioavailability to terrestrial plants at contaminated sites. In order to provide a more complete information on ecotoxicological effects of energetic contaminants in soil to risk assessors and site managers, additional studies would be required to investigate the toxicity of the EM transformation products individually or using chemical mixtures.

Measurement endpoints assessed in this study included germination measured as the number of emerged seedlings, and growth measured as fresh and dry shoot mass. Study results showed that plant growth was a more sensitive evaluation of effect than germination, therefore it should be used to set screening criteria. This supports the USEPA decision of giving a higher priority for developing Eco-SSLs for terrestrial plants to ecotoxicological benchmarks based on growth over germination endpoint (USEPA, 2000).

Toxicity limit tests with freshly amended and weathered/aged RDX or HMX amended soils showed that these two explosive compounds were not toxic to alfalfa, Japanese millet and ryegrass at concentrations of 10000 mg kg^{-1}. Growth of Japanese millet and ryegrass was significantly stimulated at these high concentrations of RDX or HMX. Dinitrotoluenes and trinitrobenzene adversely affected alfalfa, Japanese millet and ryegrass in the definitive toxicity tests performed with freshly amended and weathered/aged amended soils. Relative toxicity of nitroaromatic EMs tested in this study based on EC_{20} values for plant growth (fresh or dry shoot mass) in tests with alfalfa was (starting with the highest) 2,6-DNT > 2,4-DNT > TNB. Toxicity order for these endpoints in tests with ryegrass was 2,4-DNT > 2,6-DNT > TNB. Toxicity order varied for Japanese millet depending on exposure type and measurement endpoint used. In freshly amended soil, toxicity order was 2,6-DNT > 2,4-DNT > TNB, based on dry mass, and 2,4-DNT > 2,6-DNT > TNB, based on fresh mass. In weathered/aged amended soils, toxicity order based on fresh or dry mass was TNB > 2,4-DNT ≥ 2,6-DNT. These results show that toxicity of nitroaromatic energetics varied among the three test species and that the USEPA requirement of using multiple species for Eco-SSLs development (USEPA, 2000) is well justified.

Results of our study showed that toxicity of TNB, 2,4-DNT, and 2,6-DNT to alfalfa, Japanese millet and ryegrass generally increased in weathered/aged amended soils and that special consideration given to the effects of weathering and aging of energetic contaminants in soil for assessing phytotoxicity was well justified. Benchmark values generated in these investigations will contribute to development of Eco-SSLs that better represent the exposure conditions of terrestrial plants at contaminated sites. All ecotoxicological benchmarks determined in this study will be provided to the Ecological Soil Screening Level (Eco-SSLs) workgroup for quality control review by the Eco-SSL task group before inclusion in the Eco-SSL database, and before being used for developing Eco-SSLs for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB for terrestrial plants.

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APPENDIX B

TOXICITY OF RDX, HMX, TNB, 2,4-DNT, AND 2,6-DNT TO THE EARTHWORM, *Eisenia fetida*

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TOXICITY OF RDX, HMX, TNB, 2,4-DNT, AND 2,6-DNT TO THE EARTHWORM, *EISENIA FETIDA*

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13. ABSTRACT *(Maximum 200 words)*

Many sites associated with military operations contain elevated levels of explosives and related materials in soil. To address this problem, USEPA, in a collaborative effort with other Federal agencies, States, and private industry, is developing Ecological Soil Screening Levels (Eco-SSLs) for ecological risk assessment of energetic materials (EMs) at Superfund sites. Earthworm (*Eisenia fetida*) reproduction tests were conducted in a Sassafras sandy loam soil amended with hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), and 1,3,5-trinitrobenzene (TNB). Tests were conducted in both freshly amended soils and weathered-aged soils to assess differences in toxicity. The order of toxicity in freshly amended soils, based on EC_{20} values for *E. fetida* juvenile production derived from non-linear regression analysis was, HMX >RDX > 2,6-DNT> TNB > 2,4-DNT. The order of toxicity in weathered/aged amended soils was, RDX > 2,6-DNT > TNB > 2,4-DNT > HMX. Correlation of soil concentration with toxicity was not significantly different (P>0.05) when either acetonitrile extractable or water extractable concentrations were used in regression analysis. These results will be submitted to the Eco-SSL Workgroup for review and inclusion in their database.

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PREFACE

The work described in this report was authorized under Project No. SERDP CU-1221. The work was started in April 2001 and completed in July 2003.Subject Area.

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TOXICITY OF RDX, HMX, TNB, 2,4-DNT, AND 2,6-DNT TO THE EARTHWORM, *EISENIA FETIDA*

1. INTRODUCTION

Many sites associated with military operations that involve munitions manufacturing, disposal, testing, and training contain elevated levels of explosives and related materials in soil. Concentrations of explosives in soil were reported to exceed 87,000 mg kg^{-1} for TNT and 3,000 mg kg^{-1} for RDX and HMX (Simini *et al*., 1995). Although these energetic materials (EM) are persistent and highly mobile in the environment, their effects on soil biota have not been sufficiently investigated. As a result, no screening values, which could be used in the Ecological Risk Assessment (ERA), are available for explosives in soil. Scientifically based ecological soil screening levels (Eco-SSLs) are needed to identify contaminant explosive levels in soil that present an unacceptable ecological risk. To address this problem, the U.S. Environmental Protection Agency (USEPA) in conjunction with stakeholders is developing Eco-SSL benchmarks for contaminants most frequently found at Superfund sites. Eco-SSLs are defined as concentrations of chemicals in soil that, when not exceeded, will be protective of terrestrial ecosystems from unacceptable harmful effects. These benchmarks can be used in a screening level ERA to identify those contaminants in soil that warrant additional evaluation in a baseline ERA, and to eliminate those that do not. Eco-SSLs are derived using published data generated from laboratory toxicity tests with different test species relevant to soil ecosystems. The Eco-SSL workgroup, after an extensive literature review (USEPA, 2000), determined that there was insufficient information for explosives to generate Eco-SSL benchmarks for soil invertebrates. Our study was designed to fill this knowledge gap.

This study was designed to produce benchmark data for the development of an Eco-SSL for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), and 1,3,5-trinitrobenzene (TNB) for soil invertebrates, and meet specific criteria (USEPA, 2000), including: (1) tests were conducted in soil having physico-chemical characteristics that support relatively high bioavailability of energetics; (2) experimental designs for laboratory studies were documented and appropriate; (3) both nominal and analytically determined concentrations of chemicals of interest were reported; (4) tests included both negative and positive controls; (5) chronic or life cycle tests were used; (6) appropriate chemical dosing procedures were reported; (7) concentration-response relationships were reported; (8) statistical tests used to calculate the benchmark and level of significance were described; and (9) the origin of test species were specified and appropriate.

Several soil invertebrate toxicity tests, for which standardized protocols have been developed (ISO, 2001; ISO, 1999; ISO, 1998), can effectively be used to assess the toxicity and to derive protective benchmark values for energetic materials (Stephenson *et al*., 2002; Løkke and Van Gestel, 1998). We used the Earthworm Reproduction Test in this study. This test was selected on the bases of its ability to measure chemical toxicity to ecologically relevant test species during chronic assays, and its inclusion of at least one reproductive component among the measurement endpoints.

Special consideration in assessing chemical toxicity for Eco-SSL development was given to the effects of weathering and aging of contaminant explosives in soil, as commonly occurs at contaminated sites. Weathering/aging of chemicals in soil may reduce exposure of soil invertebrates to EMs due to photodecomposition, hydrolysis, reaction with organic matter, sorption, precipitation, immobilization, occlusion, microbial transformation and other fate processes. This may result in a dramatic reduction in the amount of chemical that is bioavailable, compared to tests conducted with freshly-amended chemicals or those tested following a short equilibration period (e.g., 24 h). Additionally, degradation products produced during the weathering and aging process may be more toxic to soil organisms than the parent material. We incorporated a weathering and aging procedure to simulate more closely the exposure effects on soil invertebrates in the field.

2. MATERIALS AND METHODS

2.1 Soil Collection and Characterization

The soil used in these studies was Sassafras sandy loam [Fine-loamy, siliceous, mesic Typic Hapludult] (SSL) collected from a grassy field (M-Field) at Aberdeen Proving Ground, MD. Vegetation and the organic horizon were removed and the top six inches of the A-horizon were then collected. Soil was sieved through a $5mm^2$ mesh screen, air-dried for at least 72h and mixed periodically to ensure uniform drying, passed through a 2-mm sieve, then stored at room temperature before use in testing. Soil was then analyzed for physical and chemical characteristics by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD. Results of these analyses are presented in Table 1.

Table 1. Physical and chemical characteristics of Sassafras sandy loam soil analyzed by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD.

Soil Parameter	Sassafras Sandy Loam
Sand %	69
Silt %	13
Clay %	17
Texture	sandy loam
CEC cmol kg^{-1}	5.49
Organic matter %	1.3
pH	5.2

2.2 Test Chemicals.

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX; CAS: 121-82-4; 99%), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX; CAS: 2691-41-0; 99%), 2,4-dinitrotoluene (2,4-DNT; CAS: 121-14-2; 98%), 2,6-dinitrotoluene (2,6-DNT; CAS: 606-20-2; 98%), and 1,3,5-

trinitrobenzene (TNB; CAS: 99-35-4; 99.7%) were obtained from the Defense Research Establishment Valcartier of the Canadian Ministry of National Defense (Val Bélair, QC, Canada). Beryllium sulfate ($BeSO_4 \cdot 4H_2O$; CAS: 7787; 99.99%) was used as the positive control in all tests. Acetone (CAS: 67-64-1; HPLC Grade) was used for preparing EM solutions during soil amendments. Acetonitrile (CAS: 75-05-8; HPLC Grade) was used for extractions for chemical analyses. Methanol (CAS: 67-56-1, Chromatography grade, 99.9%) was used in determinations by HPLC. Certified standards of the EMs (AccuStandard, Inc., New Haven, CT) were used in HPLC determinations. Unless otherwise specified, ASTM type I water (American Society of Testing and Materials, http://www.astm.org) obtained using Milli-RO® 10 Plus followed by Milli-Q® PF Plus systems (Millipore®, Bedford, MA) was used throughout the studies. Glassware was washed with phosphate-free detergent, followed by rinses with tap water, ASTM type II water, analytical reagent grade nitric acid 1% (v/v), then with ASTM type I water.

2.3 Soil Amendment Procedures.

A soil concentrate of EM for both range-finding and definitive -tests was prepared separately in glass volumetric flasks and dissolved in acetone. This was necessary to distribute the EMs to a larger soil surface area than the addition of solid chemical crystals to soil. Carrier controls were treated with acetone only. Soil was spread to a thickness of 2.5 cm. The EM/acetone solution was pipetted evenly across the soil surface, ensuring that the volume of solution added at any one time did not exceed 15% (v m^{-1}) of the dry mass soil. After addition of the EM solution, the volumetric flask was rinsed twice with a known volume of acetone and pipetted onto the soil. If the total volume of solution needed to amend the soil exceeded 15% (v m^{-1}), the solution was added in successive stages, allowing the acetone to evaporate for a minimum of 2 h under a chemical hood. Amended soil was then air-dried overnight (minimum of 18 h) in a dark chemical hood to prevent photolysis of the EM. Each soil treatment sample was transferred into fluorocarbon-coated high-density polyethylene containers and was mixed for 18 hours on a three-dimensional rotary mixer. The final nominal target treatment concentrations for definitive tests with EMs were prepared by mixing initial soil concentrate of either energetic material with clean SSL soil for 18 hours on a three-dimensional rotary mixer. After mixing, soil was hydrated with ASTM type I water to 17.1% of the soil dry weight (95% water holding capacity (WHC); 18% water) for toxicity testing in freshly amended soils, or 60% of the WHC (10.8% soil dry wt.) for the weathering/aging procedure. Hydrated soil prepared for toxicity tests was allowed to equilibrate for 24 hours before adding earthworms.

2.4 Measurement of Soil pH

The pH of the test soils were determined at the beginning and end of each definitive toxicity test using a method adapted from the Soil Survey Laboratory Methods Manual (USDA, 1996The soil slurry was vortexed for 10 seconds every five minutes for 30 minutes. The soil slurry was then vortexed for 10 seconds, one minute before pH measurement.). The pH electrode was rinsed thoroughly with ASTM type I water, blotted dry, standardized with pH 4 and pH 7 buffers, rinsed and blotted. Five grams of ASTM type I water was added to 5 g soil. The pH was measured in the solution above the soil surface while stirring gently until the reading

stabilized. The electrode was rinsed with ASTM type I water and blotted before each measurement.

2.5 Acetonitrile Extraction of Energetics in Soil

EMs were extracted from all control and treated soils, in triplicate, at the beginning and end of each definitive test using freshly amended and weathered/aged soils according to US EPA Method 8330 (USEPA, 1998). Samples for chemical analysis were hydraed for 24-h. Ten mL acetonitrile was then added to approximately 2.0 g soil from each treatment concentration in polypropylene 50 mL centrifuge tubes, sampled in triplicate. Soil dry fraction (dry wt./wet wt.) was determined in triplicate from subsamples of each treatment. Samples were vortexed for 1 min, then sonicated in the dark for 18h at 20°C. Five mL of supernatant was transferred to a glass tube, to which 5 mL of 5 g $CaCl_2$ L^{-1} solution was added. Supernatant was filtered through 0.45 µm PTFE syringe cartridges. Soil extracts were analyzed and quantified by HPLC. In the present report, acetonitrile soil extraction is referred to as total extraction (concentration).

2.6 ATCLP Extraction of Energetics in Soil

Soil samples were extracted using an Adapted Toxicity Characteristic Leaching Procedure (ATCLP) (Haley *et al*., 1993) at the beginning of each definitive test. ATCLP is a modification of the Toxicity Characteristic Leaching Procedure (TCLP) (40 CFR Part 268.41, Hazardous Waste Management, method 1311). The modification involved substitution of acetic acid for CO_2-saturated water, better simulating soil-water conditions due to respiration by soil biota. All extractions were done in triplicate. For each treatment concentration, 4 g of soil were transferred in triplicate into 20-mL vials. Sixteen mL of CO_2 saturated water (pH 3.8 to 4.0) was added and vials were immediately sealed. Soil samples were vortexed 45 sec and were mixed in the dark for 18 h on a rotary (end-over-end) mixer (30 rpm) at room temperature. Settled supernatants were filtered through 0.45 µm PTFE syringe cartridges. An equivalent volume of acetonitrile was added to filtered soil extract prior to HPLC analysis. In the present report, ATCLP soil extraction is referred to as the water-soluble fraction of EM. Nominal and determined (measured) concentrations used in the definitive tests are shown in Tables 2 through 10.

2.7 Chemical analysis

Soil extracts were analyzed by reversed-phase HPLC using a modified EPA Method 8330. The method was modified in two ways. First, the final solvent for the energetic compounds was a mixture of 60 parts water and 40 parts acetonitrile rather than a 50:50 ratio to increase peak resolution. Secondly, the flow rate of the 50:50 methanol:water mobile phase was 1.0 ml/min rather than 1.5 ml/min. A 25cm x 4.6mm x 5 micron particle size C-18 column was used for all determinations since only one energetic compound was analyzed at a time. The instrument used was a Beckman *System Gold*, consisting of a model 126 programmable solvent module, model 168 diode array detector and a model 507 automatic sampler. Calibration curves were generated before each HPLC run by dissolving certified standards (AccuStandard, Inc., New Haven, CT) of RDX and HMX in 60:40 water:acetonitrile in a range of concentrations

appropriate for each run. The method detection limit was 0.05 mg kg^{-1}. Blanks and standards were placed intermittently between unknown samples.

2.8 Weathering/Aging of Soil.

All soil treatment concentrations and negative controls that were not used for the freshly amended toxicity tests were subjected to a simulated weathering/aging procedure. This procedure consisted of alternating wetting/air-drying cycles for 90 days prior to commencement of definitive tests. Weathering/aging of test soils was conducted in Teflon–lined steel trays in a greenhouse. Soil treatments were initially hydrated to 60% (10% of the soil dry wt.) of the WHC, then placed in the greenhouse to dry. All soil treatments were weighed and adjusted to 60% of WHC twice each week, and afterward brought to 95% of WHC for initiation of bioassays. Therefore, the soil moisture used for all toxicity tests was 17.1% of the soil dry weight.

2.9 Toxicity Assessment.

The chronic test used in this study was a 56-day reproduction test (International Standardization Organization (ISO), ISO/11268-2:1998; adapted from Van Gestel et al., 1989). The endpoints of this test are number of juveniles produced, number of cocoons produced, and adult survival. Guidelines for these assays were originally developed for use with artificial soil (USEPA Standard Artificial Soil), however research in our laboratory has shown that these tests could also be successfully conducted using natural soils (Kuperman, et al. 1999).

2.9.1 Principle of the Test

Adult *E. fetida* are exposed to a range of concentrations of the test chemical added to soil. The test consists of two steps: first, a range-finding test (21 days) in which adult survival and cocoon production is assessed using few treatment concentrations (five) and replicates (two); and second, a definitive test (56 days) in which survival, live weight, dry weight, cocoon production, and juvenile production are assessed using a greater number of concentrations and replicates. Adult survival and cocoon production in the range-finding test are used to determine the range of concentrations of test chemical used in the definitive tests. In the definitive tests, adult survivors are counted and removed from the soil after 28 days. After 28 more days, cocoons and juveniles are harvested and counted. Ecotoxicological parameters are derived from regression analysis and analysis of variance. These parameters include the No Observed Effect Concentration (NOEC), the Lowest Observed Effect Concentration (LOEC) and the effective concentration that causes a x percent reduction in adults, i.e. ECx (e.g. EC_{20}, EC_{50}).

2.9.2 Validity of the Test

Validity criteria include the following performance parameters for the negative controls:

1) The mean mortality does not exceed 10% in range-finding and definitive tests;
2) The number of juveniles per five worms is $\geq$ 15.
3) The coefficient of variation for the control reproduction is $\leq$30% at the end of the test.

2.9.3 Earthworm Culture

Earthworms (*E. fetida*) were bred in plastic containers filled with approximately 14 kg of a 1:1 mixture of sphagnum PRO-GRO peat moss (Gulf Island Peat Moss Co., PEI, Canada) and BACCTO® potting soil (Michigan Peat Co., Houston, TX, USA). The pH was adjusted to 6.2 ± 0.1 by adding calcium carbonate (pulverized lime). The culture was kept moist at 21±2°C with continuous light**.** Earthworm colonies were fed biweekly with dehydrated alfalfa pellets (27% fiber, 17% protein, 1.5% fat; OB of PA, York, PA) that were fermented, dried, and ground to a course powder. Cultures were synchronized so that all worms used in each test were approximately the same age. Adult worms, 0.3g to 0.6g, with fully developed clitella were used for testing.

2.9.4 Test Conditions

Earthworms were acclimated for 48h in the test soil. Worms were selected for uniformity and placed on moist filter paper overnight to purge gut contents. Five worms were rinsed twice with ASTM type I water, blotted on paper towels, weighed on an analytical balance, and placed on the soil surface in each of four 400-mL (9 cm diameter), glass canning jars. The worms were selected randomly for placement across treatments. A 2 g bolus of alfalfa food was added to each jar and covered with the soil in the jar. Plastic film was stretched over the top of the containers and secured with the screw-on rings. The metal lids were excluded to allow light exposure. Three small holes were made in the wrap with a push-pin to allow for air exchange. Worms were incubated under a 16 h photoperiod with a mean light intensity of 12.8 µM m^{-2} sec^{-1} (SE = 0.67) and mean temperature of 21.6 ºC (SE = 0.078).

2.9.5 Endpoint Determination

After 28 days, worms were removed from the containers with blunt forceps. The number of surviving earthworms in each beaker were counted and recorded. Plastic wrap and screw rings were placed on the containers as described above. After 28 more days, cocoons and juveniles were harvested and counted. Juveniles were induced to crawl to the soil surface by immersing the sealed containers to a level just below the soil line in a heated water bath at 41ºC to 43C for 20 to25 minutes. Juveniles were removed from the soil surface with a blunt forceps and counted. Soil was then spread and examined under a 2.25x lighted magnifier to recover any additional juveniles. The number of juveniles in each container was counted and recorded. Cocoons were recovered by gently agitating the soil on a 1-mm sieve with water until only the cocoons remained on the surface of the sieve. Cocoons were placed in water in a clear glass dish. Cocoons that floated were counted as hatched, those that sank were counted as unhatched. Cocoons were then examined under the mainifier to confirm whether they were hatched or not. The number of cocoons per container was counted and recorded.

2.10 Data Analysis

Cocoon and juvenile production data were analyzed using nonlinear regression models described in Stephenson *et al*. (2000) and Kuperman *et al*. (2003). Histograms of the

residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to decide whether or not to weight the data, and to select potential models. The exponential model [1] had the best fit for all HMX data and all RDX data except weathered/aged ATCLP, which fit the logistic model (Gompertz) [2],. All data for 2,4-DNT and 2,6-DNT fit the logistic model. Data for the TNB tests fit the exponential model except for the test performed in freshly amended soil and extracted with acetonitrile. This test fit the logistic model. The fit of the lines generated by these models were closest to the data points, the variances were the smallest, and the residuals had the best appearance (i.e., most random scattering). These models were:

[1] Logistic (Gompertz) model: $Y = a \times e^{([\log(1-p)] \times [C/\mathrm{ECp}]b)}$

[2] Exponential model: $Y = a \times e^{(([\log(1-p)] / \mathrm{ECp}) \times C) + b}$

where Y is the number of juveniles produced, a is the control response, e is the base of the natural logarithm, p is the percent inhibition/100 (e.g., 0.5 for EC_{50}), C is the exposure concentration in test soil, ECp is the estimate of effect concentration for a specified percent effect, h is the hormetic effect parameter, and b is the scale parameter. The ECp parameters used in this study included the EM concentration producing a 20% (EC_{20}) or 50% (EC_{50}) reduction in the measurement endpoint. The EC_{20} parameter based on a reproduction endpoint is the preferred parameter for deriving soil invertebrate Eco-SSL benchmarks. The EC_{50}, more commonly used in the past, and survival data were included to enable comparisons of the results produced in this study with results reported by other researchers. The asymptotic standard error (a.s.e.) and 95% confidence intervals (CI) associated with the point estimates were determined.

Analysis of Variance (ANOVA) was used to determine the bounded No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values for adult survival, cocoon production, or juvenile production data. Mean separations were determined using Fisher's Least Significant Difference (LSD) pairwise comparison tests. A significance level of $p < 0.05$ was used to determine NOEC and LOEC values. All analyses were performed using measured EM concentrations. Statistical analyses were performed using SYSTAT 7.0.1 (SPSS, 1997).

3. RESULTS

3.1 Soil Analyses

Measured total (acetonitrile extractable) RDX concentrations in freshly amended soils ranged from 125 to 216 percent of nominal concentrations $\leq$ 6 mg kg^{-1}, and 91.9 to 103 percent of nominal concentrations $\geq$ 9 mg kg^{-1} (Table 2). This difference may have been due to decreased instrument accuracy at concentrations close to the method detection limit (MDL) of 0.05 mg kg^{-1}. Measured RDX water extractable (ATCLP) concentrations in freshly amended soils ranged from 44.6 to 91.4 percent of total concentrations due to low solubility of RDX in water (Table 2). Measured RDX total concentrations in weathered/aged amended soils ranged from 42.7 to 105.8 percent of nominal concentrations (Table 3). Measured RDX ATCLP extractable concentrations in weathered/aged amended soils ranged from 17.7 to 99.2 percent of total measured concentrations (Table 3). Weathering/aging of amended soils reduced total RDX concentrations on average by 20% compared with total concentrations in freshly amended soils (Table 3), whereas ATCLP extractable RDX concentrations were reduced, on average, by 7 percent compared with freshly amended soils.

Table 2. Nominal and average measured (n = 3) RDX concentrations (mg kg^{-1}) in freshly amended Sassafras sandy loam soil used in the toxicity tests with *E. fetida*. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	**Acetonitrile extraction (mg kg^{-1})**	**Standard error**	**Acetonitrile / Nominal (%)**	**ATCLP extraction (mg kg^{-1})**	**Standard error**	**ATCLP/ Acetonitrile (%)**	**Mean pH**	
							start	**end**
0.0	BDL	BDL	BDL	BDL	BDL	BDL	5.34	6.19
1.5	3.2	0.34	216.0	2.07	0.88	64.0	5.41	6.35
3.0	5.3	0.62	175.0	2.34	0.20	44.6	5.37	.
6.0	7.5	0.10	125.1	5.15	0.57	68.7	5.46	.
9.0	8.6	0.45	95.9	7.24	0.71	84.0	5.46	.
18.0	18.2	0.61	100.9	15.59	0.55	85.8	5.35	.
36.0	33.1	1.67	91.9	30.21	1.08	91.4	5.41	7.04
72.0	74.1	8.29	103.0	56.71	1.55	76.5	5.42	.
144.0	148.3	4.91	103.0	93.48	0.90	63.0	5.35	6.77

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}

Table 3. Nominal and average measured (n = 3) RDX concentrations (mg kg^{-1}) in weathered/aged amended Sassafras sandy loam soil used in the toxicity tests with *E. fetida*. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0.0	BDL			BDL			5.32	5.37
6.0	6.4	1.5	105.8	5.78	0.3	91.0	5.31	5.86
9.0	8.4	1.3	93.6	7.72	0.2	91.7	5.30	6.21
18.0	15.7	0.2	87.0	13.55	0.4	86.5	5.28	6.42
36.0	30.0	0.7	83.4	29.99	0.3	99.9	5.27	6.68
72.0	56.6	3.3	78.6	54.10	2.0	95.6	5.20	6.58
144.0	61.5	2.2	42.7	55.13	2.9	89.6	5.12	6.45
300.0	254.3	8.7	84.8	100.06	2.5	39.3	5.00	6.46
600.0	527.0	4.0	87.8	93.23	1.2	17.7	5.00	6.62

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}

Measured mean HMX total concentrations in freshly amended soils ranged from 86.7 to 124.9 percent of nominal concentrations (Table 4). Measured HMX ATCLP concentrations in freshly amended soils ranged from 8.8 to 71.9 percent of total concentrations (Table 4). Lower recovery at higher nominal concentrations is probably due to low solubility (approximately 5 mg L^{-1}) of HMX in water. Measured HMX total concentrations in weathered/aged-amended soils averaged ranged from 26.7 to 93.6) percent of nominal concentrations (Table 5). Measured HMX ATCLP extractable concentrations in weathered/aged amended soils ranged from 3.2-180.8 percent of total measured concentrations (Table 5). Percent recovery was in descending order from low to high nominal concentrations, probably due to the low solubility of HMX. Weathering/aging of amended soils reduced total HMX concentrations on average by 44% compared with total concentrations in freshly amended soils (Table 5), whereas ATCLP extractable HMX concentrations were increased, on average, by 11 percent compared with freshly amended soils. Increase in soluble HMX was greatest in nominal concentrations between 72 and 600 mg kg^{-1}. This increase in soluble HMX may have been the result of biological and/or chemical processes occurring in the soil during the weathering/aging process.

Table 4. Nominal and average measured (n = 3) HMX concentrations (mg kg^{-1}) in freshly amended Sassafras sandy loam soil used in the toxicity tests with *E. fetida*. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0.0	BDL	BDL	BDL	BDL	BDL	BDL	5.47	5.56
1.5	1.3	0.32	88.0	0.46	0.07	34.7	5.68	.
3.0	2.9	0.63	96.4	1.86	0.39	64.2	5.67	5.63
6.0	6.5	0.76	108.3	2.75	0.44	42.4	5.57	.
9.0	11.2	4.98	124.9	5.92	0.51	52.7	5.58	.
18.0	15.6	0.87	86.7	11.22	0.39	71.9	5.54	.
36.0	36.0	2.77	100.1	15.17	0.55	42.1	5.54	5.16
72.0	73.6	8.25	102.2	13.10	0.06	17.8	5.53	.
144.0	141.3	7.54	98.1	12.47	0.30	8.8	5.54	5.27

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}

Table 5. Nominal and average measured (n = 3) HMX concentrations (mg kg^{-1}) in weathered/aged amended Sassafras sandy loam soil used in the toxicity tests with *E. fetida*. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0.0	BDL	BDL	BDL	BDL	BDL	BDL	4.97	5.91
6.0	1.6	1.41	26.7	2.89	0.580	180.8	5.13	6.55
9.0	2.8	0.46	31.4	4.38	0.550	154.8	5.02	6.23
18.0	10.8	0.58	59.8	9.07	0.740	84.2	5.29	5.97
36.0	28.9	1.31	80.2	13.11	0.15	45.4	5.35	6.80
72.0	53.5	1.79	74.3	14.64	0.66	27.4	5.25	6.41
144.0	129.3	10.90	89.8	16.43	0.62	12.7	5.32	6.43
300.0	280.3	8.67	93.4	18.96	0.34	6.8	5.29	6.85
600.0	561.7	15.24	93.6	18.03	0.46	3.2	5.39	6.61

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}

Measured 2,4-DNT total concentrations in freshly amended soils ranged from 48 to 86 percent of nominal concentrations (Table 6). Measured 2,4-DNT ATCLP extractable concentrations ranged from 19 to 84 percent of total concentrations (Table 6). Measured 2,4-DNT total concentrations in weathered/aged-amended soils ranged from 37 to 56 percent of nominal concentrations (Table 7). Measured 2,4-DNT ATCLP extractable concentrations ranged

from 46 to 70 percent of total concentrations (Table 7). Weathering/aging of amended soils reduced total 2,4-DNT concentrations, on average, by 45 percent, and ATCLP extractable 2,4-DNT concentrations by 24 percent compared with respective concentrations in freshly amended soils.

Table 6. Nominal and average measured (n = 3) 2,4-DNT concentrations (mg kg^{-1}) in freshly amended Sassafras sandy loam soil used in the toxicity tests with *E. fetida*. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0	BDL	BDL	BDL	BDL	BDL	BDL	5.51	5.44
2	0.95	0.2	48	0.80	0.001	84.3	5.31	5.33
4	3.0	0.3	74	1.34	0.01	45.2	5.36	5.25
8	6.5	0.4	81	2.40	0.05	37.4	5.31	5.24
12	9.9	0.5	82	4.96	0.01	50.2	5.28	5.47
24	20.3	0.3	85	3.77	0.04	19.1	5.23	6.27
48	40.9	2.6	85	8.13	0.08	20.0	5.23	6.93
64	55.0	0.5	86	33.45	0.22	61.1	5.24	6.55
80	64.7	1.5	81	43.37	0.09	67.4	5.25	7.54

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}

Table 7. Nominal and average measured (n = 3) 2,4-DNT concentrations (mg kg^{-1}) in weathered/aged amended Sassafras sandy loam soil used in the toxicity tests with *E. fetida*. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0	BDL	BDL	BDL	BDL	BDL	BDL	5.41	5.95
8	3.0	0.5	37	1.67	0.03	56	5.39	5.35
12	5.2	0.2	43	2.42	0.06	47	5.34	5.94
24	11.5	0.2	48	5.22	0.02	46	5.40	6.05
48	21.5	0.3	45	11.77	0.12	55	5.35	6.02
64	31.0	0.8	48	15.40	0.15	50	5.35	6.82
80	37.3	0.8	47	20.47	0.37	55	5.31	6.90
160	71.7	2.3	45	46.07	0.37	64	5.37	7.72
320	178.7	8.4	56	125.00	2.00	70	5.38	7.37

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}

Measured 2,6-DNT total concentrations in freshly amended soils ranged from 80 to 267 percent of nominal concentrations (Table 8). Measured 2,6-DNT ATCLP extractable concentrations ranged from 27 to 65 percent of total concentrations (Table 8). Measured 2,6-DNT total concentrations in weathered/aged-amended soils ranged from 15 to 34 percent of nominal concentrations (Table 9). Measured 2,6-DNT ATCLP extractable concentrations ranged from 19 to 62 percent of total concentrations (Table 9). Weathering/aging of amended soils reduced total 2,6-DNT concentrations, on average, by 79% compared with total concentrations in freshly amended soils, whereas ATCLP extractable 2,6-DNT concentrations were reduced, on average, by 57 percent compared with freshly amended soils.

Table 8. Nominal and average measured (n = 3) 2,6-DNT concentrations (mg kg^{-1}) in freshly amended Sassafras sandy loam soil used in the toxicity tests with *E. fetida*. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	**Acetonitrile extraction (mg kg^{-1})**	**Standard error**	**Acetonitrile / Nominal (%)**	**ATCLP extraction (mg kg^{-1})**	**Standard error**	**ATCLP/ Acetonitrile (%)**	**Mean pH**	
							start	**end**
0	BDL	BDL	BDL	BDL	BDL	BDL	5.47	5.42
2	5.3	0.1	267	1.43	0.01	27	5.43	5.31
4	7.7	0.9	191	2.18	0.01	28	5.43	5.23
8	9.4	0.3	117	3.78	0.01	40	5.32	5.22
12	12.9	0.2	108	5.83	0.04	45	5.35	5.45
24	20.0	0.8	83	10.63	0.08	53	5.49	6.34
48	40.2	2.0	84	24.84	0.04	62	5.27	6.83
64	51.1	1.0	80	32.94	0.12	65	5.25	6.67
80	64.0	1.6	80	40.50	0.11	63	5.30	7.16

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}

Table 9. Nominal and average measured (n = 3) 2,6-DNT concentrations (mg kg^{-1}) in weathered/aged amended Sassafras sandy loam soil used in the toxicity tests with *E. fetida*. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0	BDL	BDL	BDL	BDL	BDL	BDL	5.27	5.05
8.0	1.2	0.02	15.0	0.23	0.01	19.0	5.39	4.96
12.0	1.6	0.02	13.0	0.42	0.03	27.0	5.29	5.36
24.0	3.7	0.08	15.0	1.46	0.06	40.0	5.39	5.54
48.0	9.5	0.12	20.0	4.30	0.09	45.0	5.42	5.15
64.0	13.9	0.12	22.0	6.63	0.08	48.0	5.37	5.02
80.0	18.1	0.20	23.0	9.64	0.36	53.0	5.31	5.32
160.0	37.4	0.98	23.0	17.43	3.27	47.0	5.33	6.27
320.0	108.3	1.45	34.0	66.87	2.22	62.0	5.38	.

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}

Measured TNB total concentrations in freshly amended soils ranged from 25 to 100 percent of nominal concentrations (Table 10). TNB recovery was greatly reduced in treatments below nominal 64 mg kg^{-1}. Measured TNB ATCLP extractable concentrations ranged from 56 to 86 percent of total concentrations (Table 10). These values do not include data for 8 mg kg^{-1} nominal treatment concentration, which had TNB recovery in one (0.13 mg kg^{-1}) out of three replicates producing an average ATCLP extractable value of 0.043 mg kg^{-1} (Table 10). Measured TNB total concentrations in weathered/aged amended soils ranged from 3 to 88 percent of nominal concentrations (Table 11). Measured TNB ATCLP extractable concentrations ranged from 31 to 72 percent of total concentrations (Table 11). Weathering/aging of amended soils reduced total TNB concentrations, on average, by 43% compared with total concentrations in freshly amended soils, whereas ATCLP extractable TNB concentrations were reduced, on average, by 59% compared with freshly amended soils.

Table 10. Nominal and average measured (n = 3) TNB concentrations (mg kg^{-1}) in freshly amended Sassafras sandy loam soil used in the toxicity tests with *E. fetida*. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0	BDL	BDL	BDL	BDL	BDL	BDL	5.34	6.51
4	2.3	0.08	58	BDL	BDL	BDL	5.46	5.47
8	2.6	0.11	32	0.043*	0.043*	1.7*	5.54	6.30
16	3.9	0.48	25	2.45	0.29	62	5.42	5.94
32	13.6	1.11	43	7.68	0.25	56	5.41	.
64	45.0	1.80	70	30.22	0.52	67	5.43	7.69
128	107.0	2.52	84	83.67	1.28	78	5.39	7.84
256	221.0	12.66	86	190.95	1.40	86	5.36	7.91
384	384.7	21.15	100	328.28	14.80	85	5.36	7.70

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}
* TNB was recovered in one (0.13 mg kg^{-1}) out of three replicates producing an average ATCLP extractable value of 0.043 mg kg^{-1}.

Table 11. Nominal and average measured (n = 3) TNB concentrations (mg kg^{-1}) in weathered/aged amended Sassafras sandy loam soil used in the toxicity tests with *E. fetida*.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0.0	BDL	BDL	BDL	BDL	BDL	BDL	5.01	5.86
16.0	BDL	BDL	BDL	BDL	BDL	BDL	5.00	5.41
32.0	1.0	0.09	3.0	BDL	BDL	BDL	5.03	5.55
64.0	19.9	0.31	31.0	6.16	0.12	31.0	4.83	5.55
128.0	78.7	1.44	62.0	41.83	0.85	53.1	4.67	6.56
256.0	191.0	6.93	75.0	112.00	1.53	58.6	4.72	7.25
384.0	302.0	3.06	79.0	174.30	2.60	57.7	4.64	7.16
512.0	411.0	4.36	80.0	271.33	3.84	66.0	4.69	6.72
768.0	674.0	18.36	88.0	487.00	4.16	72.3	4.76	6.54

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}

3.2 Range-Finding Toxicity Tests

Both RDX and HMX had no significant effect (0.0001) on adult survival in the range-finding tests in all treatment concentrations. Cocoon numbers were reduced by 69 ($p<0.05$) percent in 10 mg kg^{-1} RDX treatment. Twenty percent of the cocoons remained at 5,000 mg kg^{-1}

RDX compared to the control. Cocoon numbers were reduced by 60 ($p<0.05$) percent in 10 mg kg^{-1} HMX. Nine percent of the cocoons remained at 5,000 mg kg^{-1} HMX compared to the control. Results of the range finding test showed that 2,4-DNT significantly ($p<0.0001$) reduced cocoon production at 10 mg kg^{-1}. No cocoons were produced at 100 mg kg^{-1}, and no adults survived at the higher concentrations. Range-finding tests with 2,6-DNT showed that cocoon production was significantly ($p<0.01$) reduced at 10 mg kg^{-1}. There were no cocoons or adults above 10 mg kg^{-1}. Cocoon production in the range-finding test with TNB was significantly reduced at 10 mg kg^{-1} ($p<0.05$). There were no cocoons at 500 mg kg^{-1} and no adults above 500 mg kg^{-1}. Results of these range-finding tests were used to determine treatment concentrations for the definitive tests.

3.3 Definitive Toxicity Tests.

Definitive studies using the Earthworm Reproduction Tests were conducted to assess the effects of RDX, HMX, 2,4-DNT, 2,6-DNT, or TNB on the reproduction of the earthworm *E. fetida*. Adult *E. fetida* were exposed to a range of concentrations of each EM in SSL soil in independent investigations. Measurement endpoints were assessed using treatment concentrations determined using the results of the range-finding studies and included number of surviving adults after 28 days, and number of cocoons and juveniles after 56 days. All ecotoxicological parameters were estimated using measured chemical concentrations for each treatment level.

Test results complied with the validity criteria defined in the ISO test guideline. Mean adult survival in negative controls was >90% in all tests. The coefficient of variation juvenile production in control treatments did not exceed 30%. Direct comparisons of the results of positive control are not possible because no reference values for natural soils are available from the literature. Juvenile production in positive controls ranged from 54 to 98 percent reduction compared with negative controls and was within the baseline established for the laboratory culture of *E. fetida*. These results confirmed that the toxicological effects determined in the definitive tests were most likely due to test EM treatments. All reported ecotoxicological parameters have been calculated based on actual measured concentrations.

Results of RDX toxicity testing in freshly amended and weathered/aged amended SSL soils are shown in Tables 12 and 13, respectively. Adult *E. fetida* survival was not affected in all RDX concentrations producing unbounded NOEC values for RDX in freshly amended soils of 148.3 mg kg^{-1} based on total concentrations and 93.5 mg kg^{-1} based on ATCLP extractable concentrations. The unbounded NOEC value for RDX in weathered/aged-amended soils based on total concentrations was 527.0 mg kg^{-1}. The unbounded NOEC value for RDX in weathered/aged-amended soils based on ATCLP extractable concentrations was 100.1 mg kg^{-1}.

Cocoon production bounded NOEC and LOEC values based on total concentrations were, 8.6 and 18.2 mg kg^{-1} in freshly amended soil, and 56.6 and 61.5 mg kg^{-1} in weathered/aged soil, respectively (Table 14). Juvenile production bounded NOEC and LOEC values based on total concentrations were 7.5 and 8.6 mg kg^{-1} in freshly amended soil, and 8.4 and 15.7 mg kg^{-1} in weathered/aged soil, respectively (Table 14). The ATCLP based NOEC and LOEC values for cocoon production in freshly amended soils were 2.1 and 5.2 mg kg^{-1}, and 13.6

and 30.0 mg kg^{-1} in weathered/aged soils, respectively. The ATCLP based NOEC and LOEC values for juvenile production in freshly amended soils were 2.1 and 5.2, and 5.8 and 13.6 mg kg^{-1} in weathered/aged soils, respectively (Table 14).

Table 12. Mean (n = 4) adult survival, cocoon production, and juvenile production determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida* in Sassafras sandy loam soils freshly amended with RDX. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Soil concentration (mg kg^{-1})	Mean Adults	Standard Error	Mean Cocoons	Standard Error	Mean Hatched Cocoons (%)	Standard Error	Mean Juveniles	Standard Error
Negative control	5	0.0	9.3	1.5	71	23.6	12.8	5.1
Acetone control	5	0.0	13.3	0.3	83	3.7	18.3	1.1
Positive control	5	0.0	10.8	1.7	68	2.6	7.8	0.2
3	5	0.0	7.8	1.3	85	10.1	14.5	2.9
5	5	0.0	5.0	1.4	83	10.8	5.5	1.3
8	5	0.0	5.3	1.4	82	6.9	9.3	4.2
9	5	0.0	6.8	1.3	41	15.2	1.3	0.5
18	5	0.0	3.8	0.9	31	12.0	0.0	0.0
33	5	0.0	4.5	1.7	46	20.8	3.3	2.6
74	5	0.0	4.3	2.3	42	20.9	0.5	0.5
148	5	0.0	3.5	1.2	51	17.5	0.0	0.0

Table 13. Mean (n = 4) adult survival, cocoon production, and juvenile production and in weathered/aged RDX amended Sassafras sandy loam soils determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Soil concentration (mg kg^{-1})	Mean Adults	Standard Error	Mean cocoons	Standard Error	Mean hatched cocoons (%)	Standard Error	Mean juveniles	Standard Error
Negative control	4.8	0.3	17.5	1.6	58	23.6	17.3	2.7
Acetone control	4.8	0.3	18.3	1.3	54	3.7	24.3	7.5
Positive control	5.0	0.0	9.0	0.6	34	2.6	3.5	1.2
6	5.0	0.0	18.8	1.0	35	2.9	10.0	2.5
8	4.8	0.3	15.5	1.1	32	1.3	7.5	4.3
16	5.0	0.0	17.8	2.7	30	4.2	6.3	2.2
30	4.8	0.3	13.0	1.1	41	0.5	10.0	1.5
57	5.0	0.0	16.3	1.4	22	0.0	2.5	0.6
62	5.0	0.0	12.0	3.5	36	2.6	6.5	2.5
254	5.0	0.0	8.8	1.3	53	0.5	4.8	3.3
527	5.0	0.0	9.3	1.2	23	0.0	0.5	0.3

Concentration-response relationships for juvenile production in fresh and weathered/aged RDX amended soils determined by nonlinear regressions are shown in Figure 1. Data fit the exponential model best in tests with both freshly amended (Figure 1A) and weathered/aged amended (Figure 1B) soils. Overall, reproduction was higher in weathered/aged RDX amended soils (Table 13). Juvenile production EC_{20} values based on total extraction were 1.6 and 4.8 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. Juvenile production EC_{50} values were 5.0 and 14.9 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. Cocoon production EC_{20} values were 1.2 and 19.2 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively (Table 14). Cocoon production EC_{50} values were 3.7 and 59.6 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively (Table 14). Juvenile production EC_{20} values based on ATCLP extractable concentrations were 0.84, and 1.4 mg kg^{-1} in freshly amended and weathered/aged soils, respectively (Table 14). Juvenile production EC_{50} values based on ATCLP extractable concentrations were 2.6 and 14.4 mg kg^{-1} in freshly amended and weathered/aged soils, respectively (Table 16). The differences between freshly amended and weathered/aged cocoon and juvenile production were not statistically significant (95% CI; Table 14), indicating that the 3-month weathering/aging process did not affect the toxicity of RDX to *E. fetida*.

Coefficients of Determination (R^2) for total and ATCLP based extractions of RDX were calculated in nonlinear regression analyses (EC_{20} levels) to determine which chemical measure better correlates with toxicity endpoints in both fresh and weathered/aged soils. The R^2 values for juveniles in freshly amended soil were 0.84 and 0.83 in total and ATCLP based extractions, respectively (Table 14). The R^2 values for cocoons in freshly amended soil were 0.0.94 and 0.86 in total and ATCLP based extractions, respectively. The R^2 values for juveniles

in weathered/aged soil were 0.80 and 0.82 in total and ATCLP based extractions, respectively. The R^2 values for cocoons in freshly amended soil were 0.95 and 0.95 in total and ATCLP based extractions, respectively. These comparisons show that regression coefficients were very similar for both extraction methods indicating that neither extraction method had an advantage in characterizing RDX bioavailability to *E. fetida*.

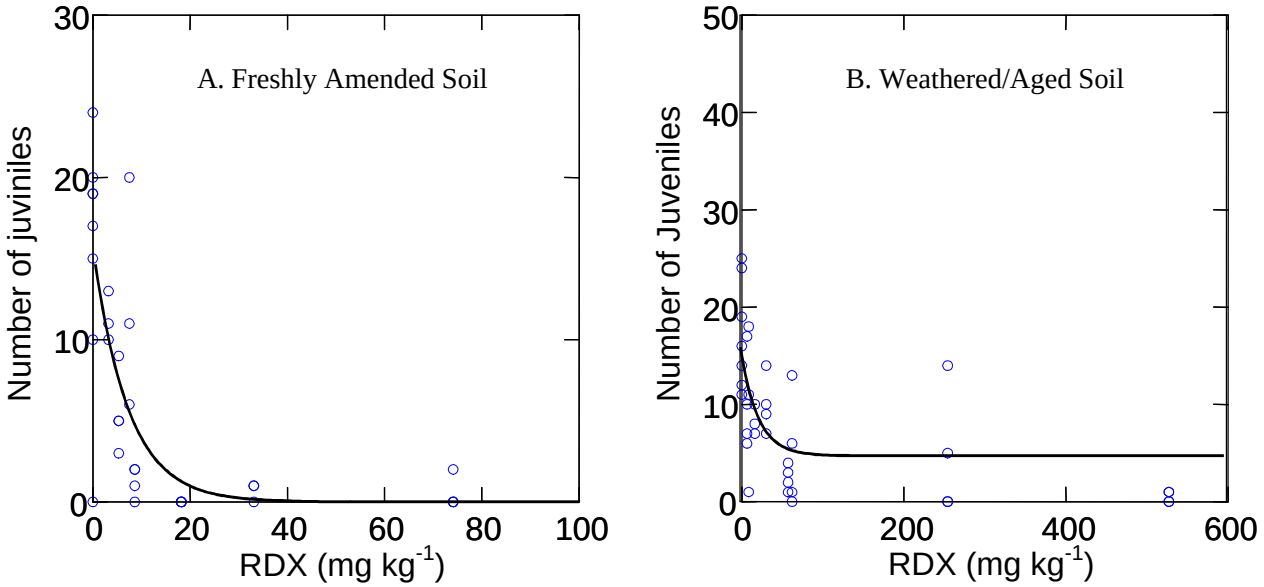

Figure 1. Non-linear regression (Exponential model: $Y = a \times e^{(([\log(1-p)] / ECp) \times C) + b}$) of RDX and *Eisenia fetida* juvenile production in freshly amended (A) and weathered/aged amended (B) Sassafras sandy loam soil. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Table 14. Ecotoxicological parameters (mg kg^{-1}) with P-value or confidence interval (C.I.) and coefficient of determination (R^2) for RDX in freshly amended and weathered/aged amended Sassafras sandy loam soil using earthworm reproduction test with *Eisenia fetida*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Exposure	Cocoon Production				Juvenile production			
	NOEC	LOEC	EC_{20}	EC_{50}	NOEC	LOEC	EC_{20}	EC_{50}
Fresh								
Total	8.6	18.2	1.2	3.7	7.5	8.6	1.6	5.0
P or 95% C.I.	0.06	0.001	0.4-2.0	1.2-6.2	0.31	0.001	0.4-2.7	1.4-8.5
R^2			0.94	0.94			0.84	0.84
ATCLP	2.1	5.2	0.46	1.4	2.1	5.2	0.84	2.6
P or 95% C.I.	0.06	0.001	.031-.89	0.1-2.8	0.19	0.0001	0.35-1.3	1.1-4.1
R^2			0.86	0.86			0.83	0.83
Aged/weathered								
Total	56.6	61.5	19.2	59.6	8.4	15.7	4.8	14.9
P or 95% C.I.	0.45	0.01	0-39	0-120	0.06	0.02	0.2-9	0.66-29
R^2			0.95	0.95			0.80	0.80
ATCLP	13.6	30.0	42.0	102.7	5.8	13.6	1.4	14.4
P or 95% C.I.	0.95	0.03	11-73	67-139	0.61	0.01	0-5	0-31
R^2			0.95	0.95			0.82	0.82

Notes:
P-value was generated by ANOVA. C.I. and R^2 were generated by nonlinear regression analysis.

Results of HMX toxicity testing in freshly amended and weathered/aged amended SSL soils are shown in Tables 15 and 16, respectively. Adult *E. fetida* survival was not affected in all HMX concentrations producing unbounded NOEC values for HMX in freshly amended soils of 141.3 mg kg^{-1} based on total concentrations and 15.2 mg kg^{-1} based on ATCLP extractable concentrations. The unbounded NOEC value for HMX in weathered/aged-amended soils based on total concentrations was 561.7 mg kg^{-1}. The unbounded NOEC value for HMX in weathered/aged-amended soils based on ATCLP extractable concentrations was 19.0 mg kg^{-1}.

Cocoon production bounded NOEC and LOEC values for HMX based on total concentrations were, 15.6 and 36.0 mg kg^{-1} in freshly amended soil (Table 17). The cocoon production unbounded NOEC in weathered/aged soil was 561.7 mg kg^{-1}. Cocoon production was not significantly reduced (P>0.46) in earthworm populations exposed to HMX in weathered/aged soil (Table 17). However, cocoon counts were reduced by 7 to 26% in treated soils compared with controls (Table 16). Juvenile production bounded NOEC and LOEC values based on total concentrations were 6.5 and 11.2 mg kg^{-1} in freshly amended soil (Table 17). The juvenile production unbounded NOEC in weathered/aged soil was 561.7 mg kg^{-1}. Juvenile production was not significantly reduced (P>0.59) for earthworm populations exposed to total HMX in weathered/aged soil (Table 17). However, juvenile counts were reduced by 2 to 35% in treated soils compared with controls (Table 16). The ATCLP based NOEC and LOEC values for cocoon production in freshly amended soils were 5.9 and 11.2 mg kg^{-1} respectively. The ATCLP based cocoon production unbounded NOEC in weathered/aged soil was 19.0 mg kg^{-1} (P>0.23) in

earthworm populations exposed to HMX in weathered/aged soil (Table 17). The ATCLP based NOEC and LOEC values for juvenile production in freshly amended soils were 5.9 and 11.2 mg kg^{-1} respectively. The ATCLP based juvenile production unbounded NOEC in weathered/aged soil was 19.0 mg kg^{-1} (P>0.68) in earthworm populations exposed to HMX in weathered/aged soil (Table 17).

Table 15. Mean (n = 4) adult survival, cocoon production, and juvenile production determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida* in Sassafras sandy loam soils freshly amended with HMX. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Soil concentration (mg kg^{-1})	Mean Adults	Standard Error	Mean Cocoons	Standard Error	Percent Hatched Cocoons	Standard Error	Mean Juveniles	Standard Error
Negative control	5	0.0	9.3	1.5	71	23.6	12.8	5.1
Acetone control	5	0.0	8.3	1.2	95	2.9	14.0	3.8
Positive control	5	0.0	3.0	1.2	65	23.6	0.3	0.3
1	5	0.0	7.8	1.7	75	9.0	8.3	1.3
3	5	0.0	4.8	2.4	89	7.9	6.3	3.2
7	5	0.0	7.0	0.7	81	8.9	7.3	4.1
11	5	0.0	5.3	1.1	50	20.4	4.0	2.3
16	5	0.0	5.5	1.0	70	14.9	5.0	2.2
36	5	0.0	2.8	0.9	82	10.7	2.8	1.8
74	5	0.0	3.5	1.0	79	15.8	4.8	2.3
141	5	0.0	4.5	1.3	92	4.9	4.3	2.6

Table 16. Mean (n = 4) adult survival and juvenile production and in weathered/aged HMX amended Sassafras sandy loam soils determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Soil concentration (mg kg^{-1})	Mean Adults	Standard Error	Mean cocoons	Standard Error	Mean hatched cocoons (%)	Standard Error	Mean juveniles	Standard Error
Negative control	5.0	0.0	20.3	1.3	70	6.2	37.5	3.4
Acetone control	5.0	0.0	20.3	1.0	70	4.3	34.0	4.4
Positive control	5.0	0.0	9.0	0.8	34	7.7	3.5	1.2
2	5.0	0.0	15.0	1.7	63	13.6	21.8	7.9
3	5.0	0.0	15.3	1.7	57	7.6	22.0	6.0
11	5.0	0.0	18.5	3.3	62	11.9	33.5	12.5
29	5.0	0.0	16.8	0.6	53	7.8	24.5	6.6
54	5.0	0.0	18.8	2.8	60	5.1	31.3	5.7
129	5.0	0.0	15.8	2.3	64	8.4	29.0	6.7
280	5.0	0.0	15.0	2.8	61	18	27.3	8.4
562	5.0	0.0	18.3	2.8	54	7.5	26.0	2.1

Concentration-response relationships for juvenile production in fresh and weathered/aged HMX amended soils determined by nonlinear regressions are shown in Figure 2. Data fit the exponential model best in tests with both freshly amended (Figure 2A) and weathered/aged amended (Figure 2B) soils. Overall, reproduction was higher in weathered/aged HMX amended soils (Tables 15 and 16). Juvenile production EC_{20} and EC_{50} values based on total extraction were 0.4 mg kg^{-1} and 1.2 mg kg^{-1}, respectively in freshly amended soil. Cocoon production EC_{20} and EC_{50} values based on total extraction were 2.7 mg kg^{-1} and 8.5 mg kg^{-1}, respectively in freshly amended soil (Table 17). Juvenile production EC_{20} and EC_{50} values based on ATCLP extraction were 0.08 mg kg^{-1} and 0.25 mg kg^{-1}, respectively in freshly amended soil. Cocoon production EC_{20} and EC_{50} values based on total extraction were 1.4 mg kg^{-1} and 4.3 mg kg^{-1}, respectively in freshly amended soil (Table 17). Toxicty data in the HMX weathered/aged studies produced a nearly straight horizontal line (Figure 2B) indicating no effect. Therefore, EC_{20} and EC_{50} values could not be calculated for toxicological endpoints in *E. fetida* exposed to HMX in weathered/aged soil. The weathering/aging process virtually eliminated the toxicity of HMX to *E. fetida*. The extent of this effect could not be directly quantified since EC values could not be calculated. Reduced toxicity could not be explained by degradation of HMX over time since an average of 75% of the HMX in the total extract was still present after weathering/aging (data not shown). ATCLP extractable HMX actually increased by an average of 124% after aging and weathering.

The R^2 values for juveniles in freshly amended soil were 0.0.74 and 0.73 in total and ATCLP based extractions, respectively (Table 17). The R^2 values for cocoons in freshly amended soil were 0.82 and 0.81 in total and ATCLP based extractions, respectively. These comparisons show that regression coefficients were very similar for both extraction methods

indicating that neither extraction method had an advantage in characterizing HMX bioavailability to *E. fetida*.

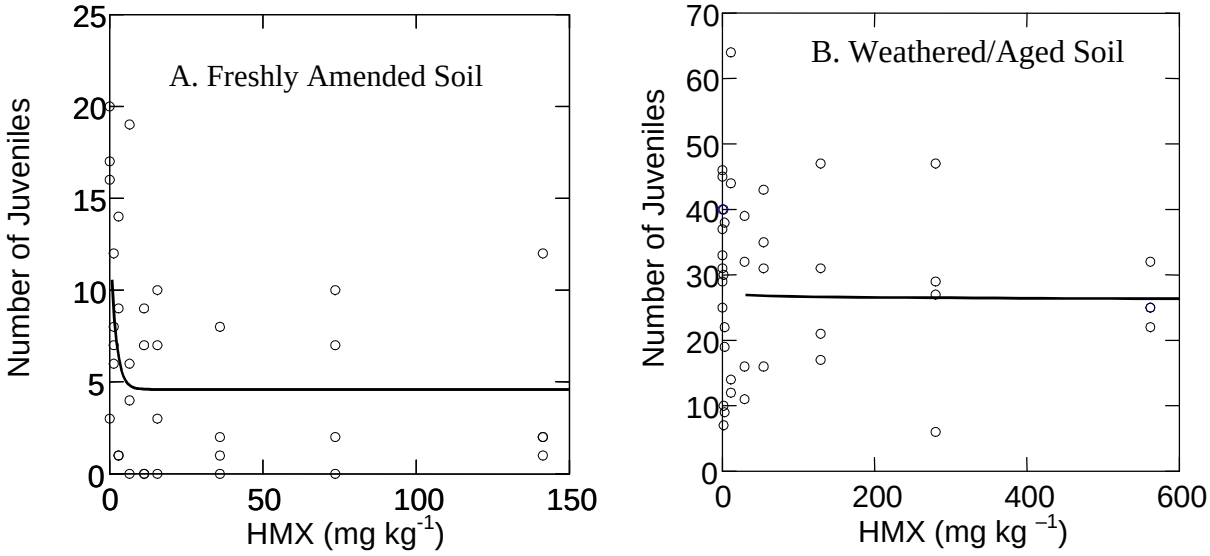

Figure 2. Non-linear regression (Exponential model: $Y = a \times e^{(([\log(1-p)] / ECp) \times C) + b}$) of HMX and *Eisenia fetida* juvenile production in freshly amended (A) and weathered/aged amended (B) Sassafras sandy loam soil. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Table 17. Ecotoxicological parameters (mg kg^{-1}) with P-value or confidence interval (C.I.) and coefficient of determination (R^2) for HMX in freshly amended and weathered/aged amended Sassafras sandy loam soil using earthworm reproduction test with *Eisenia fetida*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Exposure	Cocoon Production				Juvenile production			
	NOEC	LOEC	EC_{20}	EC_{50}	NOEC	LOEC	EC_{20}	EC_{50}
Fresh								
Total	15.6	36.0	2.7	8.5	6.5	11.2	0.4	1.2
P or 95% C.I.	.16	.007	0-7.0	0-22	0.1	0.02	0-0.9	0.5-2.8
R^2			0.82	0.82			0.74	0.74
ATCLP	5.9	11.2	1.4	4.3	5.9	11.2	.08	0.25
P or 95% C.I.	0.13	0.003	0-5.2	0-16	0.09	0.02	0-0.2	0-0.9
R^2			0.81	0.81			0.73	0.73
Aged/weathered								
Total	561.7	ND	ND	ND	561.7	ND	ND	ND
P or 95% C.I.	0.46				0.59			
R^2								
ATCLP	19.0	ND	ND	ND	19.0	ND	ND	ND
P or 95% C.I.	0.23				0.68			
R^2								

Notes:

ND, Not Determined. ECx values could not be determined because cocoon and juvenile numbers were not significantly different in all treatment concentrations compared with carrier control. P-value was generated during ANOVA. C.I. and R^2 were generated during nonlinear regression analysis.

The EM, 2,4-DNT affected adult survival, cocoon production, and juvenile production of *E. fetida* in amended SSL (Tables 18 and 19). For adult survival in freshly amended soil, the bounded NOEC and LOEC values for 2,4-DNT based on total concentrations were 55.0 and 64.7 mg kg^{-1}, respectively. The bounded NOEC and LOEC values based on ATCLP concentrations were 33.4 and 43.4 mg kg^{-1}, respectively. For adult survival in weathered/aged-amended soil, the bounded NOEC and LOEC values based on total concentrations were 37.3 and 71.7 mg kg^{-1}, respectively. No adults survived in the 179 mg kg^{-1} treatment. The bounded NOEC and LOEC values based on ATCLP concentrations were 20.5 and 46.1 mg kg^{-1}, respectively.

Cocoon production bounded NOEC and LOEC values based on total concentrations were, 20.3 and 40.9 mg kg^{-1} in freshly amended soil, and 21.5 and 31.0 mg kg^{-1} in weathered/aged soil, respectively (Table 20). Juvenile production bounded NOEC and LOEC values based on total concentrations were 55.0 and 64.7 mg kg^{-1} in freshly amended soil, and 37.3 and 71.7 mg kg^{-1} in weathered/aged soil, respectively (Table 20). The ATCLP based NOEC and LOEC values for cocoon production in freshly amended soils were 5.0 and 8.1 mg kg^{-1}, and 21.5 and 31.0 mg kg^{-1} in weathered/aged soils, respectively. The ATCLP based NOEC and LOEC values for juvenile production in freshly amended soils were 8.1 and 33.4, and 20.4 and 46.1 mg kg^{-1} in weathered/aged soils, respectively.

Table 18. Mean (n = 4) adult survival, cocoon production, and juvenile production determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida* in Sassafras sandy loam soils freshly amended with 2,4-DNT. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Soil concentration (mg kg^{-1})	Mean Adults	Standard Error	Mean Cocoons	Standard Error	Percent Hatched Cocoons	Standard Error	Mean Juveniles	Standard Error
Negative control	5	0.0	15.0	2.3	44	11.0	5.5	2.5
Acetone control	4.8	0.3	12.5	0.5	52	16.0	7.0	3.7
Positive control	5.0	0.0	6.5	1.6	36	13.1	3.0	1.9
0.95	4.8	0.3	13.3	1.5	60	16.2	12.8	5.6
3.0	5	0.0	11.0	2.3	46	7.0	2.5	1.0
6.5	4.5	0.3	13.3	2.9	28	5.6	3.3	2.0
10.0	5	0.0	12.8	1.7	45	11.5	10.0	3.4
20.3	4.3	0.3	14.0	2.7	60	9.5	2.5	0.6
40.9	5	0.0	6.5	0.6	35	2.5	5.0	1.7
55.0	4.8	0.3	3.3	0.5	48	8.6	2.0	1.1
64.7	3.0	0.4	0.8	0.5	13	12.5	0.3	0.3

Table 19. Mean (n = 4) adult survival and juvenile production and in weathered/aged 2,4-DNT amended Sassafras sandy loam soils determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Soil concentration (mg kg^{-1})	Mean Adults	Standard Error	Mean cocoons	Standard Error	Mean hatched cocoons (%)	Standard Error	Mean juveniles	Standard Error
Negative control	5.0	0.0	23.5	1.8	71.7	13.3	46.3	9.9
Acetone control	5.0	0.0	20.5	4.5	45.3	17.3	13.0	1.0
Positive control	3.5	0.0	2.8	1.0	43.3	20.8	0.8	0.5
3.0	5.0	0.0	18.8	2.3	79.7	6.3	45.3	12.9
5.2	5.0	0.0	17.3	3.8	34.7	13.1	9.8	4.8
11.5	4.8	0.0	22.0	3.2	61.3	9.6	43.5	7.6
22.0	5.0	0.0	17.5	1.0	69.9	10.6	45.8	5.4
31.0	4.8	0.0	10.3	3.5	48.6	16.7	20.0	7.6
37.0	4.8	0.0	12.8	4.0	69.0	7.7	19.0	5.1
72.0	4.0	0.0	1.3	0.5	27.5	12.5	0.3	0.3
179.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Concentration-response relationships for juvenile production in fresh and weathered/aged 2,4-DNT amended soils determined by nonlinear regressions are shown in Figure 3. Logistic (Gompertz) model had the best fit for data in tests with both freshly amended (Figure 3A) and weathered/aged amended (Figure 3B) soils. Overall, reproduction was higher in weathered/aged 2,4-DNT amended soils. Juvenile production EC_{20} values based on total extraction were 43.6 and 29.4 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. Juvenile production EC_{50} values were 51.8 and 35.7 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. Cocoon production EC_{20} values were 30.7 and 25.2 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively (Table 20). Cocoon production EC_{50} values were 42.9 and 40.5 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively (Table 20). Juvenile production EC_{20} values based on ATCLP extractable concentrations were 22.2, and 29.4 mg kg^{-1} in freshly amended and weathered/aged soils, respectively (Table 20). Juvenile production EC_{50} values based on ATCLP extractable concentrations were 29.6 and 19.2 mg kg^{-1} in freshly amended and weathered/aged soils, respectively (Table 20). Cocoon production EC_{20} values based on ATCLP extractable concentrations were 5.3 and 12.3 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively (Table 20). Cocoon production EC_{50} values based on ATCLP extractable concentrations were 14.3 and 22.3 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively (Table 20). The differences between freshly amended and weathered/aged cocoon and juvenile production were not statistically significant (95% CI; Table 20), indicating that the 3-month weathering/aging process did not affect the toxicity of 2,4-DNT to *E. fetida*.

Coefficients of Determination (R^2) for total and ATCLP based extractions of 2,4-DNT were calculated in nonlinear regression analyses (EC_{20} levels) to determine which chemical measure better correlates with toxicity endpoints in both fresh and weathered/aged soils. The R^2

values for juveniles in freshly amended soil were 0.63 and 0.57 in total and ATCLP based extractions, respectively (Table 20). The R^2 values for cocoons in freshly amended soil were 0.0.94 and 0.93 in total and ATCLP based extractions, respectively. The R^2 values for juveniles in weathered/aged soil were 0.81 and 0.84 in

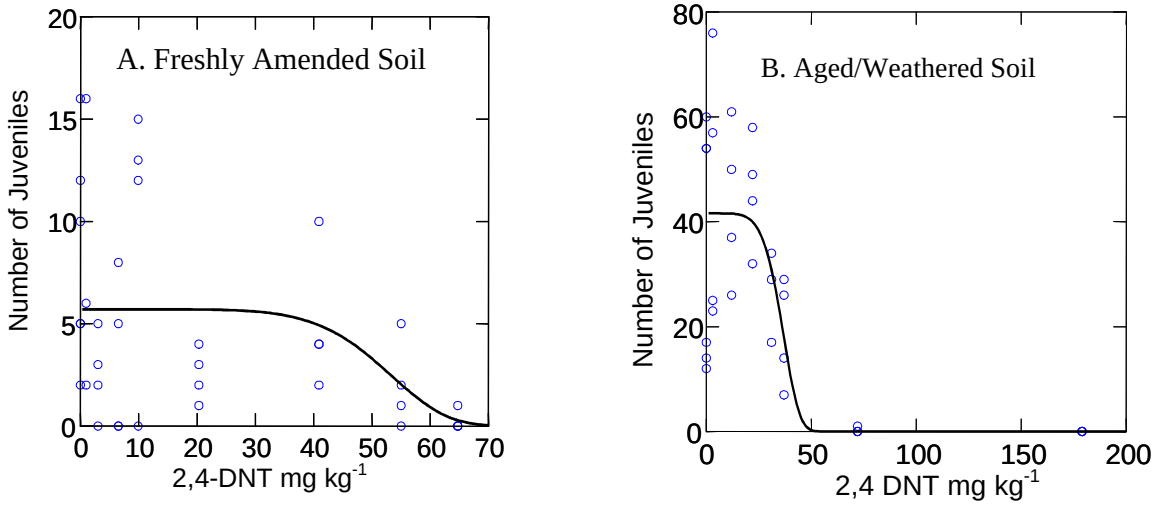

Figure 3. Non-linear regression (Logistic (Gompertz) model: $Y = a \times e^{([\log(1-p)]\times[C/ECp]b)}$) of 2,4-DNT and *Eisenia fetida* juvenile production in freshly amended (A) and weathered/aged amended (B) Sassafras sandy loam soil. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Table 20. Ecotoxicological parameters (mg kg^{-1}) with P-value or confidence interval (C.I.) and coefficient of determination (R^2) for 2,4-DNT in freshly amended and weathered/aged amended Sassafras sandy loam soil using earthworm reproduction test with *Eisenia fetida*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Exposure	Cocoon Production				Juvenile production			
	NOEC	LOEC	EC_{20}	EC_{50}	NOEC	LOEC	EC_{20}	EC_{50}
Fresh								
Total	20.3	40.9	30.7	42.9	55.0	64.7	43.6	51.8
P or 95% C.I.	0.91	0.003	17.4-44.1	34.3-51.6	.066	.021	10.7-76.5	33.2-70.4
R^2			0.94	0.94			0.63	0.63
ATCLP	5.0	8.1	5.3	14.3	8.1	33.4	22.2	29.6
P or 95% C.I.	0.65	0.003	0-10.7	5.8-22.8	0.23	.006	-34.2-78.6	0.1-59.0
R^2			0.91	0.91			0.57	0.57
Aged/weathered								
Total	21.5	31.0	25.2	40.5	37.3	71.7	29.4	35.7
P or 95% C.I.	0.1	0.01	15.9-34.5	31.6-49.4	0.12	0.002	17.4-41.5	30.0-41.5
R^2			0.93	0.93			0.81	0.81
ATCLP	11.8	15.4	12.3	22.3	20.4	46.1	15.2	19.2
P or 95% C.I.	0.10	0.001	6.7-17.9	16.1-28.4	0.5	0.001	8.1-22.3	15.2-23.3
R^2			0.94	0.94			0.84	0.84

Notes:
P-value was generated during ANOVA. C.I. and R^2 were generated during nonlinear regression analysis.

total and ATCLP based extractions, respectively. The R^2 values for cocoons in weathered/aged soil were 0.93 and 0.94 in total and ATCLP based extractions, respectively. These comparisons show that regression coefficients were very similar for both extraction methods indicating that neither extraction method had an advantage in characterizing 2,4-DNT bioavailability to *E. fetida*.

The EM, 2,6-DNT affected adult survival, cocoon production, and juvenile production of *E. fetida* in amended SSL (Tables 21 and 22). For adult survival in freshly amended soil, the bounded NOEC and LOEC values for 2,6-DNT based on total concentrations were 20.0 and 40.2 mg kg^{-1}, respectively. The bounded NOEC and LOEC values based on ATCLP concentrations were 11 and 25 mg kg^{-1}, respectively. For adult survival in weathered/aged-amended soil, the bounded NOEC and LOEC values based on total concentrations were 13.9 and 18.0 mg kg^{-1}, respectively. No adults survived in the 179 mg kg^{-1} treatment. The bounded NOEC and LOEC values for adult survival in weathered/aged soil based on ATCLP concentrations were 6.6 and 9.6 mg kg^{-1}, respectively.

Cocoon production bounded NOEC and LOEC values based on total concentrations were, 9.4 and 12.9 mg kg^{-1} in freshly amended soil, and 18.1 and 37.4 mg kg^{-1} in weathered/aged soil, respectively (Table 23). Juvenile production bounded NOEC and LOEC values based on total concentrations were 20.0 and 40.2 mg kg^{-1} in freshly amended soil, and 13.9 and 18.1 mg kg^{-1} in weathered/aged soil, respectively (Table 23). The ATCLP based NOEC and LOEC values for cocoon production in freshly amended soils were 3.8 and 5.8 mg kg^{-1}, and 9.6 and 17.4 mg kg^{-1} in weathered/aged soils, respectively. The ATCLP based NOEC and LOEC values for juvenile production in freshly amended soils were 10.6 and 24.8, and 6.6 and 9.6 mg kg^{-1} in weathered/aged soils, respectively (Table 23).

Table 21. Mean (n = 4) adult survival, cocoon production, and juvenile production determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida* in Sassafras sandy loam soils freshly amended with 2,6-DNT. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Soil concentration (mg kg^{-1})	Mean Adults	Standard Error	Mean Cocoons	Standard Error	Percent Hatched Cocoons	Standard Error	Mean Juveniles	Standard Error
Negative control	5	0.0	15.0	2.3	44	11.0	5.5	2.5
Acetone control	4.8	0.3	12.5	0.5	52	16.0	7.0	3.7
Positive control	5.0	0.0	6.5	1.6	36	13.1	3.0	1.9
5	4.8	0.3	13.3	1.5	60	16.2	12.8	5.6
7.7	5	0.0	11.0	2.3	46	7.0	2.5	1.0
9.4	4.5	0.3	13.3	2.9	28	5.6	3.3	2.0
13	5	0.0	12.8	1.7	45	11.5	10.0	3.4
20	4.3	0.3	14.0	2.7	60	9.5	2.5	0.6
40	5	0.0	6.5	0.6	35	2.5	5.0	1.7
51	4.8	0.3	3.3	0.5	48	8.6	2.0	1.1
64	3.0	0.4	0.8	0.5	13	12.5	0.3	0.3

Table 22. Mean (n = 4) adult survival and juvenile production and in weathered/aged 2,6-DNT amended Sassafras sandy loam soils determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Soil concentration (mg kg^{-1})	Mean Adults	Standard Error	Mean cocoons	Standard Error	Mean hatched cocoons (%)	Standard Error	Mean juveniles	Standard Error
Negative control	5.0	0	11.8	1.4	62	8.1	14.3	2.7
Acetone control	5.0	0	13.0	4.4	75	12.5	25.3	11.5
Positive control	5.0	0	9.0	1.4	43	18.8	5.8	3.2
1.2	5.0	0	18.8	5.2	65	17.0	29.0	11.2
1.6	4.3	1.1	12.3	4.5	65	16.0	23.8	13.8
3.7	5.0	0	16.3	2.1	52	15.4	17.8	6.7
9.5	5.0	0	20.0	3.8	45	11.1	18.0	8.1
13.9	5.0	0	13.5	2.7	40	15.5	5.0	2.8
18	3.3	1.7	10.3	3.5	15	9.8	0.8	0.8
37	1.5	1.4	0.0	0.0	0	0.0	0.0	0.0
108	0.0	0	0.0	0.0	0	0.0	0.0	0.0

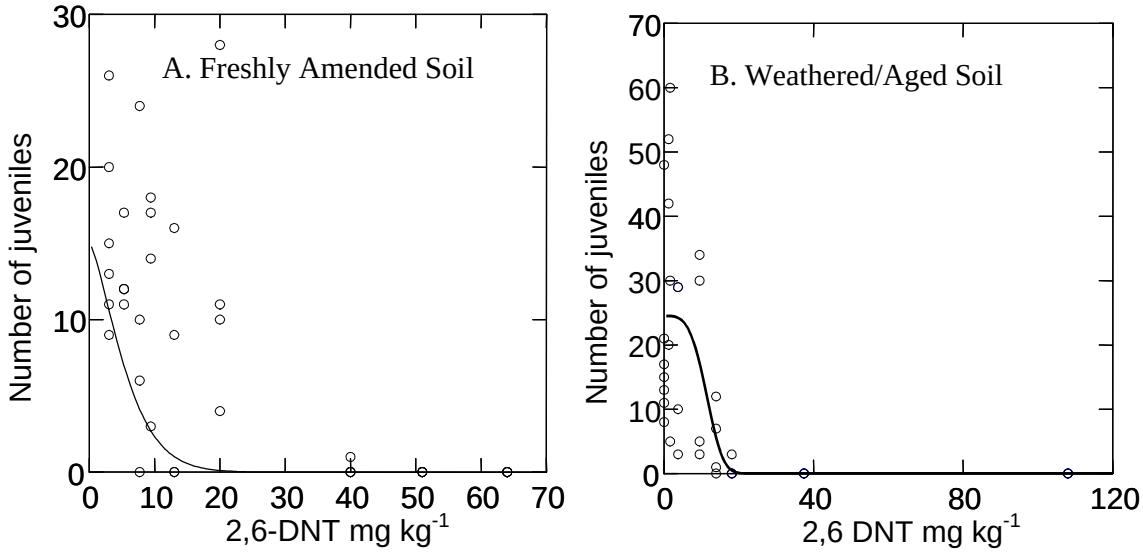

Figure 4. Non-linear regression (Logistic (Gompertz) model: $Y = a \times e^{([\log(1-p)]\times[C/ECp]b)}$) of 2,6-DNT and *Eisenia fetida* juvenile production in freshly amended (A) and weathered/aged amended (B) Sassafras sandy loam soil. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Concentration-response relationships for juvenile production in fresh and weathered/aged 2,6-DNT amended soils determined by nonlinear regressions are shown in Figure 4. Logistic (Gompertz) model had the best fit for data in tests with both freshly amended (Figure 4A) and weathered/aged amended (Figure 4B) soils. Overall, reproduction was higher in weathered/aged 2,6-DNT amended soils. Juvenile production EC_{20} values based on total extraction were 9.0 and 8.3 mg kg^{-1} in freshly amended and weathered/aged soils, respectively (Table 23). Juvenile production EC_{50} values were 27.4 and 11.3 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. Cocoon production EC_{20} values were 14.3 and 16.1 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively (Table 23). Cocoon production EC_{50} values were 24.8 and 19.3 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively (Table 23). Juvenile production EC_{20} values based on ATCLP extractable concentrations were 6.5, and 3.6 mg kg^{-1} in freshly amended and weathered/aged soils, respectively (Table 23). Juvenile production EC_{50} values based on ATCLP extractable concentrations were 20.2 and 5.2 mg kg^{-1} in freshly amended and weathered/aged soils, respectively (Table 23). Cocoon production EC_{20} values based on ATCLP extractable concentrations were 7.4 and 16.1 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively (Table 23). Cocoon production EC_{50} values based on ATCLP extractable concentrations were 14.2 and 10.5 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively (Table 23). The differences between freshly amended and weathered/aged cocoon and juvenile production were not statistically significant (95% CI; Table 22), indicating that the 3-month weathering/aging process did not affect the toxicity of 2,6-DNT to *E. fetida*.

Coefficients of Determination (R^2) for total and ATCLP based extractions of 2,6-DNT were calculated in nonlinear regression analyses (EC_{20} levels) to determine which chemical measure better correlates with toxicity endpoints in both fresh and weathered/aged soils. The R^2

values for juveniles in freshly amended soil were 0.71 and 0.73 in total and ATCLP based extractions, respectively (Table 23). The R^2 values for cocoons in freshly amended soil were 0.92 and 0.91 in total and ATCLP based extractions, respectively. The R^2 values for juveniles in weathered/aged soil were 0.72 and 0.67 in total and ATCLP based extractions, respectively. The R^2 values for cocoons in weathered/aged soil were 0.91 and 0.85 in total and ATCLP based extractions, respectively. These comparisons show that regression coefficients were very similar for both extraction methods indicating that neither extraction method had an advantage in characterizing 2,6-DNTbioavailability to *E. fetida*.

Table 23. Ecotoxicological parameters (mg kg^{-1}) with P-value or confidence interval (C.I.) and coefficient of determination (R^2) for 2,6-DNT in freshly amended and weathered/aged amended Sassafras sandy loam soil using earthworm reproduction test with *Eisenia fetida*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Exposure	Cocoon Production				Juvenile production			
	NOEC	LOEC	EC_{20}	EC_{50}	NOEC	LOEC	EC_{20}	EC_{50}
Fresh								
Total	9.4	12.9	14.3	24.8	20.0	40.2	9.0	27.4
P or 95% C.I.	.545	.035	6.6-22.1	17.4-32.1	.56	.001	0-30.5	0-91.8
R^2			0.92	0.92			0.71	0.71
ATCLP	3.8	5.8	7.4	14.2	10.6	24.8	6.5	20.2
P or 95% C.I.	.55	.035	2.9-11.9	9.3-19.1	.56	.001	0-23.8	0-74.0
R^2			0.93	0.93			0.73	0.73
Aged/weathered								
Total	18.1	37.4	16.1	19.3	13.9	18.1	8.3	11.3
P or 95% C.I.	0.58	0.002	9.9-22.2	13.4-25.3	0.09	0.03	1.6-15.1	6.9-15.8
R^2			0.91	0.91			0.71	0.71
ATCLP	9.6	17.4	8.2	10.5	6.6	9.6	3.6	5.2
P or 95% C.I.	0.58	0.002	3.9-12.6	6.2-14.7	0.09	0.03	0.04-7.2	2.8-7.6
R^2			0.85	0.85			0.67	0.67

Notes:
P-value was generated during ANOVA. C.I. and R^2 were generated during nonlinear regression analysis.

Results of TNB toxicity testing in freshly amended and weathered/aged amended SSL soils are shown in Tables 24 and 25, respectively. For adult survival in freshly amended soil, the bounded NOEC and LOEC values for TNB based on total concentrations were 45 and 107 mg kg^{-1}, respectively (Tables 26). The bounded NOEC and LOEC values based on ATCLP concentrations were 30 and 84 mg kg^{-1}, respectively (Table 26). For adult survival in weathered/aged-amended soil, the bounded NOEC and LOEC values based on total concentrations were 79 and 191 mg kg^{-1}, respectively. No adults survived in the 302 mg kg^{-1} treatment. The bounded NOEC and LOEC values for adults based on ATCLP TNB concentrations were 42 and 112 mg kg^{-1}, respectively.

Cocoon production bounded NOEC and LOEC values for based on total TNB concentrations were, 13.6 and 45.0 mg kg^{-1} in freshly amended soil, and 19.9 and 78.7 mg kg^{-1} in weathered/aged soil, respectively (Table 26). Juvenile production bounded NOEC and LOEC values based on total concentrations were 13.6 and 45.0 mg kg^{-1} in freshly amended soil, and 19.9 and 78.7 mg kg^{-1} in weathered/aged soil, respectively (Table 26). The ATCLP based NOEC and LOEC values for cocoon production in freshly amended soils were 7.7 and 30.2 mg kg^{-1}, and 6.2 and 41.8 mg kg^{-1} in weathered/aged soils, respectively. The ATCLP based NOEC and LOEC values for juvenile production in freshly amended soils were 7.7 and 30.2, and 6.2 and 41.8 mg kg^{-1} in weathered/aged soils, respectively (Table 26).

Table 24. Mean (n = 4) adult survival, cocoon production, and juvenile production determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida* in Sassafras sandy loam soils freshly amended with TNB. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Soil concentration (mg kg^{-1})	Mean Adults	Standard Error	Mean Cocoons	Standard Error	Percent Hatched Cocoons	Standard Error	Mean Juveniles	Standard Error
Negative control	5	0	14.0	1.0	63	14.8	11.3	4.1
Acetone control	4.8	0.3	13.5	1.7	50	15.4	11.0	4.2
Positive control	5	0	3.0	0.6	31	23.7	0.3	0.3
2.3	5	0	14.3	1.3	63	8.6	18.0	1.7
2.6	4.7	0.3	17.0	2.5	85	1.5	22.3	3.8
3.9	5	0	10.5	2.3	76	9.1	17.0	3.2
13.6	4.8	0.3	16.5	1.6	72	6.2	15.3	4.2
45	4.8	0.3	7.5	1.8	37	12.8	3.8	1.7
107	2.3	0.3	3.8	0.9	5	5.0	0.0	0.0
221	0.5	0.5	0.0	0.0	0	0.0	0.0	0.0
385	0	0	0.0	0.0	0	0.0	0.0	0.0

Table 25. Mean (n = 4) adult survival and juvenile production and in weathered/aged TNB amended Sassafras sandy loam soils determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330. BDL = below method detection limit of 0.05 mg kg^{-1}.

Soil concentration (mg kg^{-1})	Mean Adults	Standard Error	Mean cocoons	Standard Error	Mean hatched cocoons (%)	Standard Error	Mean juveniles	Standard Error
Negative control	5.0	0.0	20.5	0.9	81	3.5	54.8	1.9
Acetone control	5.0	0.0	23.3	1.6	77	4.2	51.8	5.1
Positive control	5.0	0.0	3.0	0.6	31	23.7	0.3	0.3
BDL (nominal 16)	4.8	0.3	20.8	0.6	79	5.5	52.3	3.1
1	5.0	0.0	22.8	1.5	65	10.8	47.3	9.4
20	5.0	0.0	24.0	3.0	75	6.3	50.0	4.8
79	3.8	1.3	5.8	2.0	56	19.1	7.8	4.4
191	4.0	0.4	0.0	0.0	0	0.0	0.0	0.0
302	0.0	0.0	0.0	0.0	0	0.0	0.0	0.0
411	0.0	0.0	0.0	0.0	0	0.0	0.0	0.0
674	0.0	0.0	0.0	0.0	0	0.0	0.0	0.0

Concentration-response relationships for juvenile production in fresh and weathered/aged TNB amended soils determined by nonlinear regressions are shown in Figure 5. Logistic (Gompertz) model had the best fit for data in tests with both freshly amended (Figure 5A) and weathered/aged amended (Figure 5B) soils. Overall, reproduction was higher in weathered/aged TNB amended soils. Juvenile production EC_{20} values based on total extraction were 21.4 and 13.2 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. Juvenile production EC_{50} values were 33.3 and 41.1 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. Cocoon production EC_{20} values were 27.2 and 18.2 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively (Table 26). Cocoon production EC_{50} values were 59.1 and 56.6 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively (Table 26). Juvenile production EC_{20} values based on ATCLP extractable concentrations were 6.6, and 5.8 mg kg^{-1} in freshly amended and weathered/aged soils, respectively (Table 26). Juvenile production EC_{50} values based on ATCLP extractable concentrations were 20.6 and 18.0 mg kg^{-1} in freshly amended and weathered/aged soils, respectively (Table 26). Cocoon production EC_{20} values based on ATCLP extractable concentrations were 13.4 and 8.4 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively (Table 26). Cocoon production EC_{50} values based on ATCLP extractable concentrations were 41.6 and 26.2 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively (Table 26). The differences between freshly amended and weathered/aged cocoon and juvenile production were not statistically significant (95% CI; Table 26), indicating that the 3-month weathering/aging process did not affect the toxicity of TNB to *E. fetida*.

Coefficients of Determination (R^2) for total and ATCLP based extractions of TNB were calculated in nonlinear regression analyses (EC_{20} levels) to determine which chemical measure better correlates with toxicity endpoints in both fresh and weathered/aged soils. The R^2 values for juveniles in freshly amended soil were 0.92 and 0.95 in total and ATCLP based extractions, respectively (Table 26). The R^2 values for cocoons in freshly amended soil were 0.94 and 0.96 in total and ATCLP based extractions, respectively. The R^2 values for juveniles in weathered/aged soil were 0.95 and 0.96 in total and ATCLP based extractions, respectively. The R^2 values for cocoons in weathered/aged soil were 0.96 and 0.97 in total and ATCLP based extractions, respectively. These comparisons show that regression coefficients were very similar for both extraction methods indicating that neither extraction method had an advantage in characterizing TNB bioavailability to *E. fetida*.

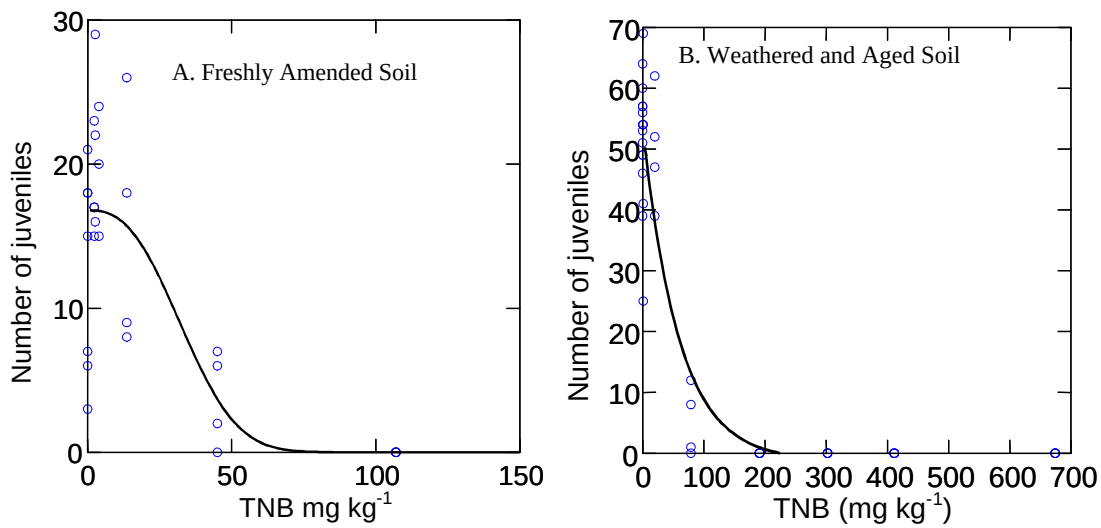

Figure 5. Non-linear regression (Logistic (Gompertz) model: $Y = a \times e^{([\log(1-p)]\times[C/ECp]b)}$) of TNB and *Eisenia fetida* juvenile production in freshly amended (A) and weathered/aged amended (B) Sassafras sandy loam soil. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Table 26. Ecotoxicological parameters (mg kg^{-1}) with P-value or confidence interval (C.I.) and coefficient of determination (R^2) for TNB in freshly amended and weathered/aged amended Sassafras sandy loam soil using earthworm reproduction test with *Eisenia fetida*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Exposure	Cocoon Production				Juvenile production			
	NOEC	LOEC	EC_{20}	EC_{50}	NOEC	LOEC	EC_{20}	EC_{50}
Fresh								
Total	13.6	45.0	27.2	59.1	13.6	45.0	21.4	33.3
P or 95% C.I.	0.09	0.0001	6.5-48.0	37.0-81.2	0.23	0.04	0-55.2	9.0-57.5
R^2			0.94	0.94			0.92	0.92
ATCLP	7.7	30.2	13.4	41.6	7.7	30.2	6.6	20.6
P or 95% C.I.	.194	.0001	6.0-20.8	18.5-64.6	.99	.002	0-13.3	0-41.3
R^2			0.95	0.95			0.81	0.81
Aged/weathered								
Total	19.9	78.7	18.2	56.6	19.9	78.7	13.2	41.1
P or 95% C.I.	0.13	0.0001	10.7-25.8	33.1-80.1	0.52	0.0001	7.3-19.1	22.6-59.5
R^2			0.96	0.96			0.95	0.95
ATCLP	6.2	41.8	8.4	26.2	6.2	41.8	5.8	18.0
P or 95% C.I.	0.17	0.0001	5.1-11.8	15.8-36.6	0.7	0.0001	3.1-8.5	9.5-26.5
R^2			0.97	0.97			0.96	0.96

Notes:
ND, Not Determined. ECx values could not be determined because cocoon and juvenile numbers were not significantly different in all treatment concentrations compared with carrier control. P-value was generated during ANOVA. C.I. and R^2 were generated during nonlinear regression analysis.

4. DISCUSSION

The present study supported the Eco-SSL requirements for establishing benchmark screening concentration levels for soil contaminants. The majority of soil toxicity test results that have been reported previously utilized standard artificial soil with high organic matter content (10%). In contrast, our toxicity studies used a natural soil that met the criteria for Eco-SSL development, in large part because it has characteristics supporting relatively high bioavailability of EMs. Most of the previous studies measured only lethal endpoints. We used reproductive as well as lethal endpoints. Our results showed that the reproductive endpoints were much more sensitive indicators of toxicity. In addition, our soil weathering/aging procedure allowed us to more realistically assess toxicity of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB under conditions more closely resembling those encountered in the field.

Definitive toxicity tests conducted with freshly amended soils showed that the order of EM toxicity, based on EC_{20} values for juvenile production with *E. fetida* was HMX >RDX > 2,6-DNT> TNB > 2,4-DNT. Definitive toxicity tests conducted with weathered/aged amended soils showed that EM toxicity order based on EC_{20} values for juvenile production in tests with *E. fetida* was RDX > 2,6-DNT > TNB > 2,4-DNT > HMX. Reproduction measurement endpoints in all tests were more sensitive compared with adult survival.

In this study, both cocoon and juvenile production were reduced at relatively low levels of RDX and HMX in freshly amended soils. Juvenile production was affected by RDX with EC_{20} estimates of 1.6 and 5 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively. However, some cocoons were still found at 148 mg kg^{-1} in the definitive tests and 5000 mg kg^{-1} in the range-finding tests. This may be due to low bioavailability of these energetic materials in soil. The solubility in water at 20°C of RDX and HMX is 42.3 and 6.63 mg L^{-1}, respectively (Roberts & Hartley 1992). The ATCLP extractable (and presumably bioavailable) fractions ranged from 100 to 18 percent of acetonitrile extractable concentration for RDX, and from >100 to 3 percent of acetonitrile extractable concentration for HMX. RDX and HMX did not affect adult *E. fetida* survival even at concentrations as high as 5,000 mg kg^{-1} in range-finding tests. Weathering and aging of RDX amended soil did not significantly (95% CI) affect its toxicity to *E. fetida*.

Results of RDX and HMX toxicity tests found in this study may not directly compare to those of other studies in the literature, since none of them were designed to meet Eco-SSL criteria of testing for soil invertebrates. Literature on the toxicity of RDX to terrestrial organisms is scant, and discrepancies are often found regarding the toxicity of the same chemical to different organisms. Significant sub lethal effects of RDX were observed on the reproduction of the earthworm *Eisenia andrei* at concentrations as low as 95 mg kg^{-1} soil (Robidoux *et al*., 2000). However, mortality and reproduction of the enchytraeid worm *E. crypticus* and collembolan *Folsomia candida* in soils spiked with up to 1000 mg kg^{-1} RDX in soil (Schafer and Achazi, 1999) were not affected. Furthermore, these studies were conducted in either standard artificial soil (Robidoux *et al*., 2000), or in soil with relatively high (2.5-3.0%) organic carbon (Schafer and Achazi, 1999), which limits their usefulness for describing natural systems or development of Eco-SSLs. The bioavailability of nonpolar organic chemicals in soil is hypothesized to be determined primarily by soil organic matter (OM) content (Belfroid et al.

1996). Sassafras sandy loam has 1.2% OM compared to 10% in artificial soil. These authors also suggest that bioaccumulation and toxicity are well correlated with the concentration of chemical in the soil solution or pore water, rather than total chemical levels. In the present study, total extractable and water extractable RDX and HMX showed no difference in correlation to toxicity.

HMX in freshly amended SSL soil was the most toxic of the five EM compounds tested in this study to *E. fetida* reproductive endpoints in freshly amended soil (cocoon EC_{20} = 2.7 mg kg^{-1}, juvenile EC_{20} = 0.4 mg kg^{-1}). HMX toxicity was greatly reduced after weathering and aging the soil. LOEC, EC_{20}, and EC_{50} could not be calculated for HMX in weathered/aged soil due to lack of significant differences among means in ANOVA and lack of fit in regression models. However, most of the HMX was still present in the acetonitrile fraction after weathering / aging (mean = 75%) and the mean HMX concentration in the ATCLP fraction actually increased slightly over time (128%). The cause of this decreased toxicity over time was beyond the scope of this study. The HMX may be in a different, less toxic form or be too tightly bound to soil or organic matter in the aged soil. Further testing is required to elucidate the cause.

Among the nitroaromatic compounds evaluated in our study, 2,6-DNT was most toxic. Comparison of our results to other studies that evaluated toxicity of nitroaromatic compounds is difficult because the toxicity of nitroaromatic energetics, including 2,4-DNT, 2,6-DNT and TNB to soil invertebrates has not been sufficiently investigated. The majority of studies reported in the available literature focused primarily on the effects of TNT and/or its degradation products (Renoux *et al*., 2000; Robidoux *et al*., 2000; 1999; Sunahara, *et al*., 2000; Rocheleau, *et al*., 1999; Schafer and Achazi, 1999; Simini, *et al*., 1995; Phillips, *et al*., 1993). Phillips *et al*. (1993) reported 100 percent mortality in the earthworm *E. fetida* growth and survival test in standard artificial soil amended with a mixture of EMs that included 30, 50, 62.5, and 20 mg kg^{-1} of TNT, TNB, 2,4-DNT and 2,6-DNT, respectively. Statistically significant ($p < 0.01$) sub lethal effects (mass loss) were reported at 6, 10, 12.5, and 4 mg kg^{-1} of TNT, TNB, 2,4-DNT and 2,6-DNT, respectively. These results are similar to findings of our investigations although direct comparisons of both studies are limited due to differences in the experimental designs.

Simini *et al*. (1995) assessed the toxicity of soil from Joliet Army Ammunition Plant contaminated with a mixture of EMs (which limits the direct comparisons with our study), including both nitroaromatic and nitro-heterocyclic compounds using earthworm *E. fetida* growth and survival test, among other bioassays. The highest soil concentrations measured at this site were 200, 117, and 8 mg $kg^{-1}$for TNB, 2,4-DNT and 2,6-DNT, respectively. The authors reported that linear regression of TNT and TNB data yielded the greatest coefficients of determination (R^2) in all bioassays, including the earthworm test. The R^2 values for TNB using earthworm test endpoints were 0.773 and 0.814 for two locations investigated at the study site. These values were 0.613 and 0.358 for 2,4-DNT, whereas 2,6-DNT had the weakest relationship with measurement points with R^2 values of 0.082 and 0.293 for the two locations, respectively. Soil TNB and 2,4-DNT concentrations found at this site were within the range of concentrations tested in our study and the results are consistent with our findings. The weak relationship between toxicity and 2,6-DNT is most likely due to very low concentrations of this EM measured at the site.

We incorporated the weathering and aging procedure to simulate more closely the exposure effects on soil invertebrates in the field. Weathering and aging of RDX amended soil for 90 days did not reduce RDX concentrations or significantly affect its toxicity to *E. fetida*. Weathering and aging soil for 90 days rendered HMX non-toxic to earthworm reproduction even though the soil concentration was not reduced. Further study is needed to elucidate the mechanisms responsible for reduced HMX toxicity in weathered/aged soils. Toxicity of 2,4-DNT, 2,6-DNT, and TNB was not altered by the weathering/aging process. Specific mechanisms of changes in the toxicity of EMs in weathered/aged amended soil are unknown. In some cases, degradation products produced during the weathering and aging process may be more toxic to soil organisms compared with the parent material. Dodard *et al*. (1999) investigated the toxic effects of 2,4-DNT and 2,6-DNT, and their respective metabolites using the 15-min Microtox (*Vibrio fischeri*) and 96-h freshwater green alga (*S. capricornutum*) growth inhibition tests. The toxicities of DNTs were species-dependent: 2,4-DNT was more toxic than 2,6-DNT to *S. capricornutum* (comports with our results for *E. crypticus*), while the reverse was true in the test with *Vibrio fischeri*. The authors reported that the reduced metabolites of 2,6-DNT tested were less toxic compared to the toxicity of parent compound. However, certain partially reduced metabolites of 2,4-DNT (4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene) were more toxic than the parent compound. Although these results cannot be directly compared to our study because the biotic reductive degradation pathway for 2,4-DNT and 2,6-DNT in aquatic environment would contrast with metabolic processes in the aerobic conditions of vadose zone simulated in our investigations, the reducing environment can exist in water-logged soil microsites, where more toxic metabolites of dinitrotoluene degradation can be present.

The exposure concentrations of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB in soil were determined using both acetonitrile extraction (total chemical measure) and water extraction methods. Water extractable portion of each EM was determined using Adapted Toxicity Characteristic Leaching Procedure (ATCLP) to establish if this technique, which is designed to measure the leachable, and presumably bioavailable fraction of chemicals in soil, could generate data that is better correlated with toxicity than total chemical measurement. Coefficients of determinations (R^2) calculated by non linear regression analysis of acetonitrile extraction data were compared to R^2 values from ATCLP extraction data. to determine which chemical measure of exposure better correlated with toxicity. These comparisons showed that R^2 values were very similar in both exposure types. Therefore, neither extraction method had an advantage in characterizing toxicity of EMs tested to *E. fetida* in this study. This result supports our decision to develop Eco-SSLs for explosives contaminants in soil on the basis of acetonitrile extraction of test compounds. Acetonitrile extraction-based Eco-SSLs will be especially useful for Ecological Risk Assessment at contaminated sites because EM concentrations determined during site characterization are usually based on total extraction by US EPA method 8330.

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APPENDIX C

TOXICITY OF NITRO-HETEROCYCLIC AND NITROAROMATIC ENERGETIC MATERIALS TO ENCHYTRAEID WORM *ENCHYTRAEUS CRYPTICUS* IN A NATURAL SANDY LOAM SOIL

ECBC-TR-XXX

TOXICITY OF NITRO-HETEROCYCLIC AND NITROAROMATIC ENERGETIC MATERIALS TO ENCHYTRAEID WORM *Enchytraeus crypticus* IN A NATURAL SANDY LOAM SOIL

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13. ABSTRACT *(Maximum 200 words)*

USEPA is developing Ecological Soil Screening Level (Eco-SSL) values for ecological risk assessment of contaminants at Superfund sites. Insufficient information for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB to generate Eco-SSLs necessitated standardized toxicity testing to fill the data gaps. We adapted the Enchytraeid Reproduction Test (ISO/16387:2001) using *Enchytraeus crypticus* in these studies. Tests were conducted in Sassafras sandy loam soil, which supports relatively high bioavailability of energetic materials. Weathering/aging procedures for amended soil were incorporated in the study to better reflect the exposure conditions in the field soils. Definitive toxicity tests conducted with both freshly amended and weathered/aged amended soils showed that EM toxicity order based on EC_{20} values for juvenile production in tests with *E. crypticus* was TNB > 2,4-DNT > 2,6-DNT > RDX with EC_{20} values of 45, 116, 194, and 585 mg kg^{-1}, respectively. HMX did not adversely affect adult survival or juvenile production up to 21750 mg kg^{-1} treatment. These study results will be provided to the Ecological Soil Screening Level (Eco-SSL) workgroup for review and inclusion in the Eco-SSL database, and for developing Ecological Soil Screening Levels (Eco-SSLs) for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB.

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PREFACE

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TOXICITY OF NITRO-HETEROCYCLIC AND NITROAROMATIC ENERGETIC MATERIALS TO ENCHYTRAEID WORM *Enchytraeus crypticus* IN A NATURAL SANDY LOAM SOIL

1. INTRODUCTION

Many sites associated with military operations that involve munition manufacturing, disposal, testing, and training contain elevated levels of explosives and related materials in soil. Concentrations of explosives in soil have been reported to exceed 87,000 mg kg^{-1} for TNT and 3,000 mg kg^{-1} for RDX and HMX (Simini *et al*., 1995). Although the energetic materials (EM) RDX and HMX are persistent and highly mobile in the environment, their effects on soil biota have not been sufficiently investigated. Scientifically based ecological soil screening levels (Eco-SSLs) are needed to identify contaminant explosive levels in soil that present an acceptable ecological risk. To address this problem, the U.S. Environmental Protection Agency (USEPA) in conjunction with stakeholders is developing Eco-SSLs for contaminants frequently found at Superfund sites. Eco-SSLs are defined as concentrations of chemicals in soil that, when not exceeded, will be protective of terrestrial ecosystems from unacceptable harmful effects. These Eco-SSL concentrations can be used in a Screening Level Ecological Risk Assessment (ERA) to identify those contaminants in soil that warrant additional evaluation in a Baseline ERA, and to eliminate those that do not. Eco-SSLs are derived using published data generated from laboratory toxicity tests with different test species relevant to soil ecosystems. The Eco-SSL workgroup, after an extensive literature review (USEPA, 2000), determined that there was insufficient information for explosives to generate Eco-SSLs for soil invertebrates, which necessitated our study to fill this knowledge gap.

This study was designed to produce benchmark data for the development of Eco-SSLs for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), and 1,3,5-trinitrobenzene (TNB) for soil invertebrates, and meet specific criteria (USEPA, 2000), including: (1) tests were conducted in soil having physico-chemical characteristics that support relatively high bioavailability of chemicals; (2) experimental designs for laboratory studies were documented and appropriate; (3) both nominal and analytically determined concentrations of chemicals of interest were reported; (4) tests included both negative and positive controls; (5) chronic or life cycle tests were used; (6) appropriate chemical dosing procedures were reported; (7) concentration-response relationships were reported; (8) statistical tests used to calculate the benchmark and level of significance were described; and (9) the origin of test species were specified and appropriate.

Several soil invertebrate toxicity tests, for which standardized protocols have been developed, could be used effectively to assess the toxicity and to derive protective benchmark values for energetic materials (Stephenson *et al*., 2002; Løkke and Van Gestel, 1998). We adapted the Enchytraeid Reproduction Test (ISO/16387: 2001) for use in these studies. This bioassay was selected on the basis of its ability to measure chemical toxicity to ecologically relevant test species during chronic assays, and its inclusion of at least one reproductive

component among the measurement endpoints. The primary objective of these studies was to quantify EM toxicities to the soil invertebrate *Enchytraeus crypticus* for production of benchmark data that can be used in development of Eco-SSLs for explosive contaminants in soil. The Enchytraeid Reproduction Test was specifically modified to comply with Eco-SSL testing conditions.

2. MATERIAL AND METHODS

2.1 Test Soil.

A natural soil, Sassafras sandy loam [Fine-loamy, siliceous, mesic Typic Hapludult] (SSL) was used in this study to assess the toxicity of test chemicals to *E. crypticus*. This soil was selected for developing ecotoxicological values protective of soil biota because it has physical and chemical characteristics supporting relatively high bioavailability of the test chemicals (low organic matter and clay contents). The SSL soil was collected from an open grassland field on the property of the U.S. Army Aberdeen Proving Ground (APG; Edgewood, MD). Vegetation and the organic horizon were removed to just below the root zone and the top six inches of the A horizon were then collected. The soil was sieved through a 5-mm^2 mesh screen, air-dried for at least 72 hours and mixed periodically to ensure uniform drying, passed through a 2-mm sieve, then stored at room temperature before use in testing. Soil was analyzed for physical and chemical characteristics by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD. Results of these analyses are presented in Table 1.

Table 1. Physical and chemical characteristics of Sassafras sandy loam soil analyzed by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD.

Soil Parameter	Sassafras Sandy Loam
Sand %	69
Silt %	13
Clay %	17
Texture	Sandy loam
CEC cmol kg^{-1}	5.5
Organic matter %	1.2
pH	5.2

2.2 Test Chemicals.

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX; CAS: 121-82-4; Purity: 99%), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX; CAS: 2691-41-0; Purity: 99%), 2,4-dinitrotoluene (2,4-DNT; CAS: 121-14-2; Purity: 97%), 2,6-dinitrotoluene (2,6-DNT; CAS: 606-

20-2; Purity: 98%), and 1,3,5-trinitrobenzene (TNB; CAS: 99-35-4; Purity: 99.7%) were obtained from the Defense Research Establishment Valcartier of the Canadian Ministry of National Defense (Val Bélair, QC, Canada). Beryllium sulfate ($BeSO_4 \cdot 4H_2O$; CAS: 7787; Purity: 99.99%) was used as the positive control in these tests. Acetone (CAS: 67-64-1; HPLC Grade) was used for preparing EM solutions during soil amendments. Acetonitrile (CAS: 75-05-8; HPLC Grade) was used for extractions for chemical analyses. Methanol (CAS: 67-56-1, Chromatography grade, Purity: 99.9%) was used in determinations by HPLC. Certified standards of the energetics (AccuStandard, Inc., New Haven, CT) were used during HPLC determinations. Unless otherwise specified, ASTM type I water (American Society of Testing and Materials, http://www.astm.org) obtained using Milli-RO® 10 Plus followed by Milli-Q® PF Plus systems (Millipore®, Bedford, MA) was used throughout the studies. Glassware was washed with phosphate-free detergent, followed by rinses with tap water, ASTM type II water, analytical reagent grade nitric acid 1% (v/v), then with ASTM type I water.

2.3 Soil Amendment Procedures.

Sassafras sandy loam soil was individually amended with RDX, HMX, 2,4-DNT, 2,6-DNT or TNB. Each treatment concentration of EM for range-finding tests was prepared separately in glass volumetric flasks and dissolved in acetone. This was necessary to dissolve the nonpolar chemicals, giving a more homogeneous mixture than the addition of solid chemical crystals to soil. Soil was spread to a thickness of 2.5 cm. The EM/acetone solution was pipetted evenly across the soil surface, ensuring that the volume of solution added at any one time did not exceed 15% (v m^{-1}) of the dry mass soil. After addition of the EM solution, the volumetric flask was rinsed twice with a known volume of acetone and pipetted onto the soil. If the acetonitrile-extractable volume of solution needed to amend the soil exceeded 15% (v m^{-1}), the solution was added in successive stages, allowing the acetone to evaporate for a minimum of 2 hours under a chemical hood. The same total EM/acetone solution volume at different EM concentrations was added to every treatment, equating the volume required to dissolve the EM at the highest concentration tested. Amended soil was then air-dried overnight (minimum of 18 hours) in a dark chemical hood to prevent photolysis of the EM. Each amended soil sample was transferred into a fluorocarbon-coated high-density polyethylene container and mixed for 18 hours on a three-dimensional rotary mixer. Initial concentrations of EMs for toxicity tests were prepared by adding test chemicals into an aliquot of SSL soil. The final nominal target treatment concentrations for definitive tests with 2,4-DNT; 2,6-DNT; or TNB were prepared by mixing initially-prepared soil amended with the appropriate EM with clean SSL soil for 18 hours on a three-dimensional rotary mixer. Treatment concentrations of RDX or HMX were prepared individually using direct amendments of EM/acetone mixtures to achieve nominal target concentrations. The exceptions were 10,000 and 20,000 mg kg^{-1} treatments, which exceeded solubility levels of RDX or HMX in acetone carrier. These were prepared by adding appropriate amounts of dry crystalline RDX or HMX to clean SSL soil. The same acetone volume was added to every RDX or HMX concentration treatment. Carrier controls were treated with the carrier solvent only. After three-dimensional mixing, soil was hydrated with ASTM type I water to 100% of the soil water holding capacity (WHC; 18% water, on a the basis of the dry soil mass) for toxicity testing, or 60% of the WHC for the weathering/aging procedure. Hydrated soil prepared for toxicity tests was allowed to equilibrate for 24 hours before exposing potworms.

2.4 Measurement of Soil pH.

The pH of the test soils were determined at the beginning of each definitive toxicity test using a method adapted from the Soil Survey Laboratory Methods Manual (USDA, 1996). The pH electrode was rinsed thoroughly with ASTM type I water, blotted dry, standardized with pH 4 and pH 7 buffers, rinsed and blotted. Five grams of ASTM type I water was added to 5 g soil. The soil slurry was Vortexed for 10 seconds every five minutes for 30 minutes. The soil slurry was then Vortexed for 10 seconds one minute before pH measurement. The pH was measured in the solution above the soil surface while stirring gently until the reading stabilized. The electrode was rinsed with ASTM type I water and blotted.

2.5 Treatment Concentrations.

2.5.1 Range-finding tests.

Range-finding tests were conducted with freshly amended soils to determine treatment concentrations for definitive tests. Soils used in range-finding tests were amended with nominal RDX or HMX concentrations of 10, 100, 500, 1000, 5000 and 10000 mg kg^{-1}. Nominal EM test concentrations selected for the range-finding tests with 2,4-DNT and 2,6-DNT were 0, 10, 50, 100, 500, and 1,000 mg kg^{-1}. Concentrations selected for the range-finding test with TNB were 0, 10, 50, 100, 500, 1000, 5000, and 10000 mg kg^{-1}.

2.5.2 Definitive tests.

Data from the range finding tests were used to determine the treatment concentrations for definitive tests. Definitive tests to assess the independent effects of EMs were conducted in freshly amended and weathered/aged amended SSL soil. Nominal RDX or HMX concentrations selected for the definitive tests in freshly amended soil included 0, 300, 600, 1200, 2400, 4800, 10000, 20000 mg kg^{-1}, and 0, 300, 600, 1200, 2500, 5000, 10000, 20000 mg kg^{-1}, respectively. Nominal concentrations selected for the definitive tests in SSL soil freshly amended with 2,4-DNT or 2,6-DNT were 0, 2, 4, 8, 12, 24, 48, 64, and 80 mg kg^{-1}. Nominal concentrations selected for the definitive test with TNB freshly amended soil were 0, 4, 8, 16, 32, 64, 128, 256, and 384 mg kg^{-1}. Nominal test chemical concentrations selected for the definitive tests in weathered/aged amended SSL soil were:

RDX (mg kg^{-1}) 0, 1200, 2400, 4800, 10000, 20000
2,4-DNT (mg kg^{-1}) 0, 8, 12, 24, 48, 64, 80, 160, 320
2,6-DNT (mg kg^{-1}) 0, 24, 48, 64, 80, 160, 320
TNB (mg kg^{-1}) 0, 16, 32, 64, 128, 256, 384

Limit test was conducted with weathered/aged HMX amended SSL soil using 0 and 20000 mg kg^{-1} treatments. All definitive tests included carrier (acetone) controls and positive controls. Positive controls were prepared as solution of beryllium sulfate in ASTM type I water

using 45 mg kg^{-1} Be nominal concentrations in all tests with 2,4-DNT, 2,6-DNT and TNB. Nominal beryllium concentration 50 or 47 mg kg^{-1} was used in freshly amended or weathered/aged amended SSL soil, respectively in tests with RDX or HMX. Nominal test concentrations of all energetic compounds were verified using USEPA Method 8330 (USEPA, 1998).

2.6 Weathering/Aging of Amended Soil.

Standardized methods for weathering/aging of explosives in soil are not available. We have developed approaches that simulate, at least partially, the weathering and aging process in soil and more closely approximate the exposure effects on soil biota in the field. This included exposing both treated and control soils, initially hydrated to 60 percent of the WHC, in open Teflon®-coated chemically inert containers in the green house to alternating wetting and drying cycles for three months. All soil treatments were weighed and readjusted to their initial mass by adding ASTM type I water twice each week. All soil treatments were brought to 100% of the WHC (18% water, on the basis of the dry soil mass) 24 hours prior to commencement of toxicity tests for initiation of bioassays. The effect of weathering and aging on EM ecotoxicity was determined by comparing test results in weathered/aged soils with those obtained using freshly amended soils.

2.7 Chemical Extractions and Analyses.

Acetonitrile extractions of soils were performed according to USEPA Method 8330 at the beginning of each definitive test, using freshly amended or weathered/aged amended soils, respectively. Samples for chemical analysis were taken after the 24-h hydration. For each treatment, 2.0 g soil was weighed in triplicate into 50-mL polypropylene centrifuge tubes, 10 mL acetonitrile was added and the samples vortexed for 1 min, then sonicated in the dark for 18 hours at 20°C. Five mL of sonicated sample were transferred to a glass tube, to which 5 mL of $CaCl_2$ solution (5 g L^{-1}) was added. Supernatant was filtered through 0.45 µm PTFE syringe cartridges. Soil extracts were analyzed and quantified using an HPLC. In this report, acetonitrile soil extraction is reported as the concentration in dry soil.

In addition to acetonitrile extraction, soil samples were extracted using an Adapted Toxicity Characteristic Leaching Procedure (ATCLP; Haley *et al*., 1993) at the beginning of each definitive test with freshly amended or weathered/aged amended soils. The ATCLP is based on modification of the Toxicity Characteristic Leaching Procedure (TCLP) (40 CFR Part 268.41, Hazardous Waste Management, Method 1311). The modification involved substitution of CO_2-saturated ASTM type I water for acetic acid, better simulating soil-water conditions due to respiration by soil biota. Prior to ATCLP extraction, soil samples were equilibrated in the dark for 24 h at room temperature, after addition of ASTM type I water (60% of WHC). All analytical measurements were done in triplicate at the beginning of each test. For each treatment concentration, 4 g of soil were transferred into 20 mL vials. Sixteen mL of CO_2-saturated water at pH 4.0 was added to the vials, then vials were rapidly sealed tight. Soil samples were vortexed 45 sec, then mixed in the dark for 18 hours using a rotary mixer (30 rpm) at room temperature. Soil solids were allowed to settle, then supernatants were filtered through

0.45 μm PTFE syringe cartridges. An equivalent volume of acetonitrile was added to filtered soil extract prior to HPLC analysis. In this report, ATCLP soil extraction is referred to as the water-soluble fraction of EM.

The soil extracts were analyzed by reversed-phase HPLC using a modified EPA Method 8330. The method was modified in two ways. First, the final solvent for the energetic compounds was a mixture of 60 parts water and 40 parts acetonitrile rather than a 50:50 ratio. Secondly, the flow rate of the 50:50 methanol:water mobile phase was 1.0 ml/min rather than 1.5 ml/min as the method calls for. A 25 cm x 4.6 mm x 5 micron particle size C-18 column was used for all determinations since only one energetic compound was analyzed at a time. The instrument used was a Beckman *System Gold*, consisting of a model 126 programmable solvent module, model 168 diode array detector and a model 507 automatic sampler. Calibration curves were generated before each HPLC run by dissolving certified standards (AccuStandard, Inc., New Haven, CT) of RDX and HMX in 60:40 water:acetonitrile in a range of concentrations appropriate for each run. The method detection limit was 0.05 mg kg^{-1}. Blanks and standards were placed intermittently between unknown samples to maintain quality assurance of the samples. All reagents used in extraction of chemicals from soils were either reagent or trace metal grade, and ASTM Type I water was used throughout the analytical studies. Glassware was washed with phosphate-free detergent followed by rinses with tap water, ASTM type II water, nitric acid 1% (v/v) and, again with ASTM type I water. Nominal and determined (measured) concentrations used in the definitive tests are shown in Tables 2 through 10.

2.8 Toxicity Assessment.

The Enchytraeid Reproduction Test (ERT) was used to assess the effects of EMs on the reproduction of the enchytraeid worm *Enchytraeus crypticus*. The test is an adaptation of an International Standardization Organization (ISO) bioassay ISO/16387 *Soil quality — Effects of pollutants on Enchytraeidae (Enchytraeus sp.) — Determination of effects on reproduction and survival* (ISO, 2001). The ERT is a Chronic/Life-Cycle Assay. The ISO Guideline for this assay was originally developed for use with Artificial Soil (USEPA Standard Artificial Soil), however our research showed that this test could also be conducted using natural soils (Kuperman *et al*., 1999; 2003). The ISO ERT was initially developed using the enchytraeid worm species *Enchytraeus albidus*. Results of our previous studies using *E. albidus* showed that this species requires soils containing high organic matter content with a soil pH 6 (±0.5) for optimal test conditions. This species performed poorly in natural soils with physical and chemical characteristics that support a higher level of EM bioavailability (Kuperman *et al*., 1999). The species of Enchytraeidae, *E. crypticus*, listed in the ISO protocol as an acceptable alternative to *E. albidus*, was selected for toxicity testing.

2.8.1 Principle of the Test.

Adult *E. crypticus* are exposed to a range of concentrations of the test chemical added to soil. The test consists of two steps. They are a range-finding test in which adult survival and total number of juveniles produced are assessed using few treatment concentrations (five) and

reduced number of replicates (two), and a definitive test in which the same endpoints are assessed using greater number of concentrations and replicates. The duration of each test is four weeks. After the first two weeks, the adult worms are removed, counted, and any morphological changes are recorded. After an additional two-week exposure, the number of juveniles produced is counted. The number of adults and juveniles in treatment concentrations are compared to numbers in the control(s) to quantify ecotoxicological parameters. These parameters include the bounded No Observed Effect Concentration (NOEC), the bounded Lowest Observed Effect Concentration (LOEC) and the effective concentration that causes a p percent reduction in juvenile numbers, ECp (e.g., EC_{20}, EC_{50}).

2.8.2 Test Validity Criteria.

The validity criteria are included in the test as part of the Quality Control procedures. They include the following performance parameters for the negative controls:

1) The adult mortality does not exceed 20% after 14 days, in the range-finding and definitive tests;
2) The average number of juvenile potworms per test container at the end of the test is greater than 2.5x the initial number of adult potworms per test container;
3) The coefficient of variation for the mean number of juveniles is ≤50% at the end of the test

2.8.3 Culturing Conditions.

Enchytraeid potworms were bred in 4.3-L clear plastic boxes (34 x 20 x 10 cm) filled with 2 kg (dry mass) SSL soil. The culture was kept in an incubator at 22±1°C with continuous light. Soil moisture level was adjusted to 100% of WHC, and was maintained by periodic (once per week) mass checks and water adjustments. Soil in the breeding culture was aerated by carefully mixing it once per week.

The potworms were fed approximately twice a week with ground oats spread on the soil surface. If food from the previous feeding date remained on the soil surface, the amount of food given was adjusted. Every 2-3 months, the worms were transferred into a freshly prepared culture substrate.

Culturing conditions were regarded satisfactory if:

- Worms did not try to leave soil
- They moved quickly through the soil
- They exhibited a shiny outer surface without soil particles clinging to it
- They were whitish in color
- Worms of different ages were present

The potworm culture was considered healthy if worms reproduced continuously.

2.8.4 Test Performance.

Glass test containers (42 mm ID; 45 mm deep) were rinsed with acetone, tap water, and ASTM type I water before the test. Twenty grams of prepared soil hydrated to 100% of the WHC were added to each test container and 0.05 g of grounded oats were mixed with soil. The mass of each container with soil was recorded. Each treatment and controls were replicated four times for definitive tests (two for range-finding tests). Limit test with weathered/aged HMX amended SSL soil included eight replicates of treatment soils and four replicates of negative or positive controls

Enchytraeid adult potworms with eggs in the clitellum region were collected from culture established in the same soil type (SSL) as soil used in the test. The selected worms were placed in a petri dish filled with a small amount of ASTM type I water for examination using a stereomicroscope. Worms with no eggs were discarded. Any invertebrates living in the cultures (such as mites) were also removed. Ten enchytraeid worms selected for uniformity (approximately 1 cm in length) were placed on top of prepared soil in each test container. Plastic wrap was stretched over the top of each container and secured with a rubber band. Three pinholes were made in the plastic wrap to facilitate air exchange. All containers were placed in an environment-controlled incubator at 22±1°C, 16 h photoperiod. The containers were weighed once a week and the mass loss was replenished with the appropriate amount of ASTM type I water. Ground oats (0.05 g) were added to each test container at that time.

After two weeks, soil in each test container was carefully searched and adult potworms were removed and counted. Potworms were examined for any morphological or behavioral changes. The remaining test substrate, including any cocoons laid during the first two weeks of the test, was incubated for additional two weeks. After four weeks from the start of the test, soil in the test containers was fixed with 70% ethanol, and seven drops of Rosebengal biological stain (1% solution in ethanol) was added. Staining continued for minimum of 24 hours. The content of each test container was wet-sieved using a No. 100 (150 μm) mesh sieve and retained contents transferred to a counting tray where potworms were counted. Measurement endpoints included number of surviving adults after 14 days and number of juveniles produced after 28 days.

2.9 Data Analysis.

Juvenile production data were analyzed using nonlinear regression models described in Stephenson *et al*. (2000) and Kuperman *et al*. (2003). Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to decide whether or not to weight the data, and to select potential models. The logistic (Gompertz) model [1] had the best fit for data in all toxicity tests except the test with SSL soil freshly amended with TNB where the logistic hormetic model [2] with an additional parameter to accommodate hormesis best fit the data. The best fit of the lines generated by these models were closest to the data points, the variances were the smallest, and the residuals had the best appearance (i.e., most random scattering). These models were:

$$Y = a \times e([\log(1\text{-}p)] \times [C/\text{ECp}]^{b}) \quad [1]$$

$$Y = (t \times [1 + hC] / \{1 + [(p + h\,\text{ECp}) / (1 - p)] \times [C/\text{ECp}]^{b}\} \quad [2]$$

where Y is the number of juveniles produced, a is the control response, e is the base of the natural logarithm, p is the percent inhibition/100 (e.g., 0.50 for EC_{50}), C is the exposure concentration in test soil, ECp is the estimate of effect concentration for a specified percent effect, t is the control response in the hormetic model, h is the hormetic effect parameter, and b is the scale parameter. The ECp parameters used in this study included the EM concentration producing a 20% (EC_{20}) or 50% (EC_{50}) reduction in the measurement endpoint. The EC_{20} parameter based on a reproduction endpoint is the preferred parameter for deriving soil invertebrate Eco-SSL values. The EC_{50}, a commonly reported value, and survival data were included to enable comparisons of the results produced in this study with results reported by other researchers. The asymptotic standard error (a.s.e.) and 95% confidence intervals (CI) associated with the point estimates were determined.

Analysis of Variance (ANOVA) was used to determine the bounded No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values for adult survival or juvenile production data. Mean separations were done using Fisher's Least Significant Difference (LSD) pairwise comparison tests. Student's *t*-Test was used in the limit test with weathered/aged HMX amended soil. A significance level of $p < 0.05$ was accepted for determining the NOEC and LOEC values. When NOAEC (bounded no observed adverse effect concentration) or LOAEC (bounded lowest observed adverse effect concentration) values were determined, the same statistical methods were used. All analyses were done using measured EM concentrations. Statistical analyses were performed using SYSTAT 7.0.1 (SPSS, 1997).

3. RESULTS

3.1 Analytical Determinations of Energetic Materials in Soil.

Concentrations of EMs in amended soils were determined at the beginning of each definitive toxicity test using both acetonitrile and ATCLP extractions. Results of these analyses are shown in Tables 2 through 10. Measured acetonitrile-extractable RDX concentrations in freshly amended soils averaged 101 (range: 92-109) percent of nominal concentrations. Measured RDX ATCLP-extractable concentrations averaged 9.6 (range: 0.4-34) percent of acetonitrile-extractable concentrations due to low solubility of RDX in water (Table 2). Measured RDX ATCLP-extractable concentrations in weathered/aged amended soils averaged 3 (range: 0.5-8.0) percent of acetonitrile-extractable concentrations (Table 3).

Weathering/aging of amended soils reduced acetonitrile-extractable RDX concentrations, on average, by 7 percent compared with acetonitrile-extractable concentrations in freshly amended soils (Table 3). ATCLP-extractable RDX concentrations were reduced, on average, by 16 percent compared with freshly amended soils.

Measured HMX acetonitrile-extractable concentrations in freshly amended soils averaged 111 (range: 88-124) percent of nominal concentrations. Measured HMX ATCLP-extractable concentrations remained relatively stable and averaged one (range: 0.5-8.0) percent of acetonitrile-extractable concentrations (Table 4).

Weathering/aging of HMX amended soil reduced acetonitrile-extractable HMX concentration by 20 percent from 21750 to 17498 mg kg^{-1} in the single treatment used in the limit test. Measured HMX ATCLP-extractable concentration in weathered/aged soil was 18 mg kg^{-1} (0.1% of acetonitrile-extractable concentration). The ATCLP-extractable portion of HMX increased by 43 percent in the single treatment used in the limit test with weathered/aged HMX amended soil.

2,4-DNT acetonitrile-extractable concentrations in freshly amended soils averaged 82 (range: 48-86) percent of nominal concentrations (Table 5). 2,4-DNT ATCLP-extractable concentrations averaged 43 (range: 19-84) percent of acetonitrile-extractable concentrations (Table 5). 2,4-DNT ATCLP-extractable concentrations averaged 55 (range: 46-70) percent of acetonitrile-extractable concentrations (Table 6).

Weathering/aging of amended soil reduced acetonitrile-extractable 2,4-DNT concentrations, on average, by 46, and ATCLP-extractable 2,4-DNT concentrations by 18 percent compared with respective concentrations in freshly amended soil.

2,6-DNT acetonitrile-extractable concentrations in freshly amended soils averaged 126 (range: 80-267) percent of nominal concentrations (Table 7). 2,6-DNT ATCLP-extractable concentrations increased proportionally with their acetonitrile-extractable concentrations and averaged 48 (range: 27-65) percent of acetonitrile-extractable concentrations (Table 7). 2,6-DNT ATCLP-extractable concentrations averaged 49 (range: 40-62) percent of acetonitrile-extractable concentrations in weathered/aged amended soil (Table 8).

Weathering/aging of amended soil reduced acetonitrile-extractable 2,6-DNT concentrations, on average, by 76% compared with acetonitrile-extractable concentrations in freshly amended soil, while ATCLP-extractable 2,6-DNT concentrations were reduced, on average, by 81 percent compared with freshly amended soil.

TNB recovery was greatly reduced in treatments below 45 mg kg^{-1}. TNB acetonitrile-extractable concentrations in freshly amended soil averaged 62 (range: 25-100) percent of nominal concentrations (Table 9). TNB ATCLP-extractable concentrations averaged 73 (range: 56-86) percent of acetonitrile-extractable concentrations (Table 9). These values do not include data for 8 mg kg^{-1} nominal treatment concentration, which had TNB recovery in one (0.13 mg kg^{-1}) out of three replicates producing an average ATCLP-extractable value of 0.043 mg kg^{-1} (Table 9). TNB ATCLP-extractable concentrations averaged 55 (range: 19-93) percent of acetonitrile-extractable concentrations in weathered/aged amended soil (Table 10).

Weathering/aging of amended soil reduced acetonitrile-extractable TNB concentrations, on average, by 54% compared with acetonitrile-extractable concentrations in

freshly amended soil, while ATCLP extractable TNB concentrations were reduced, on average, by 59 percent compared with freshly amended soil.

Table 2. Nominal and measured (mean, n = 3) RDX concentrations (mg kg^{-1}) in freshly amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (U.S. EPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
300	304	18.6	101.3	102.4	6.4	33.7
600	656	18.7	109.3	95.3	18.3	14.5
1200	1194	22.3	99.5	114.8	3.38	9.6
2400	2203	72.1	91.8	114.7	25.1	5.2
4800	4558	143.2	95.0	122.9	2.9	2.7
10000	10062	366.0	100.6	75.9	0.8	0.8
20000	21383	1205.5	106.9	107.5	15.6	0.4

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

Table 3. Nominal and measured (mean, n = 3) RDX concentrations (mg kg^{-1}) in weathered/aged amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (U.S. EPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
1200	1048	19.9	87.4	83.5	0.6	8.0
2400	2379	40.5	99.1	86.8	1.1	3.6
4800	3985	42.7	83.0	86.0	3.4	2.2
10000	9549	371.1	95.5	89.0	2.1	0.9
20000	18347	518.4	91.7	89.2	1.0	0.5

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

Table 4. Nominal and measured (mean, n = 3) HMX concentrations (mg kg^{-1}) in freshly amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (U.S. EPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
300	348	10.4	116	12.3	0.2	3.5
600	642	8.7	107	12.5	0.2	1.9
1200	1491	63.3	124	12.9	0.3	0.9
2500	2211	119.5	88	12.6	0.5	0.6
5000	5785	182.5	115	12.5	0.1	0.2
10000	10586	272.7	106	12.0	0.3	0.1
20000	21750	496.4	109	12.6	0.1	0.1

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

Table 5. Nominal and measured (mean, n = 3) 2,4-DNT concentrations (mg kg^{-1}) in freshly amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (U.S. EPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
2	0.95	0.2	48	0.80	0.001	84
4	3.0	0.3	74	1.34	0.01	45
8	6.5	0.4	81	2.40	0.05	37
12	9.9	0.5	82	4.96	0.01	50
24	20.3	0.3	85	3.77	0.04	19
48	40.9	2.6	85	8.13	0.08	20
64	55.0	0.5	86	33.45	0.22	61
80	64.7	1.5	81	43.37	0.09	67

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

Table 6. Nominal and measured (mean, n = 3) 2,4-DNT concentrations (mg kg^{-1}) in weathered/aged amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (U.S. EPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
8	3.0	0.5	37	1.67	0.03	56
12	5.2	0.2	43	2.42	0.06	47
24	11.5	0.2	48	5.22	0.02	46
48	21.5	0.3	45	11.77	0.12	55
64	31.0	0.8	48	15.40	0.15	50
80	37.3	0.8	47	20.47	0.37	55
160	71.7	2.3	45	46.07	0.37	64
320	178.7	8.4	56	125.00	2.00	70

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

Table 7. Nominal and measured (mean, n = 3) 2,6-DNT concentrations (mg kg^{-1}) in freshly amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (U.S. EPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
2	5.3	0.1	267	1.43	0.01	27
4	7.7	0.9	191	2.18	0.01	28
8	9.4	0.3	117	3.78	0.01	40
12	12.9	0.2	108	5.83	0.04	45
24	20.0	0.8	83	10.63	0.08	53
48	40.2	2.0	84	24.84	0.04	62
64	51.1	1.0	80	32.94	0.12	65
80	64.0	1.6	80	40.50	0.11	63

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

Table 8. Nominal and measured (mean, n = 3) 2,6-DNT concentrations (mg kg^{-1}) in weathered/aged amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (U.S. EPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
24	3.7	0.08	15	1.46	0.06	40
48	9.5	0.12	20	4.30	0.09	45
64	13.9	0.12	22	6.63	0.08	48
80	18.1	0.20	23	9.64	0.36	53
160	37.4	0.98	23	17.43	3.27	47
320	108.3	1.45	34	66.87	2.22	62

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

Table 9. Nominal and measured (mean, n = 3) TNB concentrations (mg kg^{-1}) in freshly amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (U.S. EPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
4	2.3	0.08	58	BDL	BDL	BDL
8	2.6	0.11	32	0.043*	0.043*	1.7*
16	3.9	0.48	25	2.45	0.29	62
32	13.6	1.11	43	7.68	0.25	56
64	45.0	1.80	70	30.22	0.52	67
128	107.0	2.52	84	83.67	1.28	78
256	221.0	12.66	86	190.95	1.40	86
384	384.7	21.15	100	328.28	14.80	85

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.
* TNB was recovered in one (0.13 mg kg^{-1}) out of three replicates producing an average ATCLP extractable value of 0.043 mg kg^{-1}.

Table 10. Nominal and measured (mean, n = 3) TNB concentrations (mg kg^{-1}) in weathered/aged amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (U.S. EPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
16	0.6	0.07	4	0.14	0.01	25
32	1.3	0.15	4	0.24	0.01	19
64	8.8	0.38	14	3.35	0.33	38
128	75.8	0.27	59	55.80	1.89	74
256	176.3	5.67	69	143.40	2.15	81
384	304.7	7.84	79	284.38	7.50	93

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

3.2 Range-Finding Toxicity Tests.

Either RDX or HMX had little or no effect on adult survival in the range-finding tests in all treatment concentrations. Juvenile numbers were reduced by 21 ($p = 0.118$) percent in 1,000 mg kg^{-1} RDX treatment and by 30 ($p < 0.041$) percent in both 5,000 and 10,000 mg kg^{-1} RDX treatments compared to control. There were no adverse effects on juvenile production in any of the HMX treatments. Results of range finding test showed that 2,4-DNT significantly ($p < 0.0001$) reduced adult survival at 100 mg kg^{-1}. No adults survived at the higher concentrations. Juvenile numbers were reduced by 19 ($p = 0.03$) and 81 ($p < 0.0001$) percent in 10 and 50 mg kg^{-1} treatments, respectively compared to control. No juveniles were produced at the higher concentrations. Range-finding tests with 2,6-DNT showed that adult survival was reduced at 50 mg kg^{-1}. No adults survived at the higher concentrations. Juvenile numbers were reduced by 25 ($p = 0.001$) and 72 ($p < 0.0001$) percent in 10 and 50 mg kg^{-1} treatments, respectively compared to control. No juveniles were produced at the higher concentrations. Adult survival in the range-finding test with TNB was significantly reduced at 100 mg kg^{-1} ($p = 0.048$). Juvenile numbers were reduced by 19 ($p = 0.153$) and 75 ($p < 0.0001$) percent in 50 and 100 mg kg^{-1} treatments, respectively compared to control. Juvenile numbers were reduced by approximately 99 percent in 500 and 1,000 mg kg^{-1} treatments. No juveniles were produced at the higher concentrations. Results of these range-finding tests allowed us to determine treatment concentrations for the definitive test shown in Tables 2-10.

3.3 Definitive Toxicity Tests.

Definitive studies using the Enchytraeid Reproduction Tests (ERT) were conducted to assess the effects of RDX, HMX, 2,4-DNT, 2,6-DNT, or TNB on the reproduction of the enchytraeid worm *E. crypticus*. Adult *E. crypticus* were exposed in SSL soil to a range of concentrations for each EM, in independent investigations. Measurement endpoints were assessed using 6-9 treatment concentrations determined from the range-finding studies and included number of surviving adults after 14 days and number of juveniles after 28 days. All ecotoxicological parameters were estimated using measured chemical concentrations for each treatment level.

Test results complied with the validity criteria defined in the ISO test guideline. Mean adult survival in negative controls was 98% in 2,6-DNT freshly amended soil and 100% in all other tests. The mean juvenile production in negative controls ranged from 809 to 1500 juveniles, and the coefficient of variation ranged from 5.4 to 16.2 percent. Direct comparisons of the results of positive control are not possible because ERT is a new test and no reference values for natural soils are available from the literature. Juvenile production in positive controls ranged from 56 to 67 percent reduction from negative controls and was within the baseline established for the laboratory culture of *E. crypticus*. These results confirmed that the toxicological effects determined in the definitive tests were most likely due to test EM treatments. All reported ecotoxicological parameters have been calculated based on actual measured concentrations.

3.3.1 Toxicity of RDX.

Results of RDX toxicity testing in freshly amended and weathered/aged amended SSL soil are shown in Table 11. Adult *E. crypticus* survival was not affected in any RDX treatment concentrations producing the unbounded NOEC values for RDX in freshly amended soils of 21,383 mg kg^{-1} based on acetonitrile-extractable concentrations and 85.8 mg kg^{-1} based on ATCLP extractable concentrations (Table 12). The unbounded NOEC value for RDX in weathered/aged amended soil based on acetonitrile-extractable concentrations was 18,347 mg kg^{-1}. The unbounded NOEC value for RDX in weathered/aged amended soil based on ATCLP extractable concentrations was 89 mg kg^{-1} (Table 12).

Juvenile production bounded NOEC and LOEC values based on acetonitrile-extractable concentrations were, respectively 1,194 and 2,203 mg kg^{-1} in freshly amended soil, and 2,379 and 3,985 mg kg^{-1} in weathered/aged amended soil (Table 12). The ATCLP based NOEC and LOEC values for juvenile production were almost identical in both freshly amended and weathered/aged amended soils because these concentrations exceeded RDX solubility in water. These values were, respectively 114.8 and 114.7, and 87 and 86 mg kg^{-1}.

Concentration-response relationships for juvenile production in fresh and weathered/aged RDX amended soils determined by nonlinear regressions are shown in Figure 1. Logistic (Gompertz) model had the best fit for data in tests with both freshly amended (Figure 1A) and weathered/aged amended (Figure 1B) soils. Overall, reproduction was higher in weathered/aged RDX amended soils (Table 11). Juvenile production EC_{20} values were 3,715 and 8,797 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. The difference between these values was not statistically significant based on 95% confidence intervals (Table 12). Juvenile production EC_{50} values were 51,413 and 142,356 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. The highest RDX concentration of 21,383 mg kg^{-1} used in the test with freshly amended soil, and 18,347 mg kg^{-1} used in the test with weathered/aged soil resulted only in 31 and 28 percent reduction in the number of juveniles produced, respectively compared to carrier control. For that reason, nonlinear regression model estimated large range for 95% C.I. in determining both EC_{50} parameters (Table 12) indicating high uncertainty in these point estimates.

All treatment concentrations used for toxicity assessments with *E. crypticus* in SSL soil were above the RDX solubility level in water (42.3 mg L^{-1} at 20°C; Roberts and Hartley, 1992) producing uniformed ATCLP concentrations across the range. This precluded determinations of the concentration-response relationship on the basis of water extractable (ATCLP) RDX portion in both freshly amended and weathered/aged amended SSL soil.

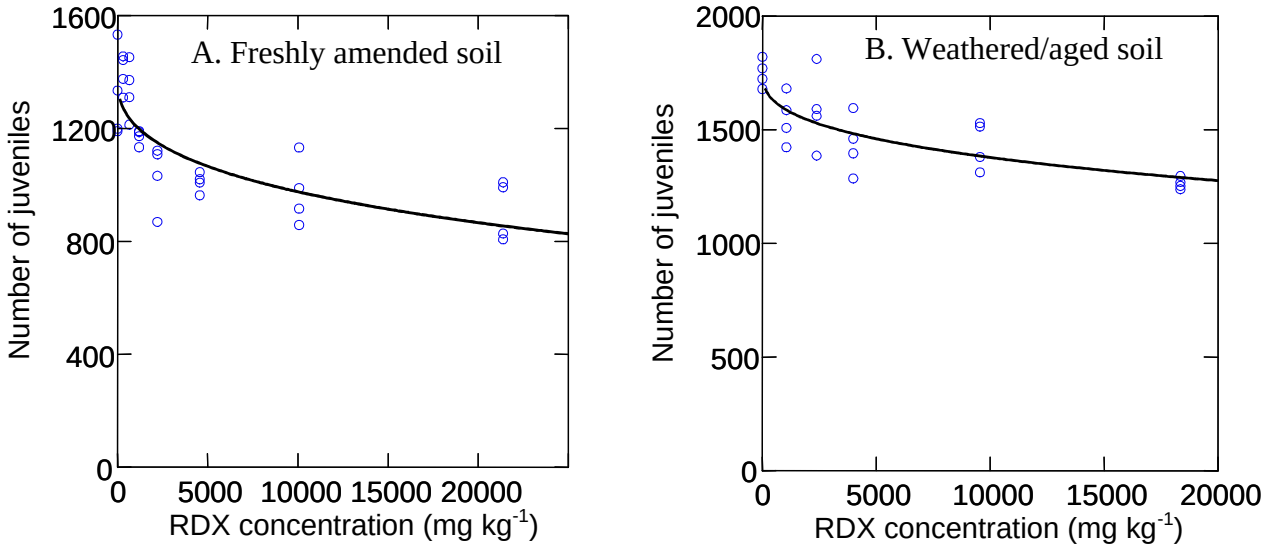
Table 11. Adult survival and juvenile production (mean, n = 4) in freshly amended and weathered/aged RDX amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Concentration in freshly amended soil (mg kg^{-1})	Number of Adults	Number of Juveniles	Standard Error	Concentration in weathered/aged amended soil (mg kg^{-1})	Number of Adults	Number of Juveniles	Standard Error
Negative control	10	1278.5	34.82	Negative control	10	1120.5	83.9
Acetone control	10	1313.8	79.86	Acetone control	10	1748.5	30.5
Positive control	10	419.8	23.62	Positive control	10	488.8	33.1
304	9.8	1395.3	33.67	1194	10	1549.8	55.2
656	10	1336.5	50.40	2203	9.8	1587.3	87.2
1194	10	1170.5	13.11	4558	10	1434.0	64.7
2203	10	1032.5	57.93	10062	10	1433.8	52.6
4558	10	1009.0	17.16	21383	9.8	1264.5	12.7
10062	10	973.8	59.17				
21383	10	908.8	52.98				

Table 12. Ecotoxicological parameters (mg kg^{-1}) for RDX determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Exposure	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh	21,383	>21,383	1,194	2,203	3,715	51,413
p or 95% C.I.			0.055	0.001	0-8,100	6,336-96,491
R^2					0.990	0.990
Weathered/aged	18,347	>18,347	2,379	3,985	8,797	142,356
p or 95% C.I.			0.056	0.001	761-16,834	0-373,753
R^2					0.995	0.995

Figure 1. Effects of RDX on juvenile production in freshly amended (A) and weathered/aged (B) RDX amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.



3.3.2 Toxicity of HMX.

Results of HMX toxicity testing in freshly amended and weathered/aged amended SSL soils are shown in Table 13. Adult *E. crypticus* survival was not affected in any HMX concentrations tested. Juvenile *E. crypticus* production was stimulated at higher HMX concentrations in freshly amended soil (Figure 2). The increase was statistically significant ($p < 0.05$) at 2,211 mg kg^{-1} and higher concentrations producing a bounded NOEC ($p = 0.109$) of 1,491 mg kg^{-1} and unbounded NOAEC (No Observed Adverse Concentration) of 21,750 mg kg^{-1} (Table 14). Results of the limit test showed that exposure of *E. crypticus* in weathered/aged HMX amended soil did not affect reproduction producing an unbounded NOEC ($p = 0.186$) of 17,498 mg kg^{-1}. Similar to RDX, all HMX treatment concentrations used for toxicity assessments were above the HMX solubility level in water (6.63 mg L^{-1} at 20°C; Roberts and Hartley, 1992).

Table 13. Adult survival and juvenile production in freshly amended and weathered/aged HMX amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Concentration in freshly amended soil (mg kg^{-1})	Mean* Adults	Mean Juveniles	Standard Error	Concentration in weathered/aged amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Standard Error
Negative control	10	808.8	31.81	Negative control	10	1116.3	33.2
Acetone control	10	737.3	45.37	Acetone control	10	1468.1	69.1
Positive control	10	292.0	19.40	Positive control	10	488.8	33.1
348	9.8	741.5	29.17	17498	10	1359.4	33.3
642	10	825.5	15.87				
1491	10	847.8	19.47				
2211	9.8	922.8	96.54				
5785	10	1151.0	19.93				
10586	10	986.8	33.89				
21750	10	1143.0	56.33				

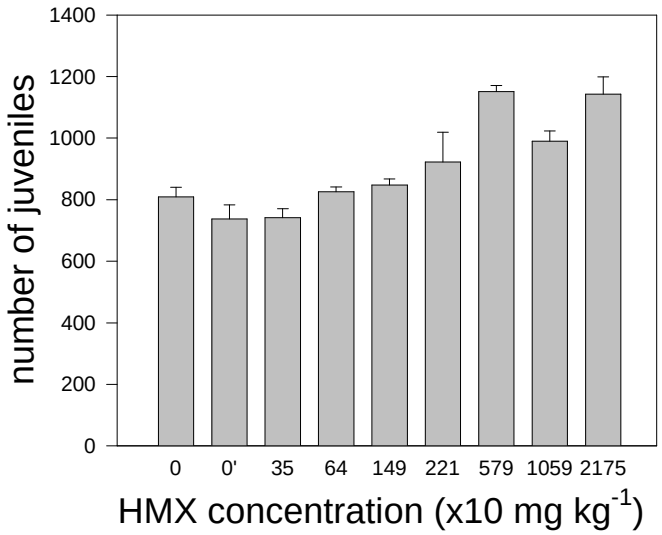
Table notes:
*Means are based on n = 4 for all treatments in freshly amended soils. Means in the limit test using weathered/aged HMX amended soil are based on n = 8 for carrier control and one treatment concentration of 17498 mg kg^{-1}; and n = 4 for the negative and positive controls.

Table 14. Ecotoxicological parameters (mg kg^{-1}) for HMX determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Exposure	Adult survival		Juvenile production			
	NOEC	LOEC	NOAEC	LOAEC	EC_{20}	EC_{50}
Fresh	21,750	>21,750	21,750	>21,750	ND	ND
Weathered/aged	17,498	>17,498	17,498	>17,498	LT	LT

Table notes:
ND, Not Determined. ECp values could not be determined due to stimulation of juvenile production in all treatment concentrations.
LT, Limit Test is based on data comparison between carrier control and one treatment concentration of 17,498 mg kg^{-1}.

Figure 2. Effect of HMX (mean and S.E., n = 4) on juvenile production by *Enchytraeus crypticus* in freshly amended Sassafras sandy loam soil. Controls shown are negative (0) and carrier (0'). All concentrations are based on acetonitrile extraction using USEPA Method 8330.



3.3.3 Toxicity of 2,4-DNT.

Adult *E. crypticus* survival and juvenile production were affected in 2,4-DNT amended SSL soil within the concentrations ranges selected from the results of range-finding test (Table 15). For adult survival in freshly amended soil, the bounded NOEC and LOEC values for 2,4-DNT based on acetonitrile-extractable concentrations were 40.9 and 55.0 mg kg^{-1}, respectively. The bounded NOEC and LOEC values based on water extractable (ATCLP) concentrations were 8.13 and 33.45 mg kg^{-1}, respectively. For adult survival in weathered/aged amended soil, the bounded NOEC and LOEC values based on acetonitrile-extractable concentrations were 37.3 and 71.7 mg kg^{-1}, respectively. No adults survived in the 178.7 mg kg^{-1} treatment. The bounded NOEC and LOEC values based on ATCLP extraction were 20.5 and 46.1 mg kg^{-1}, respectively (Table 16).

Juvenile production bounded NOEC and LOEC values based on acetonitrile-extractable concentrations were, respectively 9.9 and 20.3 mg kg^{-1} in freshly amended soils, and 5.2 and 11.5 mg kg^{-1} in weathered/aged amended soils. Juvenile production bounded NOEC and LOEC values based on ATCLP extractable concentrations were, respectively 4.96 and 8.13 mg kg^{-1} in freshly amended soils, and 2.42 and 5.22 mg kg^{-1} in weathered/aged amended soils (Table 16).

Concentration-response relationships for juvenile production in freshly amended and weathered/aged 2,4-DNT amended soils determined by nonlinear regressions are shown in Figure 3. Logistic (Gompertz) model had the best fit for data in tests with both freshly amended (Figure 3A) and weathered/aged amended (Figure 3B) soils. Overall, reproduction was higher in weathered/aged 2,4-DNT amended soils. Juvenile production EC_{20} values based on acetonitrile-extractable concentrations were 19.4, and 14.1 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively. Juvenile production EC_{50} values based on acetonitrile-extractable concentrations were 35.9 and 27.4 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively (Table 16). Juvenile production EC_{20} values based on ATCLP extractable concentrations were 3.1, and 6.6 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively (Table 16). Juvenile production EC_{50} values based on ATCLP extractable concentrations were 10.6 and 14.3 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively (Table 16). The differences among these values were not statistically significant based on 95% confidence intervals (Table 16) indicating that the 3-month weathering/aging of 2,4-DNT amended soils did not affect the toxicity of this energetic material to *E. crypticus*.

Coefficients of determinations (R^2) for acetonitrile-extractable and ATCLP based extractions determined in nonlinear regression analyses of the reproduction toxicity data (EC_{20} levels) from studies with freshly amended and weathered/aged 2,4-DNT amended soils were compared to determine which chemical measure of exposure better correlates with toxicity. The values of coefficients in freshly amended soils were 0.980 and 0.971 for acetonitrile-extractable and ATCLP based extractions, respectively. These values in weathered/aged 2,4-DNT amended soils were 0.985 and 0.983 for acetonitrile-extractable and ATCLP based extractions, respectively. These comparisons show that coefficients were very similar in both exposure types indicating that neither extraction method had an advantage in characterizing 2,4-DNT bioavailability to *E. crypticus*.

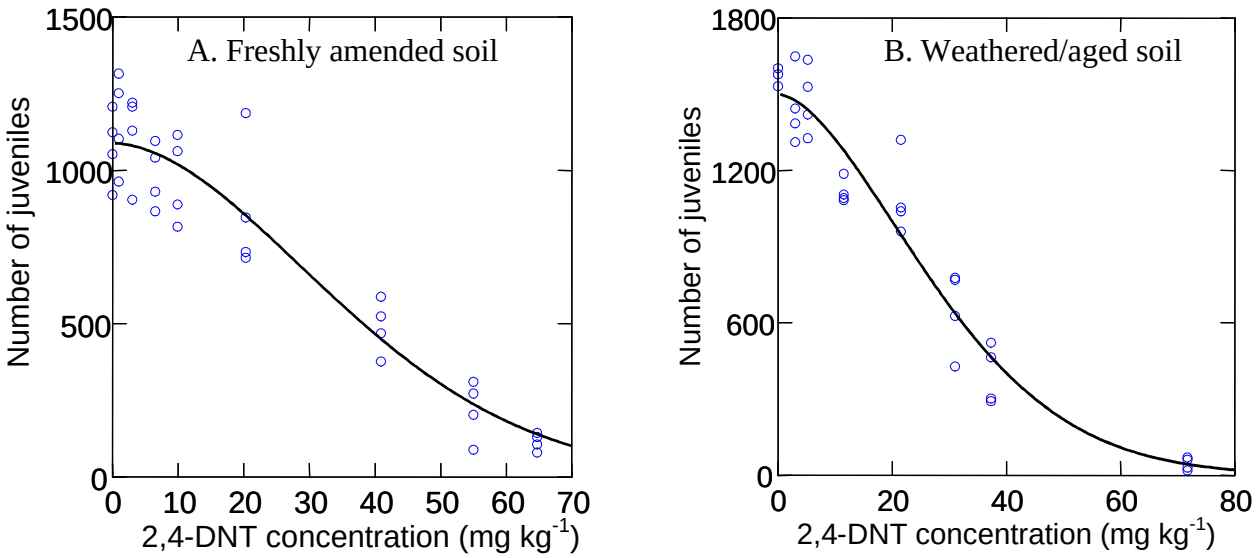
Table 15. Adult survival and juvenile production (mean, n = 4) in freshly amended and weathered/aged 2,4-DNT amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Concentration in freshly amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Standard Error	Concentration in weathered/aged amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Standard Error
Negative control	10	823.5	39.04	Negative control	10	1500.0	78.69
Acetone control	9.8	1076.5	61.03	Acetone control	10	1449.0	122.23
Positive control	9.3	351.5	23.40	Positive control	10	578.3	39.66
0.95	10	1159.0	78.71	3.0	10	1447.5	72.39
3.0	10	1116.0	73.15	5.2	10	1478.0	66.91
6.5	10	984.0	51.97	11.5	10	1116.5	23.94
9.9	10	971.3	70.67	21.5	10	1093.5	78.59
20.3	9.8	870.8	109.37	31.0	10	650.3	81.70
40.9	10	489.5	44.69	37.3	9.8	395.0	57.84
55.0	8.3	218.8	48.67	71.7	8.3	45.0	12.19
64.7	5.8	115.0	14.06	178.7	0.0	0.0	

Table 16. Ecotoxicological parameters (mg kg^{-1}) for 2,4-DNT determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330 and water extraction using Adapted Toxicity Characteristic Leaching Procedure (ATCLP).

Exposure assessment	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh						
Acetonitrile extraction	4.9	55.0	9.9	20.3	19	36
p or 95% C.I.	0.659	0.013	0.271	0.037	13 - 26	30 - 41
R^2					0.980	0.980
ATCLP extraction	8.13	33.45	4.96	8.13	3	10.6
p or 95% C.I.	0.659	0.013	0.271	<0.0001	1 - 5	7 - 14
R^2					0.971	0.971
Weathered/aged						
Acetonitrile extraction	37.3	71.7	5.2	11.5	14	27
p or 95% C.I.	0.711	0.015	0.318	<0.0001	10 - 18	24 - 31
R^2					0.985	0.985
ATCLP extraction	20.5	46.1	2.42	5.2	6.6	14
p or 95% C.I.	0.711	0.015	0.318	<0.0001	4 - 9	12 - 16
R^2					0.983	0.983

Figure 3. Effects of 2,4-DNT on juvenile production in freshly amended (A) and weathered/aged (B) 2,4-DNT amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.



3.3.4 Toxicity of 2,6-DNT.

Results of toxicity testing in 2,6-DNT freshly amended and weathered/aged amended SSL soils are shown in Table 17. Adult *E. crypticus* survival was not affected in any 2,6-DNT concentrations in freshly amended SSL soil producing the unbounded NOEC value of 64 mg kg^{-1} based on acetonitrile-extractable concentration, and 40.5 mg kg^{-1} based on ATCLP extractable concentration. For adult survival in weathered/aged amended soil, the bounded NOEC and LOEC values based on acetonitrile-extractable concentrations were 37.4 and 108.3 mg kg^{-1}, respectively. The bounded NOEC and LOEC values based on ATCLP extraction were 17.4 and 66.9 mg kg^{-1}, respectively (Table 18).

Juvenile production bounded NOEC and LOEC values based on acetonitrile-extractable concentrations were, respectively 20.0 and 40.2 mg kg^{-1} in freshly amended soils, and 18.1 and 37.4 mg kg^{-1} in weathered/aged soils. Juvenile production bounded NOEC and LOEC values based on ATCLP extractable concentrations were, respectively 10.6 and 24.8 mg kg^{-1} in freshly amended soils, and 9.6 and 17.4 mg kg^{-1} in weathered/aged soils (Table 18).

Logistic (Gompertz) model had the best fit for data in tests with both freshly amended (Figure 4 A) and weathered/aged amended (Figure 4 B) soils. Juvenile production EC_{20} values based on acetonitrile-extractable concentrations were 37.3, and 17.9 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively. Juvenile production EC_{50} values based on acetonitrile-extractable concentrations were 56.8 and 29.4 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively (Table 18). Juvenile production EC_{20} values based

on ATCLP extractable concentrations were 23.6, and 9.4 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively (Table 18). Juvenile production EC_{50} values based on ATCLP extractable concentrations were 36.2 and 14.4 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively (Table 18). The differences among these values were statistically significant based on 95% confidence intervals (Table 18) indicating that the 3-month weathering/aging of 2,6-DNT amended soils increased the toxicity of this energetic material to *E. crypticus*.

Coefficients of determinations (R^2) for acetonitrile-extractable and ATCLP based extractions determined in nonlinear regression analyses of the reproduction toxicity data (EC_{20} levels) from studies with freshly amended and weathered/aged 2,6-DNT amended soils were compared to determine which chemical measure of exposure better correlated with toxicity. The values of coefficients in freshly amended soils were 0.980 and 0.979 for acetonitrile-extractable and ATCLP based extractions, respectively (Table 18). These values in weathered/aged 2,6-DNT amended soils were 0.984 and 0.983 for acetonitrile-extractable and ATCLP based extractions, respectively (Table 18). These comparisons show that coefficients were very similar in both exposure types indicating that neither extraction method had an advantage in characterizing 2,6-DNT bioavailability to *E. crypticus*.

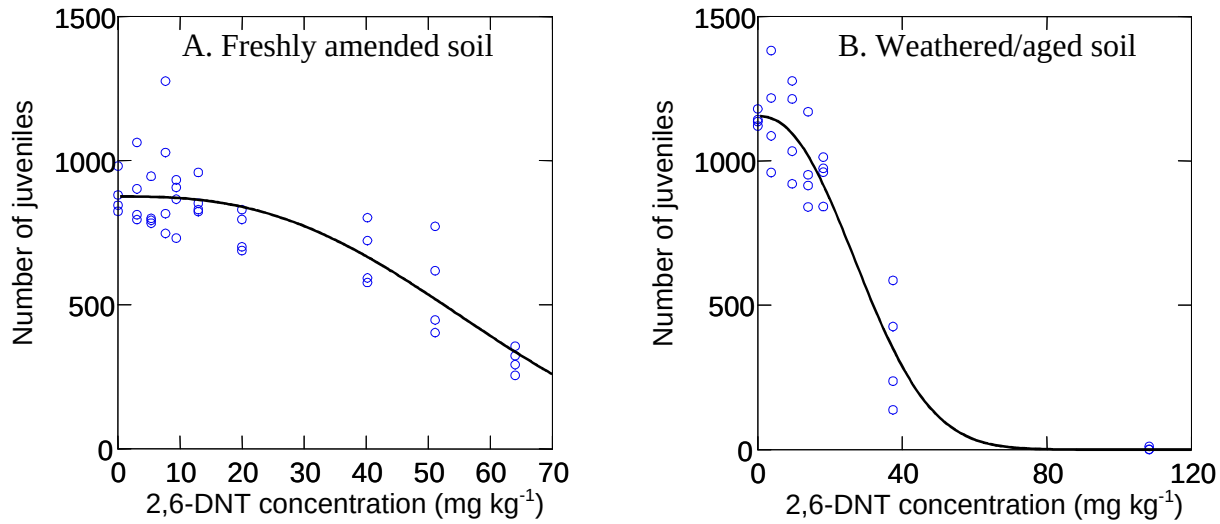
Table 17. Adult survival and juvenile production (mean, n = 4) in freshly amended and weathered/aged 2,6-DNT amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

Concentration in freshly amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Standard Error	Concentration in weathered/aged amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Standard Error
Negative control	9.8	882.8	34.80	Negative control	10	983.3	45.41
Acetone control	9.8	893.0	61.34	Acetone control	9.8	1145.0	12.54
Positive control	9.8	356.5	25.40	Positive control	9.5	411.3	17.75
5.3	10	830.5	38.66	3.7	9.8	1161.8	90.35
7.7	10	966.8	119.2	9.5	9.5	1111.8	81.85
9.4	10	859.3	44.92	13.9	9.3	969.5	70.70
12.9	10	866.0	31.61	18.1	9.8	947.5	36.86
20.0	10	753.8	35.01	37.4	9.8	346.8	99.64
40.2	10	673.8	53.82	108.3	1.7	3.0	2.68
51.1	10	560.0	84.52				
64.0	9.5	306.5	21.57				

Table 18. Ecotoxicological parameters (mg kg^{-1}) for 2,6-DNT determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330 and water extraction using Adapted Toxicity Characteristic Leaching Procedure (ATCLP).

Exposure assessment	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh						
Acetonitrile extraction	64	>64	20.0	40.2	37	57
p or 95% C.I.	0.369		0.136	0.019	28 - 47	51 - 63
R^2					0.980	0.980
ATCLP extraction	40.5	>40.5	10.6	24.8	24	36
p or 95% C.I.	0.292		0.071	0.005	17 - 30	32 - 40
R^2					0.979	0.979
Weathered/aged						
Acetonitrile extraction	37.4	108.3	18.1	37.4	18	29
p or 95% C.I.	1.000	<0.0001	0.055	<0.0001	13 - 23	25 - 34
R^2					0.984	0.984
ATCLP extraction	17.4	66.9	9.6	17.4	9	14
p or 95% C.I.	1.000	<0.0001	0.055	<0.0001	7 - 12	13 - 16
R^2					0.983	0.983

Figure 4. Effects of 2,6-DNT on juvenile production in freshly amended (A) and weathered/aged (B) 2,6-DNT amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.



3.3.5 Toxicity of TNB

TNB affected both adult *E. crypticus* survival and juvenile production in amended SSL soil within the concentrations ranges selected from the results of range-finding test (Table 19). Adult survival in freshly amended soil was not affected up to 45 mg kg^{-1} (bounded NOEC) acetonitrile extractable treatment concentration. No adults survived after a 14-day exposure to TNB in 107 mg kg^{-1} (bounded LOEC) acetonitrile extractable treatment concentration. The bounded NOEC and LOEC values based on water extractable (ATCLP) concentrations were 30.22 and 83.67 mg kg^{-1}, respectively (Table 20). Weathering/aging of TNB amended soil reduced the toxicity of TNB to *E. crypticus* adults. The bounded NOEC and LOEC values in weathered/aged soils based on acetonitrile-extractable concentrations were 75.83 and 176.33 mg kg^{-1}, respectively. The bounded NOEC and LOEC values based on ATCLP concentrations were 55.8 and 143.4 mg kg^{-1}, respectively (Table 20).

Juvenile production was stimulated at the lower treatment concentration of 2.58 mg kg^{-1} resulting in the 19 percent increase in the average number on juveniles compared with carrier control (Table 19). The increase was statistically significant ($p = 0.01$) producing a bounded NOEC value of 2.32 mg kg^{-1} and a bounded LOEC value of 2.58 mg kg^{-1} based on acetonitrile-extractable concentration. Statistically significant ($p = 0.012$) reduction in number on juveniles compared with carrier control occurred in 3.94 mg kg^{-1} treatment (Table 19), which produced a bounded NOAEC value of 2.58 mg kg^{-1} and bounded LOAEC value of 3.94 mg kg^{-1} based on acetonitrile-extractable concentration (Table 20).

TNB concentrations using ATCLP extraction were below the method detection limit (MDL) in the first two amended treatments (2.32 and 2.58 mg kg^{-1} based on acetonitrile extraction) in freshly amended soils. The first treatment with positively detected TNB concentrations using ATCLP extraction had a significantly ($p = 0.001$) decreased juvenile production compared with carrier control producing an unbounded LOEC of 2.45 mg kg^{-1} for freshly amended soils. Juvenile production bounded NOEC and LOEC values in weathered/aged soils were 1.26 and 8.75 mg kg^{-1}, respectively based on acetonitrile extraction, and 0.24 and 3.35 mg kg^{-1}, respectively based on ATCLP extraction (Table 20).

The logistic model with hormetic parameter (hormetic model) had the best fit for data from toxicity tests with TNB freshly amended SSL soil due to stimulation of juvenile production at the lower treatment concentration of 2.58 mg kg^{-1} (Figure 5 A). Juvenile production EC_{20} and EC_{50} values based on acetonitrile-extractable concentrations were 4.85, and 11.2 mg kg^{-1}, respectively (Table 20). Juvenile production EC_{20} and EC_{50} values based on ATCLP extractable concentrations were 1.33, and 8.75 mg kg^{-1}, respectively (Table 20). The logistic (Gompertz) model had the best fit for data in tests with weathered/aged TNB amended soils (Figure 5 B). Overall, reproduction was higher in weathered/aged amended soils. Juvenile production EC_{20} and EC_{50} values based on acetonitrile-extractable concentrations were 9.14 and 22.42 mg kg^{-1}, respectively (Table 20). Juvenile production EC_{20} and EC_{50} values based on ATCLP extractable concentrations were 3.45 and 11.3 mg kg^{-1}, respectively (Table 20). The differences between ECp values for freshly amended and weathered/aged TNB amended soils

were not statistically significant based on 95% confidence intervals (Table 20) indicating that the 3-month weathering/aging of TNB amended soils did not affect the toxicity of this energetic material to *E. crypticus*.

Coefficients of determinations (R^2) for acetonitrile-extractable and ATCLP based extractions determined in nonlinear regression analyses of the reproduction toxicity data (EC_{20} levels) from studies with freshly amended and weathered/aged TNB amended SSL soils were compared to determine which chemical measure of exposure better correlates with toxicity. The values of coefficients in freshly amended soils were 0.975 and 0.980 for acetonitrile-extractable and ATCLP based extractions, respectively. These values in weathered/aged TNB amended soils were 0.988 for both acetonitrile-extractable and ATCLP based extractions (Table 20). These comparisons show that coefficients were very similar in both exposure types indicating that neither extraction method had an advantage in characterizing TNB bioavailability to *E. crypticus*.

Table 19. Adult survival and juvenile production (mean, n = 4) in freshly amended and weathered/aged TNB amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

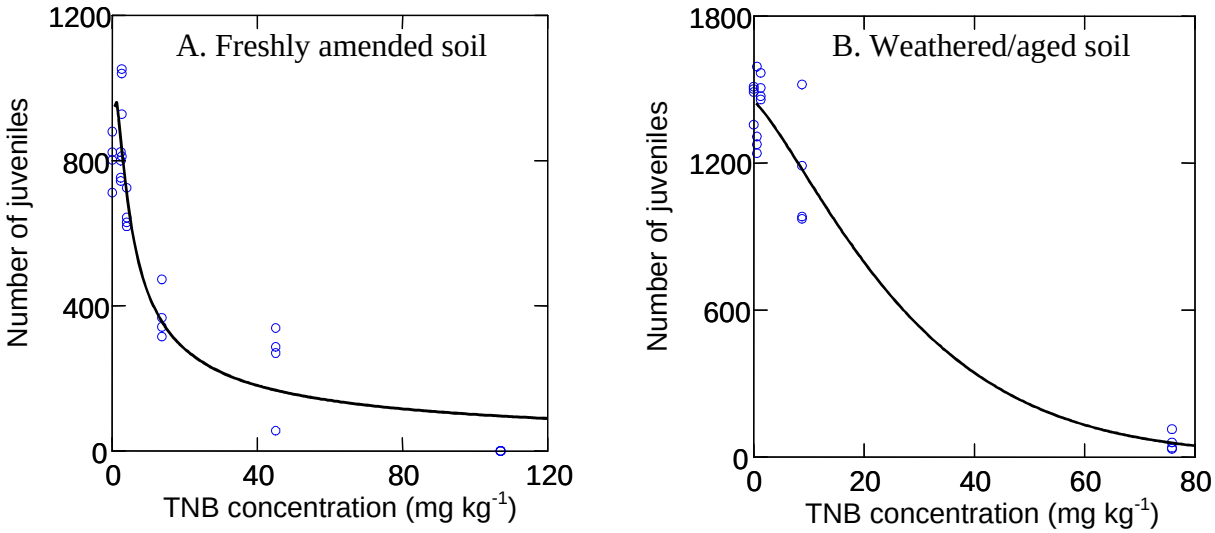
Concentration in freshly amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Standard Error	Concentration in weathered/aged amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Standard Error
Negative control	10	824.8	60.90	Negative control	10	1387.3	61.46
Acetone control	10	804.3	34.89	Acetone control	10	1465.3	36.36
Positive control	9.3	329.3	30.98	Positive control	9.0	579.0	78.40
2.3	10	780.3	19.06	0.6	9.8	1355.0	80.90
2.6	10	958.0	56.11	1.3	9.5	1501.8	24.30
3.9	10	654.3	24.09	8.8	9.3	1166.3	128.3
13.6	9.8	374.3	34.59	75.8	9.8	61.5	18.40
45.0	10	238.0	62.42	176.3	0	0	
107.0	0	0		304.7	0	0	
221.0	0	0					
384.7	0	0					

Table 20. Ecotoxicological parameters (mg kg^{-1}) for TNB determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330 and water extraction using Adapted Toxicity Characteristic Leaching Procedure (ATCLP).

Exposure assessment	Adult survival		Juvenile production			
	NOEC	LOEC	NO(A)EC[§]	LO(A)EC[§]	EC_{20}	EC_{50}
Fresh						
Acetonitrile extraction	45.03	107	2.58*	3.94*	5	11
p or 95% C.I.	1.000	<0.0001	0.010	0.012	3 - 7	7 - 16
R^2					0.975	0.975
ATCLP extraction	30.22	83.67	<2.45	2.45**	1.3	8.8
p or 95% C.I.	1.000	<0.0001		0.001	0.1 - 2.5	5.0-12.5
R^2					0.980	0.980
Weathered/aged						
Acetonitrile extraction	75.83	176.3	1.26	8.75	9	22
p or 95% C.I.	1.000	<0.0001	0.722	0.009	4 - 14	13 – 32
R^2					0.988	0.988
ATCLP extraction	55.8	143.4	0.24	3.35	3.5	11
p or 95% C.I.	1.000	<0.0001	0.722	0.009	0.9 – 6.0	5 – 17
R^2					0.988	0.988

Table notes:
[§]Values are ether No (Low) Observed Effect Concentration (NOEC and LOEC) or No (Low) Observed Adverse Effect Concentration (NOAEC and LOAEC).
*Values are NOAEC and LOAEC due to a significant (p = 0.01) increase in juvenile production in 2.58 mg kg^{-1} treatment.
**Unbounded LOEC value. TNB concentrations using ATCLP extraction were below the method detection limit (MDL) in the preceding amended treatments 2.32 and 2.58 mg kg^{-1} based on acetonitrile extraction from freshly amended soils.

Figure 5. Effects of TNB on juvenile production in freshly amended (A) and weathered/aged (B) TNB amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.



4 DISCUSSION

Development of screening level benchmarks for Ecological Risk Assessment (ERA) of contaminated soils has become a critical need in recent years (USEPA, 2000). In order to address this problem, the USEPA in conjunction with stakeholders is developing Eco-SSLs to identify concentrations of chemicals in soil that, when not exceeded, will be theoretically protective of terrestrial ecosystems within specific soil boundary conditions from unacceptable harmful effects. An extensive review of literature (USEPA, 2000) determined that there was insufficient information for energetic material contaminants in soil to generate Eco-SSL benchmarks for soil invertebrates. The majority of soil toxicity tests that were reported in literature utilized standard artificial soil with high organic matter content (10%). In contrast, our toxicity studies designed to specifically fill this knowledge gap, used a natural soil that meet the criteria for Eco-SSL development, in large part because it has characteristics supporting relatively high bioavailability of EMs. In addition, our weathering/aging procedure applied to soils loaded with the range of EM concentrations allowed us to more realistically assess the toxicity under conditions more closely resembling the potential toxic effects of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB in the field.

4.1 Chemical analysis of energetic materials in soil.

Derivation of Eco-SSL values prioritizes ecotoxicological benchmarks that are based on measured soil concentration of a chemical over those based on nominal concentrations (USEPA, 2000). In this study, the exposure concentrations of RDX, HMX, 2,4-DNT, 2,6-DNT,

and TNB in soil were analytically determined in all definitive toxicity tests. Chemical analysis utilized the USEPA Method 8330 based on acetonitrile extraction of EMs from soil and measured acetonitrile-extractable chemical concentration. Acetonitrile extraction based analysis of freshly amended soils showed good correlation between nominal and measured concentrations for the five energetic materials confirming that the soil amendment procedure used in toxicity tests was appropriate and that the USEPA Method 8330 was efficient for quantifying the amount of energetic materials in soil.

Additional procedure that measures the water extractable portion of each EM in amended soil was performed using the Adapted Toxicity Characteristic Leaching Procedure (ATCLP). This water extractable portion of each EM was perceived to measure the bioavailable fraction of chemicals in soil and could generate data that is better correlated with toxicity compared with acetonitrile-extractable chemical measure. ATCLP extractable concentrations of 2,6-DNT, and TNB freshly amended in SSL soil increased proportionally with their respective acetonitrile-extractable concentrations. In contrast, RDX and HMX ATCLP extractable concentrations decreased proportionally with their respective acetonitrile-extractable concentrations with less than one percent recovery in soils freshly amended at or above 10,000 mg kg^{-1} RDX, and at or above 1,200 mg kg^{-1} HMX. These low ATCLP-based recoveries reflected the low water solubility of both compounds, which were reported for RDX at 42 mg L^{-1} at 20°C (Sikka *et al.*, 1980) and at 60 mg L^{-1} at 25°C (Banerjee *et al.*, 1980). The water solubility of HMX was reported between 5 and 6.6 mg L^{-1} at 25°C and 20°C, respectively (Glover *et al.*, 1973; McLellan *et al.*, 1992).

Assessment of the EM toxicity to *E. crypticus* for Eco-SSL development included studies with weathered and aged EM amended soils to simulate more closely the exposure effects in the field. Weathering/aging of chemicals in soil may reduce exposure of soil invertebrates to EMs due to photodecomposition, hydrolysis, reaction with organic matter, sorption, precipitation, immobilization, occlusion, microbial transformation and other fate processes that commonly occur at contaminated sites. These fate processes can reduce the amount of chemical that is bioavailable, compared to tests conducted with freshly amended soils.

Weathering/aging of amended soils reduced acetonitrile-extractable concentrations of 2,4-DNT, 2,6-DNT, or TNB. Concentration of 2,4-DNT decreased by approximately 50 percent during the three-months procedure and was independent of the initial acetonitrile-extractable concentrations used in this study. Weathered/aged amended SSL soil used in the phytotoxicity assessment portion of this investigation were analyzed for presence on metabolic products of nitroaromatic EMs degradation. These analyses identified two metabolites of 2,4-DNT, including 2-amino-4-nitrotoluene (2-A-4 NT), and 4-amino-2-nitrotoluene (4-A-2 NT) in weathered/aged amended soil. Detection of these metabolites of 2,4-DNT confirmed that this EM was undergoing degradation. Bacteria able to mineralize 2,4-DNT, such as *Pseudomonas sp.* strain, have been isolated from a variety of contaminated soils (Spain, 1995). Both 2,4-DNT and 2,6-DNT are readily biotransformed by *Pseudomonas sp.* and eventually eliminated as nitrite (Spanggord *et al.*, 1991; Kaplan, 1992; Haidor *et al.*, 1996). Degradation of 2,6-DNT during simulated weathering/aging procedure of amended soils was greater compared with 2,4-DNT degradation, and reduced 2,6-DNT concentrations by 72-80 percent. Degradation

of TNB was inversely related to the initial acetonitrile-extractable concentration in amended soil. More than 80 percent of TNB degraded in soil amended with concentrations below 100 mg kg^{-1}, while above that treatment level, TNB degradation ranged 20-30 percent. A degradation product of TNB, 3,5-dinitroaniline (3,5-DNA), was detected in weathered/aged amended SSL soil suggesting that TNB was undergoing microbial and/or photolytic degradation. Reduction in the RDX concentrations in weathered/aged amended soil ranged 5-14 percent and was 20 percent for HMX after the 3-month weathering/aging period.

The water extractable portions of nitroaromatic EMs in weathered/aged amended soils were lower compared with freshly amended soils as a result of fate processes in the amended soils undergoing weathering and aging. In contrast to nitroaromatic EMs, the reduction in water extractable fraction of RDX was approximately one percent, and 0.1 percent for HMX, which can be attributed to limited degradation of RDX and HMX under aerobic soil conditions (Rosenblatt *et al.*, 1991; Hawari *et al.*, 2002). These findings are indirectly supported by the study results of Jones *et al*. (1995) who reported a limited, 10 percent mineralization of RDX in contaminated soil augmented with *Rhodococcus* bacterial strain. Overall, chemical analyses demonstrated that EM exposure conditions of *E. crypticus* in weathered/aged amended soils differed from those of freshly amended soils. The inclusion of weathering/aging component in the EM toxicity assessments allowed us to incorporate potential alterations in EM bioavailability at contaminated sites in the development of ecotoxicological benchmarks for soil invertebrates.

4.2 Toxicity of energetic materials to *E. crypticus* in Sassafras sandy loam soil.

Definitive toxicity tests conducted with both freshly amended and weathered/aged amended soils showed that EM toxicity order based on EC_{20} values for juvenile production in tests with *E. crypticus* was TNB > 2,4-DNT > 2,6-DNT > RDX > HMX. Reproduction measurement endpoint in all tests was more sensitive compared with adult survival. This supported the Eco-SSL requirement of the use of reproduction endpoints for benchmark development (USEPA, 2000). Nitro-heterocyclic explosives RDX and HMX did not affect adult *E. crypticus* survival even at concentrations as high as 21,383 and 21,750 mg kg^{-1}, respectively. Juvenile production was affected by RDX but the toxicity was relatively low with EC_{20} estimates of 3,715 and 8,797 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively. Weathering and aging of RDX amended soil did not significantly affect its toxicity to *E. crypticus*.

Because this study was designed to produce benchmark data for development of Eco-SSLs for explosives contaminants in soil, the results of this study may not directly compare to those of other studies in the literature, since none of them were designed to specifically quantify EM toxicity to soil invertebrates under Eco-SSL conditions of testing. Literature on the toxicity of RDX to terrestrial organisms is scant, and discrepancies are often found regarding the toxicity of the same chemical to different organisms. Significant sublethal effects of RDX were observed on the reproduction of earthworm *Eisenia andrei* at concentrations as low as 95 mg kg^{-1} soil (Robidoux *et al*., 2000). However, no effects were found on the mortality and reproduction of two terrestrial invertebrates enchytraeid worm *E. crypticus* and collembolan *Folsomia candida* in soils spiked with up to 1000 mg kg^{-1} RDX in soil (Schafer and Achazi, 1999). Furthermore,

these studies were conducted either in standard artificial soil (Robidoux *et al*., 2000), or in soil with relatively high (2.5-3.0% organic C) organic matter content (Schafer and Achazi, 1999), which limits their usefulness for describing natural systems or development of Eco-SSLs.

Exposure of *E. crypticus* to HMX in freshly amended SSL soil produced a significant stimulating effect on juvenile production (11-56% increase), which disappeared in weathered and aged soil. A hormetic response in freshly amended SSL soil, which also disappeared in weathered/aged amended soil, was observed in our toxicity test with TNB amended soil. Stevens *et al*. (2002) reported similar stimulating effect of HMX exposure on growth of midge *Chironomus tentans*. Hormetic responses were reported in explosives exposure studies for microbial nitrogen fixation activity at soil TNT concentrations of 200 and 400 mg kg^{-1} (Gong *et al*., 1999), offspring production by *Daphnia magna* exposed to 0.08 mg L^{-1} TNT (Bailey *et al*., 1985), egg production per female fathead minnow exposed to 6.3 mg L^{-1} RDX (Bentley *et al*., 1977), and density of *Selanastrum capricornutum* cells, based on acetonitrile-extractable chlorophyll measures following HMX exposure ranging 36-572 mg L^{-1} (Bentley *et al*., 1984). To date, no studies investigated the mechanisms responsible for stimulating effects of these explosives at specific concentrations. Stevens *et al*., (2002) suggested that these mechanisms could include the direct effect on test organisms through the release of metabolic products of explosives that may have a specific effect on growth and reproduction, and indirect effects through increased supply of nitrogen for bacteria, fungi, or algae (an important food source for higher trophic levels) from mineralization of explosives.

The relatively low RDX toxicity and the absence of HMX toxicity to *E. crypticus* in SSL soil at concentrations tested in our study can be related to low bioavailability of these energetic materials in soil as evidenced by low ATCLP-based recoveries of both compounds. Considering *E. crypticus* exposure to RDX and HMX in soil on the ATCLP basis provides explanation, at least partially, for the observed effects of these nitro-heterocyclic explosives. Additional research would be required to better understand the reasons for low toxicity of RDX to *E. crypticus* and elucidation of mechanisms of a stimulating response to HMX exposure.

Dinitrotoluenes (DNTs) and trinitrobenzene (TNB) are by-products of TNT production, which are present worldwide at munitions manufacturing and post-production sites. 2,4-DNT and 2,6-DNT are also aerobic metabolites of microbial degradation of TNT (Gorontzy, *et al*., 1994; Spain, 2000). Toxicity of nitraromatic EMs tested to *E. crypticus* juvenile production was considerably greater (more than two orders of magnitude) compared with RDX and even greater compared with HMX. Juvenile production EC_{20} estimates ranged from 5 to 37 mg kg^{-1} in freshly amended soils, and from 9 to 20 mg kg^{-1} in weathered/aged amended soils. Comparison of our results to other studies is difficult because the toxicity of nitroaromatic energetics, including 2,4-DNT, 2,6-DNT and TNB to soil invertebrates has not been sufficiently investigated. The majority of studies reported in the available literature focused primarily on the effects of TNT and/or its degradation products (Dodard *et al.*, 2003; Renoux *et al*., 2000; Robidoux *et al*., 2000; 1999; Sunahara, *et al*., 2001; Rocheleau, *et al*., 1999; Schafer and Achazi, 1999; Simini, *et al*., 1995; Phillips, *et al*., 1993). Dodard *et al*. (2003) in the study with *E. albidus* using OECD artificial soil determined EC_{50} value for TNT of 111 mg kg^{-1} for juvenile production. Phillips *et al*. (1993) reported 100 percent mortality in the earthworm *E. fetida*

growth and survival test in USEPA standard artificial soil fortified with a mixture of EMs that included 30, 50, 62.5, and 20 mg kg^{-1} of TNT, TNB, 2,4-DNT and 2,6-DNT, respectively. Statistically significant ($p < 0.01$) sublethal effects (mass loss) were reported at concentrations 6, 10, 12.5, and 4 mg kg^{-1} of TNT, TNB, 2,4-DNT and 2,6-DNT, respectively. These results are in general agreement with findings of our investigations although direct comparisons of both studies are limited due to differences in the experimental designs.

Simini *et al*. (1995) assessed the toxicity of soil from Joliet Army Ammunition Plant contaminated with a mixture of EMs (which limits the direct comparisons with our study), including both nitroaromatic and nitro-heterocyclic compounds using earthworm *E. fetida* growth and survival test, among other bioassays. The highest soil concentrations measured at this site for TNB, 2,4-DNT and 2,6-DNT were 200, 117, and 8 mg kg^{-1}, respectively. Authors reported that TNT and TNB had greatest coefficients of determinations in all bioassays, including the earthworm test. Liner regression analyses R^2 values for TNB using earthworm test endpoints were 0.773 and 0.814 for the two locations investigated at the study site. These values for 2,4-DNT were 0.613 and 0.358, while 2,6-DNT had the weakest relationship to measurement points used with R^2 values of 0.082 and 0.293 for the two locations, respectively. Soil TNB and 2,4-DNT concentrations found at this site were within the range of concentrations tested in our study and the results are consistent with our findings. The weak relationship determined for 2,6-DNT is most likely due to very low concentrations of this EM measured at the investigated site.

Special consideration in assessing chemical toxicity for Eco-SSL development was given to the effects of weathering and aging of contaminant explosives in soil on exposure of soil invertebrates. Weathering and aging of amended soils significantly increased the toxicity of 2,6-DNT to *E. crypticus*, while toxicity of 2,4-DNT and TNB was unaffected. Dodard *et al*. (2003) reported a decrease in TNT toxicity to *E. albidus* on the LC_{50} basis for reproduction from 44 to 89 mg kg^{-1} in OECD artificial soil following a 21-day aging period. Specific mechanisms of changes in the toxicity of EMs in weathered/aged amended soil are unknown. Degradation products produced during the weathering and aging process may be more toxic to soil organisms compared with the parent material, and can be one of the factors contributing to the increased toxicity in weathered/aged amended soil. Dodard *et al*. (1999) investigated the toxicity of 2,4-DNT and 2,6-DNT, and their respective metabolites using the 15-min Microtox (*Vibrio fischeri*) and 96-h freshwater green alga (*S. capricornutum*) growth inhibition tests. The toxicities of DNTs were species-dependent: 2,4-DNT was more toxic than 2,6-DNT to *S. capricornutum* (comports with our results for *E. crypticus*), while the reverse was true in the test with *Vibrio fischeri*. The authors reported that the reduced metabolites of 2,6-DNT tested were less toxic compared to the toxicity of parent compound. However, certain partially reduced metabolites of 2,4-DNT (4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene) were more toxic than the parent compound. Although these results cannot be directly compared to our study because the biotic reductive degradation pathway for 2,4-DNT and 2,6-DNT in aquatic environment would contrast with metabolic processes in the aerobic conditions of vadose zone simulated in our investigations, the reducing environment can exist in water-logged soil microsites, where more toxic metabolites of dinitrotoluenes degradation can be present. The higher toxicity of these metabolites would contribute to possible explanation of the increased toxicity of 2,6-DNT in weathered/aged amended SSL soil observed in our study. Overall results of our study showed

that special consideration given to the effects of weathering and aging of energetic contaminants in soil for assessing toxicity was well justified. Benchmark values generated in this study will contribute to development of Eco-SSLs that better represent the exposure conditions of soil invertebrates at contaminated sites.

Coefficients of determinations (R^2) for acetonitrile-extractable and ATCLP based extractions determined in nonlinear regression analyses of the reproduction toxicity data from studies with freshly amended and weathered/aged amended soils were compared to determine which chemical measure of exposure better correlated with toxicity. These comparisons showed that coefficients of determinations were very similar in both exposure types indicating that neither extraction method had an advantage in characterizing bioavailability of EMs tested in this study to *E. crypticus*. This result supports our decision for developing draft Eco-SSLs for explosives contaminants in soil on the basis of acetonitrile extraction of test compounds. The acetonitrile extraction-based Eco-SSLs would be especially useful for Ecological Risk Assessment at contaminated sites because EM concentrations determined during site characterization are usually based on acetonitrile extraction by US EPA Method 8330.

5 CONCLUSIONS

This study has produced ecotoxicological data for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB using ecologically relevant soil invertebrate species *E. crypticus*. Relative toxicity of the five EMs tested in this study was TNB > 2,4-DNT > 2,6-DNT > RDX > HMX. All ecotoxicological parameters were estimated using measured chemical concentrations to comply with USEPA preference for derivation of Eco-SSL values on the basis of measured soil concentration of a chemical over those based on nominal concentrations (USEPA, 2000). Chemical analyses of freshly amended soils using the USEPA Method 8330 showed good correlation between nominal and measured acetonitrile-extractable (acetonitrile extraction) concentrations for the five energetic materials confirming that the soil amendment procedure used in toxicity tests was appropriate and that this method was efficient for quantifying the amounts of energetic materials in soil. The water extractable portion of each EM, which was perceived to measure the bioavailable fraction of chemicals in soil, was determined using the Adapted Toxicity Characteristic Leaching Procedure (ATCLP). Comparisons of the results of nonlinear regression analyses of the toxicity tests data showed that neither extraction method had an advantage for characterizing bioavailability and toxicity of EMs to *E. crypticus*. This result supports our decision to recommend developing Eco-SSLs for explosives contaminants in soil on the basis of acetonitrile-extractable concentrations of test compounds.

A natural soil, Sassafras sandy loam was used in all toxicity tests. Sassafras sandy loam had low organic matter and clay contents, which fulfilled the USEPA requirement of using soil with characteristics that support relatively high contaminant bioavailability for developing conservative Eco-SSL values (USEPA, 2000). Weathering and aging of amended soils were incorporated into experimental design of toxicity testing to produce a soil microenvironment more similar to field conditions. Results of chemical analyses showed that exposure conditions of *E. crypticus* to EMs tested in weathered/aged amended soils differed from those of freshly

amended soils due to significant degradation of 2,4-DNT, 2,6-DNT, and TNB and the influx of degradation products, including 3,5-DNA, 2-A-4 NT, and 4-A-2 NT. The inclusion of weathering/aging component in the EM toxicity assessments allowed us to assess the potential alterations in EM bioavailability to *E. crypticus* at contaminated sites. Additional studies would be required to investigate the toxicity of the EM degradation products individually or using chemical mixtures to provide a more complete information on ecotoxicological effects of energetic contaminants in soil to risk assessors and site managers.

Measurement endpoints assessed in this study included adult survival and juvenile production. Study results showed that tests based on reproduction endpoint provide a more sensitive evaluation of effect than adult survival and, therefore, should be used to set screening criteria. These study results will be provided to the Ecological Soil Screening Level (Eco-SSL) workgroup for review. Results will undergo quality control review by the Eco-SSL task group before inclusion in the Eco-SSL database, and before being used for developing Ecological Soil Screening Levels (Eco-SSLs) for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB.

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APPENDIX D

TOXICITY OF NITRO-HETEROCYCLIC AND NITROAROMATIC ENERGETIC MATERIALS TO *Folsomia candida* IN A NATURAL SANDY LOAM SOIL

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TOXICITY OF NITRO-HETEROCYCLIC AND NITROAROMATIC ENERGETIC MATERIALS TO *Folsomia candida* IN A NATURAL SANDY LOAM SOIL

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13. ABSTRACT *(Maximum 200 words)*

The US Environmental Protection Agency (USEPA) in conjunction with stakeholders is developing Ecological Soil Screening Level (Eco-SSL) benchmarks for contaminants at Superfund sites. Eco-SSLs are derived using published data generated from laboratory toxicity tests with different test species relevant to soil ecosystems. The Eco-SSL workgroup, after an extensive literature review (USEPA, 2000), determined that there was insufficient information for explosive materials (EM) to generate Eco-SSL benchmarks for soil invertebrates. This study was designed to produce benchmark data for the development of an Eco-SSL for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB for soil invertebrates. We used the Folsomia Reproduction Test, which uses an ecologically relevant test species and includes at least one reproductive component among the measurement endpoints. Definitive toxicity tests conducted with freshly amended soil showed that EM toxicity order based on EC_{20} values for juvenile production was TNB > 2,6-DNT > 2,4-DNT > RDX > HMX. Definitive toxicity tests conducted with weathered/aged amended soil showed that EM toxicity order based on EC_{20} values for juvenile production was 2,6-DNT > 2,4-DNT > TNB > RDX > HMX. This supported the Eco-SSL requirement of the use of reproduction endpoints for benchmark development.

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PREFACE

The work described in this report was authorized under Project No. SERDP CU-1221. The work was started in April 2001 and completed in July 2003.

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TOXICITY OF NITRO-HETEROCYCLIC AND NITROAROMATIC ENERGETIC MATERIALS TO FOLSOMIA CANDIDA IN A NATURAL SANDY LOAM SOIL

1. INTRODUCTION

Many sites associated with military operations that involve munition manufacturing, disposal, testing, and training contain elevated levels of explosives and related materials in soil. Concentrations of explosives in soil have been reported to exceed 87,000 mg kg^{-1} for TNT and 3,000 mg kg^{-1} for RDX and HMX (Simini *et al*., 1995). Although the energetic materials (EM) RDX and HMX are persistent and highly mobile in the environment, their effects on soil biota have not been sufficiently investigated. Scientifically based ecological soil screening levels (Eco-SSLs) are needed to identify contaminant explosive levels in soil that present an acceptable ecological risk. To address this problem, the U.S. Environmental Protection Agency (USEPA) in conjunction with stakeholders is developing Eco-SSL benchmarks for contaminants frequently found at Superfund sites. Eco-SSLs are defined as concentrations of chemicals in soil that, when not exceeded, will be protective of terrestrial ecosystems from unacceptable harmful effects. These Eco-SSL concentrations can be used in a Screening Level ERA to identify those contaminants in soil that warrant additional evaluation in a Baseline ERA, and to eliminate those that do not. Eco-SSLs are derived using published data generated from laboratory toxicity tests with different test species relevant to soil ecosystems. The Eco-SSL workgroup, after an extensive literature review (USEPA, 2000), determined that there was insufficient information for explosives to generate Eco-SSL benchmarks for soil invertebrates, which necessitated our study to fill this knowledge gap.

This study was designed to produce benchmark data for the development of an Eco-SSL for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), and 1,3,5-trinitrobenzene (TNB) for soil invertebrates, and meet specific criteria (USEPA, 2000), including: (1) tests were conducted in soil having physicochemical characteristics that support relatively high bioavailability of metals; (2) experimental designs for laboratory studies were documented and appropriate; (3) both nominal and analytically determined concentrations of chemicals of interest were reported; (4) tests included both negative and positive controls; (5) chronic or life cycle tests were used; (6) appropriate chemical dosing procedures were reported; (7) concentration-response relationships were reported; (8) statistical tests used to calculate the benchmark and level of significance were described; and (9) the origin of test species were specified and appropriate.

Several soil invertebrate toxicity tests, for which standardized protocols have been developed, can effectively be used to assess the toxicity and to derive protective benchmark values for energetic materials (Stephenson *et al*., 2002; Løkke and Van Gestel, 1998). We adapted the Folsomia Reproduction Test (ISO 11267:1998) for use in these studies. This bioassay was selected on the basis of its ability to measure chemical toxicity to ecologically relevant test species during chronic assays, and its inclusion of at least one reproductive component among the measurement endpoints. The primary objective of these studies was to quantify EM toxicities to the soil invertebrate *Folsomia candida* for production of benchmark data that can be used in development of Eco-SSLs for explosive contaminants in soil.

2. MATERIAL AND METHODS

2.1 Test Soil.

A natural soil, Sassafras sandy loam [Fine-loamy, siliceous, mesic Typic Hapludult] (SSL) was used in this study to assess the toxicity of test chemicals to *F. candida*. This soil was selected for developing ecotoxicological values protective of soil biota because it has physical and chemical characteristics supporting relatively high bioavailability of the test chemicals (low organic matter and clay contents). The SSL soil was collected from an open grassland field on the property of the U.S. Army Aberdeen Proving Ground (APG; Edgewood, MD). Vegetation and the organic matter horizon were removed to just below the root zone and the top six inches of the A horizon were then collected. The soil was sieved through a 5-mm^2 mesh screen, air-dried for at least 72 hours and mixed periodically to ensure uniform drying, passed through a 2-mm sieve, then stored at room temperature before use in testing. Soil was analyzed for physical and chemical characteristics by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD. Results of these analyses are presented in Table 1.

Table 1. Physical and chemical characteristics of Sassafras sandy loam soil analyzed by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD.

Soil Parameter	Sassafras Sandy Loam
Sand %	69
Silt %	13
Clay %	17
Texture	Sandy loam
CEC cmol kg^{-1}	5.5
Organic matter %	1.2
pH	5.2

2.2 Test Chemicals.

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX; CAS: 121-82-4; Purity: 99%), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX; CAS: 2691-41-0; Purity: 99%), 2,4-dinitrotoluene (2,4-DNT; CAS: 121-14-2; Purity: 97%), 2,6-dinitrotoluene (2,6-DNT; CAS: 606-20-2; Purity: 98%), and 1,3,5-trinitrobenzene (TNB; CAS: 99-35-4; Purity: 99.7%) were obtained from the Defense Research Establishment Valcartier of the Canadian Ministry of National Defense (Val Bélair, QC, Canada). Beryllium sulfate ($BeSO_4 \cdot 4H_2O$; CAS: 7787; Purity: 99.99%) was used as the positive control in these tests. Acetone (CAS: 67-64-1; HPLC Grade) was used for preparing EM solutions during soil amendments. Acetonitrile (CAS: 75-05-8; HPLC Grade) was used for extractions for chemical analyses. Methanol (CAS: 67-56-1, Chromatography grade, Purity: 99.9%) was used in determinations by HPLC. Certified standards of the energetics

(AccuStandard, Inc., New Haven, CT) were used during HPLC determinations. Unless otherwise specified, ASTM type I water (American Society of Testing and Materials, http://www.astm.org) obtained using Milli-RO® 10 Plus followed by Milli-Q® PF Plus systems (Millipore®, Bedford, MA) was used throughout the studies. Glassware was washed with phosphate-free detergent, followed by rinses with tap water, ASTM type II water, analytical reagent grade nitric acid 1% (v/v), then with ASTM type I water.

2.3 Soil Amendment Procedures.

Sassafras sandy loam soil was individually amended with RDX, HMX, 2,4-DNT, 2,6-DNT or TNB. Each treatment concentration of EM for range-finding tests was prepared separately in glass volumetric flasks and dissolved in acetone. This was necessary to dissolve the nonpolar chemicals, giving a more homogeneous mixture than the addition of solid chemical crystals to soil. Soil was spread to a thickness of 2.5 cm. The EM/acetone solution was pipetted evenly across the soil surface, ensuring that the volume of solution added at any one time did not exceed 15% (v m^{-1}) of the dry mass soil. After addition of the EM solution, the volumetric flask was rinsed twice with a known volume of acetone and pipetted onto the soil. If the total volume of solution needed to amend the soil exceeded 15% (v m^{-1}), the solution was added in successive stages, allowing the acetone to evaporate for a minimum of 2 hours under a chemical hood. The same total EM/acetone solution volume at different EM concentration was added to every treatment, equaling the volume required to dissolve the EM at the highest concentration tested. Amended soil was then air-dried overnight (minimum of 18 hours) in a dark chemical hood to prevent photolysis of the EM. Each amended soil sample was transferred into a fluorocarbon-coated high-density polyethylene container and mixed for 18 hours on a three-dimensional rotary mixer. Initial concentrations of EMs for definitive toxicity tests were prepared by adding test chemicals into an aliquot of SSL soil, using the same procedures as in range-finding tests. The final nominal target treatment concentrations for definitive tests with EMs were prepared by mixing initially-prepared soil amended with the appropriate EM with clean SSL soil for 18 hours on a three-dimensional rotary mixer. Carrier controls were treated with the carrier solvent only. After three-dimensional mixing, soil was hydrated with ASTM type I water to 88% of the soil water holding capacity (WHC; 18% water, on a the basis of the dry soil mass) for toxicity testing, or 60% of the WHC for the weathering/aging procedure. Hydrated soil prepared for toxicity tests was allowed to equilibrate for 24 hours before introducing the Folsomia.

2.4 Measurement of Soil pH.

The pH of the test soils were determined at the beginning of each definitive toxicity test using a method adapted from the Soil Survey Laboratory Methods Manual (USDA, 1996). The pH electrode was rinsed thoroughly with ASTM type I water, blotted dry, standardized with pH 4 and pH 7 buffers, rinsed and blotted. Five grams of ASTM type I water was added to 5 g soil. The soil slurry was Vortexed for 10 seconds every five minutes for 30 minutes. The soil slurry was then Vortexed for 10 seconds one minute before pH measurement. The pH was measured in the solution above the soil surface while stirring gently until the reading stabilized. The electrode was rinsed with ASTM type I water and blotted between samples.

2.5 Treatment Concentrations.

2.5.1 Range-finding tests.

Range-finding tests were conducted with freshly amended soils to determine treatment concentrations for definitive tests. Nominal EM concentrations of 0, 10, 100, 500, 1,000, 5,000 and 10,000 mg kg^{-1} were initially used in all range-finding tests.

2.5.2 Definitive tests.

Data from the range finding tests were used to determine the treatment concentrations for definitive tests. Definitive tests to assess the independent effects of EMs were conducted in freshly amended and weathered/aged amended SSL soil. Nominal EM concentrations (mg kg^{-1}) selected for the definitive test in freshly amended soil were:

RDX - 0, 1.5, 3, 9, 18, 36, 120, 360, 720, 2,000;
HMX - 0, 9, 36, 72, 144, 300, 600, 1,200, 2,400;
2,4-DNT - 0, 0.5, 1, 2, 4, 8, 12, 24, 48;
2,6-DNT - 0, 0.5, 1, 2, 4, 8, 12, 24, 48;
TNB - 0, 8, 16, 32, 64, 128, 256, 384, 512

Nominal test chemical concentrations (mg kg^{-1}) selected for the definitive tests in weathered/aged amended SSL soil were:

RDX - 0, 6, 9, 18, 36, 72, 144, 300, 600;
HMX - 0, 36, 72, 144, 300, 600, 1,200, 2,400, 5,000;
2,4-DNT - 0, 2, 4, 8, 12, 24, 48, 64, 80, 160;
2,6-DNT - 0, 2, 4, 8, 12, 24, 48, 64, 80, 160;
TNB - 0, 6, 32, 64, 128, 256, 384, 512, 768

All definitive tests included carrier (acetone) controls and positive controls. Positive controls were prepared as a solution of beryllium sulfate in ASTM type I water using 50 mg kg^{-1} Be nominal concentrations in all tests with either freshly amended or weathered/aged amended SSL soil. Nominal test concentrations of all energetic compounds were verified using USEPA Method 8330 (USEPA, 1998).

2.6 Weathering/Aging of Amended Soil.

Standardized methods for weathering/aging of explosives in soil are not available. We have developed approaches that simulate, at least partially, the weathering and aging process in soil and more closely approximate the exposure effects on soil biota in the field. This included exposing both treated and control soils, initially hydrated to 60 percent of the WHC, in open Teflon®-coated chemically inert containers in the green house to alternating wetting and drying cycles for three months. All soil treatments were weighed and readjusted to their initial mass by

adding ASTM type I water twice each week. All soil treatments were brought to 88% of the WHC (18% water, on the basis of the dry soil mass) 24 hours prior to commencement of toxicity tests for initiation of bioassays. The effect of weathering and aging on EM ecotoxicity was determined by comparing test results of weathered/aged soils with freshly amended soils.

2.7 Chemical Extractions and Analyses.

Acetonitrile extractions of soils were performed following USEPA Method 8330. At the beginning of each definitive test, either freshly amended or weathered/aged amended soils were hydrated to 60% of the WHC. Samples for chemical analysis were taken after the 24-h hydration. For each treatment, 2.0 g soil was weighed in triplicate into 50-mL polypropylene centrifuge tubes, 10 mL acetonitrile was added and the samples vortexed for 1 min, then sonicated in the dark for 18 hours at 20°C. Five mL of sonicated sample was transferred to a glass tube, to which 5 mL of $CaCl_2$ solution (5 g L^{-1}) was added. Supernatant was filtered through 0.45 µm PTFE syringe cartridges. Soil extracts were analyzed and quantified using an HPLC. In this report, results are reported as the concentration in dry soil.

In addition to acetonitrile-extractable, soil samples were extracted using an Adapted Toxicity Characteristic Leaching Procedure (ATCLP, Haley, 1993) at the beginning of each definitive test with freshly amended or weathered/aged amended soils. The ATCLP is based on modification of the Toxicity Characteristic Leaching Procedure (TCLP) (40 CFR Part 268.41, Hazardous Waste Management, Method 1311). The modification involved substitution of CO_2-saturated ASTM type I water for acetic acid, better simulating soil-water conditions due to respiration by soil biota. All analytical measurements were done in triplicate at the beginning of each test. For each treatment concentration, 4 g of soil was transferred in triplicate into 20 mL vials. Sixteen mL of CO_2-saturated water (pH 3.8 to 4.0) was added to the vials, and the vials were rapidly sealed. Soil samples were vortexed for 45 sec, and then mixed in the dark for 18 hours using a rotary mixer (30 rpm) at room temperature. Settled supernatants were filtered through 0.45 µm PTFE syringe cartridges. An equivalent volume of acetonitrile was added to filtered soil extract prior to HPLC analysis. In this report, ATCLP soil extraction is referred to as the water-soluble fraction of EM. Nominal and determined (measured) concentrations used in the definitive tests are shown in Tables 2 through 11.

The soil extracts were analyzed by reversed-phase HPLC using a modified EPA Method 8330. The method was modified in two ways. First, the final solvent for the energetic compounds was a mixture of 60 parts water and 40 parts acetonitrile rather than a 50:50 ratio. Secondly, the flow rate of the 50:50 methanol:water mobile phase was 1.0 ml/min rather than 1.5 ml/min. A 25 cm x 4.6 mm x 5 micron particle size C-18 column was used for all determinations since only one energetic compound was analyzed at a time. The instrument used was a Beckman *System Gold*, consisting of a model 126 programmable solvent module, model 168 diode array detector and a model 507 automatic sampler. Calibration curves were generated before each HPLC run by dissolving certified standards (AccuStandard, Inc., New Haven, CT) of RDX and HMX in 60:40 water:acetonitrile in a range of concentrations appropriate for each run. The method detection limit was 0.05 mg kg^{-1}. Blanks and standards were placed intermittently between unknown samples to maintain quality assurance of the samples. All reagents used in

extraction of chemicals from soils were either reagent or trace metal grade, and ASTM Type I water was used throughout the analytical studies

2.8 Toxicity Assessment.

The Folsomia Reproduction Test (ISO 11267:1998) was used to assess the effects of EMs on the reproduction of the Collembolan *F. candida*. The test is an adaptations of an internationally standardized bioassay of the International Standardization Organization (ISO) Soil Quality – Inhibition of Reproduction of Collembola (*Folsomia candida*) by Soil Pollutants, reference number: ISO/FDIS 11267:1998(E). The measurement endpoints for the test are adult survival and production of juveniles, where juvenile production is the reproduction endpoint. The ISO Guideline for this assay was originally developed for use with OECD Artificial Soil (equivalent to USEPA Standard Artificial Soil; SAS). Our research has shown that this test can be conducted using natural soils (Phillips et al., 2002; Kuperman et al, 2003).

2.8.1 Principle of the Test.

Collembola are exposed to a range of concentrations of the test substance mixed in soil. The total number of juveniles produced (effective reproduction) and the survival of adult collembola are assessed. Test duration is 28 days. After 28 days both the number of adults and the number of juveniles are determined by counting. The effective reproduction and survival of adults exposed to the test substance is compared to that of the Control treatments to quantify ecotoxicological parameters. These parameters include the bounded No Observed Effect Concentration (NOEC), the bounded Lowest Observed Effect Concentration (LOEC) and the effective concentration that causes an x percent reduction in juvenile numbers, ECp (e.g., EC_{20}, EC_{50}).

2.8.2 Test Validity Criteria.

Validity criteria are part of Quality Control procedures. Adaptation of the Folsomia Reproduction Test for use with natural soils, included the following performance parameters for the negative controls:

1) The adult mortality should not exceed 30% at the end of the test;
2) The average number of juveniles per chamber should reach 80 instars at the end of the 28-day test;
3) The coefficient of variation for reproduction should not exceed 30%.

2.8.3 Culturing Conditions.

The U.S. Army Edgewood Chemical Biological Center (USA ECBC) laboratory culture of *F. candida* (Collembola; springtails) was established in 1994 from a stock culture obtained from the University of Illinois-Chicago. The University of Illinois-Chicago originated its culture from Collembola collected in Kane County, Illinois in 1981. The USA ECBC culture is maintained in culture jars on a mixture of charcoal and plaster of Paris in the dark at $20^{o}C$. The Collembola were fed baker's yeast and kept moist by routine misting with purified water approximately twice per week. Synchronized cultures were established for the experiments by

removing egg clusters from stock cultures and placing them into new jars. Eggs were monitored daily to determine the onset of hatching. Once hatching began, it was allowed to proceed for 2 days, after which juveniles were transferred to new jars. These synchronized juveniles were then held for 10 d, and these procedures provided the 10-12 day-old juveniles used in these tests.

2.8.4 Test Performance.

Glass test containers (42 mm ID; 45 mm deep) were rinsed with successively with acetone, tap water, and purified water before the test. In order to prepare five replicates of each treatment, 100g of each air-dried treatment soil, respectively, was hydrated to 88% of water holding capacity (WHC). Then one-fifth of each batch of hydrated treatment soil was transferred by weight into a test container and 0.05 g of baker's yeast was added to the surface of the soil. Ten 10-12-day-old juveniles were placed in each test container, followed by light misting with purified water. A piece of plastic food-wrap was placed on each container and held in place with a rubber band. The mass of each container was then recorded to monitor soil moisture loss during the test. Five replicates were used for each EM treatment concentration, as well as the control treatments.

The test containers were randomly placed in an incubator at 20$\pm$0.5C with a relative humidity of 88$\pm$5%. During the course of the study, the containers were misted weekly to maintain soil moisture level.

To terminate a test, approximately 15 mL of tap water was added to a test container and allowed to sit for several minutes to fully hydrate the soil. After gentle mixing with a spatula, an additional 10 mL of water was added. The contents of the test container were given a final mixing and examined under a dissecting microscope (15x) for the presence of juveniles and adults. The juveniles and adults that floated to the surface were counted.

Measurement endpoints were the number of surviving adults and the number of juveniles produced after 28 days. All ecotoxicological parameters were estimated using acetonitrile-extractable concentrations of each explosive for each treatment concentration.

2.9 Data Analysis.

Juvenile production data were analyzed using nonlinear regression models described in Stephenson *et al*. (2000) and Kuperman *et al*. (2003). Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to decide whether or not to weight the data, and to select potential models. The logistic (Gompertz) model [1] had the best fit for data in all toxicity tests except tests with SSL soil freshly amended with RDX and 2,6-DNT. The Exponential model best fit the data. The fit of the lines generated by these models were closest to the data points, the variances were the smallest, and the residuals had the best appearance (i.e., most random scattering). These models were:

[1] $Y = a \times e([\log(1\text{-}p)] \times [C/\mathrm{ECp}]b)$

[2] $Y = a \times e(([\log(1\text{-}p)] / \mathrm{ECp}) \times C) + \mathrm{b}$

where *Y* is the number of juveniles produced, *a* is the control response, *e* is the base of the natural logarithm, p is the percent inhibition/100 (e.g., 0.20 for EC_{20}; 0.50 for EC_{50}), *C* is the exposure concentration measured in test soil, ECp is the estimate of effect concentration for a specified percent effect, and *b* is the scale parameter. The ECp parameters used in this study included the concentrations producing a 20% (EC_{20}) or 50% (EC_{50}) reduction in the measurement endpoint, respectively. The asymptotic standard error (a.s.e.) and 95% confidence intervals (CI) associated with the point estimates were determined. The EC_{20} parameter based on a reproduction endpoint is the preferred parameter for deriving soil invertebrate Eco-SSL benchmarks. The $EC_{50,}$ a commonly reported value, was included to enable comparisons of the results produced in this study with results reported previously by other researchers.

Analysis of Variance (ANOVA) was used to determine the bounded No Observed Effect Concentration (NOEC) and bounded Lowest Observed Effect Concentration (LOEC) values for adult survival or juvenile production data. ANOVA analyses, and adult survival data, were included to enable comparisons of the results produced in this study with results previously reported by other researchers. Mean separations were determined using Fisher's Least Significant Difference (LSD) pairwise comparison tests. A significance level of $P < 0.05$ was used. All analyses were done using measured acetonitrile-extractable EM concentrations. Statistical analyses were performed using SYSTAT 7.0.1 (SPSS 1997).

3. RESULTS

3.1 Analytical Determinations of Energetic Materials in Soil.

Concentrations of EMs in amended soils were determined at the beginning of each definitive toxicity test using both acetonitrile-extractable and ATCLP extractions. Results of these analyses are shown in Tables 2 through 11. Measured RDX acetonitrile-extractable concentrations in freshly amended Sassafras sandy loam (SSL) soil averaged 112 (range: 99-131) percent of nominal concentrations. Measured RDX Adapted Toxicity Characteristic Leaching Procedure (ATCLP) water extractable concentrations ranged from 1 to 121 mg kg^{-1} and averaged 59% of acetonitrile-extractable concentrations due to low solubility of RDX in water (Table 2). Measured soil pH values among the different concentrations did not deviate more than 0.1 pH units from the control soil (Table 2).

Table 2. Nominal and average measured (n = 3) RDX concentrations (mg kg^{-1}) and mean pH values in freshly amended SSL soil. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH (water) n=3
0	BDL*			BDL			5.51
1.5	1.96	0.06	131	1.32	0.04	67	5.58
3	3.03	0.21	101	2.79	0.11	92	5.54
9	10.24	0.16	114	7.99	0.17	78	5.56
18	20.4	1.07	113	17.17	0.42	84	5.58
36	44.37	2.60	123	37.07	1.37	84	5.46
120	138.7	2.60	116	120.67	0.33	87	5.58
360	355.7	5.67	99	94.20	2.08	27	5.60
720	744.7	3.38	103	88.27	4.20	12	5.52
2000	2120.7	32.20	106	78.87	0.41	4	5.52

*BDL - Below Detection Limit is reported when no RDX was detected in the control soil.

Measured RDX acetonitrile-extractable concentrations in weathered/aged amended soils averaged 83 (range: 43-105) percent of nominal concentrations (Table 3). Measured RDX ATCLP-extractable concentrations in weathered/aged amended soils averaged 76 (range: 18-100) percent of acetonitrile-extractable concentrations (Table 3). Weathering/aging of amended soils reduced RDX acetonitrile-extractable concentrations on average by 29% compared with acetonitrile-extractable concentrations in freshly amended soils (Table 3), whereas RDX ATCLP-extractable concentrations increased, on average, by 17 percent compared with freshly amended soils. Measured soil pH values among the different concentrations did not deviate more than 0.4 pH units from the control soil (Table 3).

Table 3. Nominal and average measured (n = 3) RDX concentrations (mg kg^{-1}) and mean pH values in weathered/aged amended SSL soil. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH (water) n=3
0	BDL*			BDL			5.32
6	6.4	1.50	106	5.78	0.38	91	5.31
9	8.4	1.31	94	7.72	0.27	92	5.30
18	15.7	0.24	87	13.55	0.47	87	5.28
36	30.0	0.75	83	29.99	0.38	100	5.27
72	56.6	3.38	79	54.10	2.01	96	5.20
144	61.5	2.23	43	55.13	2.99	90	5.12
300	254.3	8.74	85	100.06	2.54	39	5.00
600	527.0	4.04	88	93.23	1.16	18	5.00

*BDL - Below Detection Limit is reported when no RDX was detected in the control soil.

Measured HMX acetonitrile-extractable concentrations in freshly amended soils averaged 108 (range: 92-125) percent of nominal concentrations. Measured HMX ATCLP-extractable concentrations remained relatively constant ranging from 6 to 15 mg kg^{-1} and averaged 16% of acetonitrile-extractable concentrations (Table 4). Measured soil pH values among the different concentrations did not deviate more than 0.1 pH units from the control soil (Table 4).

Table 4. Nominal and average measured (n = 3) HMX concentrations (mg kg^{-1}) and mean pH values in freshly amended SSL soil. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	**Acetonitrile extraction (mg kg^{-1})**	**Standard error**	**Acetonitrile/ Nominal (%)**	**ATCLP extraction (mg kg^{-1})**	**Standard error**	**ATCLP/ Acetonitrile (%)**	**Mean pH (water) n=3**
0	BDL*			BDL			5.37
9	11.2	4.98	125	5.92	0.51	53	5.41
36	36.0	2.77	100	15.17	0.55	42	5.35
72	73.6	8.25	102	13.10	0.06	18	5.35
144	141.3	7.54	98	12.47	0.30	98	5.32
300	348.0	10.44	116	12.29	0.21	45	5.35
600	641.7	8.69	107	12.48	0.19	2	5.39
1200	1491.3	63.31	124	12.94	0.32	1	5.40
2400	2211.3	119.45	92	12.58	0.45	0.6	5.38

*BDL - Below Detection Limit is reported when no HMX was detected in the control soil.

Measured HMX acetonitrile-extractable concentrations in weathered/aged amended soils averaged 90 (range: 74-99) percent of nominal concentrations (Table 5). Measured HMX ATCLP-extractable concentrations in weathered/aged amended soils averaged 12 (range: 0.4-45) percent of acetonitrile-extractable concentrations (Table 5). Weathering/aging of amended soils reduced HMX acetonitrile-extractable concentrations on average by 18% compared with acetonitrile-extractable concentrations in freshly amended soils (Table 5), whereas RDX ATCLP-extractable concentrations were reduced, on average, by 4 percent compared with freshly amended soils. Measured soil pH values among the different concentrations did not deviate more than 0.8 pH units from the control soil (Table 5).

Table 5. Nominal and average measured (n = 3) HMX concentrations (mg kg^{-1}) and mean pH values in weathered/aged amended SSL soil. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH (water) n=3
0	BDL			BDL			4.97
36	28.9	1.31	80	13.11	0.15	45	5.35
72	53.44	1.79	74	14.64	0.66	27	5.25
144	129.3	10.90	90	16.43	0.62	13	5.32
300	280.3	8.67	93	18.96	0.34	7	5.29
600	561.7	15.24	94	18.03	0.46	3	5.39
1200	1124.0	49.33	94	21.79	0.81	2	5.56
2500	2490.7	114.24	99	17.46	0.98	0.7	5.68
5000	4784.0	142.73	96	17.92	0.44	0.4	5.76

*BDL - Below Detection Limit is reported when no HMX was detected in the control soil.

Measured 2,4-DNT acetonitrile-extractable concentrations in freshly amended soils averaged 78 (range: 67-121) percent of nominal concentrations (Table 6). Measured 2,4-DNT ATCLP-extractable concentrations averaged 35 (range: 12-51.7) percent of acetonitrile-extractable concentrations (Table 6). Measured soil pH values among the different concentrations did not deviate more than 0.3 pH units from the control soil (Table 6).

Measured 2,4-DNT acetonitrile-extractable concentrations in weathered/aged amended soils averaged 56 (range: 37-120) percent of nominal concentrations (Table 7). Measured 2,4-DNT ATCLP-extractable concentrations averaged 54 (range: 47-64) percent of acetonitrile-extractable concentrations (Table 7). Weathering/aging of amended soils reduced 2,4-DNT acetonitrile-extractable concentrations, on average, by 16 percent, while 2,4-DNT ATCLP-extractable concentrations increased, on average, by 19 percent compared with freshly amended soils. Measured soil pH values among the different concentrations did not deviate more than 0.2 pH units from the control soil (Table 7).

Table 6. Nominal and average measured (n = 3) 2,4-DNT concentrations (mg kg^{-1}) and mean pH values in freshly amended SSL soil. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH (water) n=3
0	BDL*			BDL			5.51
0.5	0.6	0.0	121	0.07	0.01	12	5.24
1	0.95	0.03	95	0.15	0.01	16	5.31
2	1.44	0.06	72	0.30	0.03	21	5.31
4	3.08	0.08	77	0.88	0.01	29	5.36
8	5.41	0.15	68	2.14	0.03	40	5.31
12	8.40	0.26	70	3.56	0.11	42	5.28
24	19.73	0.66	82	9.56	0.12	48	5.23
48	42.77	0.57	89	22.13	0.52	52	5.23

*BDL - Below Detection Limit is reported when no 2,4-DNT was detected in the control soil.

Table 7. Nominal and average measured (n = 3) 2,4-DNT concentrations (mg kg^{-1}) and mean pH values in weathered/aged amended SSL soil. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH (water) n=3
0	BDL*			BDL			5.41
2	2.4	0.06	120	1.36	0.000	56	5.34
4	2.95	0.04	74	1.59	0.040	54	5.29
8	3.0	0.53	37	1.67	0.030	56	5.39
12	5.2	0.20	43	2.42	0.06	47	5.34
24	11.5	0.18	48	5.22	0.02	46	5.40
48	21.5	0.34	45	11.77	0.12	55	5.35
64	31.0	0.75	48	15.40	0.15	50	5.35
80	37.3	0.82	47	20.47	0.37	55	5.31
160	71.7	2.27	45	46.07	0.37	64	5.37

*BDL - Below Detection Limit is reported when no 2,4-DNT was detected in the control soil.

Measured 2,6-DNT acetonitrile-extractable concentrations in freshly amended soils averaged 254 (range: 83-747) percent of nominal concentrations (Table 8). Measured 2,6-DNT ATCLP-extractable concentrations averaged 39 (range: 25-62) percent of acetonitrile-extractable concentrations (Table 8). Measured soil pH values among the different concentrations did not deviate more than 0.2 pH units from the control soil (Table 8).

Table 8. Nominal and average measured (n = 3) 2,6-DNT concentrations (mg kg^{-1}) and mean pH values in freshly amended SSL soil. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH (water) n=3
0	BDL*			BDL			5.47
0.5	3.74	0.82	747	1.02	0.01	27	5.51
1	4.35	0.03	435	1.08	0.001	25	5.35
2	5.33	0.13	267	1.43	0.014	27	5.43
4	7.65	0.85	191	2.18	0.01	28	5.43
8	9.38	0.27	117	3.78	0.01	40	5.32
12	12.93	0.19	108	5.83	0.04	45	5.35
24	19.97	0.81	83	10.63	0.08	53	5.49
48	40.20	1.96	84	24.84	0.04	62	5.27

*BDL - Below Detection Limit is reported when no 2,6-DNT was detected in the control soil.

Measured 2,6-DNT acetonitrile-extractable concentrations in weathered/aged amended soils averaged 20 (range: 13-34) percent of nominal concentrations (Table 9). Measured 2,6-DNT ATCLP-extractable concentrations averaged 40 (range: 16-62) percent of acetonitrile-extractable concentrations (Table 9). Weathering/aging of amended soils reduced 2,6-DNT acetonitrile-extractable concentrations, on average, by 234% compared with acetonitrile-extractable concentrations in freshly amended soils, while 2,6-DNT ATCLP-extractable concentrations increased, on average, by one percent compared with freshly amended soils. Measured soil pH values among the different concentrations did not deviate more than 0.2 pH units from the control soil (Table 9).

Table 9. Nominal and average measured (n = 3) 2,6-DNT concentrations (mg kg^{-1}) and mean pH values in weathered/aged amended SSL soil. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH (water) n=3
0	BDL*			BDL			5.27
2	0.3	0.08	15	BDL			5.29
4	0.8	0.01	20	0.13	0.06	16	5.29
8	1.2	0.02	15	0.23	0.01	19	5.39
12	1.6	0.02	13	0.42	0.03	27	5.29
24	3.7	0.08	15	1.46	0.06	40	5.39
48	9.5	0.12	20	4.30	0.09	45	5.42
64	13.9	0.12	22	6.63	0.08	48	5.37
80	18.1	0.20	23	9.64	0.36	53	5.31
160	37.4	0.98	23	17.43	3.27	47	5.33
320	108.3	1.45	34	66.87	2.22	62	5.38

*BDL - Below Detection Limit is reported when no 2,6-DNT was detected in the control soil.

TNB recovery was greatly reduced in treatments below 64 mg kg^{-1}. Measured TNB acetonitrile-extractable concentrations in freshly amended soils averaged 68 (range: 25-101) percent of nominal concentrations (Table 10). Measured TNB ATCLP-extractable concentrations averaged 65 (range: 56-86) percent of acetonitrile-extractable concentrations (Table 10). These values do not include data for 8 mg kg^{-1} nominal treatment concentration, which had TNB recovery in one (0.13 mg kg^{-1}) out of three replicates producing an average ATCLP-extractable value of 0.043 mg kg^{-1} (Table 10). Measured soil pH values among the different concentrations did not deviate more than 0.2 pH units from the control soil (Table 10).

Table 10. Nominal and average measured (n = 3) TNB concentrations (mg kg^{-1}) and mean pH values in freshly amended SSL soil. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	**Acetonitrile extraction (mg kg^{-1})**	**Standard error**	**Acetonitrile/ Nominal (%)**	**ATCLP extraction (mg kg^{-1})**	**Standard error**	**ATCLP/ Acetonitrile (%)**	**Mean pH (water) n=3**
0	BDL*			BDL			5.34
8	2.6	0.11	32	0.043		2	5.54
16	3.9	0.48	25	2.45	0.29	62	5.42
32	13.6	1.11	43	7.68	0.25	56	5.41
64	45.0	1.80	70	30.22	0.52	67	5.43
128	107.0	2.52	84	83.67	1.28	78	5.39
256	221.0	12.66	86	190.95	1.40	86	5.36
384	385.7	21.15	100	328.28	14.80	85	5.36
512	518.0	9.17	101	439.56	9.87	85	5.44

*BDL - Below Detection Limit is reported when no TNB was detected in the control soil.

Measured TNB acetonitrile-extractable concentrations in weathered/aged amended soils averaged 53 (range: 4-97) percent of nominal concentrations (Table 11). Measured TNB ATCLP-extractable concentrations averaged 63 (range: 19-93) percent of acetonitrile-extractable concentrations (Table 11). Weathering/aging of amended soils reduced TNB acetonitrile-extractable concentrations, on average, by 15% compared with acetonitrile-extractable concentrations in freshly amended soils, while TNB ATCLP-extractable concentrations were reduced, on average, by two percent compared with freshly amended soils. Measured soil pH values among the different concentrations did not deviate more than 0.2 pH units from the control soil (Table 11).

Table 11. Nominal and average measured (n = 3) TNB concentrations (mg kg^{-1}) and mean pH values in weathered/aged amended SSL soil. Measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH (water) n=3
0	BDL*			BDL			5.38
16	0.6	0.07	4	0.14	0.010	25	5.43
32	1.3	0.15	4	0.24	0.01	19	5.43
64	8.8	0.38	14	3.35	0.33	38	5.42
128	75.8	0.27	59	55.80	1.89	74	5.31
256	176.3	5.67	69	143.40	2.15	81	5.22
384	304.7	7.84	79	284.38	7.50	93	5.28
512	491.3	9.96	96	396.38	5.05	81	5.31
768	747.7	22.60	97	665.22	6.64	89	5.35

*BDL - Below Detection Limit is reported when no TNB was detected in the control soil.

3.2 Range-Finding Toxicity Tests.

RDX in SSL soil caused a reduction in adult survival and juvenile production at the 100 mg kg^{-1} treatment level. HMX had an effect on adult survival in the range-finding test starting at the 1,000 mg kg^{-1} concentration. Juvenile numbers were reduced at the 500 mg kg^{-1} treatment level. Both 2,4-DNT and 2,6-DNT had an effect on adult survival and juvenile production starting at the 10 mg kg^{-1} treatment level. In the range-finding test with TNB, adult survival and juvenile production was reduced at 100 mg kg^{-1}. Results of these range-finding tests were used to determine treatment concentrations for the definitive tests shown in Tables 2 through 11.

3.3 Definitive Toxicity Tests.

Definitive studies using the Folsomia Reproduction Test were conducted to assess the effects of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB on the reproduction of the Collembolan *F. candida*. Juveniles were exposed in SSL soil to a range of concentrations for each EM, in independent investigations. Measurement endpoints were assessed using 6 to 10 treatment concentrations determined from the range-finding studies and included the number of surviving adults and the number of juveniles produced after 28 days. All ecotoxicological parameters were estimated using measured chemical concentrations for each treatment level.

Test results complied with the validity criteria adapted from the ISO test guideline. Mean adult survival in the negative controls ranged from 86 to 96 percent in all tests. The mean juvenile production in negative controls ranged from 134 to 566 juveniles, and the coefficient of variation ranged from 5 to 30 percent. Juvenile production in the positive controls ranged from 24 to 64 percent reduction from negative controls and was within the baseline established for the laboratory culture of *F. candida*. These results confirmed that the toxicological effects determined

in the definitive tests were most likely due to the EM treatments. All reported ecotoxicological parameters have been calculated based on actual measured concentrations. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330.

3.3.1 Toxicity of RDX.

Results of RDX toxicity testing in freshly amended and weathered/aged amended SSL soil are shown in Table 12. The bounded NOEC for adult survival in freshly amended SSL soil was 44.37 mg kg^{-1} (no significant difference compared to control, $P = 1.000$) (Table 13). Adult survival was significantly ($P \leq 0.0001$) reduced by 21% at the LOEC of 138.7 mg kg^{-1}. The bounded NOEC for juvenile production was 20.4 mg kg^{-1} (no significant difference compared to control, $P = 0.535$). The bounded LOEC for juvenile production was 44.37 mg kg^{-1} ($P = 0.005$). The EC_{20} and EC_{50} values were 27.84 and 86.48 mg kg^{-1}, respectively (Exponential model) (Table 13). The bounded NOEC for adult survival in weathered/aged amended SSL soil was 527 mg kg^{-1} (no significant difference compared to control, $P = 0.264$) (Table 13). Adult survival was not significantly ($P = 0.264$) reduced at the highest concentration used in this study, thus producing an unbounded LOEC at >527 mg kg^{-1}. The bounded NOEC for juvenile production was 56.6 mg kg^{-1} (no significant difference compared to control, $P = 0.079$). The bounded LOEC for juvenile production was 61.5 mg kg^{-1} ($P = 0.012$). The EC_{20} and EC_{50} values were 113.03 and 770.66 mg kg^{-1}, respectively (Gompertz model) (Table 13). All ecotoxicological parameters (mg kg^{-1}) for RDX determined in freshly amended and weathered/aged amended SSL soil are given in Table 13.

Table 12. Mean (n = 5) adult survival and juvenile production in freshly amended and in weathered/aged RDX-amended SSL soil.

Concentration in freshly amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error	Concentration in weathered/aged amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error
Negative control	10	295	16	Negative control	9	473	23
Acetone control	9	285	7	Acetone control	10	500	12
Positive control	6	119	10	Positive control	10	304	27
1.96	10	299	17	6.4	10	474	14
3.03	9	273	17	8.4	10	480	15
10.24	9	289	14	15.7	10	482	19
20.4	9	273	19	30.0	10	463	22
44.37	8	226	12	56.6	9	451	33
138.7	8	171	10	61.5	9	428	13
355.7	7	165	16	254.3	9	319	15
744.7	6	115	10	527.0	9	308	23
2120.7	5	116	13				

Table 13. Summary of ecotoxicological parameters (mg kg^{-1}) for adult survival and for juvenile production of *F. candida* for RDX determined in freshly amended and in weathered/aged amended SSL soil. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330 and water extraction using Adapted Toxicity Characteristic Leaching Procedure (ATCLP).

Exposure assessment	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh						
Acetonitrile extraction	44.4	138.7	20.4	44.37	28	86.5
p or 95% C.I.	1.000	<0.0001	0.535	0.005	14 – 41	45 – 128
R^2					0.982	0.982
ATCLP extraction	37	88	17	37	26	93
p or 95% C.I.	1.000	<0.0001	0.535	0.005	6 – 45	71 – 115
R^2					0.972	0.972
Weathered/aged						
Acetonitrile extraction	527	>527	56.6	61.5	113	771
p or 95% C.I.	0.264	0.264	0.079	0.012	29 – 197	444 – 1097
R^2					0.991	0.991
ATCLP extraction	93	>93	54	55	74	118
p or 95% C.I.	0.264	0.264	0.079	0.012	62 – 85	103 – 134
R^2					0.992	0.992

Concentration-response relationships for juvenile production in fresh and in weathered/aged RDX amended soil determined by nonlinear regressions are shown in Figure 1. The Exponential model had the best fit for data from the test with freshly amended soil (Figure 1A). The Gompertz model had the best fit for data from the test with weathered/aged amended soil (Figure 1B). Overall, reproduction was higher in weathered/aged soils amended with RDX (Table 12). Juvenile production EC_{20} values were 27.840 and 113.026 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. The difference between these values was not statistically significant based on 95% confidence intervals (Table 13). Juvenile production EC_{50} values were 86.479 and 770.662 mg kg^{-1} in freshly amended and weathered/aged soils, respectively.

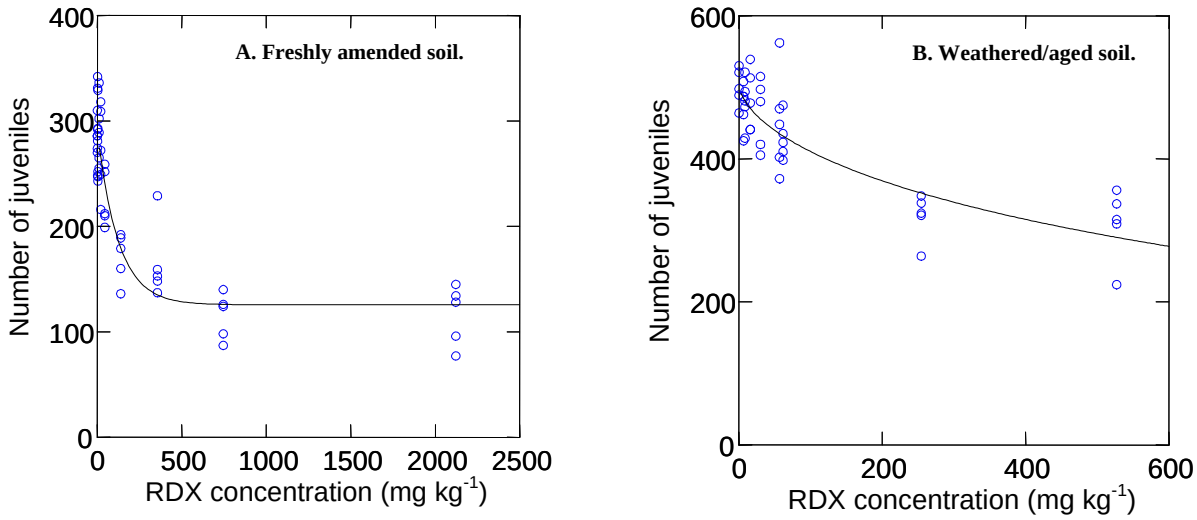

Figure 1. Effects of RDX on juvenile production in freshly amended (A) and weathered/aged (B) RDX amended SSL soil.

3.3.2 Toxicity of HMX.

Results of HMX toxicity testing in freshly amended and weathered/aged amended SSL soils are shown in Table 14. Mean adult survival and juvenile production for the HMX toxicity tests in freshly amended and in weathered/aged amended SSL soils are shown in Table 14.

Table 14. Mean (n = 5) adult survival and juvenile production in freshly amended and in weathered/aged HMX-amended SSL soil.

Concentration in freshly amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error	Concentration in weathered/aged amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error
Negative control	9.2	183	15	Negative control	10	558	34
Acetone control	9.2	190	15	Acetone control	10	570	21
Positive control	6.8	110	10	Positive control	9	296	29
11.2	8.8	177	14	28.9	10	560	16
36.0	8.6	161	12	53.44	10	529	25
73.6	8.8	153	11	129.3	10	505	16
141.3	8.4	161	10	280.3	9	485	35
348.0	8.4	142	8	561.7	10	481	24
641.7	8.2	157	10	1124.0	9	472	26
1491.3	7.6	128	15	2490.7	10	415	35
2211.3	7.8	112	9	4784.0	7	336	16

The bounded NOEC for adult survival in freshly amended SSL soil was 641.7 mg kg^{-1} (no significant difference compared to control, $P = 0.075$). Adult survival was significantly ($P = 0.006$) reduced at the LOEC of 1491.3 mg kg^{-1}. The bounded NOEC for juvenile production was 641.7 mg kg^{-1} (no significant difference compared to control, $P = 0.054$). The bounded LOEC for juvenile production was 1491.3 mg kg^{-1} ($P = 0.001$). The EC_{20} and EC_{50} values were 234.831 and 8798.571 mg kg^{-1}, respectively (Gompertz model). The bounded NOEC for adult survival in weathered/aged amended SSL soil was 2490.7 mg kg^{-1} (no significant difference compared to control, $P = 0.744$). Adult survival was significantly ($P \leq 0.0001$) reduced at the highest concentration used in this study, thus producing an unbounded LOEC at >4784 mg kg^{-1}. The bounded NOEC for juvenile production was 129.3 mg kg^{-1} (no significant difference compared to control, $P = 0.069$). The bounded LOEC for juvenile production was 280.3 mg kg^{-1} ($P = 0.019$). The EC_{20} and EC_{50} values were 1045.627 and 10,369.869 mg kg^{-1}, respectively (Gompertz model). All ecotoxicological parameters (mg kg^{-1}) for HMX determined in freshly amended and weathered/aged amended SSL soil are given in Table 15.

Table 15. Summary of ecotoxicological parameters (mg kg^{-1}) for adult survival and for juvenile production of *F. candida* for HMX determined in freshly amended and in weathered/aged amended SSL soil. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330 and water extraction using Adapted Toxicity Characteristic Leaching Procedure (ATCLP).

Exposure assessment	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh						
Acetonitrile extraction	642	1,491	642	1,491	235	8,799
p or 95% C.I.	0.075	0.006	0.054	0.001	0 - 730	0-22,648
R^2					0.975	0.975
ATCLP extraction	12.94	12.58	15.17	13.10	9.7	39
p or 95% C.I.	0.0006	0.015	0.86	0.032	1 – 18	0 – 103
R^2					0.967	0.967
Weathered/aged						
Acetonitrile extraction	2,491	4,784	129	280	1,046	10,370
p or 95% C.I.	0.744	<0.0001	0.069	0.019	58-2,033	3,156-17,583
R^2					0.989	0.989
ATCLP extraction	17.46	17.92	16.43	18.96	18	34
p or 95% C.I.	0.744	<0.0001	0.069	0.019	12 – 24	12 – 55
R^2					0.977	0.977

Concentration-response relationships for juvenile production in fresh and in weathered/aged HMX amended soil determined by nonlinear regressions are shown in Figure 2. The Gompertz model had the best fit for data from the test with freshly amended soil (Figure 2A) and with weathered/aged amended soil (Figure 2B). Overall, reproduction was higher in weathered/aged HMX amended soils (Table 14). Juvenile production EC_{20} values were 234.831 and 1045.627 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. The difference between these values was not statistically significant based on 95% confidence intervals (Table 15). Juvenile production EC_{50} values were 8798.571 and 10,369.869 mg kg^{-1} in freshly amended

and weathered/aged soils, respectively. The difference between the EC_{50} values was not statistically significant based on 95% confidence intervals (Table 15).

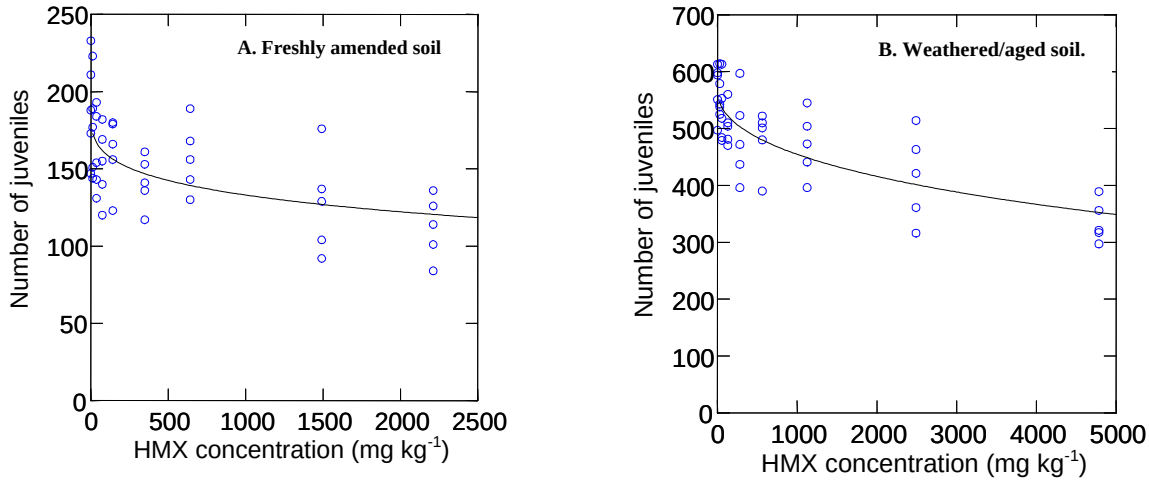

Figure 2. Effects of HMX on juvenile production in freshly amended (A) and weathered/aged (B) HMX amended SSL soil.

3.3.3 Toxicity of 2,4-DNT.

Mean adult survival and juvenile production for the 2,4-DNT toxicity tests in freshly amended and in weathered/aged amended SSL soils are shown in Table 16.

Table 16. Mean (n = 5) adult survival and juvenile production in freshly amended and in weathered/aged 2,4-DNT-amended SSL soil.

Concentration in freshly amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error	Concentration in weathered/aged amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error
Negative control	9	294	21	Negative control	9	414	18
Acetone control	10	312	14	Acetone control	9	400	19
Positive control	6	127	26	Positive control	7	223	16
0.6	10	306	16	2.4	9	407	21
0.95	9	301	18	2.95	8	399	13
1.44	9	267	23	3.0	9	358	21
3.08	10	284	15	5.2	8	315	13
5.41	7	239	23	11.5	6	299	8
8.40	7	213	9	21.5	6	217	39
19.73	6	198	20	31.0	5	50	15
42.77	2	0	0	37.3	2	3	3
				71.7	0	0	0

The bounded NOEC for adult survival in freshly amended SSL soil was 3.08 mg kg^{-1} (no significant difference compared to control, $P = 1.000$) (Table 17). Adult survival was significantly ($P \leq 0.0001$) reduced at the LOEC of 5.41 mg kg^{-1}. The bounded NOEC for juvenile production was 3.08 mg kg^{-1} (no significant difference compared to control, $P = 0.239$). The bounded LOEC for juvenile production was 5.41 mg kg^{-1} ($P = 0.004$). The EC_{20} and EC_{50} values were 9.918 and 20.661 mg kg^{-1}, respectively (Gompertz model) (Table 17). The bounded NOEC for adult survival in weathered/aged amended SSL soil was 5.2 mg kg^{-1} (no significant difference compared to control, $P = 0.325$) (Table 17). Adult survival was significantly ($P \leq 0.0001$) reduced at the LOEC of 11.5 mg kg^{-1}. The bounded NOEC for juvenile production was 3.0 mg kg^{-1} (no significant difference compared to control, $P = 0.084$). The bounded LOEC for juvenile production was 5.2 mg kg^{-1} ($P = 0.001$). The EC_{20} and EC_{50} values were 14.925 and 22.859 mg kg^{-1}, respectively (Gompertz model) (Table 17). All ecotoxicological parameters (mg kg^{-1}) for 2,4-DNT determined in freshly amended and weathered/aged amended SSL soil are given in Table 17.

Concentration-response relationships for juvenile production in fresh and in weathered/aged 2,4-DNT amended soil determined by nonlinear regressions are shown in Figure 3. The Gompertz model had the best fit for data from the test with freshly amended soil (Figure 3A) and with weathered/aged amended soil (Figure 3B). Overall, reproduction was higher in weathered/aged 2,4-DNT amended soils (Table 16). Juvenile production EC_{20} values were 9.918 and 14.925 mg kg^{-1} in freshly amended and weathered/aged soils, respectively (Table 17). The difference between these values was not statistically significant based on 95% confidence intervals (Table 17). Juvenile production EC_{50} values were 20.661 and 22.859 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. Also, the difference between the EC_{50} values was not statistically significant based on 95% confidence intervals (Table 17).

Table 17. Summary of ecotoxicological parameters (mg kg^{-1}) for adult survival and for juvenile production of *F. candida* for 2,4-DNT determined in freshly amended and in weathered/aged amended SSL soil. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330 and water extraction using Adapted Toxicity Characteristic Leaching Procedure (ATCLP).

Exposure assessment	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh						
Acetonitrile extraction	3.1	5.4	3.1	5.4	10	21
p or 95% C.I.	1.000	<0.0001	0.239	0.004	6 – 14	16 – 25
R^2					0.972	0.972
ATCLP extraction	0.9	2	0.9	2	5	10
p or 95% C.I.	1.000	<0.0001	0.239	0.004	2 – 7	8 – 13
R^2					0.971	0.971
Weathered/aged						
Acetonitrile extraction	5.2	11.5	3.0	5.2	15	23
p or 95% C.I.	0.325	<0.0001	0.143	0.004	11 – 19	20 – 25
R^2					0.980	0.980
ATCLP extraction	2.42	5.22	1.67	5.22	11	13
p or 95% C.I.	0.325	<0.0001	0.084	0.001	9 – 12	12 – 14
R^2					0.978	0.978

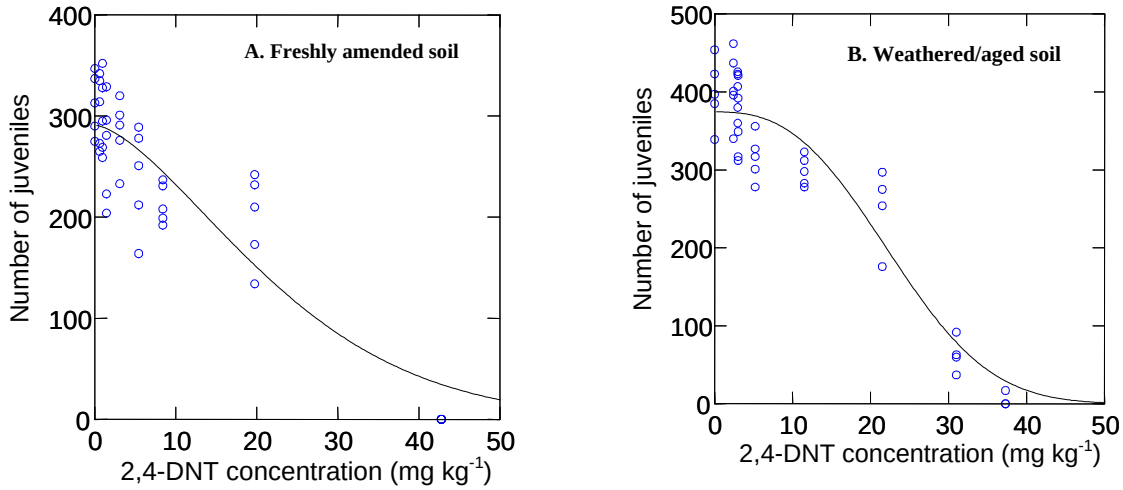

Figure 3. Effects of 2,4-DNT on juvenile production in freshly amended (A) and weathered/aged (B) 2,4-DNT amended SSL soil.

3.3.4 Toxicity of 2,6-DNT.

Results of toxicity testing in 2,6-DNT freshly amended and weathered/aged amended SSL soils are shown in Table 18. The bounded NOEC for adult survival in freshly amended SSL soil was 7.65 mg kg^{-1} (no significant difference compared to control, $P = 0.809$) (Table 19). Adult survival was significantly ($P = 0.007$) reduced at the LOEC of 9.38 mg kg^{-1}. The bounded NOEC for juvenile production was 7.65 mg kg^{-1} (no significant difference compared to control, $P = 0.073$). The bounded LOEC for juvenile production was 9.38 mg kg^{-1} ($P = 0.002$). The EC_{20} and EC_{50} values were 5.90 and 11.135 mg kg^{-1}, respectively (Exponential model). The bounded NOEC for adult survival in weathered/aged amended SSL soil was 1.6 mg kg^{-1} (no significant difference compared to control, $P = 0.285$) (Table 19). Adult survival was significantly ($P = 0.001$) reduced at the LOEC of 3.7 mg kg^{-1}. The bounded NOEC for juvenile production was 1.6 mg kg^{-1} (no significant difference compared to control, $P = 0.167$). The bounded LOEC for juvenile production was 3.7 mg kg^{-1} ($P \leq 0.0001$). The EC_{20} and EC_{50} values were 0.956 and 3.628 mg kg^{-1}, respectively (Gompertz model). All ecotoxicological parameters (mg kg^{-1}) for 2,6-DNT determined in freshly amended and weathered/aged amended SSL soil are given in Table 19.

Table 18. Mean (n = 5) adult survival and juvenile production in freshly amended and in weathered/aged 2,6-DNT amended SSL soils.

Concentration in freshly amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error	Concentration in weathered/aged amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error
Negative control	9	143	11	Negative control	9	237	38
Acetone control	9	169	11	Acetone control	9	243	33
Positive control	5	37	16	Positive control	5	56	16
3.74	9	129	14	0.3	9	240	45
4.35	8	138	17	0.8	9	220	32
5.33	9	141	31	1.2	8	225	16
7.65	7	96	27	1.6	8	192	28
9.38	5	77	21	3.7	6	46	15
12.93	3	58	35	9.5	4	67	23
19.97	1	1	1	13.9	2	30	7
40.2	0	0	0	18.1	3	22	10
				37.4	0.2	0	0

Table 19. Summary of ecotoxicological parameters (mg kg^{-1}) for adult survival and for juvenile production of *F. candida* for 2,6-DNT determined in freshly amended and in weathered/aged amended SSL soil. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330 and water extraction using Adapted Toxicity Characteristic Leaching Procedure (ATCLP).

Exposure assessment	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh						
Acetonitrile extraction	7.6	9	7.6	9	5.9	11
p or 95% C.I.	0.809	0.007	0.073	0.002	1.8 – 10	7 – 15
R^2					0.906	0.906
ATCLP extraction	2	4	2	4	2	4
p or 95% C.I.	0.809	0.007	0.073	0.002	0 – 3	2 – 7
R^2					0.907	0.907
Weathered/aged						
Acetonitrile extraction	1.6	3.7	1.6	3.7	0.96	3.6
p or 95% C.I.	0.285	0.001	0.167	<0.0001	0 - 2.1	1.4 - 5.9
R^2					0.899	0.899
ATCLP extraction	0.42	1.46	0.42	1.46	0.2	1.3
p or 95% C.I.	0.211	<0.0001	0.119	<0.0001	0 - 0.6	0.4 - 2.3
R^2					0.904	0.904

Concentration-response relationships for juvenile production in fresh and in weathered/aged 2,6-DNT amended soil determined by nonlinear regressions are shown in Figure 4. The Exponential model had the best fit for data from the test with freshly amended soil (Figure 4A). The Gompertz model had the best fit for data from the test with weathered/aged amended soil (Figure 4B). Overall, reproduction was higher in weathered/aged 2,6-DNT amended soils (Table 18). Juvenile production EC_{20} values were 5.90 and 0.956 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. The difference between these values was not statistically significant based on 95% confidence intervals (Table 19). Juvenile production EC_{50} values were 11.138 and 3.628mg kg^{-1} in freshly amended and weathered/aged soils, respectively. The difference between these values was statistically significant based on 95% confidence intervals (Table 19).

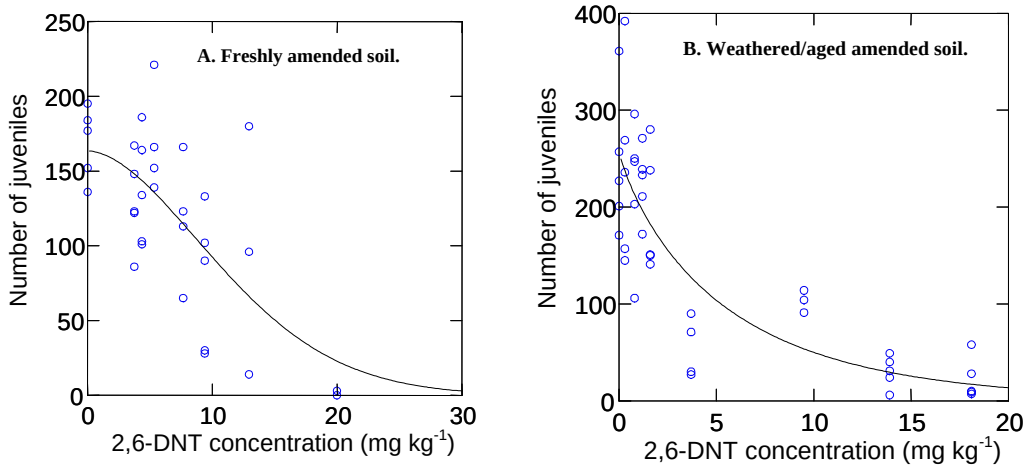

Figure 4. Effects of 2,6-DNT on juvenile production in freshly amended (A) and weathered/aged (B) 2,6-DNT amended SSL soil.

3.3.5 Toxicity of TNB

Mean adult survival and juvenile production for the TNB toxicity tests in freshly amended and in weathered/aged amended SSL soils are shown in Table 20.

The bounded NOEC for adult survival in freshly amended SSL soil was 45.0 mg kg^{-1} (no significant difference compared to control, $P = 0.279$). Adult survival was significantly ($P \leq 0.0001$) reduced at the LOEC of 107mg kg^{-1}. The bounded NOEC for juvenile production was 3.9 mg kg^{-1} (no significant difference compared to control, $P = 0.481$). The bounded LOEC for juvenile production was 13.6 mg kg^{-1} ($P = 0.002$). The EC_{20} and EC_{50} values were 4.423 and 24.695 mg kg^{-1}, respectively (Gompertz model). The bounded NOEC for adult survival in weathered/aged amended SSL soil was 75.8 mg kg^{-1} (no significant difference compared to control, $P = 0.608$). Adult survival was significantly ($P = 0.001$) reduced at the LOEC of 176.3 mg kg^{-1}. The bounded NOEC for juvenile production was 8.75 mg kg^{-1} (no significant difference compared to control, $P = 0.676$). The bounded LOEC for juvenile production was 75.8 mg kg^{-1} ($P \leq 0.0001$). The EC_{20} and EC_{50} values were 47.876 and 87.514 mg kg^{-1}, respectively (Gompertz model). All ecotoxicological parameters (mg kg^{-1}) for TNB determined in freshly amended and weathered/aged amended SSL soil are given in Table 21.

Table 20. Mean (n = 5) adult survival and juvenile production in freshly amended and in weathered/aged TNB amended SSL soils.

Concentration in freshly amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error	Concentration in weathered/aged amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error
Negative control	9	134	21	Negative control	9	566	30
Acetone control	9	168	11	Acetone control	8	557	29
Positive control	4	39	6	Positive control	8	304	27
2.6	9	143	25	0.6	10	582	29
3.9	9	149	16	1.3	7	569	14
13.6	8	79	20	8.75	8	537	48
45.0	9	88	31	75.8	7	267	72
107.0	2	17	11	176.3	0.4	15	12
221.0	0	0	0	304.7	0	0	0
385.7	0	0	0	491.3	0	0	0
518.0	0	0	0	747.7	0	0	0

Table 21. Summary of ecotoxicological parameters (mg kg^{-1}) for adult survival and for juvenile production of *F. candida* for TNB determined in freshly amended and in weathered/aged amended SSL soil. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330 and water extraction using Adapted Toxicity Characteristic Leaching Procedure (ATCLP).

Exposure assessment	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh						
Acetonitrile extraction	45	107	3.9	13.6	4.4	24.7
p or 95% C.I.	0.279	<0.0001	0.481	0.002	0 - 12	2.7 - 46.7
R^2					0.877	0.877
ATCLP extraction	30.22	83.63	2.45	7.68	3.7	22
p or 95% C.I.	0.279	<0.0001	0.481	0.002	0 – 10	1.5 – 43
R^2					0.876	0.876
Weathered/aged						
Acetonitrile extraction	76	176	8.8	75.8	48	87.5
p or 95% C.I.	0.608	0.001	0.676	<0.0001	27 - 68	70 - 105
R^2					0.985	0.985
ATCLP extraction	55.8	143.4	3.35	55.8	34	66
p or 95% C.I.	0.608	0.001	0.676	<0.0001	18 – 50	51 – 80
R^2					0.985	0.985

Concentration-response relationships for juvenile production in fresh and in weathered/aged TNB amended soil determined by nonlinear regressions are shown in Figure 5. The Gompertz model had the best fit for data from the test with freshly amended soil (Figure 5A) and for data from the test with weathered/aged amended soil (Figure 5B). Overall, reproduction

was higher in weathered/aged TNB amended soil (Table 20). Juvenile production EC_{20} values were 4.423 and 47.876 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. The difference between these values was statistically significant based on 95% confidence intervals (Table 21). Juvenile production EC_{50} values were 24.695 and 87.514 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. The difference between these values was statistically significant based on 95% confidence intervals (Table 21).

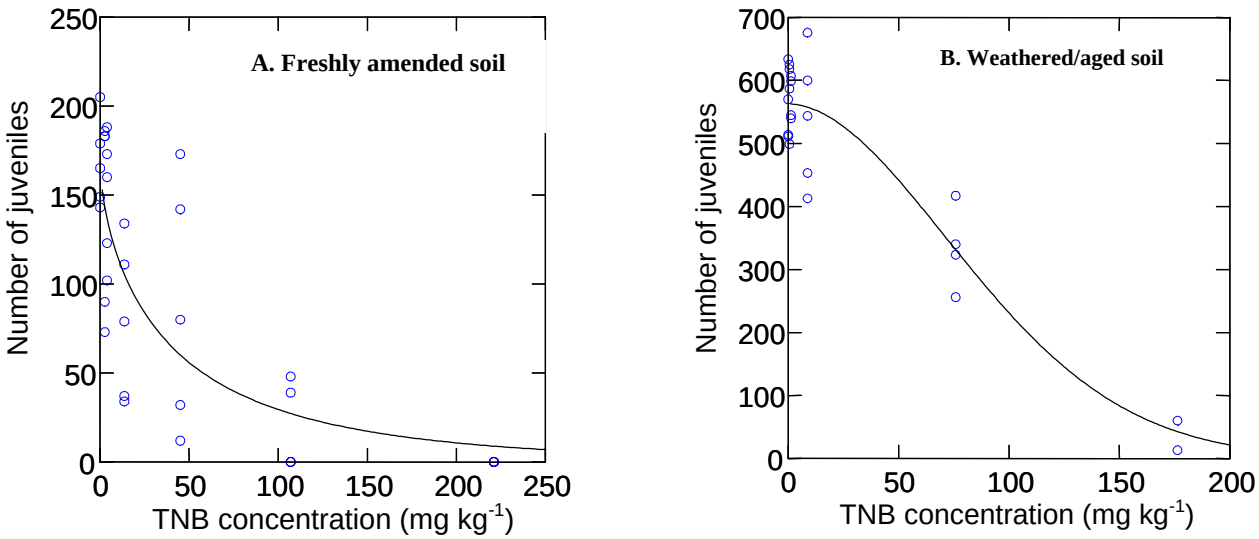

Figure 5. Effects of TNB on juvenile production in freshly amended (A) and weathered/aged (B) TNB amended SSL soil.

4. DISCUSSION

Development of screening level benchmarks for Ecological Risk Assessment (ERA) of contaminated soils has become a critical need in recent years (USEPA, 2000). In order to address this problem, the USEPA in conjunction with stakeholders is developing Eco-SSLs to identify concentrations of chemicals in soil that, when not exceeded, will be theoretically protective of terrestrial ecosystems within specific soil boundary conditions from unacceptable harmful effects. An extensive review of literature (USEPA, 2000) determined that there was insufficient information for energetic material contaminants in soil to generate Eco-SSL benchmarks for soil invertebrates. The majority of soil toxicity tests that were reported in literature utilized standard artificial soil with high organic matter content (10%). In contrast, our toxicity studies designed to specifically fill this knowledge gap, used a natural soil that meet the criteria for Eco-SSL development, in large part because it has characteristics supporting relatively high bioavailability of EMs. In addition, our weathering/aging procedure for soils amended with a range of EM concentrations allowed us to more realistically assess RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB toxicity under conditions that closely mimic field conditions.

4.1 Determination of energetic materials in soil by chemical analysis.

Derivation of Eco-SSL values prioritizes ecotoxicological benchmarks that are based on measured soil concentration of a chemical over those based on nominal concentrations (USEPA, 2000). In this study, the exposure concentrations of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB in soil were analytically determined in all definitive toxicity tests. Chemical analysis utilized the USEPA Method 8330 based on acetonitrile extraction of EMs from soil. Results from acetonitrile extraction of freshly amended soils showed good correlation between nominal and measured concentrations for the five energetic materials, confirming that the soil amendment procedure used in toxicity tests was appropriate and that the USEPA Method 8330 was efficient for quantifying the amount of energetic materials in soil.

An additional procedure that measured the water-extractable portion of each EM in amended soils was performed using the Adapted Toxicity Characteristic Leaching Procedure (ATCLP). This water extractable portion of each EM was perceived to measure the bioavailable fraction of chemicals in soil pore water that is potentially better correlated with toxicity as compared to acetonitrile extracted chemical measure. ATCLP extractable concentrations of 2,4-DNT, 2,6-DNT and TNB in freshly amended SSL soil increased proportionally with their respective acetonitrile-extractable concentrations. In contrast, RDX and HMX ATCLP-extractable concentrations decreased proportionally with their respective acetonitrile-extractable concentrations with four percent recovery in soil freshly amended at 2,000 mg kg^{-1} RDX, and less than one percent recovery at 2,400 mg kg^{-1} HMX. These low ATCLP-based recoveries reflected the low water solubility of both compounds, which were reported for RDX as 42 mg L^{-1} at 20°C (Sikka *et al.*, 1980) and as 60 mg L^{-1} at 25°C (Banerjee *et al.*, 1980). The water solubility of HMX was reported between 5 and 6.6 mg L^{-1} at 25°C and 20°C, respectively (Glover *et al.*, 1973; McLellan *et al.*, 1992).

Assessment of the EM toxicities to *F. candida* for Eco-SSL development included studies with weathered and aged EM amended soils to simulate more closely the exposure effects in the field. Weathering/aging of chemicals in soil may reduce exposure of soil invertebrates to EMs due to photodecomposition, hydrolysis, a reaction with organic matter, sorption/fixation, precipitation, immobilization, occlusion, microbial transformation and other fate processes that commonly occur at contaminated sites. These fate processes can reduce the amount of chemical that is bioavailable, compared to tests conducted with freshly amended soils, or may reveal increased toxicity due to the presence of more toxic transformation products.

Weathering/aging of amended soils caused an overall reduction in the acetonitrile-extractable portion of all energetics studied. Reduction in the RDX concentrations in weathered/aged soil had a mean loss of 24 percent. For HMX after the 3-month weathering/aging period, the reduction averaged 14 percent. The concentration of 2,4-DNT decreased by an average of 19 percent during the three-months procedure and was independent of the initial acetonitrile-extractable concentrations used in this study (only at the 2 mg L^{-1} concentration was there an increase in the acetonitrile-extractable portion). Degradation of 2,6-DNT over the 3-month weathering/aging period was greater compared with 2,4-DNT degradation, and reduced 2,6-DNT concentrations by an average of 86 percent. Degradation of TNB was inversely related to the

initial acetonitrile-extractable concentration in amended soil. There was an average reduction of 85 percent of TNB in soil amended with concentrations below 107 mg kg^{-1}. At treatment levels above that, TNB degradation averaged 19 percent.

The weathering/aging procedure caused a 15 percent reduction in the water extractable portion of RDX, which was less than the acetonitrile-extractable portion. For HMX, the weathered/aged water extractable portion increased by an average of 34%. This is contrary to the findings of Rosenblatt *et al.* (1991) and Hawari *et al.* (2002) who reported a limited degradation of RDX and HMX under aerobic soil conditions. Also, Jones *et al*. (1995) reported a limited 10 percent mineralization of RDX in contaminated soil augmented with *Rhodococcus* bacterial strain. The water extractable portions of 2,4-DNT in weathered/aged amended soils were higher at concentrations below 5 mg kg^{-1}. At concentrations above 5 mg kg^{-1}, the water extractable portions of 2,4-DNT in weathered/aged amended soils decreased by 37 percent, on average, than those for the freshly amended soils. The water extractable portion of 2,6-DNT and TNB in the weathered/aged soil had an average reduction of 90 and 52 percent, respectively. Overall, chemical analyses demonstrated that EM exposure conditions of *F. candida* in weathered/aged amended soils differed from those of freshly amended soils. The inclusion of weathering/aging component in the EM toxicity assessments allowed us to incorporate potential alterations in EM bioavailability at contaminated sites in the development of ecotoxicological benchmarks for soil invertebrates.

4.2 Toxicity of energetic materials to *F. candida* in Sassafras sandy loam soil.

Definitive toxicity tests conducted with freshly amended soil showed that EM toxicity order based on EC_{20} values for juvenile production in tests with *F. candida* was TNB > 2,6-DNT > 2,4-DNT > RDX > HMX. Definitive toxicity tests conducted with weathered/aged amended soil showed that EM toxicity order based on EC_{20} values for juvenile production in tests with *F. candida* was 2,6-DNT > 2,4-DNT > TNB > RDX > HMX. Juvenile production measurement endpoint based on EC_{20} values was more sensitive compared with adult survival in all tests except for tests with 2.4-DNT in freshly amended and weathered/aged amended soils, where adult survival LOEC values were lower compared with EC_{20} values for juvenile production. This supported the Eco-SSL requirement of using reproduction endpoints for benchmark development.

EC_{20} values for weathered/aged EM amended soil were generally higher than those in freshly amended soil. This indicates that weathering and aging caused a decrease in the toxicity of RDX, HMX, 2,4-DNT, and TNB based on EC_{20} values for juvenile production. The EC_{20} value for weathered/aged 2,6-DNT was the only EM that was lower than for the freshly amended soil (i.e. 0.956 and 5.90 mg kg^{-1}, respectively).

Because this study was designed to produce benchmark data for development of Eco-SSLs for explosives contaminants in soil, the results of this study may not directly compare to those of other studies in the literature, since none of them were designed to specifically quantify EM toxicity to soil invertebrates under Eco-SSL conditions of testing. Literature on the toxicity of RDX to terrestrial organisms is scant, and discrepancies are often found regarding the toxicity of the same chemical to different organisms. Significant sublethal effects of RDX were observed on

the reproduction of earthworm *Eisenia andrei* at concentrations as low as 95 mg kg^{-1} soil (Robidoux *et al*., 2000). However, no effects were found on the mortality and reproduction of two terrestrial invertebrates enchytraeid worm *E. crypticus* and collembolan *Folsomia candida* in soils spiked with up to 1000 mg kg^{-1} RDX in soil (Schafer and Achazi, 1999). Furthermore, these studies were conducted either in standard artificial soil (Robidoux *et al*., 2000), or in soil with relatively high (2.5-3.0% organic C) organic matter content (Schafer and Achazi, 1999), which limits their usefulness for describing natural systems or development of Eco-SSLs.

Exposure of *F. candida* to HMX in freshly amended SSL soil produced a significant effect on juvenile production (EC_{20} value of 234.831 mg kg^{-1}). The EC_{20} value increased to 1045.627 mg kg^{-1} in weathered and aged soil although the LOEC for juvenile production decreased in weathered/aged HMX amended soil.

The relatively low RDX and HMX toxicity to *F. candida* in SSL soil at concentrations tested in our study can be related to low bioavailability of these energetic materials in soil. The solubility in water at 20°C of RDX and HMX is 42.3 and 6.63 mg L^{-1}, respectively (Roberts and Hartley, 1992). These low solubility levels in water contribute to low bioavailability of RDX and HMX in soil. Considering *F. candida* exposure to RDX and HMX in soil on the ATCLP basis provides explanation, at least partially, for the observed effects of these nitro-heterocyclic explosives. The better understanding of the reasons for low toxicity of RDX to *F. candida* and elucidation of mechanisms of a stimulating response to HMX exposure will require additional research.

Dinitrotoluenes (DNTs) and trinitrobenzene (TNB) are by-products of TNT production, which are present worldwide at munitions manufacturing and post-production sites. 2,4-DNT and 2,6-DNT are also aerobic metabolites of microbial degradation of TNT (Gorontzy, *et al*., 1994; Spain, 2000). The nitroaromatics 2,4-DNT, 2,6-DNT and TNB affected adult *F. candida* survival at the range of concentrations tested in our study. In freshly amended SSL soils, the NOEC values ranged from 3 to 45 mg kg^{-1} and the LOEC values ranged from 5 to 107 mg kg^{-1} (Tables 17, 19, 21). In weathered/aged amended SSL soil, the NOEC values ranged from 1.6 to 75.8 mg kg^{-1} and the LOEC values ranged from 3.7 to 176.3 mg kg^{-1} (Tables 17, 19, 21).

Toxicity of nitroaromatic EMs tested to *F. candida* juvenile production was greater compared with RDX and even greater compared with HMX, which was not toxic to adults up to 4784 mg kg^{-1} in freshly amended SSL soil. Juvenile production EC_{20} estimates ranged from 1 to 48 mg kg^{-1} in weathered/aged amended soils. Comparison of our results to other studies is difficult because the toxicity of nitro aromatic energetics, including 2,4-DNT, 2,6-DNT and TNB to soil invertebrates has not been sufficiently investigated. The majority of studies reported in the available literature focused primarily on the effects of TNT and/or its degradation products (Renoux *et al*., 2000; Robidoux *et al*., 2000; 1999; Sunahara, *et al*., 2001; Rocheleau, *et al*., 1999; Schafer and Achazi, 1999; Simini, *et al*., 1995; Phillips, *et al*., 1993). Dodard *et al*. (2003) in the study with *E. albidus* using OECD artificial soil determined EC_{50} value for TNT of 111 mg kg^{-1} for juvenile production. Phillips *et al*. (1993) reported 100 percent mortality in the earthworm *E. fetida* growth and survival test in USEPA standard artificial soil fortified with a mixture of EMs that included 30, 50, 62.5, and 20 mg kg^{-1} of TNT, TNB, 2,4-DNT and 2,6-DNT, respectively. Statistically significant ($p < 0.01$) sublethal effects (mass loss) were reported at concentrations 6,

10, 12.5, and 4 mg kg^{-1} of TNT, TNB, 2,4-DNT and 2,6-DNT, respectively. These results are in general agreement with findings of our investigations although direct comparisons of both studies are limited due to differences in the experimental designs.

Simini *et al*. (1995) assessed the toxicity of soil from Joliet Army Ammunition Plant contaminated with a mixture of EMs (which limits the direct comparisons with our study), including both nitroaromatic and nitro-heterocyclic compounds using earthworm *E. fetida* growth and survival test, among other bioassays. The highest soil concentrations measured at this site for TNB, 2,4-DNT and 2,6-DNT were 200, 117, and 8 mg kg^{-1}, respectively. Authors reported that TNT and TNB had greatest coefficients of determinations in all bioassays, including the earthworm test. Liner regression analyses R^2 values for TNB using earthworm test endpoints were 0.773 and 0.814 for the two locations investigated at the study site. These values for 2,4-DNT were 0.613 and 0.358, while 2,6-DNT had the weakest relationship to measurement points used with R^2 values of 0.082 and 0.293 for the two locations, respectively. Soil TNB and 2,4-DNT concentrations found at this site were within the range of concentrations tested in our study and the results are consistent with our findings. The weak relationship determined for 2,6-DNT is most likely due to very low concentrations of this EM measured at the investigated site.

Special consideration was given to the effects of weathering and aging in assessing the chemical toxicity for Eco-SSL development. Weathering and aging of energetics in soil may reduce the soil invertebrates' exposure to these chemicals. This can result in a dramatic reduction in the amount of chemical that is bioavailable, compared to tests conducted with freshly amended chemicals or those tested following a short equilibration period (e.g., 24 h). Dodard *et al*. (2003) reported a decrease in TNT toxicity to *E. albidus* on the LC_{50} basis for reproduction from 44 to 89 mg kg^{-1} in OECD artificial soil following a 21-day aging period. Specific mechanisms of changes in the toxicity of EMs in weathered/aged amended soil are unknown. Degradation products produced during the weathering and aging process may be more toxic to soil organisms compared with the parent material, and can be one of the factors contributing to the increased toxicity in weathered/aged amended soil. We incorporated the weathering and aging procedure to simulate more closely the exposure effects on soil invertebrates in the field. Weathering and aging of amended soils increased the toxicity of 2,6-DNT to both adults and juvenile production of *F. candida*, while toxicity of 2,4-DNT to adults decreased but did not change for juvenile production. TNB toxicity decreased with weathering and aging for both adult survival and juvenile production. Specific mechanisms of changes in the toxicity of EMs in weathered/aged amended soil are unknown. Degradation products produced during the weathering and aging process may be more toxic to soil organisms compared with the parent material, and can be one of the factors contributing to the increased toxicity in weathered/aged amended soil. Dodard *et al*. (1999) investigated the toxic effects of 2,4-DNT and 2,6-DNT, and their respective metabolites using the 15-min Microtox (*Vibrio fischeri*) and 96-h freshwater green alga (*S. capricornutum*) growth inhibition tests. The toxicities of DNTs were species-dependent: 2,4-DNT was more toxic than 2,6-DNT to *S. capricornutum* (comports with our results for *F. candida* in freshly amended soil for both adult survival and juvenile production), while the reverse was true in the test with *Vibrio fischeri*. The authors reported that the reduced metabolites of 2,6-DNT tested were less toxic compared to the toxicity of parent compound. However, certain partially reduced metabolites of 2,4-DNT (4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene) were more toxic than the parent compound. Although these results cannot be directly compared to our study

because the biotic reductive degradation pathway for 2,4-DNT and 2,6-DNT in aquatic environment would contrast with metabolic processes in the aerobic conditions of vadose zone simulated in our investigations, the reducing environment can exist in water-logged soil microsites, where more toxic metabolites of dinitrotoluenes degradation can be present. The higher toxicity of these metabolites would contribute to possible explanation of the increased toxicity of 2,6-DNT in weathered/aged amended SSL soil observed in our study.

The exposure concentrations of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB in soil were determined by different methods, including acetonitrile extraction and water extraction. The water extractable portion of each EM was determined using an Adapted Toxicity Characteristic Leaching Procedure (ATCLP) to establish if this technique, which is perceived to measure the bioavailable fraction of chemicals in soil pore water, could generate data that is better correlated with toxicity than acetonitrile-extractable chemical measure. Coefficients of determinations (R^2) for acetonitrile-extractable and ATCLP based extractions determined in nonlinear regression analyses of the reproduction toxicity data from studies with fresh and weathered/aged amended soils were compared to determine which chemical measure of exposure better correlated with toxicity. These comparisons showed that coefficients of determinations were very similar in both exposure types indicating that neither extraction method had an advantage in characterizing bioavailability of EMs tested in this study to *F. candida*. This result supports our decision for developing Eco-SSLs for explosives contaminants in soil on the basis of acetonitrile extraction of test compounds. The acetonitrile extraction-based Eco-SSLs will be especially useful for Ecological Risk Assessment at contaminated sites because EM concentrations determined during site characterization are usually based on acetonitrile-extractable by US EPA method 8330.

5. CONCLUSIONS

This study has produced ecotoxicological data for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB using the ecologically relevant soil invertebrate species *F. candida*. Relative toxicity of the five EMs tested in this study was TNB > 2,6-DNT > 2,4-DNT > RDX > HMX for freshly amended soil and 2,6-DNT > 2,4-DNT > TNB > RDX > HMX for weathered and aged amended soil. Study results showed that tests based on reproductive endpoint provide a more sensitive evaluation of effect than adult survival and therefore should be used to set screening criteria. Tests were performed using a natural Sassafras sandy loam soil. Sassafras sandy loam has low organic matter and clay contents, low cation exchange capacity, and high sand content. Such characteristics support relatively high bioavailability of energetic contaminants in soil. Furthermore, weathering and aging of amended soils produced a soil microenvironment more similar to field conditions than previous studies where soil invertebrates were exposed immediately following spiking of soil.

A natural soil, Sassafras sandy loam was used in all toxicity tests. Sassafras sandy loam had low organic matter and clay contents, which fulfilled the USEPA requirement of using soil with characteristics that support relatively high contaminant bioavailability for developing conservative Eco-SSL values (USEPA, 2000). Weathering and aging of amended soils were incorporated into experimental design of toxicity testing to produce a soil microenvironment more similar to field conditions. Results of chemical analyses showed that exposure conditions of *F.*

candida to EMs tested in weathered/aged amended soils differed from those of freshly amended soils due to significant transformation of 2,4-DNT, 2,6-DNT, and TNB, and the formation of transformation products, including 3,5-DNA, 2-A-4 NT, and 4-A-2 NT. The inclusion of weathering/aging component in the EM toxicity assessments allowed us to assess the potential alterations in EM bioavailability to *F. candida* at contaminated sites. In order to provide a more complete information on ecotoxicological effects of energetic contaminants in soil to risk assessors and site managers, additional studies would be required to investigate the toxicity of the EM degradation products individually or using chemical mixtures.

Measurement endpoints assessed in this study included adult survival and juvenile production. Study results showed that tests based on reproduction endpoint provide a more sensitive evaluation of effect than adult survival, therefore, it should be used to set screening criteria. All ecotoxicological benchmarks determined in this study will be provided to the Ecological Soil Screening Level (Eco-SSL) workgroup for quality control review by the Eco-SSL task group before inclusion in the Eco-SSL database, and before being used for developing Eco-SSLs for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB.

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APPENDIX E

BIOACCUMULATION OF NITRO-HETEROCYCLIC AND NITROAROMATIC ENERGETIC MATERIALS IN TERRESTRIAL RECEPTORS IN A NATURAL SANDY LOAM SOIL

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BIOACCUMULATION OF NITRO-HETEROCYCLIC AND NITROAROMATIC ENERGETIC MATERIALS IN TERRESTRIAL RECEPTORS IN A NATURAL SANDY LOAM SOIL

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13. ABSTRACT *(Maximum 200 words)*

We investigated the bioaccumulation and mass balance characteristics of two nitro-heterocyclic energetic materials (EM), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) using alfalfa, Japanese millet, ryegrass, lettuce, corn, and the earthworm *Eisenia andrei*. The bioaccumulation of TNT by-products, including 1,3,5-trinitrobenzene (TNB), 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) was also investigated. The test soil supports relatively high bioavailability of these EMs. The effect of simulated weathering/aging procedure on the bioaccumulation of these EMs was incorporated in the study. Results showed that [C^{14}]-RDX and [C^{14}]-HMX were significantly accumulated by the selected plant species with maximum bioconcentration factors of 79 and 2.2, respectively. Virtually no accumulation of TNB and of the DNT's was observed in plants. Mass balance data indicate that plants accumulate only a few percent of the amended RDX, or HMX. The partitioning of RDX and HMX among plant compartments was evaluated in corn. After three weeks of exposure, leaves were found to be the major site of accumulation, as seen with other plants. In plants, most of the radiolabeled RDX and HMX were unmetabolized. In the earthworm, accumulation was low for RDX with a bioconcentration factor (BCF) of 2.9 -13, and was negligible for HMX with a BCF of 0.3 – 1.0.

14. SUBJECT TERMS

RDX, HMX, 2,4-DNT, 2,6-DNT, TNB, Bioaccumulation, Soil Invertebrates, Toxicity, Terrestrial Plants, Natural Soil, Bioavailability

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PREFACE

The work described in this report was authorized under Project No. SERDP CU-1221. The work started in April 2001 and was completed in May 2003.

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BIOACCUMULATION OF NITRO-HETEROCYCLIC AND NITROAROMATIC ENERGETIC MATERIALS IN TERRESTRIAL RECEPTORS IN A NATURAL SANDY LOAM SOIL

1. INTRODUCTION

The Strategic Environmental Research and Development Program (SERDP) has identified a research need under the FY00 Broad Agency Announcement (BAA) CUSON-SP-00-04 entitled "Development of Ecological Toxicity and Biomagnification Data for Explosives Contaminants in Soil" to extend the knowledge of toxicity and the potential for biomagnification of explosives-related soil contaminants to ecological receptors. Ecological receptors of interest included terrestrial plants and soil invertebrates. The focus of this investigation was to obtain direct experimental data on bioaccumulation potential of nitramine and nitroaromatic compounds in specific ecological receptors, and to determine whether nitramines HMX and RDX pose a potential risk for toxic effects on higher trophic levels.

Plant uptake of RDX has been assessed mostly in hydroponic or wetland systems (Harvey *et al*., 1991; Best *et al*., 1999). Recent investigations have demonstrated that RDX and HMX, two nitro-heterocyclic energetic materials (EMs), are resistant to aerobic biodegradation in soil (Singh et al., 1998; Gong, unpublished data) but both tend to accumulate in higher plants (Harvey *et al.*, 1991; Best *et al*., 1999; Groom *et al*., 2002). Therefore, phytoremediation is a potential route for removing these EMs from soil. Recently, Groom *et al.* (2002) have shown that plant uptake is much greater when plants are exposed to a field-collected contaminated soil in the greenhouse compared to plants found on the site itself. A possible explanation for this observation is that soil moisture may be an important factor driving HMX accumulation in plant tissues (*i.e.*, by enhancing dissolution of the compound present in soil). On the other hand, bioaccumulation of these two compounds may significantly impact ecological and human health through the food chain. The fate, transport and toxicity of HMX were studied by Yoon *et al.* (2002) in a system using poplar trees grown in hydroponic solutions. No toxicity was observed for the plant cuttings. These authors showed that HMX is rapidly transported throughout the entire plant, concentrating primarily in the leaves. Since no significant biodegradation was observed, they concluded that HMX accumulated in leaves could present a source of concern because it could pass into the food chain. In an earlier study conducted by Larson *et al*. (1999), it was shown that RDX could undergo extensive transformation to high molecular weight molecules and that this process leads to considerable accumulation in plants. In their study, tomato, radish and lettuce were exposed to [^{14}C]-RDX added in the irrigation water (1 ppm), but quantitative data on the transfer of explosives from soil were not provided. In a similar type of study, Checkai and Simini (1996) using RDX in irrigation water (2 – 100 µg kg^{-1}) found a low accumulation in lettuce, tomato, radish, bush bean, soybean, corn and alfalfa. For alfalfa, the maximal amount found was 186 µg kg^{-1} dry weight. Accumulation in plants was proportional to the concentration of RDX in water. No data was provided on RDX concentrations in soil.

No data on the bioaccumulation of TNB, 2,4-DNT and 2,6-DNT in plants could be located in the available literature, although an extensive literature exists for TNT. Data on TNT will not be included here, as it is well covered by several authors (Talmage *et al.*, 1999 ; Major *et al.*, 2002). The general conclusion is that TNT does not accumulate in plants grown in TNT-contaminated soil, as its uptake is limited due to extensive transformation in soil.

Accumulation of RDX or HMX in soil invertebrates has not been reported so far in the published literature. Tissue accumulation of TNB, RDX and HMX resulting from exposure to contaminated sediment in benthic invertebrates was presented by Lotufo *et al.* (2001). TNB or any related degradation products were not detected in the extracts from invertebrates tissues. These authors reported significant bioaccumulation of RDX and HMX in terms of molar-equivalent tissue concentrations, but did not provide bioaccumulation values since HMX or RDX were not detected in extracts prepared from the tissues. This suggests an appreciable transformation of the compounds in the aquatic medium, in contrast to what occurs in soil. To our knowledge, the only data available on accumulation of RDX and HMX in *E. andrei* comes from our laboratories. Studies were conducted using various exposure routes such as filter paper, OECD artificial soil and forest soil (Robidoux *et al.*, in preparation). At the tested concentrations, HMX accumulation was linear with soil concentration. However, a non-linear relationship was found between soil concentration and tissue accumulation of RDX. Transformation of TNT in the earthworm has been presented by Renoux *et al.* (2000) and Robidoux *et al.* (2002). TNT was not accumulated as such but its degradation products were found in earthworm tissues. In a study where earthworms (*Lumbricus terrestris*) were fed to salamanders, Johnson *et al.* (2000) reported that earthworm tissues contained low levels of TNT compared to TNT metabolites. No bioconcentration factor could be calculated for TNT due to its very rapid transformation. No published data on accumulation of TNB and 2,6-DNT in earthworms could be located. A study by Liu *et al.* (1983) reports some exploratory accumulations studies with 2,4-DNT on the sediment-associated oligochaete *L. variegatus.* For this species, the 4-d BCF was 58. This value is applicable only to the uptake phase and not to the clearance phase of accumulation so the value may overestimate the potential for accumulation. Some information on TNB is available from studies with small rodents that show rapid biotransformation. In field studies, TNB was not detected in tissues of terrestrial wildlife living in TNB contaminated area (reviewed by Talmage *et al.*, 1999). No bioaccumulation study was conducted for TNB at our knowledge. The present study will fill the existing knowledge gaps concerning the accumulation of TNB, 2,4-DNT and 2,6-DNT in plants, and the biomagnification potential of RDX and HMX directly from soil by higher plants and soil invertebrates.

The objectives of the present study were to evaluate the effects of exposure concentrations and weathering/aging of amended soil on the bioconcentration potential of selected energetic compounds in plants and soil invertebrates. Firstly, data collected from the terrestrial plant toxicity experiments provided an estimate of the bioconcentration potential of TNB, 2,4-DNT, 2,6-DNT, RDX and HMX in freshly amended and in weathered/aged Sassafras sandy loam soil using three selected plants, *i.e.*, alfalfa, Japanese millet and

ryegrass. Secondly, the accumulation potential of the two radiolabeled nitramines RDX and HMX was studied in plant and earthworm tissues. Mass balance studies were carried out to better assess their potential fate in the environment, using mineralization rate and organism compartment distribution pattern.

2. TECHNICAL APPROACH

To determine the bioconcentration potential of HMX and of RDX on selected ecological receptors, radiolabeled compounds were used in addition to unlabeled materials. The advantage of using labeled compounds is that mass balance analysis can include mineralisation and, if appropriate techniques are used, the production of volatile metabolites. The use of ^{14}C-labeled molecules eliminated analytical problems associated with interference from other organic compounds during the monitoring of RDX or HMX in soil and tissue samples.

Bioconcentration studies in higher plants and in earthworm (*Eisenia andrei*) were carried out using [^{14}C]-RDX or [^{14}C]-HMX-amended soils. Five plant species, alfalfa (*Medicago sativa*), perennial ryegrass (*Lolium perenne* L.) Japanese Millet (*Echinochloa crusgalli* L.), corn (*Zea mays* L.), and lettuce (*Lactuca sativa* L.) were evaluated. During the range-finding toxicity tests, alfalfa, Japanese millet and ryegrasss were determined as the most sensitive species to EM (Rocheleau *et al.*, 2003) and therefore were chosen for definitive testing of bioaccumulation. Earthworms were selected as a representative soil invertebrate as they represent 80% of the invertebrate biomass per species in soil. A system for mass balance and bioaccumulation studies was designed and used. Radioactivity in soil and tissue samples was measured with a wet combustion system. The plant tissues were extracted with acetonitrile according to a modification of US EPA Method # 8330A. Recovery studies were carried out to optimize the wet combustion system.

3. MATERIAL AND METHODS

3.1. Sassafras sandy loam Soil

A natural soil, Sassafras sandy loam [Fine-loamy, siliceous, mesic Typic Hapludult] (SSL) was used in this study to assess the toxicity of test chemicals to plants. This soil was selected for developing ecotoxicological values protective of soil biota because it has physical and chemical characteristics supporting relatively high bioavailability of the test chemicals (low organic matter and clay contents). The SSL soil was collected from an open grassland field on the property of the U.S. Army Aberdeen Proving Ground (APG; Edgewood, MD). Vegetation and the organic matter horizon were removed to just below the root zone and the top six inches of the A horizon were then collected. The soil was sieved through a 5 mm^2 (2 mm^2 for work with radiolabeled compounds) mesh screen, air-dried for at least 72 h and mixed periodically to ensure uniform drying, then stored at room temperature before use in testing. Soil was analyzed for physical and chemical characteristics by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD. Results of these analyses are presented in Table 1.

Table 1. Physical and chemical characteristics of Sassafras sandy loam soil analyzed by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD.

Soil Parameter	Sassafras Sandy Loam
Sand (%)	71
Silt (%)	18
Clay (%)	11
Texture	Sandy loam
CEC (cmol kg^{-1})	4.27
Organic matter (%)	1.3
pH	5.0
Moisture content (%)*	0.7
Water holding capacity (mL 100 g dry $soil^{-1}$) *	21.2

* Parameters measured at BRI laboratories.

3.2. Chemicals and equipment

Non radiolabeled hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX; CAS: 121-82-4; Purity: 99%), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX; CAS: 2691-41-0; Purity: 99%), and 1,3,5-trinitrobenzene (TNB; CAS: 99-35-4; Purity: 99.7%) were obtained from the Defense Research Establishment Valcartier of the Canadian Ministry of National Defence (Val Bélair, QC, Canada). 2,4-Dinitrotoluene (2,4-DNT; CAS: 121-14-2; Purity: 97%) and 2,6-dinitrotoluene (2,6-DNT; CAS: 606-20-2; Purity: 98%) were obtained from

Sigma-Aldrich Canada (Oakville, ON, Canada). The radiolabeled [^{14}C]-RDX (specific activity = 54.4 µCi mmole^{-1}) and [^{14}C]-HMX (specific activity = 101.4 µCi mmole^{-1}) were provided by Dr. Guy Ampleman (Defence Research Establishment Valcartier, Val Bélair, QC, Canada).

Acetone (CAS: 67-64-1; HPLC Grade) was used for preparing EM solutions during soil amendments. Acetonitrile (CAS: 75-05-8; HPLC Grade) was used in some experiments for preparing EM solutions and for all extractions. Both were obtained from Caledon Laboratories (Georgetown, ON, Canada). Calcium chloride ($CaCl_2 \cdot 2H_2O$; analytical grade) and sodium bisulfate ($NaHSO_4 \cdot H_2O$; certified grade) were purchased from BDH (Toronto, ON, Canada). All other reagent grade chemicals were obtained from Anachemia (St. Pierre, QC, Canada) and Aldrich (Milwaukee, WI, USA). Glassware was washed with phosphate-free detergent followed by rinses with acetone, nitric acid, and ASTM type I water (American Society of Testing and Materials, http://www.astm.org). ASTM type I water was obtained using Millipore® Super Q water purification system (Millipore®, Nepean, ON, Canada) and was used throughout the study.

3.3. Soil amendment

The following procedure was used for terrestrial plant toxicity assays from which accumulation data on non-radiolabeled EMs was derived. Sassafras sandy loam (SSL) soil was individually amended with RDX, HMX, 2,4-DNT, 2,6-DNT or TNB. SSL soil sieved on 5 mm^2 mesh screen was weighed separately for each treatment in a glass dish. For each treatment, soil was spread to a thickness of approximately 2.5 - 4 cm. Each concentration of EM was prepared separately in glass volumetric flasks and dissolved in acetone. The EM/acetone solution was poured evenly across the soil surface, ensuring that the volume of solution added at any one time did not exceed 15% (volume weight^{-1}) of the dry mass soil. After addition of the EM solution, the volumetric flask was rinsed twice with a known volume of acetone in order to transfer the entire EM weighed. If the total volume of solution needed to amend the soil exceeded 15% (volume weight^{-1}), the solution was added in successive stages, allowing the acetone to evaporate for a minimum of 2 h under a chemical hood. The same total EM /acetone solution volume was added to every concentration treatment, which was the volume required to dissolve the highest concentration tested. The amended soil was then air-dried overnight (minimum of 18 h) in a darkened chemical hood. Each soil treatment sample was transferred into high-density polyethylene containers coated with fluoropolymer (Teflon-like chemical) and covered with aluminum foil, to prevent photolysis of the EM. The sample was mixed overnight (18 ± 2 h) using a three-dimensional mixer. Soil was then ready for the phytotoxicity assay.

Weathered/aged amended soil was prepared in the same manner as the freshly amended soil. ASTM type I water was added to adjust the soil moisture to a level equivalent to 75 % of the water holding capacity. Hydrated soil was exposed to wetting and drying cycles and sunlight in a greenhouse for a period of 13 weeks. Each week, ASTM type I water was added to adjust the soil moisture to initial level, and was allowed to dry until the next addition of water. One day prior to the initiation of the plant toxicity test using

weathered/aged amended soil, each dry soil treatment was mixed overnight using a three-dimensional mixer.

For preliminary experiments with radiolabeled [^{14}C]-RDX and [^{14}C]-HMX, the soil was thoroughly mixed, air dried, and sieved through a 2-mm mesh soil sieve. For preliminary assays in earthworms, the method was as follows: concentrated stock solutions (near saturation) of radiolabeled and non-radiolabeled RDX or HMX were prepared in acetone (or acetonitrile for test with RDX at 100 and 400 mg kg^{-1}), and the necessary volume of each solution was distributed onto 201 g soil (in 15 cm glass Petri dishes) to reach the desired concentrations. Carrier was adjusted at a 4% volume weight^{-1} ratio. In plant preliminary tests, acetone was added to reach 15% volume weight^{-1} ratio in all tested concentrations. Soil samples were left for at least 24 h (generally 48 h) under a darkened chemical hood to permit the evaporation of acetone (or acetonitrile for test with RDX at 100 and 400 mg kg^{-1}). The amended soil was then transferred into 1-L (preliminary tests) or 4-L (definitive tests) jars and vigorously mixed for 2 min. When plants were used, soil was transferred into plastic pots, as described in plant bioaccumulation test section (section 3.6). Soil was then hydrated to 75% of the water holding capacity (WHC) for both plants and earthworms prior to the start of the experiment. The WHC was 21 mL per 100 g^{-1} dry weight Sassafras sandy loam soil. Carrier control groups received the acetone vehicle only. Nominal concentrations of chemicals in test soil were measured using wet combustion and in some cases HPLC analysis (section 3.10).

In all definitive experiments, except for the RDX plant accumulation assay, soil was amended with RDX or HMX following the procedure outlined above for terrestrial plant toxicity assays (*i.e.*, batch preparation using acetone as carrier and 15% volume weight^{-1} ratio). In the RDX accumulation tests using Japanese millet and alfalfa, a concentrated stock solution was prepared in acetonitrile, and completion to the standard 15% volume weight^{-1} ratio was done with acetone. The acetonitrile concentration in soil before evaporation were 0.6% and 4%, for the 100 mg kg^{-1} and 1000 mg kg^{-1} treatment groups, respectively. Carrier control groups received the acetone vehicle only.

3.4. Determination of the water holding capacity of soil

Water holding capacity of the soil was measured accordingly to the procedure provided by Dr. Ronald Checkai (U.S. Army ECBC, Aberdeen Proving Ground, MD). Briefly, SSL soil was transferred in 4-inch (10 cm) plastic pots in triplicate so that the soil surface was 2 cm below the rim of the pot. Pots were placed on 2 mm mesh sieves to allow free water drainage. A volume of ASTM Type I water equal to the soil volume was slowly added onto the settled soil. Water was allowed to dry for 24 h. A first aliquot of soil was sampled below the soil surface (below 1-3 cm). Moist soil was immediately weighed and recorded as moist weight (Mass $_{\text{moist soil}}$). Similar aliquots were taken from the two other replicates. Moist soil aliquots were dried in a 105°C oven for 18 h and transferred in a desiccator at room temperature for 30 min prior to weighing the dry weight (Mass $_{\text{dry soil}}$). This procedure was repeated after 48 h and 72 h, to ensure that a steady state for WHC had

been achieved. Water holding capacity (WHC) was calculated according to the following formula:

$$WHC\ \% = [(Mass_{moist\ soil} - Mass_{dry\ soil}) / Mass_{dry\ soil})] * 100$$

3.5. Microcosm design for bioaccumulation test

A modified clear polycarbonate vacuum desiccator (Nalgene Part No. 5311-0250) was used to construct an enclosed system, a microcosm, that can house the earthworms or plants (Figure 1). The microcosm was made pressure tight by using a metal rod and associated PTFE / rubber O-rings and nuts that joined the top and bottom parts. An access hole (3 mm) was drilled in the top to allow watering of plants, and to allow filling and emptying the KOH traps. One of the ports was used to pump air while the second was connected as an outlet to a series of 3 tubes containing 10.0 mL of 0.25 M KOH to trap CO_2. A catalytic conversion unit made of potassium permanganate mixed with activated charcoal was used to convert putative volatile organic compounds into CO_2.

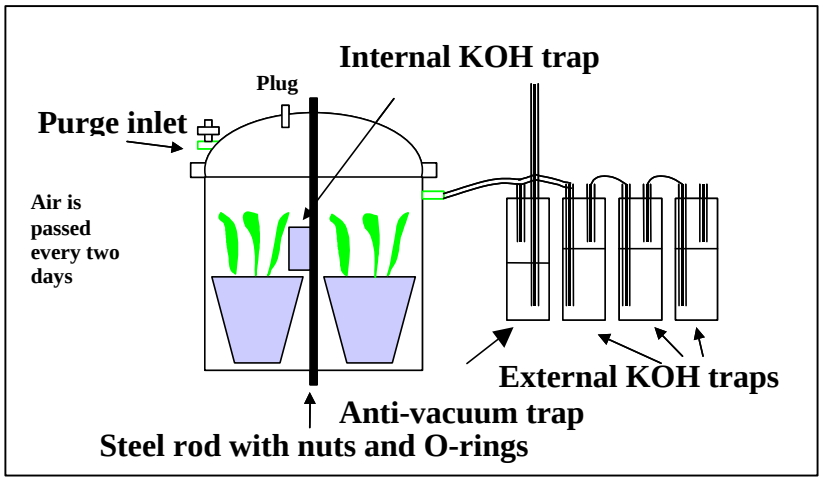

Figure 1. Microcosm design for assessing bioaccumulation of [^{14}C]-RDX or [^{14}C]-HMX in plants and earthworms.

3.6. Plant bioaccumulation test

The plant bioaccumulation tests were performed according to modified protocols of ASTM standard guide for conducting terrestrial plant toxicity tests (American Society for Testing and Materials, 1999), and USEPA early seedling growth test (United States Environmental Protection Agency (USEPA), 1982).

For assays with non-radiolabeled compounds, plant accumulation tests were performed with alfalfa (*Medicago sativa*), Japanese millet (*Echinochloa crusgalli*) and perennial ryegrass (*Lolium perenne*) which were determined in the plant toxicity study

(Rocheleau *et al*., 2003) as the three most sensitive plant species. Six to nine nominal concentrations as well as a control (ASTM type I water) and a carrier control (acetone) were tested, using four replicates. Bioaccumulation measurements were done on selected treatment for the five non-radioactive compounds and three plant species. Two concentrations of each compound were chosen at which shoot biomass was sufficient and some growth inhibition was observed.

The measured concentrations of TNB used in freshly amended soils for evaluation of tissue accumulation in alfalfa and Japanese millet were 2.6 and 67 mg kg^{-1} and for ryegrass they were 5 and 112 mg kg^{-1}. Concentrations of 2,4-DNT in freshly amended soils for alfalfa were 4.7 and 46.5 mg kg^{-1}, for Japanese millet they were 4.7 and 21.5 mg kg^{-1} and for ryegrass they were 1 and 9.1 mg kg^{-1}. Concentrations of 2,6-DNT used in freshly amended soils for alfalfa were 8 and 14 mg kg^{-1}, for Japanese millet they were 4.1 and 14 mg kg^{-1} and for ryegrass they were 4.1 and 30 mg kg^{-1}. A single concentration of 9740 mg kg^{-1} RDX and of 10411 mg kg^{-1} HMX was used for freshly amended soils for alfalfa, Japanese millet and ryegrass.

For weathered/aged soils, the measured concentrations were as follows: for TNB in alfalfa 22.1 mg kg^{-1}, in Japanese millet 5.2 mg kg^{-1}. It was found that TNB amended at a nominal soil concentration of 5 mg kg^{-1} was no longer detectable following weathering. Measured concentrations of 0.3 and 81 mg kg^{-1} were used for ryegrass in weathered/aged soils. Concentrations of 2,4-DNT used for weathered/aged soils for alfalfa were 3.7 and 14.9 mg kg^{-1}, for Japanese millet and for ryegrass they were 3.7 and 7.8 mg kg^{-1}. Concentrations of 2,6-DNT used in weathered/aged soils for alfalfa were 0.6 and 5.3 mg kg^{-1}, for Japanese millet they were 1.16 and 5.3 mg kg^{-1} and for ryegrass they were 1.16 and 14.9 mg kg^{-1}. Finally, a single concentration of 9537 mg kg^{-1} RDX and of 9341 mg kg^{-1} HMX was used for weathered/aged soils for alfalfa, Japanese millet and ryegrass.

For assays using non-radiolabeled compounds, soil was amended as stated earlier (section 3.3). In brief, twenty (20) seeds of each plant species were sown per 4-inch pot containing 201 g soil. The bottom of each plant pot was previously covered with a piece of cheesecloth to prevent soil loss. Alfalfa seeds were inoculated with nitrogen-fixing bacteria prior to its sowing by immersing the seeds in a bacterial suspension in 0.1% pyrophosphate buffer, pH = 7.0. Thirty (30) mL of ASTM type I water was added in order to obtain 75% of water holding capacity. Plant pots were placed in 1-L polyethylene bags closed with an elastic band to prevent loss of soil water content. Plant toxicity tests were performed in a temperature and light controlled growth chamber. Plants were incubated in the dark for the first two days and lights were turned on afterwards. The growth chamber conditions were set as follows: light intensity at 5000 ± 500 lux, day time at 25°C for 16 h, night time at 20°C for 8 h. Luminosity was measured weekly using a photometer and the light intensity was adjusted when needed.

For assays using radiolabeled compounds, preliminary tests were performed using alfalfa (*Medicago sativa*), Japanese millet (*Echinochloa crusgalli*), perennial ryegrass (*Lolium perenne*), corn (*Zea mays*) and lettuce (*Lactuva sativa*). The preparation of the pots

was as described for non-radiolabeled compounds, with the following modifications. Plant pots were placed in a plastic desiccator fitted with inlet, outlet, inside and outside KOH traps (described above). Plants were first incubated in the dark for 2 d at room temperature, and then transferred to the greenhouse. The light intensity was measured and was approximately 4000 ± 500 lux. Temperature was recorded using a data logger (Watchdog Model 110, precision ± 0.7°C, Spectrum Technologies, Inc., Plainfield, IL). Large variations in temperature were observed inside the microcosm when it was placed near the greenhouse windows, so for the definitive tests, the microcosms were placed away from direct sunlight.

The number of emerged seedlings was counted after 5 d for alfalfa, Japanese millet and corn, and after 7 d for lettuce and ryegrass. In the preliminary test with lettuce exposed to [^{14}C]-HMX, lettuce seeds were germinated on wet filter paper in Petri dishes, and 2-d old seedlings were transplanted in SSL soil. Transplantation success was close to 100%. Seedling counts, pooled shoot fresh mass and pooled shoot dry mass were measured at the end of the exposure period for alfalfa, lettuce and Japanese millet (6 weeks) and for corn (3 weeks). Dry mass was obtained after a lyophilization step.

The bioaccumulation definitive tests were performed using the three most sensitive plant species, alfalfa (*Medicago sativa*), Japanese millet (*Echinochloa crusgalli*) and perennial ryegrass (*Lolium perenne*). Nominal concentrations for the definitive assays were 100 and 1000 mg kg $^{-1}$. Measured concentrations were 83 - 93 mg kg $^{-1}$ and 946 – 1011 mg kg $^{-1}$ for RDX, and 101- 102 mg kg $^{-1}$ and 1123 – 1129 mg kg $^{-1}$ for HMX. Detailed values are reported in the tables. A control with EMs but no plants (to evaluate the basal mineralization rate) was included using three replicates, and the carrier control (acetone) with plants had a single replicate.

3.7. Earthworm bioaccumulation test

The earthworm bioaccumulation tests were performed according to modified protocols of ASTM standard guide for conducting laboratory soil toxicity or bioaccumulation tests with the lumbricid earthworm *Eisenia fetida* (American Society for Testing and Materials, 1998). Modifications included reducing the exposure from the recommended 28 d to 14 d to eliminate the need for feeding the earthworms, and using glass instead of plastic pots to avoid adsorption of EMs.

Earthworms *Eisenia andrei* were obtained from Carolina Biological Supply (Burlington, NC) and were maintained in earthworm bedding (Magic Products, Amherst, Jct, WI) and fed with dry cereal (Magic Worm Food, Magic Products) at 20 ± 1°C, 70-80% (w/w) moisture and a 16 h : 8 h (light:dark) cycle. Adult *E. andrei* used for the bioaccumulation tests had a well-developed clitellum and a wet mass ranging from 300 mg to 600 mg. Earthworms were acclimated for 24 h in non-amended Sassafras sandy loam soil prior to the experiment. Gut contents was not expelled prior to transfer in amended soil. Ten earthworms were then placed into each test unit (*i.e.*, 1-L glass jar), previously filled with 200 g of test soil (dry weight). Test units were prepared in triplicate for each concentration of chemical (described in section 3.3). Nominal concentrations for the definitive assays were

fixed at 10 and 100 mg kg $^{-1}$. Measured concentrations were 11 and 99 mg kg^{-1} for RDX; and 9 mg kg^{-1} and 83 mg kg^{-1} for HMX. Each test unit was covered by perforated caps and a chemically inert porous geotextile (Landscape Fabric, Select) and placed in the microcosm. After 14 d exposure, surviving earthworms were counted, rinsed with ASTM type 1 water, and depurated for 24 h on moistened (5 mL of ASTM type 1 water) filter paper. Depurated earthworms were rinsed, blotted-dry, placed into Teflon tubes and were immediately frozen at –80°C. Soil was homogenized and stored at –20°C until extracted with acetonitrile for HPLC analyses.

3.8. Wet combustion of radiolabeled samples

Plant sample preparation prior to total combustion included an additional washing step in ASTM type 1 water. Leaves were left for 5-10 min on absorbent paper before their wet weight was measured. Preparation of roots during the preliminary test involved separation of soil by soaking and repeated washing in ASTM type 1 water. Only corn roots could be separated efficiently from adhering soil. Soil and plant samples were subjected to a lyophilization step to obtain dry material for analysis. During the preliminary tests, earthworm samples were also subjected to lyophilization in order to calculate the dry mass, the dried earthworms were combusted. Larson *et al.* (1998) reported that lyophilization had many advantages over air drying, such as uniformity of extracting conditions. For the definitive tests, soil samples were combusted after lyophilization, plant samples were extracted using USEPA Method # 8330A and the radioactivity still present in the residue remaining after evaporation of acetonitrile was measured by wet combustion. Finally, earthworm samples were combusted directly without lyophilization and a factor derived from preliminary tests was used fo the conversion of wet to dry mass (see Results section).

The combustion method was derived from Allison (1960) as described by Nelson and Sommers (1982). A glass and PTFE apparatus was assembled (Figure 2), consisting of a 100 mL round bottom flask (A) with heating mantle, a gas inlet (B), a water filled jacket condenser (C) and an outlet (D) fitted with a separatory funnel (E) used for liquid transfer. The outlet was connected to a CO_2 trap consisting of 5 bubbling tubes (17 x 150 mm) attached in series. Each tube was filled with 10 mL of 0.25 M KOH containing a low concentration of thymolphtalein as pH indicator (for changes in the alkaline range).

The separatory funnel was filled with 10 mL of a mixture of 60% concentrated sulfuric acid and 40% concentrated phosphoric acid. A sample of dried material was weighed to the nearest 0.1 mg and transferred to a 100 mL flask. Sample weight was between 0.5 -1.2 g for soil, between 0.02 - 0.05 g for plants and approximately 0.2 g for earthworms (two lyophilized earthworms), respectively. One gram (± 5%) of potassium dichromate was then added, the flask was attached to the condenser, and fitted with the heating mantle. After the dropwise addition of 10 mL of the concentrated sulfuric and phosphoric acids, the flask was slowly heated. Nitrogen flow was turned on at a flow rate of approximatively 2 bubbles per second. Heating was stopped after 10 min and nitrogen was passed at an increased flow rate (10-15 bubbles per second) for another 10 min. The contents of the KOH traps were mixed (traps 1 and 2, separately from 3, 4, and 5) and duplicate 2 mL aliquots of each were counted

in 18 mL of scintillation counting fluid (ACS, Amersham, Oakville, Canada) in a Packard Tri-Carb 2100 TR scintillation counter.

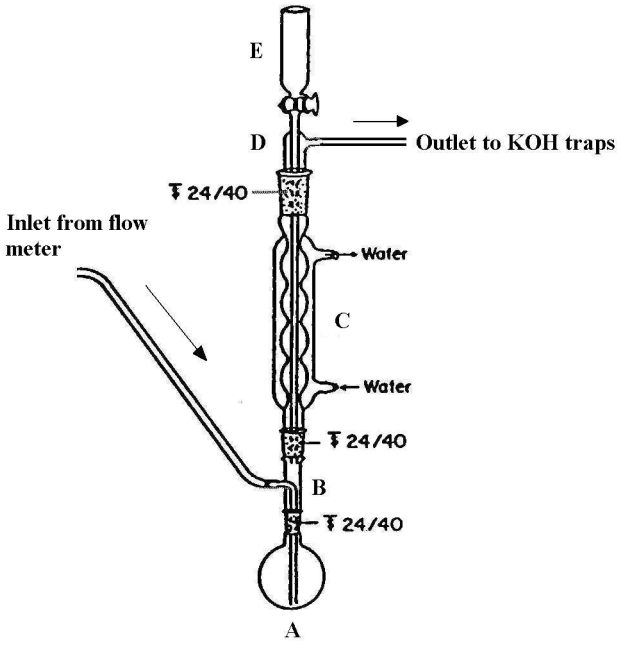

Figure 2. Apparatus used for measurement of [^{14}C]-RDX or [^{14}C]-HMX in plants and earthworms by wet combustion (modified from Nelson and Sommers, 1982).

3.9. Chemical extraction methods

3.9.1. *Soil samples*

Acetonitrile extraction procedure is a modification of US EPA Method # 8330A (United States Environmental Protection Agency, 1998). At the beginning of each toxicity test from which are derived the bioaccumulation data for non-radiolabeled compounds, soil samples were equilibrated in the dark for 24 h at room temperature, after addition of water (75% WHC). Aliquots of 2.0 g were sampled in triplicate from each treatment concentration. At the end of the toxicity tests, aliquots of 2.0 g were taken from each plant replicate exposed to different concentrations of contaminant. To each soil aliquot, 100 µL of 50 mg 1,3-dinitrobenzene (1,3-DNB) L^{-1} internal standard solution and 10 mL of acetonitrile were added. Glass tubes were vortexed for 1 min and then sonicated in the dark for 18 ± 2 h at 20 °C. Five (5) mL of sonicated sample was transferred to a new tube, to which 5 mL of 5 g $CaCl_2$ L^{-1} solution was added. For soil samples amended with TNB, a solution of 5 g $CaCl_2$ L^{-1} + 0.2 g $NaHSO_4$ L^{-1} was added to prevent TNB degradation. Supernatant was filtered on 0.45 µm Millex-HV cartridges. Soil extracts were analyzed and

quantified using an HPLC. Extraction was repeated if 1,3-DNB internal standard recovery was lower than 90%.

For radiolabeled samples, extracts were analyzed using HPLC fitted with a radioactivity detector in addition to the standard UV detector (see section 3.11). This was done to ascertain that the peaks occurring at known retention times corresponded to radiolabeled material. Extracts were also quantified using a scintillation counter.

3.9.2. *Plant samples*

In non-radiolabeled plant accumulation tests, concentrations of the EMs in plants were determined for selected treatment levels to measure bioaccumulation factors. At the end of definitive toxicity tests, two concentrations were chosen at which shoot biomass was sufficient and some growth inhibition was observed. Four replicates of the two EM concentrations and of the negative control were lyophilized in the dark for 24 h. Dried shoots were kept in a desiccator at room temperature before weighing. At least 0.02 g of finely ground shoot was transferred to a glass conical tube, to which a volume of internal standard / acetonitrile solution equivalent to 25 times dry biomass was added. For plants exposed to TNB, 2,4-DNT, or 2,6-DNT, 100 µL of 0.5 mg L^{-1} 1,3-DNB internal standard solution in acetonitrile was added. For plants exposed to RDX or HMX, the internal standard was 0.5 mg 2,4-DNT L^{-1}. Plant extracts were sonicated in the dark at 20°C for 18 h ± 2 h and then centrifuged at 1500 rpm (360 x g) for one hour. Supernatants were transferred in glass vials, to which an equivalent volume of ASTM type 1 water was added and kept at 4°C for 24 h. Supernatants were filtered on 0.45 µm cartridges and analyzed by HPLC.

During the analysis of non-radiolabeled plant extracts, it became obvious that plant matrix was interfering in the HPLC analysis of TNB, 2,4-DNT and 2,6-DNT, as evidenced by a peak eluting at the same retention time as these compounds in the negative controls. The UV absorption spectra of the interfering compounds confirmed that these were not any of the nitroaromatics. Therefore, a "**net content**" was calculated by subtracting the peak area value obtained in controls from the measured values in exposed plants. When the result of that subtraction was less than the detection limit (2.5 µg g^{-1} tissue) the value was reported as below detection limit (BDL) in the tables. This was done systematically for all EMs.

In radiolabeled plant accumulation tests, samples were processed using essentially the same methods used for non-radiolabeled samples, with the addition of special precautions related to radioactivity work (dedicated hood and analytical balance). Plant shoots were washed in distilled water and then placed on filter paper before being transferred to glass vials. Plants were then subjected to lyophilization. All calculations were expressed in terms of plant dry mass.

3.9.3. Earthworm samples

Determination of RDX and HMX concentrations in earthworms exposed to radiolabeled EMs was performed using the method described in Renoux *et al.* (2000). Whole frozen earthworms were thawed in acetonitrile at room temperature for 15 min, then homogenized for 1 min (Kinematica Model CH-6010 with 10 mm probe, Kriens-Lu, Switzerland) at 4°C and vortexed for 1 min. Samples were then sonicated (Branson 3200) for 18 h and centrifuged (12000 x g) for 10 min at 4°C (Sorval Super T21). An aliquot of the supernatant was treated with an equivalent volume of $CaCl_2$ (10 g L^{-1}) to precipitate fine particles. Samples were then left for 2 h at 4 °C prior to filtration through a 0.45-μm cartridge (Millipore) for subsequent HPLC analysis.

3.10. Chemical analysis of non-radiolabeled samples

Soil and plant extracts were analyzed using a Thermo Separation Products chromatographic system composed of model P4000 pump, a model AS1000 injector, including the temperature control for the column, and a model UV6000LP photodiode-array detector. For TNB, 2,4-DNT and 2,6-DNT analyses, a Supelcosil C8 column (25 cm x 4.6 mm ID, 5 μm particles) and a 18% 2-propanol / 82% water mobile phase were used. The flow rate was 1 mL min^{-1} and the run time was 40 min. For RDX and HMX analyses, the column used was a Supelcosil LC-CN (25 cm x 4.6 mm ID, 5 μm), which was held at 35°C. The initial solvent composition was 30% methanol / 70% water, which was held for 8 min, then a linear gradient was run from 30 to 65% methanol over 12 min. This solvent ratio was then changed to initial conditions (30% methanol) over 5 min. These initial conditions were then held for an additional 5 min. The injection volume was 50 μL. The detector was set to scan from 200 to 350 nm and chromatograms were extracted at 254 nm. The limit of quantification was 50 ppb for all chemicals. Precision was > 95% (SD < 2%, S/N = 10).

3.11. Chemical analysis of radiolabeled samples

Extracts were analyzed by HPLC using a chromatographic system composed of a Beckman System Gold Model 128 pump, a Beckman Model 166 UV detector, a Waters Model 717 Plus sample injector and a Waters temperature control module. A radiodetector (Model β-RAM, IN/US Systems Inc., Tampa, FL) was used with IN-Flow BD scintillation cocktail and Win-Flow software. A Supelcosil C8 column (25 cm × 4.6 mm i.d., 5 μm particles) was used for separation at 35°C. The mobile phase composition was 82% water and 18% 2-propanol (v/v), and the flow rate was 1 mL/min. The sample volume injected was 50 μL with a run time of 40 min. Analytes were detected by HPLC and by the radiodetector simultaneously at a wavelength of 254 nm. The limit of quantification of the instrument was 50 μg L^{-1} for RDX or HMX. The detection limit of the radiodetector was approximately 100 dpm. Chromatograms were examined for the presence of metabolites, which were identified on the basis of their retention time.

3.12. Statistical analysis

Differences in bioconcentration factors between plant species were assessed by analysis of variance. Fisher's least significant difference test was used as post-hoc test. Analysis of variance were performed using SYSTAT program (SPSS Inc., 1997). Differences in plant and earthworm tissue concentrations and effects of weathering/aging of soils were analyzed using Student's *t*-test. Non equality of variance was assumed, and was justified by the small number of replicates used, as well as null variance in some of the datasets. The significance level was fixed at $p < 0.05$. Student's *t*-test were performed using EXCEL 97 SR-2 (Microsoft Corp., 1997). Analyses were done using nominal EM concentrations.

4. RESULTS

Results of the preliminary studies are presented in sections 4.1 and 4.2. The efficiency of the wet combustion technique for measuring total radioactivity present in soil, earthworm and plant samples was evaluated. To assess plant survival in closed conditions, a prototype of the microcosm was assembled and tested with plants without radioactivity. This was followed by preliminary assays using radiolabeled compounds. Results of the definitive assays are presented in section 4.3.

4.1. Efficiency assessment of the wet combustion technique

Clean soil samples were spiked with various amounts of radiolabeled solutions, and immediately combusted. Radiolabeled HMX was used, and a satisfactory recovery was obtained for soil spiked with HMX averaging 95.9 ± 0.9% (n = 3). Results are shown in Table 2.

Table 2. CO_2 recovery from radiolabeled HMX after combustion

Amount of radioactivity added / matrix	Recovery (%)
100 dpm / soil	96.9 %
1100 dpm / soil	96.6%
11000 dpm / soil	94.2%

4.2. Preliminary tests

4.2.1. *Evaluation of plant survival*

Alfalfa, Japanese millet, ryegrass, corn and lettuce were tested using standardized conditions of terrestrial plant toxicity tests (growth chamber) and using microcosm unit. Microcosm units were placed in a greenhouse, under artificial and natural light. Conditions in growth chamber were as defined in section 3.7. In this study, single pots were used for each plant species (no replicates), 7 corn seeds were sowed per pot, and 20 seeds were sowed for the other plants. Lettuce did not grow well in the Sassafras sandy loam soil, showing variable germination rate. The microcosm gave a performance equivalent or better than plants incubated in growth chamber in terms of survival for corn, ryegrass and alfalfa after 34 d (Table 3). The final weights of the plants in the microcosm were greater than those placed in the incubator, except for alfalfa and lettuce. Fungal mycelia appeared during this period and affected the plants, alfalfa in particular. Japanese millet had a tendency to wilt prematurely in conditions of high humidity, explaining the reduced survival. Fungal growth was not controlled by using fungicides in order to avoid possible interfering effects. Corn showed very strong growth initially, but leaves started to discolor and wilt after two weeks inside the microcosm. Although corn growth and seedling emergence data were satisfactory, this species was not chosen for the toxicity assay because of its low sensitivity.

A 3-week preliminary test was carried out with corn to study the distribution of EMs in various plant compartments.

Table 3. Plant survival after 34 d exposure using microcosm unit compared to growth chamber conditions

Species	Growth chamber			Microcosm unit		
	% germinated Day 5	% survival Day 34	Final wet weight (g)	% germinated Day 5	% survival Day 34	Final wet weight (g)
Buttercrunch lettuce	45	85	0.4075	20	30	0.4107
Sweet corn	100	100	0.8041	100	100	1.2550
Japanese millet	90	90	0.2024	90	50	0.5044
Perennial ryegrass	90	100	0.3618	85	105	0.4665
Alfalfa	95	30	0.1202	90	50	0.1046

4.2.2. *Preliminary accumulation test*

Preliminary accumulation assays were carried out using alfalfa, lettuce and ryegrass under microcosm culture conditions. Plants were exposed for 6 weeks to a low concentration of 21 mg kg^{-1} [C^{14}]-HMX that was considered to be relevant to field studies. No soil or plant controls (not exposed to EM) were used in this experiment. Germination problems with lettuce were avoided using a filter paper germination step, as described in section 3.7. Fungal growth was noted and resulted in the loss of one replicate. Accumulation was similar in all plants, ranging from 23 – 27 fold (alfalfa) to 26 - 29 fold (ryegrass) based on the initial soil concentration. Approximately 65% (range of 48 –100%) of the radioactivity incorporated in plants could be extracted and identified as HMX by HPLC analysis. Energetic material conjugates were not observed in plant acetonitrile extracts, as a single peak of radioactivity was noted in each run. Highest tissue levels were found in lettuce (575 mg kg^{-1}), alfalfa and ryegrass showing similar tissue accumulation of approximatively 508 ± 13 mg kg^{-1}. Mineralization of HMX was negligible, averaging 0.23 ± 0.05% of the measured amount present in soil over a 6 week period when the data from the three species were combined. A small proportion of the radiolabeled compound was accumulated, at 0.4-1.0% of the amount present in soil. Total recovery (mass balance study) of amended radiolabeled material averaged 96 ± 3% (using measured values). Following this preliminary test, it was decided to extract all samples with acetonitrile before performing total combustion of the plant residue.

4.2.3. Distribution study using corn

This test was conducted to allow measurement of the distribution of RDX and HMX in the various compartments of the plant. Corn was chosen because its roots could

easily be washed free of adhering soil. Plants were exposed in triplicate to 27 mg kg^{-1} [C^{14}]-RDX and 25 mg kg^{-1} [C^{14}]-HMX for 19 d, but only a single replicate of roots for each RDX and HMX was analyzed. Initial concentrations of EMs in soils were determined by wet combustion. Detailed bioaccumulation data (HPLC analysis of plant tissue or wet combustion of plant residue and soil) are given for each chemical in Table 4. Bioconcentration factors (BCF) (expressed as mg kg^{-1} dry mass plant divided by mg kg^{-1} dry soil) ranged from 3.6 (roots) to 18 (leaves) in the case of RDX and from 0.5 (roots) to 3.1 (leaves) for HMX.

Table 4. Bioaccumulation-related parameters for corn exposed to 27 mg kg^{-1} [C^{14}]-RDX and 25 mg kg^{-1} [C^{14}]-HMX in freshly amended Sassafras sandy loam soil for 19d.

Parameter	Plant compartment		
	Leaves	Stems	Roots
Exposure to RDX			
RDX in plant tissue (mmol kg^{-1})	2.2 ± 0.3	0.86 ± 0.07	0.47
RDX in plant tissue (mg kg^{-1})	480 ± 56	191 ± 16	104
BCF	18 ± 1	7.1 ± 0.5	3.6
Acetonitrile extraction of RDX (%)	78 ± 1	46 ± 4	68
Exposure to HMX			
HMX in plant tissue (mmol kg^{-1})	0.27 ± 0.02	0.09 ± 0.01	0.04
HMX in plant tissue (mg kg^{-1})	79 ± 6	26 ± 3	12
BCF	3.1 ± 0.2	1.0 ± 0.1	0.5
Acetonitrile extraction of HMX (%)	82 ± 1 %	40 ± 2 %	73 %

* Values are average ± standard error.

The distribution of the [C^{14}]-label was studied by acetonitrile extraction of the plant tissues. Total combustion of the remaining residue after extraction was used to obtain the estimate of non-extractable fraction. Overall considering the whole plants, about 68% (RDX) to 72% (HMX) of the measured radioactivity was extractable in acetonitrile (expressed as µCi in extract divided by the total µCi in plant tissues). For the two chemicals, the highest proportion of acetonitrile extractable material (77 to 82%) was in the leaves (this is the amount present in leaf extract divided by total amount present in residue plus extract). Non-extractable radiolabeled material was most abundant in stems. HMX accumulation was relatively low, from 12 mg kg^{-1} in roots to 79 mg kg^{-1} in leaves (Table 4). Accumulation of RDX was approximately 8 to 10 times higher (on a molar basis) than HMX, with levels reaching 480 mg kg^{-1} in leaves.

The distribution of EMs is summarized in Table 5 for RDX and HMX in various compartments of the plant and of the whole microcosm. The bioconcentration factors (BCF) are shown as well as the percentage of EM found in each compartment (stems, leaves, roots) relative to the total mass of EM present in the plant. Mineralization was pooled for the three replicates to optimize the use of the microcosm enclosure. Mass balance is based on counting using a liquid scintillation counter (LSC) of [C^{14}]-labeled material in acetonitrile plant extracts (% in plant tissue) and from LSC counting of [C^{14}]-labeled CO_2 produced by wet combustion of plant (% in plant tissue including residue) and soil samples (% recovery). The EM uptaken by plants (or percentage of EMs in plants) was expressed as the acetonitrile extractable or total concentration in plant tissue (µCi) divided by the initial soil content (µCi).

For both EMs, the leaves were the major site of accumulation, followed by roots and stems. Approximately 62% (RDX) to 56% (HMX) of the total mass of EMs was found in leaves. This compartment represented only 26 - 31 % of the total dry biomass and suggested that evapotranspiration was a probable mechanism of accumulation. The other compartments represent the following percent of the dry plant mass; stems 14 - 17 % and roots 51 - 59 %.

Table 5. Summary of the bioconcentration factors (n =3) and mass balance studies in corn exposed to 27 mg kg^{-1} RDX and 25 mg kg^{-1} HMX in Sassafras sandy loam soil for 19d.

Plant compartment	Energetic material	
	RDX	**HMX**
BCF ± SE* in:		
Stems	7.1 ± 0.5	1.0 ± 0.1
Leaves	18 ± 1	3.1 ± 0.2
Roots	3.6	0.5
% of total radiolabel present in the plant in:		
Stems	15.0 ± 0.4	10.0 ± 0.5
Leaves	62 ± 1	56 ± 2
Roots	23	35
Microcosm compartments		
Percent Mineralization	0.73	0.054
Percent in plant tissue (CH_3CN soluble, by LSC)	5.6 ± 0.3	1.0 ± 0.1
Percent in plant tissue (including residue, LSC)	8.7 ± 0.6	1.4 ± 0.1
Percent Recovery in soil	58 ± 1	87 ± 7
Unaccounted (%)	36	12

* Values are average ± standard error

Mineralization data (Table 5) show that biotransformation of RDX was higher (ten-fold difference) than that of HMX. Overall, recovery was lower for RDX (58%) than for HMX (87%), suggesting that RDX is metabolized to intermediate metabolites that could not be trapped in the KOH solution. In this experiment, total recovery of HMX from corn was lower (87%) compared to the average 96% obtained previously in the experiment with alfalfa, lettuce and ryegrass. HPLC analysis of the plant extracts and of some soil extracts gave concentrations that were between 73% to 79% of those measured using LSC counting. Consequently, the recoveries expressed with the HPLC data are lower at 49 ± 3% (RDX) and 64 ± 5% (HMX).

4.2.4. *Accumulation test with earthworms*

Preliminary experiments using nominal concentrations of 0, 100, and 400 mg kg^{-1} RDX (as single replicates) were conducted in order to estimate the 14-d bioconcentration factor for earthworms. Mortality was not observed in any of the assays. Using wet combustion of the earthworm and soil samples, the BCF were 2.20 ± 0.20 and 0.71 ± 0.06 (µCi g^{-1} dry mass earthworm / µCi g^{-1} dry soil) for earthworms exposed to RDX at 100 and 400 mg kg^{-1}, respectively. Tissue accumulation was 240 ± 20 mg kg^{-1} (1.1 ± 0.1 mmol kg^{-1}) and 270 ± 20 mg kg^{-1} (1.2 ± 0.1 mmol kg^{-1}) at 100 and 400 mg kg^{-1} RDX, respectively. Mass balance calculations showed that 85% of the added radiolabeled RDX (at 100 mg kg^{-1}) was recovered in the soil after 14 d, and 100% was recovered in soil amended with 400 mg kg^{-1} RDX. Approximately 0.9% and to 0.3% of the added radiolabeled RDX was taken up by the earthworms following exposure to 100 and 400 mg kg^{-1} RDX, respectively.

Experiments using 400 mg kg^{-1} HMX (in triplicate) were also conducted. For HMX, a BCF of 0.09 ± 0.02 was calculated and the observed tissue accumulation was 37 ± 8 mg kg^{-1} (or 0.13 ± 0.03 mmol kg^{-1}) following exposure to HMX. Mass balance calculations showed that an average of 96 ± 14% of the added [C^{14}]-HMX was recovered in the soils after 14 d. Following exposure to HMX, only 0.024 ± 0.001% of the initial amount present in soil was present in earthworm tissues (in µCi HMX in the dry mass earthworm / initial µCi HMX in dry soil). Mineralization was measured only for HMX, and accounted for 0.026% of the total starting material. During these preliminary experiments, coefficients allowing calculation of dry mass from earthworm wet mass were calculated. The ratio dry weight / wet weight were 0.175 ± 0.015 (n = 10) for RDX and 0.151 ± 0.015 (n = 15) for HMX-exposed earthworms.

4.3. Definitive tests

4.3.1. *EM accumulation in plants using non-radiolabeled compounds*

Plant tissue taken from the phytotoxicity tests to generate ecotoxicological benchmark data for RDX, HMX, 2,4-DNT, 2,6-DNT and TNB (see Rocheleau *et al.*, 2003) was analyzed for EM concentrations. To assess the bioaccumulation potential of EM in alfalfa, Japanese millet and ryegrass, ratios (recovered EMs divided by initial EM concentrations) for all compounds were determined. Mass balance was not calculated in this study because only non-radiolabeled chemicals were used for the phytotoxicity tests. Plants were incubated in a temperature and light controlled growth chamber, as described in section 3.7.

Soil concentration values at the beginning and end of the tests are shown in Tables 6 to 15. The presence of metabolites in the acetonitrile extracts from plant tissue was measured using HPLC. No obvious extraneous peaks were found, but trace amounts of metabolites could have gone undetected.

Plants were exposed to freshly amended SSL soil at measured concentrations of 2.6, 5, 67 and 112 mg TNB kg $^{-1}$soil (Table 6). Only alfalfa accumulated detectable amounts of TNB (20 mg kg $^{-1}$), following exposure at a soil concentration of 67 mg kg $^{-1}$. The resulting BCF was 0.3, indicative of a low bioaccumulation potential. No EM was detected in the plants exposed to the weathered/aged TNB amended SSL soil (Table 7).

Following exposure to freshly amended SSL soil, 2,4-DNT was not detected in plants (Table 8). However, exposure to weathered/aged soil led to detectable tissue levels in plants (Table 9). Exposure to initial measured concentrations of 7.8 and 14.9 mg 2,4-DNT kg $^{-1}$ dry soil resulted in tissues levels of 3.4 and 2.3 mg kg $^{-1}$ tissue in ryegrass and alfalfa, respectively. The associated BCFs were 0.15 and 0.44, suggesting a low bioaccumulation potential.

Accumulation of 2,6-DNT in plants was higher than for TNB and 2,4-DNT (Table 10). Concentrations up to 24 mg kg^{-1} tissue were found in alfalfa exposed to freshly amended soils, with a BCF of 1.7. Values were lower in Japanese millet and ryegrass. When weathered/aged amended soils were used (Table 11), net concentrations were similar and BCF values were not statistically different from results with freshly amended soil (p = 0.45).

Accumulation of RDX and HMX in plants differed from that of nitroaromatic EMs tested in this study. Recovery in soil was high for RDX (96 - 105%) and for HMX (83 - 105%). RDX concentrations in plants exposed to freshly amended soil ranged from 1329 mg kg $^{-1}$ in ryegrass to 2610 mg kg $^{-1}$ in alfalfa (Table 12). Because of high EM concentrations in the soil, the calculated BCF values remained low, at 0.14 to 0.27. When plant species were compared, accumulation in alfalfa was significantly different from millet and ryegrass (p < 0.012)

Weathering/aging of amended soil enhanced the availability of RDX to the plants (Table 13). The highest net concentration found in plant tissue was 6321 mg kg $^{-1}$ in alfalfa exposed to weathered/aged amended SSL soil. A similar amount was found in Japanese millet (5047 mg kg $^{-1}$) exposed to the weathered/aged amended soil and ryegrass accumulated in a smaller amount (3747 mg kg $^{-1}$). The calculated BCFs ranged from 0.39 to 0.66, and were significantly different from those obtained in freshly amended soil ($p = 0.03$). Accumulation was significantly different between all three plant species ($p < 0.05$).

HMX was accumulated in plants following exposure in freshly amended soil (Table 14). The net tissue concentration ranged from 133 mg kg $^{-1}$ in Japanese millet to 288 mg kg $^{-1}$ in alfalfa. The calculated BCF values for HMX were low, ranging from 0.01 to 0.03, due to the high soil concentrations used in this study. Accumulation was similar in all plants ($p > 0.05$). Weathering/aging of amended soil resulted in an increased accumulation for Japanese millet (Table 15). The highest HMX net concentration determined in plant tissue was 349 mg kg $^{-1}$ in alfalfa, followed by Japanese millet (241 mg kg $^{-1}$) and ryegrass (166 mg kg $^{-1}$). After weathering/aging of the soil, accumulation was significantly different between all three plant species ($p < 0.05$). Comparison of the BCF data obtained in weathered/aged soil for all species to the pooled data obtained in freshly amended soil showed that weathering/aging of HMX amended soils had no significant effect on accumulation by plants ($p = 0.34$).

Table 6. Soil and plant tissue TNB concentrations, and bioconcentration factors (BCF) after 16-d (alfalfa and Japanese millet) and 19-d (ryegrass) exposure in freshly amended Sassafras sandy loam soil.

Plant species and Nominal concentration (mg kg^{-1})	Soil extraction at T_o mean ± SE (mg kg^{-1})	Soil extraction at T_f mean ± SE (mg kg^{-1})	Recovery (%)	Plant extraction at T_f (net) mean ± SE (mg kg^{-1})	Plant BCF mean ± SE
Alfalfa					
5	2.6 ± 0.1	BDL*	0.0	BDL	BDL
80	67 ± 2	19.6 ± 0.3	29	20 ± 5	0.3 ± 0.1
Japanese millet					
5	2.6 ± 0.1	0.1 ± 0.1	4	BDL	BDL
60	67 ± 1	7.1 ± 0.6	11	BDL	BDL
Ryegrass					
10	5.0 ± 0.2	0.2 ± 0.1	4	BDL	BDL
120	112 ± 2	61 ± 8	55	BDL	BDL

* BDL : Below detection limits. The standard error is reported when triplicate samples could be analyzed, otherwise in the case of plant samples data come from the average of duplicates, in rare cases from single measurements on pooled samples.

Table 7. Soil and plant tissue TNB concentrations, and bioconcentration factors (BCF) after 16-d (alfalfa and Japanese millet) and 19-d (ryegrass) exposure in weathered/aged Sassafras sandy loam soil.

Plant species and Nominal concentration (mg kg^{-1})	Soil extraction at T_o mean ± SE (mg kg^{-1})	Soil extraction at T_f mean ± SE (mg kg^{-1})	Recovery (%)	Plant extraction at T_f (net) mean ± SE (mg kg^{-1})	Plant BCF
Alfalfa					
5	BDL*	BDL	0.0	BDL	BDL
80	22.1 ± 0.4	10.7 ± 0.2	48	BDL	BDL
Japanese millet					
5	BDL	BDL	0.0	BDL	BDL
60	5.2 ± 0.2	1.3 ± 0.1	26	BDL	BDL
Ryegrass					
10	0.32 ± 0.01	BDL	0.0	BDL	BDL
120	81 ± 2	30 ± 3	37	BDL	BDL

* BDL : Below detection limits.

Table 8. Soil and plant tissue 2,4-DNT concentrations, and bioconcentration factors (BCF) after 16-d (alfalfa and Japanese millet) and 19-d (ryegrass) exposure in freshly amended Sassafras sandy loam soil.

Plant species and Nominal concentration (mg kg^{-1})	Soil extraction at T_o mean ± SE (mg kg^{-1})	Soil extraction at T_f mean ± SE (mg kg^{-1})	Recovery (%)	Plant extraction at T_f (net) mean ± SE (mg kg^{-1})	Plant BCF
Alfalfa					
5	4.7 ± 0.1	0.37 ± 0.02	8	BDL*	BDL
50	46.5 ± 0.5	20 ± 1	43	BDL	BDL
Japanese millet					
5	4.7 ± 0.1	0.5 ± 0.2	10	BDL	BDL
25	21.5 ± 0.6	5.8 ± 0.7	27	BDL	BDL
Ryegrass					
1	1.0 ± 0.0	BDL	6	BDL	BDL
10	9.1 ± 0.2	1.5 ± 0.04	16	BDL	BDL

* BDL : Below detection limits.

Table 9. Soil and plant tissue 2,4-DNT concentrations, and bioconcentration factors (BCF) after 16-d (alfalfa and Japanese millet) and 19-d (ryegrass) exposure in weathered/aged Sassafras sandy loam soil.

Plant species and Nominal concentration (mg kg^{-1})	Soil extraction at T_o mean ± SE (mg kg^{-1})	Soil extraction at T_f mean ± SE (mg kg^{-1})	Recovery (%)	Plant extraction at T_f (net) mean ± SE (mg kg^{-1})	Plant BCF
Alfalfa					
10	3.7 ± 0.2	2.8 ± 0.1	77	BDL*	BDL
50	14.9 ± 0.3	15.8 ± 0.8	106	2.3	0.15
Japanese millet					
10	3.7 ± 0.2	2.9 ± 0.1	77	BDL	BDL
25	7.8 ± 0.1	5.7 ± 0.1	73	BDL	BDL
Ryegrass					
10	3.7 ± 0.2	3.0 ± 0.1	80	BDL	BDL
25	7.8 ± 0.1	5.6 ± 0.2	73	3.4	0.44

* BDL : Below detection limits.

Table 10. Soil and plant tissue 2,6-DNT concentrations, and bioconcentration factors (BCF) after 16-d (alfalfa and Japanese millet) and 19-d (ryegrass) exposure in freshly amended Sassafras sandy loam soil.

Plant species and Nominal concentration (mg kg^{-1})	Soil extraction at T_o mean ± SE (mg kg^{-1})	Soil extraction at T_f mean ± SE (mg kg^{-1})	Recovery (%)	Plant extraction at T_f (net) mean ± SE (mg kg^{-1})	Plant BCF
Alfalfa					
10	8.0 ± 0.3	0.4 ± 0.3	5	4.4	0.54
20	14 ± 1	4.6 ± 0.1	33	24	1.7
Japanese millet					
5	4.1 ± 0.1	0.5 ± 0.1	11	1 ± 1	0.25
20	14 ± 1	2.7 ± 0.1	19	9 ± 3	0.64
Ryegrass					
5	4.1 ± 0.1	0.1 ± 0.1	1	BDL	BDL
40	30 ± 1	13 ± 1	43	7.7	0.26

* BDL : Below detection limits.

Table 11. Soil and plant tissue 2,6-DNT concentrations, and bioconcentration factors (BCF) after 16-d (alfalfa and Japanese millet) and 19-d (ryegrass) exposure in weathered/aged Sassafras sandy loam soil.

Plant species and Nominal concentration (mg kg^{-1})	Soil extraction at T_o mean ± SE (mg kg^{-1})	Soil extraction at T_f mean ± SE (mg kg^{-1})	Recovery (%)	Plant extraction at T_f (net) mean ± SE (mg kg^{-1})	Plant BCF mean ± SE
Alfalfa					
5	0.60 ± 0.04	0.29 ± 0.02	48	BDL	BDL
40	5.3 ± 0.1	2.7 ± 0.1	50	3.4	0.64
Japanese millet					
10	1.16 ± 0.01	0.60 ± 0.04	52	BDL	BDL
40	5.3 ± 0.1	3.3 ± 0.1	63	9.7 ± 0.9	1.8 ± 0.2
Ryegrass					
10	1.16 ± 0.01	0.57 ± 0.03	49	BDL	BDL
100	14.9 ± 0.1	8.3 ± 0.3	56	11 ± 1	0.74 ± 0.07

* BDL : Below detection limits.

Table 12. Soil and plant tissue RDX concentrations, and bioconcentration factors (BCF) after 16-d (alfalfa and Japanese millet) and 19-d (ryegrass) exposure in freshly amended Sassafras sandy loam soil.

Plant species and Nominal concentration (mg kg^{-1})	Soil extraction at T_o mean ± SE (mg kg^{-1})	Soil extraction at T_f mean ± SE (mg kg^{-1})	Recovery (%)	Plant extraction at T_f (net) mean ± SE (mg kg^{-1})	Plant BCF mean ± SE
Alfalfa					
10000	9740 ± 154	10296 ± 181	106	2610 ± 90	0.27 ± 0.01
Japanese millet					
10000	9740 ± 154	10244 ± 113	105	1658 ± 201	0.17 ± 0.02
Ryegrass					
10000	9740 ± 154	9373 ± 201	96	1330 ± 126	0.14 ± 0.013

Table 13. Soil and plant tissue RDX concentrations, and bioconcentration factors (BCF) after 16-d (alfalfa and Japanese millet) and 19-d (ryegrass) exposure in weathered/aged Sassafras sandy loam soil.

Plant species and Nominal concentration (mg kg^{-1})	Soil extraction at T_o mean ± SE (mg kg^{-1})	Soil extraction at T_f mean ± SE (mg kg^{-1})	Recovery (%)	Plant extraction at T_f (net) mean ± SE (mg kg^{-1})	Plant BCF mean ± SE
Alfalfa					
10000	9537 ± 214	9549 ± 127	100	6321	0.66
Japanese millet					
10000	9537 ± 214	9148 ± 168	96	5047 ± 251	0.53 ± 0.03
Ryegrass					
10000	9537 ± 214	9532 ± 216	100	3748	0.39

Table 14. Soil and plant tissue HMX concentrations, and bioconcentration factors (BCF) after 16-d (alfalfa and Japanese millet) and 19-d (ryegrass) exposure in freshly amended Sassafras sandy loam soil.

Plant species and Nominal concentration (mg kg^{-1})	Soil extraction at T_o mean ± SE (mg kg^{-1})	Soil extraction at T_f mean ± SE (mg kg^{-1})	Recovery (%)	Plant extraction at T_f (net) mean ± SE (mg kg^{-1})	Plant BCF mean ± SE
Alfalfa					
10000	10411 ± 807	9427 ± 385	91	288 ± 50	0.028 ± 0.005
Japanese millet					
10000	10411 ± 807	8597 ± 211	83	133 ± 6	0.013 ± 0.001
Ryegrass					
10000	10411 ± 807	9064 ± 307	87	182	0.017

Table 15. Soil and plant tissue HMX concentrations, and bioconcentration factors (BCF) after 16-d (alfalfa and Japanese millet) and 19-d (ryegrass) exposure in weathered/aged Sassafras sandy loam soil.

Plant species and Nominal concentration (mg kg^{-1})	Soil extraction at T_o mean ± SE (mg kg^{-1})	Soil extraction at T_f mean ± SE (mg kg^{-1})	Recovery (%)	Plant extraction at T_f (net) mean ± SE (mg kg^{-1})	Plant BCF
Alfalfa					
10000	9341 ± 804	9845 ± 579	105	349	0.037
Japanese millet					
10000	9341 ± 804	9048 ± 318	97	241 ± 11	0.026 ± 0.001
Ryegrass					
10000	9341 ± 804	9161 ± 389	98	166 ± 11	0.018 ± 0.001

BCFs for TNB, 2,4-DNT and 2,6- DNT in plants are presented in Table 16. In summary, the BCF values were generally close to or less than 1 (ratio of tissue against soil concentrations), indicating that either the chemicals were not accumulated, or that they were extensively (bio)transformed in plants. There was no significant difference in accumulation according to the species, as verified by Student's *t*-test ($p > 0.46$) on the whole data for each plant. An accumulation order is apparent for the nitramines RDX and HMX but the low values of BCF resulting from exposure at a single high soil concentrations do not allow any distinction between dicotyledonous and monocotyledonous species.

Table 16. Summary of nitroaromatics bioconcentration factors derived from definitive plant toxicity tests.

Measured concentration	Alfalfa	Japanese millet	Ryegrass
Exposure period (d)	16 d	16 d	19 d
TNB (F) (mg kg^{-1})			
2.6 ± 0.1	BDL *	BDL	
5.0 ± 0.2			BDL
67 ± 1		BDL	
67 ± 2	**0.3 ± 0.1**		
112 ± 2			BDL
TNB (W/A) (mg kg^{-1})			
0 **	BDL	BDL	
0.3 ± 0.1			BDL
5.2 ± 0.2		BDL	
22.1 ± 0.4	BDL		
120			BDL
2,4-DNT (F) (mg kg^{-1})			
1.0 ± 0.1			BDL
4.7 ± 0.1	BDL	BDL	
9.1 ± 0.2			BDL
21.5 ± 0.6		BDL	
46.5 ± 0.5	BDL		
2,4-DNT (W/A) (mg kg^{-1})			
3.7 ± 0.2	BDL	BDL	BDL
7.8 ± 0.1		BDL	**0.44**
14.9 ± 0.3	**0.15**		
2,6-DNT (F) (mg kg^{-1})			
4.1 ± 0.1		**0.3 ± 0.3**	BDL
8.0 ± 0.3	**0.54**		
13.9 ± 0.6	**1.7**	**0.64**	
30 ± 1			**0.26**
2,6-DNT (W/A) (mg kg^{-1})			
0.6 ± 0.1	BDL		
1.2 ± 0.1		BDL	BDL
5.4 ± 0.1	**0.64**	**1.8 ± 0.2**	
14.9 ± 0.1			**0.74 ± 0.07**

* BDL : Below detection limits. The standard error is reported when triplicate samples could be analyzed, otherwise data come from the average of duplicates, in rare cases from single measurements on pooled samples. ** This correspond to a group having a nominal concentration of 5.0 mg kg^{-1}, in which TNB was not detected following the weathering/aging process.

4.3.2. *Tissue accumulation in plants using radiolabeled compounds*

Definitive bioaccumulation tests were conducted after minor adjustments to the microcosm units. The first modification was addition of a potassium permanganate / activated charcoal trap to convert putative volatile intermediates of HMX / RDX microbial degradation (typically formaldehyde) to carbon dioxide, following a procedure described by Sekine and Nishimura (2001). The second modification was the addition of an external polyester seal to the lower part of the desiccator unit which tended to crack with time leading to gas leaks.

Plant species for definitive bioaccumulation tests were selected on the basis of the results of range-finding phytotoxicity tests presented in a separate report (Rocheleau *et al*., 2003). Alfalfa, Japanese millet and ryegrass were the more sensitive species to EMs. Two test concentrations were chosen from the sublethal portion of the range-finding toxicity tests. These included 100 mg kg^{-1} and 1000 mg kg^{-1}, since it was considered that mass balance studies would produce more reliable results by using higher soil concentrations. Background values (*i.e.* radioactivity counts) obtained from negative control plants (in single replicate) were subtracted from measured values in exposed plant and soil samples, as well as for mineralization data. Net accumulation is reported in Tables 17 and 18.

4.3.2.1. Accumulation in plants using radiolabeled RDX

Exposure of alfalfa, Japanese millet and ryegrass to RDX at nominal soil concentrations of 100 and 1000 mg kg^{-1} was conducted for 6 weeks. The measured values were between 83 and 93 mg kg^{-1} and between 946 and 1011 mg kg^{-1} (Table 17). The bioconcentration factors (BCF) are shown as well as the tissue concentrations of EM found in each species (aerial part only). The BCF data are expressed as mg kg^{-1} dry mass plant compartment divided by the measured concentration in mg kg^{-1} dry soil, based on HPLC analysis of the plant acetonitrile extract and wet combustion of soil. As shown in Table 17 accumulation of RDX was independent of the soil concentration. Tissue levels in alfalfa reached 6871 mg kg^{-1} at the lowest exposure concentration and 6820 mg kg^{-1} at the higher one, suggesting a saturation of the accumulation, as well as chemico-physical and biochemical mechanisms. Approximately 68% (range of 21 – 100%) of the radioactivity incorporated in plants was extracted and identified as RDX by HPLC analysis. Japanese millet and alfalfa accumulated more than ryegrass. ANOVA analysis showed that accumulation in ryegrass at the nominal concentration of 100 mg kg^{-1} was significantly different ($p < 0.05$) from the two other species. Bioconcentration values varied from 45 to 79 versus initial soil concentration, indicating a moderate potential of accumulation for this compound. At the nominal concentration of 1000 mg kg^{-1}, accumulation in ryegrass was significantly different from alfalfa only ($p < 0.05$).

Table 17. Bioaccumulation data for terrestrial plants exposed for 42d to RDX at nominal concentrations of 100 and 1000 mg kg^{-1} in Sassafras sandy loam soil.

Parameter	Plant species		
	Alfalfa	Japanese millet	Perennial ryegrass
Carrier controls (equivalent mg kg^{-1})	**1.1**	**1.9**	**0.3**
RDX in plant extract (mg kg^{-1})	0.0	0.0	0.0
RDX in plant residue (mg kg^{-1})	16.1	24.2	7.4
Exposure to 100 mg RDX kg^{-1}	**87 ± 2*** (measured)	**83 ± 2** (measured)	**93 ± 2** (measured)
RDX in plant tissue (mmol kg^{-1})	31 ± 1	26 ± 2	19 ± 2
RDX in plant tissue (mg kg^{-1})	6871 ± 307	5802 ± 339	4115 ± 503
BCF	79 ± 4	70 ± 2	45 ± 6
Exposure to 1000 mg RDX kg^{-1}	**998 ± 23** (measured)	**1011 ± 29** (measured)	**946 ± 24** (measured)
RDX in plant tissue (mmol kg^{-1})	31 (23 – 38)	23.4 ± 0.4	15 ± 3
RDX in plant tissue (mg kg^{-1})	6820 (5156 - 8474)	5190 ± 86	3288 ± 570
BCF	6.8 (5.0 – 8.7)	5.1 ± 0.1	3.5 ± 0.5

* Values are expressed as average ± standard error.

4.3.2.2. Accumulation in plants using radiolabeled HMX

Alfalfa, Japanese millet and ryegrass were exposed to HMX at nominal concentrations of 100 and 1000 mg kg^{-1} for 4 weeks. The measured values were between 100 and 102 mg kg^{-1} and between 1123 and 1129 mg kg^{-1} HMX as shown in Table 18. The 28-d exposure period is sufficient to allow the system to reach steady-state (Van Gestel *et al.*, 2002). The exposure time was shortened since wilting increased toward the end of the 6 weeks exposure period for RDX. Soil (amended with EM but without plants) and plant (not exposed to EM amended soil) controls were included.

As seen for RDX, HMX accumulation in plants was independent of the soil concentration (Table 18). Following exposure at concentrations varying by a factor of 10, similar amounts of HMX (from 218 to 253 mg kg^{-1} in alfalfa) were found in the plant tissue. Exposure of Japanese millet to 1000 mg kg^{-1} lasted for only 2 weeks, and the resulting increase in HMX tissue concentration of 185% may not be directly comparable with the rest of the data. HMX tissue concentrations were approximately 30-fold lower than those observed for RDX. Approximately 88% (range 49% to 100%) of the radioactivity incorporated in plants could be extracted and identified as HMX by HPLC analysis. The

radioactivity present in the residue after extraction represented 0 to 52% of the total radioactivity found in the plant. Variations in bioconcentration by the plants were moderate, Japanese millet and alfalfa accumulating more than ryegrass. Bioconcentration values for plants exposed to a nominal concentration of 100 mg kg^{-1} HMX ranged from 1.4 to 2.2 (Table 18). Analytical determinations of HMX in the acetonitrile extract using USEPA Method 8330A accounted for approximately 57 – 98% of material quantified by using radio-labeled compound. The calculated BCFs for plants exposed to a nominal concentration of 1000 mg kg^{-1} HMX ranged from 0.13 to 0.36, indicating, as found for RDX, a saturating mechanism of accumulation.

Table 18. Bioaccumulation data for terrestrial plants exposed for 28d to HMX at nominal concentrations of 100 and 1000 mg kg^{-1} in Sassafras sandy loam soil.

Parameter	Plant species		
	Alfalfa	Japanese millet	Perennial ryegrass
Carrier controls (equivalent mg kg^{-1})	**1.1**	**1.9**	**0.3**
HMX in plant extract (mg kg^{-1})	0.0	0.0	0.0
HMX in plant residue (mg kg^{-1})	16.1 (blank value)	24.2 (blank value)	7.4 (blank value)
Exposure to 100 mg HMX kg^{-1}	**101 ± 2** (measured)	**101 ± 2** (measured)	**102 ± 2** (measured)
HMX in plant tissue (mmol kg^{-1})	0.74 ± 0.05	0.73 ± 0.01	0.50 ± 0.01
HMX in plant tissue (mg kg^{-1})	218 ± 16	216 ± 3	148 ± 4
BCF	2.2 ± 0.1	2.16 ± 0.03	1.47 ± 0.04
Exposure to 1000 mg HMX kg^{-1}	**1126 ± 44** (measured)	**1123 ± 44 *** (measured)	**1129 ± 44** (measured)
HMX in plant tissue (mmol kg^{-1})	0.85 ± 0.06	1.35 ± 0.12	0.48 ± 0.01
HMX in plant tissue (mg kg^{-1})	253 ± 19	400 ± 37	141 ± 2
BCF	0.22 ± 0.01	0.36 ± 0.04	0.13 ± 0.06

* Exposure to 1123 mg kg^{-1} lasted 14 d only. Values are expressed as average ± standard error.

Again, Japanese millet and alfalfa accumulated more than ryegrass. ANOVA analysis showed that accumulation in ryegrass at the nominal concentration of 100 mg kg^{-1} was significantly different from the two other species ($p < 0.05$). There was a significant difference ($p < 0.05$) in BCF values for all species exposed at the nominal concentration of 1000 mg kg^{-1} HMX. Overall, bioconcentration values varied from 0.13 to 2.2 indicating a low potential of accumulation for HMX.

4.3.2.3. Mass balance studies for plants exposed to radiolabeled RDX and HMX

The mass balance results were obtained from one experiment done in triplicate (Table 19). The measured concentrations for each treatment are also reported. Mineralization was pooled for each of the three replicates forming a group (control group, treatment 100, treatment 1000 mg kg^{-1}).

Mass balance is based on HPLC analysis and LSC counting of [C^{14}]-labeled material in acetonitrile plant extracts (% in plant tissue) and from LSC counting of [C^{14}]-labeled CO_2 produced by wet combustion of plant (% in plant tissue non extractable) and soil samples (% recovery). The percentage of EMs in plants tissues was expressed as the acetonitrile soluble or total amount of compound present in plants (in µCi) / initial soil content (µCi). Soluble RDX indicates the proportion of extractable radioactivity that could be identified as RDX by HPLC.

Mineralization data show that biotransformation of RDX decreased proportionally as the soil concentration increased (Table 19). Mineralization in control soils without plants (0.6% and 0.1% at 100 and 1000 mg RDX kg^{-1}) was lower than in soils having plants. Approximately 6-19% of the RDX was unaccounted for at a nominal concentration of 100 mg kg^{-1}, which can be explained to some extent by transformation to various intermediate metabolites that were not trapped by KOH solution. Recovery in soil at a nominal concentration of 1000 mg RDX kg^{-1} was between 93 and 103%, which indicates that most of the EM remained in the soil. HPLC analysis of the plant extracts were in good agreement with values measured via LSC counting. The percentages of RDX found in plant as extractable in acetonitrile varied widely from 48 to 97%. A small increase in acetonitrile-insoluble material was apparent at a nominal concentration of 1000 mg kg^{-1}.

The HMX mass balance results from one experiment done in triplicate are presented in Table 20. Exposures were based on the measured concentrations for each treatment. Calculations were carried out as described in the preceeding table. Japanese millet exposure to 1000 mg kg^{-1} nominal HMX was accidentally terminated after 14 d instead of the normal exposure of 28 d.

The mineralization of HMX was low, averaging 0.07% of the measured amount present in soil at 100 mg kg^{-1} and 0.04% at 1000 mg kg^{-1} when the three species data are combined (Table 20). Mineralization decreased by only a factor of two as the soil concentration increased, in contrast to the relatively proportional trend seen for RDX. Mineralization in the control soils without plants (0.07% at 100 mg HMX kg^{-1} and 0.05% at 1000 mg HMX kg^{-1}) was similar or higher than in soil containing plants. The proportion of the radiolabeled compound that was accumulated by plants when exposed to 100 mg kg^{-1} HMX averaged 0.09 ± 0.03% of the amount present in soil. However, at nominal concentration of 1000 mg kg^{-1} HMX, uptake was reduced reaching only 0.01% of the initial amount present in soil.

Overall, recovery was lower at higher soil concentrations. As for RDX at 100 mg kg^{-1}, approximately 10% of the HMX was unaccounted for at 100 mg kg^{-1}, which can be explained also by transformation to some intermediate metabolites that cannot be trapped in a KOH solution. However some loss (> 20%) at 1000 mg kg^{-1} are not easily explained. HPLC analysis of the plant extracts were in good agreement with values measured using LSC counting, except for ryegrass that had only 63-66% of the LSC values. The percentages of HMX found in plant as extractable in acetonitrile varied from 62 to 100%, like in the RDX group, a small increase in acetonitrile-insoluble material was apparent in the high exposure treatment group.

Table 19. Summary of mass balance studies for plants exposed to 100 and 1000 mg kg^{-1} RDX in freshly amended Sassafras sandy loam soil for 42d.

Parameter	Plant species		
	Alfalfa	Japanese millet	Ryegrass
Exposure to100 mg RDX kg^{-1}	**87 ± 2*** (measured)	**83 ± 2** (measured)	**93 ± 2** (measured)
% Mineralization	1.48 %	0.78 %	1.40 %
RDX in plant tissue (% via HPLC)	1.7 ± 0.2 %	2.6 ± 0.2 %	1.1 ± 0.1 %
[^{14}C] in plant tissue (% via LSC)	1.7 ± 0.7 %	2.9 ± 0.1 %	1.2 ± 0.1 %
[^{14}C] in plant tissue (% non extractable)	0.8 ± 0.2 %	0.1 ± 0.1 %	0.5 ± 0.2
% soluble RDX in plant tissue	59 ± 18	97 ± 3	73 ± 6
% Recovery in soil	90 ± 5%	77 ± 5%	78 ± 8 %
% Unaccounted	6 ± 5%	19 ± 4%	19 ± 8 %
Exposure to1000 mg RDX kg^{-1}	**998 ± 23** (measured)	**1011 ± 29** (measured)	**946 ± 24** (measured)
% Mineralization	0.16 %	0.18 %	0.19 %
RDX in plant tissue (% via HPLC)	0.12 % (0.17 % and 0.08 %)	0.19 ± 0.01 %	0.07 ± 0.01 %
[^{14}C] in plant tissue (% via LSC)	0.15 % (0.20 % and 0.10 %)	0.20 ± 0.02 %	0.06 ± 0.03 %
[^{14}C] in plant tissue (% non extractable)	0.06 %	0.07 ± 0.02 %	0.06 ± 0.02 %
% soluble RDX in plant tissue	69 (64 -74)	75 ± 8	48 ± 18
% Recovery in soil	97 ± 5 %	103 ± 8 %	93 ± 3 %
% Unaccounted	4 ± 5 %	-3 ± 8 %	7 ± 3 %

* Values are expressed as the average ± standard error.

Table 20. Summary of mass balance studies for plants exposed to 100 and 1000 mg kg^{-1} HMX in freshly amended Sassafras sandy loam soil for 28d.

Parameter	Plant species		
	Alfalfa	Japanese millet	Ryegrass
Exposure to100 mg HMX kg^{-1}	**101 ± 2** (measured)	**101 ± 2** (measured)	**102 ± 2** (measured)
% Mineralization	0.070 %	0.060 %	0.062 %
HMX in plant tissue (% via HPLC)	0.052 ± 0.005 %	0.097 ± 0.013 %	0.060 ± 0.010 %
[^{14}C] in plant tissue (% via LSC)	0.058 ± 0.005 %	0.123 ± 0.014 %	0.095 ± 0.015 %
[^{14}C] in plant tissue (% non extractable)	0.0088 ± 0.0016 %	0.0033 ± 0.0033 %	0.0
% soluble HMX in plant tissue	88 ± 3	100	100
% Recovery in soil	89 ± 8 %	90 ± 3 %	92 ± 10 %
% Unaccounted	11 ± 8 %	9 ± 3 %	8 ± 10 %
Exposure to1000 mg HMX kg^{-1}	**1126 ± 44** (measured)	**1123 ± 44** (measured)	**1129 ± 44** (measured)
% Mineralization	0.042 %	0.021 %	0.043 %
HMX in plant tissue (% via HPLC)	0.0038 ± 0.0003 %	0.0071 ± 0.0018 %	0.0053 ± 0.0005 %
[^{14}C] in plant tissue (% via LSC)	0.004 ± 0.001 %	0.008 ± 0.002 %	0.008 ± 0.001 %
[^{14}C] in plant tissue (% non extractable)	0.0021 ± 0.0016 %	0.0011 ± 0.0008 %	0.0
% soluble HMX in plant tissue	59 ± 7	75 ± 13	100
% Recovery in soil	80 ± 7 %	84 ± 4 %	76 ± 6 %
% Unaccounted	20 ± 7 %	16 ± 4 %	24 ± 6 %

* Values are expressed as the average ± standard error.

BCFs of RDX and HMX obtained from plant accumulation definitive tests following 28 – 42 d exposure in microcosm and the 16 – 19 d exposure in growth chamber are summarized in Table 21. Measured concentrations are shown. The BCF values calculated in the definitive tests were low (0.13 – 2.2) for HMX to moderate (3.5 – 79) for RDX, the EMs were accumulated mostly in their unchanged form, (bio)transformation in plants was not observed to proceed at a significant level. HMX gave moderate BCF values (23 –29) when tested during a preliminary test at a lower soil concentration (21 mg kg^{-1}). Accumulation of nitramines RDX and HMX was slightly different when using non-radiolabeled EMs. At the tested concentrations, only ryegrass seemed to accumulate lesser amounts of the tested nitramines compared to alfalfa and Japanese millet, as evaluated by multiple pairwise Student's *t*-test ($p < 0.05$). However, when plants were exposed to RDX at

a nominal concentration of 1000 mg kg^{-1}, no significant difference was found between all species ($p > 0.09$). No distinction can be made between monocotyledonous and dicotyledonous species.

Table 21. Bioconcentration factors from definitive plant tests for nitramines.

Measured concentration		Alfalfa	Japanese millet	Ryegrass
RDX (F)	87 ± 2 mg kg^{-1}	79 (42 d)*	70 (42 d)	45 (42 d)
RDX (F)	985 ± 20 mg kg^{-1}	6.8 (42 d)	5.1 (42 d)	3.5 (42 d)
RDX (F)	9,740 ± 154 mg kg^{-1}	0.27 (16 d)	0.17 (16 d)	0.14 (16 d)
RDX (W/A)	9,537 ± 214 mg kg^{-1}	0.66 (16 d)	0.53 (16 d)	0.39 (16 d)
HMX (F)	101 ± 2 mg kg^{-1}	2.2 (28 d)	2.2 (28 d)	1.4 (28 d)
HMX (F)	1,126 ± 44 mg kg^{-1}	0.22 (28 d)	0.36 (28 d)	0.13 (28 d)
HMX (F)	10,411 ± 807 mg kg^{-1}	0.03 (16 d)	0.01 (16 d)	0.02 (16 d)
HMX (W/A)	9,341 ± 804 mg kg^{-1}	0.04 (16 d)	0.03 (16 d)	0.02 (16 d)

* Exposure periods are presented in brackets. (F): Freshly amended soil; (W/A): Weathered/aged soil.

4.3.2.4. Soil analytical data

A subset of the soil samples were analyzed by HPLC essentially in order to identify acetonitrile extractable metabolites that could have resulted from microbial activity and plant activity for T_f values. The results are presented in Table 22. Based on the soils samples analyzed, most of the EMs were unchanged. No known nitroso-metabolite of RDX (or HMX) was detected in the samples analyzed.

No clear pattern is observed in this data, none of the methods giving consistently a higher value than the other. Overall, concentrations calculated by HPLC were 7% higher than those obtained through combustion of the soils. The two methods were in fairly good agreement, but the combustion of small subsamples (approximatively 0.5 g) and the extraction of relatively small samples (approximatively 2.0 g) led to increased variability of the results.

Table 22. Soil analytical data (HPLC analysis of extracts) of selected samples from definitive tests with plants exposed to 100 and 1000 mg kg^{-1} nominal RDX or HMX in freshly amended Sassafras sandy loam soil.

Plant species and Nominal concentration (mg kg^{-1})	Soil extraction at To mean ± SE (mg kg^{-1})	Soil combustion at To mean ± SE (mg kg^{-1})	Soil extraction at Tf mean ± SE (mg kg^{-1})	Soil combustion at Tf mean ± SE (mg kg^{-1})	Recovery HPLC (%)	Recovery LSC (%)	Ratio HPLC / LSC (%)
Alfalfa RDX							
100		87 ± 2	76 ± 3	79 ± 4	87.1	90.3	96
1000		998 ± 23	886 ± 38	966 ± 25	88.8	96.8	92
Control 100 (no plant)		93 ± 2	87 ± 3	80 ± 3	93.6	85.6	109
Alfalfa HMX							
100		101 ± 2	94 ± 1	90 ± 8	92.9	88.7	105
1000		1126 ± 44	1089 ± 32	896 ± 51	96.7	79.6	122
Control 100 (no plant)		101 ± 2	91 ± 2	89 ± 6	89.3	88.0	102
Ryegrass RDX							
1000		946 ± 24	741 ± 31	880 ± 41	78.3	93.0	84
Control 1000 (no plant)	937 ± 26	1023 ± 31	879 ± 49	878 ± 11	93.9	85.9	109
Ryegrass HMX							
1000		1129 ± 44	1039 ± 80	848 ± 44	92.1	75.2	122
Control 1000 (no plant)	1018 ± 100	1129 ± 44	994 ± 32	875 ± 69	97.6	77.5	126

4.3.3. *Accumulation of radiolabeled RDX and HMX in earthworms*

Based on the results of earthworm range-finding toxicity tests with HMX or RDX, nominal concentrations of 10 and 100 mg kg^{-1} of each EM individually amended in SSL soil were selected for bioaccumulation studies. These concentrations represent the exposure conditions below the onset of toxicity to *E. andrei* reproduction and are more relevant for ecological risk assessment of soil contamination in the field. The measured values were 10 and 99 mg kg^{-1} for RDX; and 9 and 83 mg kg^{-1} for HMX. The corresponding measured values for the soil only controls (without earthworms) were 12.4 and 102 mg kg^{-1} for RDX; and 9 and 83 mg kg^{-1} for HMX.

4.3.3.1. Accumulation in earthworms using radiolabeled RDX

Following exposure to freshly amended soil, RDX was moderately accumulated in earthworm tissues (Table 23). Control earthworms were analyzed as well as

the control soil to provide analytical blanks, which were subtracted from measured values in exposed samples. The tissue concentration in the control earthworms was 1.3 mg kg $^{-1}$. The concentration in the control soil at To was 0.40 mg kg $^{-1}$. The apparent concentration in the control soil at Tf was 0.36 mg kg $^{-1}$. Accumulation in earthworm tissues was measured by total combustion. When earthworm extracts were subjected to HPLC analysis, concentrations of EMs were below the level of detection.

The highest tissue concentration measured was 283 mg kg $^{-1}$ (at a nominal soil concentration of 100 mg kg $^{-1}$. Exposure at the two different soil concentrations resulted in significantly different BCF and accumulation values ($p < 0.05$).

Table 23. Bioaccumulation-related parameters for earthworms exposed to 10 and 100 mg kg^{-1} RDX and HMX in Sassafras soil for 14d.

Parameter	Nominal concentration in soil	
	10 mg kg^{-1}	100 mg kg^{-1}
Exposure to RDX		
Accumulation in earthworm tissue (mg kg^{-1})	125 ± 11	283 ± 28 *
Accumulation of RDX in tissue (mmol kg^{-1})	0.56 ± 0.05	1.27 ± 0.13
BCF	13 ± 1	2.9 ± 0.2 *
Exposure to HMX		
Accumulation in earthworm tissue (mg kg^{-1})	9 ± 1	26 ± 3 *
Accumulation of HMX in tissue (mmol kg^{-1})	0.029 ± 0.004	0.089 ± 0.009
BCF	1.0 ± 0.2	0.32 ± 0.02

* Significant difference ($p < 0.05$) between concentrations. Values for concentrations are rounded.

4.3.3.2. Accumulation in earthworms using radiolabeled HMX

Following exposure to freshly amended soil (9 mg kg^{-1}), low levels of HMX was detected in earthworm tissues at 9 mg kg $^{-1}$ (Table 23). Controls were the same as for RDX study. The highest tissue concentration measured was more than ten times lower than that observed for RDX, at 26 mg kg $^{-1}$ (nominal soil concentration 100 mg kg^{-1}; measured concentration 83 mg kg^{-1}). Exposure of earthworms at the two different soil concentrations resulted in significantly different tissue levels ($p < 0.05$), however the BCF values were not significantly different ($p = 0.09$). BCFs values of 1 and less indicate that earthworms did not accumulate HMX over the levels present in soils under the tested conditions.

4.3.3.3. Mass balance studies for earthworms exposed to RDX and HMX

Earthworm mass balance results were obtained from one experiment done in triplicate (Table 24). Mineralization data show that biotransformation of RDX decreased proportionally as the soil concentration increase, from 0.4 to 0.2% at 10 and 99 mg kg^{-1} soil. The apparent mineralization in a control soil without EMs was 0.00064 µCi, corresponding to 6.0% of the mineralization observed in the 10 mg RDX kg^{-1} group. This background value was subtracted from all mineralization data. The presence of earthworms resulted in an increase in mineralization, from 0.6 to 1.2% in the 10 mg kg^{-1} treatment. Mass balance calculations indicated that approximately 5% and 1% of the added radiolabeled compound was taken up by the earthworms following exposure to concentrations of 10 and 99 mg kg^{-1} RDX, respectively. Recovery was similar at all soil concentrations, unaccounted RDX ranged from 4 to 17%.

Table 24. Mass balance studies in earthworms exposed to 10 and 100 mg kg^{-1} RDX and HMX in Sassafras soil for 14d.

Parameter	Nominal concentrations			
	10 mg kg^{-1}		100 mg kg^{-1}	
	Control	+ Earthworms	Control	+ Earthworms
Exposure to RDX				
Percent Mineralization	0.6	1.2	0.2	0.3
Percent in earthworm tissue (via combustion / LSC)	-	5.5	-	1.1
Soil concentration (Tf)	10.3 ± 0.4*	8.9 ± 0.7	89 ± 4	88 ± 4
Percent Recovery in soil Tf	83 ± 5	89 ± 9	88 ± 8	90 ± 8
Percent Unaccounted	17	4	12	10
Exposure to HMX				
Percent Mineralization	0.4	0.4	0.2	0.2
Percent in earthworm tissue (via combustion / LSC)	-	0.3	-	0.1
Soil concentration (Tf)	9.7 ± 0.1	9.6 ± 0.2	97 ± 5	92 ± 4
Percent Recovery in soil Tf	116 ± 12	115 ± 10	117 ± 8	111 ± 5
Percent Unaccounted	(16)**	(15)	(17)	(11)

* Values are expressed as the average ± standard error. ** Recovery was over 100%.

Mass balance data obtained following exposure of earthworms to [C^{14}]-HMX is presented in Table 24. The mineralization in the control soil (background value) without EM (0.00064 µCi) amounted to 10% of the value observed in the 9 mg HMX kg^{-1} group. The percentage of HMX mineralization was reduced two-fold when HMX concentration in soil was increased from 9 to 83 mg kg^{-1}.

This 2-fold reduction in mineralization following a 10-fold increase in soil concentration was measured previously in the plant experiments. Mineralization in control soils (0.4% at 9 and 0.2% at 83 mg HMX kg^{-1}) was similar to that of soils with earthworms. Mass balance calculations indicated that approximately 0.3% and 0.1% of the added radiolabeled compound was taken up by the earthworms following exposure to 9 and 83 mg

kg^{-1} HMX, respectively. Based on measured soil concentrations, recovery was over 100% at both soil concentrations. Recoveries between 92 – 97% were obtained when calculated against the nominal initial concentrations.

5. DISCUSSION

The bioconcentration potential of chemicals is an important property to consider when assessing potential impacts of soil contamination on ecological and human health mediated by food chain transfer. This study was designed to obtain direct experimental data on bioaccumulation potential of nitramine and nitroaromatic energetic materials in terrestrial plants and to determine whether these EMs pose a potential risk for toxic effects on higher trophic levels. Bioaccumulation potential of nitramine EMs (RDX and HMX) in earthworms was also included in this investigation to assess the potential risk of contaminant transfer in a food chain that contains a soil invertebrate consumer. Experiments were based on exposure of selected plant species to sub-lethal concentrations of RDX, HMX, 2,4-DNT, 2,6-DNT or TNB, and on exposure of earthworm *E. andrei* to sub-lethal concentrations of RDX or HMX. This study included the use of radio-labeled RDX and HMX to track the transformation products of the parent chemical.

5.1. Plant accumulation tests in Sassafras sandy loam soil

Bioaccumulation was assessed in Sassafras sandy loam soil, which has low organic matter and clay contents, low cation exchange capacity, and high sand content. Such characteristics support relatively high bioavailability of energetic contaminants. A preliminary test was carried out with HMX at a low environmentally relevant initial soil concentration (21 mg kg^{-1}) using alfalfa, lettuce and ryegrass. It was found that the plants could accumulate the EM, with resulting BCFs ranging between 23 – 27 (alfalfa) to 26 - 29 (ryegrass). These results are comparable to those reported by Groom *et al*. (2002) who reported accumulation in ryegrass of up to 459 mg kg^{-1} HMX, when the plant was grown for 77 d in a field soil containing 32 mg kg^{-1} of this EM. From their data, the calculated BCF is 14, which is at the same order of magnitude as the values obtained in our preliminary test using a 42 d exposure.

5.1.1. Accumulation of nitroaromatics in plants

Only alfalfa showed measurable tissue levels of TNB following exposure to 67 mg kg^{-1} freshly amended soil (BCF = 0.3). TNB was not detected in plants after weathering/aging of the soil. Bioavailability of TNB is thus reduced if not abolished following the weathering/aging soil process, in parallel to the reduced soil concentrations measured at beginning and end of the plant toxicity assay.

2,4-DNT was not found in plant tissues, following exposure to freshly amended soil. In contrast to the effects just described for TNB, weathering/aging of 2,4-DNT amended soil resulted in a measurable accumulation of the EM into plant tissues. The associated BCFs were 0.15 and 0.44 for alfalfa and ryegrass, respectively. Bioavailability of 2,4-DNT was thus increased following weathering/aging process, but given the low values of the BCFs, bioaccumulation of 2,4-DNT remains negligible.

2,6-DNT was found in measurable amounts in plant tissues, leading to low accumulation in all tested species. In freshly amended soil and in weathered/aged soil, the BCF values for 2,6-DNT ranged between 0.54 – 1.7 for alfalfa, 0.3 - 1.8 for Japanese millet and 0.26 – 0.74 for ryegrass. 2,6-DNT accumulation was not changed by the soil weathering/aging process in contrary to TNB and 2,4-DNT.

In summary, highest BCF values for nitroaromatic EMs were 1.7 for alfalfa exposed to freshly amended 2,6-DNT and 1.8 for Japanese millet exposed to weathered/aged 2,6-DNT. Variations in BCF values amongst plant species suggest species-specific biotransformation dynamics. Although accumulation in plants was very limited in these tests, and can be considered as negligible, concentrations in plant tissues seemed to increase concurrently with the soil concentrations. In general, accumulation in plants is dependent on the persistence of a compound in soil and on its entry/degradation rate in plants. For certain compounds (*e.g.*, TNB, 2,4-DNT and 2,6-DNT), the relatively lower accumulation by plants could result from a smaller pool of EMs in soil since these EMs are easily transformed in soil. Additionally, EMs that are taken-up by plants could undergo further degradation which could explain their low recovery from plant tissue. The combination of these two factors would decrease the ability of these EMs to accumulate in their unchanged form. Additional testing would be needed using radiolabeled chemicals to verify if biotransformation is occurring in the plants. Production of metabolites by plants was not observed by HPLC analysis.

5.1.2. Accumulation of nitramines in plants

The bioaccumulation of nitramine EMs in plants was evaluated using both microcosm and growth chamber studies. Calculated BCFs for RDX and HMX in plants decreased proportionally with soil concentration. In freshly amended soil, BCF values of RDX in alfalfa were 79, 6.8, and 0.27 at nominal concentrations of 100, 1000 and 10000 mg kg^{-1}, respectively. For HMX, these values were 2.2, 0.22 and 0.03 at nominal concentrations of 100, 1000 and 10000 mg kg^{-1}, respectively. From a preliminary test, a BCF value for HMX of 25 was obtained following exposure at 21 mg kg^{-1}. Taken together, this data shows that BCFs decrease in a correlated way as the soil concentrations increase. The data could be described by a logarithmic modelization of tissue concentrations in function of soil concentrations. Additional data obtained at lower soil concentrations will be needed to gain confidence in such a model. It is expected that the relationship between tissue concentrations and soil concentrations could be more linear at lower soil concentrations. The same type of proportional decrease in BCF with increasing soil concentrations is apparent in Japanese millet and in ryegrass. The inverse relationship between BCF values and soil concentrations is due to maximal accumulation of the EM in the tissue even when the EM concentration in soil increases. This relationship may not not apply to all plants and may be typical only of exposure to high concentrations in soil. Data from our laboratory (Dr. Pierre Yves Robidoux, personal communication) support the hypothesis of a saturation level in plants exposed to high soil concentrations of HMX, as it was found that tissue concentrations reached a plateau at 146 mg kg^{-1} in lettuce exposed to concentrations of 1110 and 2250 mg kg^{-1} dry soil for 14-d. Results presented for RDX in the present study are in good agreement with Cataldo *et al.*

(1990) data which indicated a 60-fold bioconcentration in seeds of bush beans exposed to a concentration of 10 mg RDX kg^{-1} soil. Accumulation in leaves was on the order of 22-fold following the 60-d exposure period on the soil exhibiting the highest bioavailability (sandy loam with 0.5% organic carbon). Winfield (2001) also reported a BCF value as high as 382 following exposure of sunflower plants to RDX (embryo stage) for 6 weeks, (cited in Major *et al*., 2002). The study of Checkai and Simini (1996), in which very low concentrations (2 to 100 µg L^{-1}) of RDX in irrigation water were used, also suggested that plants can accumulate nitramines. Following exposure at 100 µg L^{-1}, accumulation in alfalfa shoots was 186 µg kg^{-1} dry weight. Soil concentrations resulting from irrigation were not provided, but could have been very low, thus no BCF could be calculated.

RDX accumulation in plant tissue was significantly greater in weathered/aged soils than in freshly amended soil for alfalfa, Japanese millet and ryegrass. Weathering/aging had no significant effects on HMX amended soils when the three plants were considerd altogether, although accumulation by Japanese millet was significantly increased. Following exposure to freshly amended soil with HMX, no difference in accumulation potential between plant species was measured. No consistent pattern for plant accumulation potential regarding the various EMs was observed. The effect of weathering/aging on RDX was observed at a relatively high soil concentration, additional work is needed to confirm that this could also hapen at low soil concentrations.

It is apparent that accumulation in the plant tissues may be due to factors related to the plant itself or to the bioavailability of the compound itself. For example, transport of HMX would be governed by the transpiration stream as this compound has a sufficiently low K_{ow} value to move through the endodermis and be freely transported into the plant (Groom et al., 2002). This transport is dependent on the solubility of the compound in water, and the soil moisture would be an important factor governing accumulation. The effect of various moisture levels are discussed in the literature (Groom *et al*., 2002 ; Yoon *et al*., 2002 ; and references cited therein). It was noted by Groom *et al*. (2002) that accumulation in plants in the field is much lower than what is obtained in a greenhouse, presumably because of the drier conditions that prevail in the field.

For the purpose of the following discussion, BCF values less than 10 will be indicative of low accumulation potential, values between 10 and 50 will be indicative of a moderate potential, and between 50 and 100 will be indicative of a relatively high accumulation potential. Based on our results, bioaccumulation potential in plants is relatively high for nitramines at lower soil concentrations (25 –29 for HMX at 21 mg kg^{-1} soil ; 45 – 79 for RDX at 87 mg kg^{-1} soil) and generally low for nitroaromatics (< 0.3 for TNB at 67 mg kg^{-1} soil ; ≤ 0.44 for 2,4-DNT at 3.7 – 14.9 mg kg^{-1} soil ; ≤ 1.8 for 2,6-DNT at 4.1 – 14.9 mg kg^{-1} soil). However, the consideration of a single value BCF may not be sufficient to evaluate the risk of transfer of compounds from soil to various species of plants, invertebrates or animals. The concept of BCF developed for exposure in aquatic environment is not easily transposed in a soil exposure context, where a large difference may exist between the actual soil solution concentration and the total soil concentration, and the problems are accentuated when compounds with low water solubility are considered. The non-linear relationship

previously outlined in plants between soil concentrations and tissue concentrations shows that there are some limitations of using a BCF-based approach for ecological risk assessment. By the nature of its calculation (ratio of tissue to soil concentrations), there is a possibility that, in the case of compounds having low aqueous solubility, BCF will always be lower for more contaminated soils creating an erroneous conclusion that risks of food chain transfer are decreasing with increasing soil contamination level. As pointed out for metal-contaminated soils by Van Gestel *et al*. (2002), the use of BCF values (or BSAF; biota to soil accumulation factor) has some drawbacks. First, BSAF values might increase with decreasing soil concentrations and second, these BSAF values cannot give an indication of potential risk. In the case of metals, one solution is to use background levels. In the case of EMs, there is no obvious answer. Toxicity can be evaluated with the help of critical body residue or internal effect concentrations. The possibility of transfer through the food chain could perhaps be estimated by the use of the internal non-lethal tissue concentration determined at the lowest possible soil concentration. As an alternative, the equation describing the variation of BCF in function of soil concentration could be used, keeping in mind the limits for extrapolation to low soil concentrations.

5.1.3. Partitioning of RDX and HMX in corn

The partitioning of RDX and HMX amongst plant compartments was evaluated in corn. After three weeks of exposure, the distribution of radiolabeled RDX and HMX was similar to what is already known for other plants. Bioconcentration factors were relatively low for these two EMs, ranging from 3.6 (roots) to 18 (leaves) in the case of RDX and from 0.5 (roots) to 3.1 (leaves) for HMX. RDX accumulation ranged from 104 mg kg^{-1} in roots to 480 mg kg^{-1} in leaves. Accumulation of HMX was approximately 8 to 10 times lower (on a molar basis) than RDX, with levels ranging from 12 mg kg^{-1} in roots to 79 mg kg^{-1} in leaves. In term of mass balance, for both EMs, the leaves were the major site of accumulation, followed by roots and finally stem. Approximately 63% (RDX) to 56% (HMX) of the total mass of EMs present in the plant was found in leaves. This compartment represented only 26 - 31 % of the total dry biomass which suggests that evapotranspiration is a probable mechanism of accumulation (evapotranspiration occurs mainly in leaves, water flowing through the plant evaporates from leaves, leading to the accumulation of non volatile or non degradable compounds).

The partitioning results presented in this study are in agreement with data presented by Cataldo *et al*. (1990). These authors reported that the relative order of tissue concentration in bush beans was seed > leaves = stem > root. Another study by Larson *et al.* (1999) used RDX in irrigation water at 1 µg ml^{-1}. Accumulation in corn was detected in leaves (22 µg g^{-1}) and in tassels (22 µg g^{-1}) following a 80-d exposure. A recent study conducted by Yoon *et al*. (2002) with poplar tree cuttings showed that HMX is accumulated in plants in a manner very similar to RDX. In this hydroponic system, 70% of the [^{14}C]-HMX was taken up in the leaves, 23.6% in stems and 6.8% in roots following a 65 d exposure. Data from our experiment with corn is not directly comparable as we report HMX concentrations obtained by HPLC, and only a small fraction of total radioactivity was taken

up by the plant. Nevertheless, the data from all studies suggest that leaves are the major site of accumulation for nitramines, and a potential for transfer to a species consuming these leaves exists since approximately 80% of the EM is in the unchanged form, readily extractable with solvents and presumably bioavailable.

A significant part of the radioactivity in the plant ($\geq$ 30% averaged for the whole plant) was found to be unextractable and quantifiable only by total combustion. Analytical determinations of EMs in corn tissue using USEPA Method 8330A accounted for approximately 70 percent of material quantified by using radio-labeled compound. The remaining 30 percent detected only by using labeled EMs were either bound degradation products or CO_2 produced by mineralization and assimilated by the plant. The additional utility of labeled material-based analytical methodology lies in the possibility of identifying unknown metabolites, quantifying the percentage of material integrated in the plant as well as measuring the mineralization of a compound.

5.2. Mass balance studies in plants using radiolabeled RDX and HMX

Mineralization of RDX in soil was stimulated when plants were present, by a factor of 2 in the case of alfalfa and ryegrass. The effect was less striking at nominal soil concentration of 1000 mg kg^{-1}, but still increases of 36 – 68% were noted. This may be due to a direct effect (mineralization by plants) or as a secondary effect of accelerated microbial growth due to plant roots. Additional work should be done to confirm the capacity of plant tissue to degrade RDX, although some authors have demonstrated this possibility (Harvey *et al*., 1991; Van Aken and Schnoor, 2002). There was no obvious difference between mineralization of HMX in the control soils and the soils with plants, at the two concentrations tested, confirming that a great part of the mineralization was carried out by soil bacteria, and that the plant species used in this study had little capacity to transform HMX. In plants, most of the radiolabeled RDX or HMX was not metabolized, total recoveries between 80 % and 95 % were observed for RDX, and from 85 – 100% in the case of HMX. Mass-balance data indicate that plants accumulate less than 3% of the amended RDX, or less that 0.1% of amended HMX, and 21 to 100% and 49 to 100% of the acetonitrile extractable radioactivity was identified by HPLC as RDX and HMX, respectively. This indicates that a high percentage of the plant radioactivity could be bound to the plant residue after acetonitrile extraction. A consistently high percentage of 92 – 94 % (100% in one case) of the radioactivity present in plant extract could be identified as authentic RDX and HMX by HPLC. Only a small fraction of the soluble radioactivity was either CO_2 assimilation products or RDX/HMX derivatives.

Analysis of plants by HPLC consistently gave a lower value compared to radioactivity counting, because of the radioactivity associated with insoluble/bound material. Since this non-extractable radioactivity is not necessarily available for transfer to other trophic levels, it was not accounted for in the calculations of BCFs.

In order to increase the usefulness of acetonitrile-based BCF values for risk assessment, one could consider to use a correction factor based on the acetonitrile/

radiolabeled ratio to adjust the measured BCF. It is known that RDX and HMX are not totally extractable from plant tissue by the USEPA Method 8330A. From our definitive test, an average of 70% of the radioactive RDX was extractable and a correction factor of 1.4 would be obtained. The corresponding factor for HMX (88.5% extractable) would be 1.13.

5.3. Bioaccumulation in earthworms

This work demonstrated that nitramines can accumulate in the earthworm *E. andrei*. Following exposure to freshly amended soil, RDX was moderately accumulated in the earthworm tissues (BCF $\leq$ 12.7). The tissue concentration measured following exposure to 11 mg RDX kg $^{-1}$ soil was 125 mg kg $^{-1}$dry weight and this was only doubled (to 283 mg kg $^{-1}$) when the soil concentration was increased by 9-fold to 99 mg kg $^{-1}$, suggesting a saturation of the accumulation mechanism. Exposure at the two different soil concentrations resulted in significantly different BCF and accumulation values. Following exposure to freshly amended soil, HMX was also detected in earthworm tissues resulting in low values of BCF ($\leq$ 1.0). The tissue concentration measured following exposure to 8.5 mg kg $^{-1}$ soil was 8.5 mg kg $^{-1}$ dry weight tissue. The highest tissue concentration measured was 26 mg kg $^{-1}$ (at measured soil concentration of 83 mg kg^{-1}). Exposure of earthworms at the two different soil concentrations resulted in significantly different tissue levels. Calculated BCFs close to or less than 1, indicate that earthworms do not accumulate HMX (over the concentrations present in the surrounding soil). Acetonitrile extracts prepared from earthworm tissues contained no detectable levels of RDX or HMX.

Few data have been reported on nitramine accumulation in invertebrates. Lotufo *et al*. (2000) reported a BCF of 10 for RDX in a marine invertebrate, *Leptocheirus plumulosus.* In a more recent paper, tissue accumulation of RDX and HMX resulting from exposure to contaminated sediment in benthic invertebrates was presented by Lotufo *et al.* (2001). By using molar-equivalent tissue concentrations, these authors reported concentrations as high as 1,324 µg g $^{-1}$ tissue for RDX and 121 µg g $^{-1}$ tissue for HMX. From a linear regression fit of the data for RDX, a BCF value of 5.7 could be deduced. The authors indicated that HMX or RDX were not detected in extracts prepared from the tissues, probably due to the low levels present. Data on RDX accumulation is available from studies in aquatic species by Bentley *et al*. (1977). BCF values were approximately 5 and 10 in fish muscle and viscera, respectively. From these data, it seems that accumulation in earthworm is quite similar to what is seen in higher animals. Using various exposure routes such as filter paper, OECD artificial soil and forest soil, a non-linear relationship was found between soil concentration and tissue accumulation of RDX (Robidoux *et al.*, in preparation). The resulting maximal BCF value was 3.3 measured at 25 mg kg $^{-1}$ dry soil. The tissue concentrations ranged from 230 to 540 mg kg $^{-1}$ dry weight when exposed to 95 to 1670 mg kg $^{-1}$ dry soil showing the saturation effect as soil concentration increase. In contrast, HMX accumulation was relatively linear with soil concentration, resulting in BCF values of 0.2 to 0.13 at soil concentrations of 46 to 3013 mg kg $^{-1}$ dry soil. A maximal BCF value of 0.3 was measured at 13 mg kg $^{-1}$ dry soil.

Finally, it can be noted that the BCF for HMX was approximately 4 - 5% of the BCF for RDX. This value is close to the ratio of HMX's solubility to RDX's solubility of approximately 11%. Differences in water solubility can only partially explain the differences in accumulation.

5.4. Mass balance studies in earthworms using radiolabeled RDX and HMX

Mineralization data show that relative biotransformation of RDX in soil decreased proportionally as the soil concentration increased, similar to the results of the plant experiments. The presence of earthworms resulted in a 100% increase in mineralization at 10 mg RDX kg^{-1} in soil, but no effect was noted at a concentration of 99 mg RDX kg^{-1} in soil. Mass balance calculations indicated that approximately 5% and 1% of the added radiolabeled compound was taken up by the earthworms following exposure to concentrations of 10 and 99 mg kg^{-1} RDX, respectively. Recovery was similar at all soil concentrations, unaccounted RDX ranging from 4 to 17% and could consist of volatile products.

HMX mineralization was decreased by 2-fold when its concentration in soil was increased from 9 to 83 mg HMX kg^{-1} soil, as seen previously in the plant experiments. The presence of earthworms had no effect on HMX mineralization. Mass balance calculations indicated that approximately 0.34% and 0.10% of the added radiolabeled compound was taken up by the earthworms following exposure to 9 and 83 mg kg^{-1} HMX, respectively. Based on measured soil concentrations, recovery was over 100% at both soil concentrations, indicating a lower degradation rate for this compound as compared to RDX.

No mass balance experiment using earthworms exposed to the EMs tested in this study was found in the literature. As stated before, the ratio of HMX to RDX accumulation in earthworms (7%) is close to the ratio of solubilities of these EMs (11%).

5.5. Recommendations for future bioaccumulation studies

In order to overcome the limitations of BCF calculation, future bioaccumulation studies of nitramines and nitroaromatics in plants may include:

- Studies using lower soil concentrations which are needed to confirm the non-linear relationship between soil concentration and plant tissue levels, to measure if there is a maximal tissue concentration for plants exposed to HMX in soil and to verify how the bioaccumulation potential varies at low soil concentrations, where the highest bioconcentration could occur.

- Studies using radiolabeled nitroaromatic compounds to allow a better evaluation of the distribution and degradation of the compounds in plants and soil.

- Investigation of the usefulness of critical body residues or of internal effect concentrations for evaluating the transfer in the food chain.

- Further work to be carried out in order to verify if a significant portion of the bound radioactivity can be transferred to plant consumers, for example. This type of work has been recently realized by Driver and Fellows (2000) (as summarized in Major *et al.*, 2002) and indicates the possibility of transfer of the soluble RDX fraction in plants. Work with bound material remains to be done.

6. CONCLUSION

Bioaccumulation and mass-balance characteristics of two nitro-heterocyclic energetic materials, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) were investigated using alfalfa, Japanese millet, ryegrass, lettuce and corn, and the earthworm *Eisenia andrei*. Bioaccumulation of TNT by-products, including 1,3,5-trinitrobenzene (TNB), 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) was also investigated. Tests were conducted in Sassafras sandy loam soil which supports relatively high bioavailability of these EMs. The effect of simulated weathering/aging of the soil on the bioaccumulation of these EMs was incorporated in the study.

Results showed that [C^{14}]-RDX and [C^{14}]-HMX were accumulated in the earthworm tissues and in the selected plant species, whereas virtually no accumulation of TNB or of the DNT's was observed in plants. This study conclusively shows the difference in the determined bioaccumulation potentials between nitroamine EMs (characterized by a relatively high potential for plants and a moderate potential for earthworms), and nitroaromatic EMs (characterized by a low potential for plants).

Bioaccumulation values in plants for freshly-amended nitramines was 45 – 79 for RDX (at 87 mg kg^{-1} soil) and decreased at higher soil concentrations to 6.8, and 0.27 at the nominal concentrations of 1000 and 10000 mg RDX kg^{-1}, respectively. For HMX these values were 25 – 29, 2.2, 0.22 and 0.03 at the nominal concentrations of 21, 100, 1000 and 10000 mg kg^{-1}, respectively. For the nitroaromatics values were < 0.3 for TNB at 67 mg kg^{-1} soil; = 0.4 for 2,4-DNT at at 3.7 – 14.9 mg kg^{-1} soil and = 1.8 for 2,6-DNT at 4.1 – 14.9 mg kg^{-1} soil. Bioaccumulation in earthworm from freshly amended soil was moderate for RDX (BCF of 13 and 2.9 at concentrations of 10 and 99 mg kg^{-1} soil) and low for HMX (BCF of 1.0 and 0.32 at concentrations of 9 and 83 mg kg^{-1} soil, respectively).

Weathering/aging of amended soil abolished accumulation of TNB, increased accumulation of RDX and 2,4-DNT, and was almost without effect for 2,6-DNT and HMX accumulation in plants. These effects for the nitroaromatics can be considered as negligible given the extremnely low accumulation found for these compounds. As said earlier, the effects observed for RDX should be verified using lower soil concentrations of this compound.

Our results suggest that some limitations on the use of BCFs for plants may exist in the case of compounds having low water solubility like RDX and HMX. The nature of the simple BCF makes it difficult to correctly express the relationship between soil concentration of a compound and its concentration in the plants growing in the soil. For RDX and HMX, BCFs tend to decrease as soil concentrations increase, reflecting the saturation of tissue levels of these compounds. The use of different BCF values according to the range of soil concentrations could be considered. Alternatively, a fixed BCF value could be replaced by a detailed equation (model) that would better describe the effects governing transfer of a chemical from the soil to the organism. For ERA purposes, more research is

needed to overcome the problems associated with BCFs in cases where a non-linear relationship exists between concentrations in soil and tissue levels in target organisms. The use of a concept derived from the critical body residues could be explored.

Our results support those published recently by various authors that have previously demonstrated accumulation of RDX and HMX in plants and fill a gap that existed in the published earthworm literature.

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APPENDIX F

Genotoxicity of 2,4- and 2,6-dinitrotoluene as measured by the *Tradescantia* micronucleus (Trad-MCN) bioassay. *Mutation Research – Genetic Toxicology and Environmental Mutagenesis*

APPENDIX G

Peer-reviewed Papers Accepted for Publication

in Journal Pedobiologia

Survival and reproduction of *Enchytraeus crypticus* (Oligochaeta, Enchytraeidae) in a natural sandy loam soil amended with the nitro-heterocyclic explosives RDX and HMX.

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Summary- Munition manufacturing, disposal, testing, training and other operations at military sites produced elevated levels of explosives and related materials in soil. The effects of these persistent and highly mobile in the environment energetic materials on soil biota have not been sufficiently investigated. The U.S. Environmental Protection Agency (USEPA) in conjunction with stakeholders, is developing Ecological Soil Screening Level (Eco-SSL) benchmarks for ecological risk assessment (ERA) of contaminants at Superfund sites to identify those contaminants in soil that warrant additional evaluation in a Baseline ERA, and to eliminate those that do not. Eco-SSLs are developed from literature values whenever sufficient quantity and quality of data exist. Insufficient data were available on the toxicity of energetic compounds, RDX and HMX, to soil invertebrates, necessitating toxicity testing. Tests were conducted under conditions preferred for Eco-SSL derivation, using a Sassafras sandy loam soil that supports relatively high bioavailability of test compounds. Toxicity testing was performed using enchytraeid reproduction test (ISO/16387 2001) measuring adult survival and juvenile production by the potworm *Enchytraeus crypticus* in freshly amended soil and weathered/aged amended soil. RDX or HMX had no effect on adult survival in the definitive tests in all treatment concentrations. Juvenile production EC_{20} values were 3,715 and 8,797 mg kg^{-1} RDX in freshly amended and weathered/aged amended soils, respectively. Juvenile *E. crypticus* production was not adversely affected by exposure to HMX in freshly amended and weathered/aged amended soils. Juvenile production was stimulated in freshly amended soil up to 21,750 mg kg^{-1} HMX. Results of these toxicity studies will be submitted to the Eco-SSL Task Group for quality control review, and pending approval will be included in the Eco-SSL database for Eco-SSL derivation.

Key words: explosives, soil, enchytraeid worm, toxicity, hormesis, Eco-SSL.

Introduction

Many sites associated with military operations that involve munition manufacturing, disposal, testing, and training contain elevated levels of explosives and related materials in soil. Concentrations of explosives in soil were reported to exceed 87,000 mg kg^{-1} for TNT and 3,000 mg kg^{-1} for RDX and HMX (Simini et al. 1995). Although these energetic materials are persistent and highly mobile in the environment, their effects on soil biota have not been sufficiently investigated. As a result, no screening values, which could be used in the Ecological Risk Assessment (ERA), are available for explosives in soil. Scientifically based ecological soil screening levels (Eco-SSLs) are needed to identify explosive contaminant levels in soil that present an acceptable ecological risk. To address this problem, the U.S. Environmental Protection Agency (USEPA), in conjunction with stakeholders, is developing Eco-SSL benchmarks for contaminants most frequently found at Superfund sites. Eco-SSLs are defined as concentrations of chemicals in soil that, when not exceeded, will be protective of terrestrial ecosystems from unacceptable harmful effects. These concentrations can be used in a screening level ERA to identify those contaminants in soil that warrant additional evaluation in a Baseline ERA, and to eliminate those that do not. Eco-SSLs are derived using published data generated from laboratory toxicity tests with different test species relevant to soil ecosystems. The Eco-SSL workgroup, after an extensive literature review (USEPA 2000), determined that there was insufficient information for explosives to generate Eco-SSLs for soil invertebrates. The purpose of the present study was to fill this knowledge gap.

Materials and methods

Soil exposures and chemical analyses

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX; CAS: 121-82-4; 99%) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX; CAS: 2691-41-0; 99%) were obtained from the Defense Research Establishment Valcartier of the Canadian Ministry of National Defense (Val Bélair, QC, Canada). The positive controls used in these tests were 50 and 47 mg kg^{-1} Be ($BeSO_4{*}4H_2O$; CAS #7787; 99.99%) in freshly amended and weathered/aged soils, respectively. A natural soil Sassafras sandy loam [Fine-loamy, siliceous, mesic Typic Hapludult] (SSL; 69% sand; 13% silt; 17% clay; 1.2% organic matter; 5.2 pH; 5.5 cmol kg^{-1} Cation Exchange Capacity; 18% Water Holding Capacity, WHC) was used to assess RDX or HMX toxicity. SSL soil was selected because it has physical and chemical characteristics that support relatively high bioavailability of RDX and HMX (e.g., low organic matter, and clay contents). The soil was collected from an uncontaminated open grassland on the property of the U.S. Army Aberdeen Proving Ground, Edgewood, MD.

Treatment concentrations for definitive toxicity tests were prepared by adding test chemicals into an aliquot of soil, using an organic solvent acetone as a carrier. The carrier solvent was allowed to volatilize, and the aliquot containing RDX or HMX was mixed with appropriate amount of clean SSL soil to achieve nominal target concentrations. Carrier controls were treated with the carrier solvent only. The soil was mixed for 18 hours on a three-dimensional rotary mixer. Analytical concentrations of RDX or HMX were determined by high-performance liquid chromatography following USEPA Method 8330 (USEPA 1998). Water extractable portion of RDX and HMX was determined using Adapted Toxicity Characteristic

Leaching Procedure (ATCLP) described in Haley et al. (1993). The modification involved substitution of acetic acid extraction by CO_2-saturated water, better simulating soil-water conditions. All analytical measurements were done in triplicate at the beginning of each test. Nominal and measured concentrations used in the definitive tests are shown in Table 1.

Special consideration was given to the effects of weathering and aging of explosives in soil on the "real world" exposure of soil invertebrates as occurs at contaminated sites. Weathering/aging of chemicals in soil may reduce exposure of soil invertebrates to RDX or HMX due to photodecomposition, hydrolysis, reaction with organic matter, sorption, precipitation, immobilization, occlusion, microbial transformation and other fate processes. We used the weathering and aging procedure to simulate more closely the exposure effects on soil invertebrates in the field. This included exposing both treated and control soils, initially hydrated to 60 percent of the WHC, in open Teflon®-coated chemically inert containers in the greenhouse to alternating wetting and drying cycles for three months. All soil treatments were weighed and readjusted to their initial mass by adding ASTM type I water (American Society of Testing and Materials, http://www.astm.org) water twice each week. The effect of weathering/aging on RDX or HMX ecotoxicity was determined by comparing test results in weathered/aged and freshly amended soils.

Effects of exposure to RDX or HMX were assessed using the enchytraeid reproduction test (ISO/16387 2001), which measures adult survival and juvenile production by the potworm *Enchytraeus crypticus*. Test species came from culture maintained at the U.S. Army Edgewood Chemical Biological Center. All soil treatments hydrated to 100 percent of the WHC were allowed a 24-hour equilibration period before testing. Measurement endpoints were assessed using 6-8 treatment concentrations and four replicates per treatment. A limit test was conducted for weathered/aged HMX amended soil using treatment concentration 17,498 mg kg^{-1} and eight replicates for treatment and carrier control. Measurement endpoints in all tests included number of surviving adults after 14 days and number of juveniles produced after 28 days.

Data Analyses

Juvenile production data were analyzed using nonlinear regression models described in Stephenson et al. (2000) and Kuperman et al. (2003). Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to decide whether or not to weight the data, and to select potential models. The Gompertz model $Y = a \times e^{([\log(1-p)]\times[C/\mathrm{ECp}]\wedge b)}$ had the best fit for data (Fig. 1). The estimates of effect concentration (ECp parameters) for a specified percent effect used in this study included the explosive material concentration producing a 20% (EC_{20}) or 50% (EC_{50}) reduction in the measurement endpoint. The EC_{20} parameter based on a reproduction endpoint is the preferred parameter for deriving soil invertebrate Eco-SSL values. The EC_{50}, more commonly used in the past, and adult survival data were included to enable comparisons of the results produced in this study with results reported by other researchers. The 95% confidence intervals (CI) associated with the point estimates were determined.

Analysis of Variance (ANOVA) was used to determine the bounded No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values for adult survival or juvenile production data. Mean separations were done using Fisher's Least Significant Difference (LSD) pairwise comparison tests or Student's *t*-Test in the limit test. A significance level of $p < 0.05$ was accepted for determining the NOEC and LOEC values. When

NOAEC (no observed adverse effect concentration) or LOAEC (lowest observed adverse effect concentration) values were determined, the same statistical methods were used. All analyses were done using measured RDX, or HMX concentrations. Statistical analyses were performed using SYSTAT 7.0.1 (SPSS 1997).

Results

Measured RDX total (acetonitrile extractable) concentrations in freshly amended soils averaged 101 (range: 92-109) percent of nominal concentrations. Measured RDX ATCLP extractable concentrations ranged from 76 to 123 mg kg^{-1} and averaged 9.6% of acetonitrile-extractable concentrations due to low solubility of RDX in water (Table 1). Weathering/aging of amended soils reduced total RDX concentrations on average by 7% compared with total concentrations in freshly amended soils. Measured HMX total concentrations in freshly amended soils averaged 111 (range: 88-124) percent of nominal concentrations. Measured HMX ATCLP extractable concentrations remained relatively stable and averaged one (range: 0.5-8.0) percent of total concentrations. Weathering/aging of HMX amended soil reduced total HMX concentration by 20 percent in the single treatment used in the limit test.

Toxicity test results complied with the validity criteria for negative controls defined in the ISO test guidelines. Adult *E. crypticus* survival was not affected in the definitive tests in all RDX or HMX concentrations. Concentration-response relationships for juvenile production in freshly amended and weathered/aged RDX amended soils determined by nonlinear regressions are shown in Fig. 1. Overall, reproduction was higher in weathered/aged RDX amended soils. Juvenile production bounded NOEC and LOEC values were, respectively 1,194 and 2,203 mg kg^{-1} in freshly amended soil, and 2,379 and 3,985 mg kg^{-1} in weathered/aged soil (Table 2). Juvenile production EC_{20} values were 3,715 and 8,797 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. The difference between these values was not statistically significant based on 95% confidence intervals (Table 2). Juvenile production EC_{50} values were 51,413 and 142,356 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. The highest RDX concentration of 21,383 mg kg^{-1} used in the test with freshly amended soil, and 18,347 mg kg^{-1} used in the test with weathered/aged soil resulted only in 31% and 28% reduction in the number of juveniles produced, respectively, compared to carrier control. For that reason, nonlinear regression model estimated large range for 95% CI in determining both EC_{50} parameters (Table 2) indicating high uncertainty in these point estimates.

Juvenile *E. crypticus* production was stimulated at higher HMX exposure concentrations in freshly amended soil (Fig. 2). The increase was statistically significant ($p < 0.05$) at 2,211 mg kg^{-1}, and higher concentrations producing a bounded NOEC ($p = 0.109$) at 1,491 mg kg^{-1} and unbounded No Observed Adverse Effect Concentration (NOAEC) at 21,750 mg kg^{-1} (Table 2). Limit test showed that exposure of *E. crypticus* in weathered/aged HMX amended soil did not affect reproduction producing an unbounded NOEC ($p = 0.186$) of 17,498 mg kg^{-1}.

Discussion

Nitro-heterocyclic explosives RDX or HMX did not affect adult *E. crypticus* survival even at concentrations as high as 21,383 and 21,750 mg kg^{-1}, respectively. Juvenile production was affected by RDX but the toxicity was relatively low with EC_{20} estimates of 3,715 and 8,797 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively. Weathering and aging

of RDX amended soil did not significantly affect its toxicity to *E. crypticus*. Literature on the toxicity of RDX to terrestrial organisms is scant, and discrepancies are often found regarding the toxicity of the same chemical to different organisms. Significant sublethal effects of RDX were observed on the reproduction of earthworm *Eisenia andrei* at concentrations as low as 95 mg kg^{-1} soil (Robidoux et al. 2000). However, no effects were found on the mortality and reproduction of two terrestrial invertebrates *E. crypticus* and *Folsomia candida* in soils spiked with up to 1000 mg kg^{-1} RDX in soil (Schafer & Achazi 1999).

Exposure of *E. crypticus* to HMX in freshly amended SSL soil produced a significant stimulating effect on juvenile production (11-56% increase), which disappeared in weathered/aged soil. Stevens et al. (2002) reported a similar stimulating effect of HMX on growth of the midge *Chironomus tentans*. Hormetic responses were reported in explosives exposure studies for microbial nitrogen fixation activity at soil TNT concentrations of 200 and 400 mg kg^{-1} (Gong et al. 1999), offspring production by *Daphnia magna* exposed to 0.08 mg L^{-1} TNT (Bailey et al. 1985), egg production per female fathead minnow exposed to 6.3 mg L^{-1} RDX (Bentley et al. 1977), and density of *Selanastrum capricornutum* cells, based on total chlorophyll measures following HMX exposure ranging 36-572 mg L^{-1} (Bentley et al. 1984). To date, no studies have investigated the mechanisms responsible for hormetic effects of these explosives. Stevens et al. (2002) suggested that these mechanisms could include a direct effect on test organisms through the release of metabolic products of explosives that may have a specific effect on growth and reproduction, and indirect effects through increased supply of nitrogen for bacteria, fungi, or algae (an important food source for higher trophic levels) from the mineralization of explosives.

In this study, the relatively low RDX toxicity and the absence of HMX toxicity to *E. crypticus* in SSL soil at concentrations tested can be related to low bioavailability of these energetic materials in soil. The solubility in water at 20°C of RDX and HMX is 42.3 and 6.63 mg L^{-1}, respectively (Roberts & Hartley 1992). These low solubility levels in water contribute to low bioavailability of RDX and HMX in soil. Considering *E. crypticus* exposure to RDX and HMX in soil on the ATCLP basis provides an explanation, at least partially, for the observed effects of these nitro-heterocyclic explosives. The better understanding of the reasons for low toxicity of RDX to *E. crypticus*, and elucidation of mechanisms of a stimulating response to HMX exposure, will require additional research.

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1,3,5,7-tetrazocine in sediments to *Chironomus tentans* and *Hyalella azteca*: low-dose hormesis and high-dose mortality. Environmental Toxicology and Chemistry 21, 1475-1482.

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Table 1. Nominal and measured RDX and HMX concentrations (mg kg^{-1}) in Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Total (acetonitrile extractable) concentration values were determined using USEPA Method 8330. Water extractable concentration values were determined using Adapted Toxicity Characteristic Leaching Procedure (ATCLP).

RDX						HMX					
Freshly Amended			Weathered and Aged			Freshly Amended			Weathered and Aged		
Nominal	Total	ATCLP	Nominal	Total	ATCLP	Nominal	Total	ATCLP	Nominal	Total	ATCLP
0	ND	ND	0	ND	ND	0	ND	ND	0	ND	ND
300	304	102.4	1200	1048	83.5	300	348	12.3	20000	17498	18.1
600	656	95.3	2400	2379	86.8	600	642	12.5			
1200	1194	114.8	4800	3985	86.0	1200	1491	12.9			
2400	2203	114.7	10000	9549	89.0	2500	2211	12.6			
4800	4558	122.9	20000	18347	89.2	5000	5785	12.5			
10000	10062	75.9				10000	10586	12.0			
20000	21383	107.5				20000	21750	12.6			

Table notes:
ND- not detected. Method Detection Limit, MDL = 0.05 mg L^{-1}

Table 2. Ecotoxicological parameters (mg kg^{-1}) for RDX and HMX determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using enchytraeid reproduction test with *E. crypticus*.

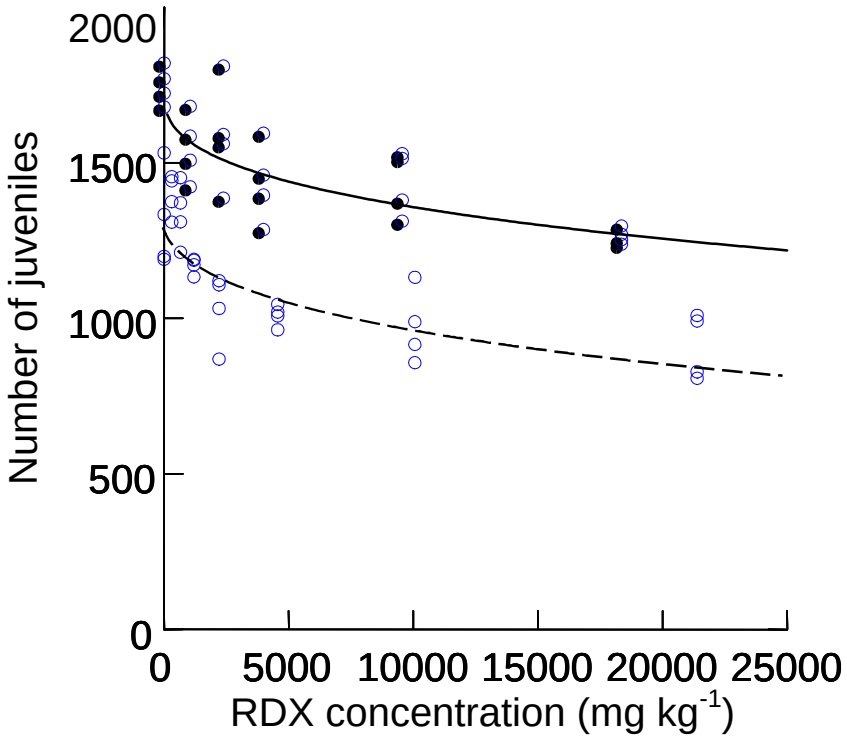
Energetic Material	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
RDX						
Fresh	21,383	>21,383	1,194	2,203	3,715	51,413
p or 95% C.I.			0.055	0.001	0-8,100	6,336-96,491
Weathered/aged	18,347	>18,347	2,379	3,985	8,797	142,356
p or 95% C.I.			0.056	0.001	761-16,834	0-373,753
HMX						
Fresh	21,750	>21,750	21,750*	>21,750*	ND	ND
Weathered/aged	17,498	>17,498	17,498	>17,498	LT	LT

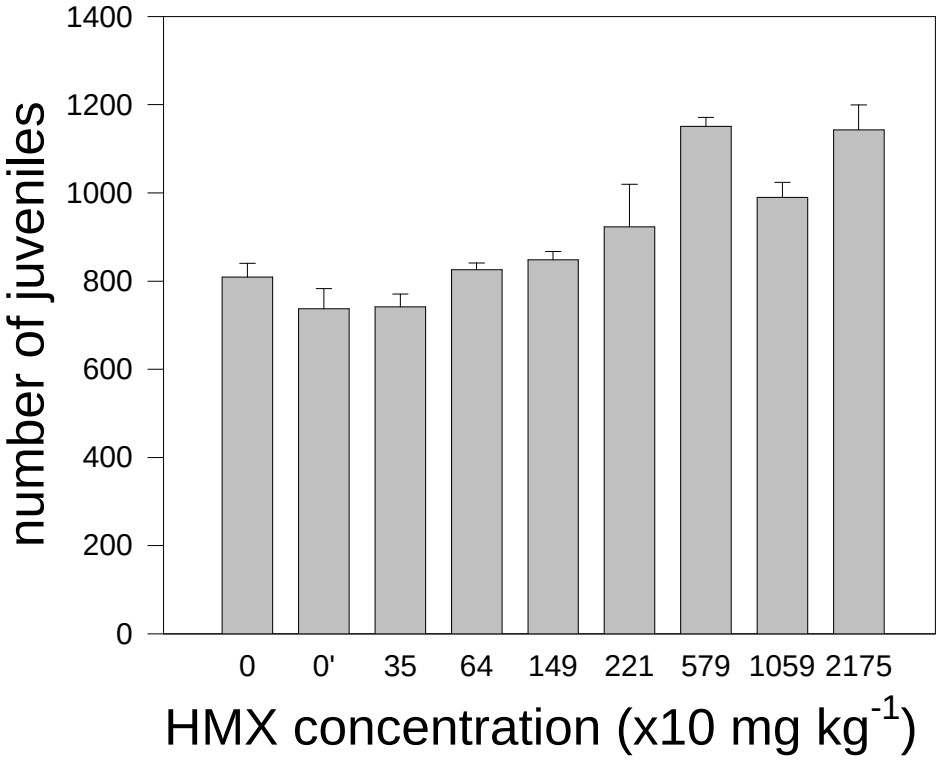
Notes:
* Value represents a No/Low Observed Adverse Effect Concentration (NOAEC/LOAEC) due to a significant ($p \leq 0.01$) increase in juvenile numbers in treatments above 2,211 mg kg^{-1}, compared with carrier control.
ND, Not Determined. ECp values could not be determined because juvenile numbers were higher in all treatment concentrations compared with carrier control.
LT, Limit Test is based on data comparison between carrier control and one treatment concentration of 17,498 mg kg^{-1}.

Figure legends

1. RDX effect on juvenile production by *Enchytraeus crypticus* in freshly amended (dashed line, open circle data points) and weathered/aged amended (solid line, filled circle data points) Sassafras sandy loam soil. Concentration-response relationships were determined using nonlinear regression model $Y = a \times e^{([\log(1-p)]\times[C/\mathrm{ECp}]\wedge b)}$. All concentrations are based on acetonitrile extraction using USEPA Method 8330.

2. HMX effect (mean and S.E.) on juvenile production by *Enchytraeus crypticus* in freshly amended Sassafras sandy loam soil. Controls shown are negative (0) and carrier (0'). All concentrations are based on acetonitrile extraction using USEPA Method 8330.


2000
1500
1000
500
0
Number of juveniles
0
5000
10000
15000
20000
25000
RDX concentration (mg kg-1)


1400
1200
1000
800
600
400
200
0
number of juveniles
0
0'
35
64
149
221
579
1059
2175
HMX concentration (x10 mg kg-1)

Reproduction and survival of *Eisenia fetida* in a sandy loam soil amended with the nitro-heterocyclic explosives RDX and HMX.

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Summary- Munitions manufacturing, disposal, testing, training and other operations at military sites have produced elevated levels of explosives and related materials in soil. Insufficient data were available on the toxicity of the explosives, RDX and HMX to soil invertebrates, necessitating toxicity testing. The U.S. Environmental Protection Agency (USEPA) in conjunction with stakeholders, is developing Ecological Soil Screening Level (Eco-SSL) benchmarks for ecological risk assessment (ERA) of contaminants at Superfund sites to identify those contaminants in soil that warrant additional evaluation in a baseline ERA, and to eliminate those that do not. Eco-SSLs are developed from literature values whenever sufficient quantity and quality of data exist. Tests were conducted under conditions preferred for Eco-SSL derivation, using a sandy loam soil that supports relatively high bioavailability of test compounds. Earthworm (*Eisenia fetida*) toxicity was assessed using a standardized earthworm reproduction test in freshly amended soil and weathered/aged amended soil. RDX or HMX had no effect on adult survival. Cocoon production EC_{20} values for RDX were 1.2 and 19 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. Juvenile production EC_{20} values were 1.6 and 0.8 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. Cocoon production and juvenile production EC_{20} values for HMX were 2.7 and 0.4 mg kg^{-1} in freshly amended soil. Both cocoon production and juvenile production in weathered/aged HMX treated soils were not significantly different ($p < 0.05$) from control soils. Results of these toxicity studies will be submitted to the Eco-SSL Task Group and will be included in the Eco-SSL database for Eco-SSL derivation.

Key words: explosives, soil, earthworm, toxicity, Eco-SSL.

Introduction

Many military sites that involve munitions manufacturing, disposal, testing, and training contain elevated levels of explosives and related materials in soil. Concentrations of explosives in soil were reported to exceed 87,000 mg kg^{-1} for TNT and 3,000 mg kg^{-1} for cyclotrimethylenetrinitramine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Simini et al., 1995). Although these energetic materials are persistent and highly mobile in the environment, their effects on soil biota have not been sufficiently investigated. As a result, screening values are not available for Ecological Risk Assessment (ERA) of explosives in soil. Scientifically based ecological soil screening level (Eco-SSL) benchmarks are needed to identify explosive contaminant levels that present an acceptable ecological risk. To address this problem, the U.S. Environmental Protection Agency (USEPA) in conjunction with stakeholders, is developing Eco-SSL benchmarks for contaminants most frequently found at Superfund sites. Eco-SSL benchmarks are defined as concentrations of chemicals in soil that, when not exceeded, will be protective of terrestrial ecosystems from unacceptable harmful effects. These benchmarks can be used in a screening level ERA to identify those contaminants in soil that warrant additional evaluation in a baseline ERA, and to eliminate those that do not. Eco-SSLs are derived using published data generated from laboratory toxicity tests with different test species relevant to soil ecosystems. The Eco-SSL workgroup, after an extensive literature review (USEPA 2000), determined that there was insufficient information for explosives to generate Eco-SSL benchmarks for soil invertebrates. The objective of this study was to determine benchmark levels of RDX and HMX for the earthworm, *Eisenia fetida* in a natural sandy loam soil that could be used to develop an Eco-SSL benchmark for soil invertebrates.

Materials and Methods

Soil Exposures and Chemical Analysis

Sassafras sandy loam (SSL) [Fine-loamy, siliceous, mesic Typic Hapludult] (69% sand; 13% silt; 17% clay; 1.2 % organic matter; 5.2 pH; 5.5 cmol kg^{-1} cation exchange capacity; 18% Water Holding Capacity, WHC) was selected for this study because it has physical and chemical characteristics (e.g., low organic matter, and clay content) that maintain relatively high bioavailability of RDX and HMX. The soil was collected from an uncontaminated open grassland on the property of Aberdeen Proving Ground, Edgewood, MD. Vegetation and surface organic matter were removed and the top 15 cm of the A horizon was collected and sieved successively through 5-mm^2 and 2-mm^2 screens and air-dried.

Crystalline RDX (CAS:121-82-4, 99.9% purity) and HMX (CAS: 2691-41-0, 99.9% purity) were obtained from the Defense Research Establishment Valcartier of the Canadian Ministry of National Defense (Val Bélair, QC, Canada). Beryllium ($BeSO_4{*}4H_2O$; CAS #7787; 99.99%) was used as the positive control. Glassware was washed with phosphate-free detergent followed by rinses with 0.1N nitric acid, deionized (ASTM type I) water, and acetone. Purified water (Millipore PF) was used throughout the study.

Chemicals were dissolved in acetone and pipetted onto a 2.5 cm thick “soil pancake” to make a soil concentrate. The acetone was volatilized in a fume hood overnight. Treatment concentrations for all tests were prepared by mixing soil concentrate of either RDX or HMX with clean soil. Carrier controls were treated with acetone only. Soil was mixed for 18 hours on a three-dimensional rotary mixer, and hydrated to 95% of the WHC (17.1% dry weight). Range-

finding tests were conducted with freshly amended soils to determine treatment concentrations for definitive tests. Both total and water extractable RDX and HMX were determined in soil. A small amount of soil was extracted after 24 h hydration to 95% WHC and prior to addition of earthworms. Total extractable RDX or HMX in soil were determined using U.S. EPA Method 8330 (USEPA 1998). This procedure involves acetonitrile extraction followed by 18 h sonication. The water extractable portion of RDX and HMX in soils was determined using Adapted Toxicity Characteristic Leaching Procedure (ATCLP) described in Haley et al. (1993). The adaptation involved substitution of acetic acid with CO_2-saturated water, better simulating soil-water conditions. RDX and HMX in soil were determined by high-performance liquid chromatography. Nominal and determined (measured) concentrations used in the definitive tests are shown in Table 1. The 1.5 and 3 mg kg^{-1} RDX and HMX soils were used for the freshly amended toxicity tests only. Soils amended with 300 and 600 mg kg^{-1} were weathered and aged and used in the final toxicity tests. This was done to account for any loss in bioavilability of the compounds during the weathering/aging process.

Toxicity and soil chemical concentrations were conducted in freshly amended soils and those subjected to a simulated weathering/aging process. The simulated weathering/aging process was performed to more closely mimick "real world" conditions. To simulate this process, all soils were initially hydrated to 60 percent of the WHC (10.8% dry weight) in open, Teflon®-coated containers in a greenhouse and alternately wetted and dried for three months. Soils were weighed twice each week to attain their initial mass. All soils were re-hydrated to 95% percent of the WHC prior to initiation of the definitive tests. Soil (800 g) was divided equally and placed in four containers per treatment level.

Toxicity Assays

Earthworms (*E. fetida*) were bred and maintained in the laboratory (ISO, 1998). Cultures were synchronized so that all worms were approximately the same age. The earthworm assay used in this study was a 56-day reproduction test (ISO 1998). Adult worms, 0.3 g to 0.6 g, with fully developed clitella were randomly selected and placed in 550 cc containers. Two grams of fermented, dried, ground alfalfa pellets were placed in each jar as worm food. Plastic wrap was stretched over the top of the containers and secured with a screw top. Three holes were made in the plastic wrap to facilitate air exchange.

All containers were placed in an environment-controlled incubator @ 22 $\pm$ 1°C, 16 h light: 8h dark photoperiod. Adults were removed and counted after 28 days. Two grams of worm food were placed in each container and the containers were sealed. Juveniles and cocoons were removed and counted after 56 days.

Data Analysis

Cocoon and juvenile production data were analyzed using nonlinear regression models (Stephenson et al. 2000; Kuperman et al. 2003). The exponential model $Y = a \times e^{([\log(1-p)]\times[C/ECp])}$ had the best fit for data (Fig. 1). The estimates of percent effect concentration (ECx parameters) used in this study were explosive material concentration producing a 20% (EC_{20}) or 50% (EC_{50}) reduction in the measurement endpoint. The EC_{20} is the preferred parameter for deriving soil invertebrate Eco-SSL benchmarks. The EC_{50}, more commonly used in the past, and survival

data were included to compare results from this study with results reported by other researchers. The 95% confidence intervals (CI) associated with the point estimates were determined.

Analysis of Variance (ANOVA) was used to determine the bounded No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values for adult survival, cocoon production or juvenile production. Mean separations ($p < 0.05$) were determined using Fisher's Least Significant Difference (LSD) pairwise comparison tests. All analyses were done using measured RDX or HMX concentrations. Statistical analyses were performed using SYSTAT 7.0.1 (SPSS, 1997).

Results

Adult *E. fetida* survival was not affected by RDX or HMX at all tested concentrations (data not shown). Concentration-response relationships for cocoon production in fresh and weathered/aged RDX amended soils are shown in Fig. 1. Overall, reproduction was higher in weathered/aged RDX amended soils. Cocoon production EC_{20} values were 1.2 and 19.2 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively (Table 2). Juvenile production EC_{20} values were 1.6 and 4.8 mg kg^{-1} in freshly amended soils and weathered/aged soils, respectively. Cocoon production EC_{50} values were 3.7 and 59.6 mg kg^{-1} in freshly amended soil and in weathered/aged soil, respectively (Table 2). Juvenile production EC_{50} values were 5.0 and 14.9 mg kg^{-1} in freshly amended soils and weathered/aged soils, respectively. The differences between freshly amended and weathered/aged cocoon and juvenile production were not statistically significant (95% CI; Table 2).

Concentration-response relationships for cocoon production in fresh and weathered/aged HMX amended soils are shown in Fig. 2. Cocoon production EC_{20} and EC_{50} values were 2.7 and 8.5 mg kg^{-1} in freshly amended soils (Table 2). Juvenile production EC_{20} and EC_{50} values were 0.4 and 1.2 mg kg^{-1} in freshly amended soils, respectively. The difference between these values was not statistically significant based on 95% confidence intervals (Table 2). In weathered/aged soils, reproduction was not significantly ($p>0.05$) reduced by HMX. Water extractable RDX and HMX EC_p (95% CI) and means comparisons ($p>0.05$) were not significantly different than total extractable RDX and HMX toxicity parameters.

Discussion

Nitro-heterocyclic explosives RDX and HMX in freshly amended soils were highly toxic to *E. fetida* reproduction but non-lethal to adults in this study. Literature on the toxicity of RDX to terrestrial organisms is scant, and discrepancies are often found regarding the toxicity of the same chemical to different organisms. Significant effects of RDX on the reproduction of the earthworm *Eisenia andrei* were observed at concentrations as low as 95 mg kg^{-1} in artificial soil (Robidoux et al., 2000). However, mortality and reproduction of two terrestrial invertebrates, *Enchytraeus albidis* and *Folsomia candida* in soils spiked with up to 1000 mg kg^{-1} RDX in artificial soil (Schafer & Achazi, 1999) were not affected. These studies were conducted in artificial soil, which limits their usefulness for describing natural systems. Greater toxicity (lower EC_{20} and EC_{50} values) in our study may be due to greater bioavailability in the natural soil. The bioavailability of nonpolar organic chemicals in soil is hypothesized to be determined primarily by soil organic matter (OM) content (Belfroid et al. 1996). Sassafras sandy loam has 1.2% OM compared to 10% in artificial soil. Belfroid et al. (1996) also suggest that bioaccumulation and toxicity are well correlated with the concentration of chemical in the soil solution or pore water,

rather than total chemical levels. In the present study, total extractable and water extractable RDX and HMX showed no difference in correlation to toxicity.

In this study, both cocoon and juvenile production were reduced at relatively low levels of RDX and HMX in freshly amended soils (Table 2). However, some cocoons were still found at 148 mg kg^{-1} in the definitive tests, and at 5000 mg kg^{-1} in the range-finding tests. This may be due to low bioavailability of these energetic materials in soil. The solubility in water at 20°C of RDX and HMX is 42.3 and 6.63 mg L^{-1}, respectively (Roberts & Hartley 1992). The ATCLP extractable (and presumably bioavailable) fractions ranged from 100 to 16 percent of acetonitrile extractable concentration for RDX, and from >100 to 3 percent of acetonitrile extractable concentration for HMX (Table 1).

Weathering and aging of RDX amended soil for 90 days did not reduce RDX concentrations (Table 1) or significantly affect its toxicity to *E. fetida* (Table 2). Weathering and aging soil for 90 days rendered HMX non-toxic to earthworm reproduction even though the soil concentration was not reduced (Tables 1 and 2). Further study is needed to elucidate the mechanisms responsible for reduced HMX toxicity in weathered/aged soils.

Acknowledgments
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Table 1. Nominal and measured (total and ATCLP) RDX and HMX concentrations (mg kg^{-1}) in Sassafras sandy loam soil used in the toxicity tests with *E. fetida*. Total (acetonitrile extractable) concentration values were determined using U.S. EPA Method 8330 (USEPA, 1998). Water extractable concentration values were determined using Adapted Toxicity Characteristic Leaching Procedure (ATCLP). Total and ATCLP concentrations are the mean of three replicate measurements. BDL = below detection limit of 0.05 mg L^{-1} in solution.

RDX						HMX					
Freshly Amended			Weathered and Aged			Freshly Amended			Weathered and Aged		
Nominal	Total	ATCLP	Nominal	Total	ATCLP	Nominal	Total	ATCLP	Nominal	Total	ATCLP
0	BDL	BDL	0	BDL	BDL	0	BDL	BDL	0	BDL	BDL
1.5	3.2	2.1	6	6.4	5.8	1.5	1.3	0.5	6	1.6	2.9
3	5.3	2.3	9	8.4	7.7	3	2.9	1.9	9	2.8	4.4
6	7.5	5.2	18	15.7	13.6	6	6.5	2.8	18	10.8	9.1
9	8.6	7.2	36	30.0	30.0	9	11.2	5.9	36	28.9	13.1
18	18.2	15.6	72	56.6	54.1	18	15.6	11.2	72	53.5	14.6
36	33.1	30.2	144	61.5	55.1	36	36.0	15.2	144	129.3	16.4
72	74.1	56.7	300	254.3	100.1	72	73.6	13.1	300	280.3	19.0
144	148.3	93.5	600	527.0	93.2	144	141.3	12.5	600	561.7	18.0

Table 2. Ecotoxicological parameters (mg kg^{-1}) for RDX and HMX determined in freshly amended and weathered/aged Sassafras sandy loam soil using earthworm reproduction test with *E. fetida* (ISO, 1998).

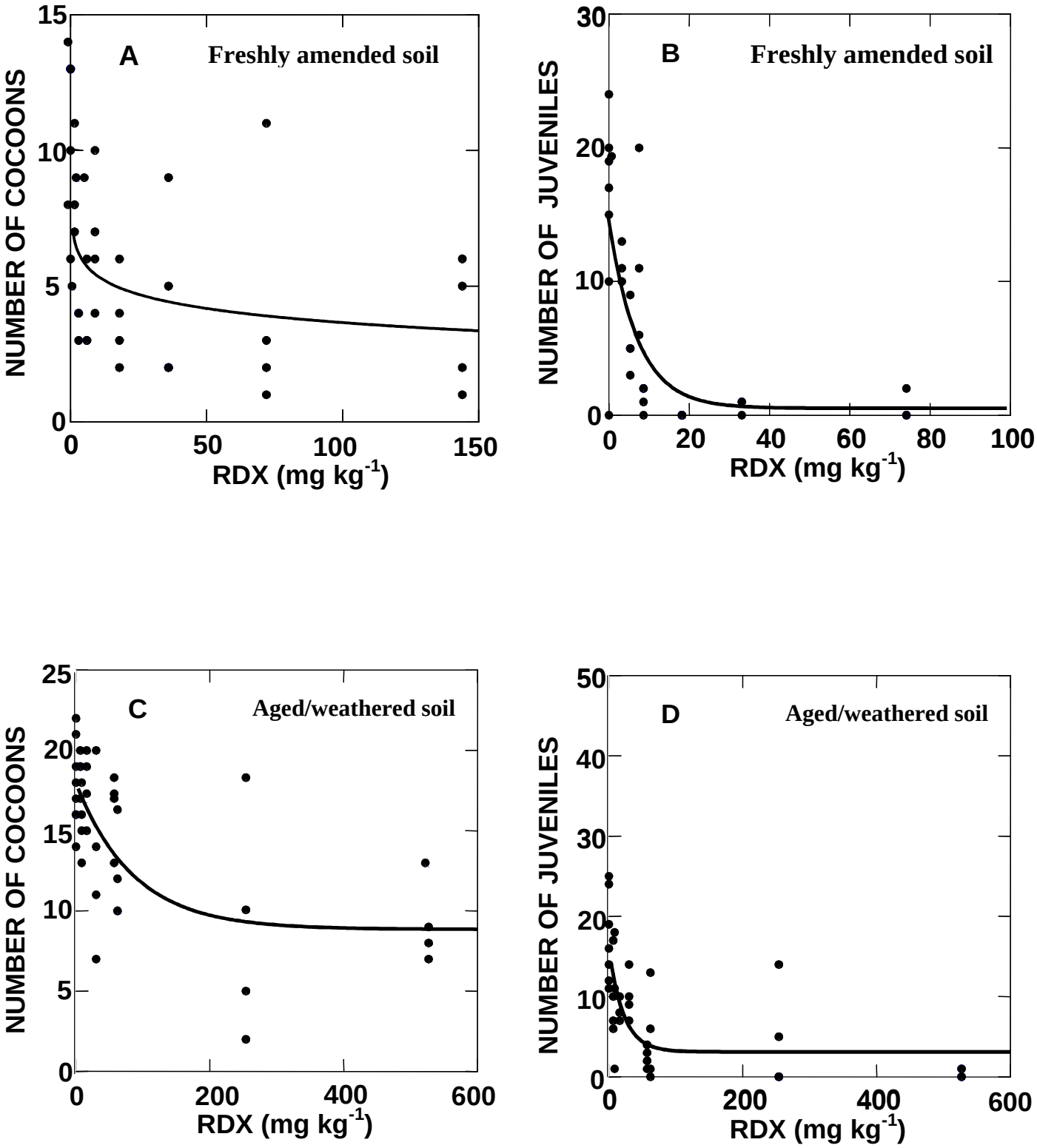
Energetic Material	Cocoon Production				Juvenile Production			
	NOEC	LOEC	EC_{20}	EC_{50}	NOEC	LOEC	EC_{20}	EC_{50}
RDX								
Fresh	8.6	18.2	1.2	3.7	7.5	8.6	1.6	5.0
P or 95% C.I.	0.06	0.001	0.4-2.0	1.2-6.2	0.31	0.001	0.4-2.7	1.4-8.5
Aged/weathered	56.6	61.5	19.2	59.6	8.4	15.7	4.8	14.9
P or 95% C.I.	0.45	0.01	0-39	0-120	0.06	0.02	0.2-9	0.7-29
HMX								
Fresh	15.6	36.0	2.7	8.5	6.5	11.2	0.4	1.2
P or 95% C.I.	0.16	0.007	0-7.0	0-22	0.095	0.016	0-0.9	0-2.8
Aged/weathered	562	>562	ND	ND	562	>562	ND	ND

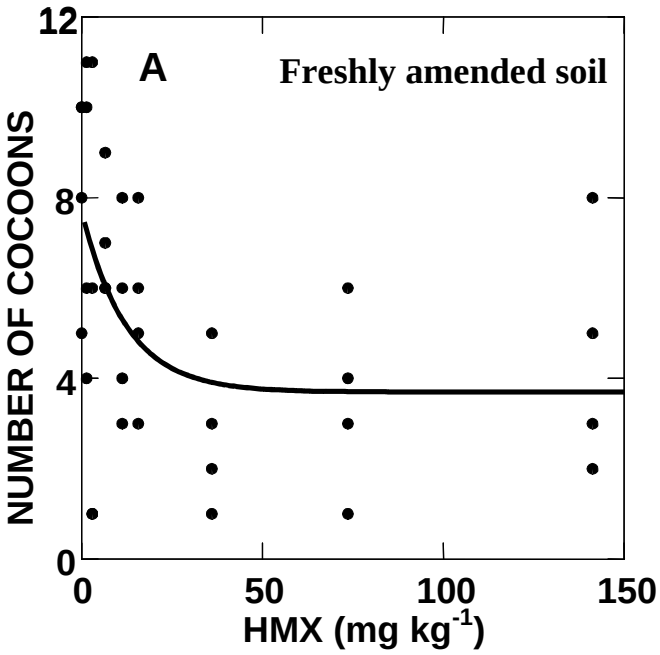
Notes:
ND, Not Determined. ECx values could not be determined because cocoon and juvenile numbers were not significantly ($p>0.05$) different in all treatment concentrations compared with carrier control.

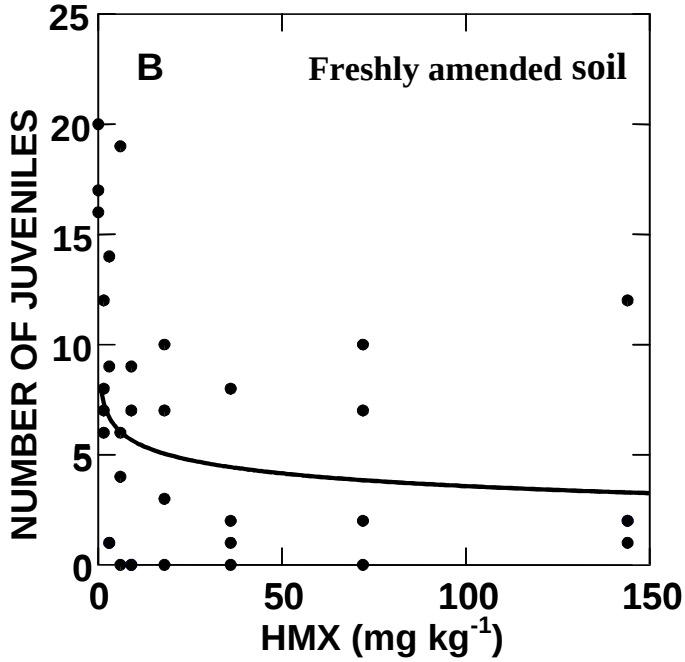
Figure legends

Figure 1. RDX effect on cocoon production and juvenile production by *Eisenia fetida* in freshly amended (A, B) and weathered/aged amended (C, D) Sassafras sandy loam soil. Concentration-response relationships were determined using exponential model $Y = a \times e^{([\log(1-p)]\times[C/ECp])}$ All concentrations are based on acetonitrile extraction using USEPA Method 8330 (USEPA, 1998).

Figure 2. HMX effect on cocoon production (A) and juvenile production (B) by *Eisenia fetida* in freshly amended Sassafras sandy loam soil. Concentration-response relationships were determined using exponential model $Y = a \times e^{([\log(1-p)]\times[C/ECp])}$ All concentrations are based on acetonitrile extraction using USEPA Method 8330 (USEPA, 1998).


A
Freshly amended soil
NUMBER OF COCOONS
RDX (mg kg-1)
B
Freshly amended soil
NUMBER OF JUVENILES
RDX (mg kg-1)
C
Aged/weathered soil
NUMBER OF COCOONS
RDX (mg kg-1)
D
Aged/weathered soil
NUMBER OF JUVENILES
RDX (mg kg-1)


A
Freshly amended soil
NUMBER OF COCOONS
HMX (mg kg-1)
0
4
8
12
0
50
100
150


B
Freshly amended soil
NUMBER OF JUVENILES
HMX (mg kg-1)
0
5
10
15
20
25
0
50
100
150

APPENDIX H

PUBLISSHED TECHNICAL ABSTRACTS

Kuperman, R.G., Simini, M., Phillips, C.T., Checkai, R.T., Kolakowski, J.E., Kurnas, C.W., and Sunahara G.I., 2003. Soil invertebrate-based Ecological Soil Screening Levels (Eco-SSL) for explosive contaminants in soil. The Soil Ecological Society 2003 Conference, Palm Springs, California, 11-14 May 2003 (published abstract).

Abstract

We investigated the toxicity of the energetic materials (EM), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT) and 1,3,5-trinitrobenzene (TNB) to soil invertebrates. The study was designed to develop Ecological Soil Screening Level (Eco-SSL) benchmarks for ecological risk assessment (ERA) of EMs at contaminated sites. Eco-SSLs are ecotoxicity values that can be used in screening ERAs to identify contaminants in soil that warrant additional evaluation in a baseline ERA, and to eliminate those that do not. Test species included soil invertebrates *Eisenia fetida* (ISO 11268-2:1998), *Enchytraeus crypticus* (ISO/16387:2001), and *Folsomia candida* (ISO 11267:1998). Tests were conducted under conditions preferred for Eco-SSL derivation using a Sassafras sandy loam soil that supports relatively high bioavailability of EM. Simulated weathering/aging of amended soil was incorporated in the experimental design to better assess the toxicity potential in the field. Exposure concentrations were measured as total (acetonitrile-extractable) chemical concentrations. These concentrations were correlated with reproduction endpoints to develop ecotoxicological parameters for EMs based on concentration-response relationships. Data were analyzed using nonlinear regression models, to produce EC_{20} and EC_{50} values based on EM concentration vs measurement endpoints. Draft Eco-SSL values for all studied EMs were determined on the basis of EC_{20} values for juvenile production by the three test species. Results of these studies will undergo quality assurance by the Eco-SSL task group before inclusion in the Eco-SSL database.

Kuperman, R.G., Checkai, R.T., Sunahara, G.I., Simini, M., Phillips, C.T., Gong, P, Rocheleau, S., Lachance, B., Kolakowski, J.E., and Kurnas, C.W., 2002. Toxicity assessment of explosive contaminants in soil for development of ecological soil screening levels (Eco-SSL). The 2002 SERDP Partners in Environmental Technology Technical Symposium & Workshop, Washington, DC, December 3-5, 2002 (published abstract).

Abstract

The goal of this research was to determine the toxicity and bioaccumulation potential of the energetic materials (EM), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT) and 1,3,5-trinitrobenzene (TNB) for soil invertebrates and plants. The study was designed to develop Ecological Soil Screening Level (Eco-SSL) benchmarks for ecological risk assessment (ERA) of EMs at contaminated sites. Eco-SSLs are ecotoxicity values that can be used in screening ERAs to identify contaminants in soil that warrant additional evaluation in a baseline ERA, and to eliminate those that do not. Test species included soil invertebrates *Eisenia fetida* (ISO 11268-2:1998), *Enchytraeus crypticus* (ISO/16387:2001), *Folsomia candida* (ISO 11267:1998), and terrestrial plants (ASTM E1963-98 and EPA 712-C-96-347) alfalfa (*Medicago sativa*), Japanese millet (*Echinochloa crusgalli*), and perennial ryegrass (*Lolium perenne*). Tests were conducted under conditions preferred for Eco-SSL derivation using a Sassafras sandy loam soil that supports relatively high bioavailability of EM. Simulated weathering/aging of soil was incorporated in the experimental design to better assess the toxicity potential in the field. Bioaccumulation potential in plants and earthworms was investigated using [^{14}C]-labeled-RDX or -HMX. Phytogenotoxicity was determined using Trad-MN assays with *Tradescantia paludosa*. Exposure concentrations were measured as total (acetonitrile-extractable) chemical concentrations and as water-extractable (water adapted-TCLP; ATCLP) portion that is presumed bioavailable. Both these chemical measures were correlated with toxicity endpoints (growth, reproduction) and bioaccumulation to develop ecotoxicological parameters for these EMs based on concentration-response relationships. Data were analyzed using nonlinear regression models, to produce EC_{20} and EC_{50} values based on EM concentration vs measurement endpoints. Results of these studies will undergo quality assurance by the Eco-SSL task group before inclusion in the Eco-SSL database. This work is funded by SERDP (CU-1221).

Kuperman, R.G., Simini, M., Phillips, C.T., Checkai, R.T., Kolakowski, J.E., Kurnas, C.W., and Sunahara, G.I., 2002. Toxicity of energetic compounds to *Enchytraeus crypticus* in amended natural sandy loam soil. The 2002 SERDP Partners in Environmental Technology Technical Symposium & Workshop, Washington, DC, December 3-5, 2002 (published abstract).

Abstract

We investigated the toxicity of energetic materials (EM) hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT) and 1,3,5-trinitrobenzene (TNB) to the soil invertebrate species *Enchytraeus crypticus.* The study was designed to develop Ecological Soil Screening Level (Eco-SSL) benchmarks for ecological risk assessment (ERA) of explosives at contaminated sites. Eco-SSLs are ecotoxicity values that can be used routinely in screening ERAs to identify those contaminants in soil that warrant additional evaluation in a baseline ERA, and to eliminate those that do not. Tests were conducted under conditions preferred for Eco-SSL derivation, using a Sassafras sandy loam soil that supports relatively high bioavailability of test compounds. Toxicity testing was performed using the enchytraeid reproduction test (ISO/16387:2001) that measures adult survival and juvenile production by the potworm *E. crypticus* in freshly amended and weathered/aged soils. Measurement endpoints were assessed using 7-8 treatment concentrations and four replicates per treatment. Appropriate negative, carrier (acetone), and positive controls were included. Adult survival and juvenile production data were analyzed using nonlinear regression models, which included ECp as a parameter to determine the EM concentration producing a specified percentage effect. These parameters included EC_{20} and EC_{50} levels. Preliminary results showed that the order of EM toxicity to *E. crypticus* juvenile production in both freshly amended and weathered/aged soils was TNB > 2,4-DNT > 2,6-DNT > RDX > HMX. The EC_{20} values for juvenile production in freshly amended and weathered/aged soils were: 5 and 9, 19 and 14, 38 and 18, 3715 and 8797 mg kg^{-1} for TNB, 2,4-DNT, 2,6-DNT, and RDX, respectively. Weathering/aging of amended soil significantly increased the toxicity of 2,6-DNT to *E. crypticus*. Toxicity of other EMs was not affected by weathering/aging of amended soils significantly based on 95% confidence intervals. There were no adverse effects on adult survival or juvenile production up to the highest HMX concentration of 21,750 mg kg^{-1}.
This work was funded by SERDP (CU-1221).

Phillips, C.T., Kuperman, R.G., Checkai, R.T., Simini, M., Kolakowski, J.E., Kurnas, C.W., and Sunahara, G.I., 2002. Survival and reproduction of collembolan *Folsomia candida* exposed to energetic materials in freshly amended and weathered/aged Sassafras sandy loam soil. The 2002 SERDP Partners in Environmental Technology Technical Symposium & Workshop, Washington, DC, December 3-5, 2002 (published abstract).

Abstract

We investigated the toxicity of the energetic materials (EM) hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT) and 1,3,5-trinitrobenzene (TNB) to the collembolan *Folsomia candida*. The purpose of the study was to develop Ecological Soil Screening Level (Eco-SSL) benchmarks for ecological risk assessment (ERA) of explosives at contaminated sites. Tests were conducted under conditions preferred for Eco-SSL derivation, using soil that supports relatively high bioavailability of test compounds (i.e., low organic matter and clay content). Toxicity testing was performed in Sassafras sandy loam soil using the Inhibition of Reproduction of Collembola by Soil Pollutants method (ISO 11267:1998). Measurement endpoints included adult survival and juvenile production after exposure to EM for 28 days. Carrier (acetone), and positive controls were included. Measured soil concentrations of EM were correlated with measurement endpoints to develop concentration-response parameters. Data were analyzed using nonlinear regression models to estimate EC_{20} and EC_{50} values. The EC_{20} values for juvenile production in freshly amended soil were: 3, 4, and 6 mg kg^{-1}, for HMX, TNB, and 2,6-DNT, respectively. Tests with freshly amended 2,4-DNT and RDX are in progress. The order of EM toxicity in weathered/aged soil to *F. candida* was 2,6-DNT > 2,4-DNT > TNB > RDX > HMX. The EC_{20} values for juvenile production were: 0.96, 15, 48, 113, and 1046 mg kg^{-1} for 2,6-DNT, 2,4-DNT, TNB, RDX, and HMX, respectively. Weathering/aging of amended soils for 3 months significantly decreased the toxicity of TNB and HMX, and increased the toxicity of 2,6-DNT.
This work is funded by SERDP (CU-1221).

Simini, M., Kuperman, R.G., Phillips, C.T., Checkai, R.T., Kolakowski, J.E., Kurnas, C.W., and Sunahara, G.I., 2002. Toxicity of energetic compounds to *Eisenia fetida* in amended natural sandy loam soil. The 2002 SERDP Partners in Environmental Technology Technical Symposium & Workshop, Washington, DC, December 3-5, 2002 (published abstract).

Abstract

We investigated the toxicity of the energetic materials (EM) hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT) and 1,3,5-trinitrobenzene (TNB) to the earthworm *Eisenia fetida.* The study was designed to develop benchmarks for deriving Ecological Soil Screening Levels (Eco-SSLs) for ecological risk assessment (ERA) of explosives at contaminated sites. Tests were conducted under conditions preferred for Eco-SSL derivation, using a Sassafras sandy loam soil that supports relatively high bioavailability of the EM compounds. Toxicity testing was performed using an earthworm reproduction test (ISO 11268-2:1998) to measure adult survival, cocoon production, and juvenile production by *E. fetida* in freshly amended and weathered/aged soils. Measurement endpoints were assessed using 7-8 treatment concentrations, with four replicates per treatment. Negative, carrier (acetone), and positive control treatments were included. Reproduction data were analyzed using nonlinear regression models to determine the EM concentrations causing a 20% (EC_{20}) or 50% (EC_{50}) reduction in the measurement endpoints. Preliminary results showed that the order of EM toxicity to *E. fetida* reproductive endpoints was RDX = HMX > 2,6-DNT > TNB > 2,4-DNT. Mean adult survival was not significantly different ($p>0.05$) for all RDX and HMX levels compared to control. The EC_{20} values for cocoon production in freshly amended soils were: 1.2, 3, 14, 27, and 31 mg kg^{-1} for RDX, HMX, 2,6-DNT, TNB, and 2,4-DNT, respectively. The EC_{20} values for juvenile production in freshly amended soils were: 1.6, 0.4, 9, 21, 44 and for RDX, HMX, 2,6-DNT, TNB, and 2,4-DNT respectively. The EC_{20} values for cocoon production in weathered/aged soils were: 19, 16, 18, and 25 for RDX, 2,6-DNT, TNB, and 2,4-DNT, respectively. The EC_{20} values for juvenile production in weathered/aged soils were: 5, 8, 13, and 29 mg kg^{-1} for RDX, 2,6-DNT, TNB, and 2,4-DNT respectively. Mean cocoon and juvenile production were not significantly different ($p>0.05$) across all HMX concentrations up to 562 mg kg^{-1} in weathered/aged soils compared to control soils. All energetics except HMX did not have significantly different EC_{20} or EC_{50} values (95% C.I.) in weathered/aged soils compared to freshly amended soils. This work was funded by SERDP (CU-1221).

Rocheleau, S., Martel, M., Bardai, G., Wong, S., Sarrazin, M., Dodard, S., Kuperman, R.G., Checkai, R.T., Hawari, J., and Sunahara, G.I., 2002. Phytotoxicity of five energetic materials in amended Sassafras sandy loam soil. The 2002 SERDP Partners in Environmental Technology Technical Symposium & Workshop, Washington, DC, December 3-5, 2002 (published abstract).

Abstract

The phytotoxicity of two explosive compounds, cyclotrimethylenetrinitramine (RDX) and 1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and three TNT by-products, 1,3,5-trinitrobenzene (TNB), 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) was determined using alfalfa, Japanese millet, ryegrass, lettuce and corn. The effect of simulated aging/weathering procedure on the toxicity was examined and two soil extraction (acetonitrile vs water) methods were compared in order to develop Ecological Soil Screening Level (Eco-SSL) benchmarks for ecological risk assessment of energetic materials (EM) at contaminated sites. Preliminary range-finding tests indicated that corn was the least sensitive species and that lettuce did not grow well in the reference soil which has high bioavailability characteristics (Sassafras sandy loam soil). Definitive toxicity tests were therefore performed using alfalfa inoculated with nitrogen fixing bacteria, millet and ryegrass with seedling emergence and growth as measurement endpoints. Exposure concentrations were measured as total (acetonitrile-extractable) chemical concentrations and as water-extractable (water adapted-TCLP; ATCLP) portion that is presumed bioavailable. Data were analyzed using nonlinear regression models to calculate EC50, EC20, LOEC and NOEC values based on growth endpoints (fresh and dry mass) and EM acetonitrile-extractable and ATCLP -extractable concentrations. Results indicated that both dinitrotoluenes were more toxic than TNB, and RDX and HMX were not toxic to these plant species. Chemicals were generally more toxic in aged/weathered soil then in freshly amended soil. Results will undergo quality assurance by the Eco-SSL task group before inclusion in the Eco-SSL database.

Lachance, B., Leduc, F., Rocheleau, S., Martel, M., Dodard, S., G. Bardai, G., Kuperman, R.G., Checkai, R.T., Hawari, J., and Sunahara, G.I., 2002. Bioaccumulation of five energetic materials in Sassafras sandy loam soil. The 2002 SERDP Partners in Environmental Technology Technical Symposium & Workshop, Washington, DC, December 3-5, 2002 (published abstract).

Abstract

We investigated the bioaccumulation and mass-balance characteristics of two nitro-heterocyclic energetic materials (EM), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) using alfalfa, Japanese millet, ryegrass, lettuce and corn, and the earthworm *Eisenia andrei*. The bioaccumulation of TNT by-products, including 1,3,5-trinitrobenzene (TNB), 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) was also investigated. Tests were conducted in Sassafras sandy loam soil that supports relatively high bioavailability of these EMs. The effect of simulated aging/weathering procedure on the bioaccumulation of these EMs was incorporated in the study. Results showed that [C^{14}]-RDX and [C^{14}]-HMX were significantly accumulated by the selected plant species at the soil concentrations tested. Virtually no accumulation of TNB and of the DNT's was observed in plants. Mass-balance data indicate that plants accumulate less than 3% of the amended RDX, or less that 1% of amended HMX. The partitioning of RDX and HMX among plant compartments was evaluated in corn. After three weeks of exposure, the distribution of RDX and HMX (as radioactive labels) was similar to what is already known for other plants, with leaves being the major site of accumulation. In plants, most of the radiolabeled RDX and HMX were unmetabolized, with up to 20% of the plant radioactivity remaining in the residue after acetonitrile extraction. In the earthworm, accumulation was low for RDX, with a bioconcentration factor of 5-10, and was negligible for HMX. After a two-week exposure period, up to 5% of initial soil RDX radioactivity was found in the worm tissues (at 10 mg kg^{-1} in soil), but less than 0.4% of radioactivity was associated with tissues of worms exposed to 10 mg kg^{-1} HMX. This work was supported, in part by SERDP project CU-1221.

Gong, P., Ambroise, E., Zhang, X.-M., Kuperman, R.G., and Sunahara, G.I., 2002. Phytogenotoxicity of 2,4-DNT and 2,6-DNT Measured by *Tradescantia* Micronucleus (Trad-MCN) Assay. The 2002 SERDP Partners in Environmental Technology Technical Symposium & Workshop, Washington, DC, December 3-5, 2002 (published abstract).

Abstract

The phytogenotoxicity of 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) was assessed using the Tradescantia micronuclei bioassay. Inflorescences of 12-15 young Tradescantia cuttings were exposed for 6 hours to 2,4-DNT or 2,6-DNT amended water solutions up to their respective solubilities. The nominal concentrations were 0, 1.875, 3.75, 7.5, 15, 30, 60, 100, 150, 200 mg/L of 2,4-DNT, and 0, 7.5, 15, 30, 60, 90, 120, 180 mg/L of 2,6-DNT. Each treatment was repeated three times. Chemical concentrations in test solutions were analyzed prior to and after the exposure. Cadmium chloride was used as the positive control. Micronuclei were scored in the tetrad-stage pollen mother cells. The micronuclei frequency (%), i.e., the number of micronuclei scored in 100 tetrads, was the measurement endpoint. Results indicate that both 2,4-DNT and 2,6-DNT are genotoxic with the lowest observed effect concentration (LOEC) of 30 mg/L and 135 mg/L, and the no observed effect concentration (NOEC) of 15 mg/L and 85 mg/L, respectively. The phytogenotoxicity of 2,4-DNT was also tested in soil slurries made of 100 ml of dechlorined tap water and 50 g of a Sassafras sandy loam soil. The soil was amended with 2,4-DNT at 25, 250, 500, 1000 and 2000 mg/kg soil. Except for the lowest amendment level, all other amended soils caused significantly higher micronuclei frequency if compared with the control.

Kuperman, R.G., Checkai, R.T., Sunahara, G.I., M. Simini, M., Phillips, C.T., Gong, P., Rocheleau, S., Lachance, B., Kolakowski, J.E., and Kurnas, C.W., 2002. Development of ecological soil screening level (Eco-SSL) benchmarks for explosive contaminants in soil. Society of Environmental Toxicology and Chemistry Meeting, Salt Lake City, UT. 16 - 20 November 2002 (published abstract).

Abstract

The goal of this research was to determine the toxicity and bioaccumulation potential of the energetic materials (EM), cyclotrimethylenetrinitramine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT) and 1,3,5-trinitrobenzene (TNB) in soil invertebrates and plants. The study was designed to develop Ecological Soil Screening Level (Eco-SSL) benchmarks for ecological risk assessment (ERA) of EM at contaminated sites. Eco-SSLs are ecotoxicity values that can be used in screening ERAs to identify contaminants in soil that warrant additional evaluation in a baseline ERA, and to eliminate those that do not. Test species included soil invertebrates Eisenia fetida (ISO 11268-2:1998), Enchytraeus crypticus (ISO/16387:2001), Folsomia candida (ISO 11267:1998), and terrestrial plants (ASTM E1963-98 and EPA 712-C-96-347) alfalfa (Medicago sativa), Japanese millet (Echinochloa crusgalli), and perennial ryegrass (Lolium perenne). Tests were conducted under conditions preferred for Eco-SSL derivation using a Sassafras sandy loam soil that supports relatively high bioavailability of EM. Simulated aging/weathering of soil was incorporated in the experimental design to better assess the toxicity potential in the field. Bioaccumulation potential in plants and earthworms was investigated using [14C]-labeled-RDX or -HMX. Phytogenotoxicity was determined using Trad-MN assays with Tradescantia paludosa. Exposure concentrations were measured as total (acetonitrile-extractable) chemical concentrations and as water-extractable (water adapted-TCLP; ATCLP) portion that is presumed bioavailable. Both these chemical measures were correlated with toxicity endpoints (growth, reproduction) and bioaccumulation to develop ecotoxicological parameters for these EMs based on concentration-response relationships. Data were analyzed using nonlinear regression models, to produce EC20 and EC50 values based on EM concentration vs measurement endpoints. Results of these studies will undergo quality assurance by the Eco-SSL task group before inclusion in the Eco-SSL database.

Kuperman, R.G., Checkai, R.T., Simini, M., Phillips, C.T., Kolakowski, J.E. Kurnas, C.W., and Sunahara, G.I., 2002. Survival and reproduction of *Enchytraeus crypticus* exposed to energetic compounds in a natural soil. Society of Environmental Toxicology and Chemistry Meeting, Salt Lake City, UT. 16 - 20 November 2002 (published abstract).

Abstract

We investigated the toxicity of energetic materials (EM) cyclotrimethylenetrinitramine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT) and 1,3,5-trinitrobenzene (TNB) to the soil invertebrate species *Enchytraeus crypticus*. The study was designed to develop benchmarks for deriving Ecological Soil Screening Levels (Eco-SSLs) for ecological risk assessment (ERA) of explosives at contaminated sites. Eco-SSLs are ecotoxicity values that can be used routinely in screening ERAs to identify those contaminants in soil that warrant additional evaluation in a baseline ERA, and to eliminate those that do not. Ecotoxicity tests were conducted under conditions maximizing compliance with Eco-SSL evaluation criteria, using a Sassafras sandy loam soil that supports relatively high bioavailability of EM compounds. Toxicity testing was performed in freshly amended soil using an enchytraeid reproduction test (ISO/16387:2001) that measures adult survival and juvenile production by the potworm *E. crypticus*. Measurement endpoints were assessed using 7-8 treatment concentrations with four replicates per treatment. Negative, carrier (acetone), and positive control treatments were included. Adult survival and juvenile production data were analyzed using nonlinear regression models, which included ECx as a parameter to determine the EM concentration producing a specified percentage effect. These parameters included EC_{20} and EC_{50} levels. Preliminary results showed that the order of EM toxicity to *E. crypticus* was TNB > 2,4-DNT > 2,6-DNT > RDX > HMX. The respective EC_{20} and EC_{50} values for juvenile production were (EM mg kg^{-1}): 4 and 11 (TNB); 19 and 36 (2,4-DNT); 38 and 57 (2,6-DNT); 4000 and 50000 (RDX). There were no adverse effects on adult survival or juvenile production up to 20,000 mg kg^{-1} HMX, the highest concentration of HMX tested. Results of these studies will undergo quality control review by the Eco-SSL task group before inclusion in the Eco-SSL database.

Phillips, C.T., Checkai, R.T., Kuperman, R.G., Simini, M, Kolakowski, J.E., Kurnas, C.W., and Sunahara, G.I., 2002. Survival and reproduction of collembolan *Folsomia candida* exposed to energetic materials in Sassafras sandy loam. Society of Environmental Toxicology and Chemistry Meeting, Salt Lake City, UT. 16 - 20 November 2002 (published abstract).

Abstract

We investigated the toxicity of energetic materials (EM) octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT) and 1,3,5-trinitrobenzene (TNB) to the soil collembolan species *Folsomia candida*. The study was designed to develop benchmarks for deriving Ecological Soil Screening Levels (Eco-SSLs) for ecological risk assessment (ERA) of explosives at contaminated sites. Ecotoxicity tests were conducted under conditions maximizing compliance with Eco-SSL evaluation criteria, using a Sassafras sandy loam soil that supports relatively high bioavailability of EM compounds. Toxicity testing was performed using the Inhibition of Reproduction of Collembola by Soil Pollutants method (ISO 11267:1998). Measurement endpoints were adult survival and juvenile production after exposure to the respective EM compounds for 28 days. Acetonitrile-extractable (total) concentrations included, for 2,4-DNT: 0, 0.46, 1.0, 3.0, 6.5, 9.9, 20, and 41 mg kg-1; for 2,6-DNT: 0, 3.0, 4.0, 4.4, 5.3, 8.0, 9.4, 13, 20, and 40 mg kg-1; for TNB: 0, 2.6, 3.9, 13, 45, 107, 220, 380, and 520 mg kg-1; for HMX: 0, 11, 36, 70, 140, 350, 640, 1500, and 2200 mg kg-1. Carrier (acetone), and positive controls were included. Measured soil concentrations of EM were correlated with measurement endpoints to develop concentration-response parameters. Data were analyzed using nonlinear regression models to estimate EC20 and EC50 values. Preliminary results showed that the order of EM toxicity to F. candida was 2,6-DNT > TNB > 2,4-DNT > HMX. The respective EC20 and EC50 values for juvenile production were (EM mg kg-1): 2.1 and 6.5 (2,6-DNT); 4.4 and 25 (TNB); 6.4 and 13.8 (2,4-DNT); 9 and 146 (HMX). Results of these studies will undergo quality control review by the Eco-SSL task group before inclusion in the Eco-SSL database.

Simini, M., Checkai, R.T., Kuperman, R.G., Phillips, C.T., Kolakowski, J.E., and Kurnas, C.W., 2002. Survival and reproduction of *Eisenia fetida* exposed to energetic compounds in a natural soil. Society of Environmental Toxicology and Chemistry Meeting, Salt Lake City, UT. 16 - 20 November 2002 (published abstract).

Abstract

We investigated the toxicity of the energetic materials (EM) cyclotrimethylenetrinitramine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT) and 1,3,5-trinitrobenzene (TNB) to the earthworm *Eisenia fetida.* The study was designed to develop benchmarks for deriving Ecological Soil Screening Levels (Eco-SSLs) for ecological risk assessment (ERA) of explosives at contaminated sites. The toxicity tests were conducted under conditions maximizing compliance with Eco-SSL evaluation criteria, using a Sassafras sandy loam soil that supports relatively high bioavailability of the EM compounds. Toxicity testing was performed using an earthworm reproduction test (ISO 11268-2:1998) to measure adult survival, cocoon production, and juvenile production by *E. fetida* in freshly amended soil. Measurement endpoints were assessed using 7-8 treatment concentrations, with four replicates per treatment. Negative, carrier (acetone), and positive control treatments were included. Survival and reproduction data were analyzed using nonlinear regression models to determine the EM concentrations causing a 20% (EC20) or 50% (EC50) reduction in the measurement endpoints. Preliminary results showed that the order of EM toxicity to *E. fetida* was RDX = HMX > 2,6-DNT > TNB > 2,4-DNT. The respective EC20 and EC50 values for cocoon production were (EM mg kg-1): 1.2 and 4 (RDX); 3 and 9 (HMX); 14 and 25 (2,6-DNT); 27 and 59 (TNB); and 31 and 43 (2,4-DNT). Juvenile production EC20 and EC50 values were (EM mg kg-1): 1.6 and 5 (RDX); 0.4 and 1.2 (HMX); 9 and 27 (2,6-DNT); 21 and 33 (TNB); and 44 and 52 (2,4-DNT), respectively. Results of these studies will undergo quality control review by the Eco-SSL task group before inclusion in the Eco-SSL database.

Simini, M., Kuperman, R., Phillips, C.T., Checkai, R.T., Kolakowski, J.E., Kurnas, C.W., and Sunahara, G.I., 2002. Concentration of total and water extractable energetic compounds in an amended natural soil. Society of Environmental Toxicology and Chemistry Meeting, Salt Lake City, UT. 16 - 20 November 2002 (published abstract).

Abstract

We measured total and water extractable concentration of energetic materials (EM) RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB in Sassafras sandy loam soil. Analyses were performed in support of soil invertebrate toxicity tests designed to develop Ecological Soil Screening Level (Eco-SSL) benchmarks for ecological risk assessment (ERA) of explosives at contaminated sites. Freshly amended soils were analyzed for total (acetonitrile extraction) and water extractable (water adapted-TCLP; ATCLP) concentrations of EM. Nominal concentrations of RDX and HMX in freshly amended soils ranged from 1.5 to 20000 mg kg-1. Mean total RDX and HMX concentrations were 99% and 105% percent of the nominal, respectively. Water extractable RDX ranged from 0.5% to 91% of the total concentration. Percent recovery was much lower above 144 mg kg-1. Water extractable HMX ranged from 0.1% to 72% of the total extractable concentration. Percentage recovery was much lower above 36 mg kg-1. Lower water extractable concentrations at higher nominal levels can be explained by the low solubility of RDX and HMX. Nominal TNB concentrations ranged from 4 to 768 mg kg-1. Total TNB averaged 46% nominal from 4 to 64 mg kg-1, whereas total TNB averaged 94% nominal from 128 to 768 mg kg-1. Water extractable TNB averaged 75% total from 16 to 64 mg kg-1. Water extractable TNB was below detection limits at nominal 4 and 8 mg kg-1. TNB degradation appears to be accelerated below 100 mg kg-1. Nominal 2,4-DNT and 2,6-DNT ranged from 0.5 to 320 mg kg-1. Total 2,4-DNT averaged 85% nominal. Water extractable 2,4-DNT averaged 60% total. Total 2,6-DNT averaged 106% nominal. Water extractable 2,6-DNT averaged 80% total. These results will be correlated with toxicity endpoints to establish Eco-SSLs for soil invertebrates exposed to energetic compounds.

Rocheleau, S., Martel, M., Bardai, G., Wong, S., Sarrazin, M., Dodard, S., Kuperman, R., Checkai, R.T., and Sunahara, G.I., 2002. Toxicity of five energetic materials to plants exposed in Sassafras sandy loam soil. Society of Environmental Toxicology and Chemistry Meeting, Salt Lake City, UT. 16 - 20 November 2002 (published abstract).

Abstract

The objectives of the present study were: a) to determine the phytotoxicity of two explosive compounds, cyclotrimethylenetrinitramine (RDX) and 1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and three TNT by-products, 1,3,5-trinitrobenzene (TNB), 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT), b) to determine which soil extraction (acetonitrile vs water) method correlates better with toxicity, c) to examine the effect of simulated aging/weathering procedure on the toxicity and d) to develop Ecological Soil Screening Level (Eco-SSL) benchmarks for ecological risk assessment of energetic materials (EM) at contaminated sites. The phytotoxicity of these five recalcitrant EMs was determined using alfalfa, Japanese millet, ryegrass, lettuce and corn. Preliminary range-finding tests indicated that corn was the least sensitive species and that lettuce did not grow well in the reference soil, which has high bioavailability characteristics (Sassafras sandy loam soil). Definitive toxicity tests were therefore performed using alfalfa inoculated with nitrogen fixing bacteria, millet and ryegrass with seedling emergence and growth as measurement endpoints. Exposure concentrations were measured as total (acetonitrile-extractable) chemical concentrations and as water-extractable (water adapted-TCLP; ATCLP) portion that is presumed bioavailable. Data were analyzed using nonlinear regression models to calculate EC20 values based on toxicity endpoints and EM acetonitrile-extractable and ATCLP -extractable concentrations. Results indicated that both dinitrotoluenes were more toxic than TNB, and RDX and HMX were not toxic to these plant species. Results will undergo quality assurance by the Eco-SSL task group before inclusion in the Eco-SSL.

Kuperman, R.G., Simini, M., Phillips, C.T., Checkai, R.T., and Sunahara, G.I., 2002. Toxicity of Energetic Compounds RDX, HMX and TNB to the Potworm *Enchytraeus crypticus* in a Sandy Loam Soil. The 7th International Symposium on Earthworm Ecology, Cardiff, Wales, UK, 1-7 September 2002 (published abstract).

Abstract

The U.S. Environmental Protection Agency (USEPA) in a collaborative effort with other Federal agencies, States, and private industry, is developing Ecological Soil Screening Level (Eco-SSL) benchmarks for ecological risk assessment (ERA) of contaminants at Superfund sites. Eco-SSLs are ecotoxicity values that can be used routinely in screening ERAs to identify those contaminants in soil that warrant additional evaluation in a baseline ERA, and to eliminate those that do not. Eco-SSLs are developed from literature values whenever sufficient quantity and quality of data exist. Insufficient data were available on the toxicity of energetic compounds, RDX, HMX and TNB to soil invertebrates, necessitating toxicity testing. Tests were conducted under conditions preferred for Eco-SSL derivation, using a Sassafras sandy loam soil that supports relatively high bioavailability of test compounds. Toxicity testing was performed using enchytraeid reproduction test (ISO/16387:2001) measuring adult survival and juvenile production by the potworm *Enchytraeus crypticus* in freshly amended soil. The treatment concentrations were determined from range-finding studies conducted earlier. Measurement endpoints were assessed using 7-8 treatment concentrations and four replicates per treatment. Nominal soil concentrations were as follows, RDX: 300, 600, 1200, 2400, 4800, 10000, 20,000 mg kg^{-1}, HMX: 300, 600, 1200, 2500, 5000, 10000, 20,000 mg kg^{-1}, and TNB: 4, 8, 16, 32, 64, 128, 256, and 387 mg kg^{-1}. Appropriate negative, carrier (acetone), and positive controls were included. RDX had no effect on adult survival in the definitive tests in all treatment concentrations. The bounded no observed effect concentration (NOEC) and lowest observed effects concentration (LOEC) values for juvenile production were 600 mg kg^{-1} and 1200 mg kg^{-1} ($p = 0.042$), respectively. There were no adverse effects on adult survival or juvenile production in any of the HMX treatment concentration. TNB did not affect adult *E. crypticus* survival up to 64 mg kg^{-1}. No adults survived at the higher concentration levels. The bounded lowest observed adverse effect concentration (LOAEC) for juvenile production was 16 mg kg^{-1} ($p = 0.02$). No juveniles were produced at treatment concentrations above 64 mg kg^{-1}, which can be attributed to 100% adult mortality at these concentrations. Results will undergo quality assurance by the Eco-SSL task group before inclusion in the Eco-SSL database.

Simini, M., Kuperman, R.G., Checkai, R.T., Phillips, C.T., and Sunahara, G.I., 2002. Reproduction and survival of *Eisenia fetida* exposed to energetic compounds in a sandy loam soil. The 7th International Symposium on Earthworm Ecology, Cardiff, Wales, UK, 1-7 September 2002 (published abstract).

Abstract

We investigated the toxicity of the energetic materials (EM) cyclotrimethylenetrinitramine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT) and 1,3,5-trinitrobenzene (TNB) to the earthworm *Eisenia fetida.* The study was designed to develop benchmarks for deriving Ecological Soil Screening Levels (Eco-SSLs) for ecological risk assessment (ERA) of explosives at contaminated sites. Tests were conducted under conditions preferred for Eco-SSL derivation, using a Sassafras sandy loam soil that supports relatively high bioavailability of the EM compounds. Toxicity testing was performed using an earthworm reproduction test (ISO 11268-2:1998) to measure adult survival, cocoon production, and juvenile production by *E. fetida* in freshly amended and aged/weathered soils. Measurement endpoints were assessed using 7-8 treatment concentrations, with four replicates per treatment. Negative, carrier (acetone), and positive control treatments were included. Reproduction data were analyzed using nonlinear regression models to determine the EM concentrations causing a 20% (EC20) or 50% (EC50) reduction in the measurement endpoints. Preliminary results showed that the order of EM toxicity to *E. fetida* was RDX = HMX > 2,6-DNT > TNB > 2,4-DNT. The EC20 values for cocoon production in freshly amended and weathered/aged soils were: 1.2 and 19, 3 and >562, 14 and 16, 27 and 18, 31 and 31 mg kg-1 for RDX, HMX, 2,6-DNT, TNB, and 2,4-DNT, respectively. The EC20 values for juvenile production in freshly amended and weathered/aged soils were: 1.6 and 5, 0.4 and >562, 9 and 8, 21 and 13, 44 and 29 mg kg-1 for RDX, HMX, 2,6-DNT, 2,4-DNT, and TNB, respectively.

Gong, P, Rocheleau, S., Lachance, B., Kuperman, R., and Sunahara, G.I., 2002. Plant Toxicity and Bioaccumulation of Energetic Compounds. The 1st International Conference on Pollution Eco-Chemistry and Ecological Processes, August 26-31, 2002, Shenyang, China (published abstract).

Abstract

The ecotoxicity of five selected energetic compounds (i.e., RDX, HMX, 2,4-dinitrotoluene, 2,6-dinitrotoluene and 1,3,5-trinitrobenzene) and the plant accumulation of RDX and HMX have been investigated using an integrated ecotoxicology and chemistry approach. This approach requires that toxicity tests be carried out in parallel with chemical analyses, allowing one to link the environmental behavior and fate of these compounds with their toxicity. Estimation of exposure to toxicants in soil is often done using methods involving aqueous or organic solvent extracts of contaminated soil. It is not known, however, whether these methods best reflect the bioavailability of the moderately hydrophobic compounds (such as RDX and HMX) in a soil matrix. The use of [^{14}C]-labeled-RDX or -HMX would allow one to study their extractability in soil in order to better understand the bioavailability as well as the biotic and abiotic pathways of these compounds (e.g., microbial degradation/mineralization, plant uptake and transformation). With this latter approach, the bioaccumulation and metabolism by higher plants and microorganisms were investigated. The measurement of different fractions (extractable vs. non-extractable) of the [^{14}C]-labeled-RDX or -HMX in soil also enables us to attribute certain "bioavailable" fraction of these compounds to the observed toxicity by establishing dose-response relationships. This information would be very useful in the derivation of ecological threshold of energetic compounds.

Rocheleau, S., Martel, M., Bardai, G., Wong, S., Sarrazin, M., Dodard, S., Kuperman, R., and Sunahara, G.I., 2002. Phytotoxicity of five energetic compounds. SRA- Society of Environmental Toxicology and Chemistry Meeting, Saint-Laurence Chapter, June 6-7, 2002, Quebec, Quebec, Canada (published abstract).

Abstract

La présente étude s'inscrit dans un projet global effectué en collaboration avec l'armée américaine et Geo-Centers Inc. visant à déterminer la toxicité de deux composés explosifs, soit le cyclotrimethylenetrinitramine (RDX) et le 1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) et de trois produits dérivés du TNT, soit le 1,3,5-trinitrobenzène (TNB), le 2,4-dinitrotoluène (2,4-DNT) et le 2,6-dinitrotoluène (2,6-DNT). Ces composés sont des contaminants récalcitrants que l'on retrouve dans les sols utilisés comme champs de tir. La phytotoxicité de ces cinq composés a été déterminée à l'aide de la luzerne, du millet Japonais, du ryegrass, de la laitue et du maïs. Des tests préliminaires ont démontré que le maïs était l'espèce la moins sensible et que la laitue ne poussait pas bien dans le sol de référence utilisé (sol Sassafras sablonneux limoneux). Les tests de toxicité définitifs ont donc été poursuivis avec la luzerne inoculée de bactéries fixatrices d'azote, le millet Japonais et le ryegrass. Des résultats préliminaires ont démontré que le RDX et le HMX sont très peu toxiques pour les plantes étudiées et que les dinitrotoluènes sont plus toxiques que le TNB. Des valeurs de CE20, de CSE et de CSSE seront déterminées pour chacun des composés et transmises à l'agence américaine de protection environnementale (US EPA) afin d'établir les critères environnementaux de qualité du sol (ecological soil screening levels - Eco-SSL).