



# EDGEWOOD

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**EFFECT OF SIMULATED WEATHERING AND  
AGING OF TNT IN AMENDED SANDY LOAM SOIL ON TOXICITY  
TO THE ENCHYTRAEID WORM, *ENCHYTRAEUS CRYPTICUS***

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14. ABSTRACT The U.S. Environmental Protection Agency is developing Ecological Soil Screening Levels (Eco-SSLs) for the ecological risk assessment of contaminants at Superfund sites. Insufficient information for TNT to generate Eco-SSL for soil invertebrates has necessitated standardized toxicity testing to fill the data gap. In these studies, the Enchytraeid Reproduction Test (ISO/16387:2001) was adapted for use with <i>Enchytraeus crypticus</i> . Tests were conducted in Sassafras sandy loam soil, which supports relatively high bioavailability of TNT. Weathering/aging procedures for TNT amended to the test soil were included to reflect the exposure conditions in field soils. Definitive toxicity tests conducted with both amended soils showed that the weathering and aging of TNT in amended soil significantly increased toxicity for <i>E. crypticus</i> juvenile production with EC <sub>20</sub> and EC <sub>50</sub> values 77 and 98 mg kg <sup>-1</sup> , respectively, in freshly amended soil, and 38 and 48 mg kg <sup>-1</sup> , respectively, for TNT weathered/aged in amended soil. These study results will be provided to the Eco-SSL workgroup for review and inclusion in the Eco-SSL database, and for developing a TNT Eco-SSL for soil invertebrates.					
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## PREFACE

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# EFFECT OF SIMULATED WEATHERING AND AGING OF TNT IN AMENDED SANDY LOAM SOIL ON TOXICITY TO THE ENCHYTRAeid WORM, *ENCHYTRAeus CRYPTICUS*

## 1. INTRODUCTION

Energetic materials (EMs) are employed in a wide range of commercial and military activities, and are often released into the environment. More than 15 million acres in the U.S. are suspected or known to be contaminated with elevated levels of explosives and related materials in soil. As of 2002, the U.S. DoD identified 2,307 sites of potential contamination by military explosives. Army ammunition plants were identified as the most heavily contaminated sites (USGS, 2002). By 2003, assessments were completed for only 558 sites of which 83 required remediation (USGAO, 2003). Estimated costs range from \$8 billion to \$35 billion and the DoD continues to identify additional EM-contaminated sites associated with military operations (USGAO, 2003).

Biodegradation of the explosives 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) in vadose soil zone is limited, and manufacture, use, or disposal practices have led to their persistence in the environment (Spain, 2000). Specific activities resulting in the significant release of military EMs include: dumping of aqueous washings during manufacture (U.S. Environmental Protection Agency, USEPA, 2003a), the failure of munitions to detonate on training ranges (Thiboutot *et al.*, 1998), deposition of ordnance in landfills (USEPA, 2003b), and the incomplete combustion of ordnance during disposal by open burning or detonation (USEPA, 2003c). These compounds have been identified in plants and earthworms exposed to contaminated soil (Groom *et al.*, 2002; Robidoux *et al.*, 2004). Concentrations of explosives in soil have been reported to exceed 87,000 mg kg<sup>-1</sup> for TNT and 3,000 mg kg<sup>-1</sup> for RDX or HMX (Simini *et al.*, 1995). The effects of several of these EMs on soil biota have not been sufficiently investigated. This presents a challenge for Site Managers who wish 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.

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 issue, 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. 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, 2003d), determined that there was insufficient

information for TNT to generate an Eco-SSL for soil invertebrates, which necessitated our study to fill this knowledge gap.

Assessment of the effects of weathering and aging of contaminant explosives in soil on the exposed soil organisms is critical for developing toxicity benchmarks that adequately reflect potential ecological risks, 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 TNT in soil may reduce exposure of soil invertebrates 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. Certain fate processes, including microbial transformation of EMs can also produce chemicals that are more bioavailable or more toxic to soil organisms compared with parent EM compounds freshly introduced into soil. Therefore, we designed our studies to include weathering and aging of TNT in the amended natural soil prior to exposing test organisms, which allowed us to more closely simulate the exposure effects in the field.

## 2. MATERIALS AND METHODS

### 2.1 Study Design.

This study was designed to produce toxicity benchmark data for use in the development of Eco-SSL for TNT for soil invertebrates, and meet specific criteria (USEPA, 2003d), 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, can 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 reproduction component among the measurement endpoints. Our objective was to assess TNT toxicity to the soil invertebrate *Enchytraeus crypticus* and to produce benchmark data that can be used in development of Eco-SSLs for TNT contaminated soil. The Enchytraeid Reproduction Test was specifically modified to comply with Eco-SSL testing conditions and to assess the effect of weathering/aging of TNT in the amended soil on its toxicity to *E. crypticus*.

## 2.2 Soil Processing.

A natural soil, Sassafras sandy loam (Fine-loamy, siliceous, mesic Typic Hapludult) (USDA/ARS, 1999; SSL) was used in this study to assess TNT toxicity 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 TNT bioavailability, including low organic matter and clay contents (USEPA, 2003d). The SSL soil was collected from an open grassland field on the property of the U.S. Army Aberdeen Proving Ground (APG, MD). Vegetation and the organic horizon were removed to just below the grass root zone and the top 15 cm of the A horizon were then collected. The soil was sieved through a 5-mm<sup>2</sup> 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.

Soil Parameter	Sassafras Sandy Loam
Sand %	69
Silt %	13
Clay %	17
Texture	Sandy loam
CEC cmol kg <sup>-1</sup>	5.5
Organic matter %	1.2
pH	5.2

## 2.3 Test Chemicals.

Crystalline TNT (CAS: 118-96-7, 99.9% purity) was obtained from the Defense Research Establishment Valcartier of the Canadian Ministry of National Defense (Val Bélair, QC, Canada). Beryllium sulfate (BeSO<sub>4</sub>·4H<sub>2</sub>O; CAS: 7787-56-6; Purity: 99.99%) was used as the positive control in these tests. Acetone (CAS: 67-64-1; HPLC Grade) was used for preparing TNT 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 TNT (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<sup>®</sup> 10 Plus followed by Milli-Q<sup>®</sup> PF Plus systems (Millipore<sup>®</sup>, 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.

## 2.4 Soil Amendment Procedures.

Preparation of test concentrations generally followed methods described in internationally accepted standardized protocols (ISO, 1998a, b), with necessary modifications. Modifications were required when a natural soil is used in the place of artificial soil in the standard protocols. Experiments were performed separately and independently for TNT in freshly amended soil and in amended and weathered/aged soil to determine toxicity benchmark values for TNT in each exposure type. During the procedure, TNT was mixed into a separate aliquot of soil, using an organic solvent as a carrier, necessary to dissolve the nonpolar chemical in order to yield a more homogeneous mixture than direct addition of solid chemical crystals to soil. TNT was dissolved in acetone and pipetted onto a 2.5 cm thick layer of soil to establish each TNT concentration in soil, ensuring that the volume of solution added at any one time did not exceed 15% ( $\text{v m}^{-1}$ ) of the dry mass soil. After addition of TNT solution, the volumetric flask was rinsed twice with a known volume of acetone and pipetted onto the soil. The same total TNT/acetone solution volume at different TNT concentrations was added to every treatment, equating the volume required to dissolve TNT at the highest concentration tested. The acetone was allowed to volatilize in a dark chemical hood to prevent photolysis of TNT. Carrier control was treated with acetone only. 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. After mixing, soil was hydrated with ASTM type I water to 100% of the soil water holding capacity (WHC; 18% water, on 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.

## 2.5 Treatment Concentrations.

### 2.5.1 Range-finding Tests.

Range-finding test was conducted with freshly amended SSL soil to estimate treatment concentrations for definitive tests. Soil used in range-finding tests was amended with nominal TNT concentrations of 10, 100, 200, 400, 800, and 1000  $\text{mg kg}^{-1}$ . Control soil was treated with acetone only as described in 2.4.

### 2.5.2 Definitive Tests.

Data from the range finding tests were used to establish the treatment concentrations for definitive tests. Definitive tests to assess the effects of TNT on *E. crypticus* were conducted with freshly amended soil, and with TNT weathered/aged in amended soil. Nominal TNT concentrations selected for the definitive tests in freshly amended soil included 0, 50, 75, 100, 150, 200, and 300  $\text{mg kg}^{-1}$ . Nominal concentrations selected for the definitive tests using TNT amended and weathered/aged SSL soil included 0, 20, 75, 100, 150, 160, and 180  $\text{mg kg}^{-1}$ . All definitive tests included carrier (acetone) control and positive control. Positive control was prepared as solution of beryllium sulfate

in ASTM type I water using 30 mg kg<sup>-1</sup> Be nominal concentration. Nominal test concentrations of TNT were verified using USEPA Method 8330 (USEPA, 1998).

## 2.6 Weathering/Aging of TNT in 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 (Kuperman *et al.*, 2003, 2004a, 2004b; Simini *et al.*, 2003). This included exposing amended soils, initially hydrated to 60 percent of the WHC, in open glass containers in the greenhouse to alternating wetting and drying cycles. Soil treatments with TNT concentrations representing low, medium and high levels shown in Figure 1 were monitored periodically during weathering/aging process to determine the time when TNT concentration would stabilize and/or would decline below five percent of the initial concentration in treatments with the highest rate of decrease. This time was designated for terminating weathering/aging procedure and for commencement of definitive toxicity test. All soil treatments were weighed and readjusted to their initial mass by adding ASTM Type I water each week. All soil treatments were brought to 100% of the WHC 24 h prior to commencement of toxicity tests. The effects of weathering/aging on TNT ecotoxicity was investigated by comparing test results for TNT weathered/aged in amended soils with results obtained using TNT freshly amended into 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, 2.3 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<sub>2</sub> solution (5 g L<sup>-1</sup>) were added. Supernatant was filtered through 0.45 µm PTFE syringe cartridges. Soil extracts were analyzed and quantified using HPLC. In this report, the result of acetonitrile soil extraction is reported as the TNT 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 ATCLP-modification involved substituting CO<sub>2</sub>-saturated ASTM type I water, instead of using acetic acid called for in TCLP, thus better simulating for toxicity testing the soil-water conditions from respiration of CO<sub>2</sub> 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 the WHC). All analytical measurements were done in triplicate. For each treatment concentration, 4 g of soil were transferred into 20 mL vials. Sixteen mL of

CO<sub>2</sub>-saturated water at pH  $\leq 4.0$  was added to the vials, and 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, and then supernatants were filtered through 0.45  $\mu\text{m}$  PTFE syringe cartridges. An equivalent volume of acetonitrile was added to filtered soil extract prior to HPLC analysis. In this report, the result of ATCLP soil extraction is referred to as water-extractable fraction of TNT in dry soil.

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 TNT 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. 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 TNT in 60:40 water:acetonitrile in a range of concentrations appropriate for each run. The method detection limit was 0.05 mg L<sup>-1</sup>, 0.5 mg kg<sup>-1</sup> soil. Blanks and standards were placed intermittently between samples having unknown concentration in order 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. Nominal and determined (measured) concentrations used in the definitive tests are shown in Tables 2 and 3.

## 2.8 Toxicity Assessment.

The Enchytraeid Reproduction Test (ERT) was used to assess the effects of TNT on the reproduction of the enchytraeid worm *Enchytraeus crypticus* (potworm). 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/OECD Standard Artificial Soil), however our research showed that this test could also be conducted using natural soils (Kuperman *et al.*, 1999; 2003; 2004a). 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 ( $\pm 0.5$ ) for optimal test conditions. This species performed poorly in natural soils having physical and chemical characteristics that support a higher level of TNT 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, EC<sub>p</sub> (e.g., EC<sub>20</sub>, and EC<sub>50</sub>).

### 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:

- a) The adult mortality does not exceed 20% after 14 days, in the range finding and definitive tests;
- b) 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;
- c) 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 the 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 added was adjusted. Every 2 months, the worms were transferred into a freshly prepared culture substrate.

The culturing process was regarded as being satisfactory under the following conditions:

- Worms did not try to leave the soil.
- Worms exhibited a shiny outer surface with no soil particles clinging to them.
- Worms 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 ground oats were mixed with the soil. The mass of each container with prepared soil was recorded. Each treatment and control were replicated four times for definitive tests (two for range-finding tests).

Adult enchytraeid worms with eggs in the clitellum region were collected from culture established in the same soil type (SSL) as soil to be 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  $22 \pm 1^\circ\text{C}$  and 16:8 h light/dark 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 nine drops of Rosebengal biological stain (1% solution in ethanol) were added. Staining continued for minimum of 24 hours. The content of each test container was wet-sieved using a No. 100 (150  $\mu\text{m}$ ) 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 Analyses.

Adult survival and juvenile production data were analyzed using nonlinear regression models described in Stephenson *et al.* (2000). 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 adult survival data in all toxicity tests. The logistic hormetic model [2] having an additional parameter to accommodate hormesis had the best fit for the juvenile production 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:

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

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

where

$Y$  = number for a measurement endpoint (e.g., number of juveniles),  
 $a$  = control response,  
 $t$  = control response in the hormetic model,  
 $e$  = base of the natural logarithm,  
 $p$  = percent inhibition/100 (e.g., 0.50 for  $EC_{50}$ ),  
 $C$  = exposure concentration in test soil,  
 $ECp$  = estimate of effect concentration for a specified percent effect,  
 $h$  = hormetic effect parameter, and  
 $b$  = scale parameter.

The  $ECp$  parameters used in this study included the TNT concentration producing a 20% ( $EC_{20}$ ) or 50% ( $EC_{50}$ ) reduction in the measurement endpoint. The  $EC_{20}$  parameter based on reproduction 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 (C.I.) 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. A significance level of  $p \leq 0.05$  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 TNT concentrations. Statistical analyses were performed using SYSTAT 7.0.1 (SPSS, 1997).

### 3. RESULTS

#### 3.1 Analytical Determinations of TNT in Soil.

Concentrations of TNT in amended soil were determined at the beginning of each definitive toxicity test using both acetonitrile and ATCLP-based extractions. Soil samples amended for weathering/aging were analyzed to determine the initial TNT concentrations using acetonitrile extraction only. These concentrations were contrasted with TNT concentrations at the end of weathering/aging procedure to assess the effect of weathering/aging on TNT degradation, transformation or other fate processes that could affect its bioavailability for *E. crypticus*. Analytically determined initial concentrations were also used for monitoring acetonitrile extractable TNT concentrations during weathering/aging process to determine the time when TNT concentrations were effectively stabilized and/or had declined to five percent of the initial concentration in treatments with the highest rate of decrease for commencement of definitive toxicity test. Percentage decrease in TNT concentrations during weathering/aging process was highest in treatment with the lowest initial amended concentration in soil. After 56 days from the start of weathering/aging procedure, TNT concentration in the 50, 100, and 200 mg kg<sup>-1</sup> nominal (46, 92, and 182 mg kg<sup>-1</sup> initial measured) treatments decreased by 95, 28, and 20%, respectively, and were 2.5, 66, and 146 mg kg<sup>-1</sup>. Analytical determinations after total of 83 days of weathering/aging process showed no further appreciable decrease in TNT concentrations in the 50 and 100 mg kg<sup>-1</sup> monitored treatments at which time toxicity testing was initiated (Figure 1). Analytically determined TNT concentrations in the 200 mg kg<sup>-1</sup> nominal treatment continued to decline resulting in an additional 17% decrease between day 56 and 83 of weathering and aging of TNT in SSL soil (Figure 1). However, this treatment was not included in definitive toxicity testing for TNT weathered and aged in soil.

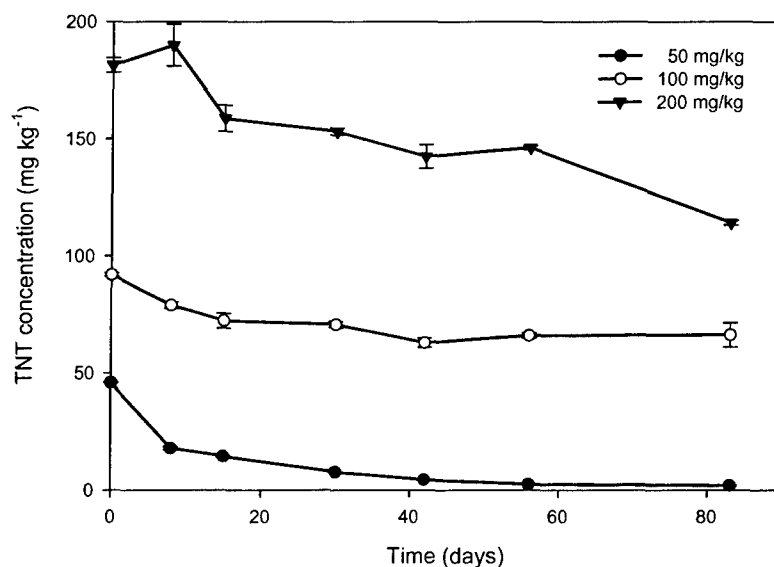


Figure 1. Changes in TNT concentrations during weathering and aging in Sassafras sandy loam soil amended with 50, 100, and 200 mg kg<sup>-1</sup> nominal concentrations.

Results of chemical analyses are shown in Tables 2 and 3. Acetonitrile-extractable TNT concentrations in freshly amended soils averaged 88 percent (range: 80-96%) of nominal concentrations indicating that a portion of TNT could be rapidly transformed/degraded during the initial 24-h period after soil hydration. The ATCLP-extractable TNT concentrations averaged 70 percent (range: 64-75%) of acetonitrile-extractable concentrations (Table 2).

Weathering/aging of TNT in amended soils used in definitive toxicity tests decreased acetonitrile-extractable TNT concentrations, on average, by 39% (recovery range from 17-78%) compared with initial concentrations in amended soils (Table 3). The greatest percentage decrease occurred in the lowest nominal TNT treatments of 20 mg kg<sup>-1</sup>. Percentage decrease in acetonitrile-extractable TNT concentrations during weathering/aging procedure was lower and more uniform at the higher nominal treatment levels 75-180 mg kg<sup>-1</sup>, averaging 70 percent (range: 63-78%) of the initial acetonitrile-extractable TNT concentrations in freshly amended soils (Table 3). Two transformation products of TNT, 2-ADNT and/or 4-ADNT, were qualitatively detected (identification confirmed by ultraviolet spectrum; data is not shown) in weathered and aged TNT treatments, indicating that TNT was partially transformed during weathering and aging in SSL soil.

The ATCLP-extractable TNT concentrations in weathered/aged amended soils averaged 28 percent (range: 13-33%) of acetonitrile-extractable concentrations (Table 3). Overall, ATCLP-extractable TNT concentrations decreased from an average of 70% of acetonitrile-extractable TNT concentrations in freshly amended soil to an average of 28% in weathered/aged soil treatments.

Table 2. Nominal and determined TNT concentrations in freshly amended Sassafras sandy loam soil.

Nominal concentration (mg kg <sup>-1</sup> )	Acetonitrile extraction (mg kg <sup>-1</sup> )	Standard error	Acetonitrile/nominal (%)	ATCLP extraction (mg kg <sup>-1</sup> )	Standard error	ATCLP/acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
50	40	0.4	80	25	0.2	64
75	62	2.1	82	40	0.5	64
100	85	0.2	85	59	1.2	69
150	134	6.1	90	98	0.6	73
200	186	2.4	93	137	0.9	73
300	287	2.5	96	215	1.0	75

Table notes: Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) mean (n=3) values. BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L<sup>-1</sup>; 0.5 mg kg<sup>-1</sup> soil.

Table 3. Recovery of acetonitrile (ACN) extractable and water (ATCLP) extractable TNT from amended Sassafras sandy loam soil used in definitive toxicity tests with weathered/aged (W-A) treatments.

Nominal concentration (mg kg <sup>-1</sup> )	Initial ACN (mg kg <sup>-1</sup> )	W-A ACN (mg kg <sup>-1</sup> )	W-A /Initial ACN (%)	W-A ATCLP (mg kg <sup>-1</sup> )	W-A ATCLP/ W-A ACN (%)
0	BDL	BDL	BDL	BDL	BDL
20	18 (0.2)	3 (0.5)	17	0.4 (0.01)	13
75	73 (0.8)	46 (0.7)	63	14.2 (0.09)	31
100	92 (0.8)	66 (2.5)	72	20.2 (0.31)	31
150	139 (5.3)	94 (0.0)	68	30.8 (0.36)	33
160	150 (0.6)	105 (2.7)	70	31.0 (1.43)	30
180	175 (8.6)	137 (3.8)	78	42.1 (1.26)	31

Table notes: Acetonitrile extraction was based on USEPA Method 8330; water extraction was based on Adapted Toxicity Characteristic Leaching Procedure (ATCLP). Values are means (n=3) and standard errors (S.E.). BDL=below detection limit. Method Detection Limit, MDL = 0.05 mg L<sup>-1</sup>; 0.5 mg kg<sup>-1</sup> soil.

### 3.2 Range-finding Toxicity Tests.

Results of range finding test showed that TNT significantly ( $p = 0.001$ ) reduced adult survival at 400 mg kg<sup>-1</sup>. No adults survived at the higher concentrations. Juvenile numbers increased by 26 percent in 10 mg kg<sup>-1</sup> treatment and were reduced by 29 ( $p = 0.203$ ) and 83 ( $p = 0.008$ ) percent in 100 and 200 mg kg<sup>-1</sup> treatments, respectively, compared to control. No juveniles were produced in 800 and 1000 mg kg<sup>-1</sup> treatments. Results of this range-finding test allowed us to establish the nominal treatment concentrations for the definitive test shown in Tables 2 and 3.

### 3.3 Definitive Toxicity Tests.

Definitive studies using the Enchytraeid Reproduction Tests (ERT) were conducted to assess the effects of TNT on the enchytraeid worm *E. crypticus*. Adult potworms were exposed to a range of TNT concentrations in freshly amended soil, and to TNT weathered/aged in amended soil in independent investigations. Measurement endpoints were assessed using six positive 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 in definitive toxicity tests.

Test results complied with the validity criteria defined in the ISO test guideline. Mean adult survival in negative controls was 93% in freshly amended soil, and 100% for TNT weathered/aged in soil. The mean number of juveniles in respective negative controls was 1202 and 1851, and the coefficients of variation were 10.3 and 2.9 percent, respectively. Direct comparisons of the results of positive control are not possible because ERT is a new test and reference values for natural soils are not yet available from

the literature. Juvenile production in positive controls was reduced by 56 and 59 percent from respective negative controls and was within  $\pm 2$  times standard error of the baseline established for the laboratory culture of *E. crypticus*. These results confirmed the power of the test, indicating that the toxicological effects determined in the definitive tests were most likely due to TNT treatments.

### 3.3.1 Toxicity of TNT in Freshly Amended Soil.

Adult *E. crypticus* survival and juvenile production were affected in TNT amended SSL soil within the concentration range selected from the results of range-finding test (Table 4). For adult survival, the bounded NOEC and LOEC values based on acetonitrile-extractable concentrations were 186 ( $p = 0.064$ ) and 287 ( $p < 0.0001$ )  $\text{mg kg}^{-1}$ , respectively. The bounded NOEC and LOEC values based on water-extractable TNT concentrations were 137 and 215  $\text{mg kg}^{-1}$ , respectively. Concentration-response relationship for adult survival determined by nonlinear regression is shown in Figure 2. Logistic (Gompertz) model had the best fit for the data. Adult survival  $\text{EC}_{20}$  and  $\text{EC}_{50}$  values based on acetonitrile-extractable concentrations were 180 and 360  $\text{mg kg}^{-1}$ , respectively. Adult survival  $\text{EC}_{20}$  and  $\text{EC}_{50}$  values based on water-extractable concentrations were 130 and 280  $\text{mg kg}^{-1}$ , respectively (Table 5).

Juvenile production was the more sensitive measurement endpoint for assessing TNT toxicity for *E. crypticus* compared with adult survival. Juvenile production was stimulated in the first positive treatment concentration of 40  $\text{mg kg}^{-1}$  resulting in the 24 percent increase in the average number on juveniles compared with carrier control (Table 4). The increase was statistically significant ( $p = 0.003$ ) producing an unbounded LOEC value of 40  $\text{mg kg}^{-1}$  and a bounded NOAEC value ( $p = 0.820$ ) of 62  $\text{mg kg}^{-1}$  based on acetonitrile-extractable concentration. Statistically significant ( $p < 0.0001$ ) reduction in number of juveniles compared with carrier control occurred in 85  $\text{mg kg}^{-1}$  treatment (Table 4), which produced a bounded LOAEC value of 85  $\text{mg kg}^{-1}$  based on acetonitrile-extractable concentration (Table 5). The respective water-based extraction values were 25, 40, and 59  $\text{mg kg}^{-1}$ .

The logistic model with hormetic parameter (hormetic model) had the best fit for the data from toxicity tests with TNT freshly amended SSL soil due to stimulation of juvenile production at the lower treatment concentration described above (Figure 3). Juvenile production  $\text{EC}_{20}$  and  $\text{EC}_{50}$  values based on acetonitrile-extractable concentrations were 77 and 98  $\text{mg kg}^{-1}$ , respectively (Table 5). Juvenile production  $\text{EC}_{20}$  and  $\text{EC}_{50}$  values based on ATCLP-extractable concentrations were 52 and 69  $\text{mg kg}^{-1}$ , respectively (Table 5).

Coefficients of determinations ( $R^2$ ) from nonlinear regression analyses of the reproduction toxicity data using acetonitrile-extractable or water-extractable TNT concentrations in freshly amended SSL soil were compared to determine which chemical measure of exposure better correlated with toxicity. The values of raw and corrected (1-Residual/Corrected) coefficients were 0.987 and 0.967 for acetonitrile extraction, and 0.985 and 0.964 for water extraction, respectively (Table 5). These comparisons show that

coefficients were very similar for both extraction types indicating that data from both extraction methods had excellent correlation with the toxicity data for juvenile production, and that neither method had an advantage in characterizing TNT bioavailability to *E. crypticus*.

Table 4. Adult survival and juvenile production by *Enchytraeus crypticus* exposed to TNT in freshly amended (F-A) soil or to TNT weathered/aged (W-A) in amended soil.

F-A treatments (mg kg <sup>-1</sup> )	Mean Adults	Mean Juveniles	Standard Error	W-A treatments (mg kg <sup>-1</sup> )	Mean Adults	Mean Juveniles	Standard Error
Negative control	9.3	1202	62	Negative control	10.0	1851	27
Acetone control	10.0	1029	73	Acetone control	10.0	2120	52
Positive control	9.5	532	84	Positive control	9.5	764	39
40	10.0	1273	49	3	9.8	2662	253
62	9.8	1013	72	46	9.5	1125	69
85	9.8	746	52	66	10.0	617	79
134	8.3	217	18	94	8.5	276	60
186	8.3	35	20	105	7.5	160	61
287	6.2	0		137	5.5	0	

Table note: Concentrations are based on acetonitrile extraction using USEPA Method 8330.

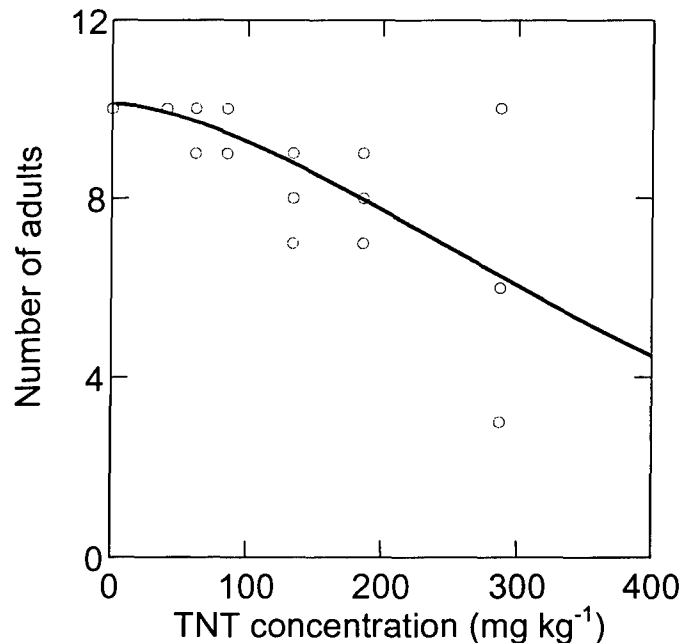


Figure 2. Effect of TNT on *Enchytraeus crypticus* adult survival in freshly amended Sassafras sandy loam soil.

Table 5. Ecotoxicological benchmarks (mg kg<sup>-1</sup>) for TNT determined in freshly amended Sassafras sandy loam soil using Enchytraeid Reproduction Test with *Enchytraeus crypticus*.

Extraction	Adult survival				Juvenile production			
	NOEC	LOEC	EC <sub>20</sub>	EC <sub>50</sub>	NOAEC	LOAEC	EC <sub>20</sub>	EC <sub>50</sub>
Acetonitrile	186	287	180	360	62	85	77	98
<i>p</i> or 95% C.I.	0.064	<0.0001	100-258	240-484	0.820	<0.0001	70-84	91-106
<i>R</i> <sup>2</sup> raw			0.985				0.987	
<i>R</i> <sup>2</sup> corrected			0.565				0.967	
ATCLP	137	215	130	280	40	59	52	69
<i>p</i> or 95% C.I.	0.064	<0.0001	72-192	177-372	0.820	<0.0001	47-58	63-75
<i>R</i> <sup>2</sup> raw			0.985				0.985	
<i>R</i> <sup>2</sup> corrected			0.568				0.964	

Table notes: Concentrations are based on acetonitrile extraction using USEPA Method 8330 and water extraction using Adapted Toxicity Characteristic Leaching Procedure (ATCLP). EC = effect concentration; C.I. = confidence intervals; NOEC = no-observed-effect concentration; NOAEC = no-observed-adverse effect concentration; LOEC = lowest-observed-effect concentration; LOAEC = lowest-observed-adverse effect concentration.

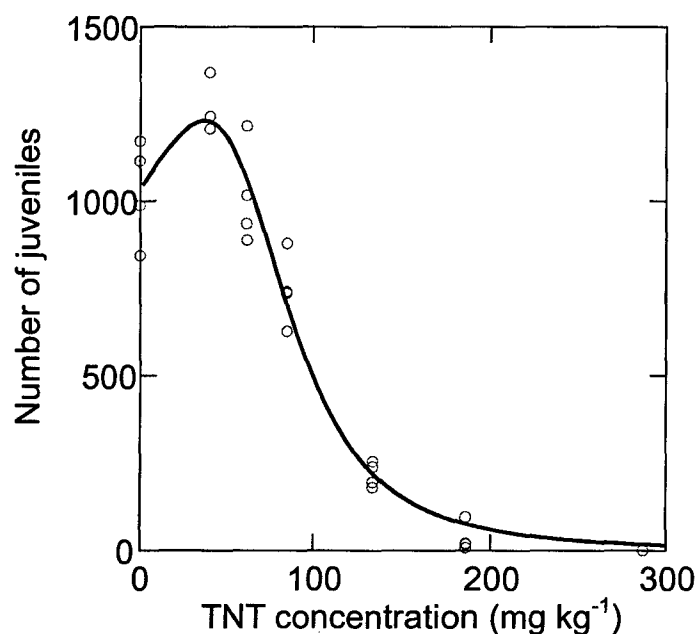


Figure 3. Effect of TNT on juvenile production by *Enchytraeus crypticus* in freshly amended Sassafras sandy loam soil.

### 3.3.2 Toxicity of TNT Weathered and Aged in Amended Soil.

Adult *E. crypticus* survival and juvenile production were affected by exposure to TNT weathered and aged in amended SSL soil (Table 4). For adult survival, the bounded NOEC and LOEC values based on acetonitrile-extractable concentrations were 94 ( $p = 0.185$ ) and 105 ( $p = 0.032$ )  $\text{mg kg}^{-1}$ , respectively (Table 6). The bounded NOEC and LOEC values based on water-extractable TNT concentrations were 30.8 and 31.0  $\text{mg kg}^{-1}$ , respectively. Concentration-response relationship for adult survival determined by nonlinear regression is shown in Figure 4. Logistic (Gompertz) model had the best fit for the data. Adult survival  $\text{EC}_{20}$  and  $\text{EC}_{50}$  values based on acetonitrile-extractable concentrations were 100 and 140  $\text{mg kg}^{-1}$ , respectively. Adult survival  $\text{EC}_{20}$  and  $\text{EC}_{50}$  values based on water-extractable TNT concentrations were 32 and 44  $\text{mg kg}^{-1}$ , respectively (Table 6). Weathering/aging of amended soil more than doubled the toxicity of TNT to *E. crypticus* adults based on  $\text{EC}_{50}$  values for survival. The differences between these values were statistically significant based on 95% confidence intervals (Tables 5 and 6).

Similar to the results of exposure in freshly amended soil, juvenile production in TNT amended and weathered/aged soil was the more sensitive indicator of TNT toxicity for *E. crypticus* compared with adult survival. Stimulation of juvenile production (observed in freshly amended soil) was also evident in weathered/aged soil but occurred at lower concentration of 3  $\text{mg kg}^{-1}$ . The 26 percent increase in the average number of juveniles compared with carrier control was similar in magnitude to the increase in freshly amended soil. The increase was statistically significant ( $p = 0.002$ ) producing an unbounded LOEC value of 3  $\text{mg kg}^{-1}$ . Considering the adverse effects only, the bounded NOAEC and LOAEC values were 3 and 46  $\text{mg kg}^{-1}$  based on acetonitrile-extractable concentrations (Table 6). The water-based ATCLP extraction values for bounded NOEC and LOEC were 0.3 and 0.4  $\text{mg kg}^{-1}$ , respectively. The bounded NOAEC and LOAEC values for water-based ATCLP extraction were 0.4 and 14.2  $\text{mg kg}^{-1}$ , respectively (Table 6).

Due to stimulation of juvenile production in the 3  $\text{mg kg}^{-1}$  treatment described above, the logistic hormetic model had the best fit for the data from toxicity tests with TNT weathered/aged in amended SSL soil (Figure 5). Juvenile production  $\text{EC}_{20}$  and  $\text{EC}_{50}$  values based on acetonitrile-extractable concentrations were 38 and 48  $\text{mg kg}^{-1}$ , respectively (Table 6). Juvenile production  $\text{EC}_{20}$  and  $\text{EC}_{50}$  values based on water-extractable concentrations were 12 and 15  $\text{mg kg}^{-1}$ , respectively (Table 6). Similar to the effect on adults, weathering/aging of amended soil doubled the toxicity of TNT to *E. crypticus* juvenile production. The differences between both exposure types based on  $\text{EC}_{20}$  and  $\text{EC}_{50}$  values were statistically significant at 95% C.I. (Tables 5 and 6).

Table 6. Ecotoxicological benchmarks ( $\text{mg kg}^{-1}$ ) for TNT weathered and aged in amended Sassafras sandy loam soil determined using the Enchytraeid Reproduction Test with *Enchytraeus crypticus*.

Extraction	Adult survival				Juvenile production			
	NOEC	LOEC	EC <sub>20</sub>	EC <sub>50</sub>	NOAEC	LOAEC	EC <sub>20</sub>	EC <sub>50</sub>
Acetonitrile	94	105	100	140	3	46	38	48
<i>p</i> or 95% C.I.	0.185	0.032	82-124	122-161	0.939	<0.0001	31-44	43-54
<i>R</i> <sup>2</sup> raw			0.977				0.980	
<i>R</i> <sup>2</sup> corrected			0.556				0.955	
ATCLP	30.8	31.0	32	44	0.4	14.2	12	15
<i>p</i> or 95% C.I.	0.185	0.032	25-38	38-50	0.939	<0.0001	10-14	13-16
<i>R</i> <sup>2</sup> raw			0.977				0.979	
<i>R</i> <sup>2</sup> corrected			0.544				0.955	

Table notes: Concentrations are based on acetonitrile extraction using USEPA Method 8330 or water extraction using Adapted Toxicity Characteristic Leaching Procedure (ATCLP). EC = effect concentration; C.I. = confidence intervals; NOEC = no-observed-effect concentration; NOAEC = no-observed-adverse effect concentration; LOEC = lowest-observed-effect concentration; LOAEC = lowest-observed-adverse effect concentration.

Coefficients of determinations ( $R^2$ ) from nonlinear regression analyses of the reproduction toxicity data using acetonitrile-extractable or water-extractable TNT concentrations in weathered/aged soil were compared to determine which chemical measure of exposure better correlated with toxicity. The values of raw and corrected (1-Residual/Corrected) coefficients were 0.980 and 0.955 for acetonitrile extraction, and 0.979 and 0.955 for water extraction, respectively (Table 6). These comparisons show that coefficients were very similar for both extraction types indicating that data from both extraction methods had excellent correlation with the toxicity data for juvenile production, and that neither method had an advantage in characterizing TNT bioavailability to *E. crypticus*.

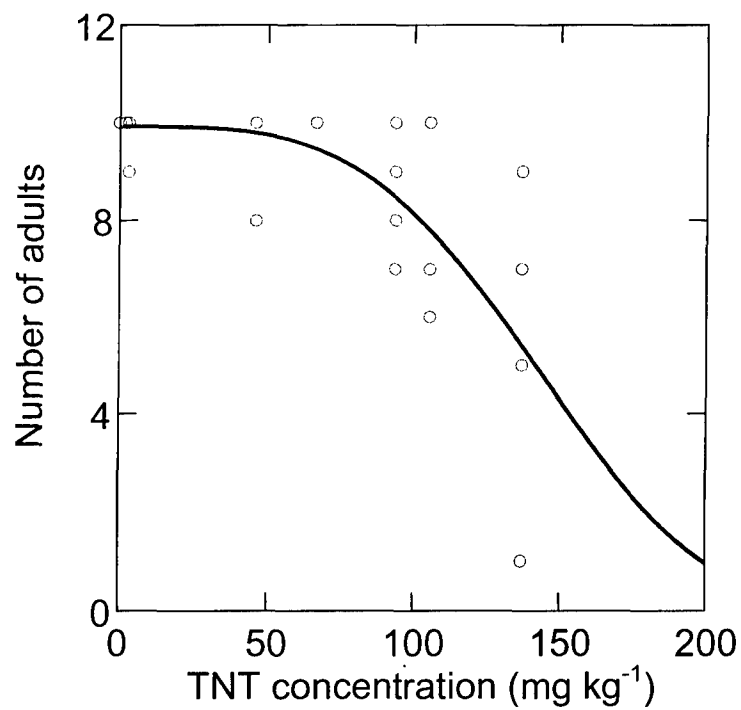


Figure 4. Effect of TNT weathered and aged in Sassafras sandy loam soil on *Enchytraeus crypticus* adult survival.

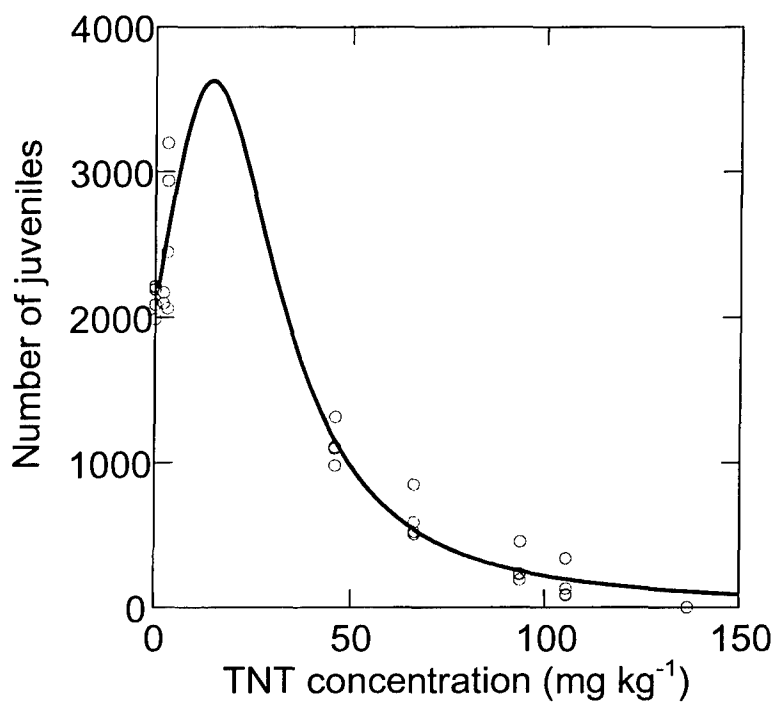


Figure 5. Effect of TNT weathered and aged in Sassafras sandy loam soil on juvenile production by *Enchytraeus crypticus*.

#### 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 (USEPA, 2003d). 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 TNT to generate Eco-SSL for soil invertebrates (USEPA, 2003d). Our toxicity studies designed to specifically fill this knowledge gap, used a natural soil that meets the criteria for Eco-SSL development, in large part because it has characteristics supporting relatively high TNT bioavailability. The weathering and aging procedure applied to soils amended with the range of TNT concentrations allowed us to determine the net ecotoxicological effect of complex fate processes in soil that affect bioavailability of TNT for the soil invertebrate *E. crypticus*, and to more realistically assess the toxicity under conditions more closely resembling the potential exposure effects in the field.

##### 4.1 Chemical Analysis for TNT 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, 2003d). In this study, the exposure concentrations of TNT in soil were analytically determined in definitive toxicity tests. Various methods are available for determining chemical concentrations in soil used for toxicity assessments. The commonly used "total-extractable" chemical measurements represent a potentially simplified estimate 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-extractable chemical levels are often not well correlated with bioaccumulation or toxicity (Linz and Nakles, 1997), until recently few alternatives to "total-extractable" bulk chemical measures were available. For this reason, identifying measures of exposure that may better represent the bioavailable fraction of TNT was included in this investigation. A better measure of the contaminant concentration immediately available to 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 TNT amended soils. These methods included acetonitrile extraction performed according to USEPA Method 8330 (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<sub>2</sub>-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 for TNT weathered/aged in amended soils.

Acetonitrile-extractable concentrations of TNT in freshly amended SSL soil averaged 88 percent of nominal concentrations indicating good correlation between nominal and measured TNT concentrations determined in our study after a 24-h equilibration period for soils hydrated to 60% of the WHC. Dodard *et al.* (2003) reported an average of 99% recovery in the study with OECD standard artificial soil immediately extracted following the amendment with nominal TNT concentrations ranging from 50 to 1000 mg kg<sup>-1</sup>. Such high recovery of TNT can be attributed to both the properties of the components of this synthetic soil, the absence or insufficient biotic transformation/degradation of TNT in dry artificial soil compared with hydrated natural SSL soil, and the absence of physical sorption processes driven by wetting/drying cycles of weathering. Lower extracted quantities of TNT compared with nominal TNT concentrations in studies with amended Lufa 2.2 soil (2.2% organic C, 5.8 pH, 78.1% sand, 15.4% silt, 6.7% clay) were observed by Schäfer (2002). The author reported that only 29 and 54 percent of the originally added TNT could be recovered using the diethyl ether extraction of 150 and 300 mg kg<sup>-1</sup> nominal soil treatments (concentrations that were also included in our study). The author attributed this decrease in recovery of TNT to a combination of factors, including sorption and subsequent binding of TNT to the soil matrix, and possible microbial degradation, which was indirectly evident from the presence of amino-dinitrotoluenes (ADNTs). Both mechanisms are corroborated by findings of Myers *et al.* (1998), who reported that sorption of TNT in soils with a wide range of physical properties was rapid, occurred on a time scale of a few minutes, and that the attainment of steady-state TNT concentrations was not possible as long as transformation continued. Major *et al.* (1992) suggested that time dependent disappearance of explosive-residues may be due to covalent or other non-equilibrium bonding to natural soil components, and therefore, analytical results for soils that were amended with explosives, air-dried, then immediately extracted, test primarily the “potential” efficiency of the extraction process. Overall, our results of chemical analysis confirmed that the soil amendment procedure used in toxicity tests was appropriate and that the USEPA Method 8330 was efficient for quantifying the amount of this energetic material in soil.

Special consideration in assessing TNT toxicity was given to the effects of weathering and aging of TNT in soil on the exposure of soil receptors. Assessment of the TNT toxicity included studies with TNT weathered and aged in amended soils to more closely simulate the exposure effects in the field locations where contaminants have been long-present. Weathering/aging of TNT in soil may reduce exposure of soil invertebrates to the parent material 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 freshly contaminated soils, or may reveal increased toxicity due to the presence of more toxic transformation products than parent compound freshly introduced into soil.

The 83-d weathering/aging of amended soils decreased overall acetonitrile-extractable TNT concentrations by approximately 40 percent compared with initial concentrations. The greatest decrease in TNT concentrations occurred in soils amended with the greatest amounts of added TNT. However, the greatest percentage decrease, averaging 80 percent, occurred in the lowest nominal treatment of 20 mg kg<sup>-1</sup>. Percentage decrease in TNT concentrations at the higher nominal treatment levels 75-180 mg kg<sup>-1</sup> was not as pronounced and was more uniform, averaging 30 percent from the initial acetonitrile-extractable TNT concentrations. Comparable results were found in studies with several nitroaromatic byproducts of TNT, including 1,3,5-trinitrobenzene (TNB); 2,4-dinitrotoluene (2,4-DNT); and 2,6-dinitrotoluene (2,6-DNT) weathered and aged in amended SSL soil under similar conditions (Kuperman *et al.*, 2004b). Recovery of TNB was inversely related to the initial acetonitrile-extractable concentration in amended soil. Greater than 80 percent decrease in TNB concentrations occurred in soils amended with concentrations below 100 mg kg<sup>-1</sup>, while above that treatment level TNB concentrations decreased by 20-30 percent. Weathering/aging of amended soil decreased acetonitrile-extractable 2,4-DNT and 2,6-DNT concentrations, on average, by 46 and 76 percent compared with respective concentrations in freshly amended soils used in that study (Kuperman *et al.*, 2004b). Considerably greater percentage decrease from the initial TNT concentrations due to ageing in amended Lufa 2.2 soil was reported by Schäfer (2002). No TNT was detected in the 150 mg kg<sup>-1</sup> nominal treatment incubated at 20°C for two months (68% of the initial TNT concentration remained in the same nominal treatment in our study), while TNT recovery from the portion of this treatment soil incubated at 4°C was 12 percent of nominal concentration. These results suggested that biotic transformation/degradation of TNT, which was indicated by the presence of ADNTs in soil, continued at a slower rate even at low temperatures (Schäfer, 2002).

TNT is resistant to electrophilic attack at the aromatic ring, as each of the three nitro groups withdraws electrons from the ring and renders the aromatic ring electron deficient (Rieger and Knackmuss, 1995). In contrast, the nitro groups are susceptible to reductive attack, leading to the reduction of the nitro groups as the major pathway for TNT degradation (Preuß and Rieger, 1995) and the formation of its associated hydroxylamino- and amino-nitrotoluene intermediates (Gorontzy *et al.*, 1994; Hawari *et al.*, 1998). As a consequence, TNT is converted in a stepwise process via the ADNTs and diaminonitrotoluenes (DANT) to triaminotoluene (TAT). Only the last step, the formation of TAT, is strictly anaerobic (Preuß and Rieger, 1995). Under aerobic conditions, different intermediates and condensation products like azoxy compounds can be formed, some of which are potentially more bioavailable and/or toxic than their precursors (Rieger and Knackmuss, 1995). Several of these products can be found in TNT-contaminated soil (Daun *et al.*, 1998; Frische, 2002), and few, including 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2,4-diamino-6-nitrotoluene (2,4-DANT) and 2,6-diamino-4-nitrotoluene (2,6-DANT) have been detected in earthworms *Eisenia andrei* and *Lumbricus terrestris* exposed to TNT contaminated soils (Johnson *et al.*, 2000; Renoux *et al.*, 2000; Robidoux *et al.*, 2000).

Identification of breakdown products of TNT in amended soils subjected to weathering/aging procedure was not included in the scope of current investigation because

we focused primarily on the net toxic effects of exposure of *E. crypticus* to TNT in an aerobic upland soil that supports relatively high bioavailability of organic compounds. However, amended SSL soil was analyzed for presence of metabolic transformation products from nitroaromatic EMs, in our studies with TNT byproducts (Rocheleau *et al.*, 2003). These analyses identified two transformation metabolites, including 2-amino-4-nitrotoluene (2-A-4 NT), and 4-amino-2-nitrotoluene (4-A-2 NT) in weathered/aged amended soil. Earlier studies showed that amino-dinitrotoluenes 4-ADNT and 2-ADNT were formed in TNT-amended OECD Standard Artificial Soil (Dodard *et al.*, 2003; 2004). The amino-nitrotoluene intermediates, which are the most commonly detected products of TNT degradation can be formed by the soil bacteria in either aerobic or anaerobic conditions (Hawari *et al.*, 1998). Bacteria capable of mineralizing nitrotoluenes, such as *Pseudomonas sp.* strain, have been isolated from a variety of contaminated soils (Spain, 1995). Nitrotoluenes are readily biotransformed by *Pseudomonas sp.* and eventually eliminated as nitrites (Spanggord *et al.*, 1991; Kaplan, 1992; Haidor and Ramos, 1996).

As nominal concentrations of TNT in soil were increased at higher treatment levels, the water-extractable concentrations of TNT increased proportionally with their respective acetonitrile-extractable concentrations in both freshly amended and in amended and weathered/aged SSL soils. Data analysis of ATCLP-based extractions of TNT in weathered/aged amended soils confirmed that the water-extractable portions of TNT in weathered/aged amended soils were considerably lower compared with freshly amended soils, a concomitant result of fate processes in the amended soils undergoing weathering and aging. Average ATCLP-extractable TNT concentrations in weathered/aged amended soils decreased from 70 in freshly amended soil to 26 in weathered/aged soil, similar to the average percent of acetonitrile-extractable TNT concentrations. Overall, chemical analyses demonstrated that TNT exposure conditions of *E. crypticus* in amended soils subjected to weathering/aging procedure differed from those of freshly amended soils. The inclusion of weathering/aging component in the TNT toxicity assessments allowed us to incorporate potential alterations in TNT bioavailability at contaminated sites in the development of ecotoxicological benchmarks for soil invertebrates.

Coefficients of determinations ( $R^2$ ) for acetonitrile 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 in order to determine which chemical measure of exposure better correlated with TNT toxicity. These comparisons showed that both extraction methods had excellent correlation with the toxicity data for juvenile production, and that neither extraction method had an advantage for characterizing bioavailability of TNT to *E. crypticus*. This was true for both freshly amended and weathered/aged amended soils. This result supports our decision of developing draft Eco-SSL for TNT for soil invertebrates on the basis of acetonitrile extraction. The acetonitrile extraction-based Eco-SSL values will be especially practical for Ecological Risk Assessment at contaminated sites because TNT concentrations determined during site characterization are typically based on acetonitrile extraction by the USEPA Method 8330.

Definitive studies using the Enchytraeid Reproduction Tests were conducted to assess the effects of TNT on the enchytraeid worm *E. crypticus*. Adult potworms were exposed to a range of TNT concentrations in freshly amended soil and to TNT weathered/aged in amended soil, in independent investigations. Results of these studies showed that weathering/aging of TNT in amended SSL soil significantly increased the toxicity of TNT to *E. crypticus*. The increase was approximately 200 percent and was evident for both the adult survival and reproduction measurement endpoints. EC<sub>20</sub> and EC<sub>50</sub> values for adult survival based on acetonitrile-extractable concentrations were 180 and 360 mg kg<sup>-1</sup>, respectively for TNT freshly amended in SSL soil, and 100 and 140 mg kg<sup>-1</sup>, respectively for TNT weathered and aged in amended SSL soil. These values for juvenile production were, respectively 77 and 98 mg kg<sup>-1</sup> for freshly amended soil, and 38 and 48 mg kg<sup>-1</sup>, respectively for weathered/aged soil. The reproduction measurement endpoint in both studies was more sensitive measure of TNT toxicity compared with adult survival, which comports with results reported in literature for potworms (Dodard *et al.*, 2003; Schäfer, 2002; Schäfer and Achazi, 1999), earthworms (Robidoux *et al.*, 2002; 2001; 2000; Phillips *et al.*, 1993), and collembola (Schäfer, 2002; Schäfer and Achazi, 1999). This supported the Eco-SSL requirement of using reproduction endpoints for toxicity benchmark development (USEPA, 2003d).

Juvenile production was stimulated at the lowest TNT concentrations tested in both freshly amended and in weathered/aged treatments. These hormetic responses (stimulatory effects caused by low levels of potentially toxic chemicals, followed by inhibitory effects at higher concentrations) of *E. crypticus* to TNT exposure were similar in magnitude and occurred at 40 and 3 mg kg<sup>-1</sup> treatment levels in freshly amended and in weathered/aged amended soils, respectively. The increase in both exposure types was statistically significant ( $p < 0.01$ ) producing the bounded NOAEC and LOAEC values of 62 and 85 mg kg<sup>-1</sup> in freshly amended soil, and 3 and 46 mg kg<sup>-1</sup> in weathered/aged amended soils, respectively based on acetonitrile-extractable TNT concentrations. Similar hormetic response by *E. crypticus* was observed in a study with TNB freshly amended in SSL soil (Kuperman *et al.*, 2004b). Stimulation of juvenile production was reported at 2.6 mg kg<sup>-1</sup> TNB concentration and resulted in the 19 percent increase in the average number on juveniles, which was comparable with results of our current study of TNT. Stimulation was also reported in TNT exposure studies for microbial nitrogen fixation activity at soil TNT concentrations of 200 and 400 mg kg<sup>-1</sup> (Gong *et al.*, 1999) and for offspring production by *Daphnia magna* exposed to 0.08 mg L<sup>-1</sup> TNT (Bailey *et al.*, 1985). Other explosives were also reported to elicit the stimulating effect on the measurement endpoints used in toxicity tests. Juvenile production by *E. crypticus* was stimulated by exposure to HMX in freshly amended SSL soil at concentrations ranging from 2,210 to 21,750 mg kg<sup>-1</sup> (Kuperman *et al.*, 2003, 2004b). Bentley *et al.* (1977), reported stimulation in egg production by fathead minnow exposed to 6.3 mg L<sup>-1</sup> RDX. Density of *Selanastrum capricornutum* cells, based on acetonitrile-extractable chlorophyll measures was increased following exposure to HMX ranging from 36-572 mg L<sup>-1</sup> (Bentley *et al.*, 1984). To date, no studies investigated the mechanisms responsible for hormetic stimulating effects of 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.

Results of our study showed that toxicity of TNT to *E. crypticus* was higher compared to nitramine explosives RDX and HMX in the same soil type. Kuperman *et al.* (2003, 2004b) reported juvenile production EC<sub>20</sub> values of 3,715 and 8,797 mg kg<sup>-1</sup> for RDX freshly amended in SSL soil and for RDX weathered/aged in amended SSL soil, respectively. HMX did not adversely affect *E. crypticus* up to the highest tested concentrations 21,750 mg kg<sup>-1</sup> in freshly amended SSL soil and 17,498 mg kg<sup>-1</sup> in weathered/aged amended SSL soil (Kuperman *et al.*, 2003, 2004b). In contrast, toxicity of RDX and HMX to the earthworm *Eisenia fetida* in freshly amended SSL soil was considerably greater compared with TNT toxicity to *E. crypticus*, with reported EC<sub>20</sub> values for earthworm cocoon and juvenile production of 1.2 and 0.4 mg kg<sup>-1</sup> in freshly amended SSL soil, respectively (Simini *et al.*, 2003). It is noteworthy that exposure of *E. fetida* to HMX weathered/aged in amended SSL soil did not affect cocoon or juvenile production up to the highest concentration 562 mg kg<sup>-1</sup> tested in that study (Simini *et al.*, 2003).

Because this study was designed to produce toxicity benchmark data for use in development of an Eco-SSL for TNT contaminated soils, the results of this study may not directly compare to those of other studies in the literature since none of those studies were designed to specifically quantify EM toxicity to soil invertebrates under Eco-SSL conditions of testing. The effects of exposure to TNT and its metabolites and byproducts in contaminated soils, composted explosives-contaminated soils, and experimentally amended soils have been investigated for a variety of terrestrial ecological receptors including soil microbial communities (Fuller and Manning, 1998; Gong *et al.*, 1999), plants (Peterson, 1996; Gong *et al.*, 1999; 2003; Robidoux *et al.*, 2003; Rocheleau, *et al.*, 2003), earthworms (Phillips *et al.*, 1993; Simini *et al.*, 1995; Simini *et al.*, 2003; Jarvis *et al.*, 1998; Renoux *et al.*, 2000; Robidoux *et al.*, 1999; 2000; 2001; 2002), enchytraeids (Schäfer and Achazi, 1999; Dodard *et al.*, 2003; Kuperman *et al.*, 2003, 2004b), collembola (Schäfer, 2002; Schäfer and Achazi, 1999), and nematode and microarthropod communities (Parmelee *et al.*, 1993). These studies have shown that soil organisms respond to TNT and/or its byproducts following exposure in soils.

Schäfer (2002) in a study with multiple soil types reported that *E. crypticus* was less sensitive to TNT exposure compared with *F. candida* and determined toxicity benchmarks for *E. crypticus* that were considerably higher compared with those determined in our studies reported herein. The respective reproduction EC<sub>50</sub> values for *E. crypticus* and *F. candida* were 501 and 64 mg kg<sup>-1</sup> in Lufa 2.2 soil (2.2% org C), and 277 and 23 mg kg<sup>-1</sup> in Lufa 2.3 soil (0.7% org C). However, these values may not be representative, due to problems encountered with the performance of both test organisms in soils used in those studies, including failure to meet validity criteria in several control treatments, high data variability, and low reproduction rates of species tested (Schäfer,

2002). Dodard *et al.* (2003) in a study with *E. albidus* using OECD standard artificial soil determined EC<sub>20</sub> and EC<sub>50</sub> values for TNT of 59 and 111 mg kg<sup>-1</sup> for juvenile production, both of which are not statistically different from those determined in our study with freshly amended SSL soil. For earthworms exposed in amended OECD standard artificial soil, TNT affected adult survival of the earthworm *E. andrei* with 14-d LC<sub>50</sub> of 365 mg kg<sup>-1</sup> (Robidoux *et al.*, 1999), and reproduction with 56-d LOEC of 111 mg kg<sup>-1</sup> (Robidoux *et al.*, 2000). Higher toxicity of TNT (compared to values in OECD artificial soil) with EC<sub>20</sub> value for juvenile production of 52 mg kg<sup>-1</sup> was determined by Robidoux *et al.* (2002) for *E. andrei* exposed in a sandy forest soil (3.8% organic matter, 83% sand, 8% clay, 7.6 pH). These results were comparable with findings for *E. crypticus* in our study with freshly amended SSL soil. The important role of soil properties in affecting bioavailability and toxicity of TNT and other EM soil contaminants to soil invertebrates has been emphasized in several studies (Kuperman *et al.*, 2003, 2004b; Phillips *et al.*, 1993; Robidoux *et al.*, 2002; Schäfer, 2002; Simini *et al.*, 2003) and is being further investigated in our ongoing studies. Lethal and sub-lethal (weight change) effects of TNT on the earthworm *E. fetida* have been reported by Phillips *et al.* (1993) for exposures in amended USEPA standard artificial soil, and a natural forest soil that had higher organic matter content compared with artificial soil. In these studies, LOEC values based on earthworm weight loss and nominal TNT concentrations were 140 and 150 mg kg<sup>-1</sup> for artificial soil and forest soil, respectively. Phillips *et al.* (1993) also reported 100 percent *E. fetida* mortality in USEPA standard artificial soil fortified with a mixture of EMs that included 30, 50, 62.5, and 20 mg kg<sup>-1</sup> of TNT, TNB, 2,4-DNT and 2,6-DNT, respectively. Statistically significant ( $p < 0.01$ ) sublethal effects on earthworm (weight loss) were observed at concentrations 6, 10, 12.5, and 4 mg kg<sup>-1</sup> of TNT, TNB, 2,4-DNT and 2,6-DNT, respectively. These results showed a higher toxicity of TNT compared with our investigations, however direct comparisons of data from among these studies are inappropriate due to differences in the experimental designs, particularly due to presence of contaminant mixtures in Phillips *et al.* (1993).

Although reported toxicological benchmarks for TNT for soil invertebrates are generally consistent, several factors contributed to variability observed among them. These include differences in physiologically related species-specific sensitivity of organisms tested, differences in the soil types used (with contrasting physical properties that affect sorption), abiotic and biological transformation/degradation pathways and resulting bioavailability of TNT in the exposure substrates, and the differences in experimental designs especially when non-standardized assays were used or when effects of contaminant mixtures were assessed. The use of Eco-SSL conditions of testing (USEPA, 2003d) employed in our investigations for generating the effects-based toxicity benchmarks for TNT minimized uncertainties that were commonly associated with selection and interpretation of reported ecotoxicological data for development of Eco-SSLs. Previous efforts of developing soil screening values that did not rely on rigorous data screening and acceptance procedures, such as those used for Eco-SSL development, may have produced data that underestimate the potential risk of TNT exposure for soil organisms. Renoux *et al.* (2001) proposed an interim Environmental Soil Quality Guideline (SQGe) for TNT of 86 mg kg<sup>-1</sup> for commercial/industrial land use. The proposed SQGe may provide inadequate protection for soil invertebrates; as is indicated by

comparison to EC<sub>20</sub> values developed in our studies based on *E. crypticus* reproduction values for TNT freshly amended soil (77 mg kg<sup>-1</sup>) and weathered/aged (38 mg kg<sup>-1</sup>) in soil. Other data also indicate that an SQGe of 86 mg kg<sup>-1</sup> proposed by Renoux *et al.* (2001) may not be sufficiently protective; including LOEC values of 2.3/3.3 mg kg<sup>-1</sup> for reproduction of *E. albidus* exposed to fresh/aged TNT, and a reproduction (fresh amendment) EC<sub>20</sub> of 59 mg kg<sup>-1</sup> for *E. albidus* (Dodard *et al.*, 2003); juvenile production EC<sub>20</sub> of 52 mg kg<sup>-1</sup> for *E. andrei* (Robidoux *et al.*, 2002); and reproduction EC<sub>50</sub> of 64 mg kg<sup>-1</sup> for *F. candida* (Schäfer and Achazi, 1999). This conclusion is also supported by a toxicity benchmark for TNT proposed by Sunahara *et al.* (2001). Using published toxicity data, those authors developed a sensitivity distribution of the sublethal effects (at the LOEC levels) of TNT on soil organisms using a cumulative frequency distribution approach. They linearized the toxicological data for the three major groups of soil biota, including microorganisms, plants, and soil invertebrates. Based on this approach, Sunahara *et al.* (2001) determined that TNT concentrations of less than 2 mg kg<sup>-1</sup> is protective for more than 90% of the soil organisms considered in their analysis. However, the authors acknowledged that more data is needed to increase confidence in this benchmark. Such approaches can be further improved if concentration-response based toxicity data, like the EC<sub>20</sub> measurement endpoints for chronic exposures employed in our investigations, are used in stead of often arbitrarily selected and unbounded LOEC values.

Very few published studies investigated the effects of weathering and aging of contaminant explosives in soil on the resulting exposure of terrestrial organisms. Kuperman *et al.* (2004b) reported that weathering/aging of amended SSL soils significantly increased the toxicity of 2,6-DNT to *E. crypticus*, while toxicities of 2,4-DNT and TNB were unaffected. In contrast, Dodard *et al.* (2003) found decreased TNT toxicity to *E. albidus* in OECD artificial soil following a 21-d aging period, and reported reproduction LOEC values of 2.3 and 3.3 mg kg<sup>-1</sup>, respectively, for freshly amended and aged soils. Direct comparison of these results to findings of our studies is difficult due to several factors, including other authors' use of different test species, artificial soil, and considerably shorter and undefined aging period. Additionally, the 21-d reproduction LC<sub>50</sub> values of 44 to 89 mg kg<sup>-1</sup> reported by the authors for freshly amended and aged soils, respectively, were estimated from only two positive TNT treatments and were based on nominal TNT concentrations. Results comparison to Dodard *et al.*, 2003 were further complicated by their respective decreases of 94 and 97 percent in measured TNT concentration even at the highest nominal TNT concentration 118 mg kg<sup>-1</sup> tested in that study. Decreased toxicity of TNT aged in Lufa 2.2 soil (considerably higher organic matter content compared with SSL) at 60% of the WHC and 20°C in the dark was reported for collembolan *F. candida* (Schäfer, 2002). Both adult 7-d LC<sub>50</sub> and juvenile 28-d EC<sub>50</sub> values increased steadily during the 2-month aging period (up to 6 months for adult mortality test). The 28-d EC<sub>50</sub> values for 0-d (freshly amended soil), 7-d, 15-d, 1-mo, and 2-mo aging periods were 64.3, 119.6, 155.3, 186.1, and 230.2 mg kg<sup>-1</sup>, respectively (Schäfer, 2002). Several factors may contribute to differential effects of TNT weathering/aging in amended soils on the toxicity to *E. crypticus* observed in our study, and the toxicity to *E. crypticus* and *F. candida* determined by Dodard *et al.* (2003) and Schäfer (2002). These include differences in properties of soils used in the studies, the weathering/aging procedures employed, and resulting effects on bioavailability of TNT

and its transformation/degradation products for the organisms tested. The effects of weathering and aging of amended soils on toxicity of TNT byproducts were also investigated for terrestrial plants. Rocheleau *et al.* (2003) reported significantly increased toxicity for Japanese millet (*Echinochloa crusgalli*) of 2,4-DNT, 2,6-DNT, or TNB weathered/aged in amended SSL soils under conditions similar to our study, and for perennial ryegrass (*Lolium perenne*) of 2,4-DNT weathered/aged in amended soil. Weathering and aging of either of these nitroaromatic EMs in amended soils did not affect their toxicity to alfalfa (*Medicago sativa*) (Rocheleau *et al.*, 2003).

Both the results of our studies and data available from the reviewed literature show that degradation/transformation of TNT weathered/aged in amended soil is affected by the initial concentration of TNT in soils, frequency of wetting/drying cycles, exposure to light, soil properties, temperature, and the duration of the weathering and/or aging processes. Specific mechanisms of changes in the toxicity of TNT in weathered/aged amended soil are unproven. Compounds produced due to TNT degradation or transformation 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. Lachance *et al.* (2004) investigated toxicities of the TNT reduction products 2-ADNT; 4-ADNT; 2,4-DANT; and 2,6-DANT for adult earthworm *E. andrei* using a 14-d exposure in amended sandy loam forest soil. The authors reported adult mortality LC<sub>50</sub> values for TNT, 4-ADNT, and 2-ADNT of 132, 105, and 215 mg kg<sup>-1</sup>, respectively, and gave the following order of toxicity: 4-ADNT > TNT > 2-ADNT. Dodard *et al.* (1999) investigated the aquatic 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*, while the reverse was true for *Vibrio fischeri*. Those 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-A-2-NT and 2-A-4-NT) 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 may exist in intermittently water-logged soil microsites, where more toxic metabolites of nitrotoluenes transformation may then be present. The higher toxicity of these metabolites may in part explain the increased toxicity of TNT weathered/aged in amended SSL soil that was observed in our study.

Overall results of our study showed that special consideration given to the effects of weathering and aging of TNT in soil for assessing toxicity was well justified. Benchmark values generated in this study will contribute to development of an Eco-SSL that better represents the exposure conditions of soil invertebrates at contaminated sites. Our findings of increased toxicity to *E. crypticus* of TNT weathered/aged in amended soil, and findings reported in the literature clearly show that additional studies are required to more completely investigate and resolve the toxicity of the TNT transformation and degradation products. Analogously, further investigation of the more toxic transformation

compounds that arise within soils amended with 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 also be designed to generate benchmark data for EM breakdown/transformation products so results may be used in 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.

## 5. CONCLUSIONS

This study has produced ecotoxicological data for TNT using ecologically relevant soil invertebrate species *E. crypticus*. The ecotoxicological parameters were determined using measured TNT 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, 2003d). Chemical analyses of freshly amended soils using the USEPA Method 8330 showed good correlation between nominal and measured acetonitrile-extracted concentrations confirming that the soil amendment procedure used in definitive toxicity tests was appropriate, and this method was efficient for quantifying the amounts of TNT in soil. The water-extractable portion of TNT, which was perceived to measure the immediately bioavailable fraction of chemical 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 both extraction methods had excellent correlation with the *E. crypticus* toxicity data for juvenile production, and that neither extraction method had a statistical advantage for characterizing bioavailability and toxicity of TNT to *E. crypticus*. This result supports our decision to recommend developing Eco-SSL 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 bioavailability of organic pollutants, for developing conservative Eco-SSL values (USEPA, 2003d). 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 TNT weathered/aged in amended soils differed from those of freshly amended soil. The inclusion of weathering/aging component in the toxicity assessment allowed us to assess the potential alterations in TNT bioavailability to *E. crypticus* 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 are required to investigate the toxicity of the TNT transformation products individually, or using chemical mixtures.

Measurement endpoints investigated in this study included adult survival and juvenile production. Study results showed that reproduction was 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-SSLs) workgroup for quality control review by the Eco-SSL task group before inclusion in the Eco-SSL database, and before being used in the development of an Eco-SSL for TNT for soil invertebrates.

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