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DEVELOPMENT OF TOXICITY BENCHMARKS FOR NITROGEN-BASED ENERGETIC MATERIALS FOR THE ENCHYTRAEID WORM, *ENCHYTRAEUS CRYPTICUS*

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14. ABSTRACT We investigated individual toxicities of nitrogen-based energetic materials (EMs) 2,4-dinitrotoluene (2,4-DNT), 2-amino-3,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), nitroglycerin (NG), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) to the potworm <i>Enchytraeus crypticus</i> using the Enchytraeid Reproduction Test (ISO 16387:2004). Studies were designed to generate ecotoxicological benchmarks for developing the ecological soil screening levels (Eco-SSLs) for risk assessments of contaminated soils, and to identify and characterize the predominant soil physicochemical parameters that affect the toxicity of 2,4-DNT to <i>E. crypticus</i> . Soils with a wide range of physicochemical parameters included: Teller sandy loam, Sassafra sandy loam, Kirkland loam, and Webster clay loam. Reproduction EC ₅₀ values (mg kg ⁻¹) for EMs weathered-and-aged (W-A) in sandy loam soils were 27 (2,4-DNT), 37 (4-ADNT), 103 (2-ADNT), 146 (NG), and >10,208 (HMX). Reproduction toxicity of 2,4-DNT W-A in soil, based on EC ₅₀ values, correlated strongly with soil organic matter and clay contents. Toxicity benchmarks established in TSL and SSL have been submitted to the U.S. Environmental Protection Agency Eco-SSL Workgroup for use in developing soil invertebrate-based Eco-SSLs for 2,4-DNT, 2-ADNT, 4-ADNT, and NG.					
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PREFACE

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DEVELOPMENT OF TOXICITY BENCHMARKS FOR NITROGEN-BASED ENERGETIC MATERIALS FOR THE ENCHYTRAeid WORM, *ENCHYTRAeus CRYPTICUS*

1. INTRODUCTION

The substantially increased demand for training resources is usually associated with increased environmental impacts at testing and training ranges due, in part, to the release of energetic materials (EMs). Consequently, soil contamination with explosives, propellants, and related materials at many U.S. military installations is widespread. By some accounts, more than 15 million acres of land have been contaminated with energetic materials (GAO, 2003). Among the common energetic residues found in soil are 2,4-dinitrotoluene (2,4-DNT), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and nitroglycerin (NG). 2,4-DNT does not mineralize once exposed to the environment either aerobically or anaerobically, but it can be environmentally transformed to a variety of nitroaromatic species (Jenkins, 2007; Monteil-Rivera et al., 2009). HMX does not degrade aerobically to any extent and is persistent in surface soils (Jenkins, 2007; Monteil-Rivera et al., 2009). Consequently, concentrations of these EMs in soil have been reported to exceed 117 mg kg⁻¹ for 2,4-DNT, and 3,000 mg kg⁻¹ for HMX (Simini et al., 1995). Partially reduced degradation products of 2,4,6-trinitrotoluene (TNT) and DNTs, 2-amino-4,6-dinitrotoluene (2-ADNT), and 4-amino-2,6-dinitrotoluene (4-ADNT) frequently co-occur in soil contaminated with nitroaromatic EMs (Kuperman et al., 2009a). NG can be released into the environment from the nitrocellulose matrix of solid propellants used in rockets and artillery ammunitions. It is mobile in soil due to its moderate aqueous solubility of 1.8 g L⁻¹ at 20 °C (Verscheuren, 1983; Pal and Ryon, 1986), and low partition coefficient values such as log K_{ow} of 1.62 (Sunahara et al., 2009) and log K_{oc} of 2.77 (Spanggard et al., 1980). Environmental assessments conducted at 23 military firing ranges in the United States and Canada identified NG as a soil contaminant at antitank rocket ranges, with concentrations in soil as high as 4700 mg kg⁻¹ (Jenkins et al., 2006). Notwithstanding the persistence of these EMs in soil, their effects on soil invertebrates have not been sufficiently investigated (Kuperman et al., 2009a). As a result, scientifically defensible screening values, which could be used in ecological risk assessment (ERA), are not currently available for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG in soil.

Assessment and protection of the terrestrial environment at defense installations can be advanced by developing and applying scientifically based ecological soil screening levels (Eco-SSLs; <http://www.epa.gov/ecotox/ecossl/>; last accessed December 2012) for energetic materials released into upland aerobic soil environments (USEPA, 2005). The Eco-SSLs are concentrations of contaminants in soil that are protective of ecological receptors that commonly come into contact with soil or ingest biota that live in or on such soils. These values can be used in the screening level ERA (SLERA) to identify those contaminants that are not of potential ecological concern in soils and thus do not require further evaluation in the baseline ecological risk assessment (BERA). Eco-SSLs are consistent national screening values, potentially resulting in cost savings during ecologically based site assessments and remedial investigations. Use of Eco-SSL values can help site managers to distinguish those sites that do not pose significant environmental risks from those that do, prioritize contaminated sites by the level of risk posed, quantify the relative risks at each site, and decide whether further investigation in a BERA is merited to determine appropriate remedial actions.

Eco-SSLs are derived using published data generated from laboratory toxicity tests with different test species that are relevant to soil ecosystems. Our extensive literature review (Kuperman et al., 2009a) showed that, despite considerable attention to assessing ecotoxicity of energetics, the available data for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG were insufficient to generate Eco-SSL values for soil invertebrates. To fill the existing data gaps, we conducted definitive studies designed to specifically meet the U.S. Environmental Protection Agency (USEPA) criteria (USEPA, 2005) for derivation of toxicity benchmarks acceptable for Eco-SSL development and expand the ecotoxicological data set, which can aid site managers in the knowledge-based decision-making process of securing the sustainable use of testing and training installations.

2. MATERIALS AND METHODS

2.1 Soil Collection and Characterization

Soils utilized in these studies included: Teller sandy loam (TSL) (fine-loamy, mixed, active, thermic Udic Argiustoll) collected from agricultural land of the Oklahoma State University Perkins Experiment Station, Payne County, OK; Sassafra sandy loam (SSL) (fine-loamy, siliceous, semiactive, mesic Typic Hapludult) collected from an open grassland field in the coastal plain on the property of the U.S. Army Aberdeen Proving Ground in Harford County, MD; Kirkland loam (KL) (fine, mixed, superactive, thermic Udertic Paleustoll) collected in Payne County, OK; and Webster clay loam (WCL) (fine-loamy, mixed, superactive, mesic Typic Endoaquoll) collected in Story County, IA. Several batches of SSL soil were used throughout the SERDP-funded projects. The SSL2000 soil batch was used for toxicity testing with 2,4-DNT during SERDP CU-1221 project. The SSL2007 soil was used for toxicity testing with 2-ADNT, 4-ADNT, and NG. The TSL2002 soil was used for toxicity testing with HMX. The qualitative relative bioavailability (QRB) scores for organic chemicals in natural soils were considered very high for TSL and SSL and medium for the KL and WCL utilized in these studies, according to the Eco-SSL criteria (USEPA, 2005).

During soil collection in the field, vegetation and the organic horizon were removed, and the top 12 cm of the A-horizon were then collected. Soil was sieved through a 5 mm screen, air-dried for at least 72 h, mixed periodically to ensure uniform drying, passed through a 2 mm sieve, then stored at room temperature before use in testing. The respective soils were then analyzed for physical and chemical characteristics (Table 1).

The SSL and TSL soils had sufficiently low organic matter and clay contents to fulfill the USEPA requirement for using soils with characteristics that support high relative bioavailability of organic pollutants, for developing realistic conservative Eco-SSL values (USEPA, 2005). Although ecotoxicological data determined in these soils can be representative of potential exposure effects in soils with similar chemical bioavailability conditions, such data can overestimate or underestimate the toxicities of EM in soil types with properties that contrast with those of SSL or TSL. Therefore, studies with additional natural soils (KL, and WCL) were conducted with 2,4-DNT to extend the range of soil physico-chemical characteristics,

hypothesized to affect EM toxicity (USEPA, 2005), in order to ascertain the relationships among predominant soil physico-chemical parameters and the toxicity of 2,4-DNT for soil invertebrates.

Table 1. Physical and Chemical Characteristics of Soils Used in Toxicity Testing

Soil Parameter	Teller Sandy Loam (TSL2002)	Sassafras Sandy Loam (SSL2000)	Sassafras Sandy Loam (SSL2007d)	Kirkland Loam (KL2006)	Webster Clay Loam (WCL2001)
Sand (%)	65	70	62	39	33
Silt (%)	22	13	25	42	39
Clay (%)	13	17	13	19	28
Texture	Sandy loam	Sandy loam	Sandy loam	Loam	Clay loam
Cation Exchange Capacity (CEC)(cmol kg ⁻¹)	4.3	5.5	7.8	13	21
Organic matter (%)	1.4	1.2	2.2	1.5	5.3
pH	4.4	5.2	5.0	5.7	5.9
Water Holding Capacity (WHC) (%)	13	18	18	20	23
QRB*	Very high	Very high	Very high	Medium	Medium

*Based on QRB scores for nonionizing organic contaminants in natural soils (USEPA, 2005).

2.2 Chemicals and Reagents

Nitrogen-based EMs used in the studies included the nitroaromatics 2,4-DNT [Chemical Abstracts Service (CAS) no. 121-14-2; purity 97%], 2-ADNT (CAS no. 35572-78-2; purity 99%), 4-ADNT (CAS no. 19406-51-0; purity 99%), NG (CAS no. 55-63-0; purity 99%), as well as the nitramine HMX (CAS no. 2691-41-0; purity 99%). These EMs were obtained from Defense Research and Development Canada-Valcartier (Quebec City, Quebec, Canada). High-performance liquid chromatography (HPLC)-grade acetone (CAS no. 67-64-1) was used to prepare individual EM solutions prior to amending soils. Acetonitrile (ACN; CAS no. 75-05-8; HPLC grade), methanol (CAS no. 67-56-1; chromatography grade; purity 99.9%), and calcium chloride (CaCl₂; CAS no. 10043-52-4; reagent grade) were used for the soil extractions and in analytical determinations by HPLC. Certified standards of EMs (AccuStandard, Inc., New Haven, CT) were used in HPLC determinations. ASTM Type I water (18 MΩ cm at 25 °C; ASTM, 2004a) was used throughout the toxicity studies. It was obtained using Milli-RO 10 Plus followed by Milli-Q PF Plus systems (Millipore, Bedford, MA). The same grade of water was used throughout the analytical determinations. Glassware was washed with phosphate-free detergent and sequentially rinsed with tap water, ASTM Type II water (>5 MΩ cm at 25 °C), analytical reagent-grade nitric acid 1% (v/v), and ASTM Type I water.

2.3 Soil Amendment Procedures

Studies were performed separately and independently for 2,4-DNT or NG in freshly amended (FA) soil and for 2,4-DNT or NG weathered-and-aged (W-A) in soil, to determine toxicity benchmark values for 2,4-DNT or NG in each exposure type. Studies were also performed separately and independently for 2-ADNT, 4-ADNT, and HMX W-A in soil, to determine the net effects of *Enchytraeus crypticus* exposure to each EM. During the soil amendment procedure, each EM was amended into separate aliquots of soil, using an organic solvent (acetone) as a carrier. This was necessary to distribute the EMs evenly and uniformly to a large soil surface area. Carrier control soils were amended with acetone only. Soil was spread to a thickness of 2.5 cm. Individual EMs were dissolved in acetone in glass volumetric flasks then pipetted across the soil surface, ensuring that the volume of solution added at any one time did not exceed 15% ($v w^{-1}$) of the soil dry mass. After the solution was added, the volumetric flask was rinsed twice with a known volume of acetone, and the acetone rinsate was also pipetted onto the soil. If the total volume of solution needed to amend the soil exceeded 15% ($v w^{-1}$), the solution was added in successive stages. The acetone was allowed to evaporate between additions for a minimum of 2 h within a darkened chemical hood. The same total EM/acetone solution volume at different EM concentrations was added to every treatment, to equal the volume required to dissolve EM at the greatest dissolved concentration amended. To prevent photolysis of the EM, amended soil was air-dried overnight (minimum of 18 h) in a darkened chemical hood. Each soil treatment sample was then transferred into a fluorocarbon-coated high-density polyethylene container and mixed for 18 h on a three-dimensional rotary soil mixer. After three-dimensional mixing, samples of FA soil were collected from each soil treatment batch and were sent overnight to Biotechnology Research Institute (BRI) for analytical determinations of the initial EM concentrations using USEPA Method 8330A (USEPA, 2007). Those soil treatments containing the FA EM were hydrated with ASTM Type I water to 100% of the respective water holding capacity (WHC) of each soil, and allowed to moisture-equilibrate for 24 h. Enchytraeid worms were then added for commencement of toxicity testing.

2.4 Weathering-and-Aging EMs in Soil

Explosives in soils at many contaminated sites have been subjected to weathering-and-aging processes onsite for many years. Therefore, special consideration was given to weathering-and-aging of EMs in soil for assessing their toxicities to *E. crypticus*, to provide appropriate benchmark data for Eco-SSL development. Standardized methods for weathering-and-aging of explosives in soil are not available. We have developed procedures that simulate, at least partially, the weathering-and-aging processes for chemicals in soil. These procedures allowed us to more accurately approximate the exposure conditions for soil biota in the field, compared with tests conducted with FA chemicals or tests conducted following a short equilibration period (e.g., 24 h) (Kuperman et al., 2003, 2005, 2006a, 2006b, 2006c, 2006d; Simini et al., 2003, 2006). Prior to additional toxicity testing, samples of each FA soil were initially hydrated with ASTM Type I water to 60% of the respective WHC to initiate weathering-and-aging of EMs in soil in open glass containers. Soil was then subjected to alternating hydrating and air-drying cycles at ambient temperatures in a greenhouse. All soil treatments were weighed and readjusted to their initial mass by adding ASTM Type I water each week. Any soil surface crusting that formed during the week was broken with a spatula before water was added. After completion of the EM weathering-and-aging procedures, all soil treatments were brought

to 100% of the WHC of each soil 24 h before commencement of toxicity tests. Soil samples collected from each treatment after the weathering-and-aging procedure, which corresponded to the beginning of the definitive toxicity tests, were sent overnight to BRI for analytical determinations of EM concentrations. The effects of weathering-and-aging of EMs in soil on toxicity to *E. crypticus* were investigated by comparing test results for EMs W-A in soils with results obtained using soils with FA EMs.

A pilot study of weathering-and-aging of NG in the SSL2007d soil was initiated prior to the definitive toxicity tests. Concentrations of NG were analytically determined at General Dynamics at the beginning, and after 1 and 2 months of the weathering-and-aging process to establish the times when NG concentrations were effectively stabilized. Due to rapid degradation of NG, the weathering-and-aging procedure was terminated after 30 d.

2.5 Extraction of EMs from Soil

Concentrations of EMs were analytically determined in all control and treated soils, in triplicate, at the beginning of each definitive test using ACN extraction and USEPA Method 8330A (USEPA, 2007). Samples for chemical analysis were hydrated for 24 h in accordance with Soil Amendment Procedures (Section 2.3) prior to extraction. Soil dry-fraction (dry weight/wet weight) was determined in triplicate from subsamples of each treatment concentration. For extraction, 2 g treatment and control samples were collected from each soil batch, and respective samples placed into 50 mL polypropylene centrifuge tubes, then 10 mL of ACN was added to each tube. Internal standards were added (100 μ L) to each tube to evaluate the extraction efficiency. Internal standards were 1,3-dinitrobenzene (1,3-DNB) for 2,4-DNT, 2-ADNT, and 4-ADNT; 2,4-DNT for HMX; and HMX for NG. Soil extraction was repeated if internal standard recovery was less than 90%. Samples were vortexed with ACN for 1 min, then sonicated in darkness for 18 h at 20 °C. After the sonicated samples had settled for 1 h at room temperature, 5 mL of each supernatant was transferred into glass tubes that contained 5 mL of CaCl_2 solution (5 g L^{-1}) as a flocculent. Supernatant was filtered through a 0.45 μm polytetrafluoroethylene (PTFE) syringe cartridge, and 1 mL of each filtered solution was transferred into an HPLC vial. Soil extracts were analyzed and quantified by HPLC.

2.6 Analytical Determinations of EMs in Soil

Soil extracts were analyzed and EM concentrations were quantified using a Waters (Milford, MA) HPLC system composed of a model 600 pump, a model 717 Plus injector, a model 2996 photodiode-array, and a temperature control module. Calibration curves were generated before each HPLC analysis using certified standards of each EM (AccuStandard, New Haven, CT or Cerilliant, Round Rock, TX), in a range of concentrations appropriate for each set of determinations. The respective limits of detection were 0.01, 0.005, 0.005, 0.034, and 0.05 mg L^{-1} for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG, corresponding to 0.1, 0.05, 0.05, 0.34, and 0.5 mg kg^{-1} (dry soil mass). All chemical concentrations in soil were expressed on dry mass basis. Nominal and analytically determined (measured) concentrations used in the definitive tests are shown in Tables 2–9.

Several soil invertebrate toxicity tests, for which standardized protocols have been developed by the International Organization for Standardization (ISO, 1998a, 1998b), can effectively be used to assess toxicity and derive protective benchmark values for EMs (Stephenson et al., 2002; Løkke and Van Gestel, 1998). We adapted the ISO 16387 bioassay, *Soil Quality: Effects of Pollutants on Enchytraeidae (Enchytraeus sp)—Determination of Effects on Reproduction and Survival* (ISO, 2004) to assess the effects of 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG on the enchytraeid worm *E. crypticus*. This test was selected on the basis of its use measuring chemical toxicity to ecologically relevant test species during chronic assays, and also because of its inclusion of at least one reproduction component among the measurement endpoints. Our adaptation of ISO 16387 consisted of its use with natural soils, and the enchytraeid worm *E. crypticus* as the test species. The ISO guideline for this assay was originally developed for use with Organisation for Economic Co-operation and Development (OECD, 1984) artificial soil [a similar soil formulation was later adapted for USEPA standard artificial soil (SAS) (USEPA, 1996); and for ASTM artificial soil (AS) (ASTM E1676-04, 2004b)]. However, several studies demonstrated that this test could also be conducted using natural soils (Amorim et al., 2005a, 2005b, 2009; Kuperman et al., 2003, 2004a, 2004b, 2005, 2006a–2006d). The ISO 16387 bioassay was initially developed using the enchytraeid worm species *Enchytraeus albidus*. Results of our previous studies using *E. albidus* showed that for optimal test conditions, this species requires soils containing high organic matter (OM) content with pH 6 (± 0.5). *E. albidus* performed poorly in natural soils having physical and chemical characteristics that support a higher level of EM bioavailability (Amorim et al., 2005a, 2005b, 2009; Kuperman et al., 1999, 2006a). *E. crypticus*, which is listed in the ISO protocol as an acceptable alternative to *E. albidus*, was therefore selected for toxicity testing.

Potworms were bred in 4.3 L clear plastic boxes (34 cm \times 20 cm \times 10 cm) filled with 2 kg (dry mass) moist SSL soil. The culture was kept in an environment-controlled incubator under a 16 h light–8 h dark photoperiod cycle with a mean photosynthetically active radiation (PAR) light intensity of $12.8 \pm 0.7 \mu\text{M m}^{-2} \text{s}^{-1}$ (985 ± 52 lux), and mean temperature of 21.6 ± 0.1 °C. Soil moisture level was adjusted to 100% of the WHC of SSL soil, 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 each week. The potworms were fed approximately twice each week with ground oats spread onto the soil surface. If food from the previous feeding date remained on the soil surface, the amount of food added was adjusted. Every 6 weeks, the worms were transferred into a freshly prepared culture substrate. Cultures were synchronized so that all worms used in each toxicity test were approximately the same age. The potworm culture was deemed healthy if worms were whitish in color, reproduced continuously, did not try to leave the soil, and exhibited a shiny outer surface with no soil particles clinging to them.

Glass vessels (jars; 42 mm i.d. \times 45 mm height) were used as test containers. Before the jars were used in the toxicity tests, they were cleaned with acetone, rinsed successively with tap water and ASTM Type I water, and then air-dried. Twenty grams (dry mass basis) of prepared treatment soil and 0.05 g of ground oats were added to each test container, which was then mixed, and hydrated to 100% of the WHC of each soil. The mass of each container with hydrated soil was recorded.

Adult potworms with eggs in the clitellum region were used for testing. They were collected from culture and placed in a Petri dish filled with a small amount of ASTM Type I water for examination with a stereomicroscope. Potworms with no eggs were discarded; any invertebrates living in the cultures (e.g., mites) were also removed. Ten potworms, selected for uniformity (approximately 1 cm in length), were placed on top of the prepared hydrated treatment soil in each test container. Transparent 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 test containers were placed in an environment-controlled incubator under a 16 h light–8 h dark photoperiod cycle with a mean PAR light intensity of $12.8 \pm 0.7 \mu\text{M m}^{-2} \text{sec}^{-1}$ (985 ± 52 lux) and a mean temperature of 21.6 ± 0.1 °C for the duration of the 28 d test. The containers were weighed once each week, and the mass loss was replenished with the appropriate amount of ASTM Type I water. At that time, 0.05 g of ground oats were added atop the soil within each test container.

After 14 d, 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 2 weeks of the test, was incubated for additional 14 d. After 28 d from the start of the test, soil in the test containers was fixed with 70% ethanol, and 9 drops of Rose Bengal biological stain (1% solution in ethanol) were added. Staining continued for at least 24 h. The content of each test container was then wet-sieved using a no. 100 mesh (150 μm) sieve, and retained contents were transferred to a counting tray where potworms were counted. Measurement endpoints included the number of adults surviving after 14 d and the number of juveniles produced after 28 d.

Treatment concentrations for each definitive test were selected based on the results of the range-finding tests, which were performed in order to bracket the 20 and 50% inhibition in juvenile production, compared with juvenile production in carrier controls for each soil. Based on the results of previous tests with SSL soil, the composite toxicity/Limit Test was conducted to assess the effects of HMX W-A in TSL soils on adult survival and production of juveniles by *E. crypticus*. The Limit Test is a definitive test variant to statistically compare treatment effects between the carrier (acetone) control treatment and the single greatest treatment concentration. It is performed when statistical analysis of the range-finding test data shows no significant differences among all treatment concentrations of a test chemical. Definitive tests included negative control (no chemicals added), carrier (acetone) control, and toxicity tests with a reference toxicant. Validity criteria for the negative controls in all tests included the following performance parameters (ISO, 2004):

- The adult mortality does not exceed 20% after 14 d,
- The average number of juvenile potworms per test container at the end of the test is greater than 2.5-fold the initial number of adult potworms per test container, and
- The coefficient of variation for the mean number of juveniles is $\leq 50\%$.

Toxicity tests with reference toxicant, boric acid (positive control), were conducted throughout the project using SSL soil to assess changes in sensitivity, health, and performance of *E. crypticus* maintained in U. S. Army Edgewood Chemical Biological Center (ECBC)

laboratory cultures. Test treatments were prepared by adding appropriate solutions of boric acid in ASTM Type I water to SSL soil to obtain nominal concentrations of 0 (negative control), 20, 30, 50, 80, 100, and 200 mg kg⁻¹. Nonlinear regression analyses of toxicity data from independent studies were used to establish the respective median effect concentration (EC₅₀) values and corresponding 95% confidence limits (CL) for juvenile production. These values were plotted on a Boric Acid Warning Chart, using modified procedures described by Environment Canada (EC, 2005), in order to monitor the condition of the potworms and precision within laboratory culture. The modification included using calculations based on untransformed EC₅₀ values for boric acid concentrations, instead of logarithmic concentrations.

2.8 Data Analyses

Data for adult survival and production of juveniles were analyzed separately using regression models selected from among those described in an EC guidance document (EC, 2005). During the model selection process, compliance with the normality assumptions and homoscedasticity of the residuals were determined by examining the stem-and-leaf graphs and histograms of the residuals. The best fit was evident when the regression lines generated by the models were closest to the data points; the regression coefficients for point estimates were the greatest; the residuals were homoscedastic (i.e., had most random scattering); and the means, standard errors, and variances of the residuals were the smallest. The models selected for data analyses in these studies were logistic (Gompertz; eq 1) or logistic hormetic (eq 2):

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

$$Y = \frac{a \times [1 + (h \times C)]}{1 + [(p + (h \times C)) \div (1 - p)] \times [C + EC_p]^b} \quad (2)$$

where

- Y* is the dependent variable for a measurement endpoint (e.g., number of juveniles or adults);
- a* is the y-axis intercept (i.e., the control response);
- e* is the exponent of the base of the natural logarithm;
- p* is the desired value for “*p*” effect (e.g., 0.50 for a 50% decrease from the control response; EC₅₀);
- C* is the exposure concentration in test soil;
- EC_p is the estimate of concentration for a specified percent effect;
- h* is the hormetic effect parameter; and
- b* is a scale parameter that defines the shape of the equation.

Data that exhibited hormesis, a concentration-response phenomenon characterized by a low-dose stimulation and high-dose inhibition (Calabrese, 2008), were fitted to the hormetic model. The EC_p (LC_p for lethal effects) parameters used in these studies included the EM concentration producing a 20% (EC₂₀ and LC₂₀) or 50% (EC₅₀ and LC₅₀) decrease in the measurement endpoint compared with the carrier control. The LC₅₀ and EC₅₀ parameters, commonly reported values, were included to enable comparisons of the results produced in these studies with results reported by other researchers. The 95% confidence intervals (CIs) associated with the point estimates were determined.

Analysis of variance was used to determine the bounded (when possible) no-observed-effect concentration (NOEC) and the lowest-observed-effect-concentration (LOEC) values for adult survival and juvenile production data, respectively. When no-observed-adverse-effect concentration (NOAEC) or lowest-observed-adverse-effect concentration (LOAEC) values were determined, the same statistical methods were used. Mean separations were done using Fisher's least-significant difference (FLSD) pairwise comparison tests. Student's *t*-test (two tailed) was used for pairwise comparisons in the limit tests. The relationships among the selected soil parameters and 2,4-DNT toxicity data were determined using Pearson's correlation analysis. All analyses were performed using untransformed data and analytically determined EM concentrations. A significance level of $p \leq 0.05$ (95% confidence level) was accepted for all statistical tests. Statistical analyses were performed using SYSTAT 11.0 (Systat Software, Inc., Chicago, IL).

3. RESULTS

3.1 Analytical Determinations of EMs in Soil

Concentrations of 2,4-DNT within amended soil were determined in TSL, KL, and WCL at the beginning of each definitive toxicity test using ACN-based extraction (Tables 2–4). Samples prepared for weathering-and-aging of 2,4-DNT in test soils were analyzed to determine the initial 2,4-DNT concentrations. These concentrations were then contrasted with 2,4-DNT concentrations at the end of the weathering-and-aging procedure to assess the net effect of weathering-and-aging of 2,4-DNT in soil on the exposure conditions for *E. crypticus* during respective toxicity tests, and to determine 2,4-DNT FA versus W-A in soil.

Mean values for 2,4-DNT W-A in soil and remaining ACN-extractable, calculated as percentages of corresponding initial concentrations of ACN-extractable 2,4-DNT in FA soils, were 71% in TSL, 49% in KL, and 61% in WCL. This indicates that a portion of 2,4-DNT was transformed/degraded, strongly sorbed onto soil, or affected by a combination of these processes during the weathering-and-aging period. Trace amount of 2,4-DNT was detected in TSL carrier control, likely resulting from cross-contamination of the sample.

Concentrations of 2-ADNT within amended SSL soil were determined at the beginning of each definitive toxicity test using ACN-based extraction (Table 5). Samples prepared for weathering-and-aging of 2-ADNT in soil were analyzed to determine the initial 2-ADNT concentrations. These concentrations were then contrasted with 2-ADNT concentrations at the end of the weathering-and-aging procedure to assess the net effect of weathering-and-aging of 2-ADNT in soil on the exposure conditions for *E. crypticus* during respective toxicity tests, and to determine 2-ADNT FA versus W-A in soil.

Table 2. Concentrations of 2,4-DNT FA and W-A in TSL Soil Used in Definitive Toxicity Tests with *E. crypticus*

Nominal Concentration (mg kg ⁻¹)	Initial (mg kg ⁻¹)			W-A (mg kg ⁻¹)			W-A/Initial (%)
		BDL			BDL		
Negative control		BDL			BDL		NA
Carrier control		BDL		0.4	±	0.1	NA
5	5.3	±	0.1	3.7	±	0.1	70
10	11	±	0.1	7.5	±	0.3	72
15	15	±	0.5	11	±	0.5	71
20	21	±	0.3	15	±	0.4	69
40	42	±	1	29	±	1	69
60	64	±	0.3	44	±	1	69
80	84	±	3	63	±	2	76
160	170	±	3	127	±	1	74

Note: Analytically determined concentrations (means and Standard Deviations [SDs]; $n = 3$) are based on ACN extraction (USEPA Method 8330A) of 2,4-DNT from soil.

NA, not applicable.

BDL, below detection limit (0.1 mg kg⁻¹ in soil).

Table 3. Concentrations of 2,4-DNT FA and W-A in KL Soil Used in Definitive Toxicity Tests with *E. crypticus*

Nominal Concentration (mg kg ⁻¹)	Initial (mg kg ⁻¹)			W-A (mg kg ⁻¹)			W-A/Initial (%)
		BDL			BDL		
Negative control		BDL			BDL		NA
Carrier control		BDL			BDL		NA
10	11	±	0.2	4.6	±	0.1	42
20	20	±	0.3	8.7	±	0.1	43
40	42	±	0.3	18	±	0.4	43
60	63	±	1	29	±	0.5	46
80	82	±	13.0	36	±	13	44
100	110	±	10	51	±	1.5	46
160	160	±	2	88	±	2	55
200	212	±	2	115	±	1	54
300	304	±	6	175	±	3	58
400	391	±	0.3	239	±	6	61

Note: Analytically determined concentrations (means and Standard Deviations [SDs]; $n = 3$) are based on ACN extraction (USEPA Method 8330A) of 2,4-DNT from soil.

NA, not applicable.

BDL, below detection limit (0.1 mg kg⁻¹ in soil).

Mean values for 2-ADNT W-A in SSL soil and remaining ACN-extractable, calculated as percentages of corresponding initial concentrations of ACN-extractable 2-ADNT in FA soil, ranged from 60% to 87%. The greatest percentage decrease (40%) occurred in the lowest 2-ADNT treatment, nominal 40 mg kg⁻¹.

Table 4. Concentrations of 2,4-DNT FA and W-A in WCL Soil Used in Definitive Toxicity Tests with *E. crypticus*

Nominal Concentration (mg kg ⁻¹)	Initial (mg kg ⁻¹)			W-A (mg kg ⁻¹)			W-A/Initial (%)
		BDL			BDL		
Negative control		BDL			BDL		NA
Carrier control		BDL			BDL		NA
20	21	±	2	14	±	0.2	67
60	64	±	2	39	±	0.2	61
80	80	±	5	54	±	2	68
100	109	±	3	65	±	1	60
160	175	±	7	97	±	2	55
200	219	±	11	115	±	4	53
300	339	±	9	189	±	5	56
400	440	±	26	260	±	11	59
600	677	±	19	447	±	4	67

Note: Analytically determined concentrations (means and Standard Deviations [SDs]; *n* = 3) are based on ACN extraction (USEPA Method 8330A) of 2,4-DNT from soil.

NA, not applicable.

BDL, below detection limit (0.1 mg kg⁻¹ in soil).

Table 5. Concentrations of 2-ADNT FA and W-A in SSL Soil Used in Definitive Toxicity Tests with *E. crypticus*

Nominal Concentration (mg kg ⁻¹)	Initial (mg kg ⁻¹)			W-A (mg kg ⁻¹)			W-A/Initial (%)
		BDL			BDL		
Negative control		BDL			BDL		NA
Carrier control		BDL			BDL		NA
40	35	±	2	21	±	4	60
60	53	±	4	34	±	1	64
100	90	±	4	58	±	7	64
120	105	±	2	76	±	3	72
140	131	±	5	90	±	4	69
160	142	±	3	99	±	2	70
180	166	±	2	120	±	1	72
400	359	±	10	314	±	8	87

Note: Analytically determined concentrations (means and Standard Deviations [SDs]; $n = 3$) are based on ACN extraction (USEPA Method 8330A) of 2-ADNT from soil.

NA, not applicable.

BDL, below detection limit (0.05 mg kg⁻¹ in soil).

Concentrations of 4-ADNT within amended SSL soil were determined at the beginning of each definitive toxicity test (Table 6). Samples prepared for weathering-and-aging of 4-ADNT in soil were analyzed to determine the initial 4-ADNT concentrations. These concentrations were then contrasted with 4-ADNT concentrations at the end of the weathering-and-aging procedure to assess the net effect of weathering-and-aging of 4-ADNT in soil on the exposure conditions for *E. crypticus* during respective toxicity tests, and to determine 4-ADNT FA versus W-A in soil.

Mean values for 4-ADNT W-A in SSL soil and remaining ACN-extractable, calculated as percentages of corresponding initial concentrations of ACN-extractable 4-ADNT in FA soil, ranged from 19% to 41%. This indicates that a greater portion of 4-ADNT (compared with 2-ADNT) was transformed/degraded, strongly sorbed onto soil, or affected by a combination of these processes during weathering-and-aging of 4-ADNT in SSL soil. The greatest percentage decrease (81%) occurred in the lowest 4-ADNT treatment, nominal 60 mg kg⁻¹. The percentage decrease in ACN-extractable 4-ADNT during the weathering-and-aging procedure was lower at greater nominal concentrations of 300–400 mg kg⁻¹ (Table 6).

Table 6. Concentrations of 4-ADNT FA and W-A in SSL Soil Used in Definitive Toxicity Tests with *E. crypticus*

Nominal Concentration (mg kg ⁻¹)	Initial (mg kg ⁻¹)			W-A (mg kg ⁻¹)			W-A/Initial (%)
Negative control		BDL			BDL		NA
Carrier control		BDL			BDL		NA
60	69	±	0.4	13	±	0.5	19
100	118	±	7	28	±	2	24
160	204	±	8	63	±	8	31
200	275	±	34	75	±	3	27
300	404	±	69	150	±	19	37
400	590	±	65	243	±	12	41

Note: Analytically determined concentrations (means and Standard Deviations [SDs]; $n = 3$) are based on ACN extraction (USEPA Method 8330A) of 4-ADNT from soil.

NA, not applicable.

BDL, below detection limit (0.05 mg kg⁻¹ in soil).

Definitive toxicity testing was conducted to assess the effects of HMX W-A in TSL soil on *E. crypticus*. Based on *E. crypticus* responses at HMX concentrations shown in Table 7, limit test design was selected for toxicity assessment. Mean values for HMX W-A in TSL soil and remaining ACN-extractable, calculated as percentages of corresponding initial concentrations of HMX in FA soil, ranged from 98% to 122%.

Table 7. Concentrations of HMX FA and W-A in TSL Soil Used in Definitive Toxicity Tests with *E. crypticus*

Nominal Concentration (mg kg ⁻¹)	Initial (mg kg ⁻¹)			W-A (mg kg ⁻¹)			W-A/Initial (%)
Negative control		BDL			BDL		NA
Carrier control		BDL			BDL		NA
100	70	±	15	72	±	44	103
1000	933	±	131	913	±	133	98
5000	4017	±	35	4888	±	328	122
10000	8833	±	522	10208	±	1143	116

Note: Analytically determined concentrations (means and Standard Deviations [SDs]; $n = 3$) are based on ACN extraction (USEPA Method 8330A) of HMX from soil.

NA, not applicable.

BDL, below detection limit (0.34 mg kg⁻¹ in soil).

Nominal concentrations selected for toxicity tests with NG FA in SSL soil were 0, 100, 200, 400, and 600 mg kg⁻¹. The measured NG concentrations FA in SSL soil at the beginning of the toxicity tests are presented in Table 8. There was a good correspondence between nominal and measured NG concentration in FA SSL soil.

Table 8. Concentrations of NG FA in SSL Soil Used in Definitive Toxicity Tests with *E. crypticus*

Nominal Concentration (mg kg ⁻¹)	Measured FA (mg kg ⁻¹)			Measured/Nominal (%)
Negative control		BDL		NA
Carrier control		BDL		NA
100	92	±	3	92
200	202	±	6	101
400	404	±	10	101
600	564	±	33	94

Note: Analytically determined concentrations (means and Standard Deviations [SDs]; $n = 3$) are based on ACN extraction (USEPA Method 8330A) of NG from soil. NA, not applicable. BDL, below detection limit (0.5 mg kg⁻¹ in soil).

Among all EMs tested in the present studies, NG W-A in SSL soil had the lowest recovery rate after 1 month of weathering-and-aging. Mean values for NG W-A in SSL soil, calculated as percentages of corresponding initial concentrations of NG in FA soil, ranged from 2% to 34%. This indicates that a substantial portion of NG was transformed/degraded, strongly sorbed onto soil, or affected by a combination of these processes during weathering-and-aging of NG in SSL soil. Percent recovery was directly related to nominal NG concentration in FA soil (Table 9). The greatest percentage decrease in NG concentrations (98%) occurred in the two lowest nominal NG treatments 100 and 140 mg kg⁻¹. Traces of a NG metabolite (1,2-DNG) were detected at nominal NG concentrations ≥ 200 mg kg⁻¹.

Table 9. Initial Concentrations of NG in FA SSL Soil and Concentrations of NG W-A in SSL Soil Used in Definitive Toxicity Test with *E. crypticus*

Nominal Concentration (mg kg ⁻¹)	Initial (mg kg ⁻¹)			W-A (mg kg ⁻¹)			W-A/Initial (%)
Negative control		BDL			BDL		NA
Carrier control		BDL			BDL		NA
100	96	±	2	1.8	±	0.1	2
140	124	±	3	2.4	±	0.1	2
160	160	±	2	6.2	±	0.4	4
250	242	±	2	22	±	0	9
300	299	±	6	36	±	1	12
400	404	±	8	122	±	3	30

Note: Analytically determined concentrations (means and Standard Deviations [SDs]; $n = 3$) are based on ACN extraction (USEPA Method 8330A) of NG from soil.

NA, not applicable.

BDL, below detection limit (0.5 mg kg⁻¹ in soil).

3.2 Toxicity of Reference Toxicant, Boric Acid, to the Potworm *E. crypticus*

Toxicity tests with reference toxicant, boric acid (positive control), were conducted throughout the project to assess changes in sensitivity, health and performance of *E. crypticus* maintained in ECBC laboratory cultures. Nonlinear regression analyses of toxicity data from independent studies with SSL soil were used to establish the respective EC₅₀ values and corresponding 95% CL for juvenile production. All determined EC₅₀ values were within both the Warning Limits of ± 2 SD and 95% CL established for *E. crypticus* culture in tests with boric acid (Figure 1). The coefficient of variation (CV) was 8.4% (less than CV of 20% suggested as reasonable by EC, 2005). These results confirmed that the condition of *E. crypticus* culture met the validity requirements of the test protocol.

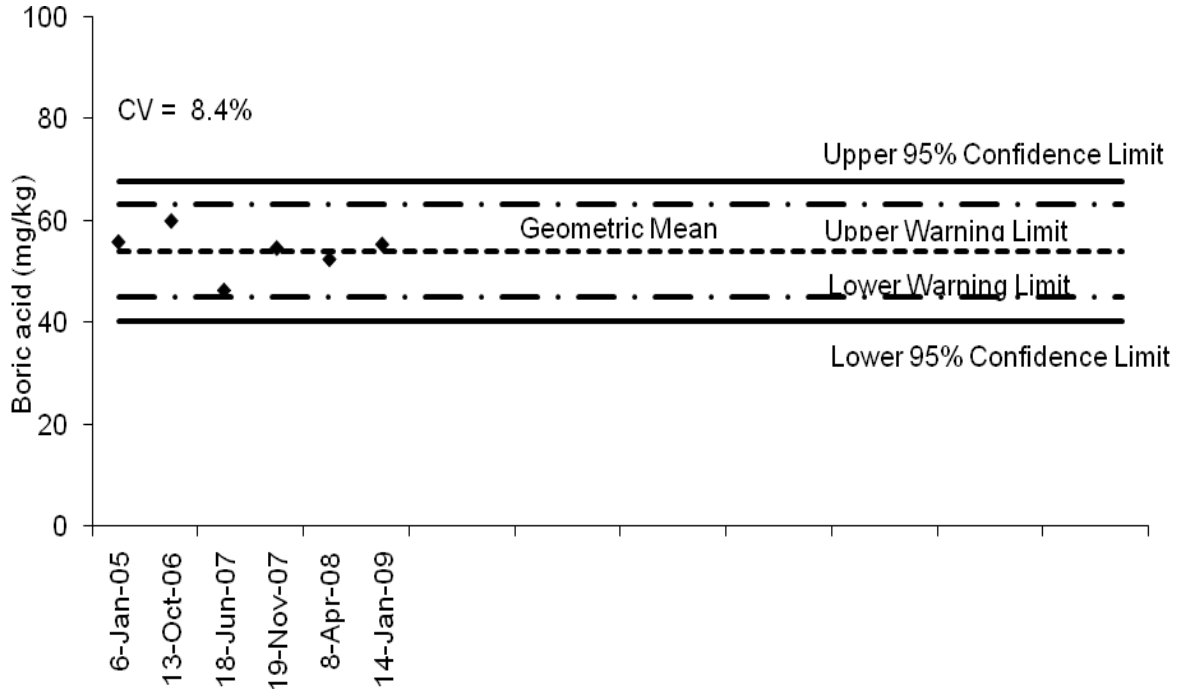


Figure 1. Warning Chart for the *E. crypticus* culture showing the EC₅₀ values for juvenile production established in the definitive tests with the reference toxicant, boric acid in Sassafras sandy loam soil.

3.3 Effects of 2,4-DNT on the Potworm *E. crypticus*

Definitive studies using the enchytraeid toxicity test in ISO 16387 (2004) were conducted to assess the acute (adult mortality) and chronic (juvenile production) effects of 2,4-DNT on the potworm *E. crypticus* in TSL, KL, and WCL soils. Toxicity data for 2,4-DNT established in our studies with SSL soil (corresponding soil batch designation SSL2000) were reported previously in Kuperman et al. (2006b) and are included in Tables 10 and 11 of this report for convenience of comparison with other soils reported herein. Within each type of soil, in independent investigations, adult potworms were exposed to a range of 2,4-DNT concentrations. Measurement endpoints were assessed using treatment concentrations that were based on the results of the range-finding studies. Measurement endpoints included number of surviving adults after 14 d and number of juveniles produced after 28 d. Exposure concentrations for each soil were selected for definitive tests to achieve bracketing of significant effects on reproduction endpoints (i.e., production of juveniles). Reproduction endpoints are preferred for the development of Eco-SSL values for soil invertebrates (USEPA, 2005), and were therefore the main focus of these studies. The ranges of exposure concentrations were expanded to allow determination of the concentrations that caused lethal effects to adults. All ecotoxicological

parameters were estimated using these measurement endpoint values and concentrations of 2,4-DNT in soil that were analytically determined utilizing USEPA Method 8330A (USEPA, 2007).

Treatment concentrations of 2,4-DNT in each soil were prepared as single batches for toxicity studies (Tables 2–4). Each batch was analyzed to determine 2,4-DNT concentration at the time of introducing the test species. Following treatment batch preparation (described earlier in this report), 100-g samples of FA soil were collected from each soil treatment batch and stored at $-80\text{ }^{\circ}\text{C}$ for three months prior to toxicity testing. Definitive toxicity testing with the FA TSL, KL, and WCL soils were conducted at approximately the same time (within one week) as testing of 2,4-DNT W-A in the respective soils to minimize potential seasonal variability in reproduction rates of *E. crypticus*. The TSL, KL, and WCL soil samples used in definitive toxicity testing with 2,4-DNT, either FA or W-A in soil, were hydrated with ASTM Type I water to 100% of the respective soil WHC (13, 20, and 23% of the TSL, KL, and WCL soil dry mass, respectively), and were allowed to equilibrate for 24 h before exposing the potworms.

Test results complied with the validity criteria defined in the ISO 16387 (2004) test guideline and those stipulated in Section 2.9 of this report. The respective validity criteria for test results from a single set of replicate negative control treatments used for concurrent studies of FA 2,4-DNT and for 2,4-DNT W-A in soil, were, respectively: TSL, mean adult survival 98%, mean number of juveniles produced 334, CV 24%; KL, mean adult survival 100%, mean number of juveniles produced 1921, CV 6 %; and WCL, mean adult survival 95%, mean number of juveniles produced 1857, CV 11%. Results of definitive tests with a reference toxicant, boric acid (positive control), are shown in Figure 1 and discussed in Section 3.2. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the 2,4-DNT treatments.

Ecotoxicological responses of *E. crypticus* to 2,4-DNT FA and to 2,4-DNT W-A in each soil are shown in Tables 10 and 11, respectively. Both adult survival and juvenile production were affected in 2,4-DNT-amended soils within the concentration ranges selected for definitive tests. Juvenile production was the more sensitive measurement endpoint for assessing 2,4-DNT toxicity to *E. crypticus* in all soil types tested, compared with adult survival, which comports with results of our previous studies. The logistic Gompertz model had the best fit for data in all toxicity tests, except for juvenile production data in test with 2,4-DNT W-A in TSL, where the logistic hormetic model had the best fit (Figures 2–7). Values for regression coefficients (R^2) determined for toxicity endpoints were ≥ 0.916 in tests with 2,4-DNT FA into soils, and ≥ 0.977 in tests with 2,4-DNT W-A in soils (Tables 10 and 11), indicating good fit of the models used for toxicity data.

Weathering-and-aging 2,4-DNT in sandy loam soils significantly (95% CI basis) decreased the toxicity to *E. crypticus* based on the EC_{20} values for juvenile production in TSL and the LC_{50} values for adult survival in SSL, compared to these effects levels in respective FA soils. In contrast, weathering-and-aging 2,4-DNT in clay loam soils significantly (95% CI basis) increased the toxicity to *E. crypticus* based on the EC_{50} values for juvenile production in KL and the LC_{50} values for adult survival in WCL, compared to these effects levels in respective FA soils (Tables 10 and 11).

Table 10. Ecotoxicological Benchmarks for Adult Survival and Juvenile Production by *E. crypticus* Exposed to 2,4-DNT FA in TSL, SSL, KL, and WCL Soils

Ecotoxicological Parameter	TSL (mg kg ⁻¹)	SSL* (mg kg ⁻¹)	KL (mg kg ⁻¹)	WCL (mg kg ⁻¹)
<i>Adult survival</i>				
NOEC	64	40.9	110	440
<i>p</i>	0.275	0.659	0.618	0.085
LOEC	84	55	160	677
<i>p</i>	0.004	0.013	<0.0001	<0.0001
LC ₂₀	32	57	207	473
CI (95%)	0-83	54-60	194-221	443-503
LC ₅₀	157	67	243	554
CI (95%)	42-272	64-70	230-257	529-579
Model used	Gompertz	Gompertz	Gompertz	Gompertz
<i>R</i> ²	0.942	0.994	0.992	0.996
<i>Juvenile production</i>				
NOEC	10.5	9.9	20	64
<i>p</i>	0.151	0.271	0.879	0.073
LOEC	15.4	20.3	42	80
<i>p</i>	0.010	0.037	0.022	0.004
EC ₂₀	9	19	52	189
CI (95%)	1-17	13-26	45-60	139-239
EC ₅₀	28	36	106	287
CI (95%)	15-40	30-41	98-113	249-325
Model used	Gompertz	Gompertz	Gompertz	Gompertz
<i>R</i> ²	0.916	0.980	0.994	0.967

*Modified from Kuperman et al. (2006b).

Notes: Concentrations of 2,4-DNT are based on ACN extraction (USEPA Method 8330A).

*R*², coefficient of determination.

Table 11. Ecotoxicological Benchmarks for Adult Survival and Juvenile Production by *E. crypticus* Exposed to 2,4-DNT W-A in TSL, SSL, KL, and WCL Soils

Ecotoxicological Parameter	TSL (mg kg ⁻¹)	SSL [†] (mg kg ⁻¹)	KL (mg kg ⁻¹)	WCL (mg kg ⁻¹)
<i>Adult survival</i>				
NOEC	44	37	87	260
<i>p</i>	0.697	0.711	0.591	0.664
LOEC	63	72	115	447
<i>p</i>	0.010	0.015	<0.0001	<0.0001
LC ₂₀	74	74*	184	404
CI (95%)	59–89	65–84	161–207	288–519
LC ₅₀	112	101	203	467*
CI (95%)	102–123	62–140	173–233	410–524
Model used	Gompertz	Gompertz	Gompertz	Gompertz
<i>R</i> ²	0.992	0.994	0.972	0.994
<i>Juvenile production</i>				
NOEC	14.5	5.2	4.6	65
<i>p</i>	0.174	0.318	0.732	0.254
LOEC	29	11.8	8.7	97
<i>p</i>	<0.0001	<0.0001	0.009	<0.0001
EC ₂₀	28*	14	38	122
CI (95%)	21–34	10–18	29–47	90–155
EC ₅₀	41	27	80*	228
CI (95%)	35–46	24–31	70–89	198–258
Model used	Hormetic	Gompertz	Gompertz	Gompertz
<i>R</i> ²	0.977	0.983	0.987	0.984

*Statistically significant (95% CI basis) change in toxicity following weathering-and-aging of 2,4-DNT in soil.

[†]Modified from Kuperman et al. (2006b).

Notes: Concentrations of 2,4-DNT are based on ACN extraction (USEPA Method 8330A). *R*², coefficient of determination.

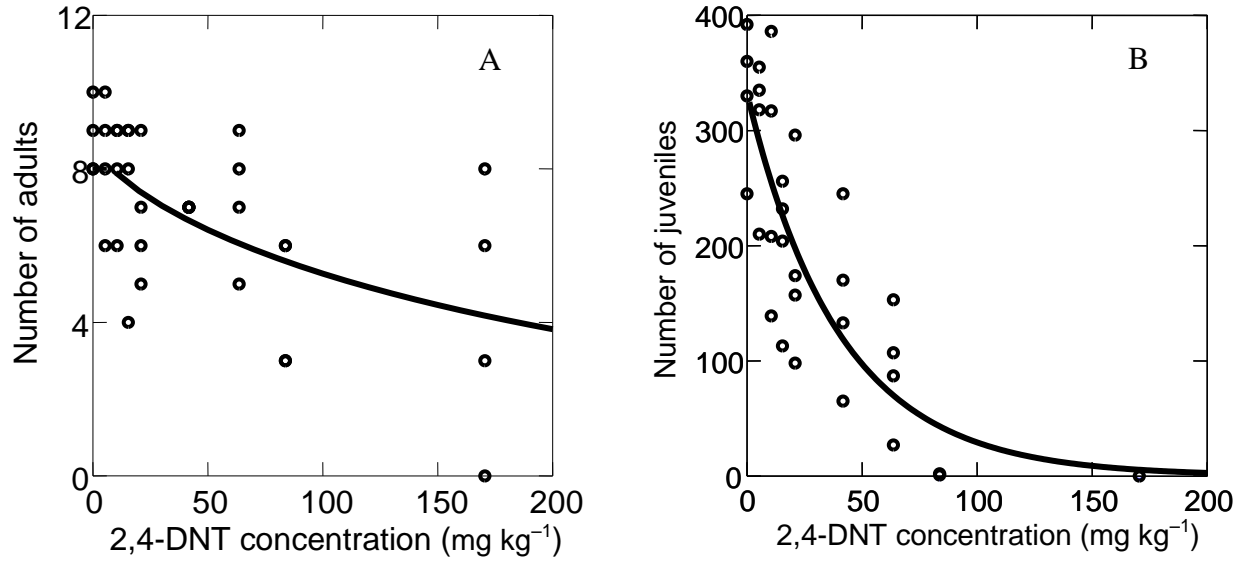


Figure 2. Effect of 2,4-DNT FA in TSL on adult survival (A) and on production of juveniles (B) by *E. crypticus*.

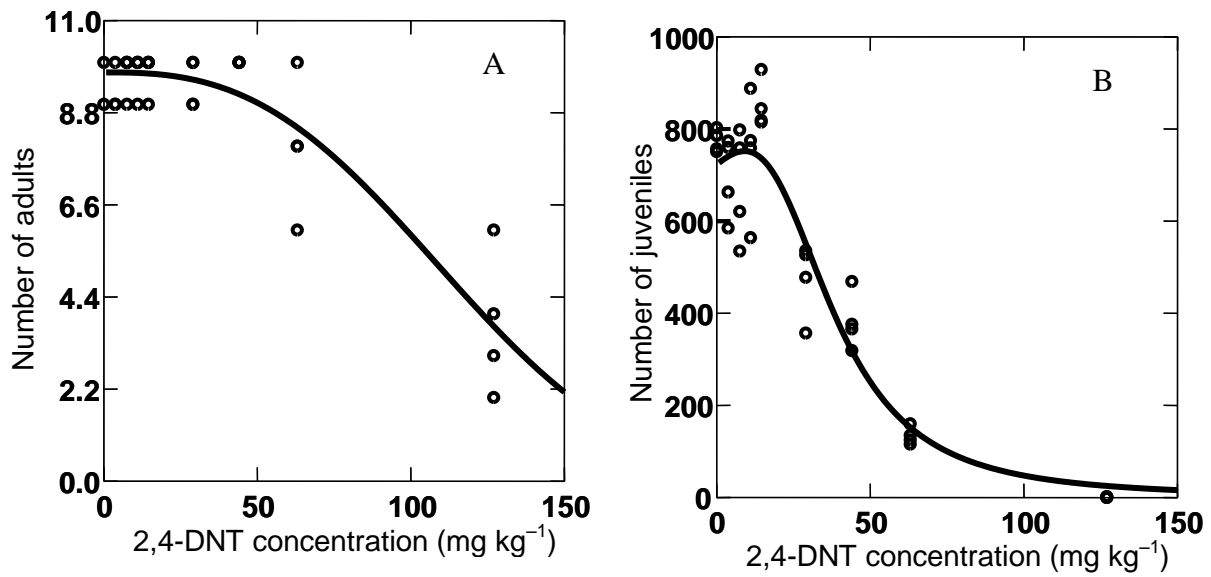


Figure 3. Effect of 2,4-DNT W-A in TSL on adult survival (A) and on production of juveniles (B) by *E. crypticus*.

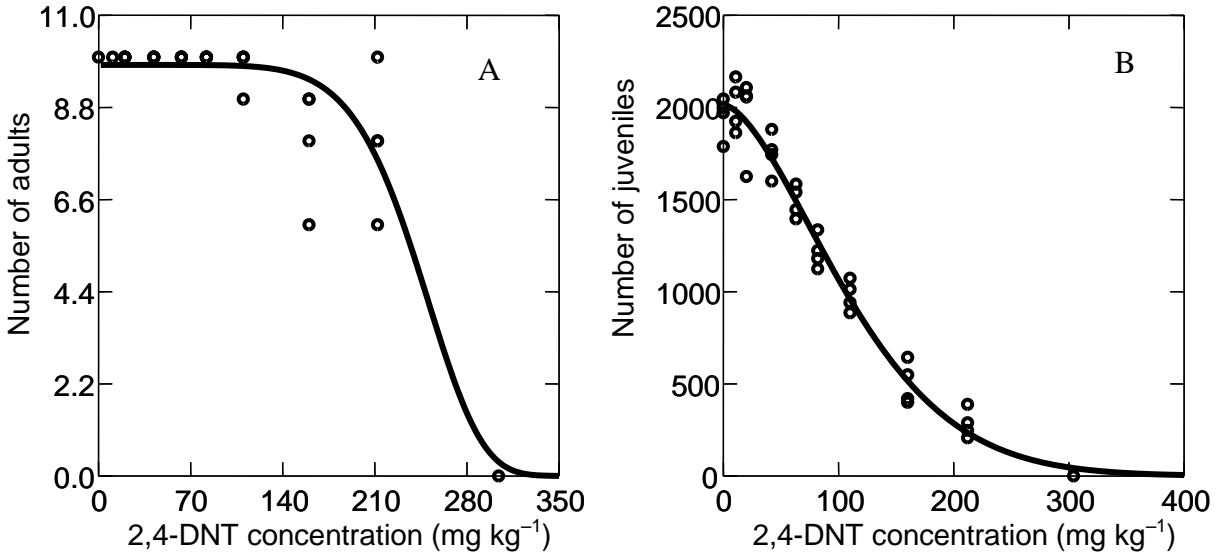
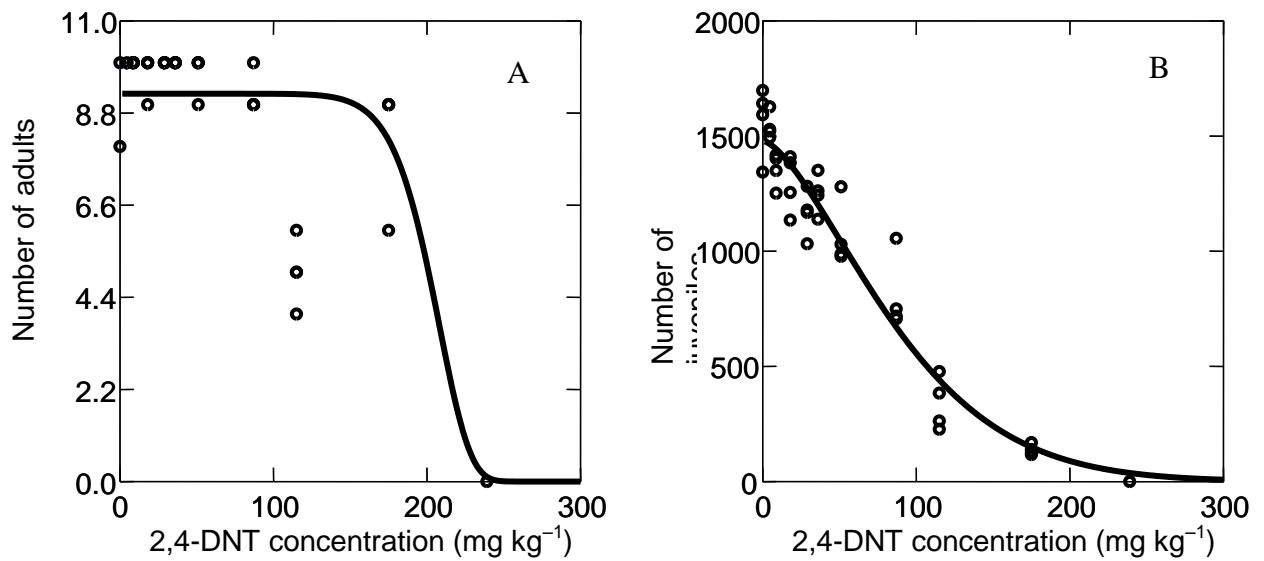


Figure 4. Effect of 2,4-DNT FA in KL on adult survival (A) and on production of juveniles (B) by *E. crypticus*.



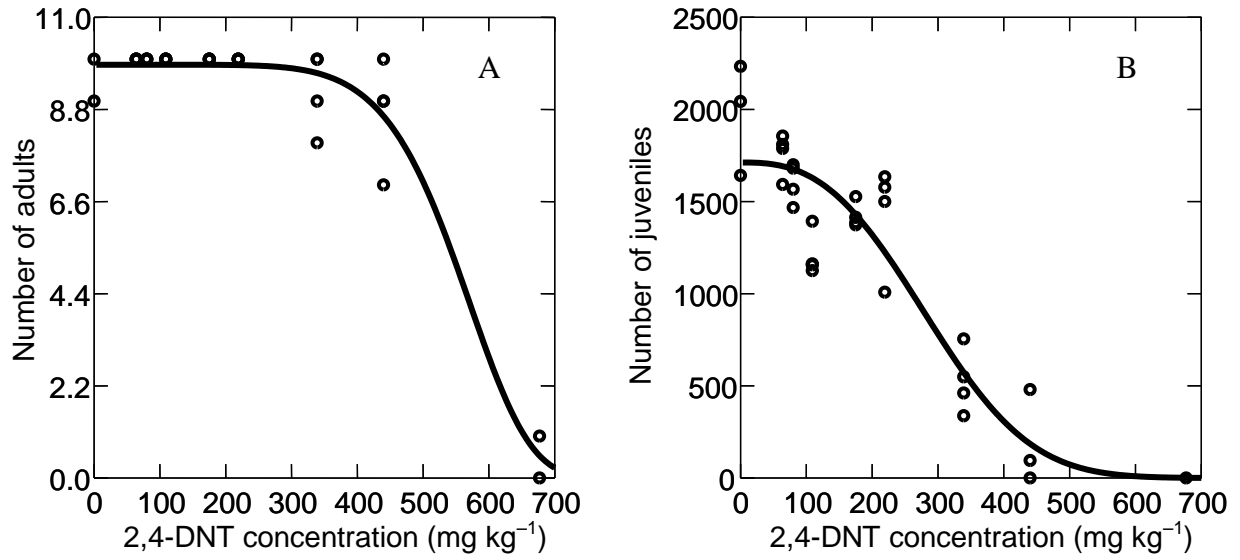


Figure 6. Effect of 2,4-DNT FA in WCL on adult survival (A) and on production of juveniles (B) by *E. crypticus*.

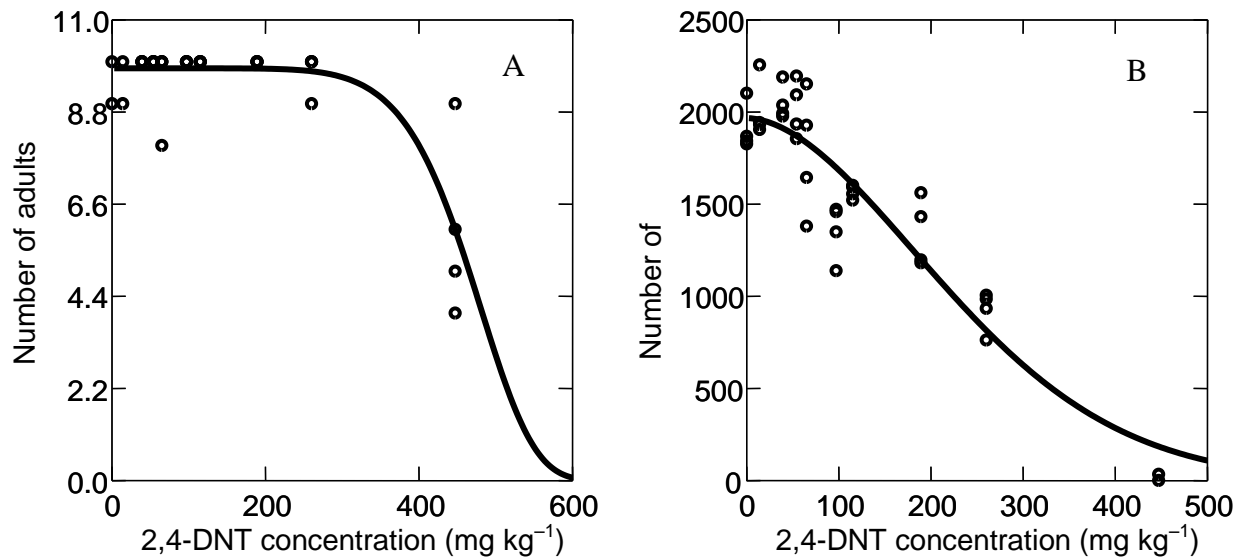


Figure 7. Effect of 2,4-DNT W-A in WCL on adult survival (A) and on production of juveniles (B) by *E. crypticus*.

3.4

Effects of Soil Properties on 2,4-DNT Toxicity

Toxicity of 2,4-DNT varied across the selected soils. Soil-related differences were evident in acute (adult survival) and chronic (juvenile production) toxicity benchmarks for 2,4-DNT FA or W-A in each of the four natural soils tested in these studies. Chronic toxicity (the main focus of these studies) to *E. crypticus*, based on the EC₅₀ values for 2,4-DNT FA into soil was in the order (from greatest to least toxicity; smallest to greatest EC₅₀ values): TSL > SSL > KL > WCL. The order for 2,4-DNT W-A in soil (a primary experimental design factor for developing Eco-SSL data) was: SSL > TSL > KL > WCL.

The effect of soil on 2,4-DNT toxicity was investigated by determining quantitative relationships between the concentration-response-based toxicity benchmark estimates for acute or chronic endpoints and soil property measurements, shown in Table 1. The relationships among the 2,4-DNT toxicity benchmarks for *E. crypticus* and soil properties were determined using Pearson's correlation analysis. All linear correlations were performed on the original (untransformed) data. Toxicity data for *E. crypticus* established in our previous studies with SSL (SSL2000) soil (Kuperman et al., 2006b) were included in this analysis. Pearson's linear correlation coefficients (*r*) and their respective probability (*p*) values are summarized in Table 12. There was no statistically significant collinearity (*p* = 0.076) between soil organic matter and clay measurements, which are key soil constituents that could affect bioavailability of 2,4-DNT. There was significant correlation between soil cation exchange capacity (CEC) and clay content (*r* = 0.960, *p* = 0.040; data not shown).

Table 12. Pearson Correlation Coefficients for Key Soil Properties and 2,4-DNT Toxicity Benchmarks for Acute (Adult Survival) and Chronic (Juvenile Production) Endpoints Determined in Definitive Tests with *E. crypticus*

Parameter	Clay Content		OM Content		pH		CEC	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
LC ₂₀ FA _{acute}	0.975	0.025	0.940	0.060	0.648	0.352	0.992	0.008
LC ₅₀ FA _{acute}	0.907	0.093	0.960	0.040	0.494	0.506	0.949	0.051
LC ₂₀ W-A _{acute}	0.966	0.034	0.958	0.042	0.596	0.404	0.982	0.018
LC ₅₀ W-A _{acute}	0.958	0.042	0.976	0.024	0.536	0.464	0.966	0.034
EC ₂₀ FA _{chronic}	0.973	0.027	0.983	0.017	0.523	0.477	0.955	0.045
EC ₅₀ FA _{chronic}	0.973	0.027	0.969	0.031	0.573	0.427	0.974	0.026
EC ₂₀ W-A _{chronic}	0.919	0.081	0.990	0.010	0.418	0.582	0.923	0.077
EC ₅₀ W-A _{chronic}	0.946	0.054	0.982	0.018	0.497	0.503	0.953	0.047

Notes: The *r* values with corresponding probabilities were determined using data from the definitive toxicity tests with SSL, TSL, KL, and WCL soils. Estimates of effect producing a 20% or 50% decrease in the measurement endpoint compared with acetone control were determined for 2,4-DNT FA and W-A in soil
CEC, cation exchange capacity.

Organic matter content of the soil was strongly ($r \geq 0.969$) and significantly ($p \leq 0.031$) correlated with all reproduction toxicity benchmarks for 2,4-DNT, and with adult survival benchmarks ($r \geq 0.958$; $p \leq 0.042$), except for the LC₂₀ benchmark in FA soil (Table 12). Soil clay content was strongly ($r \geq 0.907$) correlated with all toxicity benchmarks for 2,4-DNT. These correlations were statistically significant ($p < 0.05$), except for adult survival LC₅₀ benchmark in FA soil ($p = 0.093$), and the reproduction EC₂₀ or EC₅₀ benchmarks for 2,4-DNT W-A in soil ($p \leq 0.081$). Strong correlations were also detected for toxicity benchmarks and soil CEC ($r \geq 0.923$) (Table 12), which are likely a result of significant collinearity between clay and CEC found in soils used in the present studies. No significant ($p \geq 0.352$) correlations were found among any toxicity benchmarks for 2,4-DNT and soil pH. These results identified soil organic matter and clay contents as the dominant properties mitigating 2,4-DNT toxicity to *E. crypticus*.

3.5 Effects of Aminodinitrotoluenes on the Potworm *E. crypticus*

Definitive studies using the enchytraeid toxicity test in ISO 16387 (2004) were conducted to assess the acute (adult mortality) and chronic (juvenile production) effects of 2-ADNT or 4-ADNT on the potworm *E. crypticus* in SSL (SSL2007d) soil. Adult potworms were exposed to concentration ranges of each EM W-A for three months in SSL soil in independent investigations. Measurement endpoints were assessed using treatment concentrations that were based on the results of the range-finding studies. Measurement endpoints included number of surviving adults after 14 d and number of juveniles produced after 28 d. Exposure concentrations for each soil were selected for definitive tests to achieve bracketing of significant effects on reproduction endpoints (i.e., production of juveniles). Reproduction endpoints are preferred for the development of Eco-SSL values for soil invertebrates (USEPA, 2005), and were therefore the main focus of these studies. The ranges of exposure concentrations were expanded to allow determination of the concentrations that caused lethal effects to adults. All ecotoxicological parameters were estimated using these measurement endpoint values and concentrations of 2,4-DNT in soil that were analytically determined utilizing USEPA Method 8330A (USEPA, 2007).

Treatment concentrations of 2-ADNT or 4-ADNT in SSL soil were prepared as single batches for toxicity studies (Tables 5 and 6). Each batch was analyzed to determine EM concentrations at the time of introducing the test species. Test results complied with the validity criteria defined in the ISO 16387 (2004) test guideline and those stipulated in Section 2.9 of this report. The respective validity criteria for test results from negative control treatments used in individual studies of 2-ADNT or 4-ADNT W-A in soil, were: mean adult survival 100% and 98%, mean number of juveniles produced 941 and 304, and CV 11.8% and 17.3%. Results of definitive tests with a reference toxicant, boric acid, are shown in Figure 1, and discussed in Section 3.2. Compliance with the test validity criteria confirmed that the toxicological effects determined in respective definitive tests were attributable to the 2-ADNT or 4-ADNT treatments.

Ecotoxicological responses of *E. crypticus* to 2-ADNT or 4-ADNT W-A in SSL soil are shown in Table 13. Both adult survival and juvenile production were affected in EM-amended soils within the concentration ranges selected for definitive tests. Juvenile production was the more sensitive measurement endpoint for assessing 2-ADNT or 4-ADNT toxicity to *E. crypticus*, compared with adult survival. The logistic Gompertz model had the best fit for data in both toxicity tests (Figures 8-9). Values for R^2 determined for toxicity endpoints were ≥ 0.995 in

the test with 2-ADNT, and ≥ 0.979 in the test with 4-ADNT (Table 13), indicating good fit of the models used for toxicity data.

Table 13. Ecotoxicological Benchmarks for Adult Survival and Juvenile Production by *E. crypticus* Exposed to 2-ADNT or 4-ADNT W-A in SSL Soil

Ecotoxicological Parameter	2-ADNT (mg kg ⁻¹)	4-ADNT (mg kg ⁻¹)
<i>Adult survival</i>		
NOEC	120	75
<i>p</i>	0.166	0.749
LOEC	314	150
<i>p</i>	0.003	0.002
LC ₂₀	332	137
CI (95%)	199–465	115–159
LC ₅₀	878	185
CI (95%)	43–1713	169–202
Model used	Gompertz	Gompertz
<i>R</i> ²	0.995	0.988
<i>Juvenile production</i>		
NOEC	76	13
<i>p</i>	0.686	0.127
LOEC	90	63
<i>p</i>	0.001	<0.0001
EC ₂₀	76	21
CI (95%)	63–88	9–32
EC ₅₀	103	37
CI (95%)	95–110	26–47
Model used	Gompertz	Gompertz
<i>R</i> ²	0.972	0.979

Notes: Concentrations of 2-ADNT and 4-ADNT are based on ACN extraction (USEPA Method 8330A).
*R*², coefficient of determination.

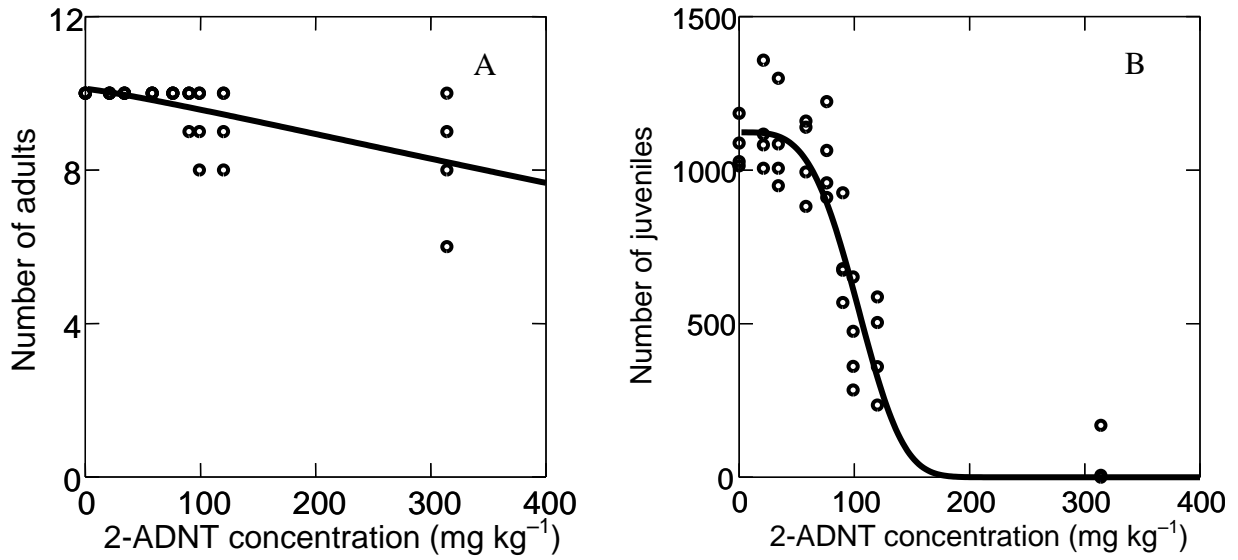


Figure 8. Effect of 2-ADNT W-A in SSL on adult survival (A) and on production of juveniles (B) by *E. crypticus*.

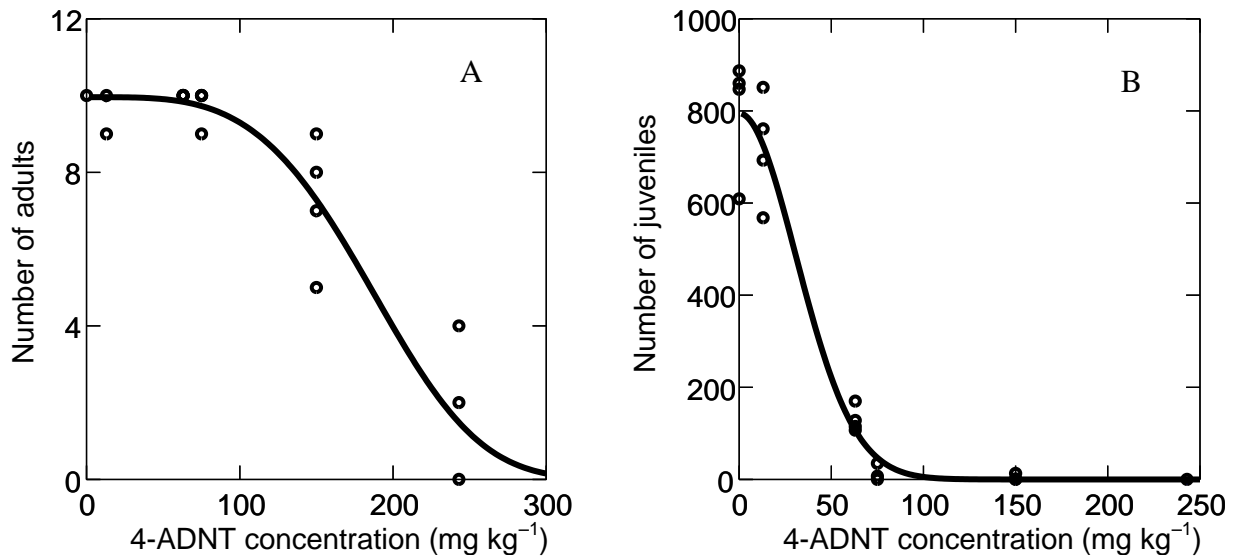


Figure 9. Effect of 4-ADNT W-A in SSL on adult survival (A) and on production of juveniles (B) by *E. crypticus*.

3.6

Effects of HMX on the Potworm *E. crypticus*

Definitive studies using the enchytraeid toxicity test in ISO 16387 (2004) were conducted to assess the effects of HMX on the potworm *E. crypticus* in TSL (TSL2002) soil. Adult potworms were exposed to HMX W-A for three months in TSL soil using the composite toxicity/limit test. Nominal HMX concentrations included 0 (negative control), 0' (acetone control), 100, 1000, 5000, and 10,000 mg kg⁻¹. The respective analytically determined HMX concentrations at commencement of the test are shown in Table 7. The following replication was used: eight replicates of 0' (acetone carrier control) and 10,208 mg kg⁻¹ treatments, and four replicates of the 0 (negative control), 72, 913, and 4888 mg kg⁻¹ treatments. Measurement endpoints included number of surviving adults after 14 d and number of juveniles produced after 28 d. All ecotoxicological parameters were estimated using these measurement endpoint values and HMX concentrations in soil that were analytically determined utilizing USEPA Method 8330A (USEPA, 2007).

Test results complied with the validity criteria defined in the ISO 16387 (2004) test guideline and those stipulated in Section 2.9 of this report. The validity criteria for test results from negative control treatment used in studies with HMX W-A in TSL soil, were: mean adult survival 93%, mean number of juveniles produced 412, and CV 27%. Results of definitive tests with a reference toxicant, boric acid, are shown in Figure 1, and discussed in Section 3.2. Compliance with the test validity criteria confirmed that the toxicological effects determined in this test were attributable to the HMX treatments.

Test results showed no statistically significant ($p > 0.05$) effect of HMX on adult potworm survival in any of the treatments tested in TSL soil, compared with the acetone carrier control. Respective adult potworm survival rates were 93, 98, 93, 100, 100, and 100% in HMX treatments 0, 0', 72, 913, 4888, and 10,208 mg kg⁻¹. Production of juveniles by *E. crypticus* in the greatest HMX treatment of 10,208 mg kg⁻¹ was not significantly different (t -Test; $p = 0.535$) from that in acetone carrier control. These results showed that exposure to HMX W-A in TSL for three months did not affect either adult potworm survival or the production of juveniles at $\leq 10,208$ mg kg⁻¹ (unbounded NOEC), thus confirming the toxicity data previously established in our studies with SSL soil (Kuperman et al., 2003).

3.7

Effects of NG on the Potworm *E. crypticus*

Definitive studies using the enchytraeid toxicity test in ISO 16387 (2004) were conducted to assess the acute and chronic effects of NG FA or W-A in SSL (SSL2007d) soil on the potworm *E. crypticus*. Measurement endpoints were assessed using treatment concentrations that were based on the results of the range-finding studies. Measurement endpoints included number of surviving adults after 14 d and number of juveniles produced after 28 d. Exposure concentrations for each soil were selected for definitive tests to achieve bracketing of significant effects on reproduction endpoints (i.e., production of juveniles). All ecotoxicological parameters were estimated using these measurement endpoint values and concentrations of NG in soil that were analytically determined utilizing USEPA Method 8330A (USEPA, 2007).

Test results complied with the validity criteria for negative controls defined in the ISO 16387 (2004) test guideline and those stipulated in Section 2.9 of this report. The respective

validity criteria for test results from negative control treatments used in individual studies of NG FA or NG W-A in soil, were: mean adult survival 98% and 98%, mean number of juveniles produced 948 and 912, and CV 14% and 19%. Results of definitive tests with a reference toxicant, boric acid, are shown in Figure 1, and discussed in Section 3.2. Compliance with the test validity criteria confirmed that the toxicological effects determined in respective definitive tests were attributable to the NG treatments.

Table 14. Ecotoxicological Benchmarks for Adult Survival and Juvenile Production by *E. crypticus* Exposed to NG FA or NG W-A in SSL Soil

Ecotoxicological Parameter	NG FA (mg kg ⁻¹)	NG W-A (mg kg ⁻¹)
<i>Adult survival</i>		
NOEC	202	122 [†]
<i>p</i>	1.0	0.056
LOEC	404	>122
<i>p</i>	<0.0001	ND
<i>Juvenile production</i>		
NOEC	<92	36
<i>p</i>	ND	0.136
LOEC	92	122
<i>p</i>	<0.0001	<0.0001
EC ₂₀	30	44
CI (95%)	13–46	11–77
EC ₅₀	76	146
CI (95%)	59–93	82–211
Model used	Gompertz	Gompertz
<i>R</i> ²	0.988	0.986

Notes: Concentrations of NG are based on ACN extraction (USEPA Method 8330A).

[†]Unbounded NOEC.

ND, not determined; could not be determined within the concentration range tested.

*R*², coefficient of determination.

Ecotoxicological responses of *E. crypticus* to NG FA or W-A in SSL soil are shown in Table 14. Results of the test with FA NG showed that numbers of surviving adult *E. crypticus* were not significantly ($p > 0.05$) different among the acetone carrier control, 92, and 202 mg kg⁻¹ treatments. No adults survived in the FA NG treatment concentrations ≥ 404 mg kg⁻¹. Juvenile production was significantly ($p < 0.0001$) decreased in the first positive FA NG concentration compared with the number of juveniles in the carrier control, producing an unbounded LOEC of 92 mg kg⁻¹ (Table 14). The range of FA NG concentrations selected for the test was sufficient to establish the concentration-response relationship based on juvenile production by *E. crypticus* (Figure 10). Nonlinear regression analysis of toxicity data yielded the EC₂₀ and EC₅₀ values and corresponding 95% CIs for juvenile production of 30 (13–46) and 76

(59–93) mg kg^{-1} , respectively. These results were used to determine concentrations for the definitive testing of NG W-A in SSL soil.

Definitive studies using the enchytraeid toxicity test in ISO 16387 (2004) were conducted to assess the acute and chronic effects of NG W-A in SSL soil on the potworm *E. crypticus*. Number of surviving adult *E. crypticus* was not affected by NG W-A in SSL up to and including the greatest concentration tested in this study, producing an unbounded NOEC of 122 mg kg^{-1} . The range of NG concentrations selected for the test was sufficient to establish the concentration-response relationships for juvenile production by *E. crypticus* (Figure 10). Juvenile production was the more sensitive measurement endpoint for assessing NG toxicity to *E. crypticus*, compared with adult survival, which comports with results of our previous studies. The logistic Gompertz model had the best fit for data in both toxicity tests (Figure 10). Values for R^2 determined for reproduction toxicity endpoints were 0.988 in test with NG FA into soil, and 0.986 in test with NG W-A in soils (Table 14), indicating good fit of the models used for toxicity data. Weathering-and-aging NG in SSL soil did not significantly affect the toxicity to *E. crypticus* based on the EC_{20} or EC_{50} values (and respective 95% CIs) for juvenile production (Table 14).

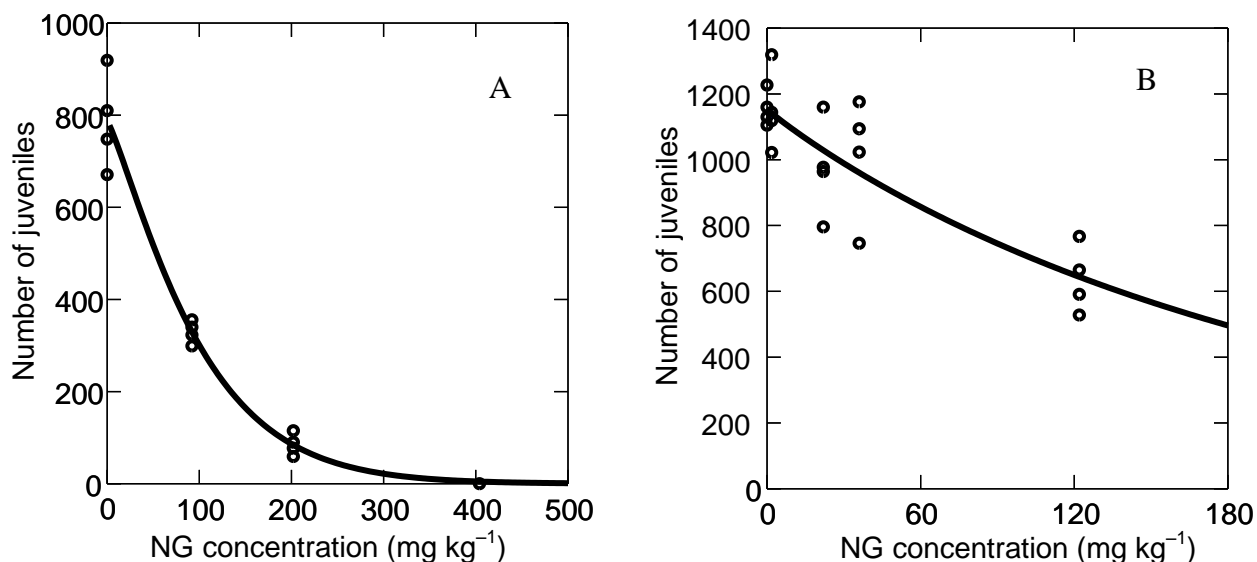


Figure 10. Effect of NG FA (A) and W-A (B) in SSL on production of juveniles by *E. crypticus*.

4. DISCUSSION

Development of ecotoxicological benchmarks for energetic soil contaminants has become a critical need in recent years (Kuperman et al., 2009b). These benchmarks are required for derivation of Eco-SSLs for use in ERA of contaminated sites (USEPA, 2005). The 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. These values can be used in the SLERA to identify those contaminants that are not of potential ecological concern in soils, and thus do not require further evaluation in the BERA, potentially resulting in cost-savings during ecologically based site assessments and remedial investigations. An extensive review of literature (Kuperman et al., 2009a) provided evidence that there was insufficient information existing for HMX and NG to generate Eco-SSLs for soil invertebrates. Furthermore, findings reported in the literature of increased reproduction toxicities for *E. crypticus* from TNT (Kuperman et al., 2012; 2005) and 2,6-dinitrotoluene (2,6-DNT) (Kuperman et al., 2006b) after weathering-and-aging these EMs in soil, clearly showed that additional studies were required to investigate the toxicities of the reduction products of these nitroaromatic EMs, including 2-ADNT and 4-ADNT. The present toxicity studies were designed to specifically fill these knowledge gaps, and to meet the criteria for Eco-SSL development. The weathering-and-aging procedure applied to EMs amended into soils, with a range of EM concentrations, allowed us to determine the net ecotoxicological effect of complex fate processes in soil that affect bioavailability of 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG for the soil invertebrate *E. crypticus*. This also allowed more realistic assessments of the respective toxicities under conditions more closely resembling the potential exposure situations in the field.

4.1 Analytical Determinations of EMs in Soil

The exposure concentrations of each EM in soil were analytically determined at the beginning of each definitive toxicity test using ACN extraction and USEPA Method 8330A (USEPA, 2007). Recovery of FA 2,4-DNT was $105 \pm 0.4\%$ (mean \pm SE; $n = 8$) of nominal concentrations in TSL, $103 \pm 1.1\%$ ($n = 10$) in KL, and $108 \pm 1.5\%$ ($n = 9$) in WCL soil. Recoveries in FA SSL soil were $90 \pm 0.8\%$ ($n = 8$) for 2-ADNT, $129 \pm 3.3\%$ ($n = 8$) for 4-ADNT, and $97 \pm 1.4\%$ ($n = 10$) for NG. Recovery of HMX was $83 \pm 5\%$ ($n = 4$) of nominal concentrations in FA TSL soil. These recovery rates indicated good correlations between nominal and measured EM concentrations determined in our studies after a 24 h moisture equilibration period for soils hydrated to 60% of the WHC. Overall, the results of analyses confirmed that the soil amendment procedure used in these toxicity tests was appropriate, and that USEPA Method 8330A was efficient for quantifying the respective EMs in soil.

The 3-month weathering-and-aging of 2,4-DNT in soils decreased 2,4-DNT concentrations in all soils tested. The residual concentrations were representative of 2,4-DNT concentrations found in contaminated soils at some former ammunition plants (Simini et al., 1995). The overall recovery of 2,4-DNT was 60% of initial concentrations in hydrated FA soils. The recovery of 2,4-DNT was in the order of: TSL (71%) > WCL (61%) > KL (49%). Within the soils, percent recovery was not affected by the initial 2,4-DNT concentration in amended treatments. This contrasted with the results found in studies with TNT and other nitroaromatic compounds (NACs), including 2,6-DNT, and 1,3,5-trinitrobenzene (TNB) W-A in natural soils under similar conditions (Kuperman et al., 2004a, 2005, 2006b; Renoux et al., 2000; Rocheleau et al., 2006, 2010). The recoveries of dinitrotoluenes were 70% for 2-ADNT and 29% for 4-ADNT. NG had the lowest recovery rate among all EMs tested in the present studies with an average of 10% remaining after 1 month of weathering-and-aging in SSL soil. These results indicate that a portion of EMs was transformed/degraded, strongly sorbed onto soil, or affected by a combination of these processes during the weathering-and-aging period. Overall, analyses

demonstrated that EM exposure conditions of *E. crypticus* in amended soils subjected to weathering-and-aging procedures differed from those of FA soils. The inclusion of these procedures in the assessments of 2,4-DNT, ADNTs, and NG toxicities to *E. crypticus* allowed us to incorporate potential alterations in chemical bioavailability and resulting toxicities at contaminated sites into the development of toxicological benchmarks for soil invertebrates.

In contrast with the fate of 2,4-DNT, ADNTs, and NG in amended soils, the HMX concentrations in TSL soil did not decrease during the 3-month weathering-and-aging process. These results are consistent with other studies that investigated fate and ecotoxicological effects of HMX in soils under aerobic conditions. Respective HMX recoveries averaging 80, 97, and 56%, after similarly performed weathering-and-aging procedures using SSL soil, were determined by Kuperman et al. (2003), Rocheleau et al. (2005), and Simini et al. (2006). These results confirm conclusions by Rosenblatt et al., (1991), and Hawari and Halasz (2002), that HMX transformation is limited under aerobic conditions.

4.2 Toxicities of EMs in Natural Soils

This project was undertaken to produce scientifically defensible toxicity data for the development of soil invertebrate-based Eco-SSL values for 2,4-DNT, 2-ADNT, 4-ADNT, NG, and HMX, and to investigate and characterize predominant soil physicochemical parameters that can affect the bioavailability and resulting toxicity of 2,4-DNT to soil invertebrates. To achieve the first objective, studies were designed to meet specific criteria (USEPA, 2005). Eco-SSL test acceptance criteria were met or exceeded in these investigations by ensuring that:

- (1) Tests were conducted in natural soils having physicochemical characteristics that support high relative bioavailability of EMs,
- (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 was specified and appropriate.

Definitive studies using the potworm *E. crypticus* exposures in TSL and SSL soils established new ecotoxicological data for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG effects on soil invertebrates under conditions of very high relative bioavailability for organic chemicals in soil (as defined in USEPA, 2005). The present studies also confirmed the toxicity data established for 2,4-DNT and HMX in our previous studies with SSL soil (Kuperman et al., 2003, 2004a, 2006b). Toxicological benchmarks for 2,4-DNT, and HMX, established in the present studies were generally consistent with data for soil invertebrates reported in a comprehensive review by Kuperman et al. (2009a). Acute toxicities of 2,4-DNT (either FA or W-A in soil, respectively) were not statistically different between TSL and SSL, based on the LC₂₀ or LC₅₀

values and respective 95% CIs for adult survival (Tables 10 and 11). Reproduction toxicity of 2,4-DNT was similar in FA TSL and SSL soils, but greater for 2,4-DNT W-A in SSL soil, based on the EC₂₀ or EC₅₀ values (Tables 10 and 11). Greater toxicity of 4-ADNT compared with toxicity of 2-ADNT determined in the present studies with *E. crypticus* comports with findings of Lachance et al. (2004), who established the following order of toxicity (from greatest to least): 4-ADNT > TNT > 2-ADNT in a 14-d study with earthworm *Eisenia andrei* in a sandy loam forest soil.

The juvenile production endpoint used in the present studies was a more sensitive measure of EM toxicity to *E. crypticus* in all soils tested, compared with the adult survival endpoint. This comports with results reported in literature for potworms (Dodard et al., 2003; Schäfer, 2002; Schäfer and Achazi, 1999; Kuperman et al., 2003, 2004a, 2004b, 2005, 2006b, 2006c), earthworms (Robidoux et al., 2000, 2001, 2002; Simini et al., 2003, 2006), and Collembola (Schäfer, 2002; Schäfer and Achazi, 1999). This finding supported the Eco-SSL requirement of using reproduction endpoints for toxicity benchmark development (USEPA, 2005). Overall, the present definitive studies using *E. crypticus* exposures in TSL or SSL soils established ecotoxicological benchmarks for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG, in compliance with Eco-SSL test acceptance criteria (USEPA, 2005), thus achieving the first objective of this investigation. These benchmarks will contribute to the dataset that will be used to derive the soil invertebrate-based draft Eco-SSL values for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG.

4.3 Effects of Soil Properties on 2,4-DNT Toxicity

The important role of soil properties in affecting bioavailability and toxicities of energetic soil contaminants to soil invertebrates has been emphasized in several studies (Kuperman et al., 2003, 2004a, 2005, 2006b, 2006c; Schäfer, 2002; Simini et al., 2003, 2006; Phillips et al., 1993; Robidoux et al., 2002). To achieve the second objective of the present studies, toxicity testing was conducted with additional natural soils (i.e., in addition to soils that support high relative bioavailability of EMs), including KL, and WCL, to extend the range of soil physicochemical characteristics that were hypothesized to affect the 2,4-DNT toxicity to soil invertebrates. The QRB scores for organic chemicals in natural soils were considered very high for TSL and SSL, and medium for KL and WCL soil, according to the Eco-SSL criteria (USEPA, 2005). Soil-related differences were evident in both acute (adult survival) and chronic (juvenile production) toxicity benchmarks established in the present studies with potworms exposed to 2,4-DNT W-A in each of the natural soils tested. Toxicity of 2,4-DNT for *E. crypticus* was greater in the light-textured sandy loam soils, compared with the more heavy-textured loam and clay loam soils. The order of acute and chronic toxicities for *E. crypticus* based on the LC₅₀ or the EC₅₀ values for 2,4-DNT W-A in soil was (from greatest to least toxicity): SSL ≥ TSL > KL > WCL, and generally paralleled the QRB scores.

The quantitative analyses of relationships among the acute or chronic toxicity benchmarks for 2,4-DNT and soil property measurements revealed that both the clay and OM contents of the soil affected the toxicity of 2,4-DNT to *E. crypticus*. Strong correlations were also detected for several survival and reproduction endpoints and soil CEC, which are likely a result of significant collinearity between clay and CEC found in soils used in the present studies.

No significant correlations were found among any toxicity benchmarks for 2,4-DNT and soil pH. These results identified soil organic matter and clay contents as the dominant properties mitigating 2,4-DNT toxicity to *E. crypticus*.

Results of the present studies comport with the findings of several published studies that suggest sorption of 2,4-DNT and related NACs in soil is a function of clay content (Emery et al., 2001; Haderlein et al., 1996; Singh et al., 2008), OM content (Anzhi et al., 1997; Eriksson and Skyllberg, 2001; Singh et al., 2010), or a combination of the two (Jaenig, 2006). Sorption of NACs to constituents of natural soils is not linear, and is dominated by strong and specific interactions with certain matrix components, rather than by hydrophobic partitioning (Monteil-Rivera et al., 2009). Among all matrix components commonly found in soils including clays, carbonates, quartz, aluminum, iron (hydr)oxides, and OM; clays were found to be strong sorbents for NACs (Daun et al., 1998; Esteve-Núñez et al., 2001; Haderlein et al., 1996; Weissmahr et al., 1997, 1998). NACs can be sorbed to uncharged regions of phyllosilicate clays through electron donor-acceptor complexes between oxygen atoms of the siloxane surface and the six-carbon ring of TNT, through pi-bonding (Haderlein et al., 1996; Weissmahr et al., 1998). Consequently, the adsorption of NACs can be strongly affected by exchangeable cations (Haderlein et al., 1996), which may partially explain significant correlation between the toxicity benchmarks for 2,4-DNT and soil CEC found in the present studies. In aqueous environments, adsorption of the NACs to clays is high when the exchangeable cations at the clays are a mixture that includes K^+ and NH_4^+ , but is negligible for homoionic Na^+ , Ca^{2+} , Mg^{2+} , and Al^{3+} clays (Haderlein et al. 1996; Weissmahr et al., 1997). Furthermore, the affinity and the adsorption capacity of the clays for NACs increase in the order kaolinite < illite < montmorillonite. Thus, clay minerals, plus their abundance and degree of K^+ - and NH_4^+ - saturation, can control the phase distribution and bioavailability of NACs in soils (Haderlein et al., 1996).

NACs and their metabolites were also shown to react and sorb to OM in the soil (Eriksson and Skyllberg, 2001; Esteve-Núñez et al., 2001; Singh et al., 2010; Thorn and Kennedy, 2002; Xing and Pignatello, 1997; Weiß et al., 2004). Sorption studies with low-polarity organic compounds, including nitroaromatic energetic materials, have shown that binding of these compounds to both soil OM (Xing and Pignatello, 1997) and silicate clays (Haderlein et al., 1996) is competitive, selective, nonlinear, and frequently reversible. Overall, the aforementioned mechanisms affecting the fate of 2,4-DNT in soil are consistent with findings of the present studies that identified soil organic matter and clay as the dominant properties mitigating 2,4-DNT toxicity for potworms.

4.4 Effects of Weathering-and-Aging EMs in Soil on Toxicity

In addition to contaminant loading, movement toward sorption-desorption equilibria for EM, and their transformation in contaminated soils, are time-dependent processes that ultimately determine EM bioavailability to soil organisms. These processes can decrease the amount of chemical that is bioavailable as compared with freshly contaminated soils, or may increase toxicity due to the presence of more toxic transformation products than parent compound freshly introduced into soil (Hawari et al., 2000; Preuß and Rieger, 1995; Spain, 2000; Schäfer, 2002; Sunahara et al., 2001). Therefore, the present studies included weathering-and-aging of EMs in soil in the experimental designs to determine the net ecotoxicological

effects of these complex processes and to more closely approximate the exposure effects in the field. These studies revealed alterations in toxicity for *E. crypticus* after weathering-and-aging of 2,4-DNT in soil, and these alterations were soil- and endpoint-specific. Weathering-and-aging 2,4-DNT in sandy loam soils significantly (95% CI basis) decreased the toxicity, based on the EC₂₀ values for juvenile production in TSL and the LC₅₀ values for adult survival in SSL, compared to these effects levels in respective freshly amended soils. In contrast, weathering-and-aging 2,4-DNT in the more heavy-textured loam and clay loam soils significantly (95% CI basis) increased the toxicity to *E. crypticus*, based on the EC₅₀ values for juvenile production in KL and the LC₅₀ values for adult survival in WCL, compared to these effects levels in respective freshly amended soils. Weathering-and-aging NG in SSL soil did not significantly affect the toxicity to *E. crypticus* based on the EC₂₀ or EC₅₀ values (and respective 95% CIs) for juvenile production.

The net effects of weathering-and-aging of contaminant EM in soil on the resulting exposure effects for soil invertebrates were investigated in several studies (Kuperman et al., 2003, 2004a, 2005, 2006b, 2006c, 2012; Schäfer, 2002; Simini et al., 2003, 2006). Kuperman et al. (2005, 2006b, 2006c) reported that weathering-and-aging in SSL soil significantly increased the toxicities of TNT; 2,6-DNT; and 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (CL-20) to *E. crypticus*, whereas the toxicities of 2,4-DNT or TNB were unaffected. Weathering-and-aging the nitroaromatic TNT in soil significantly decreased acute toxicity for *E. crypticus* in TSL soil but significantly increased acute toxicity in SSL and Richfield clay loam soils, compared with respective toxicities in FA soils (Kuperman et al., 2012). A decreased toxicity of TNT after aging in soil was also reported for *E. albidus* in OECD artificial soil (Dodard et al., 2003) and for *Folsomia candida* in Lufa 2.2 soil (Schäfer, 2002). Weathering-and-aging in soil significantly decreased both acute and chronic toxicities of the nitramine hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) to *E. crypticus* in TSL, but did not affect RDX toxicity in other soils tested in those studies (Kuperman et al., 2012). No effects of weathering-and-aging of the nitramine HMX in SSL soil on toxicity were reported for *E. crypticus* (Kuperman et al., 2003) and *Eisenia fetida* (Simini et al., 2003), which comports with findings of the present studies.

5. CONCLUSIONS

Present studies were designed to develop scientifically defensible toxicity data required for successful management of defense testing and training ranges in a sustainable manner, and for the knowledge-based decision making. Generating toxicity data to establish benchmarks that are appropriate for utilization in deriving the soil invertebrate-based Eco-SSLs for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG was among the main objectives of the studies conducted in this project. Ecotoxicological testing was specifically designed to successfully meet the criteria for Eco-SSL derivation outlined in the Eco-SSL Guideline (USEPA, 2005). The natural soils TSL and SSL used in the toxicity tests herein had low OM and clay contents, which fulfilled the USEPA requirement of using soil with characteristics that support high relative bioavailability of organic contaminants for developing realistic yet conservative Eco-SSL values (USEPA, 2005).

Definitive studies using *E. crypticus* exposures in upland aerobic sandy loam soils established new ecotoxicological data for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG effects on soil invertebrates under conditions of very high relative bioavailability for organic chemicals in soil (as defined in USEPA, 2005). The preference for reproduction benchmarks, and for low effect level (i.e., EC₂₀), was justified to ensure that Eco-SSL values would be protective of populations of the 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 (i.e., during SLERA).

The exposure concentrations of 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG in soil were analytically determined at the beginning of each definitive toxicity test; consequently, the ecotoxicological benchmarks were determined using measured EM concentrations. This complied with the USEPA preference for derivation of Eco-SSL values on the basis of measured concentrations of a chemical in soil, over those based on nominal concentrations (USEPA, 2005). Analyses of FA soils using USEPA Method 8330A showed good correlation between nominal and measured ACN-extracted concentrations, which confirmed that the soil amendment procedures used in the definitive toxicity tests were appropriate, and that this method was efficient for quantifying the amounts of 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG in soil. Overall, the definitive studies using *E. crypticus* exposures in TSL or SSL soils supported the development of ecotoxicological benchmarks for EMs in compliance with Eco-SSL test acceptance criteria (USEPA, 2005); thus, the first objective of this investigation was achieved. All ecotoxicological benchmarks determined in these studies have been provided to the USEPA Eco-SSL Work Group for quality-control review before inclusion in the Eco-SSL database and subsequent use in the development of individual soil invertebrate-based Eco-SSL values for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG.

Toxicity testing was also conducted with additional natural soils to extend the range of investigation of soil physico-chemical characteristics that were hypothesized to affect the 2,4-DNT toxicity to soil invertebrates. Soil-related differences were evident in both acute (adult survival) and chronic (juvenile production) toxicity benchmarks established in present studies with potworms exposed to 2,4-DNT W-A in each of the natural soils tested. The quantitative analyses of relationships among the acute or chronic toxicity benchmarks for 2,4-DNT and soil property measurements revealed that both clay and organic matter contents of the soil affected toxicity of 2,4-DNT to the potworms. Strong correlations were also detected for several potworm toxicity endpoints and soil CEC (which depends upon both soil clay and soil organic matter contents). No significant correlations were found among any toxicity benchmarks for 2,4-DNT and soil pH. These results identified soil organic matter and clay contents as the dominant soil properties mitigating 2,4-DNT toxicity to potworms.

The present studies included weathering-and-aging of EMs in soil in the experimental designs to produce a soil microenvironment more similar to field conditions, and thus more closely approximate the exposure effects in contaminated sites. Results of analyses showed that exposure conditions of *E. crypticus* to EMs W-A in soils differed from those of FA soils. Toxicity alterations after weathering-and-aging of 2,4-DNT or NG in soil were soil- and endpoint-specific. Overall, the results of the present studies showed that special consideration given to the effects of weathering-and-aging of EM in soil for assessing toxicity was well-

justified. Toxicity benchmarks generated in the present studies will contribute to development of Eco-SSL values that better represent the exposure conditions of soil invertebrates at contaminated sites. Additional studies would be required to resolve the current uncertainties in our understanding of the mechanisms contributing to the increased or decreased toxicities of EMs following their weathering-and-aging in soil. These studies should be conducted with soil types selected for a wide range of properties, particularly clay type and content and OM, that affect the fate and bioavailability of EMs, in order to better understand the complex interactions among physical, chemical, and biological components that jointly contribute to the outcome of ecotoxicity testing.

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ABBREVIATIONS AND ACRONYMS

ACN	acetonitrile
2-ADNT	2-amino-4,6-dinitrotoluene
4-ADNT	4-amino-2,6-dinitrotoluene
AS	artificial soil
ATCLP	adapted toxicity characteristic leaching procedure
BDL	below detection limit
BERA	baseline ecological risk assessment
BRI	Biotechnology Research Institute
BSAF	biota soil accumulation factor
CaCl ₂	calcium chloride
CAS	Chemical Abstracts Service
CEC	cation exchange capacity
CI	confidence interval
CL	confidence limits
CL-20	2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (China Lake compound 20)
CV	coefficient of variation
2,4-DANT	2,4-diaminotoluene
2,6-DANT	2,6-diaminotoluene
1,3-DNB	1,3-dinitrobenzene
1,2-DNG	1,2-dinitroglycerin
3,5-DNA	3,5-dinitroaniline
2,4-DNT	2,4-dinitrotoluene
2,6-DNT	2,6-dinitrotoluene
DNX	hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine
DOD	U.S. Department of Defense
EC	Environment Canada
ECBC	U.S. Army Edgewood Chemical Biological Center
Eco-SSL	ecological soil screening level
ECp	Effective concentration for a specified percent effect
EM	energetic material
ERA	ecological risk assessment
FA	freshly amended
FLSD	Fisher's least-significant difference
HMX	octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (high melting explosive)
HPLC	high-performance liquid chromatography
ISO	International Organization for Standardization
KCL	Kirkland clay loam
KL	Kirkland loam
K_d^s	soil adsorption coefficient
LCp	Lethal concentration for a specified percent lethality
LOAEC	lowest-observed-adverse-effect concentration
LOEC	lowest-observed-effect-concentration

MNX	hexahydro-3,5-dinitro-1-nitroso-1,3,5-triazine
NA	not applicable
NAC	nitroaromatic compound
ND	not determined
NG	nitroglycerin
NOAEC	no-observed-adverse-effect concentration
NOEC	no-observed-effect concentration
OC	organic carbon
OECD	Organisation for Economic Co-operation and Development
OM	organic matter
<i>p</i>	probability value
PAR	photosynthetically active radiation
PTFE	polytetrafluoroethylene
QRB	qualitative relative bioavailability
<i>r</i>	correlation coefficient
R^2	coefficient of determination
RCL	Richfield clay loam
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine (royal demolition explosive)
SAS	standard artificial soil
SD	standard deviation
SE	standard error
SERDP	Strategic Environmental Research and Development Program
SLERA	screening level ecological risk assessment
SSC	soil screening concentration
SSL	Sassafras sandy loam
TCLP	toxicity characteristic leaching procedure
TNB	1,3,5-trinitrobenzene
TNP	2,4,6-trinitrophenol
TNT	2,4,6-trinitrotoluene
TNX	hexahydro-1,3,5-trinitroso-1,3,5-triazine
TSL	Teller sandy loam
USEPA	U.S. Environmental Protection Agency
W-A	weathered-and-aged
WCL	Webster clay loam
WHC	water holding capacity

