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TOXICITIES OF TNT AND RDX TO THE EARTHWORM *EISENIA FETIDA* IN FIVE SOILS WITH CONTRASTING CHARACTERISTICS

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Studies were designed to characterize soil physicochemical parameters that can affect the toxicities of 2,4,6-trinitrotoluene (TNT) or hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) to *Eisenia fetida* earthworms and also to generate ecotoxicological benchmarks for development of ecological soil screening levels (Eco-SSLs) for ecological risk assessments of contaminated soils. Soils with varied physicochemical properties were tested, including Teller sandy loam (TSL), Sassafras sandy loam (SSL), Richfield clay loam (RCL), Kirkland clay loam (KCL), and Webster clay loam (WCL). Reproduction toxicity of TNT to *E. fetida* in freshly amended soils was in the order (greatest to least) of TSL > SSL = KCL = RCL > WCL. Weathering-and-aging of TNT in SSL, KCL, RCL, and WCL soils increased the toxicity to *E. fetida* compared with corresponding freshly amended treatments. Reproduction toxicity of RDX weathered-and-aged (W-A) in soil was comparable with that of TNT W-A in TSL, SSL, RCL, and WCL soils. No clear relationships were identified between TNT or RDX toxicities and the soil organic matter or clay contents or the pH levels. Toxicity benchmarks established utilizing TSL and SSL will be submitted to the U.S. Environmental Protection Agency Eco-SSL Workgroup for developing soil invertebrate-based Eco-SSLs for TNT and RDX.

15. SUBJECT TERMS

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Earthworm	RDX	*Eisenia fetida*	Natural soil
Ecological soil screening level			

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PREFACE

The work described in this report was authorized under Strategic Environmental Research and Development Program project no. SERDP CU-1210. The work was started in April 2001 and completed in November 2004.

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CONTENTS

FIGURES

TABLES

TOXICITIES OF TNT AND RDX TO THE EARTHWORM *EISENIA FETIDA* IN FIVE SOILS WITH CONTRASTING CHARACTERISTICS

1. INTRODUCTION

Many sites associated with military operations involving munitions manufacturing, disposal, testing, and training have been contaminated with elevated levels of explosives and related materials in soil. Concentrations of explosives in soil have been reported to exceed 87,000 mg kg^{-1} for 2,4,6-trinitrotoluene (TNT) (Simini et al., 1995) and 74,000 mg kg^{-1} of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Best et al., 2006). Although these energetic materials (EMs) can be persistent in the environment, their effects on soil biota have not been sufficiently investigated. As a result, scientifically defensible screening values, which could be used in ecological risk assessment (ERA), are not currently available for explosives in soil. Scientifically based ecological soil screening level values (Eco-SSLs) are needed to identify contaminant explosives levels in soil that do not present a potential ecological concern onsite and, therefore, do not need to be considered in baseline ecological risk assessment (BERA). To address this problem, the U.S. Environmental Protection Agency (U.S. EPA), in conjunction with stakeholders, is developing Eco-SSL values for contaminants most frequently found at Superfund sites (U.S. EPA, 2005). Eco-SSLs are defined as the respective concentrations of chemicals in soil that, when not exceeded, will be protective of terrestrial ecosystems from unacceptable harmful effects. These Eco-SSL values can be used in a screening level ERA (SLERA) to identify those contaminants in soil that warrant additional evaluation in a BERA 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. After an extensive literature review (U.S. EPA, 2005), the Eco-SSL workgroup determined that there was insufficient information regarding explosives to support the derivation of Eco-SSL benchmarks for soil invertebrates. Our studies were designed to fill this knowledge gap.

Several soil invertebrate toxicity tests, for which standardized protocols have been developed (International Organization for Standardization [ISO], 1998a, 1998b, 2004), can effectively be used to assess toxicity and to derive protective benchmark values for EMs (Stephenson et al., 2002; Løkke and Van Gestel, 1998). We adapted the earthworm reproduction test (ISO, 1998a) for these studies. This test was selected for its ability to measure chemical toxicity to ecologically relevant test species during chronic assays and its inclusion of at least one reproductive component among the measurement endpoints.

At many contaminated sites, explosives in soils have been subjected to weathering-and-aging processes for years. Therefore, to provide appropriate benchmark data for Eco-SSL development, special consideration was given to assessing the toxicity of EMs to soil invertebrates. Weathering-and-aging of chemicals in soil may reduce the exposure of soil invertebrates to EMs. Photodecomposition, hydrolysis, reactions with organic matter (OM), sorption, precipitation, immobilization, occlusion, microbial transformation, and other fate processes may reduce the amount of chemical that is bioavailable. Conversely, transformation products produced during weathering-and-aging processes may be more toxic to soil organisms than the parent material (Kuperman et al., 2005). We incorporated a weathering-and-aging

procedure in our tests to more accurately simulate the field conditions that may affect exposure of soil invertebrates to EMs, compared with tests conducted with freshly amended chemicals or tests conducted after a short equilibration period (e.g., 24 h).

Studies reported herein were designed to produce scientifically defensible benchmark data for the development of Eco-SSL values for TNT and RDX used with soil invertebrates in aerobic upland soils that meet specific criteria (U.S. EPA, 2005). Eco-SSL test acceptance criteria were met or exceeded in these investigations by ensuring that:

- Experimental designs for laboratory studies were documented and appropriate;
- Both nominal and analytically determined concentrations of chemicals of interest were reported;
- Tests included both negative and positive controls;
- Chronic or life cycle tests were used;
- Appropriate chemical dosing procedures were reported;
- Concentration-response relationships were reported;
- Statistical tests used to calculate the benchmarks and levels of significance were described; and
- The origins of test species were specified and appropriate.

Tests were also conducted in five different field soils having different physicochemical characteristics that may alter the bioavailability of TNT and RDX, including soils that sustain high relative bioavailability of EMs.

2. MATERIALS AND METHODS

2.1 Soil Collection and Characterization

The soils used in these studies included the following:

- Teller sandy loam (TSL), a fine loamy, mixed, active, thermic Udic Argiustoll collected from agricultural land of the Oklahoma State University Perkins Experiment Station, Payne County, OK;
- Sassafras sandy loam (SSL), a 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, Harford County, MD;
- Kirkland clay loam (KCL), a fine, mixed, superactive, thermic Udertic Paleustoll collected from Payne County, OK;
- Richfield clay loam (RCL), a fine, smectitic, mesic Aridic Argiustoll collected from Texas County, OK; and
- Webster clay loam (WCL), a fine-loamy, mixed, superactive, mesic Typic Endoaquoll collected from Story County, IA.

The qualitative relative bioavailability (QRB) scores for organic chemicals in natural soils were considered “very high” for TSL and SSL, “medium” for KCL and WCL, and

"low" for RCL according to Eco-SSL criteria (U.S. EPA, 2005). During soil collection in the field, vegetation and the organic horizon were removed, and the top 15.2 cm of the A-horizon were then collected. Soil was sieved through a 5 mm mesh screen, air-dried for at least 72 h, mixed periodically to ensure uniform drying, passed through a 2 mm sieve, and stored at room temperature. Soil was then analyzed for physical and chemical characteristics (Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD). Results of these analyses are presented in Table 1.

Table 1. Mean Physical and Chemical Characteristics of Five Field Soils (n = 3)

Soil Property	TSL Soil	SSL Soil	KCL Soil	RCL Soil	WCL Soil
Sand (%)	65 (1.0)	70 (0.7)	37 (0.33)	30 (30.3)	33 (0.6)
Silt (%)	22 (1.0)	13 (0.9)	34 (0.33)	42 (1.7)	39 (0.3)
Clay (%)	13 (0.0)	17 (0.3)	28 (0.33)	28 (0.9)	28 (0.7)
Texture	Sandy loam	Sandy loam	Clay loam	Clay loam	Clay loam
Cation exchange capacity (cmol kg^{-1})	4.3 (0.03)	5.5 (0.1)	10.3 (0.09)	27.6 (1.40)	20.8 (0.1)
Organic matter (%)	1.4 (0.03)	1.3 (0.06)	2.6 (0.06)	3.3 (0.03)	5.3 (0.09)
pH	4.4 (0.03)	5.2 (0.03)	6.4 (0.03)	7.4 (0.06)	5.9 (0.03)
Water-holding capacity (%)	13 (0.6)	18 (4.0)	20 (1.0)	21 (1.5)	23 (0.18)

Notes: Analyses were performed by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD. Standard errors of the means are shown in parentheses.

2.2 Test Chemicals

The EMs 2,4,6-trinitrotoluene (TNT; Chemical Abstracts Service [CAS] no. 118-96-7; 99.9%) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX; CAS no. 121-82-4; purity, 99%) were obtained from the Defence Research Establishment Valcartier of the Canadian Ministry of National Defence (Val Bélair, QC, Canada). Beryllium sulfate ($BeSO_4 \cdot 4H_2O$; CAS no. 7787-56-6; purity, 99.99%) was used as the positive control in all tests. High-performance liquid chromatography (HPLC)-grade acetone (CAS no. 67-64-1) was used to prepare TNT and RDX solutions for soil amendment. Acetonitrile (ACN; CAS no. 75-05-8; HPLC grade), methanol (CAS no. 67-56-1; chromatography grade; purity, 99.9%), and calcium chloride ($CaCl_2$; CAS no. 10043-52-4; reagent grade), were used for the soil extractions and in analytical HPLC determinations. Certified standards of TNT and RDX (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 TNT or RDX in freshly amended (FA) and weathered-and-aged (W-A) soil to determine toxicity benchmark values for TNT or RDX in each exposure type. During the soil amendment procedure, TNT or RDX was amended into separate aliquots of soil using an organic solvent (acetone) as a carrier. This was necessary to distribute the TNT or RDX evenly and uniformly to a large soil surface area, which would have been difficult to achieve if solid chemical crystals had been added to soil. Carrier control soils were amended with acetone only. Soil was spread to a thickness of 2.5 cm. The TNT or RDX solution was pipetted evenly across the soil surface, and the volume of solution added at any one time did not exceed 15% (v/w) of the soil dry mass. After the solution was added, the volumetric flask was rinsed twice with a known volume of acetone, which was also pipetted onto the soil. If the total volume of solution required to amend the soil exceeded 15% (v/w), the solution was added in successive stages. Between additions, the acetone was allowed to evaporate for a minimum of 2 h in a darkened chemical hood. Amended soil was air-dried overnight (minimum of 18 h) in a darkened chemical hood to prevent photolysis of the EM. 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 mixer.

2.4 Weathering-and-Aging of TNT and RDX in Soil

Standardized methods for weathering-and-aging of explosives in soil are not available. We have developed approaches that simulate, at least in part, the weathering-and-aging processes in soil to more closely approximate the exposure effects on soil biota in the field (Kuperman et al., 2003, 2005; Simini et al., 2003, 2006). Air-dried soil batches were amended with several concentrations of TNT or RDX. In a greenhouse, the dried soil batches were initially hydrated in open glass containers with ASTM Type I water to 60% of the water-holding capacity (WHC) of each soil. Soil was then subjected to alternating cycles (up to 3 months duration) of hydration and air-drying at ambient temperature in a greenhouse. Each soil treatment was weighed and readjusted to its initial mass by weekly addition of ASTM Type I water. Any soil surface crust that formed during the week was broken with a spatula before water was added. After the conclusion of the EM weathering-and-aging procedures, each soil treatment was brought to 95% of its WHC 24 h before toxicity tests were started.

Soil treatments with TNT concentrations representing low, intermediate, and high levels were monitored periodically during the weathering-and-aging process to determine the time when TNT concentrations were effectively stabilized or had declined to ≤5% of the initial concentration in FA soil treatments with the highest rate of decrease. Nominal TNT concentrations selected for monitoring in these studies were: 20, 100, 200, and 300 mg kg^{-1} in TSL; 50, 100, 200, and 400 mg kg^{-1} in SSL or KCL; 5, 25, 100, and 500 mg kg^{-1} in RCL; and 40, 100, 200, and 400 mg kg^{-1} in WCL. The respective times determined for each TNT–soil pairing were then designated for termination of the weathering-and-aging procedures for that soil and commencement of the corresponding definitive toxicity tests. The effects of weathering-and-aging of TNT in soil on toxicity to *E. fetida* were investigated by comparing test results for TNT W-A in amended soils with results obtained using soils with FA TNT.

Previous studies have shown that RDX did not significantly degrade under aerobic conditions and that toxicity to soil invertebrates did not significantly change ($p \leq 0.05$) when RDX-amended soils were subjected to the weathering-and-aging process (Simini et al., 2003; Kuperman et al., 2003; Dodard et al., 2005). Therefore, after soils were amended with RDX, concentrations in soils were not monitored until the RDX weathering-and-aging procedures were concluded after 90 days. RDX concentrations were analytically determined in each soil immediately before toxicity testing was started.

2.5 Measurement of Soil pH

The pH values of the test soils were determined at the beginning of each definitive toxicity test using a method adapted from the *Soil Survey Laboratory Methods Manual* (U.S. Department of Agriculture [USDA], 2004). Five grams of ASTM Type I water was added to 5 g of soil. The soil slurry was vortexed for 10 s out of every 5 min period over a 30 min duration. Then 1 min before pH measurement, the soil slurry was vortexed again for 10 s. While the slurry was gently stirred, the soil pH was analytically determined in the solution above the soil surface until the reading stabilized. Before measurement of soil pH for each definitive test, the pH electrode was rinsed thoroughly with ASTM Type I water, blotted dry, standardized with pH 4 and pH 7 buffers, rinsed, and blotted again. The electrode was also rinsed with ASTM Type I water and blotted before each pH measurement.

2.6 ACN Extraction of TNT and RDX from Soil

At the beginning of each definitive test, each batch of control soils and the RDX- or TNT-treated soils were subsampled in triplicate. ACN was used to extract RDX or TNT from each sample, then EM concentrations were analytically determined in accordance with U.S. EPA Method 8330A (U.S. EPA, 2007). Before extraction, soil subsamples for analytical determination were hydrated to 60% of their respective WHCs for 24 h, in accordance with the procedures in "Weathering-and-Aging of TNT or RDX in Soil" (Section 2.4). The soil dry fraction (dry weight/wet weight) was determined in triplicate from subsamples of each treatment concentration. For extraction, 2 g soil samples were collected from the soil batch treatments and controls and placed into respective 50 mL polypropylene centrifuge tubes, and 10 mL of ACN was added to each tube. Samples were vortexed with the ACN for 1 min, then sonicated in darkness for 18 h at 20 °C. Five milliliters of each supernatant was transferred into glass tubes that contained 5 mL of $CaCl_2$ solution (5 g/L). The supernatant was then 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 concentrations were quantified by HPLC.

2.7 Adapted Toxicity Characteristic Leaching Procedure (ATCLP) Extraction of TNT from Soil

During ACN extraction, both the nonaccessible (nondissolved crystalline plus adsorbed) and the water-soluble fractions of TNT or RDX are measured. Consequently, although conservative values are obtained, use of U.S. EPA Method 8330A can result in overestimation of the amount of explosive available to an exposed organism because the bioavailability of an

organic compound having an octanol–water partition coefficient (log K_{ow}) of <5 (1.6 for TNT and 0.90 for RDX; Monteil-Rivera et al., 2009) for uptake by a soil organism is primarily determined by the fraction dissolved in the soil interstitial water (Belfroid et al., 1994, 1996; Savard et al., 2010). Therefore, in addition to ACN extraction, the water-soluble fraction of TNT was extracted from soil using an ATCLP (Haley et al., 1993).

At the beginning of each definitive test, in addition to extraction with ACN, TNT was extracted from each batch of control soils and TNT-treated soils using the ATCLP method. The ATCLP is a modification of the toxicity characteristic leaching procedure (TCLP; 40 CFR Part 268.41, Hazardous Waste Management, Method 1311). The procedure was modified by substituting CO_2-saturated water for acetic acid to acidify the water used for extraction, and thereby simulate the soil–water conditions that exist as a result of respiration by soil biota and retain the effects of the natural buffering capacity of the soil. The CO_2-saturated water was not recharged once it was added to the soil. All ATCLP extractions were performed in triplicate. For each subsample replicate from the treatment concentration batches for TNT, 4 g of soil were transferred in triplicate into 20 mL vials. Sixteen milliliters of CO_2-saturated water (pH 3.8–4.0) was added to each vial, and the vials were immediately sealed. Each soil sample was vortexed for 45 s before being mixed for 18 h on a rotary (end-over-end) mixer (30 rpm) at room temperature in darkness (40 CFR Part 268.41). The solutions were allowed to settle for at least 2 h, and supernatants were filtered through 0.45 µm PTFE syringe cartridges. An equal volume of ACN was added to each filtered soil extract before HPLC analysis was performed. Herein, TNT concentrations determined using the ATCLP soil extraction procedure are referred to as the EM water-soluble fractions. Nominal and analytically determined concentrations from the definitive tests are shown in Tables 3 through 12.

ATCLP-based extractions were not conducted in studies with RDX because multiple concentrations selected for definitive toxicity tests exceeded the aqueous solubility of RDX (42 mg L^{-1} at 20 °C; Monteil-Rivera et al., 2004).

2.8 Analytical Determinations

Soil extracts were analyzed and EM concentrations were quantified by reversed-phase HPLC using a modified U.S. EPA Method 8330A. The method was modified by adjusting the flow rate of the 50/50 methanol–water mobile phase to 1.0 mL min^{-1} rather than 1.5 mL min^{-1}. A 25 cm × 4.6 mm × 5 µm particle size C-18 column was used for all determinations. For HPLC, Beckman System Gold analytical instrumentation (Beckman Coulter; Brea, CA) was used, which consists of a model 126 programmable solvent module, a model 168 diode array detector, and a model 507 automatic sampler. Calibration curves were generated before each HPLC run by dissolving certified standards (AccuStandard) of each EM in a 50/50 water–ACN solution in a range of concentrations appropriate for each set of determinations. Blanks and standards were placed intermittently between samples. The method detection limits were 0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil. All chemical concentrations in soil were expressed on a dry-mass basis.

2.9 Toxicity Assessment

A 56 day earthworm reproduction assay was used to assess the effects of TNT and RDX on the earthworm *Eisenia fetida*. The test is an adaptation of ISO bioassay 11268-2:1998 (ISO, 1998a). Guidelines for this assay were originally developed for use with Organisation for Economic Co-operation and Development (OECD, 1984) artificial soil (a similar soil formulation was later adapted for U.S. EPA Standard Artificial Soil [SAS]; U.S. EPA, 1996; and for ASTM Artificial Soil [AS]; ASTM E1676-04, 2004b). However, research in our laboratory has shown that this assay can also be successfully conducted using natural soils (Simini et al., 2003, 2006), and this adaptation was used in these studies.

Earthworms were bred in plastic containers filled with approximately 14 kg of a 1/1 mixture of Pro-Gro sphagnum peat moss (Gulf Island Peat Moss Co.; Prince Edward Island, Canada) and Baccto potting soil (Michigan Peat Co.; Houston, TX). The pH was adjusted to 6.2 ± 0.1 by addition of $CaCO_3$ (pulverized lime). The culture was kept moist at 21 ± 2 °C under continuous light. Earthworm colonies were fed biweekly with alfalfa food consisting of dehydrated alfalfa pellets (27% fiber, 17% protein, 1.5% fat; Ohio Blenders of PA; York, PA). Before use, the alfalfa pellets were hydrated, fermented for at least 14 days, air-dried, and ground to a course powder. Earthworm cultures were synchronized so that all worms used in each test were approximately the same age. Adult worms that weighed 0.3 to 0.6 g and had fully developed clitella were used for testing. Earthworms were acclimated for 48 h in unamended test soils. Earthworms were selected for uniformity and depurated on moist filter paper overnight. The worms were then randomly selected for placement across treatments. After weathering-and-aging in soil of the respective TNT and RDX amendments, 200 g of soil (dry-weight basis) per treatment level was placed into each of four 400 mL (9 cm diameter) glass jars (for each treatment, a set of four replicates was prepared). For each replicate, five worms were rinsed twice with ASTM Type I water, blotted on paper towels, weighed on an analytical balance, and placed on the soil surface in each glass jar. For both the range-finding and definitive assays, a 2 g bolus of prepared alfalfa food was added to each jar, moistened with an atomizer, and covered with soil from within the jar. Clear plastic film was stretched across the top of each jar and secured with the screw-on rings to allow light exposure. Three pinholes were made in the plastic film to allow for air exchange. The earthworm treatment jars were incubated under a 16 h light–8 h dark photoperiod with a mean photosynthetically active radiation light intensity of 12.8 ± 0.7 μM m^{-2} s^{-1} (985 ± 52 lux) and mean temperature of 21.6 ± 0.1 °C.

After 21 days in the range-finding tests and 28 days in the definitive tests, worms were removed with blunt forceps from the jars. The number and mass of surviving earthworms in each jar were determined and recorded. Cocoons were counted after 21 days in the range-finding tests, as described below, and the tests were ended. In the definitive tests, 2 g of prepared alfalfa food was again added to each jar, and clear plastic film and screw rings were again placed on the jars. After 28 more days, cocoons and juveniles in each treatment replicate were harvested, counted, and recorded. Juveniles were induced to crawl to the soil surface by immersing the containers to a level just below the soil surface in a heated water bath at 41–43 °C for 20–25 min. Juveniles were removed from the soil surface with a blunt forceps, counted, and recorded. Soil was then spread and examined under a 2.25× lighted magnifier to recover and count any additional juveniles. The total number of juveniles in each container was then recorded. Cocoons were recovered by gently

agitating the soil from each treatment on a 1 mm sieve under a stream of water until only the cocoons remained on the sieve surface. Cocoons were placed in water in a clear glass dish. Cocoons that floated were counted as hatched; those that sank were counted as unhatched. Cocoons were examined under the magnifier to confirm whether they had hatched or not. The numbers of hatched, unhatched, and total cocoons per container were recorded.

Treatment concentrations for each definitive test were selected on the basis of the range-finding test results. Concentrations in the definitive tests were selected on the basis of bracketing significant effects on reproduction endpoints (i.e., production of cocoons and juveniles for each soil type). Reproduction endpoints are preferred Eco-SSL benchmarks for the development of Eco-SSL values that are based on soil invertebrate toxicity data (U.S. EPA, 2005).

Definitive tests included negative controls (no chemicals added), carrier (acetone) controls, and positive controls; each of these controls was replicated four times per test. Positive controls were prepared as a solution of $BeSO_4$ in ASTM Type I water added to the soil to obtain a nominal Be concentration of 77 mg kg^{-1}. Validity criteria for the negative controls included the following performance parameters (ISO, 1998a):

- The mean mortality should not exceed 10% in range-finding and definitive tests;
- The number of juveniles produced per 5 worms should be ≥15; and
- The coefficient of variation for the number of juveniles in the control should be ≤50%.

2.10 Data Analysis

Cocoon and juvenile production and adult survival data were analyzed independently using nonlinear or linear regression models described in Stephenson et al. (2000) and Kuperman et al. (2003). Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to determine whether to weight the data and to help select the type of regression model to be used for each data set. The models selected had the best fit of the data points to curves generated by the respective models, the smallest variances, and the residuals with the best appearance (i.e., most random scattering). The models selected for data comparisons in these studies were:

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

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

Hormetic model: $$Y = a \times [1 + (h \times C)]/\{1 + [(p + (h \times C))/(1-p)] \times [C/\mathrm{ECp}]^b\} \quad (3)$$

Linear model: $$Y = [(-a \times p)/\mathrm{ECp}] \times C + a \quad (4)$$

where

Y is the measurement endpoint (e.g., number of juveniles);
a is the *y*-axis intercept (e.g., control response);
e is the base of the natural logarithm;
p is the percent inhibition/100 (e.g., 0.5 for the EC_{50});
C is the exposure concentration in test soil;
ECp is the estimate of effective concentration for a specified percent effect;
b is the scale parameter; and
h is the hormetic effect parameter.

Data that exhibited hormesis, a concentration-response phenomenon characterized by low-dose stimulation and high-dose inhibition (Calabrese, 2008), were fitted to the hormetic model. The ECp parameters used in this study included the concentrations of TNT or RDX that produced 20% (EC_{20}) and 50% (EC_{50}) reductions in the selected measurement endpoints compared with carrier controls. The EC_{20} parameter based on a reproduction endpoint is the preferred parameter for Eco-SSL benchmarks for deriving soil invertebrate Eco-SSL values (U.S. EPA, 2005). The EC_{50} (more commonly used in the past) and survival data were included to enable comparisons of results produced in these studies with results reported by other researchers. The asymptotic standard errors (SEs) and 95% confidence intervals (CIs) associated with the point estimates were determined.

Analysis of variance (ANOVA) was used to determine the no-observed-effect (NOEC) and lowest-observed-effect (LOEC) concentration values for adult survival, cocoon production, or juvenile production data. Mean separations were determined using Fisher's least-significant difference (FLSD) pairwise-comparison tests. A significance level of $p \leq 0.05$ was used to determine NOEC and LOEC values. Pearson's correlation analysis was used to estimate the contributions of OM, clay content, and pH to the relative toxicities of TNT or RDX to earthworms in the five soils. Analysis of covariance was used to determine the NOEC and LOEC values for final adult mass.

All statistical analyses were performed on untransformed toxicity data and analytically determined EM concentrations using SYSTAT 11.0 (Systat Software; Chicago, IL).

3. RESULTS

3.1 Measurement of pH in Soils Amended with TNT

Results of pH analyses are presented in Table 2. The pH values for soils amended with TNT did not vary greatly from the control soils.

Table 2. Mean pH Values at Start of Earthworm Reproduction Testing with TNT FA or W-A in All Soils

Nominal TNT Concentration (mg kg^{-1})	Mean pH (n = 3)									
	TSL Soil		SSL Soil		KCL Soil		RCL Soil		WCL Soil	
	FA	WA	FA	WA	FA	WA	FA	WA	FA	WA
0	4.83	4.89	5.36	5.55	6.48	ND	7.79	7.56	ND	6.34
5	4.92	—	—	—	—	—	7.81	7.69	—	—
10	4.93	—	5.48	5.59	6.53	—	7.80	7.69	—	—
15	—	4.77	—	—	—	—	—	—	—	—
20	4.91	4.94	—	—	6.51	5.89	—	—	—	—
25	—	—	—	5.57	—	—	7.81	7.69	—	—
30	—	4.76	—	—	—	—	—	—	—	—
40	4.92	—	—	—	6.55	6.18	—	—	—	—
50	—	4.87	5.46	5.57	6.53	6.38	7.86	7.66	—	—
60	4.84	—	—	—	6.58	6.37	—	—	—	—
75	—	—	5.42	5.58	—	—	—	—	—	—
80	4.78	—	—	—	6.64	6.41	—	—	—	—
100	4.82	4.92	5.41	5.57	6.72	6.53	7.89	7.59	—	—
120	4.70	4.76	—	—	—	—	—	—	—	—
125	—	—	—	5.57	—	—	7.84	—	—	—
140	—	4.78	—	—	—	—	—	—	—	—
150	—	—	5.41	5.46	6.68	6.51	7.85	7.64	—	—
160	—	4.82	—	—	—	—	—	—	—	—
180	—	4.74	—	—	—	—	—	—	—	—
200	—	4.72	5.37	5.54	6.68	6.93	7.85	7.71	ND	6.59
225	—	—	—	—	—	—	—	—	ND	6.57
250	—	4.78	—	—	6.59	6.73	—	—	ND	6.68
275	—	—	—	—	—	—	—	—	ND	6.65
300	—	—	5.39	—	—	—	7.88	7.79	ND	6.75
325	—	—	—	—	—	—	—	—	ND	6.71
350	—	—	—	—	—	—	—	—	ND	6.74
400	—	—	—	—	—	—	—	—	ND	6.69
500	—	—	—	—	—	—	—	—	ND	6.65
600	—	—	—	—	—	—	—	—	ND	6.54
Mean	4.85	4.81	5.41	5.56	6.59	6.44	7.84	7.67	ND	6.63
SE	0.03	0.02	0.01	0.01	0.02	0.10	0.01	0.02	ND	0.04

—, Treatment level not used.
ND, not determined.

3.2 Analytical Determination of TNT in Soil

3.2.1 TNT in TSL Soil

Mean values of ACN-extractable TNT FA in TSL soil, expressed as percentages of amendments, ranged from 33% at nominal 5 mg kg^{-1} to 91% at nominal 120 mg kg^{-1} (Table 3). Mean values of ATCLP-extractable TNT within FA TSL soil ranged from 20 to 60% of ACN-extractable concentrations (Table 3). Mean values of ACN-extractable TNT W-A in TSL soil, calculated as percentages of corresponding initial concentrations of ACN-extractable TNT in FA soils, ranged from 52 to 78% of nominal concentrations (Table 4). Mean values of ATCLP-extractable TNT W-A in soil ranged from 19 to 56% of ACN-extractable concentrations (Table 4).

Table 3. Concentrations of TNT FA in TSL Soil Used in Toxicity Tests with *E. fetida*

Nominal Concentration (mg kg^{-1})	ACN Extraction (mg kg^{-1})	SE	ACN/Nominal (%)	ATCLP Extraction (mg kg^{-1})	SE	ATCLP/ACN (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
5	2	0.2	33	0.4	0.1	20
10	4	0.1	35	1	0.1	25
20	9	0.1	43	2	0.2	22
40	29	0.5	72	11	0.1	38
60	49	1.0	81	24	0.1	49
80	69	0.7	86	37	0.1	54
100	88	1.3	88	50	0.5	57
120	109	1.6	91	65	1.4	60

Note: Analytically determined concentrations (means and SEs, $n = 3$) included ACN-extractable (U.S. EPA Method 8330A) and water-extractable (ATCLP; Haley et al., 1993) concentration values.
BDL, below detection limit (0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil).

Table 4. Concentrations of TNT W-A in TSL Soil Used in Definitive Toxicity Tests with *E. fetida*

Nominal Concentration (mg kg^{-1})	Initial ACN (mg kg^{-1})	W-A ACN (mg kg^{-1})	W-A/ Initial ACN (%)	W-A ATCLP (mg kg^{-1})	W-A ATCLP/ W-A ACN (%)
0	BDL	BDL	BDL	BDL	BDL
15	ND	12 (0.3)	ND	3 (0.1)	25
30	ND	16 (0.3)	ND	3 (0.2)	19
50	52 (5)	27 (0.7)	52	11 (0.03)	41
100	54 (4)	42 (1.5)	78	21 (0.4)	50
120	138 (4)	101 (2.4)	73	56 (0.3)	55
140	159 (3)	122 (1)	77	64 (1.1)	52
160	181 (13)	135 (2)	75	76 (0.5)	56
180	212 (4)	159 (5)	75	86 (0.4)	54

Note: Analytically determined concentrations (means and SEs, $n = 3$) include ACN-extractable (U.S. EPA Method 8330A, ACN) and water-extractable (ATCLP; Haley et al., 1993) concentration values.
BDL, below detection limit (0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil).
ND, not determined.

3.2.2 TNT in SSL Soil

Mean values of ACN-extractable TNT FA in SSL soil, expressed as percentages of amendments, ranged from 67% at nominal 10 mg kg^{-1} to 96% at nominal 300 mg kg^{-1} (Table 5). Mean values of ATCLP-extractable TNT within FA SSL soil ranged from 43 to 75% of ACN-extractable concentrations (Table 5). Mean values of ACN-extractable TNT W-A in SSL soil, calculated as percentages of corresponding initial concentrations of ACN-extractable TNT in FA soils, ranged from 24 to 91% of nominal concentrations (Table 6). Mean values of ATCLP-extractable TNT W-A in soil ranged from 40 to 72% of ACN-extractable concentrations (Table 6).

Table 5. Concentrations of TNT FA in SSL Soil Used in Toxicity Tests with *E. fetida*

Nominal Concentration (mg kg^{-1})	ACN Extraction (mg kg^{-1})	SE	ACN/Nominal (%)	ATCLP Extraction (mg kg^{-1})	SE	ATCLP/ACN (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
10	7	0.1	67	3	0.1	43
25	21	0.9	82	11	0.03	52
50	40	0.4	80	26	0.2	65
75	62	2.1	82	40	0.5	65
100	85	0.2	85	59	1.3	69
150	134	6.1	90	98	0.6	73
200	186	2.4	93	137	0.7	74
300	287	2.5	96	215	1.0	75

Note: Analytically determined concentrations (means and SEs, $n = 3$) include ACN-extractable (U.S. EPA Method 8330A) and water-extractable (ATCLP; Haley et al., 1993) concentration values.
BDL, below detection limit (0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil).

Table 6. Concentrations of TNT W-A in SSL Soil Used in Definitive Toxicity Tests with *E. fetida*

Nominal Concentration (mg kg^{-1})	Initial ACN (mg kg^{-1})	W-A ACN (mg kg^{-1})	W-A/ Initial ACN (%)	W-A ATCLP (mg kg^{-1})	W-A ATCLP/ W-A ACN (%)
0	BDL	BDL	BDL	BDL	BDL
10	7 (0.1)	2 (0.2)	29	1 (0.03)	50
25	21 (1)	5 (0.4)	24	2 (0.03)	40
50	40 (0.4)	15 (0.3)	38	7 (0.05)	47
75	62 (2)	36 (1.4)	58	21 (0.3)	58
100	85 (0.2)	64 (0.3)	75	38 (0.4)	59
125	109 (2)	94 (3)	86	61 (1.5)	65
150	134 (6)	119 (2)	89	77 (1.3)	65
200	186 (2)	170 (3)	91	122 (4.1)	72

Note: Analytically determined concentrations (means and SEs, $n = 3$) include ACN-extractable (U.S. EPA Method 8330A) and water-extractable (ATCLP; Haley et al., 1993) concentration values.
BDL, below detection limit (0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil).

3.2.3 TNT in KCL Soil

Mean values of ACN-extractable TNT FA in KCL soil, expressed as percentages of amendments, ranged from 70% at nominal 5 mg kg^{-1} to 90% at nominal 250 mg kg^{-1} (Table 7). Mean values of ATCLP-extractable TNT within FA KCL soil ranged from 28 to 62% of ACN-extractable concentrations (Table 7). Mean values of ACN-extractable TNT W-A in KCL soil, calculated as percentages of corresponding initial concentrations of ACN-extractable TNT in FA soils, ranged from 2 to 10% of nominal concentrations (Table 8). Mean values of ATCLP-extractable TNT W-A in soil ranged from below the detection limit to 36% of ACN-extractable concentrations (Table 8).

Table 7. Concentrations of TNT FA in KCL Soil Used in Definitive Toxicity Tests with *E. fetida*

Nominal Concentration (mg kg^{-1})	ACN Extraction (mg kg^{-1})	SE	ACN/Nominal (%)	ATCLP Extraction (mg kg^{-1})	SE	ATCLP/ACN (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
10	7	0.1	70	2	0.1	28
20	15	0.2	73	5	0.1	33
40	34	0.4	86	13	0.2	39
50	41	0.3	82	18	0.2	43
60	50	1.3	84	22	0.5	43
80	65	1.7	81	34	0.7	52
100	88	0.8	88	45	1.9	51
150	132	3.6	88	75	1.9	57
200	179	6.6	90	112	1.4	62
250	224	9.2	90	125	10.1	56

Note: Analytically determined concentrations (means and SEs, $n = 3$) include ACN-extractable (U.S. EPA Method 8330A) and water-extractable (ATCLP; Haley et al., 1993) concentration values.
BDL, below detection limit (0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil).

Table 8. Concentrations of TNT W-A in KCL Soil Used in Definitive Toxicity Tests with *E. fetida*

Nominal Concentration (mg kg^{-1})	Initial ACN (mg kg^{-1})	W-A ACN (mg kg^{-1})	W-A/ Initial ACN (%)	W-A ATCLP (mg kg^{-1})	W-A ATCLP/ W-A ACN (%)
0	BDL	BDL	BDL	BDL	BDL
20	15 (0.2)	0.5 (0.03)	3	BDL	BDL
40	34 (0.4)	2 (0.14)	5	0.44 (0.08)	28
50	41 (0.3)	1 (0.03)	2	0.04 (0.04)	5
60	50 (1.3)	4 (0.15)	9	1 (0.01)	25
80	65 (1.7)	5 (0.08)	8	2 (0.36)	36
100	88 (0.8)	2 (0.10)	2	0.5 (0.02)	25
150	132 (3.6)	12 (0.38)	9	4 (0.06)	36
200	179 (6.6)	6 (0.16)	3	2 (0.09)	33
250	224 (9.2)	26 (0.54)	10	7 (1.55)	25

Note: Analytically determined concentrations (means and SEs, $n = 3$) include ACN-extractable (U.S. EPA Method 8330A) and water-extractable (ATCLP; Haley et al., 1993) concentration values.
BDL, below detection limit (0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil).

3.2.4 TNT in RCL Soil

Mean values of ACN-extractable TNT FA in RCL soil, expressed as percentages of amendments, ranged from 40% at nominal 5 mg kg^{-1} to 89% at nominal 300 mg kg^{-1} (Table 9). Mean values of ATCLP-extractable TNT within FA RCL soil ranged from 10 to 57% of ACN-extractable concentrations (Table 9). Mean values of ACN-extractable TNT W-A in RCL soil, calculated as percentages of corresponding initial concentrations of ACN-extractable TNT in FA soils, ranged from 20 to 50% of nominal concentrations (Table 10). Mean values of ATCLP-extractable TNT W-A in soil ranged from below the detection limit to 49% of ACN-extractable concentrations (Table 10).

Table 9. Concentrations of TNT FA in RCL Soil Used in Definitive Toxicity Tests with *E. fetida*

Nominal Concentration (mg kg^{-1})	ACN Extraction (mg kg^{-1})	SE	ACN/Nominal (%)	ATCLP Extraction (mg kg^{-1})	SE	ATCLP/ACN (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
5	2	0.05	40	0.2	0.03	10
10	4	0.1	40	0.4	0.03	10
25	15	0.2	60	3.1	0.11	21
50	35	0.2	70	11.5	0.03	33
100	81	1.6	81	33.9	0.57	42
125	103	0.9	82	47.1	1.11	46
150	126	1.5	84	59.0	0.43	47
200	168	2.0	84	85.0	2.49	51
300	267	5.7	89	152.2	3.27	57

Note: Analytically determined concentrations (means and SEs, $n = 3$) include ACN-extractable (U.S. EPA Method 8330A) and water-extractable (ATCLP; Haley et al., 1993) concentration values.
BDL, below detection limit (0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil).

Table 10. Concentrations of TNT W-A in RCL Soil Used in Definitive Toxicity Tests with *E. fetida*

Nominal Concentration (mg kg^{-1})	Initial ACN (mg kg^{-1})	W-A ACN (mg kg^{-1})	W-A/ Initial ACN (%)	W-A ATCLP (mg kg^{-1})	W-A ATCLP/ W-A ACN (%)
0	BDL	BDL	BDL	BDL	BDL
5	2 (0.05)	1 (0.02)	50	BDL	BDL
10	4 (0.1)	1 (0.15)	25	BDL	BDL
25	15 (0.2)	3 (0.77)	20	0.4 (0.01)	13
50	35 (0.2)	7 (0.19)	20	2 (0.02)	24
100	81 (1.6)	16 (0.52)	20	5 (0.11)	33
150	126 (1.5)	31 (1.00)	25	11 (0.10)	35
200	168 (2.0)	42 (0.09)	25	16 (0.21)	39
400	330 (1.8)	103 (1.35)	31	50 (0.53)	49

Note: Analytically determined concentrations (means and SEs, $n = 3$) include ACN-extractable (U.S. EPA Method 8330A) and water-extractable (ATCLP; Haley et al., 1993) concentration values.
BDL, below detection limit (0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil).

3.2.5 TNT in WCL Soil

Mean values of ACN-extractable TNT FA in WCL soil, expressed as percentages of amendments, ranged from 77% at nominal 200 mg kg^{-1} to 105% at nominal 500 mg kg^{-1} (Table 11). Mean values of ATCLP-extractable TNT within FA WCL soil ranged from 26 to 48% of ACN-extractable concentrations (Table 11). Mean values of ACN-extractable TNT W-A in WCL soil, calculated as percentages of corresponding initial concentrations of ACN-extractable TNT in FA soils, ranged from 0.6 to 32% of nominal concentrations (Table 12). Mean values of ATCLP-extractable TNT W-A in soil ranged from 2 to 32% of ACN-extractable concentrations (Table 12).

Table 11. Concentrations of TNT FA in WCL Soil Used in Definitive Toxicity Tests with *E. fetida*

Nominal Concentration (mg kg^{-1})	ACN Extraction (mg kg^{-1})	SE	ACN/Nominal (%)	ATCLP Extraction (mg kg^{-1})	SE	ATCLP/ACN (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
200	155	7.2	77	41	0.6	26
225	199	10.0	88	55	1.9	28
250	245	10.8	98	83	1.2	34
275	235	9.2	85	72	5.1	31
300	284	16.5	95	100	4.5	35
325	302	9.3	93	118	0.9	39
350	334	6.3	96	126	3.8	36
400	387	6.3	97	159	1.3	40
500	523	22.5	105	200	6.6	38
600	564	21.2	94	272	2.3	48

Note: Analytically determined concentrations (means and SEs, $n = 3$) include ACN-extractable (U.S. EPA Method 8330A) and water-extractable (ATCLP; Haley et al., 1993) concentration values.
BDL, below detection limit (0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil).

Table 12. Concentrations of TNT W-A in WCL Soil Used in Definitive Toxicity Tests with *E. fetida*

Nominal Concentration (mg kg^{-1})	Initial ACN (mg kg^{-1})	W-A ACN (mg kg^{-1})	W-A/ Initial ACN (%)	W-A ATCLP (mg kg^{-1})	W-A ATCLP/ W-A ACN (%)
0	BDL	BDL	BDL	BDL	BDL
200	155	1 (0.1)	0.6	0.02 (0.02)	2
225	199	16 (1.4)	8	2 (0.12)	13
250	245	23 (1.3)	9	3 (0.03)	13
275	235	41 (0.9)	17	7 (0.11)	17
300	284	30 (0.3)	11	5 (0.55)	17
325	302	49 (11)	16	8 (0.18)	16
350	334	57 (0.1)	17	11 (0.13)	19
400	387	11 (0.3)	3	2 (0.10)	18
500	523	165 (2.3)	32	45 (0.19)	27
600	564	182 (1.9)	32	58 (0.26)	32

Note: Analytically determined concentrations (means and SEs, $n = 3$) include ACN-extractable (U.S. EPA Method 8330A) and water-extractable (ATCLP; Haley et al., 1993) concentration values. BDL, below detection limit (0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil).

3.3 Effects of Weathering-and-Aging on TNT Concentrations in Soils

The weathering-and-aging procedures performed in these studies resulted in differential rates of decreases in extractable TNT concentrations in soil over time according to soil type. TNT concentrations decreased more rapidly over time in the three clay loam soils (RCL, KCL, and WCL) than in the two sandy loam soils, TSL and SSL. As weathering-and-aging progressed, the changes in the analytically determined TNT concentrations in the nominal 100 mg kg^{-1} treatment level in the five soils are shown as a function of time in Figure 1. These changes in soil TNT concentrations were typical of other nominal TNT treatments measured periodically over the 82 day weathering-and-aging procedures, and are shown in Figure 1 as examples because the nominal 100 mg kg^{-1} was the only treatment level that was selected for monitoring in all five soils tested in the present studies. Based on the results for each TNT-soil pairing, day 82 "from the initial hydration of each test soil to 60% of the WHC" was designated for terminating the weathering-and-aging procedures, and for beginning definitive toxicity tests with *E. fetida* in each of the five soils. Figure 2 illustrates this general trend across the other treatments, showing the mean TNT concentrations used in the definitive toxicity tests utilizing positive TNT treatments that were either below or above nominal 100 mg kg^{-1} in the five soils after 82 days of weathering-and-aging.

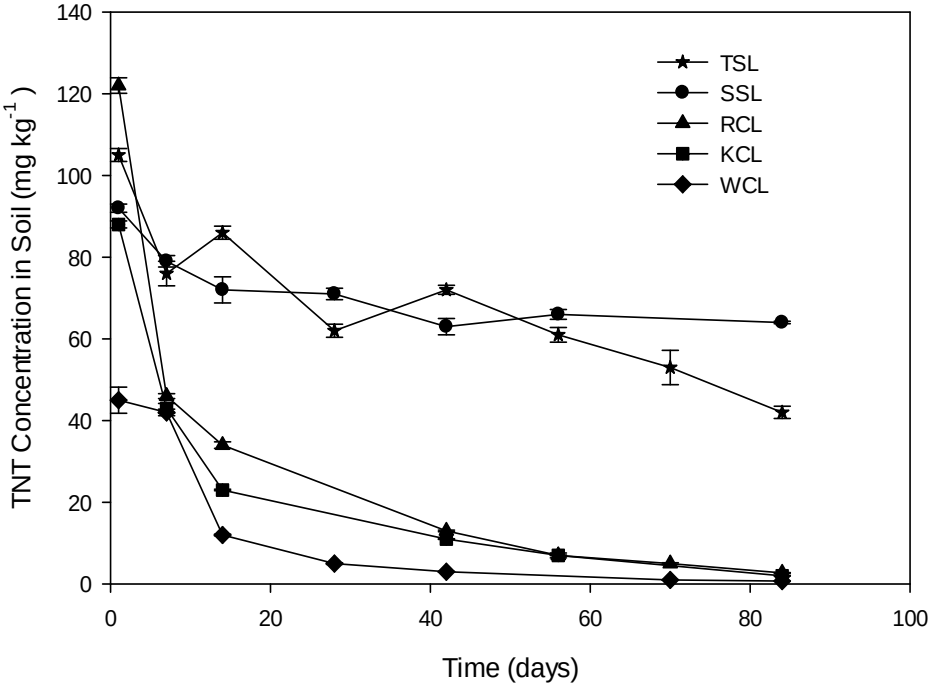

Figure 1. Analytically determined mean TNT concentrations (±SE, n = 3) in soils initially amended with a nominal concentration of 100 mg kg^{-1} TNT, as affected by weathering-and-aging for 82 days. Error bars show SEs of the means. Initial concentrations were determined after a 24 h moisture equilibration of FA soils hydrated to 60% of the WHC of each soil.

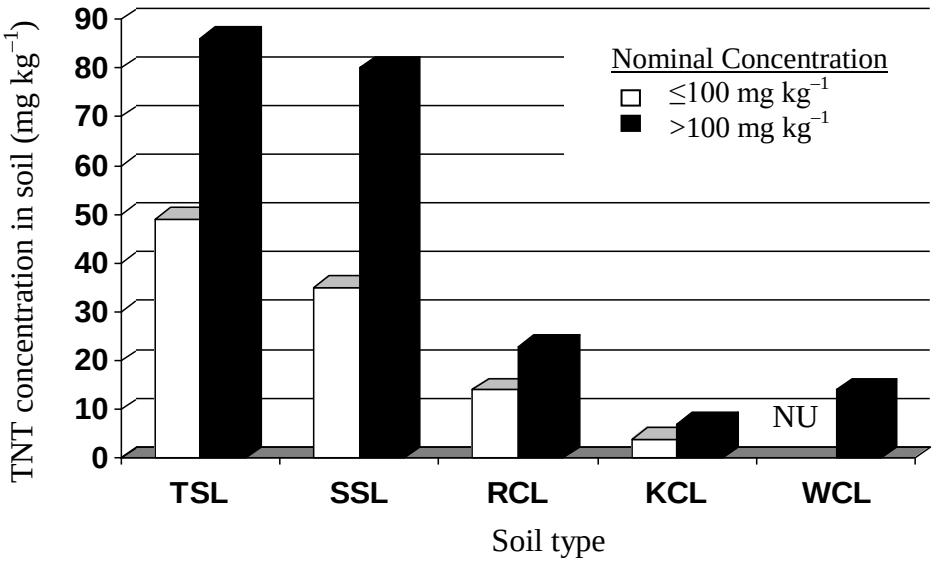

Figure 2. Analytically determined TNT concentrations in the five soils after weathering-and-aging for 82 days. Mean concentrations are shown below and above initial nominal concentrations, either ≤100 or >100 mg kg^{-1}. NU, not used in the WCL definitive toxicity test.

3.4 Range-Finding Toxicity Tests with TNT

Adult *E. fetida* were exposed to TNT concentrations in each of the five test soils in separate experiments to determine the range of concentrations to be used in the definitive reproduction tests. Soils were prepared and amended as described for the FA soils in "Materials and Methods" (Section 2). Nominal TNT concentrations used in each of the range-finding studies were: 0, 10, 100, 200, 400, 800, and 1000 mg kg^{-1} in soil. There were three replicate containers per treatment level and five worms per replicate. Toxicity testing was performed as described in Section 2.9, and assays were terminated after 21 days. All surviving adults and cocoons were then harvested and counted.

Results showed that in all soils tested, there was no cocoon production at nominal 400 mg kg^{-1} TNT and above, and there were no surviving adults at nominal 800 mg kg^{-1} TNT and above. Cocoon production was significantly reduced ($p \leq 0.05$) at nominal 100 mg kg^{-1} TNT in SSL, TSL, KCL, and RCL soils, and at nominal 400 mg kg^{-1} TNT in WCL soil. Treatment concentrations of TNT for the definitive toxicity tests were selected on the basis of the results of the range-finding tests in each of the soils.

3.5 Definitive Toxicity Tests with TNT

Independent definitive studies were conducted using the Earthworm Reproduction Test (ISO, 1998a) to assess the effects of TNT on the reproduction and adult survival of the earthworm *E. fetida* in TSL, SSL, KCL, RCL, and WCL soils. Adult earthworms were exposed to a range of TNT concentrations in each soil. Measurement endpoints were assessed using treatment concentrations that were based on the results of the range-finding studies. Measurement endpoints included numbers of cocoons and juveniles after 56 days and number and mass of surviving adults after 28 days. Concentrations used for definitive toxicity tests for each soil were selected on the basis of bracketing significant effects on reproduction endpoints (i.e., production of cocoons and juveniles). Because reproduction endpoints are the preferred Eco-SSL benchmarks for the development of Eco-SSL values for soil invertebrates (U.S. EPA, 2005), they were the main focus of these studies. The ranges of test concentrations were expanded to determine the concentration that caused a lethal effect to adult earthworms. Significant effects on the adult survival endpoint were determined when possible but were not critical to the success of these studies. All ecotoxicological parameters were estimated using these measurement endpoint values and analytically determined concentrations of TNT in soil utilizing U.S. EPA Method 8330A.

Test results complied with the validity criteria defined in the ISO test guideline. In all tests, mean adult survival in negative controls was >90%. The coefficient of variation for production of juveniles in control treatments did not exceed 50%. Juvenile production in positive controls ranged from 54 to 98% reduction compared with negative controls and was within the baseline established for the laboratory culture of *E. fetida*. These results confirmed that the significant toxicological effects determined in the definitive tests were attributable to the TNT treatments. All reported ecotoxicological parameters were calculated on the basis of analytically determined TNT concentrations in the respective soils.

3.5.1 TNT Toxicity to *E. fetida* in TSL Soil

Ecotoxicological responses of *E. fetida* to TNT FA or W-A in TSL soil are shown in Tables 13 and 14, respectively. As shown in Table 13, LOEC values for TNT FA in TSL soil were: 109 mg kg^{-1} (adult survival), 29 mg kg^{-1} (total cocoons produced), 29 mg kg^{-1} (hatched cocoons), and 29 mg kg^{-1} (total juveniles produced). Adult dry mass per worm was not significantly ($p \leq 0.05$) affected by TNT FA in TSL soil. As shown in Table 14, LOEC values for TNT W-A in soil were: 122 mg kg^{-1} (adult survival), 159 mg kg^{-1} (adult dry mass per worm), 42 mg kg^{-1} (total cocoons produced), 42 mg kg^{-1} (hatched cocoons), and 42 mg kg^{-1} (total juveniles produced).

Table 13. Ecotoxicological Responses of Earthworm *E. fetida* to TNT FA in TSL Soil

Parameter	Nominal TNT Concentration[a] (mg kg^{-1})									
	0 Negative Control	0 Carrier Control[b]	5	10	20	40	60	80	100	120
Analytically determined TNT concentration, initial mean (mg kg^{-1} dry soil)	BDL	BDL	2 (0.2)	4 (0.1)	9 (0.1)	29 (0.4)	49 (1.0)	69 (0.7)	88 (1.3)	109 (1.6)
Adult survival/ replicate (%)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	93 (7)	100 (0)	25[d] (19)
Adult final dry mass/worm[c] (mg)	50 (2)	44 (3)	53 (3)	44 (3)	48 (3)	46 (3)	45 (3)	43 (3)	44 (3)	41 (4)
Cocoons, mean total (no.)	14.8 (2.3)	19.5 (0.6)	16.5 (1.2)	22.0 (3.4)	18.0 (1.7)	9.8[d] (0.8)	5.3[d] (1.5)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)
Cocoons, mean hatched (no.)	9.8 (2.1)	15.8 (2.0)	11.0 (0.7)	14.5 (3.3)	13.3 (2.2)	5.3[d] (0.9)	2.3[d] (1.3)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)
Juveniles, mean (no.)	30.8 (7.6)	49.0 (6.9)	34.3 (6.3)	51.5 (11.8)	49.0 (7.1)	13.5[d] (2.5)	6.5[d] (3.8)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)

Note: All soils were hydrated to 95% WHC (12.4% dry mass soil) 24 h before start of test; $n = 4$ replicates, 5 adult worms per replicate.

[a] Values in parentheses are SEs.

[b] For carrier control, acetone was added as carrier solvent and evaporated before rehydration of soil.

[c] Adult final dry mass/worm = least-square mean adjusted for the covariate, initial live mass/worm, as determined by analysis of covariance.

[d] Significantly less ($p \leq 0.05$) than carrier controls within respective rows according to ANOVA and FLSD means comparison.

BDL, below detection limit of 0.5 mg kg^{-1}.

Table 14. Ecotoxicological Responses of Earthworm *E. fetida* to TNT W-A in TSL Soil

Parameter	Nominal TNT Concentration[a] (mg kg^{-1})									
	0 Negative Control	0 Carrier Control[b]	15	30	50	100	120	140	160	180
Analytically determined TNT concentration, initial mean (mg kg^{-1} dry soil)	BDL	BDL	12 (0.3)	16 (0.3)	27 (0.7)	42 (1.5)	101 (2.4)	122 (1.4)	135 (2.2)	159 (4.8)
Adult survival/ replicate (%)	100 (0)	100 (0)	100 (0)	95 (5)	100 (0)	95 (5)	90 (6)	30[d] (17)	30[d] (24)	35[d] (15)
Adult final dry mass/worm[c] (mg)	72 (2)	72 (2)	66 (3)	70 (2)	65 (2)	75 (3)	66 (2)	59 (3)	63 (3)	49[d] (12)
Cocoons, mean total (no.)	12.8 (1.2)	15.5 (0.9)	8.0 (2.9)	11.8 (2.7)	9.3 (1.6)	2.5[d] (0.6)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)
Cocoons, mean hatched (no.)	8.5 (1.6)	10.8 (0.9)	5.8 (2.2)	8.0 (2.3)	5.3 (0.9)	1.0[d] (0.4)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)
Juveniles, mean (no.)	30.8 (4.0)	30.7 (4.0)	15.5 (7.0)	20.3 (8.9)	19.3 (4.4)	1.3[d] (0.8)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)

Note: All soils were hydrated to 95% WHC (12.4% dry mass soil) 24 h before start of test; n = 4 replicates, 5 adult worms per replicate.

[a] Values in parentheses are SEs.

[b] For carrier control, acetone was added as carrier solvent and evaporated before rehydration of soil.

[c] Adult final dry mass/worm = least-square mean adjusted for the covariate, initial live mass/worm, as determined by analysis of covariance.

[d] Significantly less ($p \leq 0.05$) than carrier controls within respective rows, according to ANOVA and FLSD means comparison.

BDL, below detection limit of 0.5 mg kg^{-1}.

3.5.2 TNT Toxicity to *E. fetida* in SSL Soil

Ecotoxicological responses of *E. fetida* to TNT FA and W-A in SSL soil are shown in Tables 15 and 16, respectively. Analytically determined LOEC values for important ecotoxicological responses to TNT in soil by *E. fetida* were significantly reduced ($p \leq 0.05$) in FA SSL soil compared with corresponding responses in SSL control soils. The LOEC values were: 85 mg kg^{-1} (adult survival), 134 mg kg^{-1} (adult dry mass per worm), 62 mg kg^{-1} (total cocoons produced), 40 mg kg^{-1} (hatched cocoons), and 40 mg kg^{-1} (total juveniles produced) (Table 15). The LOEC values for responses to TNT W-A in soil by *E. fetida* were: 94 mg kg^{-1} (adult survival), 119 mg kg^{-1} (adult dry mass per worm), 15 mg kg^{-1} (total cocoons produced), 15 mg kg^{-1} (hatched cocoons), and 15 mg kg^{-1} (total juveniles produced) (Table 16).

Table 15. Ecotoxicological Responses of Earthworm *E. fetida* to TNT FA in SSL Soil

Parameter	Nominal TNT Concentration[a] (mg kg^{-1})									
	0 Negative Control	0 Carrier Control[b]	10	25	50	75	100	150	200	300
Analytically determined TNT concentration, initial mean (mg kg^{-1} dry soil)	BDL	BDL	7 (0.1)	21 (0.9)	40 (0.4)	62 (2.1)	85 (0.2)	134 (6.1)	186 (2.4)	287 (2.5)
Adult survival/ replicate (%)	100 (0)	100 (0)	100 (0)	100 (0)	95 (5)	85 (10)	26[d] (26)	46[d] (26)	0.0[d] (0.0)	0.0[d] (0.0)
Adult final dry mass/worm[c] (mg)	46 (2)	42 (1)	40 (3)	44 (2)	45 (3)	40 (3)	38 (0)	28[d] (1)	0.0[d] (0.0)	0.0[d] (0.0)
Cocoons, mean total (no.)	9.0 (1.2)	8.5 (1.0)	17.0 (4.2)	13.3 (2.0)	7.8 (2.1)	4.8[d] (2.3)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)
Cocoons, mean hatched (no.)	4.3 (1.7)	6.3 (0.6)	13.0 (4.0)	9.7 (2.2)	3.3[d] (0.5)	3.3[d] (2.3)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)
Juveniles, mean (no.)	14.7 (7.1)	17.0 (1.7)	44.7 (16.9)	33.0 (9.7)	4.8[d] (1.3)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)

Note: All soils were hydrated to 95% WHC (17.1% dry mass soil) 24 h before start of test; $n = 4$ replicates, 5 adult worms per replicate.

[a] Values in parentheses are SEs.

[b] For carrier control, acetone was added as carrier solvent and evaporated before rehydration of soil.

[c] Adult final dry mass/worm = least-square mean adjusted for the covariate, initial live mass/worm, as determined by analysis of covariance.

[d] Significantly less ($p \leq 0.05$) than carrier controls within respective rows according to ANOVA and FLSD means comparison.

BDL, below detection limit of 0.5 mg kg^{-1}.

Table 16. Ecotoxicological Responses of Earthworm *E. fetida* to TNT W-A in SSL Soil

Parameter	Nominal TNT Concentration[a] (mg kg^{-1})									
	0 Negative Control	0 Carrier Control[b]	10	25	50	75	100	125	150	200
Analytically determined TNT concentration, initial mean (mg kg^{-1} dry soil)	BDL	BDL	2 (0.2)	5 (0.4)	15 (0.3)	36 (1.4)	64 (0.3)	94 (2.7)	119 (1.2)	170 (2.7)
Adult survival/ replicate (%)	100 (0)	100 (0)	100 (0)	100 (0)	95 (5)	100 (0)	85 (10)	20[d] (12)	0.0[d] (0)	0.0[d] (0)
Adult final dry mass/worm[c] (mg)	42 (2)	40 (3)	43 (3)	44 (3)	50 (3)	48 (3)	40 (3)	35 (4)	0.0[d] (0)	0.0[d] (0)
Cocoons, mean total (no.)	17.3 (0.5)	16.3 (4.5)	17.8 (2.3)	14.5 (2.2)	7.3[d] (2.3)	3.5[d] (2.0)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)
Cocoons, mean hatched (no.)	9.3 (1.0)	8.5 (1.3)	12.0 (2.2)	10.0 (0.7)	2.3[d] (1.4)	1.3[d] (1.3)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)
Juveniles, mean (no.)	18.0 (4.1)	25.3 (5.5)	26.5 (6.0)	24.0 (3.0)	4.5[d] (2.3)	3.0[d] (3.0)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)

Note: All soils were hydrated to 95% WHC (17.1% dry mass soil) 24 h before start of test; n = 4 replicates, 5 adult worms per replicate.

[a] Values in parentheses are SEs.

[b] For carrier control, acetone was added as carrier solvent and evaporated before rehydration of soil.

[c] Adult final dry mass/worm = least-square mean adjusted for the covariate, initial live mass/worm, as determined by analysis of covariance.

[d] Significantly less ($p \leq 0.05$) than carrier controls within respective rows according to ANOVA and FLSD means comparison.

BDL, below detection limit of 0.5 mg kg^{-1}.

3.5.3 TNT Toxicity to *E. fetida* in KCL Soil

Ecotoxicological responses of *E. fetida* to TNT FA and W-A in KCL soil are shown in Tables 17 and 18, respectively. Analytically determined LOEC values for important ecotoxicological responses to TNT in soil by *E. fetida* were significantly reduced ($p \leq 0.05$) in FA KCL soils compared with corresponding responses in KCL control soils. The LOEC values were: 179 mg kg^{-1} (adult survival), 132 mg kg^{-1} (adult dry mass per worm), 88 mg kg^{-1} (total cocoons produced), 88 mg kg^{-1} (hatched cocoons), and 65 mg kg^{-1} (total juveniles produced) (Table 17). The LOEC values for responses to TNT W-A in soil by *E. fetida* were: 5 mg kg^{-1} (total cocoons produced), 5 mg kg^{-1} (hatched cocoons), and 2 mg kg^{-1} (total juveniles produced) (Table 18). Values for adult survival and adult dry mass per worm were not significantly reduced ($p > 0.05$).

Table 17. Ecotoxicological Responses of Earthworm *E. fetida* to TNT FA in KCL Soil

Parameter	Nominal TNT Concentration[a] (mg kg^{-1})											
	0 Negative Control	0 Carrier Control[b]	10	20	40	50	60	80	100	150	200	250
Analytically determined TNT concentration, initial mean (mg kg^{-1} dry soil)	BDL	BDL	7 (0.1)	15 (0.2)	34 (0.4)	41 (0.3)	50 (1.3)	65 (1.7)	88 (0.8)	132 (3.6)	179 (6.6)	224 (9.2)
Adult survival/ replicate (%)	100 (0)	100 (0)	95 (5)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	95 (5)	65[d] (24)	50[d] (21)
Adult final dry mass/worm[c] (mg)	44 (2)	51 (2)	45 (3)	46 (0.4)	50 (0.5)	45 (2)	52 (1)	49 (5)	54 (2)	41[d] (6)	32[d] (1)	38[d] (5)
Cocoons, mean total (no.)	10.3 (1.7)	12.0 (2.0)	12.5 (3.0)	10.8 (1.0)	6.5 (1.8)	8.5 (1.9)	10.0 (2.1)	10.8 (2.1)	1.5[d] (1.2)	0.8[d] (0.5)	0.0[d] (0.0)	0.0[d] (0.0)
Cocoons, mean hatched (no.)	6.3 (1.7)	7.3 (1.1)	9.5 (2.4)	7.8 (0.9)	5.5 (1.9)	6.0 (2.0)	8.0 (1.5)	5.5 (1.0)	1.5[d] (1.2)	0.8[d] (0.5)	0.0[d] (0.0)	0.0[d] (0.0)
Juveniles, mean (no.)	16.0 (3.2)	18.8 (3.0)	22.8 (7.8)	25.5 (2.2)	14.0 (3.3)	14.8 (4.9)	24.3 (2.7)	11.8[d] (3.1)	6.5[d] (2.7)	1.5[d] (1.2)	0.0[d] (0.0)	0.0[d] (0.0)

Note: All soils were hydrated to 95% WHC (20.0% dry mass soil) 24 h before start of test; $n = 4$ replicates, 5 adult worms per replicate.

[a] Values in parentheses are SEs.

[b] For carrier control, acetone was added as carrier solvent and evaporated before rehydration of soil.

[c] Adult final dry mass/worm was the least-square mean adjusted for the covariate, initial live mass/worm, as determined by analysis of covariance.

[d] Significantly less ($p \leq 0.05$) than carrier controls within respective rows according to ANOVA and FLSD means comparison.

BDL, below detection limit of 0.5 mg kg^{-1}.

Table 18. Ecotoxicological Responses of Earthworm *E. fetida* to TNT W-A in KCL Soil

Parameter	Nominal TNT Concentration[a] (mg kg^{-1})										
	0 Negative Control	0 Carrier Control[b]	20	40	50	60	80	100	150	200	250
Analytically determined TNT concentration, initial mean (mg kg^{-1} dry soil)	BDL	BDL	0.5 (0.03)	2 (0.1)	1 (0.03)	4 (0.2)	5 (0.1)	2 (0.1)	12 (0.4)	6 (0.2)	26 (0.5)
Adult survival/ replicate (%)	100 (0)	100 (0)	100 (0)	100 (5)	95 (5)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)
Adult final dry mass/worm[c] (mg)	45 (3)	48 (1)	49 (2)	43 (3)	49 (2)	44 (1)	46 (1)	50 (4)	47 (2)	49 (3)	47 (7)
Cocoons, mean total (no.)	15.0 (3.4)	22.0 (1.4)	19.8 (0.9)	22.8 (7.1)	15.8 (2.3)	20.0 (2.6)	14.0[d] (1.3)	9.5[d] (2.7)	3.5[d] (0.6)	0.0[d] (0.0)	0.0[d] (0.0)
Cocoons, mean hatched (no.)	9.8 (1.8)	15.5 (1.0)	15.3 (1.6)	16.0 (2.7)	12.3 (1.8)	15.8 (3.0)	6.5[d] (1.7)	3.5[d] (1.3)	2.0[d] (0.4)	0.0[d] (0.0)	0.0[d] (0.0)
Juveniles, mean (no.)	28.3 (8.6)	42.8 (3.4)	44.3 (4.3)	37.3 (9.6)	24.8 (5.2)	53.0 (8.6)	12.5[d] (7.9)	2.8[d] (3.1)	1.0[d] (0.6)	0.0[d] (0.0)	0.0[d] (0.0)

Note: All soils were hydrated to 95% WHC (20.0% dry mass soil) 24 h before start of test; n = 4 replicates, 5 adult worms per replicate.

[a] Values in parentheses are SEs.

[b] For carrier control, acetone was added as carrier solvent and evaporated before rehydration of soil.

[c] Adult final dry mass/worm = least-square mean adjusted for the covariate, initial live mass/worm, as determined by analysis of covariance.

[d] Significantly less ($p \leq 0.05$) than carrier controls within respective rows according to ANOVA and FLSD means comparison.

BDL, below detection limit of 0.5 mg kg^{-1}.

3.5.4 TNT Toxicity to *E. fetida* in RCL Soil

Ecotoxicological responses of *E. fetida* to TNT FA and W-A in RCL soil are shown in Tables 19 and 20, respectively. Analytically determined LOEC values for important ecotoxicological responses to TNT in soil by *E. fetida* were significantly reduced ($p \leq 0.05$) in FA RCL soils compared with corresponding responses in RCL control soils. The LOEC values were: 267 mg kg^{-1} (adult survival), 168 mg kg^{-1} (adult dry mass per worm), 35 mg kg^{-1} (total cocoons produced), 35 mg kg^{-1} (hatched cocoons), and 80 mg kg^{-1} (total juveniles produced) (Table 19). The LOEC values for responses to TNT W-A in soil by *E. fetida* were: 103 mg kg^{-1} (adult survival), 103 mg kg^{-1} (adult dry mass per worm), 16 mg kg^{-1} (total cocoons produced), 7 mg kg^{-1} (hatched cocoons), and 7 mg kg^{-1} (total juveniles produced) (Table 20).

Table 19. Ecotoxicological Responses of Earthworm *E. fetida* to FA TNT in RCL Soil

Parameter	Nominal TNT Concentration[a] (mg kg^{-1})										
	0 Negative Control	0 Carrier Control[b]	5	10	25	50	100	125	150	200	300
Analytically determined TNT concentration, initial mean (mg kg^{-1} dry soil)	BDL	BDL	2 (0.1)	4 (0.1)	15 (0.2)	35 (0.2)	80 (1.6)	103 (0.9)	126 (1.5)	168 (2.0)	267 (5.7)
Adult survival/ replicate (%)	100 (0)	95 (5)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	95 (5)	80 (20)	0.0[d] (0.0)
Adult final dry mass/worm[c] (mg)	59 (4)	58 (4)	57 (1)	54 (2)	54 (3)	54 (2)	59 (2)	53 (1)	55 (3)	41[d] (2)	0.0[d] (0.0)
Cocoons, mean total (no.)	17.3 (2.4)	16.0 (3.5)	18.3 (2.3)	18.5 (2.0)	17.3 (2.6)	11.5[d] (2.5)	3.3[d] (0.0)	0.8[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)
Cocoons, mean hatched (no.)	10.5 (0.9)	11.5 (2.5)	11.8 (1.6)	12.8 (2.3)	10.8 (1.0)	7.5[d] (1.3)	1.8[d] (1.2)	0.3[d] (0.3)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)
Juveniles, mean (no.)	33.3 (4.9)	39.0 (8.3)	34.8 (8.0)	46.8 (5.5)	33.0 (3.5)	34.8 (6.2)	5.0[d] (3.3)	1.0[d] (1.0)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)

Note: All soils were hydrated to 95% WHC (21.0% dry mass soil) 24 h before start of test; n = 4 replicates, 5 adult worms per replicate.

[a] Values in parentheses are SEs.

[b] For carrier control, acetone was added as carrier solvent and evaporated before rehydration of soil.

[c] Adult final dry mass/worm = least-square mean adjusted for the covariate, initial live mass/worm, as determined by analysis of covariance.

[d] Significantly less ($p \leq 0.05$) than carrier controls within respective rows according to ANOVA and FLSD means comparison.

BDL, below detection limit of 0.5 mg kg^{-1}.

Table 20. Ecotoxicological Responses of Earthworm *E. fetida* to TNT W-A in RCL Soil

Parameter	Nominal TNT Concentration[a] (mg kg^{-1})									
	0 Negative Control	0 Carrier Control[b]	5	10	25	50	100	150	200	400
Analytically determined TNT concentration, initial mean (mg kg^{-1} dry soil)	BDL	BDL	1 (0.1)	1 (0.1)	3 (0.2)	7 (0.2)	16 (1.6)	31 (0.9)	42 (1.5)	103 (2.0)
Adult survival/ replicate (%)	100 (0)	100 (5)	100 (0)	100 (0)	100 (0)	96 (0)	96 (0)	100 (0)	86 (5)	0[d] (20)
Adult final dry mass/worm[c] (mg)	79 (2)	76 (9)	82 (7)	82 (4)	91 (11)	85 (3)	78 (3)	71 (9)	79 (3)	55[d] (4)
Cocoons, mean total (no.)	20.8 (3.3)	21.8 (2.5)	26.3 (2.9)	19.5 (3.8)	18.8 (2.3)	20.5 (1.2)	12.0[d] (4.9)	3.5[d] (1.2)	1.0[d] (0.7)	0.0[d] (0.0)
Cocoons, mean hatched (no.)	13.0 (1.5)	13.8 (2.1)	16.5 (1.2)	12.3 (3.2)	14.8 (2.0)	12.8[d] (1.5)	6.8[d] (3.0)	0.5[d] (0.3)	0.8[d] (0.8)	0.0[d] (0.0)
Juveniles, mean (no.)	32.8 (6.3)	41.8 (6.5)	37.8 (9.0)	41.3 (9.1)	35.3 (5.5)	22.8[d] (4.3)	19.8[d] (8.8)	0.8[d] (0.5)	0.3[d] (0.3)	0.0[d] (0.0)

Note: All soils were hydrated to 95% WHC (21.0% dry mass soil) 24 h before start of test; n = 4 replicates, 5 adult worms per replicate.

[a] Values in parentheses are SEs.

[b] For carrier control, acetone was added as carrier solvent and evaporated before rehydration of soil.

[c] Adult final dry mass/worm = least-square mean adjusted for the covariate, initial live mass/worm, as determined by analysis of covariance.

[d] Significantly less ($p \leq 0.05$) than carrier controls within respective rows according to ANOVA and FLSD means comparison.

BDL, below detection limit of 0.5 mg kg^{-1}.

3.5.5 TNT Toxicity to *E. fetida* in WCL Soil

Ecotoxicological responses of *E. fetida* to TNT FA and W-A in WCL soil are shown in Tables 21 and 22, respectively. Analytically determined LOEC values for important ecotoxicological responses to TNT in soil by *E. fetida* were significantly reduced ($p \leq 0.05$) in FA WCL soils compared with corresponding responses in WCL control soils. The LOEC values were: 387 mg kg^{-1} (adult survival), 522 mg kg^{-1} (adult dry mass per worm), 198 mg kg^{-1} (total cocoons produced), 198 mg kg^{-1} (hatched cocoons), and 198 mg kg^{-1} (total juveniles produced) (Table 21). The LOEC values for responses to TNT W-A in soil by *E. fetida* were: 16 mg kg^{-1} (total cocoons produced), 16 mg kg^{-1} (hatched cocoons), and 16 mg kg^{-1} (total juveniles produced) (Table 22). The values for adult survival and adult dry mass per worm were not significantly reduced ($p > 0.05$).

Table 21. Ecotoxicological Responses of Earthworm *E. fetida* to TNT FA in WCL Soil

Parameter	Nominal TNT Concentration[a] (mg kg^{-1})											
	0 Negative Control	0 Carrier Control[b]	200	225	250	275	300	325	350	400	500	600
Analytically determined TNT concentration, initial mean (mg kg^{-1} dry soil)	BDL	BDL	154 (7)	198 (10)	245 (11)	235 (9)	283 (16)	302 (9)	334 (6)	387 (6)	522 (23)	563 (21)
Adult survival/ replicate (%)	95 (5)	100 (0)	100 (0)	100 (5)	95 (5)	95 (5)	95 (5)	85 (10)	80 (11)	20[d] (20)	0.0[d] (0)	0.0[d] (0)
Adult final dry mass/worm[c] (mg)	62 (1)	61 (1)	62 (1)	59 (3)	58 (4)	57 (2)	62 (2)	57 (5)	58 (5)	69 (0)	0.0[d] (0.0)	0.0[d] (0.0)
Cocoons, mean total (no.)	14.0 (2.0)	15.8 (0.9)	11.3 (1.5)	8.8[d] (1.1)	1.5[d] (0.5)	3.5[d] (1.9)	1.5[d] (0.9)	1.0[d] (0.6)	0.8[d] (0.6)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)
Cocoons, mean hatched (no.)	11.3 (2.0)	10.3 (2.3)	8.3 (1.2)	7.0[d] (0.7)	1.0[d] (0.7)	2.8[d] (1.5)	0.5[d] (0.3)	0.5[d] (0.5)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)
Juveniles, mean (no.)	20.5 (0.6)	24.3 (2.6)	21.7 (0.9)	15.0[d] (3.8)	1.0[d] (1.0)	3.5[d] (2.0)	0.5[d] (0.3)	0.3[d] (0.3)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)	0.0[d] (0.0)

Note: All soils were hydrated to 95% WHC (23.0% dry mass soil) 24 h before start of test; n = 4 replicates, 5 adult worms per replicate.

[a] Values in parentheses are SEs.

[b] For carrier control, acetone was added as carrier solvent and evaporated before rehydration of soil.

[c] Adult final dry mass/worm = least-square mean adjusted for the covariate, initial live mass/worm, as determined by analysis of covariance.

[d] Significantly less ($p \leq 0.05$) than carrier controls within respective rows according to ANOVA and FLSD means comparison.

BDL, below detection limit of 0.5 mg kg^{-1}.

Table 22. Ecotoxicological Responses of Earthworm *E. fetida* to TNT W-A in WCL Soil

Parameter	Nominal TNT Concentration[a] (mg kg^{-1})											
	0 Negative Control	0 Carrier Control[b]	200	225	250	275	300	325	350	400	500	600
Analytically determined TNT concentration, initial mean (mg kg^{-1} dry soil)	BDL	BDL	1.4 (0.1)	16 (1.4)	22 (1.3)	41 (0.9)	30 (0.3)	49 (10.5)	57 (0.1)	11 (0.3)	165 (2.3)	182 (1.9)
Adult survival/ replicate (%)	95 (5)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	95 (5)	90 (6)	95 (5)	85 (15)	95 (5)
Adult final dry mass/worm[c] (mg)	50 (2)	53 (2)	49 (3)	47 (1)	48 (4)	52 (2)	47 (3)	50 (4)	49 (9)	46 (6)	48 (1)	46 (3)
Cocoons, mean total (no.)	16.5 (1.3)	19.8 (3.8)	14.8 (2.3)	9.5[d] (1.3)	14.8 (3.3)	12.0 (3.8)	9.8[d] (1.9)	6.0[d] (2.0)	1.5[d] (0.9)	5.3[d] (1.0)	0.0[d] (0.0)	0.0[d] (0.0)
Cocoons, mean hatched (no.)	11.3 (2.5)	12.5 (3.5)	8.3 (3.2)	3.8[d] (0.8)	8.8[d] (4.1)	6.8[d] (1.8)	6.8[d] (1.3)	3.5[d] (1.2)	1.3[d] (0.9)	1.3[d] (0.6)	0.0[d] (0.0)	0.0[d] (0.0)
Juveniles, mean (no.)	21.3 (9.6)	25.3 (6.8)	20.5 (12.0)	3.0[d] (2.0)	15.8 (10.3)	13.8[d] (15.8)	15.8 (9.3)	8.8[d] (3.7)	1.5[d] (0.5)	0.8[d] (0.5)	0.0[d] (0.0)	0.0[d] (0.0)

Note: All soils were hydrated to 95% WHC (23.0% dry mass soil) 24 h before start of test; $n = 4$ replicates, 5 adult worms per replicate.

[a] Values in parentheses are SEs.

[b] For carrier control, acetone was added as carrier solvent and evaporated before rehydration of soil.

[c] Adult final dry mass/worm = least-square mean adjusted for the covariate, initial live mass/worm, as determined by analysis of covariance.

[d] Significantly less ($p \leq 0.05$) than carrier controls within respective rows according to ANOVA and FLSD means comparison.

BDL, below detection limit of 0.5 mg kg^{-1}.

3.6 Development of Soil Toxicity Benchmark Values and Comparison of TNT Toxicities to *E. fetida* in the Five Soil Types

Toxicological benchmark values (NOEC, LOEC, EC_{20}, and EC_{50} values) for *E. fetida* exposed to TNT, either FA or W-A in each of five natural soils with contrasting physical and chemical properties, are shown in Table 23. NOEC and LOEC values were determined by ANOVA and means separation by FLSD at $p \leq 0.05$ to test for significant differences of treatment means compared with carrier control means. The NOEC and LOEC values showed that cocoon production and juvenile production were more-sensitive indicators of TNT toxicity to earthworms than adult survival (Table 23). In general, the NOEC and LOEC values were greater (toxicity was less) in KCL and WCL soils (Table 23). Concentration-response relationships were generated by nonlinear regression of analytically determined TNT concentrations with cocoon production and juvenile production, in either FA or W-A TNT treatments in each soil. Representative concentration-response relationships for production of cocoons and juveniles by *E. fetida* in each soil are shown in Figures 3–7. Concentration-response relationships were also determined for surviving adults (not shown). Benchmark values for the effective TNT concentrations that caused 20 or 50% reductions in the measurement endpoints (the EC_{20} and EC_{50} values, respectively) compared with the carrier control were derived from the concentration-response relationships (Table 23). Examination of the 95% CI estimates for the EC_{20} and EC_{50} values showed that, in soil FA with TNT, toxicity was reduced in KCL or WCL soils compared with SSL, TSL, and RCL soils. In general, toxicity of TNT to *E. fetida* in FA soil was in the order (from greatest to least) of TSL > SSL = RCL > KCL > WCL (Table 23). On the basis of either the EC_{20} or EC_{50} values and the corresponding 95% CIs (Table 23), weathering-and-aging of TNT in soil significantly increased the toxicity of TNT to both reproduction endpoints in all soils except for TSL.

3.7 Effects of Selected Soil Properties on Toxicity of TNT to *E. fetida* Reproduction

Pearson's correlation analysis was used to determine the relationships among selected soil properties and the respective EC_{20} and EC_{50} values for TNT FA and W-A in the five soils. Results of these analyses are shown in Tables 24 and 25. For FA TNT, soil OM content significantly ($p \leq 0.05$) correlated with EC_{50} for cocoon production and EC_{20} and EC_{50} values for juvenile production, with respective r values of 0.88, 0.92, and 0.93 (Table 24). In contrast, there were no significant ($p \leq 0.05$) correlations among any of the measurement endpoints and soil properties that were analyzed in studies with TNT W-A in soil (Table 25).

Table 23. Summary: Toxicological Benchmarks for TNT Determined in Definitive Tests with *E. fetida* for TNT FA or W-A in TSL, SSL, KCL, RCL, and WCL Soils

Ecotoxicological Parameter	TSL Soil		SSL Soil		KCL Soil		RCL Soil		WCL Soil	
	FA	W-A	FA	W-A	FA	W-A	FA	W-A	FA	W-A
Adult Survival										
NOEC	88	101	62	64	132	26	35	31	284	182
p	1.0	0.408	0.364	0.090	0.659	1.0	0.455	1.0	0.802	0.723
LOEC	109	123	85	94	179	>26	81	42	302	>183
p	0.001	0.001	0.001	0.001	0.004	1.0	0.001	0.002	0.008	0.723
LC_{20}	91	91	48	68	160	>26	169	43	335	>183
95% CI	76–106	71–111	39–57	60–76	123–197	ND	152–187	33–529	281–389	ND
LC_{50}	104	122	120	82	219	>26	195	53	366	>183
95% CI	98–110	110–134	97–143	77–87	193–246	ND	111–279	28–79	337–394	ND
Model used	Gompertz	Gompertz	Linear	Gompertz	Gompertz	ND	Gompertz	Gompertz	Gompertz	ND
R^2	0.908	0.943	0.867	0.987	0.970	ND	0.984	0.984	0.919	0.939
Cocoon Production										
NOEC	9	16	40	5	65	5	15	7	155	23
p	0.270	0.753	0.001	0.146	0.586	0.249	0.798	0.820	0.100	0.247
LOEC	29	27	62	15	88	12	35	16	199	30
p	0.001	0.001	0.048	0.001	0.001	0.001	0.042	0.008	0.001	0.006
EC_{20}	18	26	38	4	71	6	27	9	157	27
95% CI	12–23	15–38	28–49	1–7	53–88	3–9	8–45	3–16	134–181	15–40
EC_{50}	32	35	53	12	79	9	48	18	201	42
95% CI	27–37	27–42	36–70	2–21	68–90	6–11	33–63	12–24	187–215	33–51
Model used	Gompertz	Gompertz	Hormetic	Exponential	Gompertz	Gompertz	Gompertz	Gompertz	Gompertz	Gompertz
R^2	0.997	0.848	0.881	0.921	0.902	0.935	0.923	0.919	0.935	0.870
Juvenile Production										
NOEC	9	16	62	5	65	2	35	7	155	49
p	0.174	0.096	0.314	0.882	0.199	0.758	0.814	0.061	0.575	0.102
LOEC	29	27	85	15	88	5	81	16	199	57
p	0.001	0.001	0.032	0.001	0.016	0.001	0.001	0.026	0.007	0.017
EC_{20}	9	6	33	3	53	1	45	4	190	19
95% CI	0.5–18	3–10	20–46	0.5–5	24–81	0.4–2	16–74	1–7	178–202	6–31
EC_{50}	29	20	40	9	78	4	61	12	212	33
95% CI	2–56	8–22	20–60	2–16	55–100	1–6	40–82	3–22	205–220	23–42
Model used	Exponential	Exponential	Hormetic	Hormetic	Gompertz	Exponential	Gompertz	Gompertz	Gompertz	Gompertz
R^2	0.867	0.785	0.749	0.749	0.810	0.810	0.903	0.903	0.955	0.833

Notes: All concentrations are expressed as milligrams of TNT per kilogram of dry soil mass and were based on ACN extraction and analyses by HPLC using U.S. EPA Method 8330A. NOEC and LOEC values were derived from ANOVA procedures and FLSD pairwise-means comparison test. EC_{20} and EC_{50} values were calculated from regression models that were fit to data from each soil type (see Figures 4–8).

R^2, regression sum of squares divided by total sum of squares.

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

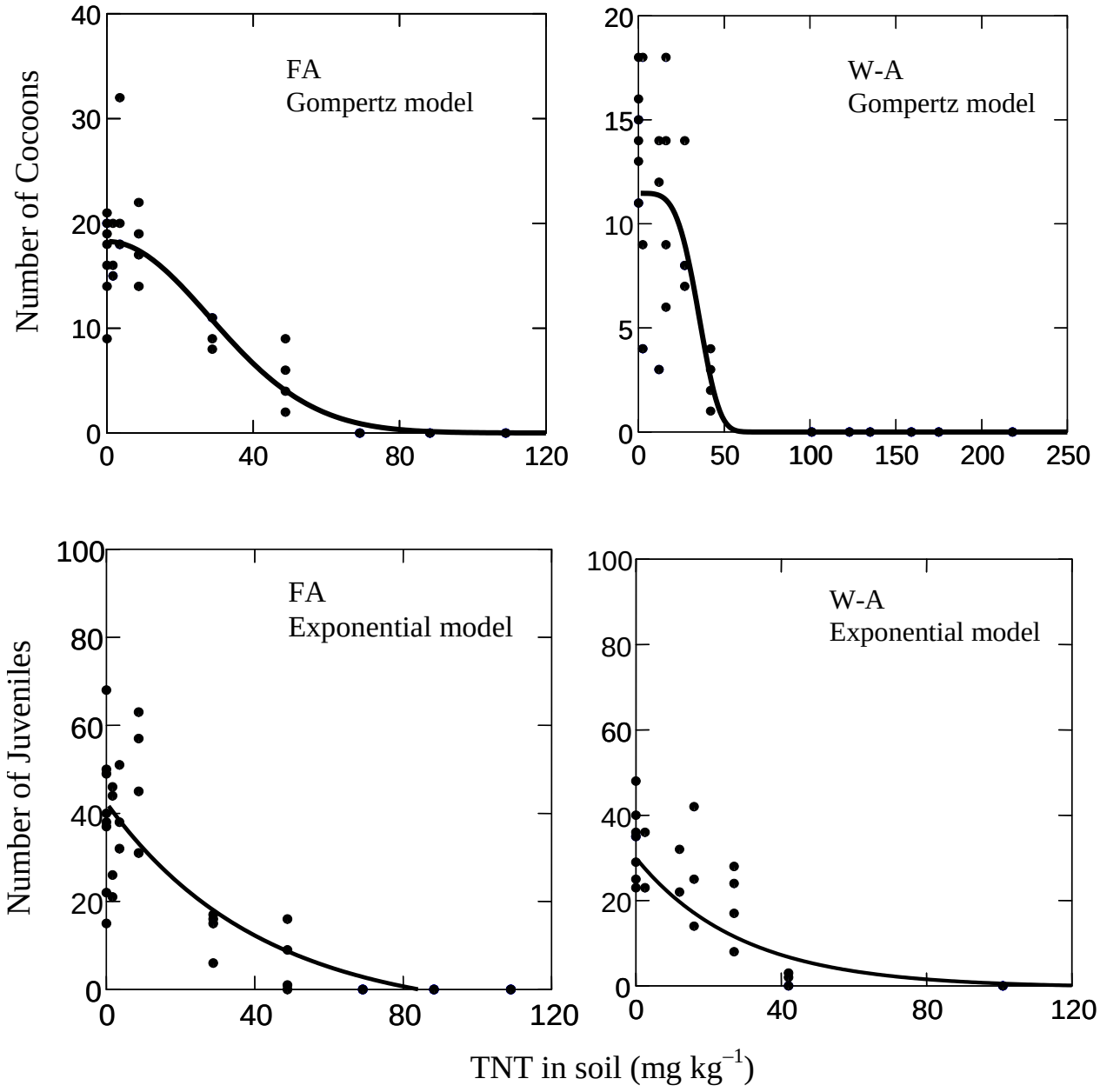

Figure 3. Nonlinear regressions of TNT FA (left) and W-A (right) in TSL soil with number of cocoons (top) and juveniles (bottom) produced per five *E. fetida* adults. TNT concentrations in soil were determined by HPLC analyses after extraction with ACN in accordance with U.S. EPA Method 8330A.

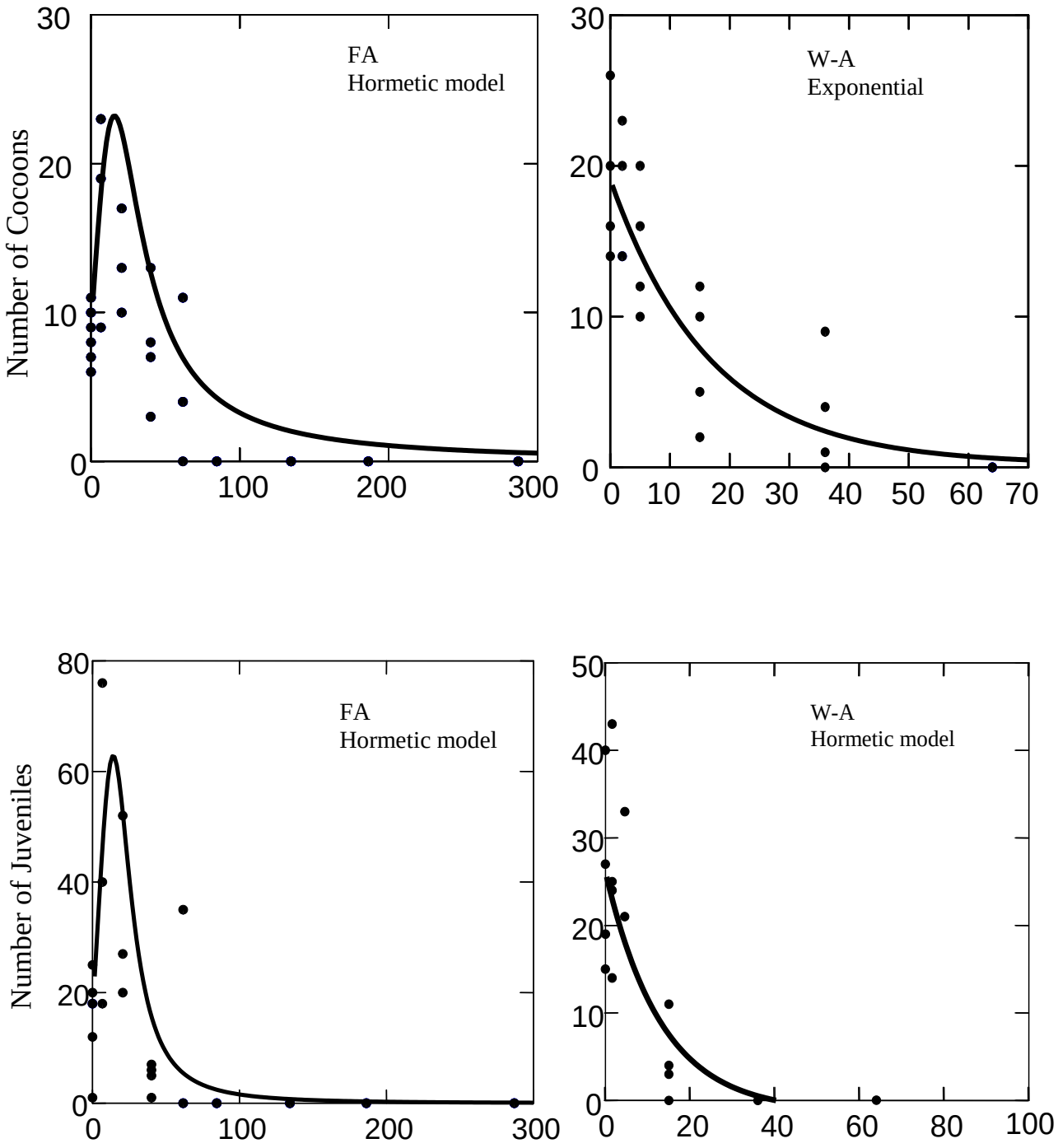

Figure 4. Nonlinear regressions of TNT FA (left) and W-A (right) in SSL soil with number of cocoons (top) and juveniles (bottom) produced per five *E. fetida* adults. TNT concentrations in soil were determined by HPLC analyses after extraction with ACN in accordance with U.S. EPA Method 8330A.

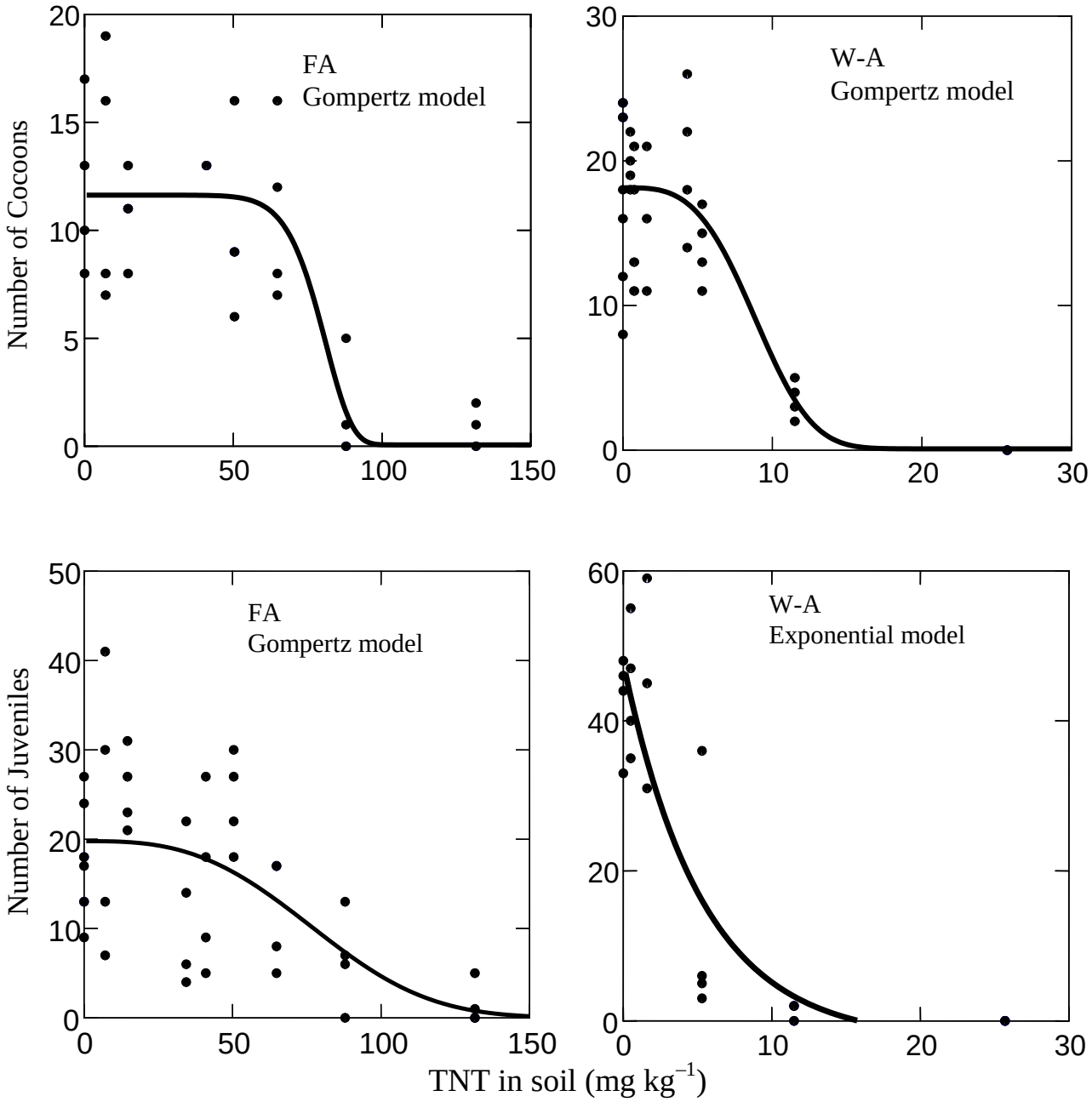

Figure 5. Nonlinear regressions of TNT FA (left) and W-A (right) in KCL soil with number of cocoons (top) and juveniles (bottom) produced per five *E. fetida* adults. TNT concentrations in soil were determined by HPLC analyses following ACN extraction in accordance with U.S. EPA Method 8330A.

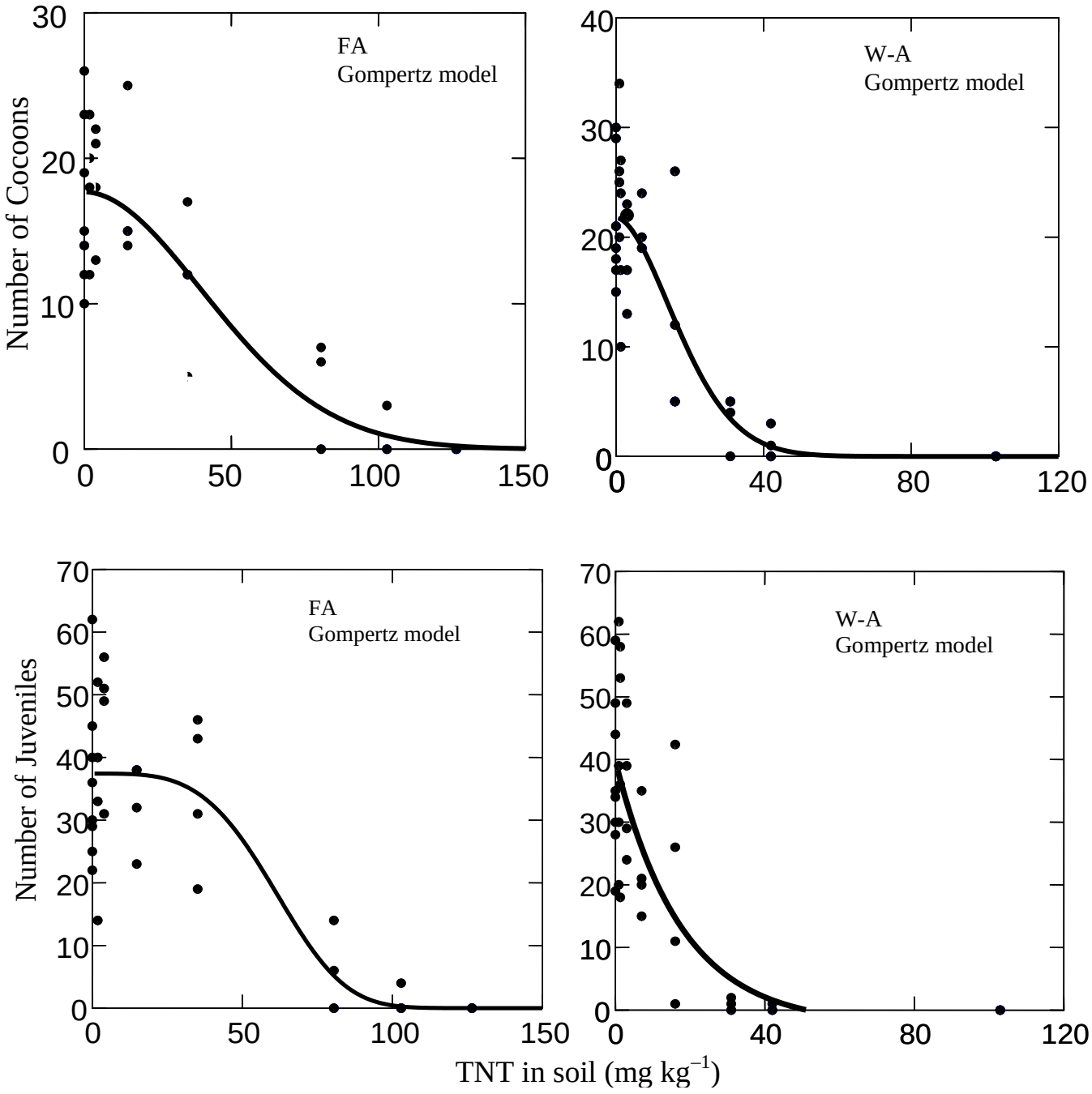

Figure 6. Nonlinear regressions of TNT FA (left) and W-A (right) in RCL soil with number of cocoons (top) and juveniles (bottom) produced per five *E. fetida* adults. TNT concentrations in soil were determined by HPLC analyses following ACN extraction in accordance with U.S. EPA Method 8330A.

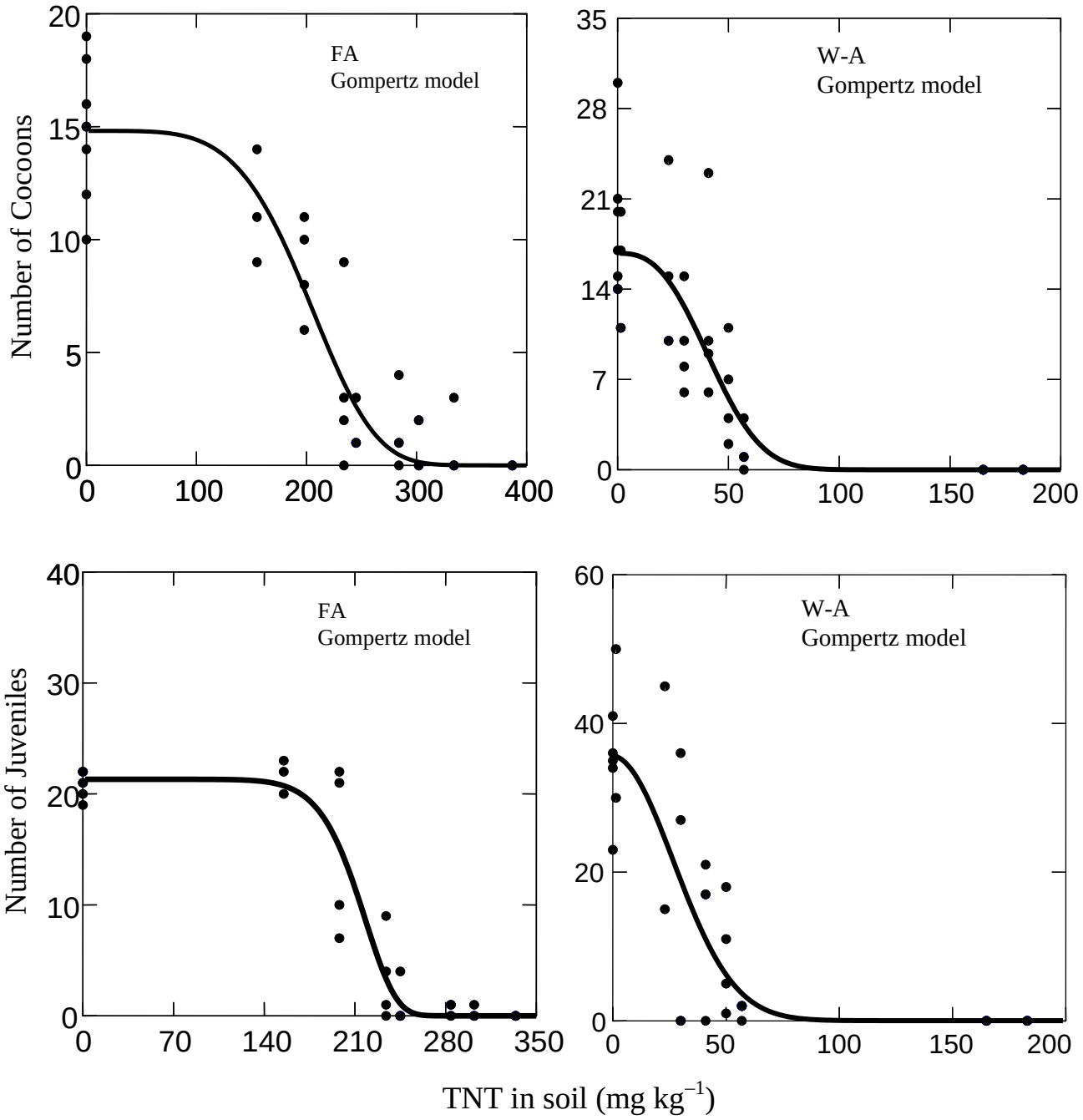

Figure 7. Nonlinear regressions of TNT FA (left) and W-A (right) in WCL soil with number of cocoons (top) and juveniles (bottom) produced per five *E. fetida* adults. TNT concentrations in soil were determined by HPLC analyses following ACN extraction in accordance with U.S. EPA Method 8330A.

Table 24. Pearson's Correlation Coefficients and Probability Values for Key Soil Properties and *E. fetida* Reproduction Endpoints (EC_{20} and EC_{50} Levels) for TNT FA in Soil

Soil Property	Cocoon Production				Juvenile Production			
	EC_{20}		EC_{50}		EC_{20}		EC_{50}	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Clay (%)	0.57	0.32	0.55	0.34	0.59	0.30	0.61	0.28
OM (%)	0.85	0.07	0.88[a]	0.05	0.92[a]	0.03	0.93[a]	0.02
pH	0.13	0.83	0.14	0.83	0.22	0.73	0.22	0.72
Cation exchange capacity (cmol kg^{-1})	0.33	0.58	0.39	0.51	0.50	0.39	0.50	0.40

[a] Statistically significant ($p \leq 0.05$).

Table 25. Pearson's Correlation Coefficients and Probability Values for Key Soil Properties and *E. fetida* Reproduction Endpoints (EC_{20} and EC_{50} Levels) for TNT W-A in Soil

Soil Property	Cocoon Production				Juvenile Production			
	EC_{20}		EC_{50}		EC_{20}		EC_{50}	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Clay (%)	–0.18	0.77	–0.13	0.84	0.23	0.71	0.02	0.97
OM (%)	0.41	0.50	0.50	0.39	0.79	0.11	0.64	0.24
pH	–0.44	0.46	–0.37	0.54	–0.10	0.87	–0.25	0.68
Cation exchange capacity (cmol kg^{-1})	0.04	0.95	0.15	0.81	0.36	0.54	0.27	0.66

3.8 Measurement of pH in Soils Amended with RDX

Results of pH analyses are presented in Table 26. The pH values in individual soil treatments were similar to pH values determined in unamended soils, as presented in Table 1 for each of the five soils. Soils amended with RDX, even after weathering-and-aging of RDX within these soils, did not cause soil pH values to vary greatly from those of the respective control soils.

3.9 Analytical Determination of RDX in Soil

RDX concentrations in soil were analytically determined at the beginning of each definitive toxicity test using ACN extraction and HPLC analyses in accordance with U.S. EPA Method 8330A. Results are presented in Table 27 for the HPLC analyses of RDX in control and treatment levels after 90 days of weathering-and-aging. Within the positive treatments, average analytically determined RDX concentrations, expressed as percentages of nominal concentrations, ranged from 60 to 102% in TSL soil, 43 to 100% in SSL soil, 101 to 158% in KCL soil, 61 to 115% in RCL soil, and 80 to 93% in WCL soil (Table 27). No RDX was identified in any of control soils above the detection limit of 0.5 mg kg^{-1}. Overall, RDX was relatively stable in all of these soils, including those subjected to the weathering-and-aging process (Table 27), and RDX was extremely stable compared with TNT W-A in soils (Tables 4, 6, 8, 10, and 12).

Table 26. Soil pH Values at Start of Earthworm Reproduction Tests with RDX W-A in All Soils

Nominal RDX Concentration (mg kg^{-1})	pH				
	TSL Soil	SSL Soil	KCL Soil	RCL Soil	WCL Soil
0	4.79	5.32	5.45	7.63	6.54
1	4.79	—	—	ND	—
2.5	—	—	—	ND	—
5	—	—	—	ND	
6	—	5.31	—	—	—
9	—	5.58	—	—	—
10	4.76	—	ND	ND	ND
18	—	5.28	—	—	—
20	4.74	—	—	—	—
25	—	—	—	ND	—
30	4.8	—	—	—	—
36	—	5.27	—	—	—
40	4.74	—	—	—	—
50	4.78	—	ND	ND	ND
72	—	5.20	—	—	—
100	4.98	—	5.46	ND	—
144	—	5.12	—	—	—
200	4.91	—	—	—	—
250	—	—	5.33	7.79	6.68
300	—	5.00	—	—	—
500	—	—	5.39	7.69	6.65
600	—	5.00	—	—	—
750	—	—	—	—	6.54
1000	—	—	5.38	—	6.62
2000	—	—	5.65	—	6.66
3000	—	—	—	—	6.67
4000	—	—	5.53	—	—
8000	—	—	ND	—	—
Mean	4.81	5.20	5.46	7.70	6.66
SE	0.03	0.04	0.04	0.05	0.02

—, Treatment level not used.
ND, not determined.

Table 27. Concentrations of RDX W-A in Five Soils Used in Definitive Toxicity Tests with *E. fetida*

RDX Concentration (mg kg^{-1})														
TSL Soil			SSL Soil			KCL Soil			RCL Soil			WCL Soil		
Nominal	Measured	% of Nominal	Nominal	Measured	% of Nominal	Nominal	Measured	% of Nominal	Nominal	Measured	% of Nominal	Nominal	Measured	% of Nominal
0	BDL	BDL	0	BDL	BDL	0	BDL	BDL	0	BDL	BDL	0	BDL	BDL
1	0.7 (0.04)	70	6	6 (1.5)	100	10	16 (0.9)	158	1	1.2 (0.03)	115	10	8 (0.3)	80
10	6 (0.1)	60	9	8 (1.3)	94	50	51 (0.4)	101	2.5	1.5 (0.03)	61	50	41 (0.2)	83
20	13 (0.3)	65	18	16 (0.3)	87	100	111 (0.4)	111	5	4.5 (0.06)	89	100	88 (2.5)	88
30	24 (0.7)	80	36	30 (0.8)	83	250	285 (23)	114	10	11 (0.2)	106	250	211 (1.6)	84
40	34 (1.5)	85	72	57 (3.4)	70	500	556 (29)	111	25	34 (1.5)	85	500	458 (6)	92
50	43 (2.4)	86	144	62 (2.2)	43	1000	1054 (147)	105	50	43 (0.2)	87	750	671 (17)	89
100	94 (1.4)	94	300	254 (8.8)	85	2000	2781 (430)	139	100	88 (9)	88	1000	885 (7)	89
200	204 (2.2)	102	600	527 (4.2)	88	4000	6252 (1028)	156	250	247 (19)	99	2000	1800 (2.2)	90
						8000	10161 (826)	127	500	464 (18)	93	3000	2780 (120)	93

Note: Analytically determined ACN-extractable concentration means and SEs (n = 3; U.S. EPA Method 8330A). SEs are shown in parentheses.
BDL, below detection limit (0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil).

3.10 Range-Finding Toxicity Tests with RDX

In separate experiments, adult *E. fetida* were exposed to RDX concentrations in each of the five test soils to determine the range of concentrations to be used in the definitive reproduction tests. Soils were prepared and amended as described in "Materials and Methods" (Section 2) for soils FA with RDX. Nominal RDX concentrations used in the range-finding studies were: 0, 1, 10, 100, 500, 400, and 1000 mg kg^{-1} in TSL, KCL, RCL, and WCL soils, and 0, 10, 100, 500, 1000, and 5000 mg kg^{-1} in SSL soil. Three replicate containers were used for each treatment level with five worms in each container. Toxicity testing was performed as described in "Materials and Methods", except that the range-finding tests were terminated after 21 days; at that time, adults and cocoons were harvested and counted.

Results of the range-finding tests showed that the numbers of cocoons produced after 21 days (cocoon production) were reduced by 50 and 81% as compared with carrier controls at nominal RDX concentrations of 100 and 1000 mg kg^{-1}, respectively, and with the carrier control in the TSL soil. Cocoon production in SSL soil was reduced by ≥69% at nominal RDX concentrations of 10, 100, 500, 1000, and 5000 mg kg^{-1}. Cocoon production in KCL soil was reduced by 14, 49, and 14% at nominal RDX concentrations of 100, 500, and 1000 mg kg^{-1}, respectively. Cocoon production in RCL soil was reduced by 19, 63, 78, 81, and 74% at nominal RDX concentrations of 1, 10, 100, 500, and 1000 mg kg^{-1}, respectively. Cocoon production in WCL soil was reduced by 9, 17, 3, 34, and 57% at nominal RDX concentrations of 1, 10, 100, 500, and 1000 mg kg^{-1}, respectively. RDX treatment concentrations for the definitive toxicity tests were selected on the basis of the results of these range-finding tests for each soil used in this study.

3.11 Definitive Toxicity Tests with RDX

Independent definitive studies using the Earthworm Reproduction Test (ISO, 1998a) were conducted to assess the effects of RDX on the reproduction and adult survival of *E. fetida* earthworms in TSL, SSL, KCL, RCL, and WCL soils. In independent investigations, adult *E. fetida* earthworms were exposed to a range of RDX concentrations W-A for 90 days in each soil. Measurement endpoints were assessed using treatment concentrations that were based on the results of the range-finding studies. Measurement endpoints included the number of cocoons and juveniles after 56 days and the mass and number of surviving adults after 28 days. Concentrations for each soil type were selected on the basis of bracketing significant effects on reproduction endpoints (i.e., production of cocoons and juveniles). Because reproduction endpoints are the preferred Eco-SSL benchmarks for the development of Eco-SSL values for soil invertebrates (U.S. EPA, 2005) they were the main focus of these studies. The ranges of test concentrations were expanded to determine the concentration that caused a lethal effect to adult earthworms. Significant effects on the adult survival endpoint were determined when possible but were not critical to the success of these studies. All ecotoxicological parameters were estimated using these measurement endpoint values and RDX concentrations in soil that were analytically determined in accordance with U.S. EPA Method 8330A.

Test results complied with the validity criteria defined in the ISO test guideline. Mean adult survival in the negative controls was >90% in all tests. The coefficient of variation

for production of juveniles in control treatments did not exceed 50%. As compared with the negative controls, juvenile production in the positive controls was reduced by 54 to 98% and was within the baseline established for laboratory culture of *E. fetida*. These results confirmed that the significant toxicological effects identified in the definitive tests were attributable to the RDX treatments. All reported ecotoxicological parameters were calculated on the basis of analytically determined RDX concentrations in the respective soils.

3.11.1 RDX Toxicity to *E. fetida* in TSL Soil

Ecotoxicological responses of *E. fetida* to RDX W-A in TSL soil are shown in Table 28. The analytically determined LOEC values for RDX in TSL soil, based on total cocoons produced, hatched cocoons, and total juveniles produced by *E. fetida*, were 96, 96, and 13 mg kg^{-1}, respectively (Table 28). Adult survival and dry mass per worm were not significantly ($p > 0.05$) reduced by RDX W-A in TSL soil at concentrations up to and including 205 mg kg^{-1}, the greatest exposure concentration tested in TSL soil in this study.

3.11.2 RDX Toxicity to *E. fetida* in SSL Soil

Ecotoxicological responses of *E. fetida* to RDX W-A in SSL soil are shown in Table 29. The analytically determined LOEC values for important ecotoxicological responses to RDX in soil at which the responses were significantly ($p \leq 0.05$) reduced by RDX in SSL soil (compared with the carrier control soil) were 62 mg kg^{-1} (total cocoons produced), 6 mg kg^{-1} (hatched cocoons), and 16 mg kg^{-1} (total juveniles produced) (Table 29). Adult dry mass per worm and adult survival were not significantly ($p > 0.05$) reduced by RDX W-A in SSL soil at concentrations up to and including 527 mg kg^{-1}, the greatest exposure concentration tested in this study.

3.11.3 RDX Toxicity to *E. fetida* in KCL Soil

Ecotoxicological responses of *E. fetida* to RDX W-A in KCL soil are shown in Table 30. The analytically determined RDX concentration at which total cocoons, hatched cocoons, and total juveniles were significantly ($p \leq 0.05$) reduced in KCL soil compared with carrier control soil was 1055 mg kg^{-1} (Table 30). However, values for these three measurement endpoints were not significantly ($p \leq 0.05$) different from those for controls at RDX concentrations of 2781 and 6252 mg kg^{-1}. Adult survival was significantly ($p \leq 0.05$) reduced at a soil RDX concentration of 10,161 mg kg^{-1}. At all concentrations tested, adult dry mass per worm did not vary significantly ($p \leq 0.05$) from the carrier control value, up to and including the 10,161 mg kg^{-1} concentration, the greatest exposure concentration tested in this study.

3.11.4 RDX Toxicity to *E. fetida* in RCL Soil

Ecotoxicological responses of *E. fetida* to RDX W-A in RCL soil are shown in Table 31. The analytically determined LOEC values for important ecotoxicological responses to RDX in soil at which the responses were significantly ($p \leq 0.05$) reduced by RDX in RCL soil (compared with the carrier control soil) were 43 mg kg^{-1} (total cocoons produced), 43 mg kg^{-1} (hatched cocoons), and 43 mg kg^{-1} (total juveniles produced) (Table 31). Adult dry mass per

worm and adult survival were not significantly ($p \leq 0.05$) reduced by RDX W-A in RCL soil at concentrations up to and including 464 mg kg^{-1}, the greatest exposure concentration tested in this study.

3.11.5 RDX Toxicity to *E. fetida* in WCL Soil

Ecotoxicological responses of *E. fetida* to RDX W-A in WCL soil are shown in Table 32. Analytically determined LOEC values for important ecotoxicological responses to RDX in soil at which the responses were significantly ($p \leq 0.05$) reduced by RDX in WCL soil (compared with carrier control soil) were 41 mg kg^{-1} (total cocoons produced), 41 mg kg^{-1} (hatched cocoons), and 41 mg kg^{-1} (total juveniles produced) (Table 32). Adult dry mass per worm and adult survival were not significantly ($p \leq 0.05$) reduced by RDX W-A in WCL soil at concentrations up to and including 2780 mg kg^{-1}, the greatest exposure concentration tested in this study.

Table 28. Ecotoxicological Responses of Earthworm *E. fetida* to RDX W-A in TSL Soil

Parameter	Nominal RDX Concentration[a] (mg kg^{-1})									
	0 Negative Control	0 Carrier Control[b]	1	10	20	30	40	50	100	200
Analytically determined RDX concentration, initial mean (mg kg^{-1} dry soil)	BDL	BDL	0.7 (0.3)	6 (0.3)	13 (0.7)	24 (1.5)	34 (2.4)	43 (1.4)	96 (2.1)	205 (1.4)
Adult survival/ replicate (%)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	95 (5)	100 (0)	100 (0)
Adult final dry mass/worm[c] (mg)	44 (9)	47 (2)	47 (3)	41 (1)	42 (2)	44 (3)	42 (3)	49 (3)	45 (2)	43 (5)
Cocoons, mean total (no.)	15.3 (1.4)	13.5 (1.7)	13.5 (3.5)	16.3 (1.8)	15.0 (2.6)	9.5 (2.4)	10.5 (1.5)	13.3 (2.2)	6.3[d] (1.8)	6.5[d] (1.5)
Cocoons, mean hatched (no.)	8.8 (1.3)	6.8 (1.3)	8.5 (2.9)	5.8 (2.1)	6.5 (2.3)	4.5 (1.0)	4.3 (1.3)	4.3 (1.9)	1.5[d] (1.2)	2.0[d] (1.2)
Juveniles, mean (no.)	23.0 (3.4)	11.8 (3.8)	12.5 (5.5)	9.8 (4.0)	5.3[d] (1.9)	4.0[d] (2.4)	4.5[d] (1.7)	4.5[d] (1.7)	1.3[d] (0.9)	0.3[d] (0.3)

Note: All soils were hydrated to 95% WHC (12.4% dry mass soil) 24 h before start of test; n = 4 replicates, 5 adult worms per replicate.

[a] Values in parentheses are SEs.

[b] For carrier control, acetone was added as carrier solvent and evaporated before rehydration of soil.

[c] Adult final dry mass/worm = least-square mean adjusted for the covariate, initial live mass/worm, as determined by analysis of covariance.

[d] Significantly less ($p \leq 0.05$) than carrier controls within respective rows according to ANOVA and FLSD means comparison.

BDL, below detection limit of 0.5 mg kg^{-1}.

Table 29. Ecotoxicological Responses of Earthworm *E. fetida* to RDX W-A in SSL Soil

Parameter	Nominal RDX Concentration[a] (mg kg^{-1})									
	0 Negative Control	0 Carrier Control[b]	6	9	18	36	72	144	300	600
Analytically determined RDX concentration, initial mean (mg kg^{-1} dry soil)	BDL	BDL	6 (1.5)	8 (1.3)	16 (0.3)	30 (0.8)	57 (3.4)	62 (2.2)	254 (8.8)	527 (4.2)
Adult survival/ replicate (%)	95 (5)	95 (5)	100 (0)	95 (5)	100 (0)	95 (5)	100 (0)	100 (0)	100 (0)	100 (0)
Adult final dry mass/worm[c] (mg)	49 (1)	53 (4)	50 (2)	54 (4)	45 (0.4)	53 (3)	46 (2)	46 (3)	45 (2)	45 (1)
Cocoons, mean total (no.)	18.3 (1.3)	17.5 (1.6)	18.8 (0.6)	15.5 (1.0)	17.8 (1.1)	13.0 (2.7)	16.3 (1.1)	12.0[d] (1.4)	8.8[d] (3.5)	9.3[d] (1.3)
Cocoons, mean hatched (no.)	10.0 (2.0)	10.0 (0.4)	6.5[d] (1.4)	5.0[d] (1.1)	5.5[d] (1.4)	4.8[d] (0.3)	3.5[d] (0.6)	4.3[d] (1.2)	4.0[d] (1.8)	1.8[d] (1.1)
Juveniles, mean (no.)	24.3 (7.5)	17.3 (2.7)	10.0 (2.5)	7.5 (4.3)	6.3[d] (2.2)	10.0 (1.5)	2.5[d] (0.6)	5.0[d] (3.0)	4.8[d] (3.3)	0.5[d] (0.3)

Note: All soils were hydrated to 95% WHC (17.1% dry mass soil) 24 h before start of test; n = 4 replicates, 5 adult worms per replicate.

[a] Values in parentheses are SEs.

[b] For carrier control, acetone was added as carrier solvent and evaporated before rehydration of soil.

[c] Adult final dry mass/worm = least-square mean adjusted for the covariate, initial live mass/worm, as determined by analysis of covariance.

[d] Significantly less ($p \leq 0.05$) than carrier controls within respective rows according to ANOVA and FLSD means comparison.

BDL, below detection limit of 0.5 mg kg^{-1}.

Table 30. Ecotoxicological Responses of Earthworm *E. fetida* to RDX W-A in KCL Soil

Parameter	Nominal RDX Concentration[a] (mg kg^{-1})										
	0 Negative Control	0 Carrier Control[b]	10	50	100	250	500	1000	2000	4000	8000
Analytically determined RDX concentration, initial mean (mg kg^{-1} dry soil)	BDL	BDL	16 (0.9)	51 (0.4)	110 (0.4)	285 (23)	555 (29)	1055 (147)	2781 (430)	6252 (1028)	10161 (826)
Adult survival/ replicate (%)	100 (0)	100 (0)	100 (0)	100 (5)	100 (0)	95 (5)	95 (5)	100 (0)	100 (0)	100 (0)	85[d] (5)
Adult final dry mass/worm[c] (mg)	36 (6)	34 (3)	37 (5)	36 (1)	34 (1)	38 (2)	34 (1)	37 (2)	41 (2)	33 (5)	38 (7)
Cocoons, mean total (no.)	12.5 (1.4)	7.8 (0.9)	8.0 (1.8)	6.7 (0.6)	5.3 (1.2)	6.3 (1.9)	6.3 (2.1)	3.0[d] (0.4)	6.5 (2.1)	5.3 (1.7)	2.0[d] (1.4)
Cocoons, mean hatched (no.)	8.0 (1.6)	4.8 (1.1)	4.0 (1.2)	3.3 (1.0)	2.0 (0.7)	4.0 (1.4)	4.3 (1.5)	1.8[d] (0.5)	4.8 (1.4)	2.8 (0.9)	0.3[d] (0.3)
Juveniles, mean (no.)	26.0 (4.8)	8.3 (4.3)	6.3 (2.8)	5.5 (2.9)	2.8 (2.2)	4.0 (1.6)	4.5 (1.7)	1.5[d] (0.5)	7.0 (3.2)	6.3 (2.5)	0.0[d] (0.0)

Note: All soils were hydrated to 95% WHC (19.0% dry mass soil) 24 h before start of test; n = 4 replicates, 5 adult worms per replicate.

[a] Values in parentheses are SEs.

[b] For carrier control, acetone was added as carrier solvent and evaporated before rehydration of soil.

[c] Adult final dry mass/worm = least-square mean adjusted for the covariate, initial live mass/worm, as determined by analysis of covariance.

[d] Significantly less ($p \leq 0.05$) than carrier controls within respective rows according to ANOVA and FLSD means comparison.

BDL, below detection limit of 0.5 mg kg^{-1}.

Table 31. Ecotoxicological Responses of Earthworm *E. fetida* to RDX W-A in RCL Soil

Parameter	Nominal RDX Concentration[a] (mg kg^{-1})										
	0 Negative Control	0 Carrier Control[b]	1	2.5	5	10	25	50	100	250	500
Analytically determined RDX concentration, initial mean (mg kg^{-1} dry soil)	BDL	BDL	1.1 (0.03)	1.5 (0.03)	4.5 (0.06)	10.5 (0.15)	22 (0.17)	43 (0.23)	88 (8.7)	247 (19.2)	464 (18.1)
Adult survival/ replicate (%)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	95 (5)	100 (0)	100 (0)	95 (5)
Adult final dry mass/worm[c] (mg)	68 (3)	67 (4)	67 (2)	66 (5)	69 (3)	68 (4)	73 (5)	70 (1)	76 (2)	64 (5)	72 (3)
Cocoons, mean total (no.)	17.3 (1.7)	11.8 (2.1)	11.5 (3.9)	10.8 (3.3)	11.5 (2.3)	11.3 (3.3)	7.5 (4.2)	1.0[d] (0.6)	0.8[d] (0.5)	0.0[d] (0.0)	0.0[d] (0.0)
Cocoons, mean hatched (no.)	8.5 (2.6)	6.3 (1.5)	5.5 (2.2)	6.0 (3.2)	5.5 (1.3)	5.5 (1.6)	5.3 (2.9)	0.0[d] (0.0)	0.3[d] (0.3)	0.0[d] (0.0)	0.0[d] (0.0)
Juveniles, mean (no.)	14.3 (1.3)	8.5 (2.6)	11.0 (7.1)	5.8 (4.4)	9.5 (4.3)	13.3 (4.4)	10.0 (5.8)	0.0[d] (0.0)	0.3[d] (0.3)	0.0[d] (0.0)	0.0[d] (0.0)

Note: All soils were hydrated to 95% WHC (20.0% dry mass soil) 24 h before start of test; n = 4 replicates, 5 adult worms per replicate.

[a] Values in parentheses are SEs.

[b] For carrier control, acetone was added as carrier solvent and evaporated before rehydration of soil.

[c] Adult final dry mass/worm = least-square mean adjusted for the covariate, initial live mass/worm, as determined by analysis of covariance.

[d] Significantly less ($p \leq 0.05$) than carrier controls within respective rows according to ANOVA and FLSD means comparison.

BDL, below detection limit of 0.5 mg kg^{-1}.

Table 32. Ecotoxicological Responses of Earthworm *E. fetida* to RDX W-A in WCL Soil

Parameter	Nominal RDX Concentration[a] (mg kg^{-1})										
	0 Negative Control	0 Carrier Control[b]	10	50	100	250	500	750	1000	2000	3000
Analytically determined RDX concentration, initial mean (mg kg^{-1} dry soil)	BDL	BDL	8 (0.25)	41 (0.20)	88 (2.5)	211 (1.6)	458 (6.3)	671 (16.9)	885 (7.2)	1800 (12.9)	2780 (120.1)
Adult survival/ replicate (%)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	95 (5)	100 (0)	100 (0)	100 (5)
Adult final dry mass/worm[c] (mg)	25 (0)	28 (0.4)	31 (2)	28 (0.6)	34 (3)	24 (1)	28 (3)	30 (0.2)	32 (3)	32 (0.3)	34 (2)
Cocoons, mean total (no.)	15.5 (2.2)	20.5 (0.5)	16.8 (1.9)	10.3[d] (3.2)	5.3[d] (2.1)	12.8 (3.9)	7.8[d] (2.3)	7.8[d] (3.7)	11.0[d] (3.4)	5.8[d] (2.2)	4.5[d] (0.9)
Cocoons, mean hatched (no.)	8.8 (1.5)	14.0 (0.4)	12.5 (2.2)	6.8[d] (2.1)	3.5[d] (1.6)	5.8[d] (2.2)	5.5[d] (0.9)	4.5[d] (2.3)	6.8[d] (2.1)	3.0[d] (1.1)	2.5[d] (0.9)
Juveniles, mean (no.)	20.5 (6.0)	40.3 (3.4)	32.5 (5.0)	15.3[d] (5.5)	7.8[d] (4.5)	13.5[d] (5.7)	9.8[d] (2.0)	11.5[d] (4.6)	12.8[d] (2.7)	4.3[d] (1.5)	3.3[d] (2.0)

Note: All soils were hydrated to 95% WHC (22% dry mass soil) 24 h before start of test; $n = 4$ replicates, 5 adult worms per replicate.

[a] Values in parentheses are SEs.

[b] For carrier control, acetone was added as carrier solvent and evaporated before rehydration of soil.

[c] Adult final dry mass/worm = least-square mean adjusted for the covariate, initial live mass/worm, as determined by analysis of covariance.

[d] Significantly less ($p \leq 0.05$) than carrier controls within respective rows according to ANOVA and FLSD means comparison.

BDL, below detection limit of 0.5 mg kg^{-1}.

3.12 Development of Soil Toxicity Benchmark Values and Comparison of RDX Toxicities to *E. fetida* in the Five Soil Types

Toxicological benchmark values (NOEC, LOEC, EC_{20}, and EC_{50} values) for *E. fetida* exposed to RDX W-A in five natural soils with contrasting physical and chemical properties are shown in Table 33. NOEC and LOEC values were determined by ANOVA and means separation by FLSD at $p \leq 0.05$ to test for significant differences of treatment means compared with carrier control means. The benchmark values showed that cocoon production and juvenile production were more-sensitive indicators of RDX toxicity to earthworms than adult survival (Table 33). Concentration-response relationships were generated by nonlinear regression of analytically determined RDX concentrations with toxicological data for RDX W-A in each soil type. Representative concentration-response relationships for *E. fetida* cocoon production and juvenile production are shown in Figures 8–12. In general, benchmark values were greater (toxicity was less) in KCL soil than in the other four soils (Table 33).

The NOEC and LOEC values for adult survival were 6252 and 10161 mg kg^{-1}, respectively, for RDX W-A in KCL soil. Toxicity benchmarks for *E. fetida* adults could not be determined within the RDX concentration ranges tested in TSL, SSL, RCL, and WCL soils. As noted previously in this report, concentrations for each soil type were selected on the basis of bracketing of significant effects on reproduction endpoints (i.e., production of cocoons and juveniles). Reproduction endpoints are the preferred Eco-SSL benchmarks for the development of Eco-SSL values for soil invertebrates (U.S. EPA, 2005); therefore, reproduction endpoints were the main focus of these studies.

3.13 Effects of Selected Soil Properties on Toxicity of RDX to *E. fetida* Reproduction

Pearson's correlation analysis was used to determine the relationships among selected soil properties and the respective EC_{20} and EC_{50} values for RDX W-A in the five soils. Results of these analyses are shown in Table 34. There were no significant ($p \leq 0.05$) correlations among any of the soil properties and the respective EC_{20} or EC_{50} values determined for RDX in the present studies (Table 34).

Table 33. Summary: Toxicological Benchmarks Determined in Definitive Tests with *E. fetida* for RDX W-A in TSL, SSL, KCL, RCL, and WCL Soils

Ecotoxicological Parameter	TSL Soil	SSL Soil	KCL Soil	RCL Soil	WCL Soil
Adult Survival					
NOEC	206	527	6252	464	2780
p			0.272		
LOEC	>206	>527	10161	>464	>2780
p			0.033		
EC_{20}	>206	>527	>10161	>464	>2780
EC_{50}	>206	>527	>10161	>464	>2780
Cocoon Production					
NOEC	43	57	6252	22	8
p	0.937	0.631	0.269	0.204	0.701
LOEC	96	62	10161	43	41
p	0.028	0.042	0.015	0.003	0.220
EC_{20}	9	19	3632	8	6
95% CI	1–16	0–39	616–6650	3–13	0.9–12
EC_{50}	27	60	9082	24	20
CI (95%)	4–50	0–120	1540–16624	9–40	3–37
Model used	Exponential	Exponential	Linear	Exponential	Exponential
R^2	0.951	0.951	0.739	0.885	0.979
Juvenile Production					
NOEC	6	8	6252	22	8
p	0.136	0.060	0.289	0.435	0.719
LOEC	13	16	10161	43	41
p	0.009	0.022	0.018	0.013	0.014
EC_{20}	4	5	3448	9	7
95% CI	0.3–7	0.2–9	0–7785	0–19	0.8–12
EC_{50}	12	15	8620	29	20
95% CI	0.9–23	0.7–29	0–19462	0–59	2–38
Model used	Exponential	Exponential	Linear	Exponential	Exponential
R^2	0.812	0.804	0.6002	0.739	0.846

Notes: All concentrations are expressed as milligrams RDX per kilogram of soil and are based on ACN extraction and HPLC using U.S. EPA Method 8330A. NOEC and LOEC values were derived from ANOVA procedures and FLSD pairwise-means comparison test.

R^2, regression sum of squares divided by total sum of squares.

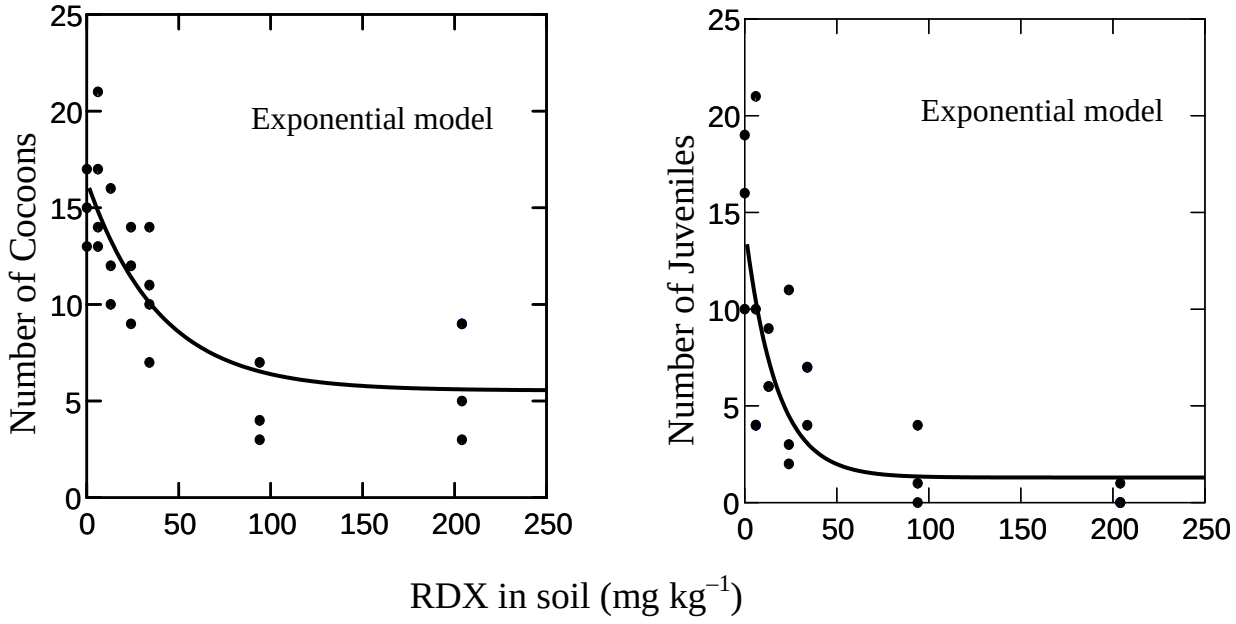

Figure 8. Nonlinear regressions of RDX W-A in TSL soil with number of cocoons (left) and juveniles produced (right) per five *E. fetida* adults. RDX concentrations in soil were determined by HPLC analyses after extraction with ACN using U.S. EPA Method 8330A.

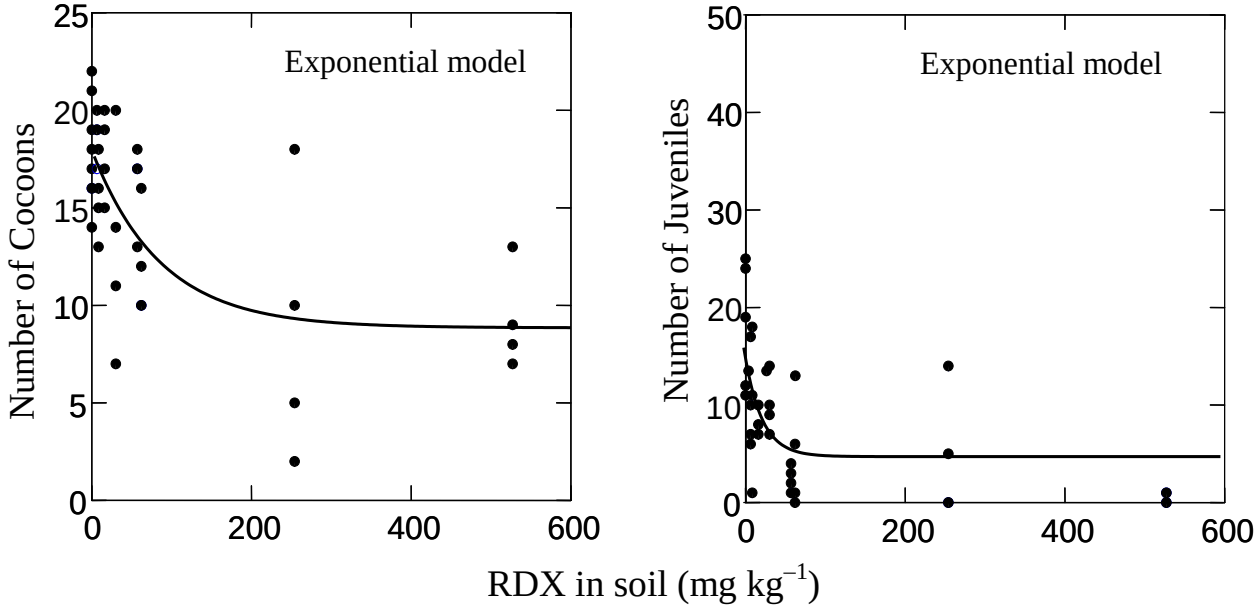

Figure 9. Nonlinear regressions of RDX W-A in SSL soil with number of cocoons (left) and juveniles produced (right) per five *E. fetida* adults. RDX concentrations in soil were determined by HPLC analyses after extraction with ACN in accordance with U.S. EPA Method 8330A.

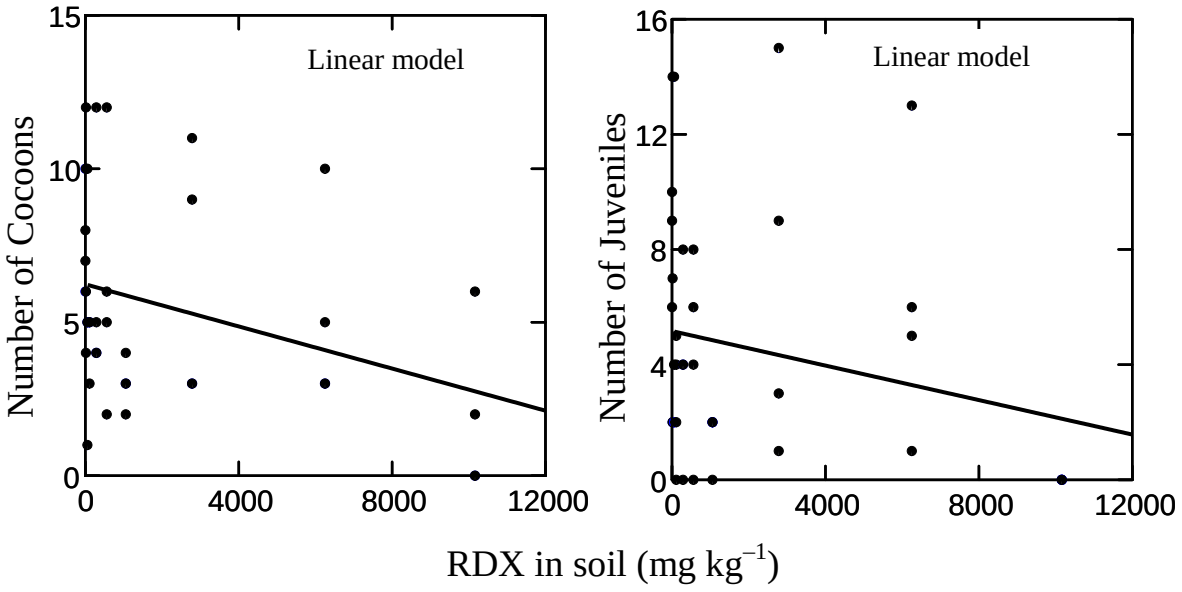

Figure 10. Linear models of effects of RDX W-A in KCL soil on number of cocoons (left) and juveniles (right) produced per five *E. fetida* adults. RDX concentrations in soil were determined by HPLC analyses after extraction with ACN in accordance with U.S. EPA Method 8330A.

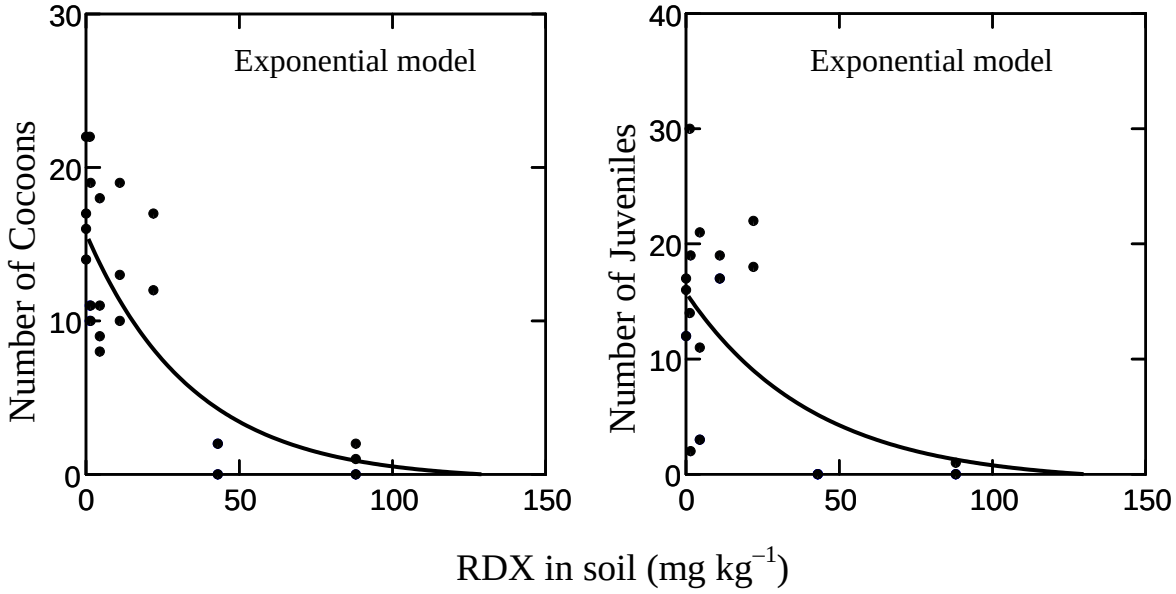

Figure 11. Nonlinear regressions of RDX W-A in RCL soil with number of cocoons (left) and juveniles produced (right) per five *E. fetida* adults. RDX concentrations in soil were determined by HPLC analyses after extraction with ACN in accordance with U.S. EPA Method 8330A.

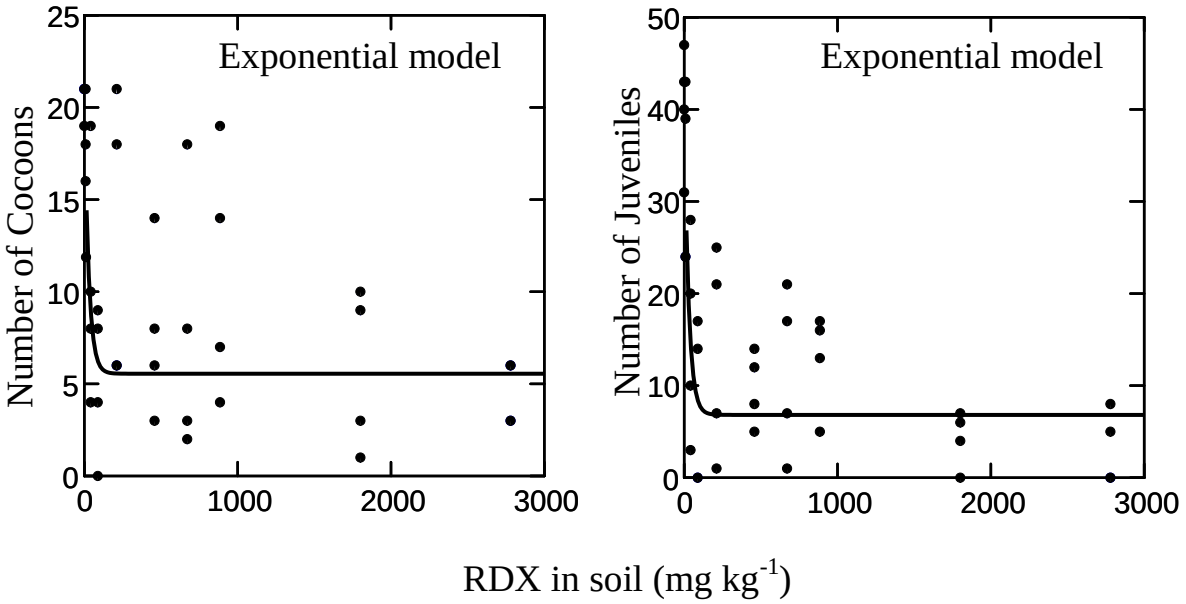

Figure 12. Nonlinear regressions of RDX W-A in WCL soil with number of cocoons (left) and juveniles produced (right) per five *E. fetida* adults. RDX concentrations in soil were determined by HPLC analyses after extraction with ACN in accordance with U.S. EPA Method 8330A.

Table 34. Pearson's Correlation Coefficients and Probability Values for Key Soil Properties and *E. fetida* Reproduction Endpoints (EC_{20} and EC_{50} Levels) for RDX W-A in Soil

Soil Property	Cocoon Production				Juvenile Production			
	EC_{20}		EC_{50}		EC_{20}		EC_{50}	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Clay (%)	0.40	0.51	0.40	0.51	0.40	0.50	0.40	0.50
OM (%)	–0.06	0.92	–0.06	0.92	–0.06	0.92	–0.06	0.92
pH	0.26	0.67	0.26	0.67	0.26	0.67	0.27	0.67
Cation exchange capacity (cmol kg^{-1})	–0.19	0.76	–0.19	0.76	–0.19	0.76	–0.19	0.76

Note: There were no significant ($p \leq 0.05$) correlations among any of the soil properties and the respective EC_{20} or EC_{50} values determined for RDX.

4. DISCUSSION

The goals of this research were to determine individual toxicity benchmarks for TNT and RDX for earthworms in field soils using accepted toxicity testing protocols, and to characterize predominant soil physicochemical parameters that may modify toxicity. Ecotoxicological benchmarks are required for derivation of Eco-SSLs for use in screening procedures (SLERAs) during ERA of contaminated sites (U.S. EPA, 2005). Individual benchmarks for earthworm toxicity were determined for TNT and RDX either FA (TNT only) or W-A (TNT and RDX) in five soils with differing physical and chemical characteristics. In the majority of soil toxicity test results reported in the literature, standard artificial soil with high OM content (10%) was used. In contrast, we utilized natural soils for the toxicity studies

reported herein. In most of the previous studies, only lethal endpoints were measured. We focused on reproduction endpoints for assessing toxicity as well as the determination of lethal endpoints. Our results showed that reproduction endpoints were much more sensitive indicators of toxicity. In addition, the inclusion of weathering-and-aging of TNT or RDX in natural soils with a wide range of physical and chemical characteristics allowed us to assess the toxicity to *E. fetida* under conditions more closely resembling those existing in the field.

On the basis of the EC_{20} values and 95% CIs for the number of juveniles produced, the toxicity of TNT to *E. fetida* in FA soils in the present studies was determined to be in the order, from greatest to least, of TSL > SSL = KCL = RCL > WCL soil. Toxicity ranged from 3- to 21-fold greater in TSL, SSL, KCL, and RCL soils than in WCL soil. WCL soil had the greatest percentage of OM (5%) compared with TSL (1.4%), SSL (1.2%), KCL (2.6%), and RCL (3.3%) soils. Concentrations of TNT extractable from FA soils decreased within 24 h after soil hydration in all soils included in our studies. In general, the percentages of ACN-extractable TNT exposure concentrations in soil, compared with the initially amended soil concentrations, were lowest in the present studies at nominal amended concentrations of ≤100 mg kg^{-1}. These results were similar to those determined in recent earthworm and enchytraeid studies with field-collected soils (Renoux et al., 2000; Kuperman et al., 2005), in which TNT concentrations were observed to be less than the initial amended concentrations at 5 min and 24 h after soil hydration, respectively. TNT was rapidly transformed into 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2-6-dinitrotoluene (4-ADNT) in a natural, temperate-region, sandy (82% sand) forest soil (Renoux et al., 2000). A subsequent study showed that toxicity to *E. fetida* adults was, from greatest to least, 4-ADNT > TNT > 2-ADNT, based on the LC_{50} values (the chemical concentrations that result in 50% mortality) in a forest sandy loam soil (Lachance et al., 2004). Several studies have shown that TNT is readily transformed to both mono- and di-amino toluenes through biotic and abiotic processes under anaerobic and aerobic conditions (Sunahara et al., 2001). A number of soil-borne bacteria and fungi are known to transform the nitro groups to amino groups (Fernando et al., 1990; Kaplan and Kaplan, 1982; McCormick et al., 1976).

On the basis of the EC_{50} values and respective 95% CIs for either juvenile or cocoon production and compared with respective toxicities in FA soils, weathering-and-aging of TNT in soil significantly increased chronic toxicity for *E. fetida* in all soils except TSL. ACN-extractable TNT decreased quickly during weathering-and-aging in soils, especially at concentrations ≤100 mg kg^{-1} (Figures 1 and 2). The rate of decline in TNT concentrations was greatest in the three clay loam soils (RCL, KCL, and WCL). Therefore, it appears that the increase in toxicity was due to the formation and persistence of TNT metabolites. The presence of 2-ADNT and 4-ADNT was detected in our previous, similarly designed studies at all concentrations of TNT W-A in SSL soil (Rocheleau et al., 2006); however, no quantitative analyses were performed in the present studies to identify TNT metabolites in the soils. Typically, the aromatic ring of the original TNT molecule remains intact except under highly reducing environments (Sunahara et al., 2001; Esteve-Núñez, 2001). Transformation products form as the result of biotic and abiotic TNT degradation during weathering-and-aging, typically resulting in the formation of 2-ADNT; 4-ADNT; 2,4-diaminotoluene (2,4-DANT); 2,6-diaminotoluene (2,6-DANT); and 1,3,5-trinitrobenzene (TNB) (Ainsworth et al., 1993; McCormick et al., 1976; Fernando et al., 1990; Esteve-Núñez et al., 2001; Hawari et al., 2000). In addition, 2,4-dintrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) are common

byproducts found in munitions as impurities resulting from TNT manufacturing (Major et al., 2002). Kuperman et al. (2006) reported greater toxicity from TNB; 2,6-DNT; and 2,4-DNT than from TNT to the potworm *Enchytraeus crypticus* in SSL soil. Lachance et al. (2004) reported greater adult mortality of *E. fetida* exposed to 4-ADNT than to TNT in soil, but less mortality of *E. fetida* exposed to 2-ADNT; 2,4-DANT; and 2,6-DANT in a sandy field-collected forest soil. All of the above-mentioned studies, as well the present studies, were performed with single contaminants. Furthermore, increased toxicity in the W-A soils may have been due to synergistic or additive effects of the TNT and its transformation products. Additional toxicological studies that are timed to begin at different stages of the weathering-and-aging process are needed to delineate the combined effects of these compounds. Our ongoing studies with amino-nitrotoluene intermediates of TNT transformation products will provide additional information toward definitively resolving existing uncertainties of the relative toxicities of TNT and its transformation products, especially as they relate to chronic exposure effects for soil invertebrates.

Others have hypothesized that the bioavailability of TNT and related nitroaromatic compounds (NACs) in soil is determined by the soil OM content (Anzhi et al., 1997; Eriksson and Skyllberg, 2001), or clay content (Emery et al., 2001; Haderlein et al., 1996), or a combination of the two (Jaenig, 2006). The WCL soil had the greatest percentages of both OM (5.3%) and clay (28%) among the five soils used in this study. In the present studies, analytically determined TNT concentrations in KCL, RCL, and WCL soils (all soils with clay contents of 28% dry mass) decreased to ≤10, 26, and 33% of initial concentrations in FA soils, respectively, after 82 days of weathering-and-aging. Conversely, TNT concentrations in the two sandy loam soils were 15 to 87% in TSL soil (13% clay) and 16 to 85% in SSL soil (17% clay) of initial concentrations in the respective FA soils after 82 days of weathering-and-aging in the present studies. TNT, its metabolites, and other NACs have been shown to bond with the clay minerals in soil (Daun et al., 1998; Esteve-Núñez et al., 2001). NACs react with the siloxane surface of clays to yield electron donor-acceptor complexes. In aqueous environments, adsorption of NACs to clays is high when the exchangeable cations in the clays include 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). Adsorption is also dependant on the type of clay in the soil. A subsequent study has shown that the affinity and adsorption capacity of TNT and other NACs is in the order of kaolinite > illinite > montmorillonite. TNT and its metabolites also react and sorb to OM in the soil (Achtnich et al., 1999; Anzhi et al., 1997; Dryzyzga et al., 1998; Eriksson and Skyllberg, 2001; Esteve-Núñez et al., 2001; Simpson, 2006; Thorn and Kennedy, 2002; Xing et al., 1997). The clay loam soils used in these studies, KCL, RCL, and WCL, had greater OM contents than TSL and SSL. This may partially explain the lower TNT concentrations over time compared with the sandy loams due to binding to the OM. TNT has also been shown to be transformed by abiotic and biotic processes in soil (Monteil-Rivera et al., 2009). Abiotic reactions include alkaline hydrolysis, photolysis, and reduction by iron.

Degradation of TNT by soil microorganisms may also have been a contributing factor to differences in TNT concentrations between the clay loams and sandy loams over time. Biodegradation of TNT occurs under aerobic conditions to a limited extent (Monteil-Rivera et al., 2009). Aerobic bacteria generally transform the TNT molecule by reducing one or more nitro groups to hydroxyamino or amino groups and producing different isomers of amino-

nitroaromatic compounds, which in turn usually accumulate without further metabolism (Esteve-Núñez, 2001). Ring cleavage rarely occurs, and then only under highly reducing anaerobic conditions (Esteve-Núñez et al., 2001). In kinetic experiments with ^{14}C-labeled TNT, the free concentration of TNT decreased with time, with the decrease divided into an exponential phase grading into a linear phase after less than 24 h (Eriksson and Skyllberg, 2001). Free ADNT (1% of added TNT) was detected after 24 h and increased to 10% of added TNT after 7 days. The authors interpreted the rapid exponential decrease in TNT as mainly an effect of sorption to OM and the slower decrease as an effect of TNT degradation. The authors found that TNT sorption increased as soil OM increased, and they suggested that increased concentrations of OM and its associated microorganisms resulted in increased rates of formation of degradation products, and that these resulting compounds associated more strongly with OM than did TNT. Results of our studies herein comport with those of Eriksson and Skyllberg. We also found that analytically determined TNT decreased exponentially in the first 7 days, and the rate substantially slowed thereafter. We found a much greater decrease in TNT in the soils with the greatest OM (KCL, RCL, and WCL). The binding of OM to clay varied with soil type, depending on the nature and relative concentrations of the different types of OM and clays within the soils. In addition, environmental conditions in the soil, such as moisture level, pH, ionic strength, and soil temperature, affected bioavailability (Haderlein et al., 1996). To further complicate matters, results of research showed that binding sites in OM and clays were finite, and that anthropogenically produced and naturally occurring organic compounds competed for these sites (Haderlein et al., 1996).

The effects of soil properties on TNT toxicity to *E. fetida* mortality and reproduction endpoints in the present studies were dependent upon the duration of weathering-and-aging of TNT in soil. According to Pearson's correlation analysis, OM was significantly ($p \leq 0.05$) correlated with the TNT EC_{50} value for *E. fetida* juvenile production in the FA treatments. However, following weathering-and-aging of TNT in soil, no statistically significant ($p \leq 0.05$) correlations were found. As stated above, TNT concentrations greatly decreased in all soils, especially in the three clay loams (KCL, RCL, and WCL), following weathering-and-aging in respective soils. The reason for this phenomenon could not be ascertained under the scope of the present studies. One possible explanation is that TNT transformation products may be more biologically available than the parent compound. Sorption studies with low-polarity organic compounds, including nitroaromatic EMs, have shown that binding of these compounds to soil OM (Xing and Pignatello, 1997) and silicate clays (Haderlein et al., 1996) was competitive, selective, nonlinear, and reversible. The physical and chemical nature of OM and clays, as well as the soil microenvironment, control the sorption/desorption potential of NACs (Luthy et al., 1997). Adsorption constants (K_d values) for homoionic K^+-montmorillonite clays were 125 for 4-ADNT; 21,500 for TNT; and 2,900 for 2-ADNT (Hederlein et al., 1996). The nature of the OM and clay, as well as the total amount of these soil components, play roles in the effective bioavailability of NACs. Recent studies have shown that linear concentration-partition models do not apply to or account for just a small fraction of sorption/desorption of nitroaromatics to OM in soils (Eriksson and Skyllburg, 2001). The authors concluded that TNT and its degradation products bind to both particulate OM (POM) and dissolved OM (DOM), and that the nature of the binding mechanisms is different. The association of TNT to DOM was strongly pH dependent and followed a nonlinear isotherm, whereas the TNT-to-POM bonding was less pH dependent, and data were equally well fitted by linear and nonlinear isotherms. These sorption

models may also partially explain the nonlinear decrease in extractable TNT concentrations in soil as well as the nonlinearity of toxicity relationships seen in the present study and in other studies (Xing and Pignatello, 1997).

Coefficients of determination (R^2) for ACN- and ATCLP-based extractions determined in nonlinear regression analyses of the reproduction toxicity data from studies with TNT FA and W-A in soils were compared to determine which chemical measure of exposure correlated better with TNT toxicity (Kuperman et al., 2012). 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 result supports a decision to develop draft Eco-SSL values for TNT for soil invertebrates on the basis of ACN extraction. The ACN extraction-based Eco-SSL values will be especially practical for ERAs at contaminated sites because TNT concentrations determined during site characterization are typically based on ACN extraction in accordance with U.S. EPA Method 8330A.

In these present studies, the nitramine explosive RDX was highly toxic to the reproductive capacity of *E. fetida* after the 90 day weathering-and-aging in TSL, SSL, RCL, and WCL soils, with respective EC_{20}/EC_{50} values of 4/13, 5/15, 9/29, and 7/20 mg kg^{-1} for production of juveniles. Cocoon production was affected as well, with respective EC_{20}/EC_{50} values of 7/27, 18/60, 8/24, and 6/20 mg kg^{-1}. Conversely, RDX was not highly toxic after 90 days of weathering-and-aging in KCL soil, with respective EC_{20}/EC_{50} values of 3448/8620 mg kg^{-1} for production of juveniles and 3632/9082 mg kg^{-1} for production of cocoons. RDX toxicity to the reproductive capacity of *E. fetida* in these studies was not well correlated with any of the soil properties selected for this investigation. Cocoon and juvenile production were reduced in a previous study with RDX W-A in SSL soil (Simini et al., 2003). The authors reported the EC_{20}/EC_{50} values for cocoon production and juvenile production of 19/60 and 5/15 mg kg^{-1}, respectively. The authors reported increased EC_{20} and EC_{50} values (reduced toxicity) with RDX W-A for 3 months in SSL soil compared with FA RDX (within 24 h), although the difference was not significant based on the 95% CI. Adult survival of *E. fetida* was not significantly reduced ($p > 0.05$) in any of the five soils following exposure to analytically determined RDX concentrations up to and including 206; 527; 10,161; 464; and 2780 mg kg^{-1} in TSL, SSL, KCL, RCL, and WCL soils, respectively. Robidoux et al. (2000) reported the LOEC values of 189 and 95 mg kg^{-1} for cocoon production and juvenile production, respectively, by *Eisenia andrei* in a standard artificial soil (OECD, 1984), but adult survival and the mass of *E. andrei* adults were not affected by RDX concentrations up to and including 756 mg kg^{-1}. Kuperman et al. (2003) determined an EC_{50} value of 51,413 mg kg^{-1} for the potworm *E. crypticus* in SSL soil. Dodard et al. (2005) reported that exposure of a different potworm species, *Enchytraeus albidus*, in a composite agricultural forest soil (23% OM, 2% clay, pH 7.9) produced the EC_{20} and EC_{50} values of 161 and 444 mg kg^{-1}, respectively, for production of juveniles. However, the authors reported that juveniles of the species *E. crypticus* were not significantly reduced ($p > 0.05$) in the same soil type containing up to and including 658 mg kg^{-1} RDX under similar conditions in a separate test. Adult survival of the potworms *E. albidus* and *E. crypticus* was not affected by RDX or octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) concentrations of 658 and 918 mg kg^{-1}, respectively, in an agricultural soil (42% OM, 1% clay, pH 8.2), a composite agricultural forest soil (23% OM, 2% clay, pH 7.9), and SSL soil (Dodard et al., 2005).

RDX concentrations in all soil types tested in our studies reported herein were not appreciably reduced after the weathering-and-aging process, compared with concentrations in FA soils. These results are consistent with studies of RDX in soils under aerobic conditions. Simini et al. (2006) reported RDX concentrations of 91.9 to 216% of nominal concentrations in FA soils, and 42.7 to 105.8% of the initial concentrations in FA soils for RDX W-A for 90 days. Sheremata et al. (2001) reported little degradation of RDX under aerobic conditions in batch cultures in a natural soil. Extensive degradation occurred only under anaerobic conditions after several weeks; RDX metabolites hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine; hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine; and hexahydro-1,3,5-trinitroso-1,3,5-triazine (MNX, DNX, and TNX, respectively) were not identified until after extensive anaerobic degradation had occurred. The authors also measured relatively low sorption (K_d^s) values (0.83 L/kg), although the sorption that occurred was nearly irreversible (Sheremata et al., 2001). Sorption of RDX to soils is low, as demonstrated by low K_d values; therefore, RDX is typically highly mobile in soils. In a separate study, the authors concluded that sorption of RDX to soils is governed by interactions with soil minerals rather than by association with soil OM (Monteil-Rivera, 2009). Consequently, RDX is readily leached through the vadose zone, which presents a high potential for groundwater contamination.

5. CONCLUSIONS

This project was undertaken to produce scientifically defensible toxicity data for the development of soil invertebrate-based Eco-SSL benchmark values for TNT and RDX, and to investigate and characterize predominant soil physicochemical parameters that can affect the bioavailability and resulting toxicities of TNT or RDX to soil invertebrates. The present studies produced ecotoxicological data for TNT and RDX using the ecologically relevant soil invertebrate species *E. fetida*. Reproduction was a more-sensitive endpoint for evaluation of the exposure effects on the soil invertebrate *E. fetida* compared with adult survival; therefore, reproduction endpoint-based toxicity benchmarks should be used to establish soil invertebrate screening criteria for TNT and RDX. This finding also supports the Eco-SSL requirement of using reproduction endpoints for toxicity benchmark development (U.S. EPA, 2005).

The natural soils TSL and SSL were used in toxicity tests reported herein to develop ecotoxicological benchmark data for use in derivation of soil invertebrate Eco-SSL values. These soils had low OM and clay contents, which fulfilled the U.S. EPA requirement of using soil with characteristics that support high relative bioavailability of organic contaminants, for developing realistic yet conservative Eco-SSL values (U.S. EPA, 2005). Concentrations of TNT or RDX in soil were analytically determined at the beginning of each definitive toxicity test; consequently, the ecotoxicological benchmarks were determined using measured TNT or RDX concentrations. This complied with the U.S. EPA preference for establishing benchmarks for derivation of Eco-SSL values on the basis of measured soil concentration of a chemical (U.S. EPA, 2005).

The definitive studies using *E. fetida* exposures in TSL or SSL soils developed ecotoxicological benchmarks for TNT and RDX in compliance with Eco-SSL test acceptance criteria (U.S. EPA, 2005), thereby achieving the first objective of this investigation. All

ecotoxicological benchmarks determined in these studies will be provided to the Ecological Soil Screening Level Work Group for quality-control review prior to inclusion in the Eco-SSL database and for subsequent use in the derivation of individual soil invertebrate-based Eco-SSL values for TNT and RDX, respectively.

On the basis of EC_{20} values and 95% CIs for juvenile production, toxicity of the nitroaromatic explosive TNT to *E. fetida* in FA soils in these studies was in the order, from greatest to least, of TSL > SSL = KCL = RCL > WCL. In comparison with FA TNT, toxicity to *E. fetida* exposed to TNT W-A for 82 days increased in all soil types except for TSL. ACN-extractable TNT decreased quickly during weathering-and-aging in soils, especially at concentrations ≤100 mg kg^{-1}. The rate of decline in TNT concentrations was greatest in the three clay loam soils (RCL, KCL, and WCL). Therefore, it appears that the increase in toxicity was due to the presence and persistence of TNT metabolites; however, no quantitative analyses were performed to identify TNT metabolites in the soils. In the present studies, the nitramine explosive RDX, W-A in soil for 90 days, was highly toxic to the reproductive capacity of *E. fetida* in TSL, SSL, RCL, and WCL soils. Conversely, RDX was not highly toxic after the 90 day weathering-and-aging in KCL soil. Adult survival was not affected by exposure to RDX in any of the soils tested in the present studies. In contrast with the fate of TNT in soil, RDX was relatively stable and resistant to degradation under aerobic conditions. In all soil types in these studies, RDX concentrations were not appreciably reduced after the weathering-and-aging process, compared with concentrations in FA soils. Therefore, in our studies, toxicity to the earthworms in soil contaminated with RDX was caused mainly by the RDX itself, rather than by the RDX transformation products.

Results of the present studies with TNT showed that soil OM content was significantly ($p \leq 0.05$) correlated with the EC_{50} values for *E. fetida* juvenile production in the FA soils. However, following weathering-and-aging of TNT in soils, no statistically significant ($p \leq 0.05$) correlations were found. There were no significant ($p \leq 0.05$) correlations among any of the key soil properties quantified in the present studies and the EC_{20} or EC_{50} values determined for RDX. As discussed, this does not necessarily mean that these properties are not important in determining the bioavailability and potential toxicity of TNT and RDX to *E. fetida*. Bioavailability and potential toxicity of TNT, RDX, and related NACs in soil depend on highly complex physical and chemical properties and environmental conditions. In-depth investigations to determine the extent of the influence of these factors on bioavailability and toxicity of TNT, RDX, and related contaminants in the soils were beyond the scope of the present studies. In addition, very little research has been performed to determine the toxic effects of mixtures of NACs; hence, virtually nothing is known about synergistic or additive effects of these chemicals. More-extensive analyses of soil chemical and physical properties, with many more soil types and under differing environmental conditions, are necessary to determine the role of these properties in determining bioavailability and toxicity of NACs to *E. fetida* and other soil invertebrates.

Overall results of the present studies showed that giving special consideration to the effects of weathering-and-aging of EMs 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. Our findings of increased reproduction toxicity to *E. fetida* of TNT W-A in 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, additional investigation of the more-toxic transformation compounds that arise within soils amended with TNT should also have a weathering-and-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 transformation products, so that research results may be used in deriving draft Eco-SSL values for these chemicals while providing more complete information on the ecotoxicological effects of energetic contaminants in soil for risk assessors and site managers.

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ACRONYMS AND ABBREVIATIONS

ACN	acetonitrile
2-ADNT	2-amino-4,6-dinitrotoluene
4-ADNT	4-amino-2-6-dinitrotoluene
ANOVA	analysis of variance
AS	artificial soil
ATCLP	adapted toxicity characteristic leaching procedure
BDL	below detection limit
BERA	baseline ecological risk assessment
CAS	Chemical Abstracts Service
CI	confidence interval
2,4-DANT	2,4-diaminotoluene
2,6-DANT	2,6-diaminotoluene
2,4-DNT	2,4-dintrotoluene
2,6-DNT	2,6-dinitrotoluene
DNX	hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine
DOM	dissolved organic matter
EC	effective concentration
EC_{20}	concentration that produces 20% decrease in measurement endpoint
EC_{50}	concentration that produces 50% decrease in measurement endpoint
ECp	estimate of effective concentration for a specified percent effect
Eco-SSL	ecological soil screening level
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
HPLC	high-performance liquid chromatography
ISO	International Organization for Standardization
KCL	Kirkland clay loam
K_d	adsorption constant
K_d^s	sorption value
K_{ow}	octanol–water partition coefficient
LOEC	lowest observed-effect concentration
MNX	hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine
NAC	nitroaromatic compound
ND	not determined
NOEC	no-observed-effect concentration
NU	not used
OECD	Organisation for Economic Co-operation and Development
OM	organic matter
p	probability
POM	particulate organic matter
PTFE	polytetrafluoroethylene

QRB	qualitative relative bioavailability
r	Pearson's correlation coefficient
R^2	coefficient of determination
RCL	Richfield clay loam
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine
SAS	standard artificial soil
SE	standard error
SLERA	screening level ecological risk assessment
SSL	Sassafras sandy loam
TCLP	toxicity characteristic leaching procedure
TNB	1,3,5-trinitrobenzene
TNT	2,4,6-trinitrotoluene
TNX	hexahydro-1,3,5-trinitroso-1,3,5-triazine
TSL	Teller sandy loam
USDA	U.S. Department of Agriculture
U.S. EPA	U.S. Environmental Protection Agency
W-A	weathered-and-aged
WCL	Webster clay loam
WHC	water-holding capacity


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