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TOXICITY OF NITRO-HETEROCYCLIC AND NITROAROMATIC ENERGETIC MATERIALS TO *FOLSOMIA CANDIDA* IN A NATURAL SANDY LOAM SOIL

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In conjunction with stakeholders, the U.S. Environmental Protection Agency (USEPA) developed ecological soil screening level (EcoSSL) values for contaminants at Superfund sites. EcoSSL values are derived by using published data that is generated from laboratory toxicity tests with test species relevant to soil ecosystems. After performing an extensive literature review (USEPA, 2005), the EcoSSL workgroup determined that insufficient information was available on energetic materials (EMs) to generate EcoSSL values for soil invertebrates. In this study, we produced benchmark data to support the development of soil invertebrate EcoSSL values for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB. We used the *Folsomia* Reproduction Test, which uses an ecologically relevant test species and includes at least one reproductive measurement endpoint. Definitive toxicity tests conducted with freshly amended EMs in soil showed that EM toxicity, based on 20% effect concentration values for the production of juveniles, was TNB > 2,6-DNT > 2,4-DNT > RDX > HMX; with weathered-and-aged EMs in soil, EM toxicity was 2,6-DNT > 2,4-DNT > TNB > RDX > HMX. These study results will be provided to the EcoSSL workgroup for review and inclusion in the EcoSSL database, and for developing EcoSSLs for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB..

15. SUBJECT TERMS

hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)	2,4-dinitrotoluene (2,4-DNT)	*Folsomia candida*
octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)	2,6-dinitrotoluene (2,6-DNT)	Bioavailability
1,3,5-trinitrobenzene (TNB)	Natural soil	
Ecological soil screening level (EcoSSL)	Sassafras sandy loam (SSL)	

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PREFACE

The work described in this report was authorized under project no. SERDP CU-1221. The work was started in April 2001 and completed in July 2003.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for public release.

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1. INTRODUCTION

The soil of many military-operation sites that involve munition manufacturing, disposal, testing, and training contains elevated levels of explosives and related materials. Concentrations of explosives in soil have been reported to exceed 87,000 mg kg^{-1} for TNT and 3,000 mg kg^{-1} for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Simini et al., 2003). Although the energetic materials (EMs), RDX and HMX, are persistent and highly mobile in the environment, their effects on soil biota have not been sufficiently investigated.

Scientifically based ecological soil screening levels (EcoSSLs) are needed to identify contaminant explosive levels that present an acceptable ecological risk. In conjunction with stakeholders, the U.S. Environmental Protection Agency (USEPA) addressed this problem by developing EcoSSLs for contaminants frequently found at Superfund sites. EcoSSLs are defined as concentrations of chemicals in soil that, when not exceeded, protect terrestrial ecosystems from unacceptable harmful effects. These EcoSSL concentrations can be used in a screening level ecological risk assessment (ERA) to identify those contaminants in soil that warrant additional evaluation in a baseline ERA and to eliminate those that do not. Usually, EcoSSLs are derived by using published data that are generated from laboratory toxicity tests with different test species relevant to soil ecosystems. After performing an extensive literature review (USEPA, 2005), the EcoSSL workgroup determined that insufficient information existed on explosives to generate EcoSSLs for soil invertebrates. This study was initiated as part of the research effort to fill this knowledge gap.

This study was designed to produce benchmark data to support the development of individual EcoSSLs, respectively, for RDX, HMX, 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), and 1,3,5-trinitrobenzene (TNB) for soil invertebrates. This study addresses the following criteria (USEPA, 2005):

1. Testing was conducted in soil having physicochemical characteristics that supported the relatively high bioavailability of chemicals;

2. Experimental designs for laboratory studies were documented and appropriate;

3. Nominal and analytically determined concentrations of chemicals of interest were reported;

4. Tests included negative and positive controls;

5. Chronic or life cycle tests were used;

6. Appropriate chemical dosing procedures were reported;

7. Concentration-response relationships were reported;

8. Statistical tests used to calculate the benchmark and level of significance were described; and

9. The origin of test species was specified and appropriate.

Several soil invertebrate toxicity tests, for which standardized protocols have been developed, can effectively be used to assess EM toxicity and to derive protective EM benchmark values (Stephenson et al., 2002; Løkke and Van Gestel, 1998). We adapted the *Folsomia* Reproduction Test (ISO 11267:1998) for use in these studies. This bioassay was selected on the basis of its ability to measure chemical toxicity to ecologically relevant test species during chronic assays and its inclusion of at least one reproduction component among the measurement endpoints. The primary objective of these studies was to quantify EM toxicities to the soil invertebrate, *Folsomia candida,* for establishing benchmark data that can be used in the development of EcoSSLs for explosive contaminants in soil.

Explosives in soils at many contaminated sites have been subjected to natural weathering-and-aging processes onsite for many years. Therefore, special consideration was given to including weathering-and-aging of EM in soil as an integral part of the process of assessing the toxicities of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB on *F. candida.* The weathering-and-aging process could reduce EM exposure of soil invertebrates because of photodecomposition, hydrolysis, reaction with organic matter, sorption, precipitation, immobilization, occlusion, microbial transformation, and other fate processes. This could result in a dramatic reduction in the amount of bioavailable chemicals, when compared with tests conducted with freshly amended chemicals or tests conducted after a short equilibration period (e.g., 24 h). Additionally, degradation products, produced during the weathering-and-aging process, could be more toxic to soil organisms than the parent material. We incorporated a weathering-and-aging procedure to closely simulate the exposure effects on soil invertebrates in the field.

2. MATERIALS AND METHODS

2.1 Test Soil

In this study, we used a natural soil, Sassafras sandy loam (SSL [fine-loamy, siliceous, semiactive, mesic Typic Hapludult]; U.S. Department of Agriculture, Natural Resources Conservation Service, Agricultural Research Service, 1975). This soil was selected because it has physical and chemical characteristics that support relatively high bioavailability of the test chemicals (low organic matter and clay contents). The SSL soil was collected from an open grassland field in Edgewood, MD. Vegetation and organic matter horizon were removed from the soil and the top 6 in. of the A horizon were collected. The soil was sieved through a 5 mm^2 mesh screen, air-dried for at least 72 h, mixed periodically during the drying process to ensure uniform drying, passed through a 2 mm sieve, and stored at room temperature. The soil

was analyzed for physical and chemical characteristics by the Cooperative Extension Service (University of Maryland Soil Testing Laboratory, College Park, MD). The results of these analyses are presented in Table 1.

Table 1. Physical and Chemical Characteristics of SSL Soil

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

*Values were determined by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD.

2.2 Test Chemicals

The following chemicals were obtained from Defense Research Establishment Valcartier (Canadian Ministry of National Defense, Val Bélair, QC, Canada):

- RDX (Chemical Abstracts Service [CAS]: 121-82-4; purity: 99%),
- HMX (CAS: 2691-41-0; purity: 99%),
- 2,4-DNT (CAS: 121-14-2; purity: 97%),
- 2,6-DNT (CAS: 606-20-2; purity: 98%), and
- TNB (CAS: 99-35-4; purity: 99.7%)

Beryllium sulfate ([$BeSO_4 \cdot 4H_2O$] CAS: 7787-56-6; purity: 99.99%) was used as the positive control compound in these tests. Acetone (CAS: 67-64-1; high-performance liquid chromatography [HPLC] grade) was used for preparing EM solutions during the soil amendments. Acetonitrile (CAS: 75-05-8; HPLC grade) was used for extractions and during analytical determinations by HPLC. Methanol (CAS: 67-56-1, chromatography grade, purity: 99.9%) was used in the HPLC determinations. Certified standards of the energetics (AccuStandard, Inc., New Haven, CT) were used during the HPLC determinations.

Unless otherwise specified, ASTM type I water (18 MΩ cm at 25 °C; ASTM, 2004), obtained using Milli-RO 10 Plus followed by Milli-Q PF Plus systems (Millipore, Bedford, MA), was used for all the tests in this study. Glassware was washed with phosphate-free detergent followed by rinses with tap water, ASTM type II water, analytical reagent-grade nitric acid 1% (v/v), and ASTM type I water.

2.3 Soil Amendment Procedures

SSL soil was individually amended with RDX, HMX, 2,4-DNT, 2,6-DNT or TNB. Each treatment concentration of EM for the range-finding tests was prepared separately in

glass volumetric flasks and dissolved in acetone. This procedure was necessary to dissolve the nonpolar chemicals, which yielded a more homogeneous mixture than the addition of solid chemical crystals to soil. The amended soil was spread to a thickness of 2.5 cm. The EM and acetone solution was pipetted evenly across the soil surface, ensuring that the volume of solution added at any given time did not exceed 15% (v m^{-1}) of the dry soil mass. After the addition of the EM solution, the volumetric flask was rinsed twice with a known volume of acetone and pipetted onto the soil. If the total volume of solution needed to amend the soil exceeded 15% (v m^{-1}), the solution was added in successive stages, allowing the acetone to evaporate for a minimum of 2 h under a chemical hood. The same total EM and acetone solution volume at a different EM concentration was added to every treatment, equaling the volume required to dissolve the EM at the highest concentration tested. The amended soil was air-dried overnight (for a minimum of 18 h) in darkness within a chemical hood to prevent photolysis of the EM. Each amended soil sample was transferred into a fluorocarbon-coated high-density polyethylene container and mixed for 18 h on a three-dimensional rotary mixer.

Initial EM concentrations for the definitive toxicity tests were prepared by adding test chemicals into an aliquot of SSL soil, using the same procedures as in the range-finding tests. The final nominal target treatment concentrations for the definitive EM tests were prepared by mixing initially prepared soil amended with the appropriate EM, with clean SSL soil, for 18 h on a three-dimensional rotary mixer. Carrier controls were treated only with the carrier solvent. After three-dimensional mixing, treated soils were hydrated with ASTM type I water to 88% of the SSL water-holding capacity ([WHC] 18% water for SSL, on the basis of dry soil mass) for toxicity testing, or 60% of the WHC for the weathering-and-aging procedure. Hydrated soil prepared for the toxicity tests was allowed to equilibrate for 24 h before *F. candida* were introduced.

2.4 Measurement of Soil pH

The pH of the test soils was determined at the beginning of each definitive toxicity test using a method adapted from the Soil Survey Laboratory Methods Manual (USDA, 1996). The pH electrode was rinsed thoroughly with ASTM type I water, blotted dry, standardized with pH 4 and pH 7 buffers, rinsed, and finally blotted again. Five grams of ASTM type I water were added to 5 g of soil. The soil slurry was vortexed for 10 s every 5 min for 30 min. The soil slurry was then vortexed a final time for 1 min before pH measurements were taken. The pH was continuously measured in the solution above the soil surface. The solution was gently stirred until the reading stabilized. The electrode was rinsed with ASTM type I water and blotted between samples.

2.5 Treatment Concentrations

2.5.1 Range-Finding Tests

Range-finding soil tests with freshly amended EMs were conducted to determine treatment concentrations for the definitive tests. Nominal EM concentrations of 0; 10; 100; 500; 1,000; 5,000; and 10,000 mg kg^{-1} were initially used in each EM toxicity test.

2.5.2 Definitive Tests

Data from the range-finding tests were used to determine the treatment concentrations for the respective definitive EM toxicity tests. To assess the independent effects of EMs, definitive tests were conducted using EM that was freshly amended or weathered-and-aged in SSL soil. The following nominal concentrations (milligrams per kilogram) were selected for the definitive tests for freshly amended EM in SSL soil:

- RDX: 0; 1.5; 3, 9; 18; 36; 120; 360; 720; 2,000
- HMX: 0, 9; 36; 72; 144; 300; 600; 1,200; 2,400
- 2,4-DNT: 0; 0.5; 1; 2; 4; 8; 12; 24; 48
- 2,6-DNT: 0; 0.5; 1; 2; 4; 8; 12; 24; 48
- TNB: 0; 8; 16; 32; 64; 128; 256; 384; 512

The following nominal test chemical concentrations (milligrams per kilogram) were selected for the definitive tests for each EM weathered-and-aged in SSL soil:

- RDX: 0; 6; 9; 18; 36; 72; 144; 300; 600
- HMX: 0; 36; 72; 144; 300; 600; 1,200; 2,400; 5,000
- 2,4-DNT: 0; 2; 4; 8; 12; 24; 48; 64; 80; 160
- 2,6-DNT: 0; 2; 4; 8; 12; 24; 48; 64; 80; 160; 320
- TNB: 0; 16; 32; 64; 128; 256; 384; 512; 768

All definitive tests included carrier (acetone) controls and positive controls. Positive controls were prepared from a solution of $BeSO_4 \cdot 4H_2O$ in ASTM type I water to produce 50 mg kg^{-1} Be nominal concentrations in all the positive controls for the definitive tests. Test concentrations of the EMs in individual definitive tests were analytically determined using USEPA Method 8330 (USEPA, 1998).

2.6 Weathering-and-Aging of Each EM in Soil

Standardized methods for weathering-and-aging of EM in soil were not available. We developed approaches that partially simulated the weathering-and-aging process in soil to closely approximate the exposure effects on soil biota in the field. This weathering-and-aging process included exposing treated and control soils, initially hydrated to 60% of the WHC in open Teflon-coated chemically inert containers in the greenhouse, to alternating moistening and drying cycles for 3 months. All soil treatments were weighed and readjusted to their initial mass by adding ASTM type I water twice a week. All soil treatments were eventually brought to 88% of the WHC 24 h before the toxicity tests were started. The effect of weathering-and-aging on EM ecotoxicity was determined by comparing respective results obtained for freshly amended EM to those obtained for EM weathered-and-aged within soil.

2.7 Chemical Extractions and Analyses

Acetonitrile extractions of soil were performed using USEPA Method 8330. At the beginning of each definitive bioassay, soil treatments containing either freshly amended or

weathered-and-aged EM in soil were hydrated to 60% of the WHC. Samples for chemical analyses were taken after 24 h of equilibration following this hydration, just prior to introduction of the test organisms. For each treatment, 2.3 g of soil was weighed in triplicate into 50 mL polypropylene centrifuge tubes, 10 mL of acetonitrile was added, and the samples were vortexed for 1 min and sonicated in darkness for 18 h at 20 °C. Five milliliters of sonicated sample were transferred into a glass tube to which 5 mL of $CaCl_2$ solution (5 g L^{-1}) was added. Supernatant was filtered through 0.45 µm polytetrafluoroethylene (PTFE) syringe cartridges. Soil extracts were analyzed and quantified by HPLC. All analytical results are reported as concentrations in dry soil.

In addition to acetonitrile-extractable extractions, soil samples were extracted using an adapted toxicity characteristic leaching procedure ([ATCLP] Haley et al., 1993) at the beginning of each definitive test. The ATCLP is a modified version of the toxicity characteristic leaching procedure (40 CFR Part 268.41, Hazardous Waste Management, Method 1311). The modification involved substitution of CO_2-saturated ASTM type I water for acetic acid and better simulation of soil-water conditions from respiration by soil biota. All analytical measurements were done in triplicate at the beginning of each test. For each treatment concentration, 4 g of soil was transferred in triplicate into 20 mL vials. Sixteen milliliters of CO_2-saturated water (pH 3.8 to 4.0) were added into the vials, which were then immediately sealed. The soil samples were vortexed for 45 s and then mixed in darkness for 18 h at 30 rpm using a rotary (end-over-end) mixer (Lars Lande, Whitmore Lake, MI) at room temperature. Settled supernatants were filtered through 0.45 µm PTFE syringe cartridges. An equivalent volume of acetonitrile was added to filtered soil extract before HPLC analysis was performed. In this report, ATCLP soil extraction is referred to as the water-soluble fraction of EM. Nominal and analytically determined (measured) concentrations used in the definitive tests are shown in Tables 2–11.

The soil extracts were analyzed by reversed-phase HPLC using a modified version of USEPA Method 8330. The method was modified in two ways:

- First, the final solvent for the EM was a mixture of 60 parts water and 40 parts acetonitrile rather than a 50:50 ratio.

- Second, the flow rate of the 50:50 methanol/water mobile phase was 1.0 mL/min rather than 1.5 mL/min.

A 25 cm × 4.6 mm × 5 µm particle size C-18 column was used for all analytical determinations. A Beckman System Gold (GMI, Inc., Willoughby, OH) consisting of a model 126 programmable solvent module, a model 168 diode array detector, and a model 507 automatic sampler was used for the analyses. Calibration curves were generated before each HPLC run by dissolving certified standards (AccuStandard, Inc., New Haven, CT) of RDX and HMX in 60:40 water/acetonitrile in a range of concentrations appropriate for each run. The method detection limit was 0.05 mg kg^{-1} in solution, corresponding to 0.5 mg kg^{-1} in soil. Blanks and standards were placed intermittently between unknown samples to maintain quality assurance of the samples. All reagents used in the extraction of chemicals from soils were either reagent or trace-metal grade, and ASTM type I water was used throughout the analytical process.

2.8 Toxicity Assessment

The *Folsomia* Reproduction Test (ISO 11267:1998) was used to assess the effects of each EM on the reproduction of the collembolan, *F. candida*. The test is an adaptation of an internationally standardized bioassay of the International Organization for Standardization (ISO). The measurement endpoints for the test are adult survival and juvenile production, in which the production of juveniles is the reproduction endpoint. The ISO guideline for this assay was originally developed for use with Organisation for Economic Co-operation and Development **(**OECD) artificial soil (equivalent to USEPA standard artificial soil). Our research has shown that this test can be conducted using natural soils (Phillips et al., 2002; Kuperman et al., 2004).

2.8.1 Test Principle

Collembola are exposed to a range of concentrations of the test substance mixed in soil. The total number of juveniles produced (effective reproduction) and the survival of adult collembola are assessed after 28 days, when the number of adults and juveniles are counted separately. The effective reproduction and survival of adults exposed to the test substance are compared with the control treatments to quantify ecotoxicological parameters. These parameters include the bounded no observed effect concentration (NOEC), the bounded lowest observed effect concentration (LOEC), and the effective concentration that causes a *p* percent reduction (EC_p) in juvenile numbers (e.g., EC_{20} and EC_{50}).

2.8.2 Test Validity Criteria

Validity criteria are part of quality-control procedures. The adaptation of the *Folsomia* Reproduction Test for use with natural soils included the following performance parameters for the negative controls:

1. Adult mortality should not exceed 30% at the end of the test.

2. The average number of juveniles per chamber should reach 80 instars at the end of the 28 day test.

3. The coefficient of variation for reproduction should not exceed 30%.

2.8.3 Culturing Conditions

The U.S. Army Edgewood Chemical Biological Center (ECBC) laboratory culture of *F. candida* (collembola; springtails) was established in 1994. The ECBC culture was maintained in culture jars on a mixture of charcoal and plaster of Paris in darkness at 20 ^{o}C. The collembola were fed baker's yeast and kept moist by routine misting with distilled water approximately twice a week. Synchronized cultures were established for the experiments by removing egg clusters from stock cultures and placing them into new jars. The eggs were monitored daily to determine the onset of hatching. Two days after the eggs hatched, the juveniles were transferred into new jars. The synchronized juveniles were held for 10 days and then used in these tests.

2.8.4 Test Performance

Glass test containers (42 mm i.d. and 45 mm deep) were rinsed successively with acetone, tap water, and ASTM type I water before the test. To prepare five replicates of each treatment, 100 g of each air-dried treatment soil was hydrated to 88% of WHC. One-fifth of each batch of hydrated treatment soil was transferred by weight into a test container, and 0.05 g of baker's yeast was added to the surface of the soil. Ten 10–12 day old juveniles were placed in each test container, followed by light misting with ASTM type I water. Each container was sealed with plastic wrap that was held in place with a rubber band. The mass of each container was then recorded to monitor soil-moisture loss during the test. Five replicates were used for each EM-treatment concentration and control treatments.

The test containers were randomly placed in an incubator at 20 ± 0.5 °C with a relative humidity of 88 ± 5%. During the course of the study, the containers were weighed and misted weekly to maintain soil moisture levels.

To terminate a test, approximately 15 mL of tap water was added to a test container and allowed to sit for several minutes to fully hydrate the soil. After gentle mixing with a spatula, an additional 10 mL of water was added. The contents of the test container were given a final mixing and were examined under a dissecting microscope (15×) for the presence of juveniles and adults. The respective juveniles and adults that floated to the surface were counted.

Measurement endpoints were the numbers of surviving adults and juveniles that were produced after 28 days. All ecotoxicological parameters were estimated using measured acetonitrile-extractable concentrations of each explosive for each treatment concentration.

2.9 Data Analyses

The juvenile production data were analyzed using nonlinear regression models described in Stephenson et al. (2000) and Kuperman et al. (2004). Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to decide whether or not to weight the data and to select potential models. The logistic (Gompertz) model (eq 1) had the best fit for data in all the toxicity tests except for the freshly amended RDX and freshly amended 2,6-DNT, for which the exponential model (eq 2) had the best fit. The fit of the lines generated by these models were closest to the data points, the variances were the smallest, and the residuals had the best appearance (i.e., most random scattering). These models were

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

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

where

- Y is the number of juveniles produced,
- a is the control response,
- e is the base of the natural logarithm,
- p is the percent inhibition/100 (e.g., 0.20 for EC_{20}; 0.50 for EC_{50}),

- *C* is the exposure concentration measured in the test soil,
- EC*p* is the estimate of effect concentration for a specified percent effect, and
- *b* is the scale parameter.

The EC*p* parameters used in this study included the concentrations producing a 20% (EC_{20}) or 50% (EC_{50}) reduction in the measurement endpoint, respectively. The asymptotic standard error and 95% confidence intervals (CIs) associated with the point estimates were determined. The EC_{20} parameter based on a reproduction endpoint is the preferred parameter for deriving soil invertebrate EcoSSL benchmarks. The EC_{50}, a commonly reported value, was included to enable comparisons of the results produced in this study with results reported previously by other researchers.

Analysis of variance (ANOVA) was used to determine the bounded NOEC and LOEC values for adult survival or the juvenile production data. ANOVA analyses and adult survival data were included to enable comparisons of the results produced in this study with results previously reported by other researchers. Mean separations were determined using Fisher's least-significant difference pairwise comparison tests. A significance level of $P \leq 0.05$ was used. All analyses were done using measured acetonitrile-extractable EM concentrations. Statistical analyses were performed using SYSTAT 7.0.1 (SPSS, 1997).

3. RESULTS

3.1 Analytical Determinations of EMs in Soil

EM concentrations were determined using acetonitrile and ATCLP extractions at the beginning of each definitive toxicity test. The results of analytical determinations by HPLC are shown in Tables 2–11. Measured acetonitrile-extractable concentrations of RDX, freshly amended in SSL soil, averaged 111% (range: 99–127%) of nominal concentrations. Measured ATCLP-extractable RDX concentrations ranged from 1.3 to 121 mg kg^{-1} and averaged 60% of acetonitrile-extractable concentrations (Table 2). The relatively low solubility of RDX in water (56.3 mg L^{-1} at 25 °C) may have been responsible for the decrease in the ratio ATCLP/ acetonitrile percent as treatment concentrations increased, while the acetonitrile and nominal percentages remained relatively constant. Measured soil pH values among the different RDX concentrations did not deviate more than 0.1 pH per unit from the control soil (Table 2).

Table 2. Nominal and Average Measured RDX Concentrations, Freshly Amended in SSL Soil, and Mean pH Values

Nominal Concentration (mg kg^{-1})	Acetonitrile Extraction (mg kg^{-1})	Standard Error	Acetonitrile/ Nominal (%)	ATCLP Extraction (mg kg^{-1})	Standard Error	ATCLP/ Acetonitrile (%)	Mean pH n = 3
0	BDL*	–	–	BDL	–	–	5.5
1.5	1.9	0.06	127	1.3	0.04	68	5.6
3	3	0.21	100	2.8	0.11	93	5.5
9	10.2	0.16	113	8	0.17	78	5.6
18	20	1.07	111	17	0.42	85	5.6
36	44	2.60	122	37	1.37	84	5.5
120	139	2.60	116	121	0.33	87	5.6
360	356	5.67	99	94	2.08	26	5.6
720	745	3.38	103	88	4.20	12	5.5
2000	2121	32.20	106	79	0.41	4	5.5

*BDL (below detection limit) was reported when no RDX was detected in the control soil. Method detection limit (MDL) = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.
–: not available
Note: Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (ATCLP) concentration values.

Measured acetonitrile-extractable concentrations of RDX, weathered-and-aged in SSL soil, averaged 82% (range: 42–100%) of nominal concentrations (Table 3). Measured ATCLP-extractable concentrations of RDX weathered-and-aged in soil averaged 79% (range: 18–100%) of acetonitrile-extractable concentrations (Table 3). Measured soil pH values among the different concentrations of RDX weathered-and-aged in soil did not deviate more than 0.3 pH per unit from the control soil (Table 3).

Table 3. Nominal and Average Measured RDX Concentrations, Weathered-and-Aged in SSL Soil, and Mean pH Values

Nominal Concentration (mg kg^{-1})	Acetonitrile Extraction (mg kg^{-1})	Standard Error	Acetonitrile/ Nominal (%)	ATCLP Extraction (mg kg^{-1})	Standard Error	ATCLP/ Acetonitrile (%)	Mean pH n = 3
0	BDL*	–	–	BDL	–	–	5.3
6	6	1.50	100	6	0.38	100	5.3
9	8	1.31	89	8	0.27	100	5.3
18	16	0.24	89	13.6	0.47	85	5.3
36	30	0.75	83	30	0.38	100	5.3
72	57	3.38	79	54	2.01	95	5.2
144	60	2.23	42	55	2.99	92	5.1
300	250	8.74	83	100	2.54	40	5.0
600	530	4.04	88	93	1.16	18	5.0

*BDL was reported when no RDX was detected in the control soil. MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.
–: not available
Note: Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (ATCLP) concentration values.

Measured acetonitrile-extractable concentrations of HMX, freshly amended in SSL soil, averaged 106% (range: 92–125%) of nominal concentrations. Measured HMX ATCLP-extractable concentrations remained relatively constant, ranging from 6 to 15 mg kg^{-1} (Table 4). Measured soil pH values among the different concentrations did not deviate more than 0.1 pH per unit from the control soil (Table 4).

Table 4. Nominal and Average Measured HMX Concentrations, Freshly Amended in SSL Soil, and Mean pH Values

Nominal Concentration (mg kg^{-1})	Acetonitrile Extraction (mg kg^{-1})	Standard Error	Acetonitrile/ Nominal (%)	ATCLP Extraction (mg kg^{-1})	Standard Error	ATCLP/ Acetonitrile (%)	Mean pH n = 3
0	BDL*	–	–	BDL	–	–	5.4
9	10	4.98	111	6	0.51	53	5.4
36	36	2.77	100	15	0.55	42	5.4
72	70	8.25	97	13.1	0.06	18	5.4
144	140	7.54	97	12.5	0.30	98	5.3
300	350	10.44	117	12	0.21	45	5.4
600	640	8.69	107	12.5	0.19	2	5.4
1200	1500	63.31	125	13	0.32	1	5.4
2400	2200	119.45	92	12.6	0.45	0.6	5.4

*BDL was reported when no HMX was detected in the control soil. MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.
–: not available
Note: Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (ATCLP) concentration values.

Measured acetonitrile-extractable concentrations of HMX, weathered-and-aged in SSL soil, averaged 90% (range: 76–100%) of nominal concentrations (Table 5). Measured ATCLP-extractable concentrations of HMX, weathered-and-aged in soil, averaged 12% (range: 0.4–45%) of acetonitrile-extractable concentrations (Table 5). Measured soil pH values among the different concentrations did not deviate more than 0.5 pH per unit from the control soil (Table 5).

Measured acetonitrile-extractable concentrations of 2,4-DNT, freshly amended in soil, averaged 83% (range: 68–120%) of nominal concentrations (Table 6). Measured 2,4-DNT ATCLP-extractable concentrations averaged 34% (range: 17–51%) of acetonitrile-extractable concentrations (Table 6). Measured soil pH values among the different concentrations did not deviate more than 0.3 pH per unit from the control soil (Table 6).

Measured acetonitrile-extractable concentrations of 2,4-DNT, weathered-and-aged in soil, averaged 56% (range: 37–120%) of nominal concentrations (Table 7). Measured 2,4-DNT ATCLP-extractable concentrations averaged 54 (range: 45–64) percent of acetonitrile-extractable concentrations (Table 7). Measured soil pH values among the different concentrations did not deviate more than 0.1 pH per unit from the control soil (Table 7).

Table 5. Nominal and Average Measured HMX Concentrations, Weathered-and-Aged in SSL Soil, and Mean pH Values

Nominal Concentration (mg kg^{-1})	Acetonitrile Extraction (mg kg^{-1})	Standard Error	Acetonitrile/ Nominal (%)	ATCLP Extraction (mg kg^{-1})	Standard Error	ATCLP/ Acetonitrile (%)	Mean pH n = 3
0	BDL	–	–	BDL	–	–	5.0
36	29	1.31	81	13	0.15	45	5.4
72	55	1.79	76	14	0.66	25	5.3
144	130	10.90	90	16	0.62	12	5.3
300	280	8.67	93	19	0.34	7	5.3
600	560	15.24	93	18	0.46	3	5.4
1200	1100	49.33	92	22	0.81	2	5.4
2500	2500	114.24	100	17	0.98	0.7	5.5
5000	4800	142.73	96	18	0.44	0.4	5.5

*BDL was reported when no HMX was detected in the control soil. MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.
–: not available
Note: Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (ATCLP) concentration values.

Table 6. Nominal and Average Measured 2,4-DNT Concentrations, Freshly Amended in SSL Soil, and Mean pH Values

Nominal Concentration (mg kg^{-1})	Acetonitrile Extraction (mg kg^{-1})	Standard Error	Acetonitrile/ Nominal (%)	ATCLP Extraction (mg kg^{-1})	Standard Error	ATCLP/ Acetonitrile (%)	Mean pH n = 3
0	BDL*	–	–	BDL	–	–	5.4
0.5	BDL	0.0	120	0.1	0.01	17	5.6
1	0.5	0.03	90	0.2	0.01	22	5.6
2	1.0	0.06	70	0.3	0.03	21	5.6
4	3	0.08	75	0.9	0.01	30	5.6
8	6.5	0.15	68	2.1	0.03	39	5.6
12	10	0.26	70	3.6	0.11	42	5.6
24	20	0.66	83	9.6	0.12	48	5.7
48	40	0.57	90	22	0.52	51	5.6

*BDL was reported when no 2,4-DNT was detected in the control soil. MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

–: not available

Note: Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (ATCLP) concentration values.

Measured acetonitrile-extractable concentrations of 2,6-DNT, freshly amended in SSL soil, averaged 237% (range: 83–600%) of nominal concentrations (Table 8). Measured 2,6-DNT ATCLP-extractable concentrations averaged 39% (range: 25–62%) of acetonitrile-extractable concentrations (Table 8). Measured soil pH values among the different concentrations did not deviate more than 0.2 pH per unit from the control soil (Table 8).

Table 7. Nominal and Average Measured 2,4-DNT Concentrations, Weathered-and-Aged in SSL Soil, and Mean pH Values

Nominal Concentration (mg kg^{-1})	Acetonitrile Extraction (mg kg^{-1})	Standard Error	Acetonitrile/ Nominal (%)	ATCLP Extraction (mg kg^{-1})	Standard Error	ATCLP/ Acetonitrile (%)	Mean pH n = 3
0	BDL*	–	–	BDL	–	–	5.4
2	2.4	0.06	120	1.4	0.000	58	5.3
4	2.9	0.04	73	1.6	0.040	55	5.3
8	3.0	0.53	37	1.7	0.030	57	5.4
12	5	0.20	42	2.4	0.06	48	5.3
24	11.5	0.18	48	5.2	0.02	45	5.4
48	21	0.34	44	11.8	0.12	57	5.4
64	31	0.75	48	15.4	0.15	50	5.4
80	37	0.82	46	20.5	0.37	55	5.3
160	70	2.27	44	46	0.37	64	5.4

*BDL was reported when no 2,4-DNT was detected in the control soil. MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

–: not available

Note: Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (ATCLP) concentration values.

Table 8. Nominal and Average Measured 2,6-DNT Concentrations, Freshly Amended in SSL Soil, and Mean pH Values

Nominal Concentration (mg kg^{-1})	Acetonitrile Extraction (mg kg^{-1})	Standard Error	Acetonitrile/ Nominal (%)	ATCLP Extraction (mg kg^{-1})	Standard Error	ATCLP/ Acetonitrile (%)	Mean pH n = 3
0	BDL*	–	–	BDL	–	–	5.5
0.5	3	0.82	600	1.02	0.01	33	5.5
1	4.4	0.03	440	1.08	0.001	25	5.4
2	5.3	0.13	223	1.43	0.014	26	5.4
4	8	0.85	200	2.18	0.01	28	5.4
8	9.4	0.27	118	3.78	0.01	40	5.3
12	13	0.19	108	5.83	0.04	45	5.4
24	20	0.81	83	10.63	0.08	53	5.5
48	40	1.96	83	24.84	0.04	62	5.3

*BDL was reported when no 2,6-DNT was detected in the control soil. MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-} soil.
–: not available.
Note: Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (ATCLP) concentration values.

Measured acetonitrile-extractable concentrations of 2,6-DNT, weathered-and-aged in SSL soil, averaged 20% (range: 13–34%) of nominal concentrations (Table 9). Measured 2,6-DNT ATCLP-extractable concentrations averaged 39% (range: 13–60%) of acetonitrile-extractable concentrations (Table 9). Measured soil pH values among the different concentrations did not deviate more than 0.1 pH per unit from the control soil (Table 9).

Table 9. Nominal and Average Measured 2,6-DNT Concentrations, Weathered-and-Aged in SSL Soil, and Mean pH Values

Nominal Concentration (mg kg^{-1})	Acetonitrile Extraction (mg kg^{-1})	Standard Error	Acetonitrile/ Nominal (%)	ATCLP Extraction (mg kg^{-1})	Standard Error	ATCLP/ Acetonitrile (%)	Mean pH n = 3
0	BDL*	–	–	BDL	–	–	5.3
2	0.3	0.08	15	BDL	–	–	5.3
4	0.8	0.01	20	0.1	0.06	13	5.3
8	1.2	0.02	15	0.2	0.01	17	5.4
12	1.6	0.02	13	0.4	0.03	25	5.4
24	3.7	0.08	15	1.5	0.06	40	5.4
48	9.5	0.12	20	4.3	0.09	45	5.3
64	14	0.12	22	6.6	0.08	43	5.3
80	18	0.20	23	9.6	0.36	53	5.4
160	37	0.98	23	20	3.27	54	5.4
320	108	1.45	34	65	2.22	60	5.4

*BDL was reported when no 2,6-DNT was detected in the control soil. MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.
–: not available.
Note: Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (ATCLP) concentration values.

Measured acetonitrile-extractable concentrations of TNB, freshly amended in SSL soil, averaged 69% (range: 25–104%) of nominal concentrations (Table 10). When data for the 8 mg kg^{-1} nominal treatment were excluded, measured TNB ATCLP-extractable concentrations averaged 65% (range: 55–86%) of acetonitrile-extractable concentrations (Table 10); the 8 mg kg^{-1} nominal treatment had detectable TNB (0.13 mg kg^{-1}) in one out of three replicates, producing an average ATCLP-extractable value of 0.043 mg kg^{-1} (Table 10). TNB percentage of extracted versus nominal was substantially lower in treatments below 64 mg kg^{-1} compared with the other acetonitrile/nominal values for freshly amended EMs used in this study. Measured soil pH values among the different concentrations did not deviate more than 0.2 pH per unit from the control soil (Table 10).

Table 10. Nominal and Average Measured TNB Concentrations, Freshly Amended in SSL Soil, and Mean pH Values

Nominal Concentration (mg kg^{-1})	Acetonitrile Extraction (mg kg^{-1})	Standard Error	Acetonitrile/ Nominal (%)	ATCLP Extraction (mg kg^{-1})	Standard Error	ATCLP/ Acetonitrile (%)	Mean pH n = 3
0	BDL*	–	–	BDL	–		5.3
8	2.6	0.11	33	0.043	–	2	5.5
16	4	0.48	25	2.5	0.29	62	5.4
32	14	1.11	44	7.7	0.25	55	5.4
64	45	1.80	70	30	0.52	67	5.4
128	107	2.52	84	84	1.28	79	5.4
256	220	12.66	86	190	1.40	86	5.4
384	400	21.15	104	330	14.80	83	5.4
512	520	9.17	102	440	9.87	85	5.4

*BDL was reported when no TNB was detected in the control soil. MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.
–: not available.
Note: Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (ATCLP) concentration values.

Measured acetonitrile-extractable concentrations of TNB, weathered-and-aged in SSL soil, averaged 53% (range: 4–98%) of nominal concentrations (Table 11). Measured TNB ATCLP-extractable concentrations averaged 60% (range: 15–93%) of acetonitrile-extractable concentrations (Table 11). Measured soil pH values among the different concentrations did not deviate more than 0.2 pH per unit from the control soil (Table 11).

3.2 Range-Finding Toxicity Tests

All range-finding toxicity tests were conducted on individual EMs, each freshly amended in SSL soil. RDX in SSL soil caused reductions in adult survival and juvenile production at the 100 mg kg^{-1} treatment level as compared with control treatments. HMX had an effect on adult survival in the range-finding test, starting at the 1000 mg kg^{-1} concentration, while juvenile production numbers were reduced at the 500 mg kg^{-1} treatment level. 2,4-DNT and 2,6-DNT had an effect on adult survival and juvenile production starting at the 10 mg kg^{-1} treatment level. In the range-finding test with TNB, both adult survival and juvenile production were reduced at 100 mg kg^{-1}. Results of these range-finding tests were used to determine

nominal treatment concentrations for the definitive tests, and corresponding measured values are shown in Tables 2–11.

Table 11. Nominal and Average Measured TNB Concentrations, Weathered-and-Aged in SSL Soil, and Mean pH Values

Nominal Concentration (mg kg^{-1})	Acetonitrile Extraction (mg kg^{-1})	Standard Error	Acetonitrile/ Nominal (%)	ATCLP Extraction (mg kg^{-1})	Standard Error	ATCLP/ Acetonitrile (%)	Mean pH n = 3
0	BDL*	–	–	BDL	–	–	5.4
16	0.6	0.07	4	0.1	0.010	17	5.4
32	1.3	0.15	4	0.2	0.01	15	5.4
64	8.8	0.38	14	3.0	0.33	34	5.4
128	76	0.27	59	56	1.89	74	5.3
256	176	5.67	69	143	2.15	81	5.2
384	300	7.84	78	280	7.50	93	5.3
512	500	9.96	98	400	5.05	80	5.3
768	750	22.60	98	660	6.64	88	5.4

*BDL was reported when no TNB was detected in the control soil. MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.
–: not available.
Note: Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (ATCLP) concentration values.

3.3 Definitive Toxicity Tests

Definitive studies using the *Folsomia* Reproduction Test were conducted to assess the effects of RDX; HMX; 2,4-DNT; 2,6-DNT; and TNB on the reproduction of the collembolan *F. candida*. Juveniles were exposed in SSL soil to a range of concentrations for each EM in independent investigations. Measurement endpoints were assessed using 6–10 treatment concentrations, determined from the range-finding studies, and they included the number of surviving adults and the number of juveniles produced after 28 days. All ecotoxicological parameters were estimated using measured chemical concentrations for each treatment level.

Test results complied with the validity criteria adapted from the ISO test guideline (Section 2.8.2). Mean adult survival in the negative controls ranged from 86 to 96% in all tests. The mean production of juveniles in negative controls ranged from 134 to 566 juveniles, and the coefficient of variation ranged from 5 to 30%. The production of juveniles in the positive controls was reduced by 24–64% compared with negative controls and was within the baseline established for the laboratory culture of *F. candida*. These results confirmed that the toxicological effects determined in the definitive tests were most likely due to the EM treatments. All reported ecotoxicological parameters were calculated based on actual measured concentrations.

3.3.1 Toxicity of RDX

Results of the toxicity testing of RDX, freshly amended or weathered-and-aged in SSL soil, are shown in Table 12. The bounded NOEC for adult survival in SSL soil freshly amended with RDX was 44 mg kg^{-1} (no significant difference compared to control, $P = 1.000$)

(Table 13). Adult survival was significantly ($P \leq 0.0001$) reduced by 21% at the LOEC of 139 mg kg^{-1}. The bounded NOEC for the production of juveniles was 20 mg kg^{-1} ($P = 0.535$). The bounded LOEC for the production of juveniles was 44 mg kg^{-1} ($P = 0.005$). The EC_{20} and EC_{50} values were 28 and 86 mg kg^{-1}, respectively (exponential model) (Table 13). The bounded NOEC for adult survival in RDX weathered-and-aged amended in SSL soil was 530 mg kg^{-1} ($P = 0.264$) (Table 13). Adult survival was not significantly ($P = 0.264$) reduced at the highest concentration used in this study, which produced an unbounded LOEC at >530 mg kg^{-1}. The bounded NOEC for the production of juveniles was 57 mg kg^{-1} ($P = 0.079$). The bounded LOEC for the production of juveniles was 60 mg kg^{-1} ($P = 0.012$). The EC_{20} and EC_{50} values were 110 and 770 mg kg^{-1}, respectively (Gompertz model) (Table 13). All ecotoxicological parameters determined for RDX, freshly amended or weathered-and-aged in SSL soil, are given in Table 13.

Concentration-response relationships for the production of juveniles in RDX, freshly amended or weathered-and-aged in SSL soil, and determined by nonlinear regression, are shown in Figure 1. The exponential model had the best fit for data from the test with freshly amended RDX in soil (Figure 1A). The Gompertz model had the best fit for data from the test with RDX, weathered-and-aged in soil (Figure 1B). Overall, juvenile reproduction was greater for RDX weathered-and-aged in soil than for juvenile reproduction in soil containing freshly amended RDX (Table 12). The EC_{20} values for the production of juveniles for RDX freshly amended and weathered-and-aged in soil were 28 and 110 mg kg^{-1}, respectively. The difference between these values was not statistically significant based on 95% CIs (Table 13). The EC_{50} values for the production of juveniles for RDX freshly amended and weathered-and-aged in soil were 86 and 770 mg kg^{-1}, respectively.

Table 12. Mean Adult Survival and Production of Juveniles Exposed to RDX, Freshly Amended or Weathered-and-Aged in SSL Soil

Concentration† of RDX Freshly Amended in Soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error	Concentration† of RDX Weathered-and-Aged in Soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error
Negative control	10	295	16	Negative control	9	473	23
Acetone control	9	285	7	Acetone control	10	500	12
Positive control	6	119	10	Positive control	10	304	27
1.9	10	299	17	6	10	474	14
3	9	273	17	8	10	480	15
10.2	9	289	14	16	10	482	19
20	9	273	19	30	10	463	22
44	8	226	12	57	9	451	33
139	8	171	10	60	9	428	13
356	7	165	16	250	9	319	15
745	6	115	10	530	9	308	23
2121	5	116	13	–		–	

†: Concentrations were based on acetonitrile extraction using USEPA Method 8330.
–: not available.

Coefficients of determination (R^2) for acetonitrile- and ATCLP-based extractions of RDX were calculated in nonlinear regression analyses (EC_{20} levels) to determine which chemical measure better correlated with toxicity endpoints for RDX freshly amended or weathered-and-aged in soil. The R^2 values for juveniles in soil freshly amended with RDX were 0.982 and 0.972 for the acetonitrile- and ATCLP-based extractions, respectively (Table 13). The R^2 values for juveniles produced in soil containing RDX weathered-and-aged within the soil were 0.991 and 0.992 for the acetonitrile- and ATCLP based extractions, respectively. These comparisons show that regression coefficients were very similar for both extraction methods, indicating that neither extraction method had an advantage in characterizing RDX bioavailability to *F. candida*.

Table 13. Summary of Ecotoxicological Parameters (mg kg^{-1})† for Adult Survival and Production of *F. candida* Juveniles Exposed to RDX, Freshly Amended or Weathered-and-Aged in SSL Soil

Exposure Assessment	Adult Survival		Juvenile Production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh						
Acetonitrile extraction	44	139	20	44	28	86
P or 95% CI	1.000	<0.0001	0.535	0.005	14–41	45–128
R^2					0.982	0.982
ATCLP extraction	37	88	17	37	26	93
P or 95% CI	1.000	<0.0001	0.535	0.005	6–45	71–115
R^2					0.972	0.972
Weathered-and-Aged						
Acetonitrile extraction	530	>530	57	60	110	770
P or 95% CI	0.264	0.264	0.079	0.012	29–197	444–1097
R^2					0.991	0.991
ATCLP extraction	93	>93	54	55	74	120
P or 95% CI	0.264	0.264	0.079	0.012	62–85	103–134
R^2					0.992	0.992

†Concentrations were based on acetonitrile extraction using USEPA Method 8330 and water extraction using ATCLP.

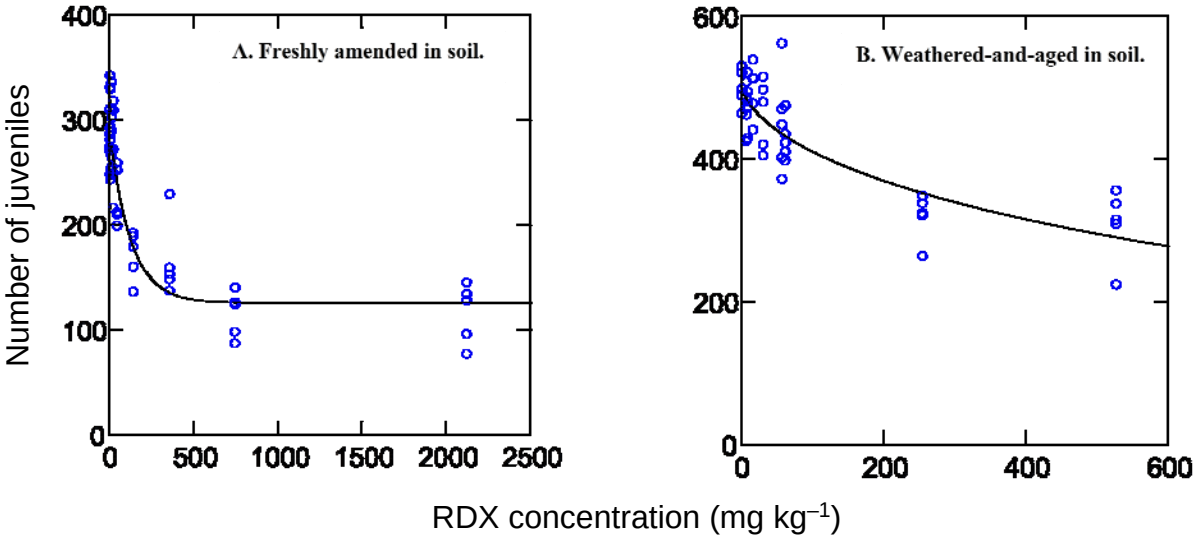

Figure 1. Production of juveniles exposed to RDX, (A) freshly amended or (B) weathered-and-aged in SSL soil.

3.3.2 Toxicity of HMX

Results of the toxicity testing of HMX, freshly amended or weathered-and-aged in SSL soil, are shown in Table 14. The bounded NOEC for adult survival for HMX freshly amended in SSL soil was 640 mg kg^{-1} ($P = 0.075$). Adult survival was significantly ($P = 0.006$) reduced at the LOEC of 1,500 mg kg^{-1}. The bounded NOEC for the production of juveniles was 640 mg kg^{-1} ($P = 0.054$). The bounded LOEC for the production of juveniles was 1,500 mg kg^{-1} ($P = 0.001$). The EC_{20} and EC_{50} values were 235 and 8,800 mg kg^{-1}, respectively (Gompertz model). The bounded NOEC for adult survival in HMX weathered-and-aged in SSL soil was 2,500 mg kg^{-1} ($P = 0.744$). Adult survival was significantly ($P \leq 0.0001$) reduced at the highest concentration used in this study, thus producing the bounded LOEC at 4,800 mg kg^{-1}. The bounded NOEC for the production of juveniles was 130 mg kg^{-1} ($P = 0.069$). The bounded LOEC for the production of juveniles was 280 mg kg^{-1} ($P = 0.019$). The EC_{20} and EC_{50} values were 1,000 and 10,400 mg kg^{-1}, respectively (Gompertz model). All ecotoxicological parameters determined for HMX, freshly amended or weathered-and-aged in SSL soil, are given in Table 15.

Table 14. Mean Adult Survival and Production of Juveniles Exposed to HMX, Freshly Amended or Weathered-and-Aged in SSL Soil

Concentration† of HMX Freshly Amended in Soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error	Concentration† of HMX Weathered-and-Aged in Soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error
Negative control	9.2	183	15	Negative control	10	558	34
Acetone control	9.2	190	15	Acetone control	10	570	21
Positive control	6.8	110	10	Positive control	9	296	29
10	8.8	177	14	29	10	560	16
36	8.6	161	12	55	10	529	25
70	8.8	153	11	130	10	505	16
140	8.4	161	10	280	9	485	35
350	8.4	142	8	560	10	481	24
640	8.2	157	10	1100	9	472	26
1500	7.6	128	15	2500	10	415	35
2200	7.8	112	9	4800	7	336	16

† Concentrations were based on acetonitrile extraction using USEPA Method 8330.

Concentration-response relationships determined by nonlinear regressions for the production of juveniles exposed to HMX, freshly amended or weathered-and-aged in soil, are shown in Figure 2. The Gompertz model had the best fit for data from the tests with HMX, freshly amended (Figure 2A) or weathered-and-aged (Figure 2B) in soil. Overall, reproduction was greater for HMX weathered-and-aged in soil compared to results for freshly amended HMX (Table 14). The EC_{20} values for the production of juveniles were 235 and 1,000 mg kg^{-1} of HMX either freshly amended or weathered-and-aged in SSL, respectively. The difference between these values was not statistically significant based on 95% CIs (Table 15). The respective EC_{50} values for the production of juveniles were 8,800 and 10,400 mg kg^{-1} of HMX either freshly amended or weathered-and-aged in SSL. The difference between the EC_{50} values was not statistically significant based on 95% CIs (Table 15).

Toxicological data for HMX were not analyzed in relation to ATCLP-extractable HMX concentrations because the concentration range tested in the definitive study was outside the water-soluble limit for HMX.

Table 15. Summary of Ecotoxicological Parameters (mg kg^{-1})[†] for Adult Survival and Production of *F. candida* Juveniles Exposed to HMX, Freshly Amended or Weathered-and-Aged in SSL Soil

Exposure Assessment	Adult Survival		Juvenile Production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh						
Acetonitrile extraction	640	1,500	640	1,500	235	8,800
P or 95% CI	0.075	0.006	0.054	0.001	0–730	0–22,648
R^2					0.975	0.975
Weathered-and-Aged						
Acetonitrile extraction	2,500	4,800	130	280	1,000	10,400
P or 95% CI	0.744	<0.0001	0.069	0.019	58–2,033	3,156–17,583
R^2					0.989	0.989

[†]Concentrations were based on acetonitrile extraction using USEPA Method 8330.

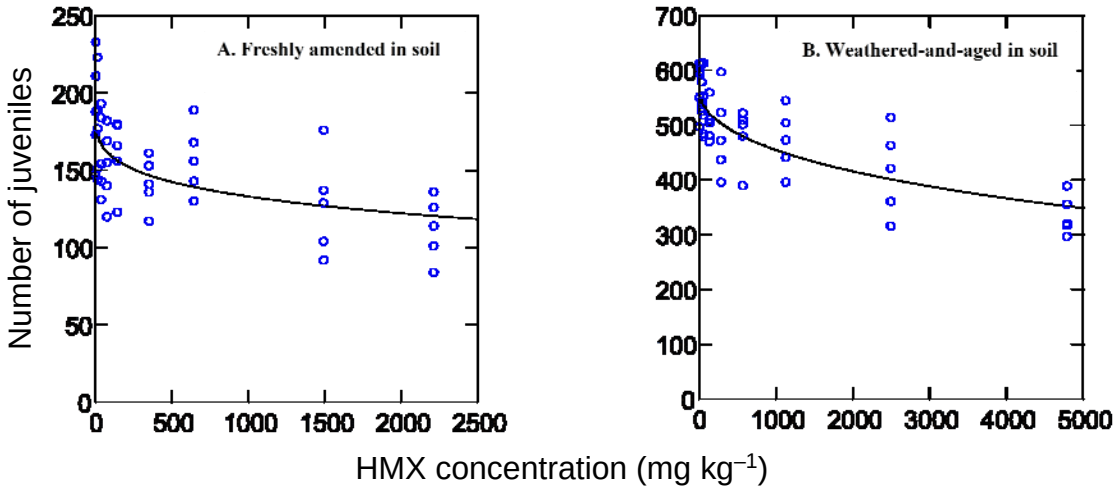

Figure 2. Production of juveniles exposed to HMX, (A) freshly amended or (B) weathered-and-aged in SSL soil.

3.3.3 Toxicity of 2,4-DNT

Results of the toxicity testing of 2,4-DNT, freshly amended or weathered-and-aged in SSL soil, are shown in Table 16. The bounded NOEC for adult survival in 2,4-DNT freshly amended in SSL soil was 3 mg kg^{-1} ($P = 1.000$) (Table 17). Adult survival was significantly ($P < 0.0001$) reduced at the LOEC of 5.4 mg kg^{-1}. The bounded NOEC for the production of juveniles was 3 mg kg^{-1} ($P = 0.239$). The bounded LOEC for the production of juveniles was 5.4 mg kg^{-1} ($P = 0.004$). The EC_{20} and EC_{50} values were 10 and 21 mg kg^{-1}, respectively (Gompertz model) (Table 17). The bounded NOEC for adult survival in 2,4-DNT weathered-and-aged in SSL soil was 5 mg kg^{-1} ($P = 0.325$) (Table 17). Adult survival was significantly ($P < 0.0001$) reduced at the LOEC of 11.5 mg kg^{-1}. The bounded NOEC for the production of juveniles was 3 mg kg^{-1} ($P = 0.143$). The bounded LOEC for the production of juveniles was 5 mg kg^{-1} ($P = 0.001$). The EC_{20} and EC_{50} values were 15 and 23 mg kg^{-1},

respectively (Gompertz model) (Table 17). All ecotoxicological parameters determined for 2,4-DNT, freshly amended or weathered-and-aged in SSL soil, are given in Table 17.

Table 16. Mean Adult Survival and Production of Juveniles Exposed to 2,4-DNT, Freshly Amended or Weathered-and-Aged in SSL Soil

Concentration† of 2,4-DNT, Freshly Amended in Soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error	Concentration† of 2,4-DNT Weathered-and-Aged in Soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error
Negative control	9	294	21	Negative control	9	414	18
Acetone control	10	312	14	Acetone control	9	400	19
Positive control	6	127	26	Positive control	7	223	16
0.6	10	306	16	2.4	9	407	21
0.9	9	301	18	2.9	8	399	13
1.4	9	267	23	3	9	358	21
3	10	284	15	5	8	315	13
5.4	7	239	23	11.5	6	299	8
8.4	7	213	9	21.5	6	217	39
20	6	198	20	31	5	50	15
43	2	0	0	37	2	3	3
–	–	–	–	70	0	0	0

†Concentrations were based on acetonitrile extraction using USEPA Method 8330.
–: not available.

Concentration-response relationships determined by nonlinear regression for the production of juveniles in soil where 2,4-DNT was either freshly amended or weathered-and-aged within the soil are shown in Figure 3. The Gompertz model had the best fit for data from the tests with 2,4-DNT, freshly amended (Figure 3A) or weathered-and-aged in SSL (Figure 3B). Overall, reproduction was greater for 2,4-DNT weathered-and-aged in soils compared to the results for freshly amended 2,4-DNT (Table 16). The EC_{20} values for the production of juveniles were 10 and 15 mg kg^{-1} of 2,4-DNT, when either freshly amended or weathered-and-aged in soil, respectively (Table 17). The difference between these values was not statistically significant based on 95% CIs (Table 17). The respective EC_{50} values for the production of juveniles were 21 and 23 mg kg^{-1} of 2,4-DNT, when either freshly amended or weathered-and-aged in soil. Also, the difference between the EC_{50} values was not statistically significant based on 95% CIs (Table 17).

Coefficients of determination (R^2) for acetonitrile- and ATCLP-based extractions of 2,4-DNT were calculated in nonlinear regression analyses to determine which chemical measure better correlated with toxicity endpoints for 2,4-DNT freshly amended and weathered-and-aged within soil. The R^2 values for juveniles produced in SSL soil freshly amended with 2,4-DNT were 0.972 and 0.971 for acetonitrile- and ATCLP-based extractions, respectively (Table 17). The R^2 values for juveniles produced in treatment containing 2,4-DNT weathered-and-aged within SSL soil were 0.980 and 0.978 for the acetonitrile- and ATCLP-based extractions, respectively. These comparisons show that regression coefficients were very similar

for both extraction methods, indicating that neither extraction method had an advantage in characterizing 2,4-DNT bioavailability to *F. candida*.

Table 17. Summary of Ecotoxicological Parameters (mg kg^{-1})† for Adult Survival and Production of *F. candida* Juveniles Exposed to 2,4-DNT, Freshly Amended or Weathered-and-Aged in SSL Soil

Exposure Assessment	Adult Survival		Production of Juveniles			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh						
Acetonitrile extraction					10	21
P or 95% CI	3	5.4	3	5.4	6–14	16–25
R^2	1.000	<0.0001	0.239	0.004	0.972	0.972
ATCLP extraction					5	10
P or 95% CI	0.9	2.1	0.9	2.1	2–7	8–13
R^2	1.000	<0.0001	0.239	0.004	0.971	0.971
Weathered-and-Aged						
Acetonitrile extraction					15	23
P or 95% CI	5	11.5	3	5	11–19	20–25
R^2	0.325	<0.0001	0.143	0.004	0.980	0.980
ATCLP extraction					11	13
P or 95% CI	2.4	5.1	1.67	2.4	9–12	12–14
R^2	0.325	<0.0001	0.084	0.001	0.978	0.978

†Concentrations are based on acetonitrile extraction using USEPA Method 8330, or water extraction using ATCLP.

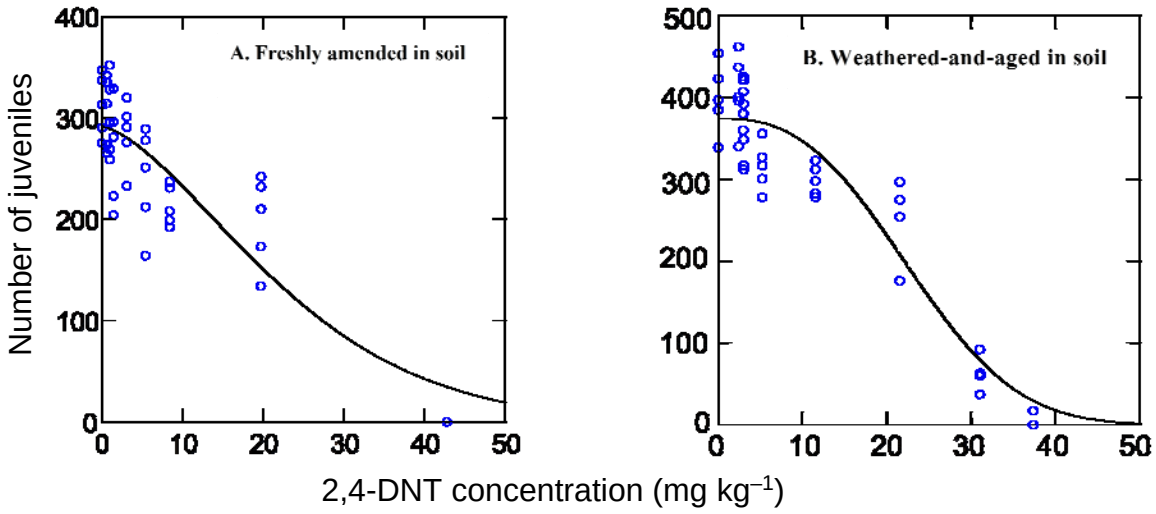

Figure 3. Production of juveniles exposed to 2,4-DNT, (A) freshly amended or (B) weathered-and-aged in SSL soil.

3.3.4 Toxicity of 2,6-DNT

Results of the toxicity testing of 2,6-DNT, freshly amended or weathered-and-aged in SSL soil, are shown in Table 18. The bounded NOEC for adult survival in 2,6-DNT freshly amended in SSL soil was 8 mg kg^{-1} ($P = 0.809$) (Table 19). Adult survival was significantly ($P = 0.007$) reduced at the LOEC of 9.4 mg kg^{-1}. The bounded NOEC for the production of juveniles was 8 mg kg^{-1} ($P = 0.073$). The bounded LOEC for the production of juveniles was 9.4 mg kg^{-1} ($P = 0.002$). The EC_{20} and EC_{50} values were 5.9 and 11 mg kg^{-1}, respectively (Exponential model). The bounded NOEC for adult survival in soil containing 2,6-DNT weathered-and-aged within SSL soil was 1.6 mg kg^{-1} ($P = 0.285$) (Table 19). Adult survival was significantly ($P = 0.001$) reduced at the LOEC of 3.7 mg kg^{-1}. The bounded NOEC for the production of juveniles was 1.6 mg kg^{-1} ($P = 0.167$). The bounded LOEC for juvenile production was 3.7 mg kg^{-1} ($P \leq 0.0001$). The EC_{20} and EC_{50} values were 0.96 and 3.6 mg kg^{-1}, respectively (Gompertz model). All ecotoxicological parameters for 2,6-DNT, freshly amended or weathered-and-aged in SSL soil, are given in Table 19.

Table 18. Mean Adult Survival and Production of Juveniles Exposed to 2,6-DNT, Freshly Amended or Weathered-and-Aged in SSL Soil

Concentration† of 2,6-DNT Freshly Amended in Soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error	Concentration† of 2,6-DNT Weathered-and-Aged in Soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error
Negative control	9	143	11	Negative control	9	237	38
Acetone control	9	169	11	Acetone control	9	243	33
Positive control	5	37	16	Positive control	5	56	16
3	9	129	14	0.3	9	240	45
4.4	8	138	17	0.8	9	220	32
5.3	9	141	31	1.2	8	225	16
8	7	96	27	1.6	8	192	28
9.4	5	77	21	3.7	6	46	15
13	3	58	35	9.5	4	67	23
20	1	1	1	14	2	30	7
40	0	0	0	18	3	22	10
–	–	–	–	37	0.2	0	0

†Concentrations are based on acetonitrile extraction using USEPA Method 8330.
–: not available.

Concentration-response relationships determined by nonlinear regression for the production of juveniles exposed to 2,6-DNT, freshly amended or weathered-and-aged in soil, are shown in Figure 4. The exponential model had the best fit for data from the test with 2,6-DNT freshly amended into soil (Figure 4A). The Gompertz model had the best fit for data from the test with 2,6-DNT weathered-and-aged within the SSL soil (Figure 4B). Overall, reproduction was greater in the SSL soil containing 2,6-DNT weathered-and-aged within the soil, compared to the results for SSL soil containing freshly amended 2,6-DNT (Table 18). The EC_{20} values for 2,6-DNT were 5.9 mg kg^{-1} for freshly amended treatments and 0.96 mg kg^{-1} for

treatments containing 2,6-DNT weathered-and-aged within soil. The difference between these values was not statistically significant based on 95% CIs (Table 19). The EC_{50} values for the production of juveniles were 11 and 3.6 mg kg^{-1} for 2,6-DNT freshly amended or weathered-and-aged within soils, respectively. The difference between these values was statistically significant based on 95% CIs (Table 19).

Coefficients of determination (R^2) for acetonitrile- and ATCLP-based extractions of 2,6-DNT were calculated in nonlinear regression analyses to determine which chemical measure better correlated with 2,6-DNT toxicity measurement endpoints for 2,6-DNT freshly amended and weathered-and-aged within soil. The R^2 values for production of juveniles in freshly amended soil were 0.906 and 0.907 for the acetonitrile- and ATCLP-based extractions, respectively (Table 19). The R^2 values for production of juveniles in treatment containing 2,6-DNT weathered-and-aged within SSL soil were 0.899 and 0.904 for the acetonitrile- and ATCLP-based extractions, respectively. These comparisons show that regression coefficients were very similar for both extraction methods, indicating that neither extraction method had an advantage in characterizing 2,6-DNT bioavailability to *F. candida*.

Table 19. Summary of Ecotoxicological Parameters (mg kg^{-1})† for Adult Survival and Production of *F. candida* Juveniles Exposed to 2,6-DNT, Freshly Amended or Weathered-and-Aged in SSL Soil

Exposure Assessment	Adult Survival		Juvenile Production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh						
Acetonitrile extraction					5.9	11
P or 95% CI	8	9.4	8	9.4	1.8–10	7–15
R^2	0.809	0.007	0.073	0.002	0.906	0.906
ATCLP extraction					2	4
P or 95% CI	2.2	3.8	2.2	3.8	0–3	2–7
R^2	0.809	0.007	0.073	0.002	0.907	0.907
Weathered-and-Aged						
Acetonitrile extraction					0.96	3.6
P or 95% CI	1.6	3.7	1.6	3.7	0–2.1	1.4–5.9
R^2	0.285	0.001	0.167	<0.0001	0.899	0.899
ATCLP extraction					0.2	1.3
P or 95% CI	0.4	1.5	0.4	1.5	0–0.6	0.4–2.3
R^2	0.211	<0.0001	0.119	<0.0001	0.904	0.904

†Concentrations were based on acetonitrile extraction using USEPA Method 8330 or water extraction using ATCLP.

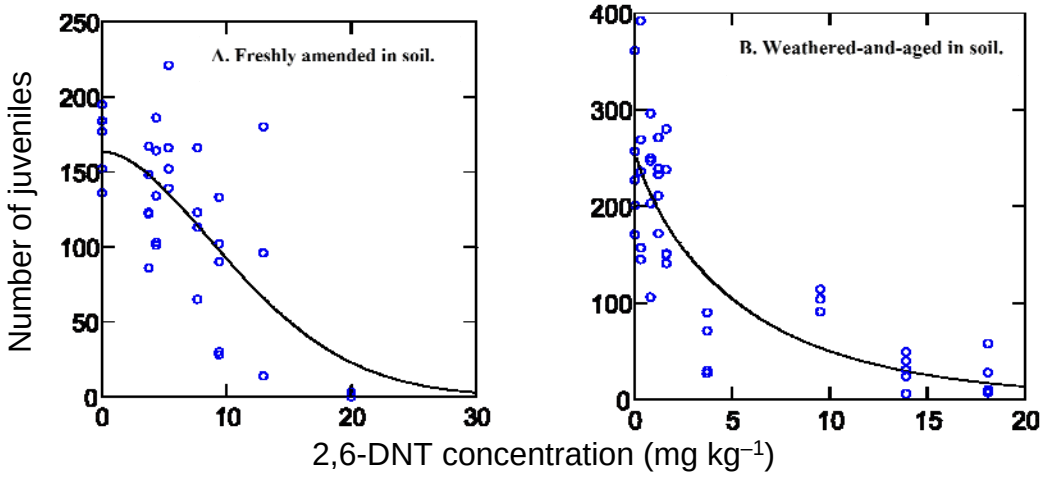

Figure 4. Production of juveniles exposed to 2,6-DNT, (A) freshly amended or (B) weathered-and-aged in SSL soil.

3.3.5 Toxicity of TNB

Results of the toxicity testing of TNB, freshly amended or weathered-and-aged in SSL soil, are shown in Table 20. Ecotoxicological parameters for TNB are summarized in Table 21. The bounded NOEC for adult survival in freshly amended SSL soil was 45 mg kg^{-1} ($P = 0.279$). Adult survival was significantly ($P \leq 0.0001$) reduced at the LOEC of 107 mg kg^{-1}. The bounded NOEC for the production of juveniles was 4 mg kg^{-1} ($P = 0.481$). The bounded LOEC for the production of juveniles was 14 mg kg^{-1} ($P = 0.002$). The EC_{20} and EC_{50} values were 4.4 and 24.7 mg kg^{-1}, respectively (Gompertz model). The bounded NOEC for adult survival for TNB weathered-and-aged in SSL soil was 76 mg kg^{-1} ($P = 0.608$). Adult survival was significantly ($P = 0.001$) reduced at the LOEC value of 176 mg kg^{-1}. The bounded NOEC value for the production of juveniles was 8.8 mg kg^{-1} ($P = 0.676$). The bounded LOEC for the production of juveniles was 76 mg kg^{-1} ($P \leq 0.0001$). The EC_{20} and EC_{50} values were 48 and 87.5 mg kg^{-1}, respectively (Gompertz model).

Table 20. Mean Adult Survival and Production of Juveniles Exposed to TNB, Freshly Amended or Weathered-and-Aged in SSL Soil

Concentration† of TNB Freshly Amended in Soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error	Concentration† of TNB Weathered-and-Aged in Soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Mean Juvenile Standard Error
Negative control	9	134	21	Negative control	9	566	30
Acetone control	9	168	11	Acetone control	8	557	29
Positive control	4	39	6	Positive control	8	304	27
2.6	9	143	25	0.6	10	582	29
4	9	149	16	1.3	7	569	14
14	8	79	20	8.8	8	537	48
45	9	88	31	76	7	267	72
107	2	17	11	176	0.4	15	12
220	0	0	0	300	0	0	0
400	0	0	0	500	0	0	0
520	0	0	0	750	0	0	0

†Concentrations are based on acetonitrile extraction using USEPA Method 8330.

Concentration-response relationships determined by nonlinear regressions for the production of juveniles exposed to TNB, freshly amended or weathered-and-aged in soil, are shown in Figure 5. The Gompertz model had the best fit for data from the tests with freshly amended TNB (Figure 5A) or for TNB weathered-and-aged within soil (Figure 5B). Overall, reproduction was greater for weathered-and-aged TNB compared to the results for freshly amended TNB (Table 20). The EC_{20} values for the production of juveniles were 4.4 and 48 mg kg^{-1} for TNB freshly amended and for TNB weathered-and-aged within soil, respectively. The difference between these values was statistically significant based on 95% CIs (Table 21). The EC_{50} values for the production of juveniles were 24.7 and 87.5 mg kg^{-1} for TNB freshly amended and for TNB weathered-and-aged within SSL soil, respectively. The difference between these values was statistically significant based on 95% CIs (Table 21).

Coefficients of determination (R^2) for acetonitrile- and ATCLP-based extractions of TNB were calculated in nonlinear regression analyses to determine which chemical measure better correlated with toxicity endpoints for freshly amended TNB and for TNB weathered-and-aged within SSL soil. The R^2 values for juveniles in freshly amended soil were 0.877 and 0.876 for the acetonitrile- and ATCLP-based extractions, respectively (Table 21). The R^2 values for juveniles in treatment containing TNB weathered-and-aged within SSL soil were 0.985 and 0.985 in the acetonitrile- and ATCLP-based extractions, respectively. These comparisons show that regression coefficients were very similar for both extraction methods, indicating that neither extraction method had an advantage in characterizing TNB bioavailability to *F. candida*.

Table 21. Summary of Ecotoxicological Parameters (mg kg^{-1})† for Adult Survival and Production of *F. candida* Juveniles Exposed to TNB, Freshly Amended or Weathered-and-Aged in SSL Soil

Exposure Assessment	Adult Survival		Production of Juveniles			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh						
Acetonitrile extraction					4.4	24.7
P or 95% CI	45	107	4	14	0–12	2.7–46.7
R^2	0.279	<0.0001	0.481	0.002	0.877	0.877
ATCLP extraction					3.7	22
P or 95% CI	30	84	2.5	7.7	0–10	1.5–43
R^2	0.279	<0.0001	0.481	0.002	0.876	0.876
Weathered-and-Aged						
Acetonitrile extraction					48	87.5
P or 95% CI	76	176	8.8	76	27–68	70–105
R^2	0.608	0.001	0.676	<0.0001	0.985	0.985
ATCLP extraction					34	66
P or 95% CI	56	143	3.0	56	18–50	51–80
R^2	0.608	0.001	0.676	<0.0001	0.985	0.985

†Concentrations were based on acetonitrile extraction using USEPA Method 8330 or water extraction using ATCLP.

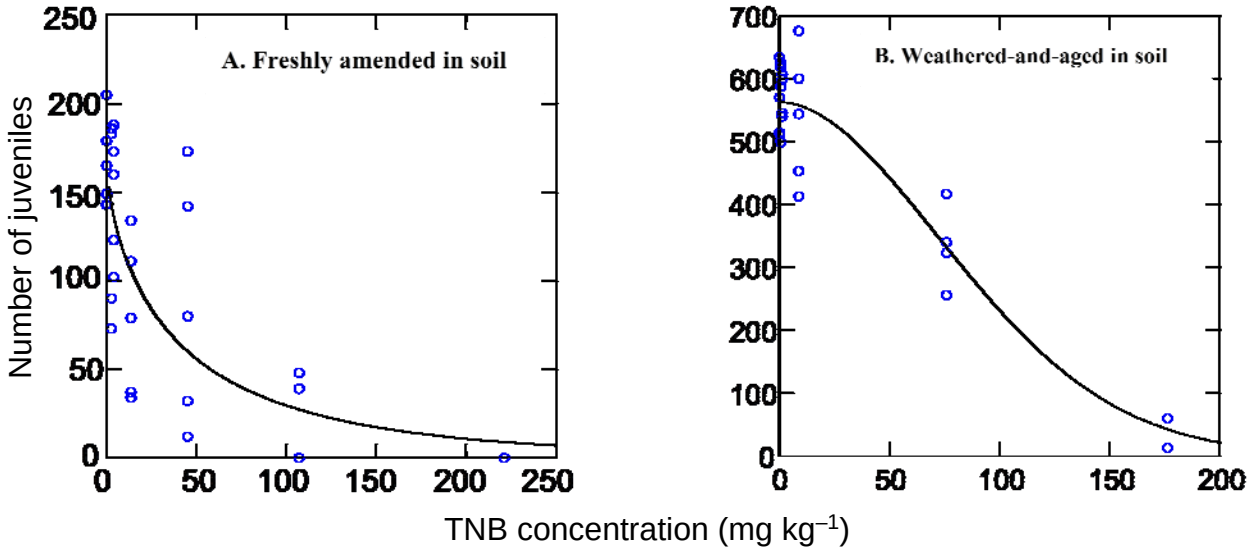

Figure 5. Production of juveniles exposed to TNB, (A) freshly amended or (B) weathered-and-aged in SSL soil.

4. DISCUSSION

The majority of soil toxicity tests that were reported in literature were performed using standard artificial soil with high organic matter content (10%). In contrast, our toxicity studies focused on using a natural soil that met the criteria for ecological soil screening level

development. The characteristics of this soil support the relatively high bioavailability of EMs. A weathering-and-aging procedure was applied to the range of EMs that were each amended into soil in independent studies, thus allowing us to assess RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB toxicities under conditions that closely mimic field conditions.

4.1 Chemical Analyses of EMs in Soil

The concentrations of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB were analytically determined for all definitive toxicity tests. Chemical analysis utilized the USEPA Method 8330 based on acetonitrile extraction of EMs from soil. Results from acetonitrile extraction of freshly amended soils showed good correlation between nominal and measured concentrations for the five EMs, confirming that the soil amendment procedure used in toxicity tests was appropriate, and that the USEPA Method 8330 was efficient for quantifying the amount of energetic materials in soil.

The water-extractable portion of each EM in an amended soil was determined using ATCLP. This water-extractable portion, believed to represent the part of the EM that is present in soil pore water, was hypothesized to better correlate with toxicity than the acetonitrile-extractable portion. The ATCLP-extractable concentrations of 2,4-DNT, 2,6-DNT, and TNB in freshly amended SSL soil increased proportionally with their respective acetonitrile-extractable concentrations. In contrast, RDX and HMX ATCLP-extractable concentrations decreased proportionally with their respective acetonitrile-extractable concentrations. Relatively low ATCLP-based concentrations for RDX and HMX can be explained on the basis of the relatively low water solubility of both compounds. The aqueous solubility of RDX was reported as 42 mg L^{-1} at 20 °C (Sikka et al., 1980) and as 60 mg L^{-1} at 25 °C (Banerjee et al., 1980). The aqueous solubility of HMX was reported to be between 5 and 6.6 mg L^{-1} at 25 and 20 °C, respectively (Glover and Hoffsommer, 1973; McLellan et al., 1992).

Assessment of toxicity to *F. candida* for EcoSSL development included studies with individual EMs that were weathered-and-aged in soils to more closely simulate the exposure effects on soil biota in the field (Kuperman et al., 2003; Kuperman et al., 2004; Kuperman et al., 2005; Simini et al., 2003). Weathering-and-aging of chemicals in soil may reduce the amount of chemical that is bioavailable to soil organisms because of hydrolysis, photodecomposition, sorption, and other fate processes that occur at contaminated sites. Also, it may result in increased toxicity because of the presence of more toxic transformation products.

The inclusion of the weathering-and-aging component in the EM toxicity assessments allowed us to incorporate potential alterations in EM bioavailability at contaminated sites in the development of ecotoxicological benchmarks for soil invertebrates.

4.2 Toxicity of EMs to *F. candida* in SSL Soil

The order of toxicity based on EC_{20} values for the production of juveniles in toxicity tests conducted with freshly amended soil was TNB > 2,6-DNT > 2,4-DNT > RDX > HMX. The order of EM toxicity to *F. candida* based on EC_{20} values for the production of juveniles for individual EM weathered-and-aged in soil was 2,6-DNT > 2,4-DNT > TNB > RDX > HMX.

These studies were designed to produce benchmark data for inclusion in the development of EcoSSLs for soil contaminated with explosives. In the literature, we often found discrepancies regarding the toxicity of the same chemical to different organisms. For the earthworm *Eisenia andrei* exposed to RDX in artificial soil, Robidoux et al. (2000) discovered that the LOEC for reproduction was 95 mg kg^{-1}. However, no effects were found on the mortality and reproduction of two terrestrial invertebrates, the enchytraeid worm (*Enchytraeus crypticus*) and the collembolan (*F. candida*), in soils amended with up to 1,000 mg kg^{-1} of either RDX or HMX (Schafer and Achazi, 1999). These studies were conducted either in standard artificial soil (Robidoux et al., 2000) or in soil with relatively high (2.5–3.0% organic C) organic matter content (Schafer and Achazi, 1999), which limits their usefulness for describing natural systems or the development of ecological soil screening levels.

Exposure of *F. candida* to HMX freshly amended into SSL soil negatively affected the production of juveniles (EC_{20} value of 235 mg kg^{-1}). The EC_{20} value was 1,000 mg kg^{-1} for the production of juveniles in soil containing HMX weathered-and-aged within the soil. Although the LOEC for the production of juveniles was less for soil containing HMX that was weathered-and-aged within soil, compared to that for freshly amended HMX, the quantity of juveniles produced in SSL containing weathered-and-aged HMX was 3–5 times as great as the numbers produced in SSL containing freshly amended HMX.

The relatively low RDX and HMX toxicities to *F. candida* in SSL soil at the concentrations tested in our studies can be related to low bioavailability of these EMs in soil. The solubilities of RDX and HMX in water at 20 °C are 42.3 and 6.63 mg L^{-1}, respectively (Roberts and Hartley, 1992). These low solubility levels in water contribute to low bioavailability of RDX and HMX in soil. Considering *F. candida* exposure to RDX and HMX in soil on the basis of ATCLP data provided herein yields an explanation for the observed effects of these nitro-heterocyclic explosives, thus providing a better understanding of an exposure mechanism that affects the relatively low toxicities to *F. candida* of RDX and HMX, compared to the toxicities of the nitroaromatic explosives tested.

The nitroaromatic EMs, 2,4-DNT, 2,6-DNT, and TNB, affected adult *F. candida* survival at the range of concentrations tested in our studies. In freshly amended SSL soils, the NOEC values for these nitroaromatic EMs ranged from 3 to 45 mg kg^{-1} and the LOEC values ranged from 5 to 107 mg kg^{-1} (Tables 17, 19, and 21). In soil containing the individual nitroaromatics weathered-and-aged within SSL soil, the NOEC values ranged from 1.6 to 76 mg kg^{-1}, and the LOEC values ranged from 3.7 to 176 mg kg^{-1} (Tables 17, 19, and 21).

The nitroaromatic EMs, 2,4-DNT, 2,6-DNT, and TNB, produced greater toxicological effects on the production of juveniles compared with the nitro-heterocyclic EMs RDX or HMX; furthermore, HMX was not toxic to adult *Eisenia fetida* at up to 640 mg kg^{-1} in freshly amended SSL soil, and not toxic up to 2,500 mg kg^{-1} for HMX weathered-and-aged in SSL. The EC_{20} estimates for these nitroaromatic EMs based on production of juveniles ranged from 1 to 48 mg kg^{-1} for EM weathered-and-aged within SSL soil. Comparison of our results with other studies is difficult because the toxicities of 2,4-DNT, 2,6-DNT, and TNB to soil invertebrates have not been sufficiently investigated. The majority of studies reported in the literature focused primarily on the effects of TNT and its degradation products (Renoux et al., 2000; Robidoux et al., 2000; Sunahara et al., 2001; Rocheleau et al., 1991; Schafer and Achazi,

1999; Simini et al., 1995; Phillips et al., 1993). In the study with *E. albidus* using OECD artificial soil, Dodard et al. (2003) determined an EC_{50} value of 111 mg kg^{-1} for TNT for the production of juveniles. Phillips et al. (1993) reported 100% mortality in the earthworm *Eisenia fetida* growth and survival test using USEPA standard artificial soil fortified with a mixture of EMs that included 30, 50, 62.5, and 20 mg kg^{-1} of TNT, TNB, 2,4-DNT, and 2,6-DNT, respectively. Statistically significant ($p < 0.01$) sublethal effects (mass loss) were reported at concentrations of 6, 10, 12.5, and 4 mg kg^{-1} of TNT, TNB, 2,4-DNT, and 2,6-DNT, respectively. These results are in general agreement with our findings, although direct comparisons of both studies are limited because of differences in the experimental designs.

Phillips et al. (1994) assessed the toxicity of soil from Joliet Army Ammunition Plant contaminated with a mixture of EMs (which limits direct comparisons with our study), including nitroaromatic and nitro-heterocyclic compounds using the earthworm *Eisenia fetida* growth and survival test among other bioassays. The greatest soil concentrations measured at this site for TNB, 2,4-DNT, and 2,6-DNT were 200, 117, and 8 mg kg^{-1}, respectively. TNT and TNB had the greatest coefficients of determination in all bioassays including the earthworm growth and survival test. Linear regression analyses of R^2 values for TNB using earthworm test measurement endpoints were 0.773 and 0.814 for the two locations investigated at the study site. These values for 2,4-DNT were 0.613 and 0.358, while 2,6-DNT had the weakest relationship to measurement points used with R^2 values of 0.082 and 0.293 for the two locations, respectively. Soil TNB and 2,4-DNT concentrations found at this site were within the range of concentrations tested in our studies, and the results are consistent with our findings. The weak relationship determined for 2,6-DNT is most likely the result of very low concentrations of this EM measured at the investigated site.

A weathering-and-aging procedure was incorporated into the design of current studies to produce toxicity data that more closely simulate the exposure effects of field conditions. Weathering-and-aging of EMs in soil may reduce the soil invertebrates' exposure to these chemicals through physico-chemical and biological processes. This can result in a dramatic reduction in the amount of chemical that is bioavailable, compared with tests conducted with freshly amended chemicals or those tested following a short equilibration period (e.g., 24 h). Dodard et al. (2003) reported a decrease in TNT toxicity to *E. albidus* from 44 to 89 mg kg^{-1} for OECD artificial soil, on the LC_{50} basis for reproduction, following a 21 day aging period after initial TNT amendment. In current studies, weathering-and-aging of EM in amended soils increased the toxicity of 2,6-DNT to adults and the production of *F. candida* juveniles, whereas toxicity of 2,4-DNT to adults decreased but did not change for juvenile production. TNB toxicity decreased with weathering-and-aging for adult survival and the production of juveniles. Specific mechanisms for changes in the toxicities of EMs weathered-and-aged in soil are unknown. EM transformation products or degradation material produced during the weathering-and-aging process may be more toxic to soil organisms compared with the parent material, and these could be contributing to increased toxicity of EM following weathering-and-aging in soil. Dodard et al. (1999) investigated the toxic effects of 2,4-DNT and 2,6-DNT, and their respective metabolites using the 15 min Microtox (*Vibrio fischeri*), and 96 h freshwater green alga (*Selenastrum capricornutum*), growth inhibition tests. The toxicities of DNTs were species-dependent; 2,4-DNT was more toxic than 2,6-DNT to *S. capricornutum* (comports with our results for *F. candida* in freshly amended soil for adult survival and the production of juveniles). The reverse was true for the test with *V. fischeri*. The authors reported that the reduced metabolites of

2,6-DNT that were tested were less toxic compared with the toxicity of the parent compound. However, certain partially reduced metabolites of 2,4-DNT (4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene) were more toxic than the parent compound. Although these results cannot be directly compared with our studies because the biotic reductive degradation pathway for 2,4-DNT and 2,6-DNT in an aquatic environment contrasts with metabolic processes in the aerobic conditions of the vadose zone simulated in our investigations; although a reducing environment can exist within water-logged soil microsites, where more toxic metabolites of dinitrotoluene degradation can be present. The greater toxicities of these metabolites may in part explain the increased toxicity of 2,6-DNT weathered-and-aged within SSL soil in our study.

The concentrations of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB in soil were determined by analyzing the water-extractable and acetonitrile-extractable portions. The water extract of each EM was obtained using ATCLP (Haley et al., 1993). This technique, perceived to measure the bioavailable fraction of chemicals within soil pore water, could generate data that is better correlated with toxicity than is the acetonitrile-extractable portion. Coefficients of determination (R^2) for acetonitrile-extractable and ATCLP-based extractions, determined in nonlinear regression analyses of the reproduction toxicity data from studies with EM compounds freshly amended and weathered-and-aged within amended soils, were compared to determine the chemical measure of exposure that better correlated with respective toxicities. These comparisons showed that coefficients of determination were very similar for both extraction types, indicating that neither extraction method had an advantage in characterizing bioavailability to *F. candida* of EMs tested in this study. This result supports our decision for developing benchmark data for energetics in soil, to be used in the establishment of EcoSSL values, on the basis of acetonitrile extraction of these test compounds. Acetonitrile extraction-based EcoSSLs will be especially useful for Ecological Risk Assessment at contaminated sites because EM concentrations determined during site characterization are usually based on data for acetonitrile extractable EMs analytically determined by USEPA Method 8330.

5. CONCLUSIONS

In this study, we produced ecotoxicological data for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB using the ecologically relevant soil invertebrate species *F. candida*. The relative toxicities of the five EMs were TNB > 2,6-DNT > 2,4-DNT > RDX > HMX for freshly amended soil, and 2,6-DNT > 2,4-DNT > TNB > RDX > HMX for EM weathered-and-aged within soil. The tests were performed using a natural SSL soil, which has low organic matter and clay contents, low cation exchange capacity, and high sand content. These characteristics support high relative bioavailability of EMs in soil. The inclusion of a weathering-and-aging procedure was designed to produce toxicity data that better reflect field conditions compared with previous studies, where soil invertebrates were exposed immediately following soil amendments.

SSL is a natural soil that fulfills the USEPA requirement of using soil with characteristics that support high relative bioavailability of contaminants for producing data to be used in developing conservative but realistic EcoSSL values (USEPA, 2005). The weathering-and-aging procedure was incorporated into our experimental design to produce exposure conditions similar to field conditions. Results of chemical analyses showed that exposure conditions of *F. candida* to EMs weathered-and-aged within soil differed from those of freshly

amended soil. This may be because of the transformation of 2,4-DNT; 2,6-DNT; and TNB, and the formation of degradation or transformation daughter products. The inclusion of a weathering-and-aging component allowed us to assess the potential alterations in EM bioavailability to *F. candida* at contaminated sites. To provide more complete information to risk assessors and site managers on ecotoxicological effects of EMs in soil, additional studies are required to determine the toxicities of the EM degradation and transformation products.

All ecotoxicological benchmarks determined in this study will be submitted to the Ecological Soil Screening Level (EcoSSLs) workgroup for quality control review by the EcoSSL task group before inclusion in the EcoSSL database, and before using for developing EcoSSLs for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB for soil invertebrates.

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ACRONYMS AND ABBREVIATIONS

2,4-DNT	2,4-dinitrotoluene
2,6-DNT	2,6-dinitrotoluene
ANOVA	analysis of variance
ASTM	American Society for Testing and Materials
ATCLP	Adapted Toxicity Characteristic Leaching Procedure
CAS	Chemical Abstracts Service
CI	confidence interval
ECBC	U.S. Army Edgewood Chemical Biological Center
EcoSSL	Ecological Soil Screening Level
EC*p*	Effective concentration for a specified percent effect
EM	explosive (or energetic) material
ERA	ecological risk assessment
HMX	high melting point explosive (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine)
HPLC	high performance liquid chromatography
ISO	International Organization for Standardization
LOEC	lowest observed effect concentration
MDL	method detection limit
NOEC	no observed effect concentration
OECD	Organisation for Economic Co-operation and Development
P	probability value
R^2	coefficient of determination
RDX	rapid detonation explosive (hexahydro-1,3,5-trinitro-1,3,5-triazine)
SERDP	Strategic Environmental Research and Development Program
SSL	sassafras sandy loam
TNB	1,3,5-trinitrobenzene
TNT	2,4,6-trinitrotoluene
USEPA	U.S. Environmental Protection Agency
WHC	water-holding capacity

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