U.S. ARMY COMBAT CAPABILITIES DEVELOPMENT COMMAND
CHEMICAL BIOLOGICAL CENTER

ABERDEEN PROVING GROUND, MD 21010-5424

Determination of Fate and Toxicological Effects of Insensitive Munitions Compounds in Terrestrial Ecosystems

Roman G. Kuperman
Michael Simini
Jeffrey McGuire
Ronald T. Checkai

RESEARCH AND TECHNOLOGY DIRECTORATE

Guilherme R. Lotufo
Afrachanna Butler
Robert Boyd
US Army Engineer Research and Development Center
Vicksburg, MS

Geoffrey Sunahara
Department of Natural Resource Sciences,
McGill University, Ste-Anne-de-Bellevue, Canada

Jalal Hawari
Department of Civil, Geological and Mining Engineering,
Ecole Polytechnique de Montreal, Montreal, Canada

Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
09-23-2022	Final	April 2017–January 2020

4. TITLE AND SUBTITLE
Determination of Fate and Toxicological Effects of Insensitive Munitions Compounds in Terrestrial Ecosystems.

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Kuperman, Roman G.; Simini, Michael; McGuire, Jeffrey; Checkai, Ronald T. (CCDC CBC); Lotufo, Guilherme R.; Boyd, Robert; and Butler, Afrachanna (US Army Engineer Research and Development Center); Sunahara, Geoffrey, I. (McGill University); Hawari, Jalal (Ecole Polytechnique de Montreal).

5d. PROJECT NUMBER
SERDP ER-2724

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Director, DEVCOM CBC, ATTN: FCDD-CBR-TM, APG, MD 21010-5424; US Army Engineer Research and Development Center, 3909 Halls Ferry Road, Vicksburg, MS 39180; McGill University, Ste-Anne-de-Bellevue, Canada; Ecole Polytechnique de Montreal, Montreal, Canada.

8. PERFORMING ORGANIZATION REPORT NUMBER
DEVCOM CBC

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Strategic Environmental Research and Development Program
4800 Mark Center Drive, Suite 16F16, Alexandria, VA 22350-3605

10. SPONSOR/MONITOR'S ACRONYM(S)
SERDP

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
ER-2724

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release: distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT: (Limit 200 words)
The military services have been developing and evaluating several insensitive munitions (IMs) for future weapon systems, to replace present munitions that contain highly sensitive explosives. The overall objective of the present research was to develop soil invertebrate-based toxicity benchmarks acceptable for deriving Draft Ecological Soil Screening Levels (Eco-SSL) for IM compounds 2,4-dinitroanisole (DNAN) and 3-nitro-1,2,4-triazol-5-one (NTO). The present research also developed empirical data to characterize the uptake dynamics DNAN and NTO from the soil into plants and earthworms. Toxicity studies were conducted using three soil invertebrate species the earthworm *Eisenia andrei*, the enchytraeid worm *Enchytraeus crypticus*, and the collembolan *Folsomia candida*. Draft Eco-SSLs and toxicokinetics data developed in this research will provide critical information for the installation managers to gauge the ecotoxicological impacts of military operations that involve the use of DNAN and NTO, thus ultimately promoting the sustainable use of testing and training ranges.

15. SUBJECT TERMS

2,4-dinitroanisole	DNAN	Soil invertebrate toxicity	Insensitive munitions
3-nitro-1,2,4-triazol-5-one	NTO	Uptake in ryegrass	Eco-SSL

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Roman G. Kuperman
U	U	U	UU	158	**19b. TELEPHONE NUMBER** *(include area code)* (410) 436-4697

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Blank

PREFACE

The work described in this report was authorized under project no. SERDP ER2724. The work was started in April 2017 and completed in January 2020.

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.

Acknowledgements

This project was completed in cooperation with and funded by the Strategic Environmental Research and Development Program (SERDP).

Blank

ABSTRACT

Unintended detonation of munitions and munition stockpiles has caused losses of human life, infrastructure, and materiel. The military services, therefore, have been developing and evaluating several insensitive munitions (IMs) for future weapon systems, to replace present munitions that contain highly sensitive explosives, and improve the safety of munitions. The overall objective of the present research was to develop scientifically defensible soil invertebrate-based toxicity benchmarks acceptable for deriving Ecological Soil Screening Levels (Eco-SSL) for IM compounds 2,4-dinitroanisole (DNAN) and 3-nitro-1,2,4-triazol-5-one (NTO). The ecological effects data for each IM compound were determined in compliance with the U.S. Environmental Protection Agency (USEPA) criteria for developing Eco-SSL that meet regulatory requirements for screening level ecological risk assessments.

Natural soil, Sassafras sandy loam, was used in the present studies. This soil was selected because it is an upland aerobic soil that has physical and chemical characteristics supporting the very high relative bioavailability of DNAN and NTO. The weathering-and-aging of DNAN and NTO in soil was performed in preparation for the definitive toxicity testing with soil invertebrates. It was conducted to simulate, at least partially, the weathering-and-aging process in field soils, and to more closely approximate the potential exposure effects on soil biota at contaminated sites. The exposure concentrations of DNAN and NTO in soil were analytically determined in all definitive studies. Toxicity studies were conducted using three soil invertebrate species according to the International Organization for Standardization methods for the earthworm *Eisenia andrei*, the enchytraeid worm *Enchytraeus crypticus*, and the collembolan *Folsomia candida*. Toxicity data were analyzed using appropriate regression models to establish concentration–response relationships for each IM compound–test species measurement endpoint pairing. The draft Eco-SSL values detailed in this report were derived using EC20 benchmark values for EM effects on soil invertebrate reproduction. The preference for reproduction benchmarks and for the low effect level (*i.e.*, EC20) was justified to ensure that Eco-SSL values for DNAN and NTO would be protective of populations of the majority of ecological receptors in soil. The Draft Eco-SSL values would also provide confidence that IM compound concentrations posing an unacceptable risk are not screened out early in the ecological risk assessment (ERA) process. The present research also developed empirical data to characterize the uptake dynamics of DNAN and NTO from the soil into plants and earthworms and determined the bioconcentration factors (BCFs) and bioaccumulation factors (BAFs), respectively, for DNAN and NTO.

Following acceptance by the USEPA, the Draft Eco-SSL values developed in the present research will allow screening of site-soil data during the Screening Level Ecological Risk Assessment to identify those IM that are not of potential ecological concern and do not need to be considered in the Baseline Ecological Risk Assessment, resulting in significant cost-savings during site assessments. These Eco-SSL will also provide an indispensable tool for the installation managers to gauge the ecotoxicological impacts of military operations that involve the use of DNAN and NTO, thus ultimately promoting the sustainable use of testing and training ranges. The toxicokinetics data will provide critical information for use in wildlife exposure models to assess the potential risks for food-chain transfer of DNAN and NTO to higher-trophic-level receptors and their corresponding potential for biomagnification.

EXECUTIVE SUMMARY

INTRODUCTION

Unintended detonation of munitions and munition stockpiles has caused losses of human life, infrastructure, and materiel. The military services, therefore, are developing and evaluating several insensitive munitions (IMs) for future weapon systems, to replace present munitions that contain highly sensitive explosives such as 1,3,5-trinitro-1,3,5-triazine (RDX) and 2,4,6-trinitrotoluene (TNT), and improve the safety of munitions (Powell 2016). Among the IM compounds being developed are 2,4-dinitroanisole (DNAN; CAS: 119-27-7) and 3-nitro-1,2,4-triazole-5-one (NTO; CAS: 932-64-9) used as components of several Insensitive Munition eXplosive compositions (IMXs). IMX-101 contains significant proportions of 2,4-dinitroanisole (DNAN), NTO, as well as other explosives compounds. Information on concentrations of IMs in natural soils was not publicly available at the initiation of the present research, and no real-world exposure information could be inferred. Therefore, the present investigation was warranted due to the manufacturing and use practices of the IM materials coupled with the chemical properties conducive to environmental transport. Ecotoxicological data were also needed to address a clear gap in knowledge of the potential risks associated with the release of DNAN and NTO into terrestrial environments.

OBJECTIVES

The Strategic Environmental Research and Development Program (SERDP) has identified a research need to determine the toxicity and uptake of IM compounds in soil biota. The overall objective of the present research was to develop ecotoxicological data for DNAN and NTO that can be used to derive risk-based regulatory levels for selected key soil ecological receptors. The ecological effects data for each IM compound were expected to comply with the U.S. Environmental Protection Agency (USEPA) criteria for developing Ecological Soil Screening Levels (Eco-SSL) and meet regulatory requirements for screening level ecological risk assessments (SLERA).

The research also aimed at developing empirical data to characterize the uptake dynamics of these IM compounds from the soil into plants and earthworms and determine the bioconcentration factors (BCFs) and bioaccumulation factors (BAFs), respectively, for DNAN and NTO. These data were needed to provide critical information for use in wildlife exposure models to assess the potential risks for food-chain transfer of DNAN and NTO to higher-trophic-level receptors and their corresponding potential for biomagnification.

TECHNICAL APPROACH

The present studies were designed to determine the ecological effects data for each IM compound in compliance with the USEPA criteria for developing Eco-SSL. For

Ecological Risk Assessment (ERA), particularly for developing ecotoxicological values protective of soil biota, we used a natural soil Sassafras sandy loam (SSL). This soil was selected because it is an upland aerobic soil that has low organic matter and clay contents. Additional studies were conducted using Webster clay loam (WCL) with greater organic matter and clay contents. The qualitative relative bioavailability (QRB) scores for organic chemicals in natural soils were considered "very high" for SSL, and "medium" for WCL, according to the USEPA Eco-SSL criteria. Studies with WCL soil were used to confirm that toxicity benchmarks developed for DNAN or NTO using SSL soil that supports higher relative bioavailability of IM compounds were sufficiently conservative for use in SLERA. The physical and chemical characteristics of test soils are shown in Table 1.

Table 1. Physical and Chemical Characteristics of Sassafras sandy loam and Webster clay loam Soils.

Soil Parameter	Sassafras Sandy Loam (SSL)	Webster Clay Loam (WCL)
Sand (%)	55	33
Silt (%)	28	39
Clay (%)	17	28
Texture	Sandy loam	Clay loam
Cation Exchange Capacity (CEC) (cmol kg^{-1})	9.3	21
Organic matter (%)	2.3	5.3
pH	4.9	5.9
Water Holding Capacity (WHC) (%)	18	23
QRB*	Very high	Medium

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

Both the 2,4-dinitroanisole (DNAN) and the 3-nitro-1,2,4-triazole-5-one (NTO) were obtained from BAE Systems, Ordnance Systems Inc. with a listed purity of 99.9%. Special consideration in assessing IM compound toxicity for Eco-SSL development was given to the inclusion of weathering-and-aging of contaminant explosives in soil in the assessment of the IM effects on terrestrial receptors. This more closely approximates the exposure effects in the field and is more relevant for ERA because Eco-SSL development by USEPA was specifically undertaken for use at Superfund sites (locations where contaminants have been long-present). The weathering-and-aging procedure of DNAN and NTO in soil was performed in preparation for the definitive toxicity testing with soil invertebrates. The procedure included exposing amended and control soils, initially hydrated to 60% of the WHC of SSL or WCL soil, in open glass containers in the greenhouse at ambient temperature to alternating moistening and air-drying cycles for 10 d. Soil samples collected from each treatment after the weathering-and-aging procedure, which corresponded to the beginning of the definitive toxicity tests, were processed for analytical determinations of DNAN and NTO concentrations.

The concentrations of DNAN and NTO were analytically determined in all definitive toxicity and uptake studies. Soil and tissue extracts were analyzed for DNAN, 2-amino-4-nitroanisole (2-ANAN, also known as 2-methoxy-5-nitroaniline), and 4-amino-2-nitroanisole (4-ANAN, 4-methoxy-3-nitroaniline) or for NTO, using a modified USEPA method 8330B.

Toxicity studies were conducted using three soil invertebrate species according to the International Organization for Standardization methods for the earthworm *Eisenia andrei*, the enchytraeid worm *Enchytraeus crypticus*, and the collembolan *Folsomia candida*. Toxicity data were analyzed using appropriate regression models to establish concentration–response relationships for each IM compound–test species measurement endpoint pairing. The draft Eco-SSL values detailed in this report were derived using EC20 benchmark values for EM effects on soil invertebrate reproduction.

IM uptake and elimination kinetics in the earthworms were assessed using amended SSL soil at a target concentration of 50 mg/kg. The experimental jars with amended soil were kept at 22 °C for 7 d to allow IM partitioning to soil constituents before receiving earthworms and to achieve more stable IM soil concentrations during the earthworm exposure period. A separate subset of jars was prepared and destructively sampled in triplicate after 1, 3, and 7 d for chemical analysis of soil. After the 7-d pre-exposure period, eight earthworms were added to each jar. Jars were destructively sampled in triplicate after 0, 1 h, 6 h, 3 d, 7 d, and 14 d for chemical analyses of earthworms and soils. Samples of earthworms exposed to DNAN- or NTO- amended soils and to control soil were frozen at –80 °C and lyophilized in a freeze dryer for at least 72 h. Dried earthworm samples were homogenized with a blender. The samples were then extracted by sonication (60 Hz) overnight (18 h) in acetonitrile (ACN) using a ratio of 1 mL ACN per 0.1 g of tissue for DNAN-exposed earthworms, or in 1% trifluoroacetic acid (TFA) in water using a ratio of 1 mL of 1% TFA per 0.1 g of tissue for NTO-exposed earthworms.

Soil and tissue extracts were analyzed for DNAN, 2-ANAN, 4-ANAN, or NTO, using a modified USEPA method 8330B. An Agilent 1200 high-performance liquid chromatograph (HPLC; Palo Alto, CA) equipped with a Phenomenex Synergi 4 µm HydroRP (80Å, 250 × 4.6 mm) LC column was used for these DNAN and NTO analyses. For analyte confirmation, a Restek (Pinacle II Biphenyl, 5 µm, 150 × 4.6 mm) reversed-phase column was used. Isocratic elution of analytes was accomplished using methanol:ultrapure water: ACN (45:51:4, v/v/v, respectively) at 0.9 mL/min. Detection and quantification were performed at 254 nm on a diode array detector.

RESULTS AND DISCUSSION

Draft Eco-SSL values were developed as geometric means of EC20 concentrations for reproduction endpoints determined for DNAN or NTO weathered-and-aged in SSL soil. Soil invertebrate toxicity benchmarks used for the derivation of the soil invertebrate-based draft Eco-SSL values for DNAN and NTO are presented in Tables 2 and 3. A total of four toxicity benchmarks developed in the present studies were used to derive draft Eco-SSL for

DNAN, and three toxicity benchmarks were used to derive draft Eco-SSL for NTO. These toxicity benchmarks yielded the draft Eco-SSL values of 46 and 53 mg/kg, respectively for DNAN and NTO (Tables 2 and 3).

Table 2. Derivation of Draft Eco-SSL values for DNAN weathered-and-aged in Sassafras sandy loam (SSL) soil using reproduction benchmarks for earthworm *Eisenia andrei*, potworm *Enchytraeus crypticus,* and collembolan *Folsomia candida*.

Receptor Group	Soil	EC20 (mg/kg)	95% Confidence Internals (mg/kg)	Draft Eco-SSL (mg/kg)
Earthworm				
Cocoon production	SSL	55	43–68	
Juvenile production	SSL	42	26–58	
Potworm				46
Juvenile production	SSL	70	46–93	
Collembola				
Juvenile production	SSL	27	20–33	

Table 3. Derivation of Draft Eco-SSL values for NTO weathered-and-aged in Sassafras sandy loam (SSL) soil using reproduction benchmarks for earthworm *Eisenia andrei*, potworm *Enchytraeus crypticus,* and collembolan *Folsomia candida*.

Receptor Group	Soil	EC20 (mg/kg)	95% Confidence Internals (mg/kg)	Draft Eco-SSL (mg/kg)
Earthworm				
Cocoon production	SSL	145	77–213	
Potworm				53
Juvenile production	SSL	16	8–24	
Collembola				
Juvenile production	SSL	63	32–95	

The preference for reproduction benchmarks and for the low effect level (*i.e.*, EC20) was justified to ensure that Eco-SSL values for DNAN and NTO would be protective of populations of the majority of ecological receptors in soil. The Eco-SSL values would also provide confidence that IM compound concentrations posing an unacceptable risk were not screened out early in the ecological risk assessment (ERA) process.

The present research also developed empirical data to characterize the uptake dynamics of DNAN and NTO from the soil into plants and earthworms. The bioaccumulation factor (BAF) values for SumDNAN (sum of DNAN, 2-ANAN, and 4-ANAN) and DNAN were generated for the uptake kinetics experiment as the concentration in tissue divided by the concentration in soil for different exposure periods shown in Table 4. For SumDNAN, the highest BAF value was 6.2 ± 1.0 kg/kg (dry wt) on day 3 and decreased to 3.8 ± 0.8 kg/kg by day 14. The highest BAF value for DNAN was 5.4 ± 0.9 kg/kg (dry wt), determined on day 3. In contrast to DNAN, the BAFs of NTO values were < 0.2 because the tissue concentrations were ≤

5 times lower than the exposure soil concentration, therefore indicating that NTO poses a low risk of transfer via the food chain compared with DNAN.

Table 4. Mean bioaccumulation factors for DNAN and SumDNAN for each time point of the uptake experiment. Numbers in parenthesis are 1 standard deviation.

Time point	DNAN	SumDNAN
1 h	0.7 (0.2)	0.7 (0.2)
6 h	2.1 (0.2)	2.2 (0.2)
1 d	3.4 (1.0)	3.6 (1.1)
3 d	5.4 (0.9)	6.2 (1.0)
7 d	2.4 (0.2)	3.5 (0.3)
14 d	2.3 (0.6)	3.8 (0.8)

RESEARCH BENEFITS

This project was undertaken specifically to develop scientifically defensible soil invertebrate-based benchmarks acceptable for deriving draft Eco-SSL values for DNAN and NTO. These draft Eco-SSL values were derived using the EC20 level toxicity benchmarks for the IM compounds effects on soil invertebrate reproduction endpoints determined in standardized toxicity tests. Ecotoxicological testing was specifically designed to meet the criteria for Eco-SSL derivation outlined in the Eco-SSL Guideline (USEPA, 2005). Following acceptance by the USEPA, these Eco-SSL values will allow screening of site-soil data during the Screening Level Ecological Risk Assessment to identify those IM compounds that are not of potential ecological concern and do not need to be considered in the Baseline Ecological Risk Assessment, resulting in significant cost-savings during site assessments. These Eco-SSLs will also provide an indispensable tool for the installation managers to gauge the ecotoxicological impacts of military operations that involve the use of DNAN and NTO, thus ultimately promoting the sustainable use of testing and training ranges. This project also determined that DNAN has a relatively low potential to bioaccumulate in soil invertebrates, while uptake of NTO into earthworm tissues was negligible. Bioconcentration of NTO in ryegrass was negligible or nonexistent. These data indicate that the studied IM compounds have a lower potential to bioaccumulate or bioconcentrate from soil compared to conventional munition compounds, and therefore pose a lower ecotoxicological risk to upper trophic level organisms.

Blank

CONTENTS

FIGURES

TABLES

Blank

DETERMINATION OF FATE AND TOXICOLOGICAL EFFECTS OF INSENSITIVE MUNITIONS COMPOUNDS IN TERRESTRIAL ECOSYSTEMS.

1. INTRODUCTION

Unintended detonation of munitions and munition stockpiles has caused losses of human life, infrastructure, and materiel. The military services, therefore, are developing and evaluating several insensitive munitions (IMs) for future weapon systems, to replace present munitions that contain highly sensitive explosives such as 1,3,5-trinitro-1,3,5-triazine (RDX) and 2,4,6-trinitrotoluene (TNT), and improve the safety of munitions (Powell 2016). Among the IM compounds being developed are 2,4-dinitroanisole (DNAN; CAS: 119-27-7) and 3-nitro-1,2,4-triazol-5-one (NTO; CAS: 932-64-9) used as components of several Insensitive Munition eXplosive compositions (IMXs). IMX-101 contains significant proportions of 2,4-dinitroanisole (DNAN), NTO, as well as other explosives compounds.

DNAN was historically used as an explosive in warheads containing Amatol 40 and is being investigated as a substitute for 2,4,6- trinitrotoluene (TNT) in IM formulations (Viswanath et al. 2018). It DNAN is a nitroaromatic compound with physicochemical properties, such as solubility in water and hydrophobicity similar to those of TNT (Viswanath et al. 2018). DNAN also has the potential to undergo photochemical transformation (Taylor et al. 2017a). DNAN It was is toxic to a wide variety of aquatic species (Dodard et al. 2013; Kennedy et al. 2015; Stanley et al. 2015; Gust et al. 2016) and soil invertebrates (Dodard et al. 2013; Gong et al. 2018). Lotufo et al. (2016) conducted bioaccumulation studies involving the uptake of DNAN by earthworms in amended soil and showed that the DNAN concentration in tissue exceeded the concentration of DNAN in soil. The bioaccumulation factors ranged from 1.2 to 4.3 kg dry soil/kg wet tissue.

3-Nitro-1,2,4-triazol-5-one (NTO) has been used since the 1980s in multiple energetic formulations. It has excellent qualities as an explosive such as insensitivity and thermal and mechanical stability, and stability and is considered a potential insensitive replacement for hexahydro-1,3,5-triazine (RDX) in various formulations (Viswanath et al. 2018). NTO has relatively low toxicity to tadpoles, fish, aquatic invertebrates, and soil nematodes and was considerably less toxic than TNT and DNAN in comparative studies (Lotufo et al. 2015; Stanley et al. 2015; Kennedy et al. 2017; Pillard et al. 2017; Gust et al. 2018; Gong et al. 2018; Madeira et al. 2018). The low Kow values predicted for NTO (from 0.89 to -1.19; Toghiani et al. 2008) indicate a low propensity to adsorb to organic C through hydrophobic interactions. NTO adsorbs very weakly to soils (Mark et al. 2017), as expected for a negatively charged compound in a matrix that also possesses a net negative charge, and the organic C content in soils has been shown not to be a good predictor of NTO adsorption (Taylor et al. 2017b). Hawari et al. (2014) reported that earthworms accumulated NTO from amended soil at lower concentrations than those found in the soil. Other studies have shown that NTO was not detected in plant roots and shoots in exposures to amended soils (Richard and Weidhaas 2014).

During live-fire training IMs are scattered by partial detonations and may weather and dissolve once on the soil surface (Taylor et al., 2017a), thereby posing a potential risk to soil

invertebrates and terrestrial plants. Although the information on concentrations of IMs in natural soils is not currently publicly available, and no real-world exposure information can be presently inferred, the manufacturing and use practices of the materials coupled with the chemical properties conducive to environmental transport warranted further investigation. Furthermore, ecotoxicological data were needed to address a clear gap in current knowledge of the potential risks associated with the release of DNAN and NTO into terrestrial environments. The overall objectives of the present research were to: 1) develop ecotoxicological data in soil for the IM compounds DNAN and NTO that can be used to derive risk-based levels that meet regulatory requirements for developing Ecological Soil Screening Levels (Eco-SSL) for use in screening-level ecological risk assessment (SLERA) for key soil ecological receptors, and 2) investigate the soil bioaccumulation and bioconcentration potentials for sublethal concentrations of DNAN and NTO in amended natural soil using the earthworm *Eisenia andrei* (this portion of the project is presented in Appendix A) and terrestrial plant ryegrass *Lolium perenne*.

2. MATERIALS AND METHODS

2.1 Reagents and Chemicals

Both the 2,4-dinitroanisole (DNAN) and the 3-nitro-1,2,4-triazol-5-one (NTO) were obtained from BAE Systems, Ordnance Systems Inc. with a listed purity of 99.9%. Acetonitrile, high-performance liquid chromatography (HPLC) grade (certificate of analysis listed the purity as ≥ 99.9%) was obtained from Fisher Scientific (Fair Lawn, NJ). Methanol (HPLC grade). Formic acid was obtained from Sigma-Aldrich (St. Louis, MO), product number 33015 (lot SZBD2830V; purity as 99.3%). 2-Amino-4-nitroanisole (2-ANAN) was obtained from Sigma-Aldrich (St. Louis, MO), product number 161195 (lot S76153V). The certificate of analysis listed the purity (GC area percentage) as 99.6%. 4-Amino-2-nitroanisole (4-ANAN) was obtained from Apollo Scientific (Stockport, U.K.), product number OR0934 (lot AS433424). The certificate of analysis listed the purity as 95+%. 1,3-Dinitrobenzene (1,3-DNB) was obtained from Avocado Research Chemicals LTD, (Heysham, UK), product number A11518 (lot B7488C) with a listed purity of 97%.

2.2 Soil Collection and Characterization

Soil clay and organic matter content have been identified as the key soil constituents that adsorb explosives, and thus affect the bioavailability and toxicity of energetic materials (EMs) for soil organisms (Haderlein et al., 1996; Pennington and Brannon, 2002; Jaenig, 2006; Singh et al., 2008, 2010; Dontsova et al., 2009; Kuperman et al., 2013; Arthur et al., 2017). For Ecological Risk Assessment (ERA), particularly for developing ecotoxicological values protective of soil biota, we used a natural soil Sassafras sandy loam (fine-loamy, siliceous, semiactive, mesic Typic Hapludult). Additional soil invertebrate studies were conducted in Webster clay loam (Fine-loamy, mixed, superactive, mesic Typic Endoaquoll). The qualitative relative bioavailability (QRB) scores for organic chemicals in natural soils were considered “very high” for Sassafras sandy loam, and “medium” for Webster clay loam, according to the United States Environmental Protection Agency (USEPA) Eco-SSL criteria (USEPA, 2005). Studies with Webster clay loam soil were used to confirm that toxicity

benchmarks developed using a soil type that supports the high relative bioavailability of IM compounds are sufficiently conservative for use in SLERA.

Sassafras sandy loam (SSL) was collected from an open grassland field in the coastal plain on the property of the U.S. Army Aberdeen Proving Ground in Harford County, MD. Webster clay loam (WCL) was collected in Story country, Iowa. During soil collection in the field, vegetation and the organic horizon were removed, and the top 12 cm of the A-horizon was then collected. The soil was sieved through a 5 mm screen, air-dried for at least 72 h, mixed periodically to ensure uniform drying, passed through a 2 mm sieve, then stored at room temperature before use in testing. Random soil samples were collected and analyzed for physical and chemical characteristics by The Agricultural Analytical Services Laboratory, The Pennsylvania State University, College of Agricultural Sciences (Table 1).

Table 1. Physical and Chemical Characteristics of Sassafras Sandy Loam and Webster Clay Loam Soils

Soil Parameter	Sassafras Sandy Loam (SSL)	Webster Clay Loam (WCL)
Sand (%)	55	33
Silt (%)	28	39
Clay (%)	17	28
Texture	Sandy loam	Clay loam
Cation Exchange Capacity (CEC) (cmol kg^{-1})	9.3	21
Organic matter (%)	2.3	5.3
pH	4.9	5.9
Water Holding Capacity (WHC) (%)	18	23
QRB*	Very high	Medium

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

The selected SSL soil had sufficiently low organic matter and clay contents to fulfill the USEPA requirement for using soils with characteristics that support high or very high relative bioavailability of organic pollutants, for developing realistic conservative Eco-SSL values (USEPA, 2005).

2.3 Soil Amendment Procedure for Toxicity Studies

Soil was weighed separately in a glass container for each IM compound treatment, and was spread from 2.5 to 4 cm thickness. Each IM compound amendment was prepared separately in a glass volumetric flask and dissolved in acetone as the carrier in studies with DNAN and methanol in studies with NTO, to produce a more homogeneous mixture in soil than the addition of solid DNAN or NTO. Soil treatments were individually amended with

DNAN or NTO. The IM compound/carrier solution was quantitatively transferred to the soil, adding it evenly across the soil surface, ensuring that the volume of solution added at any one time does not exceed 15% (v/w) of the dry mass soil. The same total volume of the carrier was added to every DNAN or NTO treatment, equaling the volume of carrier required to dissolve DNAN or NTO at the highest concentration tested. The amended soils were then air-dried for a minimum of 18 h in a darkened chemical fume hood. Each amended soil sample was transferred into a high-density polyethylene container coated with a fluoro-polymer and mixed for 18 h using a three-dimensional soil mixer in darkness to prevent photolysis of the IM compounds. Four to nine concentrations of each IM compound were used in addition to controls (negative, positive, and carrier). All treatments were appropriately replicated. After three-dimensional mixing, samples of freshly amended soil were collected from each soil treatment for analytical determinations of the initial DNAN or NTO concentrations. The remaining soil in each batch was hydrated with American Society for Testing and Materials (ASTM) Type I water to 60% of the respective soil's water holding capacity (WHC) initiating the IM compound weathering-and-aging procedure in soil.

2.4 Weathering-and-aging of energetic materials in Soils for Toxicity Studies

Special consideration in assessing IM compound toxicity for Eco-SSL development was given to the inclusion of weathering-and-aging of contaminant explosives in soil in the assessment of the IM compound effects on terrestrial receptors. This more closely approximates the exposure effects in the field and is more relevant for ERA because Eco-SSL development by the USEPA was specifically undertaken for use at Superfund sites (locations where contaminants have been long-present). Weathering/aging of chemicals in soil may reduce exposure of terrestrial plants and soil invertebrates to EMs due to photodecomposition, hydrolysis, reaction with organic matter, sorption/fixation, precipitation, immobilization, occlusion, microbial transformation, and other fate processes that commonly occur at contaminated sites. This can result in a dramatic decrease in the amount of parent compound that is bioavailable, compared to tests conducted with recently-amended chemicals or those tested following a short equilibration period (*e.g.*, 24 h), and can affect the IM compound toxicity as was demonstrated in our previous studies.

The weathering-and-aging procedure of DNAN and NTO in soil was performed at the U.S. Army Chemical Biological Center (CBC) in preparation for the definitive toxicity testing with soil invertebrates. It was conducted to simulate, at least partially, the weathering-and-aging process in field soils, and to more closely approximate the exposure effects on soil biota at contaminated sites. These procedures included exposing amended and control soils, initially hydrated to 60% of the WHC of SSL or WCL soil, in open glass containers in the greenhouse at ambient temperature to alternating moistening and air-drying cycles for 10 d. During the weathering-and-aging procedure, all soil treatments were weighed and readjusted to their initial mass by periodically adding ASTM Type I water to the soil. Soil samples collected from each treatment after the weathering-and-aging procedure, which corresponded to the beginning of the definitive toxicity tests, were processed for analytical determinations of DNAN, and NTO concentrations.

2.5 Extraction and Analytical Determinations of DNAN and NTO in Toxicity Studies

2.5.1 Extraction of DNAN from SSL or WCL Soil

DNAN was extracted from SSL or WCL soil by placing 1 g soil samples from the soil batch treatments and controls into respective 50 mL polypropylene centrifuge tubes, then adding 5 mL of acetonitrile (ACN) to each tube. Samples were vortexed with the ACN for 1 min, then sonicated in darkness for 18 h at 20 °C. After sonication, 5 mL of $CaCl_2$ solution (5 g/L) was added to each of the 50 mL polypropylene centrifuge tubes. The tubes were vortexed for 1 min and then centrifuged for an additional 1 min. 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. One or 3 µL of the internal standard (4.97 mg 1,3-DNB/L) was added to each HPLC vial. The vials were vortexed for 15 sec, then analyzed and quantified by HPLC with confirmation conducted on GC-MS-MS.

2.5.2 Analytical Methods: DNAN and Transformation Products in SSL soil

The analytical determinations of DNAN were performed using an Agilent High Performance Liquid Chromatograph (HPLC; 1100 Series, Agilent Corp., Wilmington, DE) equipped with Diode Array Detection (DAD). Separation was achieved using a 250 × 4.6 mm Supelco Discovery C18 Column at 35 °C. The isocratic mobile phase consisted of 50% water and 50% methanol running at a rate of 1 mL/min. The injection volume was 10 µL. Detection and subsequent concentration determinations of DNAN were performed at 298 nm wavelength. The Limit of Detection (LOD) under these experimental conditions was 0.049 µg/mL and a limit of quantitation (LOQ) of 0.173 µg/mL. A 10-point calibration curve (0.173, 0.347, 0.695, 1.39, 2.79, 5.58, 11.17, 22.35, 44.70, and 89.40 µg/mL) was used to determine DNAN concentrations in soil extracts. The calibration curve correlation yielded an $R^2 = 0.9999$.

DNAN transformation products included 2-ANAN and 4-ANAN. The analytical determinations of 2-ANAN and 4-ANAN were performed using an Agilent High Performance Liquid Chromatograph (HPLC; 1100 Series, Agilent Corp., Wilmington, DE) equipped with DAD. Separation was achieved using a 250 × 4.6 mm Supelco Discovery C18 Column at 35 °C. A gradient mobile phase (1 mL/min) was used for the separation of the two transformation products. The injection volume was 25 µL. Detection and subsequent concentration determinations of 2-ANAN and 4-ANAN were performed at 240 nm wavelength. The LOD for 2-ANAN under these experimental conditions was 0.01 µg/mL and an LOQ of 0.146 µg/mL. A stock solution of 2-ANAN (1.1164 mg/mL in ACN) was diluted with 50% ACN to develop a 7-point calibration curve (0.146, 0.291, 0.582, 2.91, 5.82, 29.10, and 58.20 µg/mL) used to determine 2-ANAN concentrations in soil extracts. The LOD for 4-ANAN under these experimental conditions was 0.011 µg/mL and an LOQ of 0.075 µg/mL. A stock solution of 4-ANAN (1.2108 mg/mL in ACN) was diluted with 50% ACN to develop an 8-point calibration curve (0.075, 0.151, 0.302, 0.605, 3.03, 6.05, 30.27, and 60.54 µg/mL) used to determine 4-ANAN concentrations in soil extracts. The calibration curve correlation for 2-ANAN and 4-ANAN yielded an R^2 of 1.0000 and 0.9999, respectively.

2.5.3 GC-MS-MS Confirmation for Analytes in SSL soil

Stock solutions of DNAN (1.788 mg/mL in ACN), 2-ANAN (1.116 mg/mL in ACN), and 4-ANAN (1.211 mg/mL in ACN) were used to prepare the calibration standards. Samples of the stock solutions were diluted with 50% ACN to obtain the following six nominal concentration points: 1, 5, 10, 25, 50, and 100 µg/mL. Each calibration standard also contained 25 µg/mL 1, 3-DNB diluted from the stock solution as internal standard. All calibration standards were stored at –20 °C until needed.

Analytical confirmation was achieved using an Agilent 7000A Triple Quad GC/MS instrument (Agilent Technologies; Santa Clara, CA). Gas chromatographic separations were achieved using an RTx-5MS column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Restek Corporation; Bellefonte, PA). The carrier gas was helium, with a flow rate of 1 mL/min. Injections of 1.0 or 3.0 µL were made using an Agilent 7693 ALS autoinjector into a splitless injector port at a temperature of 300 °C. The initial oven temperature of 100 °C was held for 2 min, then ramped to 280 °C at 20 °C/min, and held for 1.5 min. After each analysis was complete, the column was back flushed at 280 °C for 2.1 min at the reduced inlet pressure (0.5 psi).

One mL samples of the soil extracts were spiked with 5.2 µL of the 1,3-DNB (used as the internal standard) stock solution (4.798 mg/mL in ACN) to yield a final concentration internal standard concentration of 25 µg/mL.

Samples were ionized by positive-ion chemical ionization with methane reagent gas. Chemical ionization source conditions were optimized using Fluoroether E3 (Chemical Abstracts Service [CAS] registry number 3330-16-3; Agilent Technologies) tuning compound. Mass spectra were obtained at a dwell time of 0.1 s for each transition in the multiple reaction monitoring (MRM) mode. Helium was used as the collision gas with a collision energy of 20 V. The collision energy was optimized for the mass-to-charge ratio (m/z) 199 > 182 transition for DNAN, the m/z 169 > 108 transition for 2-ANAN, the m/z 169 > 94 transition for 4-ANAN, and the m/z 169 > 122 transition for 1,3-DNB. The MassHunter software provided with the Agilent 7000A system was used to process and analyze the data. The software provides automated peak detection, calibration, and quantitation.

2.5.4 Extraction of NTO from SSL or WCL Soil

NTO was extracted from SSL or WCL soil by placing 1 g soil samples from the soil treatments and controls into respective 50 mL polypropylene centrifuge tubes, then adding 5 mL of 50% methanol to each tube. The tubes were vortexed for 1 min, then placed into an ultrasonic water bath in darkness for 18 h at 20 °C. After sonication, 5 mL of $CaCl_2$ solution (5 g/L) was added to each of the 50 mL polypropylene centrifuge tubes. The tubes were vortexed for 1 min and then centrifuged for an additional 1 min. The supernatant was then filtered through a 0.45 µm PTFE syringe cartridge, and 1 mL of each filtered solution was transferred into an HPLC vial. Three µL of the internal standard (4.97 mg 1,3-DNB/L) was added to each HPLC vial just before analytical determination. The vials were vortexed for 15 sec, then analyzed and quantified by HPLC.

2.5.5 Analytical Methods: NTO and Transformation Products in SSL Soil

A primary stock solution of NTO was prepared by dissolving crystalline NTO in 50% ACN with 0.1% trifluoroacetic acid (TFA). This stock solution was then serially diluted using 50% ACN with 0.1% TFA to create analytical standards of 0.401, 0.802, 1.61, 3.21, 6.42, 12.84, 25.68, 51.37, 102.75, and 205.5 µg/mL (R^2 values for NTO standards ranged from 0.9998 to 0.9999). The standards were stored in darkness at 4 °C.

The analytical determinations of NTO in soil extract were conducted using HPLC (1100 Series, Agilent Corp., Wilmington, DE) with UV DAD with the wavelength set at 315 nm for NTO or 230 nm for 1,3-DNB. Separation was achieved using a 150 × 4.6 mm Hypercarb column (Thermo Scientific, Waltham, MA). The initial mobile phase was isocratic consisting of 25:75 ratio of ACN containing 0.1% TFA and water containing 0.1% TFA. The flow rate was 1 mL/min and the injection volume was 25 µL. The Limits of Detection (LOD) for NTO under these experimental conditions was 0.005 µg/mL (peak to peak). The isocratic mobile phase was run for 4 min to elute the NTO. The mobile phase was then changed to a gradient to remove the internal standard (1,3-DNB). Extraction and analytical determinations of DNAN and NTO in bioaccumulation studies are described elsewhere in this report.

2.5.6 Analytical Methods: DNAN, NTO, and Transformation Products in WCL Soil

The analytical determinations of DNAN, 2-ANAN, 4-ANAN, and NTO in WCL soil were performed using an Agilent 6410 Triple Quadrupole LC/MS System equipped with DAD. Separation was achieved using a ZORBAX Eclipse XDB-C18 column (150 mm × 4.6 mm, 5 µm pore size) at a temperature of 25 °C. The isocratic mobile phase consisted of 60% aqueous (0.1% formic acid in deionized water) and 40% organic (0.1% formic acid in methanol) with a flow rate of 1 mL/min. Injection volumes of either 10 µL or 50 µL were made. Detection and subsequent concentration determinations of DNAN were performed at 300 nm wavelength, with determinations of 2-ANAN and 4-ANAN performed at 240 nm wavelength. The internal standard 1,3-DNB was also detected and determined at 240 nm wavelength. Detection and concentration determination of NTO was performed at 315 nm wavelength. Analytical confirmation was accomplished using positive electrospray ionization mass spectrometry with a fragmentor voltage of 125 V. Mass spectra were acquired by scanning from 100 amu to 500 amu with a capillary voltage of 4000 V and a drying gas flow rate of 12 L/min at 350 °C.

One mL of the soil extracts was taken for analysis and 5.2 µL of the internal standard was added and vortexed for 15 sec. A 10 µL or 50 µL (for greater sensitivity) injection was made and analyzed as described for the SSL soil. Replicate analyses were done for each extract. Select blank extracts, both negative controls and method controls were spiked with DNAN and NTO at high levels (100 ng/µL), medium levels (10 ng/µL), and low levels (1 ng/µL). For DNAN, recoveries were 97 ± 1.2% for the high level, 99 ± 1.3% for the medium level, and 129 ± 5.5% for the low level. For NTO, recoveries were 83 ± 1.2% for the high level, 104 ± 8.9% for the medium level, and 77 ± 10.5% for the low level.

Separate linear internal standard calibration curves were generated for DNAN and its transformation products 2-ANAN and 4-ANAN, and NTO using calibration standard concentrations of 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/µL. Each calibration standard also contained 25 ng/µL 1,3-DNB as an internal standard. Relative responses (λ $Response_{Analyte}$/λ $Response_{Internal\ Standard}$) were plotted against relative concentrations ($Conc_{Analyte}$/$Conc_{Internal\ Standard}$), where λ Response equals the area under the peak at the detection wavelength. The calibration curve correlations typically yielded an $R^2 = 0.9978$. Under the experimental conditions previously described and with 50 µL injections, the limit of detection (LOD) for DNAN and its transformation products was 0.03 ng/µL, while the LOD for NTO was 0.1 ng/µL.

2.5.7 Quality Control

For quality control, USEPA Method 8000C was used for guidance in determining acceptable calibration and quality control requirements. Briefly, each set of 20 samples of either DNAN or NTO contained a blank, laboratory control sample (LCS), a matrix spike (MS), and a matrix spike duplicate (MSD). The LCS consisted of hydromatrix (diatomaceous earth sorbent) spiked with the analytes of interest. The MS consisted of an actual sample spiked with the analytes of interest. Sets of samples with very limited volume did not have MS and MSD. In this case, LCS and LCS duplicates were used as substitutes. The LCS, MS, and MSD of a given set were all spiked at the same concentration. Spike recoveries were considered acceptable between 70–130%.

2.6 Soil Invertebrate Toxicity Studies

2.6.1 Earthworm Toxicity Test

A 56-day earthworm reproduction assay was used to assess the effects of each IM compound on the earthworm *Eisenia andrei*. The test is an adaptation of an International Organization for Standardization (ISO) bioassay, ISO 11268-2:1998 (ISO, 1998). Guidelines for this assay were originally developed for use with Organization for Economic Co-operation and Development (OECD, 1984) artificial soil. However, research in our laboratory has shown that this assay can also be successfully conducted using natural soils (Simini et al. 2003; Simini et al. 2013), and this adaptation was utilized 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., PEI, Canada) and BACCTO potting soil (Michigan Peat Co., Houston, TX, USA). The pH was adjusted to 6.2 ± 0.1 by adding calcium carbonate (pulverized lime). The culture was kept moist at 21 ± 2 °C, 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) that were prepared by hydrating, fermenting for at least 14 d, air-dried, and then ground to a coarse powder.

Earthworm cultures were synchronized so that all worms used in each test were approximately the same age. Adult worms, 0.3 to 0.6 g, with fully developed clitella were

utilized for testing. Earthworms were acclimated for 48 h in respective unamended test soils. Earthworms were selected for uniformity and depurated on moist filter paper overnight. The worms were then selected randomly for placement across treatments. Following weathering-and-aging in the soil of the respective DNAN and NTO 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. Then 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 of the glass jars. For both 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 the jar. Clear plastic film was stretched across the top of each glass container and secured with the screw-on rings, allowing exposure to light. Three small holes were then made in the plastic film with a push-pin to allow for air exchange. Earthworm treatments were incubated under a 16 h light, 8 h dark photoperiod, with a mean photosynthetically active radiation (PAR) light intensity of 12.8 ± 0.7 (SE) µM sec/m^2 (985 ± 52 lux), and mean temperature of 21.6 ± 0.1 °C. Toxicity data obtained in all soil invertebrate studies are reported in Appendix B.

After 21 d in the range-finding tests and 28 d in the definitive tests, worms were removed with blunt forceps from the containers. The number and mass of surviving earthworms in each container were determined and recorded. Cocoons were counted after 21 d in the range-finding tests, as described below, and the tests were terminated. In the definitive tests, two grams of prepared alfalfa food was again added to each container, and clear plastic film and screw rings were again placed on the containers, as described above.

After 28 additional 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. The soil was then spread and examined under a 2.25x 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 surface of the sieve. Cocoons were placed in water in a clear glass dish. Cocoons that floated were counted as hatched; those that sank were counted as unhatched. Cocoons were then examined under the magnifier to confirm whether they had hatched or not; the numbers of hatched, unhatched, and total cocoons per container were then recorded.

Treatment concentrations for each definitive test were selected based on the results of the range-finding tests. Concentrations in the definitive tests were selected based on 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 (USEPA, 2005). Definitive tests included negative controls (no chemicals added), carrier controls, and positive controls, with each of these controls replicated four times per test. Positive control (reference toxicant, boric acid) tests were conducted using SSL soil to assess changes in sensitivity, health, and performance of *E. andrei* maintained in CBC laboratory cultures. Test treatments were

prepared by adding appropriate solutions of boric acid in ASTM (American Society for Testing and Materials, ASTM 2004) type I water to SSL soil to obtain nominal concentrations of 0 (negative control), 20, 30, 50, 80, 100, and 200 mg/kg. Nonlinear regression analyses of toxicity data from independent studies were used to establish the respective EC50 values and corresponding 95% CL for juvenile production. These values were plotted on a Boric Acid Warning Chart, using modified procedures described by Environment Canada (EC, 2005), to monitor the condition of the potworms and precision within laboratory culture. The modification included using calculations based on arithmetic (untransformed) EC50 values for boric acid concentrations instead of logarithmic concentrations.

Validity criteria for the negative controls included the following performance parameters (ISO, 1998):

1) The mean mortality does not exceed 10% in range-finding and definitive tests;
2) The number of juveniles produced per five worms is ≥15; and
3) The coefficient of variation for the number of juveniles in the control is ≤50%.

2.6.2 Potworm Toxicity Test

The Enchytraeid Toxicity Test was used to assess the individual effects of IM compounds on the enchytraeid worm (potworm) *Enchytraeus crypticus*. The test is an adaptation of an International Organization for Standardization (ISO) bioassay ISO/16387 *Soil quality — Effects of pollutants on Enchytraeidae (Enchytraeus sp.) — Determination of effects on reproduction and survival* (ISO, 2004). This test was selected based on 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 ISO Guideline for this assay was originally developed for use with Organization for Economic Co-operation and Development (OECD, 1984) artificial soil (similar soil formulation was later adapted for USEPA Standard Artificial Soil, SAS; USEPA, 1996). However several studies demonstrated that this test could also be conducted using natural soils (Amorim et al., 2005a,b; 2009; Dodard et al., 2005; Kuperman *et al.,* 2003; 2004; 2005; 2006a,b). The ISO/16387 was initially developed using the enchytraeid worm species *E. albidus.* Results of our previous studies using *E. albidus* showed that this species requires soils containing high organic matter content with a soil pH 6 (± 0.5) for optimal test conditions. This species performed poorly in natural soils having physical and chemical characteristics that support a higher level of EM bioavailability (Amorim et al., 2009; 2005a; Kuperman et al., 1999; 2006a). The species of the genus Enchytraeidae*, E. crypticus*, listed in the ISO protocol as an acceptable alternative to *E. albidus*, was selected for toxicity testing.

Potworms were bred in 4.3-L clear plastic boxes (34 × 20 × 10 cm) filled with 2 kg (dry mass) SSL soil. The culture was kept in an environment-controlled incubator under a 16 h light, 8 h dark photoperiod cycle, with a mean photosynthetically active radiation (PAR) light intensity of 12.8 ± 0.7 (standard error, SE) μM/m/sec (985 ± 52 lux) and mean temperature of 21.6 ± 0.1 °C. The soil moisture level was adjusted to 100% of the WHC of SSL soil and was maintained by periodic (once per week) mass checks and water adjustments. The soil in the breeding culture was aerated by carefully mixing it once each week. The potworms were fed

approximately twice each week with ground oats spread onto the soil surface. If food from the previous feeding date remained on the soil surface, the amount of food added was adjusted. Every 6 weeks, the worms were transferred into a freshly prepared culture substrate. Cultures were synchronized so that all worms used in each test were approximately the same age. The potworm culture was considered healthy if worms were whitish, reproduced continuously, did not try to leave the soil, and exhibited a shiny outer surface with no soil particles clinging to them.

Glass jars (42 mm i.d.; 45 mm deep) were used as test containers. They were rinsed with carrier, tap water, and ASTM Type I water (ASTM 2004) before the tests. Twenty grams (dry mass basis) of test soil and 0.05 g of ground oats were added to each test container, then mixed and hydrated to 100% of the WHC of each soil. The mass of each container with soil was recorded.

Adult potworms with eggs in the clitellum region were used for testing. They were collected from culture and were placed in a Petri dish filled with a small amount of ASTM Type I water for examination using a stereomicroscope. Potworms with no eggs were discarded. Any invertebrates living in the cultures (such as mites) were also removed. Ten potworms selected for uniformity (approximately 1 cm in length) were placed on top of the soil in each test container. Plastic wrap was stretched over the top of each container and secured with a rubber band. Three pinholes were made in the plastic wrapping to facilitate air exchange. All containers were placed in an environment-controlled incubator under the same conditions as described above for the maintenance of the potworm culture. The containers were weighed once a week and the mass loss was replenished with the appropriate amount of ASTM Type I water. Ground oats (0.05 g) were added to each test container at that time.

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

Treatment concentrations for definitive tests with each IM compound were selected based on the results of the range-finding tests to bracket the 20% and 50% inhibition in the production of juveniles, compared with the production of juveniles in carrier control for each soil. All definitive tests included negative controls (no chemicals added) and carrier controls.

Toxicity tests with reference toxicant boric acid (positive control) were conducted using SSL soil to assess changes in sensitivity, health, and performance of *E. crypticus* maintained in CBC laboratory culture. Test treatments were prepared by adding appropriate

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

Four replicates of each IM compound treatment or controls were used in the definitive tests. Validity criteria for the negative controls in toxicity tests included the following performance parameters (ISO/16387, 2004):

1. The adult mortality does not exceed 20% after 14 d;
2. The average number of juveniles is greater than 25 per test container at the end of the test, assuming that 10 adult worms per test container were used;
3. The coefficient of variation for the mean number of juveniles is ≤ 50%

2.6.3 Folsomia Toxicity Test

The Folsomia toxicity test was used to assess the individual effects of DNAN and NTO on the reproduction of the collembolan *Folsomia candida*. The test is an adaptation of an International Organization for Standardization (ISO) bioassay ISO 11267 (ISO, 1999). The measurement endpoints for the test included the production of juveniles and the survival of *F. candida* as adults. Collembolans were exposed to a range of DNAN or NTO concentrations, which were mixed into the soil. The total number of juvenile *F. candida* produced (*i.e.*, an indicator of effective reproduction) and the number that survive as adult *F. candida* were determined by counting the live organisms after the 28-d test duration. The effective reproduction and the survival of adult *F. candida* exposed to DNAN or NTO were compared with that of the carrier (solvent) control treatment to quantify ecotoxicological parameters. These parameters included the no-observed-effect concentration (NOEC), the lowest-observed-effect concentration (LOEC), and the effective concentration that causes a *p* percentage reduction (EC*p*) in the production of juveniles compared with those in the carrier controls (e.g., EC20 or EC50; 20 or 50% effect concentration, respectively), and the number of *F. candida* surviving as adults on Day 28.

Toxicity tests with reference toxicant boric acid (positive control) were conducted using SSL soil to assess changes in sensitivity, health, and performance of *F. candida* maintained in CBC laboratory cultures. Test treatments were prepared by adding appropriate solutions of boric acid in ASTM Type I water to SSL soil to obtain nominal concentrations of 0 (negative control), 30, 50, 80, 100, and 200 mg/kg. Nonlinear regression analyses of toxicity data from independent studies were used to establish the respective EC50 values and corresponding 95% CL for juvenile production. These values were plotted on a Boric Acid Warning Chart, using modified procedures described by Environment Canada (EC, 2005), to monitor the condition of the potworms and precision within laboratory culture. The modification included using

calculations based on arithmetic (untransformed) EC50 values for boric acid concentrations instead of logarithmic concentrations.

Five replicates of each IM compound treatment or controls were used in the definitive tests. Validity criteria for the negative controls in toxicity tests included the following performance parameters (ISO/11267, 1999):

1. The adult *F. candida* mortality should not exceed 30% at the end of the test.
2. The average number of juvenile *F. candida* per chamber should reach 80 instars (nymphs) at the end of the 28-d test.
3. The coefficient of variation for reproduction should not exceed 30% at the end of the test.

Glass jars (42 mm i.d.; 45 mm deep) were used as test containers. They were rinsed with ASTM Type I water before the tests. To prepare each treatment in the range-finding tests, 100 g of each air-dried treatment soil was hydrated to 88% of the WHC of SSL soil. Then one-fifth of the weight of each batch of hydrated treatment soil was transferred into a test container, and 0.05 g of baker's yeast was added to the surface of the soil. In the definitive tests with weathered-and-aged treatments, twenty grams of test soil hydrated to 60% of the WHC and 0.05 g of baker's yeast were added to each test container, then mixed and hydrated to 88% of the WHC of SSL soil by adding 1 g of ASTM Type I water. The mass of each container with soil was recorded to monitor soil moisture loss during the test. Ten 10–12 d old *F. candida* juveniles were placed in each test container. A piece of plastic food wrap was placed on each container and held in place with a rubber band. Five replicates were used for each treatment concentration and for the control treatments.

All containers were placed in an environment-controlled incubator under a 16 h light, 8 h dark photoperiod cycle, with a mean photosynthetically active radiation (PAR) light intensity of 12.8 ± 0.7 (SE) $\mu mol/m^2/sec^{-1}$ (985 ± 52 lux) and mean temperature of 21.6 ± 0.1 °C. The containers were weighed once a week and the mass loss was replenished with the appropriate amount of ASTM Type I water. Baker's yeast (0.05 g) was added to each test container at that time.

To terminate a test, water was added to a test container then gently mixed with a spatula and examined under a dissecting microscope (at 15× magnification) for the presence of *F. candida* juveniles and adults. The juvenile and adult *F. candida* that floated to the surface were counted. Toxicity data obtained in all soil invertebrate studies are reported in Appendix B.

2.7 Data Analyses

Ecotoxicological data were analyzed using regression models selected among those described in the Environment Canada Guidance Document (EC, 2005) to estimate the effective concentration for a specified percent effect (ECp) and corresponding 95% confidence intervals (CI). During the model selection process, compliance with the normality assumptions and homoscedasticity of the residuals were determined by examining the stem-and-leaf graphs

and histograms of the residuals. The best fit was evident when the regression lines generated by the models were closest to the data points, the regression coefficients for point estimates were the greatest, the residuals were homoscedastic (*i.e.,* had the most random scattering), and the means, standard errors, and variances of the residuals were the smallest. The ECp parameters included the IM concentration producing a 20% (EC20) and 50% (EC50) decrease in the measurement endpoint compared with carrier control. Measurement endpoints included adult survival and reproduction in studies with soil invertebrates. The EC20 parameter based on reproduction (soil invertebrates) endpoint is the preferred parameter for deriving Eco-SSL values (USEPA, 2005). The models selected for data analyses in these studies were logistic (Gompertz; eq 1), logistic hormetic (eq 2), and linear (eg 3):

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

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

$$Y = [(-a \times p) \div \mathrm{ECp}] \times \mathrm{C} + a \quad (3)$$

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

Data that exhibited hormesis, a concentration-response phenomenon characterized by a low-dose stimulation followed by a high-dose inhibition (Calabrese, 2008), were fitted to the hormetic model. The ECp parameters used in these studies included the EM concentration producing a 50% (EC50) decrease in the measurement endpoint compared with the negative control. The 95% confidence intervals (CIs) associated with the point estimates were determined.

Analysis of Variance (ANOVA) was used to determine the bounded (when possible) no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) values for survival and reproduction data using SYSTAT 13 software. Mean separations in the invertebrate tests were determined using Fisher's Least Significant Difference (FLSD) pairwise comparison test. A significance level of $p \leq 0.05$ (95% confidence level) was accepted for all statistical tests. All toxicological benchmarks were developed on the measured chemical concentration basis.

3. RESULTS

3.1 DNAN Concentrations in SSL Soil Used in the Soil Invertebrate Toxicity Studies

Nominal DNAN concentrations selected for the definitive tests in SSL soil across all test organisms were 0, 30, 40, 60, 80, 120, 160, 320, and 640 mg/kg (Table 2). DNAN

concentrations were analytically determined in all treatment and control soils at the beginning and end of the weathering and aging period. Initial analytically determined soil concentrations of DNAN before weathering-and-aging in SSL were 31, 44, 63, 82, 125, 158, 334, and 648 mg/kg, respectively. DNAN concentrations were fairly stable during the 10-d weathering-and-aging in SSL soil. The percentage of DNAN remaining in the soil at the end of the weathering-and-aging period was 81, 78, 88, 89, 87, 92, 88, and 100, respectively, compared with initial concentrations. Statistical analysis showed a significant (t-Test $p < 0.02$) decrease in DNAN concentration in 30 mg/kg, 80 mg/kg, and 120 mg/kg nominal treatments. DNAN concentrations were not statistically different in the remaining treatments on day 10 compared with initial concentrations on day 0. DNAN was not detected above the LOQ in the control soils.

DNAN concentrations in SSL soil were also determined in low, medium, and high treatment concentrations on day 3 and day 7 of the weathering-and-aging period to determine trends in transformation rates (Table 2, Figure 1). For 10 d of weathering-and-aging DNAN in SSL, all but the 640 mg/kg treatment group showed a loss in extractable DNAN detected in the soil extracts. The average amount of DNAN loss was 14% (SE = 1.9%), while the 640 mg/kg treatment group did not show any losses (based on the SE). The transformation products of DNAN (2-ANAN and 4-ANAN) at T=0 and T=10 d were below detection limits for HPLC and GC-MS-MS.

Table 2. DNAN Concentrations during Weathering-and-Aging in Amended SSL Soil[†]

Nominal Concentration (mg/kg)	DNAN (SE) T=0 d (mg/kg)	DNAN (SE) T=3 d (mg/kg)	DNAN (SE) T=7 d (mg/kg)	DNAN (SE) T=10 d (mg/kg)
30	31 (0.39)	28 (0.79)	31 (0.43)	25 (1.07)*
40	44 (1.76)	ND[††]	ND	34 (1.48)
60	63 (1.65)	ND	ND	55 (1.69)
80	82 (1.91)	ND	ND	73 (1.92)*
120	125 (0.95)	116 (4.01)	102 (9.42)	108 (1.81)*
160	158 (6.95)	ND	ND	145 (5.21)
320	334 (7.10)	ND	ND	295 (9.87)
640	648 (39.49)	621 (29.1)	609 (16.61)	649 (6.96)

[†]2-ANAN and 4-ANAN were below detection limits for HPLC and GC-MS-MS methods. [††]ND, Not Determined. Treatments were not used for analytical determinations. *Significant decrease (t-Test $p < 0.02$)

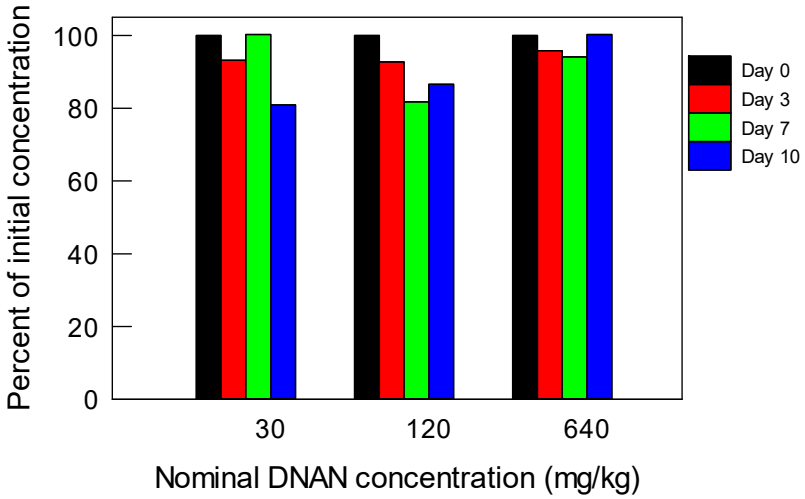

Figure 1. DNAN Recovery from three treatments monitored over 10 d period during weathering-and-aging in SSL soil.

The GC confirmation analysis of DNAN and 2-ANAN were comparable to the HPLC analysis. However, the 4-ANAN was not detected in any of the extract samples when analyzed on GC-MS-MS.

3.2 NTO Concentrations in SSL Soil Used in the Soil Invertebrate Toxicity Studies

Concentrations of NTO in the SSL soil treatments used in the soil invertebrate studies are shown in Table 3. Results of chemical analyses showed a rapid decrease (mean of 68% from initial on Day 0) in NTO concentrations during the first 24 h. The decrease was likely due to the formation of non-identified transformation products. NTO concentrations were relatively stable for the rest of the 8-d weathering-and-aging process in SSL soil (Figure 2) suggesting that chemical exposure conditions did not change appreciably during the toxicity tests.

Table 3. NTO Concentrations during Weathering-and-Aging in Amended SSL Soil

Nominal Concentration (mg/kg)	NTO T=0 d mg/kg	NTO T=1 d mg/kg	NTO T=4 d mg/kg	NTO T=8 d mg/kg
50	42.72 (1.08)	34.07 (0.77)	36.40 (1.23)	31.29 (0.57)
178	176.83 (4.94)	48.43 (7.19)	47.32 (7.98)	39.58 (1.22)
300	293.95 (3.83)	107.02 (11.15)	119.86 (7.36)	91.64 (5.40)
400	387.78 (20.40)	162.57 (6.61)	146.15 (23.98)	124.99 (9.23)
600	567.71 (10.69)	232.65 (20.25)	187.03 (8.70)	218.85 (17.90)

800	819.84 (6.75)	293.93 (35.13)	252.33 (5.11)	272.50 (64.11)
1600	1634.07 (45.89)	727.95 (72.29)	467.64 (34.49)	547.35 (37.01)
2400	2359.57 (167.05)	1199.04 (55.82)	1029.57 (100.80)	785.09 (139.23)

Numbers in parenthesis are standard errors (n = 3)

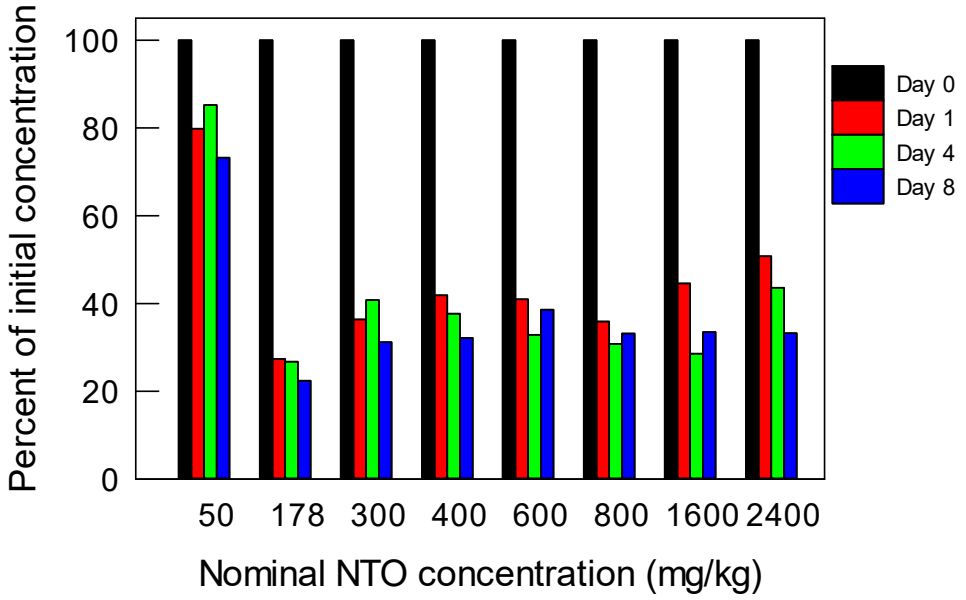

Figure 2. NTO recovered during weathering-and-aging in SSL soil used in the soil invertebrate studies.

3.3 DNAN Concentrations in WCL Soil Used in the Soil Invertebrate Toxicity Studies

Nominal DNAN concentrations selected for the definitive tests in WCL soil for all test invertebrate species were 0, 32, 42, 63, 84, 126, 168, 337, and 674 mg/kg (Table 4). DNAN concentrations were analytically determined in all treatment and control soils at the beginning and end of the weathering and aging period. Initial analytically determined soil concentrations of DNAN before weathering-and-aging in SSL were 0, 34, 42, 59, 92, 142, 174, 403, and 774 mg/kg, respectively. The percentage of DNAN remaining in the soil at the end of the weathering-and-aging period was 70, 88, 73, 76, 73, 86, 78, and 90, respectively, compared with initial concentrations (Figure 3). The average amount of DNAN loss was 21% (SE = 1.9%). DNAN was not detected above the detection limits in the control soils. The transformation products of DNAN (2-ANAN and 4-ANAN) were below detection limits for GC-MS-MS at T=0. 4-ANAN was not detected at T=10 d.

Table 4. Concentrations of DNAN and Transformation Products Extracted from WCL Soil Before (T=0) and After (T=10) the Weathering-and-Aging Procedure[†]

Nominal Concentration (mg/kg)	DNAN T = 0 d mg/kg (SE)	DNAN T = 10 d mg/kg (SE)	2-ANAN T = 10 d mg/kg (SE)	4-ANAN T = 10 d mg/kg (SE)
32	34 (1.49)	24 (1.67)	BLQ[*]	BLQ
42	42 (1.85)	37 (0.77)	0.44 (0.07)	BLQ
63	59 (1.40)	43 (2.78)	1.03 (0.10)	BLQ
84	92 (1.81)	70 (0.39)	1.76 (0.14)	BLQ
126	142 (6.94)	103 (2.34)	3.38 (0.10)	BLQ
168	174 (3.44)	150 (5.98)	4.59 (0.09)	BLQ
337	403 (19.28)	313 (9.44)	7.99 (0.12)	BLQ
674	774 (18.90)	693 (42.61)	13.85 (1.11)	BLQ

[*]BLQ, Below Limit of Quantitation; [†]2-ANAN and 4-ANAN were BLQ at T = 0 d

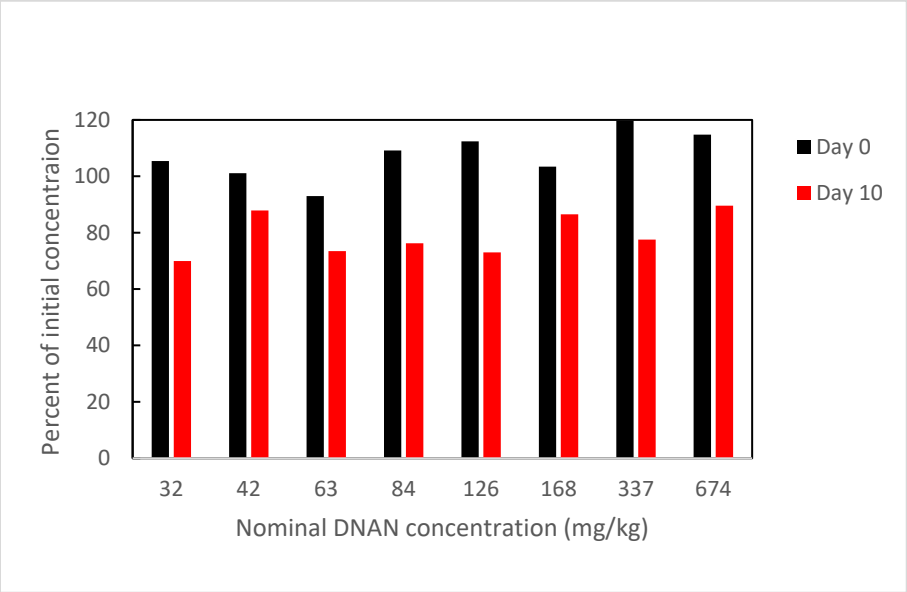

Figure 3. Recovered DNAN concentrations following weathering-and-aging in WCL soil.

The potential contribution of the earthworms to the fate of DNAN was examined in WCL soil. The concentrations of DNAN and its transformation products after the 56-d study with earthworms present in the test containers are shown in Table 5. The concentrations of DNAN and its transformation products in the test containers without earthworms are shown in

Table 6. The mean concentration of DNAN remaining in the soil after 56 d was on average 44% greater in the test containers without earthworms, while the formation of 2-ANAN decreased on average by 148% in the absence of the earthworms. Small amounts of 4-ANAN were detected in the two greatest treatment concentrations in the presence of the earthworms (Table 5) and none were detected in any treatments without the earthworms (Table 6). These findings strongly suggest that the presence of the earthworms directly or indirectly (through activation of microbial activity in soil) stimulated the transformation of DNAN to its metabolites.

Table 5. Concentrations of DNAN and the Transformation Products 2-ANAN and 4-ANAN Extracted from WCL Soil at the Conclusion of the 56-D Study with Worms.

Nominal Concentration (mg/kg)	DNAN mg/kg (SE)	2-ANAN mg/kg (SE)	4-ANAN mg/kg (SE)
63	19 (1.30)	3 (1.06)	BLQ*
84	33 (2.02)	7 (1.40)	BLQ
126	46 (2.00)	14 (1.44)	BLQ
168	60 (4.03)	17 (1.13)	BLQ
337	142 (5.37)	28 (1.28)	2.7 (1.40)
674	362 (16.66)	51 (1.77)	6.2 (1.91)

* BLQ, Below Limit of Quantitation

Table 6. Concentrations of DNAN and the Transformation Products 2-ANAN and 4-ANAN Extracted from WCL Soil at the Conclusion of the 56-D Study without Worms.

Nominal Concentration (mg/kg)	DNAN mg/kg (SE)	2-ANAN mg/kg (SE)	4-ANAN mg/kg (SE)
63	39 (0.86)	1.7 (0.13)	BLQ *
84	58 (1.54)	2.7 (0.10)	BLQ
126	90 (2.25)	4.5 (0.13)	BLQ
168	125 (5.65)	6.8 (0.34)	BLQ
337	239 (4.50)	10.5 (0.29)	BLQ
674	493 (15.94)	22.5 (0.82)	BLQ

* BLQ, Below Limit of Quantitation

3.4 NTO Concentrations in WCL Soil Used in the Soil Invertebrate Toxicity Studies

Results of chemical analyses showed a rapid decrease (mean of 76% from initial on Day 0) in NTO concentrations during the 10-d weathering-and-aging in WCL soil (Table 7, Figure 4).

Table 7. NTO Concentrations During Weathering-and-Aging in Amended WCL Soil

Nominal Concentration (mg/kg)	NTO (SE) T=0 d (mg/kg)	NTO (SE) T=10 d (mg/kg)
100	101.48 (6.05)	21.08 (0.38)
200	206.66 (8.52)	45.78 (0.49)
300	289.73 (9.60)	71.94 (1.53)
400	417.06 (16.66)	93.59 (0.70)
600	645.60 (18.41)	151.96 (2.93)
800	799.55 (30.16)	201.04 (6.64)
1200	1161.79 (70.96)	296.11 (8.27)
1600	1728.66 (75.79)	459.34 (7.28)

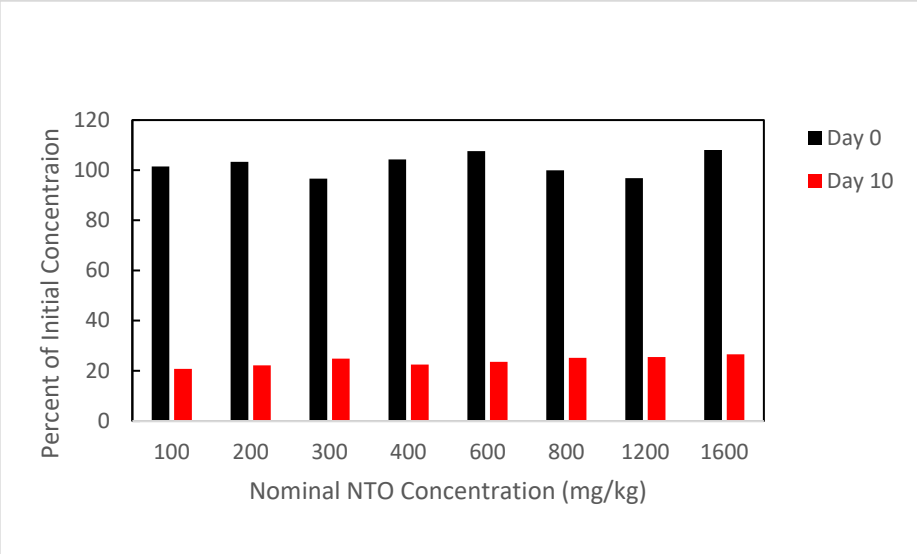

Figure 4. Recovered NTO concentrations following weathering-and-aging in WCL soil

The potential contribution of the earthworms to the fate of NTO was examined in WCL soil. The concentrations of NTO at the conclusion of the 56-d study with earthworms present in the test containers are shown in Table 8. The concentrations of NTO in the test containers without earthworms are shown in Table 9. The mean concentration of NTO remaining in the soil after 56 d was, on average, 11% greater in the test containers without earthworms, suggesting that presence of the earthworms contributed to the disappearance of NTO, although to a lesser extent compared with the contribution of earthworms to the disappearance of DNAN.

Table 8. Concentrations of NTO Extracted from WCL Soil at the Conclusion of the 56-D Study with Worms

Nominal Concentration (mg/kg)	NTO (SE) (mg/kg)
400	36 (0.54)
600	60 (0.44)
800	93 (7.32)
1200	146 (1.13)
1600	221 (10.62)

Table 9. Concentrations of NTO Extracted from WCL Soil at the Conclusion of the 56-D Study without Worms

Nominal Concentration (mg/kg)	NTO (SE) (mg/kg)
400	43 (2.4)
600	72 (4.1)
800	122 (9.9)
1200	159 (4.1)
1600	201 (11.5)

3.5 Earthworm Toxicity Studies

3.5.1 Positive Control

Toxicity tests with reference toxicant boric acid (positive control) were conducted throughout the project using SSL soil to assess changes in sensitivity, health, and performance of *E. andrei* maintained in ECBC laboratory cultures. Test treatments were prepared by adding appropriate solutions of boric acid in ASTM type I water to SSL soil. Regression analyses of toxicity data from independent studies were used to establish the respective EC50 values and corresponding 95% Confidence Limits (CL) for adult survival and production of juveniles. These values were plotted on a Boric Acid Warning Chart using modified procedures described in a previous report (EC, 2005). The modification included using calculations based on arithmetic (untransformed) EC50 values for boric acid concentrations instead of logarithmic concentrations.

Toxicity tests with boric acid (reference toxicant) were conducted in SSL soil to obtain EC50 values and the corresponding 95% confidence limits (CL). Nonlinear regression analyses of reproduction toxicity data established for six testing dates produced the following EC50 values and their corresponding CLs (in parentheses) for cocoon production: 181 (140–222), 175 (66–283), 160 (101–219), 189 (32–346), 170 (124–215), 191 and (131–250) mg H_3BO_3/kg soil. These EC50 values were plotted on a Boric Acid Warning Chart to monitor the condition of the collembolan culture. All resulting EC50 values were within both the Warning Limits and the 95% CL that were established for the *E. andrei* culture in tests with boric acid (Figure 5). These charted results confirmed that the condition of the *E. andrei* culture met the validity requirements of the test protocol.

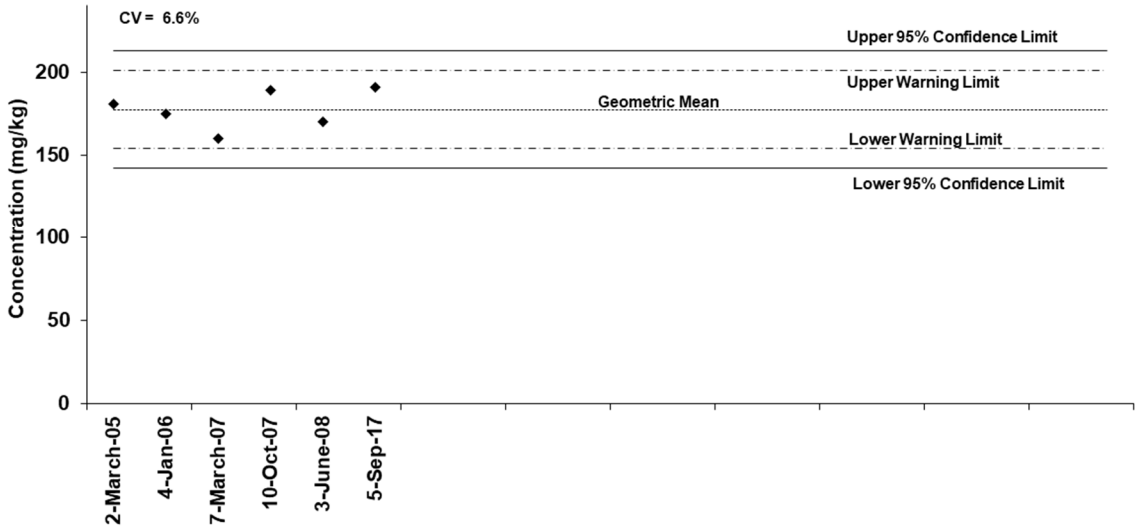

Figure 5. Warning chart for the *Eisenia andrei* culture showing the EC50 values for cocoons

3.5.2 Ecotoxicological Effects of DNAN on the Earthworm *Eisenia andrei* in SSL Soil

The bounded NOEC and LOEC values for DNAN effects on adult survival were respectively 158 and 334 mg/kg in freshly amended soil, and 55 and 145 mg/kg for DNAN weathered-and-aged in SSL soil (Table 10). The linear model had the best fit for the adult survival (acute toxicity) data (Figure 6). The EC20 and EC50 values were 111 and 278 mg/kg, respectively, for DNAN freshly amended in SSL, and 112 and 281 mg/kg, respectively, for DNAN weathered-and-aged in SSL soil (Table 10). Evaluation of these data showed that weathering-and-aging of DNAN in SSL soil did not significantly (95% CI) affect acute toxicity for *E. andrei.*

Chronic toxicity benchmarks for DNAN freshly amended (FA) and weathered-and-aged (W-A) in SSL soil are summarized in Table 11. The bounded NOEC and LOEC values for DNAN effects on the production of cocoons were 63 and 82 mg/kg, in FA and W-A SSL soil, respectively. The production of juveniles was significantly (p = 0.011) lower in the lowest positive DNAN concentration compared with carrier control, producing an unbounded LOEC of 44 mg/kg.

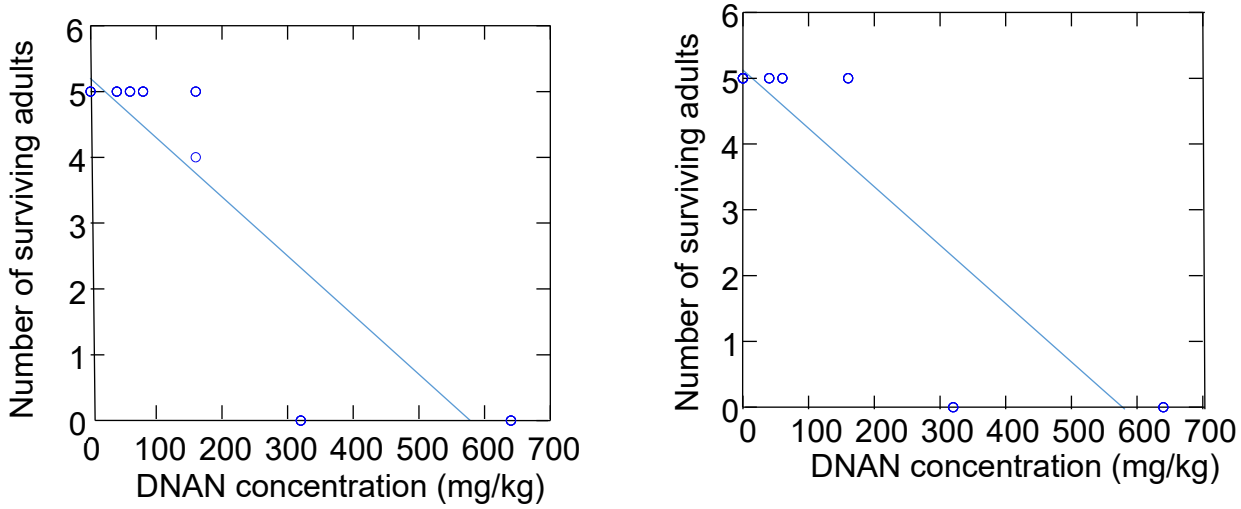

Figure 6. Effect of DNAN freshly amended (left) or weathered-and-aged (right) in Sassafras sandy loam on number of surviving *Eisenia andrei* adults

The logistic Gompertz model had the best fit for the cocoon production data (Figure 7). The EC20 and EC50 values were 99 and 128 mg/kg, respectively, for cocoon production in freshly amended soil and 55 and 91 mg/kg, respectively, in weathered-and-aged soil. The EC20 and EC50 values for production of juveniles were 102 and 256 mg/kg in freshly amended soil, and 42 and 67 mg/kg in weathered-and-aged treatment. The linear model had the best fit for the juvenile production data in the freshly amended soil whereas the Gompertz model was the best fit for the

juvenile production data from weathered-and-aged treatment. Weathering-and-aging increased the toxicity to both cocoon and juvenile production.

Table 10. Acute Ecotoxicological Benchmarks for DNAN Freshly Amended (FA) or Weathered-and-Aged (W-A) in SSL Soil Determined for the Survival of Adult *Eisenia andrei*

Ecotoxicological Parameter	DNAN FA (mg/kg)	DNAN W-A (mg/kg)
NOEC	158	55
p	0.075	0.205
LOEC	334	145
p	0.001	0.002
EC20	111	112
CI (95%)	94–129	91-134
EC50	278	281
CI (95%)	235–322	227-335
Model used	Linear	Linear
R^2	0.941	0.918

Table 11. Chronic Ecotoxicological Benchmarks for DNAN Freshly Amended (FA) or Weathered-and-Aged (W-A) in SSL Soil Determined for the Reproduction of *Eisenia andrei*

Ecotoxicological Parameter	DNAN FA (mg/kg)	DNAN W-A (mg/kg)
NOEC Cocoons	63	34
p	0.144	1.00
LOEC Cocoons	82	54
p	0.026	0.001
EC20 Cocoons	99	55
CI (95%)	63–135	43–68
EC50 Cocoons	128	91†
CI (95%)	104–251	79–103
Model used	Gompertz	Gompertz
R^2	0.963	0.882
NOEC Juveniles	<44	34
p	0.011	0.205
LOEC Juveniles	44	5
p	0.011	0.002
EC20 Juveniles	102	42
CI (95%)	38–167	26–58
EC50 Juveniles	256	67†
CI (95%)	94–417	47–87
Model used	Linear	Gompertz
R^2	0.463	0.964

†Significant increase in toxicity following weathering-and-aging of DNAN in SSL

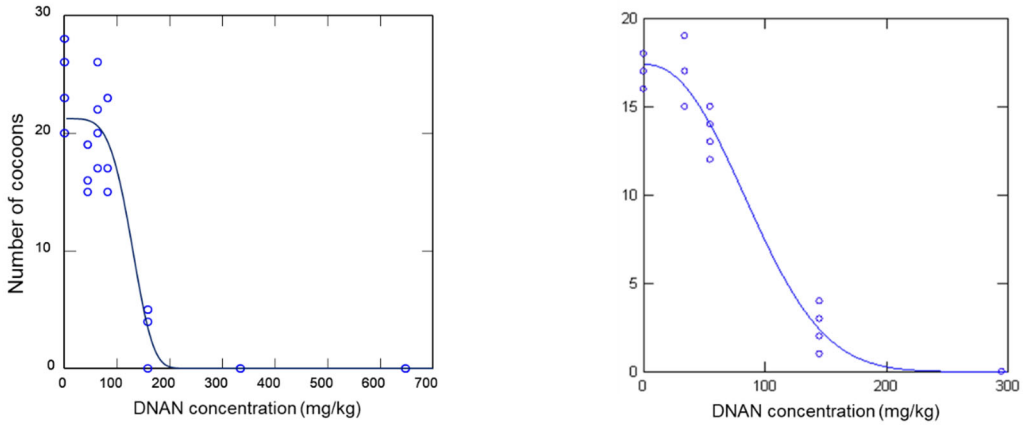

Figure 7. Effect of DNAN freshly amended (left) or weathered-and-aged (right) in Sassafras sandy loam soil on the production of cocoons by *Eisenia andrei*.

3.5.3 Ecotoxicological Effects of NTO on the earthworm *Eisenia andrei* in SSL soil

There were no significant differences ($p = 0.093$) in adult survival of *E. andrei* between solvent controls and any of the treatment concentrations, up to and including 2359 mg/kg, for NTO freshly amended or 785 mg/kg for NTO weathered-and-aged in soil. Data for the production of cocoons was sufficient to determine ecotoxicological benchmarks (Table 12). Production of cocoons by *E. andrei* for NTO freshly amended in SSL soil was significantly less ($p \leq 0.05$) compared with carrier control, resulting in bounded NOEC of 388 mg/kg and LOEC of 568 mg/kg. The bounded NOEC and LOEC values for NTO weathered-and-aged in SSL were 125 and 219 mg/kg, respectively. The logistic Gompertz model had the best fit ($R^2 = 0.882$ and 0.947, respectively) for cocoon production data determined in both exposure types (Figure 8). The EC20 and EC50 values for the production of cocoons by *E. andrei* were 429 and 596 mg/kg, respectively for NTO freshly amended in SSL, and 145 and 242 mg/kg, respectively for NTO weathered-and-aged in SSL soil, based on the measured NTO concentrations. Weathering-and-aging of NTO in SSL soil significantly increased cocoon production toxicity for *E. andrei* based on the EC20 or EC50 values and their respective 95% CIs (Table 12).

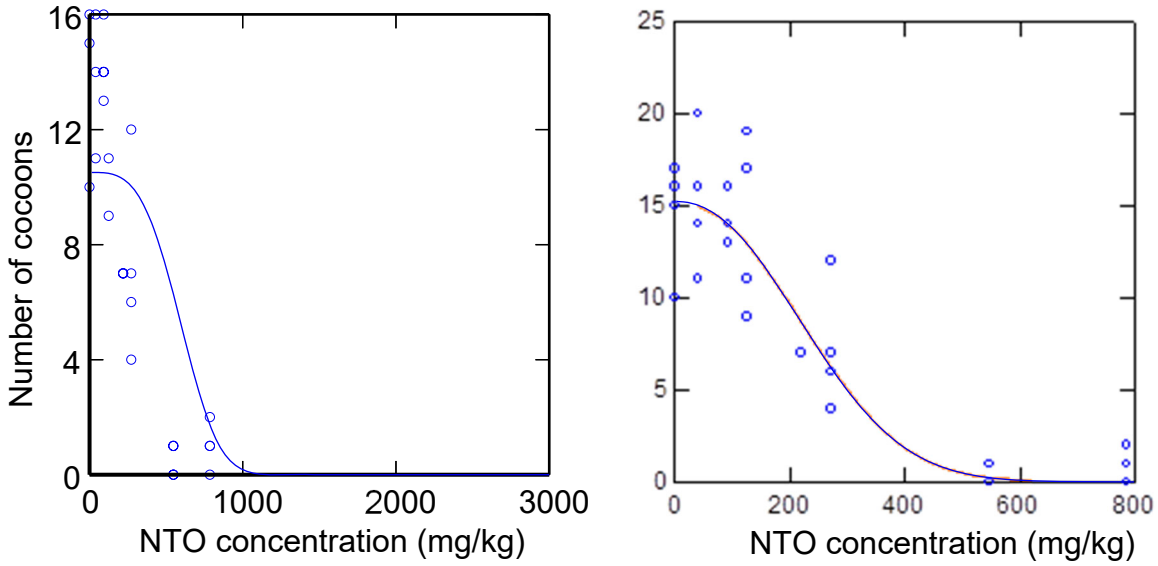

Figure 8. Effect of NTO freshly amended (left) or weathered-and-aged (right) in Sassafras sandy loam soil on the production of cocoons by *Eisenia andrei*.

Table 12. Chronic Ecotoxicological Benchmarks for NTO Freshly Amended (FA) or Weathered-and-Aged (W-A) in SSL Soil Determined for the Reproduction of *Eisenia andrei*

Ecotoxicological Parameter	NTO FA (mg/kg)	NTO W-A (mg/kg)
NOEC	388	125
p	0.458	0.087
LOEC	568	219
p	0.001	0.001
EC20 Cocoons	429	145†
CI (95%)	250–607	77–213
EC50 Cocoons	596	242†
CI (95%)	465–726	196–289
Model used	Gompertz	Gompertz
R^2	0.882	0.947

†Statistically significant (95% CI basis) increase in toxicity following weathering-and-aging of NTO in soil.

3.5.4 Ecotoxicological Effects of DNAN on the Earthworm *Eisenia andrei* in WCL Soil

There were no significant differences ($p > 0.05$) in adult survival of *E. andrei* between solvent controls and any of the DNAN treatment concentrations, up to and including 693 mg/kg in WCL soil. Chronic toxicity benchmarks for DNAN freshly amended and

weathered-and-aged in WCL soil are summarized in Table 13. The bounded NOEC and LOEC values for DNAN effects on the production of cocoons and juveniles were 150 and 313 mg/kg, respectively. The logistic Gompertz model had the best fit for the cocoon and juvenile production data (Figure 9). The EC20 and EC50 values were 188 and 282 mg/kg, respectively, for cocoon production in weathered-and-aged treatment. The EC20 and EC50 values for the production of juveniles were 125 and 196 mg/kg in weathered-and-aged treatment fitted to the Gompertz model (Table 13).

Table 13. Chronic Ecotoxicological Benchmarks for DNAN Weathered-and-Aged in WCL Soil Determined for Reproduction of *Eisenia Andrei*

Ecotoxicological Parameter	DNAN (mg/kg)
NOEC Cocoons	150
p	0.207
LOEC Cocoons	313
p	<0.0001
EC20 Cocoons	188
CI (95%)	111-266
EC50 Cocoons	282
CI (95%)	236-329
Model used	Gompertz
R^2	0.973
NOEC Juveniles	150
p	0.073
LOEC Juveniles	313
p	<0.0001
EC20 Juveniles	125
CI (95%)	71-180
EC50 Juveniles	196
CI (95%)	142-249
Model used	Gompertz
R^2	0.973

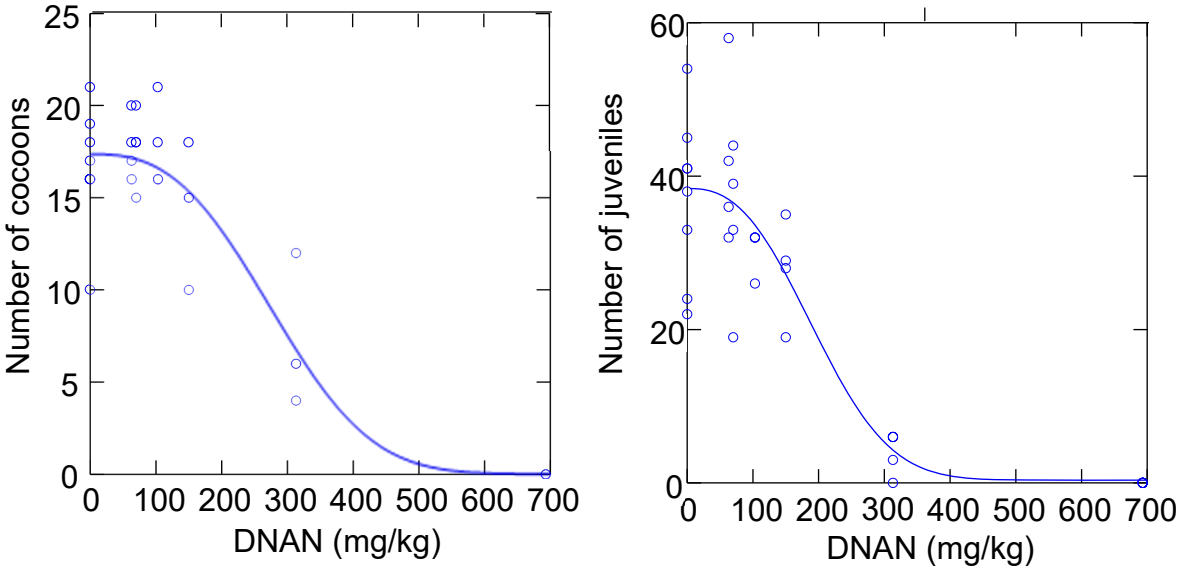

Figure 9. Effect of DNAN weathered-and-aged in Webster clay loam soil on the production of cocoons and juveniles by *Eisenia andrei*.

3.5.5 Ecotoxicological Effects of NTO on the Earthworm *Eisenia andrei* in WCL Soil

There were no significant differences ($p = 0.093$) in adult survival of *E. andrei* between solvent controls and any of the treatment concentrations, up to and including 459 mg/kg, for NTO weathered-and-aged in WCL. Production of cocoons by *E. andrei* was significantly ($p < 0.05$) affected by NTO, resulting in bounded NOEC of 296 mg/kg and LOEC of 459 mg/kg (Table 14). The bounded NOEC and LOEC values for juvenile production exposed to NTO weathered-and-aged in WCL soil were 94 and 152 mg/kg, respectively.

The linear model was used for cocoon production data and the logistic Gompertz model was used for juvenile production data ($R^2 = 0.570$ and 0.600, respectively) for cocoon and juvenile production data (data not shown). The respective EC20 and EC50 values for the production of cocoons and juveniles by *E. andrei* were 97 and 244 mg/kg, and 95 and 223 mg/kg, respectively, for NTO weathered-and-aged in WCL soil, based on analytically determined NTO concentrations.

Table 14. Chronic Ecotoxicological Benchmarks for NTO Weathered-and-Aged in WCL Soil Determined for the Reproduction of *Eisenia andrei*

Ecotoxicological Parameter	NTO (mg/kg)
NOEC Cocoons	94
p	0.051
LOEC Cocoons	152
p	0.010
EC20 Cocoons	97
CI (95%)	29-167
EC50 Cocoons	244
CI (95%)	72-417
Model used	Linear
R^2	0.570
NOEC Juveniles	94
p	0.137
LOEC Juveniles	152
p	0.008
EC20 Juveniles	95
CI (95%)	7-184
EC50 Juveniles	223
CI (95%)	116-331
Model used	Gompertz
R^2	0.600

3.6 Enchytraeid (Potworm) Toxicity Studies

3.6.1 Positive Control

Toxicity tests with boric acid (reference toxicant) were conducted in SSL soil to obtain EC50 values and the corresponding 95% confidence limits (CL). Nonlinear regression analyses of reproduction toxicity data established for seven testing dates produced the following EC50 values and their corresponding CLs (in parentheses) for juvenile production: 56 (48–63), 60 (47–77), 46 (36–56), 55 (44–65), 52 (39–66), 55 (46–65) and 55 (46–64) mg of H_3BO_3/kg of soil. These EC50 values were plotted on a Boric Acid Warning Chart to monitor the condition of the potworm culture. All resulting EC50 values were within both the Warning Limits and the 95% CL that were established for the *E. crypticus* culture in tests with boric acid (Figure 10). These charted results confirmed that the condition of the *E. crypticus* culture met the validity requirements of the test protocol.

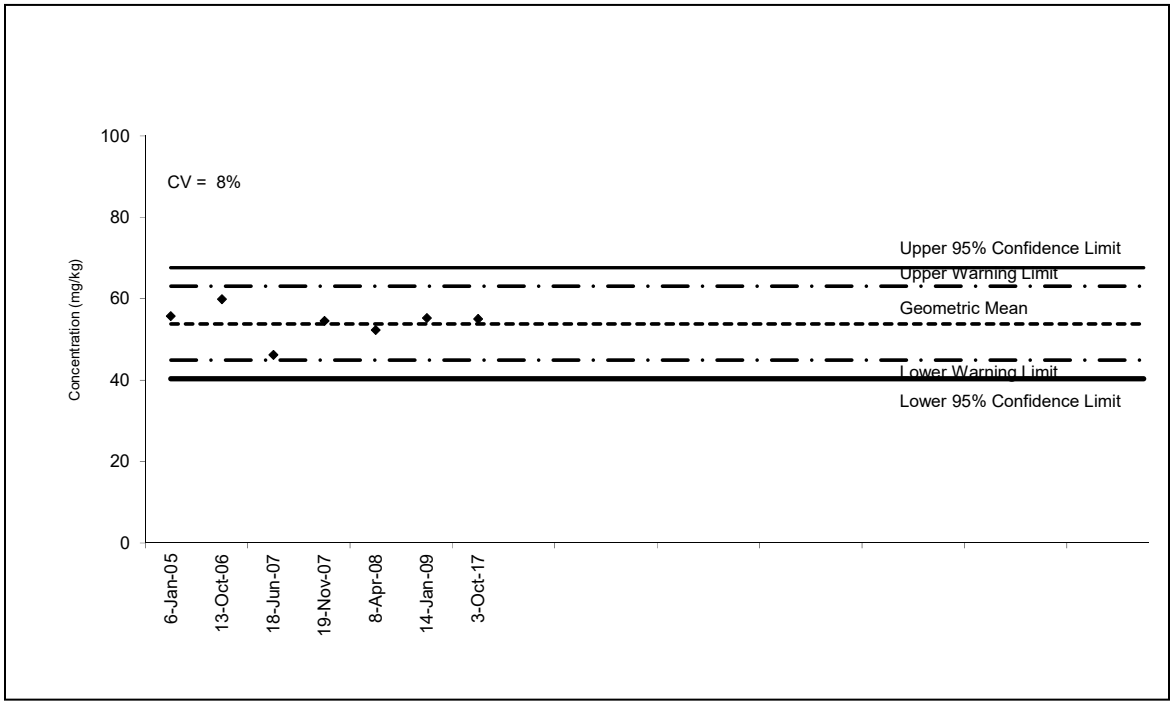

Figure 10. Warning chart for the *E. crypticus* culture showing the EC50 values for juvenile production established in definitive tests with the reference toxicant (boric acid) in SSL soil.

3.6.2 Ecotoxicological Effects of DNAN on the Potworm *Enchytraeus crypticus* in SSL Soil

The definitive toxicity test with soil invertebrate *E. crypticus* exposed to DNAN in freshly amended SSL soil was conducted to assess the acute (adult mortality) and chronic (juvenile production) effects of DNAN on *E. crypticus* in SSL soil and to determine test concentrations for the definitive study using DNAN weathered-and-aged in SSL 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 surviving adults after 14 d and the number of juveniles produced after 28 d. Exposure concentrations for each soil were selected for definitive tests to achieve bracketing of significant effects on reproduction endpoints (*i.e.*, production of juveniles).

Results of toxicity tests with DNAN freshly amended or weathered-and-aged in SSL soil complied with the validity criteria defined in the ISO 16387 test guideline. The validity criteria (mean adult survival, the mean number of juveniles produced, and CV) for test results from negative control treatment were: 98%, 354, and 21%, respectively in freshly amended treatment, and 93%, 776, and 17%, respectively, in weathered-and-aged treatment. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the DNAN treatments.

The bounded NOEC and LOEC values for DNAN effects on adult survival were respectively 63 and 82 mg/kg in freshly amended soil, and 73 and 108 mg/kg for DNAN weathered-and-aged in SSL soil. The logistic Gompertz model had the best fit for the adult survival data (acute toxicity). The EC20 and EC50 values were 82 and 225 mg/kg, respectively, for DNAN freshly amended in SSL, and 128 and 180 mg/kg, respectively, for DNAN weathered-and-aged in SSL soil (Table 15). Evaluation of these data showed that weathering-and-aging of DNAN in SSL soil did not significantly (95% CI basis) affect acute toxicity for *E. crypticus.*

Table 15. Acute Ecotoxicological Benchmarks for DNAN Freshly Amended (FA) or Weathered-and-Aged (W-A) in SSL Soil Determined for Survival of Adult *Enchytraeus crypticus*

Ecotoxicological Parameter	DNAN FA (mg/kg)	DNAN W-A (mg/kg)
NOEC	63	73
p	0.145	0.205
LOEC	82	108
p	0.004	<0.0001
EC20	83	128
CI (95%)	34–131	108–147
EC50	225[†]	180
CI (95%)	57–392	156–203
Model used	Gompertz	Gompertz
R^2	0.988	0.989

[†]Estimated outside the tested range

Table 16. Chronic Ecotoxicological Benchmarks for DNAN Freshly Amended (FA) or Weathered-and-Aged (W-A) in SSL Soil Determined for Production of Juveniles by *Enchytraeus crypticus*

Ecotoxicological Parameter	DNAN FA (mg/kg)	DNAN W-A (mg/kg)
NOAEC	44	73
p	0.126	0.205
LOAEC	63	108
p	0.029	<0.0001
EC20	40	70
CI (95%)	21–59	46–93
EC50	57	106[†]
CI (95%)	44–70	89–123
Model used	Gompertz	Gompertz
R^2	0.881	0.956

[†]Statistically significant (95% CI basis) decrease in toxicity following weathering-and-aging of DNAN in soil.

Juvenile production was the more sensitive measurement endpoint for assessing DNAN toxicity for *E. crypticus* compared with adult survival, based on the EC50 values. These data for DNAN freshly amended or weathered-and-aged in SSL soil are summarized in Table 16. The logistic Gompertz model had the best fit for reproduction data ($R^2 = 0.922$) from toxicity tests with DNAN freshly amended in SSL soil (Figure 11). The numbers of juveniles were significantly ($p = 0.016$) greater in the first positive treatment concentration, producing an unbounded LOEC value of 31 mg/kg and bounded NOAEC and LOAEC values of 44 and 63 mg/kg, respectively.

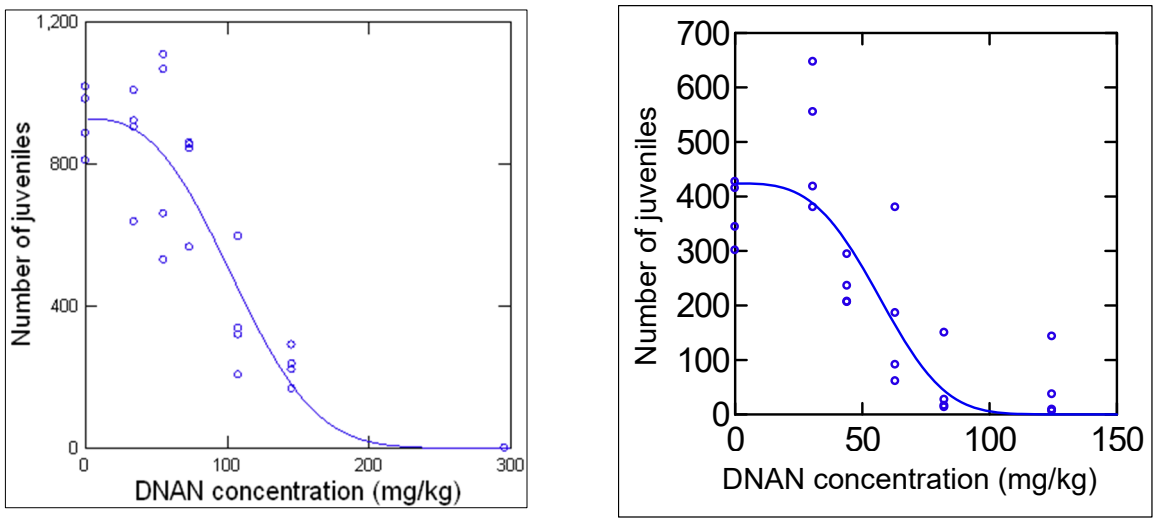

Figure 11. Effect of DNAN freshly amended (right) or weathered-and-aged (left) in Sassafras sandy loam soil on the production of juveniles by *Enchytraeus crypticus*.

The bounded NOAEC and LOEC values for DNAN weathered-and-aged in SSL soil were 73 and 108 mg/kg, respectively. The logistic Gompertz model had the best fit for the reproduction data in the study. The EC50 and EC20 values (and corresponding 95% CI) for the production of juveniles in weathered-and-aged treatment of SSL soil were 106 (89–123) and 70 (46–93) mg/kg, based on analytically determined DNAN concentrations. Weathering-and-aging of DNAN in SSL soil significantly decreased reproduction toxicity for *E. crypticus* based on the EC50 values and their respective 95% CIs (Table 16).

3.6.3 Ecotoxicological Effects of NTO on the Potworm *Enchytraeus crypticus* in SSL Soil

There were no statistically significant differences between the numbers of surviving adults or juveniles produced in negative and solvent (methanol) controls in studies with NTO freshly amended or weathered-and-aged in SSL soil (*t*-Test $p = 0.470$ and $p = 0.069$, respectively, for adults, and $p = 0.942$ and $p = 0.648$, respectively, for juveniles). Therefore, the results for the two control treatments were combined to evaluate the compliance with the validity criteria defined in the ISO 16387 test guideline. Results of toxicity tests with NTO freshly amended or weathered-and-aged in SSL soil complied with the validity criteria. The mean adult

survival, mean number of juveniles produced, and CV for combined controls results were: 84%, 274, and 47%, respectively in freshly amended treatment, and 99%, 857, and 12%, respectively, in weathered-and-aged treatment. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the NTO treatments.

The bounded NOEC and LOEC values for NTO effects on adult survival were 388 and 568 mg/kg, respectively in freshly amended soil. The logistic Gompertz model had the best fit for the adult survival data in the study with NTO freshly amended in SSL and established the EC20 and EC50 values 540 and 620 mg/kg, respectively.

The numbers of surviving adults were not significantly different from carrier control up to and including 272 mg/kg treatment for NTO weathered-and-aged in SSL, followed by 100% mortality in the next tested concentration of 547 mg/kg. This lack of a gradual concentration-response relationship precluded the determination of the adult survival EC20 and EC50 values for NTO weathered-and-aged in SSL. The NOEC and LOEC values for NTO weathered-and-aged in SSL were 272 and 547 mg/kg, respectively (Table 17).

Table 17. Acute Ecotoxicological Benchmarks for NTO Freshly Amended (FA) or Weathered-and-Aged (W-A) in SSL Soil Determined for Survival of Adult *Enchytraeus crypticus*

Ecotoxicological Parameter	NTO FA (mg/kg)	NTO W-A (mg/kg)
NOEC	388	272
p	0.303	0.106
LOEC	568	547
p	0.002	≤0.0001
EC20	540	>272
CI (95%)	420–661	ND
EC50	620	>272
CI (95%)	407–834	ND
Model used	Gompertz	ND
R^2	0.968	ND

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

Chronic ecotoxicological benchmarks for NTO freshly amended or weathered-and-aged in SSL soil are summarized in Table 18. The bounded NOEC and LOEC values were 294 and 388 mg/kg, respectively for NTO freshly amended in SSL soil. Production of juveniles by *E. crypticus* was significantly decreased in the first positive concentration of NTO weathered-and-aged in SSL, compared with carrier control, producing a NOEC of < 31 mg/kg and an unbounded LOEC of 31 mg/kg.

Juvenile production was the more sensitive measurement endpoint for assessing NTO toxicity for *E. crypticus* compared with adult survival, based on the EC20 values determined in freshly amended treatments. The logistic hormetic model had the best fit (R^2 = 0.881) for reproduction data determined in toxicity tests with NTO freshly amended in SSL soil,

due to stimulation of juvenile production at the lower treatment concentrations (Figure 12). The hormetic model yielded the EC20 and EC50 values of 197 mg/kg and 310 mg/kg, respectively. The logistic Gompertz model had the best fit ($R^2 = 0.951$) for the reproduction data in the study with NTO weathered-and-aged in SSL. The respective EC20 and EC50 values for the production of juveniles by *E. crypticus* in weathered-and-aged treatment of SSL soil were 16 and 39 mg/kg, based on analytically determined NTO concentrations. Weathering-and-aging of NTO in SSL soil significantly increased (by one order of magnitude) reproduction toxicity for *E. crypticus* based on the EC20 and EC50 values, and their respective 95% CIs (Table 18).

Table 18. Chronic Ecotoxicological Benchmarks for NTO Freshly Amended (FA) or Weathered-And-Aged (W-A) in SSL Soil Determined for the Production of Juveniles by *Enchytraeus crypticus*

Ecotoxicological Parameter	NTO FA (mg/kg)	NTO W-A (mg/kg)
NOEC	294	<31
p	0.227	ND
LOEC	388	31[§]
p	0.002	0.001
EC20	197	16[†]
CI (95%)	126–268	8–24
EC50	310	39[†]
CI (95%)	213–407	30–49
Model used	Hormetic	Gompertz
R^2	0.881	0.951

ND, Not Determined; could not be determined within the concentration range tested. [§]Unbounded LOEC. [†]Statistically significant (95% CI basis) increase in toxicity following weathering-and-aging of NTO in soil.

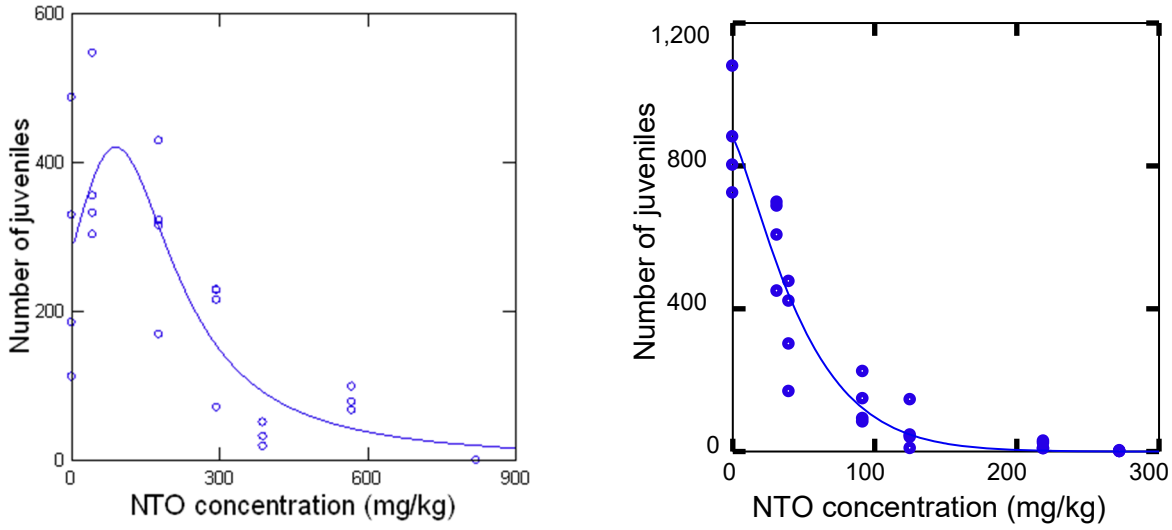

Figure 12. Effect of NTO freshly amended (left) or weathered-and-aged (right) in Sassafras sandy loam soil on the production of juveniles by *Enchytraeus crypticus*.

3.6.4 Ecotoxicological Effects of DNAN on the Potworm *Enchytraeus crypticus* in WCL Soil

Results of toxicity tests with DNAN weathered-and-aged in WCL soil complied with the validity criteria defined in the ISO 16387 test guideline. The validity criteria (mean adult survival, the mean number of juveniles produced, and CV) for test results from the negative control treatment were: 100%, 3841, and 10%, respectively. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the DNAN treatments.

There was no effect ($p > 0.05$) on adult survival in any of the DNAN concentrations tested in WCL soil, producing an unbounded NOEC of 693 mg/kg. Juvenile production was the more sensitive measurement endpoint for assessing DNAN toxicity for *E. crypticus* compared with adult survival. These data for DNAN weathered-and-aged in WCL soil are summarized in Table 19. The numbers of juveniles were significantly ($p \leq 0.0001$) greater in the first positive treatment concentration compared with carrier control, producing an unbounded LOEC value of 70 mg/kg, and the bounded NOAEC and LOAEC values of 150 and 313 mg/kg, respectively. The logistic Gompertz model had the best fit ($R^2 = 0.947$) for reproduction data (Figure 13). The EC50 and EC20 values (and corresponding 95% CI) for the production of juveniles in WCL soil were 318 (220–415) and 182 (67–297) mg/kg, respectively, based on analytically determined DNAN concentrations.

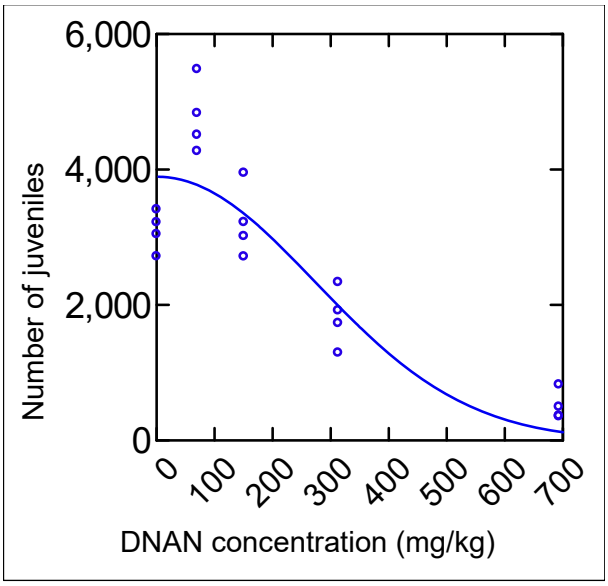

Figure 13. Effect of DNAN weathered-and-aged in Webster clay loam soil on the production of juveniles by *Enchytraeus crypticus*.

Table 19. Chronic Ecotoxicological Benchmarks for DNAN Weathered-and-Aged (W-A) in WCL Soil Determined for the Production of Juveniles by *Enchytraeus crypticus*

Ecotoxicological Parameter	DNAN (mg/kg)
NOAEC	150
p	0.672
LOAEC	313
p	0.001
EC20	182
CI (95%)	67–297
EC50	318
CI (95%)	220–415
Model used	Gompertz
R^2	0.947

3.6.5 Ecotoxicological Effects of NTO on the Potworm *Enchytraeus crypticus* in WCL Soil

Results of toxicity tests with NTO weathered-and-aged in WCL soil complied with the validity criteria defined in the ISO 16387 test guideline. The validity criteria (mean adult survival, the mean number of juveniles produced, and CV) for test results in the negative control treatment were: 100%, 2233, and 7%, respectively. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the NTO treatments.

Table 20. Chronic Ecotoxicological Benchmarks for NTO Weathered-and-Aged in WCL Soil Determined for the Production of Juveniles by *Enchytraeus crypticus*

Ecotoxicological Parameter	NTO (mg/kg)
NOEC	46
p	0.981
LOEC	152
p	≤0.0001
EC20	135
CI (95%)	104–166
EC50	255
CI (95%)	209–302
Model used	Gompertz
R^2	0.997

Numbers of surviving adults were not affected by NTO up to and including the greatest concentration tested in WCL soil producing an unbounded NOEC of 201 mg/kg. Chronic ecotoxicological benchmarks for NTO weathered-and-aged in WCL soil are summarized in Table 20. The bounded NOEC and LOEC values were 46 and 152 mg/kg, respectively. The logistic Gompertz model had the best fit ($R^2 = 0.997$) for reproduction data, yielding the EC20 and EC50 values of 135 mg/kg and 255 mg/kg, respectively, based on analytically determined NTO concentrations.

3.7 Folsomia Toxicity Studies

3.7.1 Positive Control

Toxicity tests with boric acid (reference toxicant) were conducted in SSL soil to obtain the EC50 values and the corresponding 95% CL. Nonlinear regression analyses of reproduction toxicity data established for seven testing dates produced the following EC50 values and their corresponding CLs (in parentheses) for juvenile production: 72 (68–77), 63 (53–73), 60 (53–67), 69 (61–76), 60 (51-68), 70 (59-82) and 58 (39–78) mg of H_3BO_3/kg of soil. These EC50 values were plotted on a Boric Acid Warning Chart to monitor the condition of the collembolan culture. All resulting EC50 values were within both the Warning Limits and the 95% CL that were established for the *F. candida* culture in tests with boric acid (Figure 14). These charted results confirmed that the condition of the *F. candida* culture met the validity requirements of the test protocol.

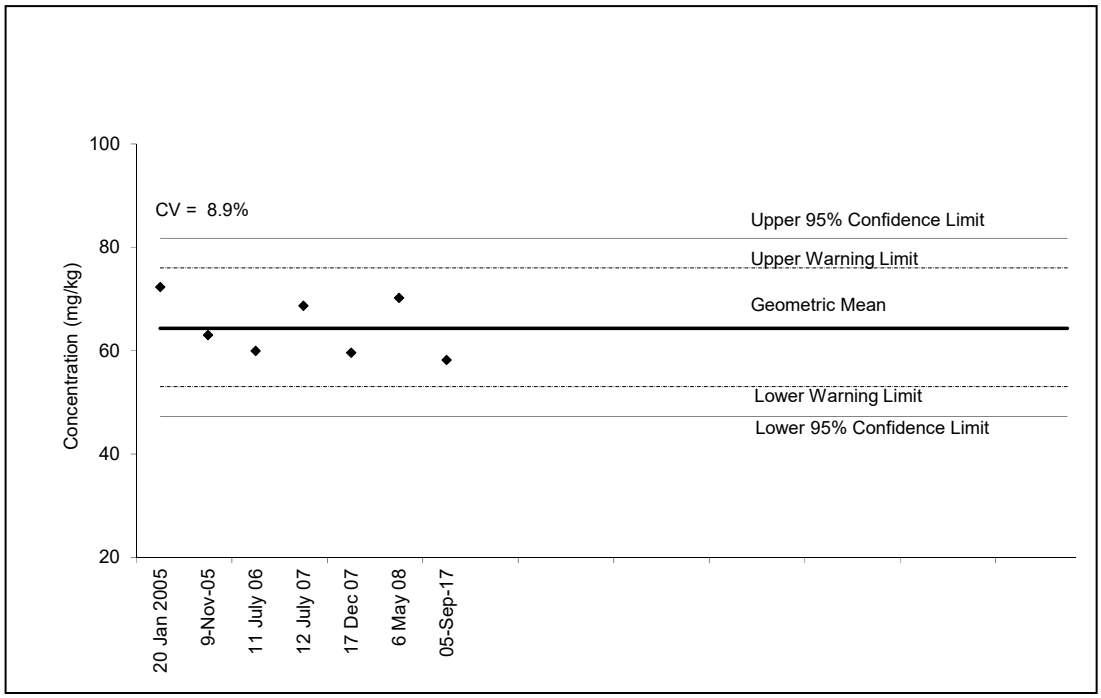

Figure 14. Warning chart for the *Folsomia candida* culture showing the EC50 values for juvenile production established in definitive tests with the reference toxicant (boric acid) in SSL soil.

3.7.2 Ecotoxicological Effects of DNAN on the Collembolan *Folsomia candida* in SSL Soil

The definitive toxicity test with collembolan *F. candida* exposed to DNAN weathered-and-aged in SSL soil was conducted to assess the acute (adult mortality) and chronic (juvenile production) effects of DNAN on *F. candida* in SSL soil. Measurement endpoints included the number of surviving adults and the number of juveniles produced after 28 d. Exposure concentrations for definitive tests were selected to achieve bracketing of significant effects on reproduction endpoints (*i.e.*, production of juveniles).

Results of the definitive toxicity test with DNAN weathered-and-aged in SSL soil complied with the validity criteria defined in the ISO 11267 (ISO, 1999) test guideline. The validity criteria for test results in negative control treatment for mean adult survival, the mean number of juveniles produced, and CV were: 76%, 83, and 15%, respectively. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the DNAN treatments.

Toxicity benchmarks for DNAN weathered-and-aged in SSL soil are summarized in Table 21. The number of surviving adults was significantly ($p = 0.009$) lower in the lowest positive DNAN concentration compared with carrier control, producing an unbounded LOEC of

25 mg/kg. The bounded NOEC and LOEC values for DNAN effects on the production of juveniles were 25 and 34 mg/kg, respectively. The logistic Gompertz model had the best fit for both, the adult survival data (acute toxicity) and juvenile production data (chronic toxicity) in SSL soil (Figure 15). The EC20 and EC50 values were respectively 11 and 28 mg/kg, and 27 and 32 mg/kg for the survival of adults for the production of juveniles.

Table 21. Ecotoxicological Benchmarks for DNAN Weathered-and-Aged in SSL Soil Determined for the Survival of Adults and Production of Juveniles by *Folsomia candida*

Ecotoxicological Parameter	Survival (mg/kg)	Reproduction (mg/kg)
NOEC	<25	25
p	ND	0.234
LOEC	25[†]	34
p	0.009	<0.0001
EC20	11	27
CI (95%)	4–19	20–33
EC50	28	32
CI (95%)	20–36	29–35
Model used	Gompertz	Gompertz
R^2	0.917	0.924

ND, not determined; could not be determined within the concentration range tested.
[†]Unbounded LOEC

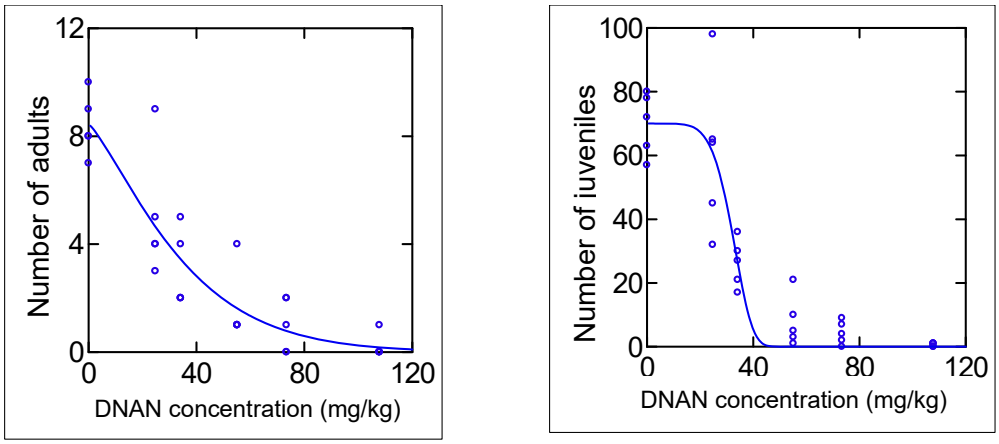

Figure 15. Effect of DNAN weathered-and-aged in SSL soil on the survival of adults (left) and production of juveniles (right) by *Folsomia candida*.

3.7.3 Ecotoxicological Effects of NTO on the Collembolan *Folsomia candida* in SSL Soil

Results of collembolan toxicity tests with NTO freshly amended or weathered-and-aged in SSL soil complied with the validity criteria defined in the ISO 11267 test guideline.

The validity criteria for mean adult survival, the mean number of juveniles produced, and CV for test results from negative control treatment were: 98%, 50, and 30%, respectively in freshly amended treatment, and 100%, 81, and 15%, respectively, in weathered-and-aged treatment. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the NTO treatments.

The bounded NOEC and LOEC values for NTO effects on adult survival were 177 and 294 mg/kg, respectively in freshly amended soil, and 40 and 92 mg/kg for NTO weathered-and-aged in SSL soil. The logistic Gompertz model had the best fit for the adult survival data and established the EC20 and EC50 values 141 and 306 mg/kg, respectively, for NTO freshly amended in SSL, and 72 and 138 mg/kg, respectively, for NTO weathered-and-aged in SSL. Weathering-and-aging of NTO in SSL soil significantly increased acute toxicity for *F. candida* based on the EC50 values and their respective 95% CIs (Table 22).

Table 22. Acute Ecotoxicological Benchmarks for NTO freshly amended (FA) or weathered-and-aged (W-A) in SSL Soil Determined for the Survival of Adult *Folsomia candida*

Ecotoxicological Parameter	NTO FA (mg/kg)	NTO W-A (mg/kg)
NOEC	177	40
p	0.204	0.570
LOEC	294	92
p	<0.0001	0.019
EC20	141	72
CI (95%)	73–209	40–104
EC50	306	138[†]
CI (95%)	237–374	108–168
Model used	Gompertz	Gompertz
R^2	0.942	0.939

[†]Statistically significant (95% CI basis) increase in toxicity following weathering-and-aging of NTO in soil.

Chronic ecotoxicological benchmarks for NTO freshly amended or weathered-and-aged in SSL soil are summarized in Table 23. Production of juveniles by *F. candida* was significantly decreased in the first positive concentration of NTO freshly amended in SSL, compared with carrier control, producing a NOEC of < 177 mg/kg and an unbounded LOEC of 177 mg/kg. The bounded NOEC and LOEC values for NTO weathered-and-aged in SSL were 40 and 92 mg/kg, respectively.

The logistic Gompertz model had the best fit (R^2 = 0.920 and 0.902, respectively) for reproduction data determined in both exposure types (Figure 16). The EC20 and EC50 values for production of juveniles by *F. candida* were 156 and 239 mg/kg for NTO freshly amended in SSL, and 63 and 111 mg/kg for NTO weathered-and-aged in SSL soil, based on analytically determined NTO concentrations. Weathering-and-aging of NTO in SSL soil significantly

increased reproduction toxicity for *F. candida* based on the EC20 or EC50 values and their respective 95% CIs (Table 23).

Table 23. Chronic Ecotoxicological Benchmarks for NTO Freshly Amended (FA) or Weathered-and-Aged (W-A) in SSL Soil Determined for Production of Juveniles by *Folsomia candida*

Ecotoxicological Parameter	NTO FA (mg/kg)	NTO W-A (mg/kg)
NOEC	<177	40
p	ND	0.621
LOEC	177[§]	92
p	0.032	0.008
EC20	156	63[†]
CI (95%)	103–209	32–95
EC50	239	111[†]
CI (95%)	199–280	85–138
Model used	Gompertz	Gompertz
R^2	0.920	0.905

ND, Not Determined; could not be determined within the concentration range tested. [§]Unbounded LOEC. [†]Statistically significant (95% CI basis) increase in toxicity following weathering-and-aging of NTO in soil.

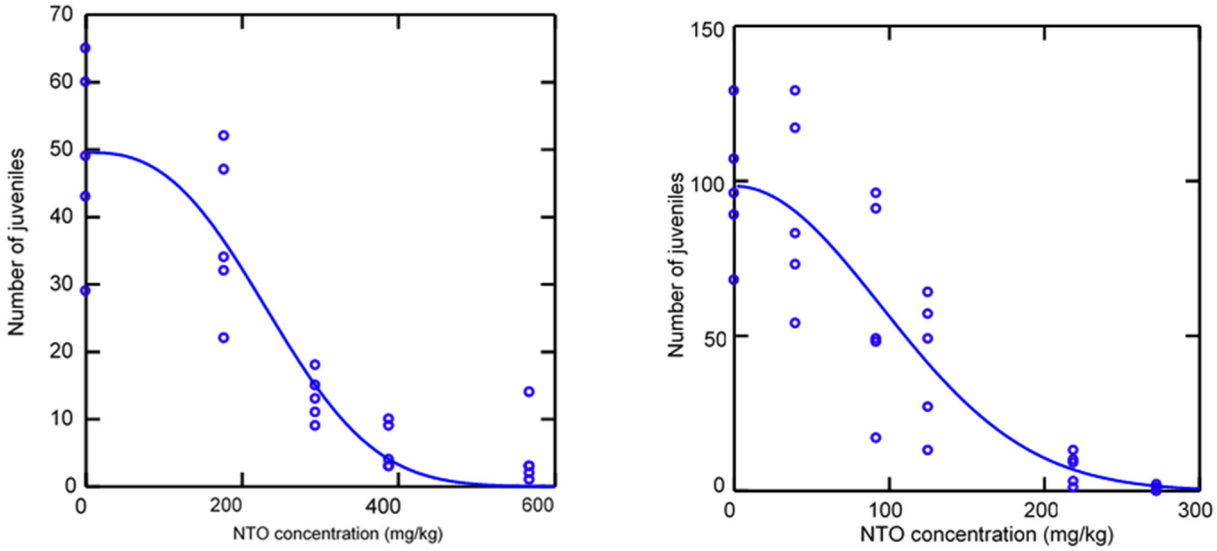

Figure 16. Effect of NTO freshly amended (left) or weathered-and-aged (right) in Sassafras sandy loam soil on the production of juveniles by *Folsomia candida*.

3.7.4 Ecotoxicological Effects of DNAN on the Collembolan *Folsomia candida* in WCL Soil

The definitive toxicity test with collembolan *F. candida* exposed to DNAN weathered-and-aged in WCL soil was conducted to assess the acute (adult mortality) and chronic (juvenile production) effects of DNAN on *F. candida* in WCL soil. Measurement endpoints included the number of surviving adults and the number of juveniles produced after 28 d. Exposure concentrations for definitive tests were selected to achieve bracketing of significant effects on reproduction endpoints (*i.e.*, production of juveniles).

Results of the definitive toxicity test with DNAN weathered-and-aged in WCL soil complied with the validity criteria defined in the ISO 11267 (ISO, 1999) test guideline. The validity criteria for test results in negative control treatment for mean adult survival, the mean number of juveniles produced, and CV were: 96%, 96, and 19%, respectively. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the DNAN treatments.

Toxicity benchmarks for DNAN weathered-and-aged in WCL soil are summarized in Table 24. The bounded NOEC and LOEC values for DNAN were 24 and 37 mg/kg, respectively, for the survival of adults, and 37 and 43 mg/kg, for the production of juveniles. The logistic Gompertz model had the best fit for both, the adult survival data (acute toxicity) and juvenile production data (chronic toxicity) in SSL soil (Figure 17). The EC20 and EC50 values were 11 and 60 mg/kg, respectively, for the survival of adults, and 23 and 52 mg/kg, for the production of juveniles.

Table 24. Ecotoxicological Benchmarks for DNAN Weathered-and-Aged in WCL Soil Determined for the Survival of Adults and Production of Juveniles by *Folsomia candida*

Ecotoxicological Parameter	Survival (mg/kg)	Reproduction (mg/kg)
NOEC	24	37
p	0.619	0.155
LOEC	37	43
p	0.026	0.001
EC20	11	23
CI (95%)	4–19	7–39
EC50	60	52
CI (95%)	44–76	34–70
Model used	Gompertz	Gompertz
R^2	0.932	0.882

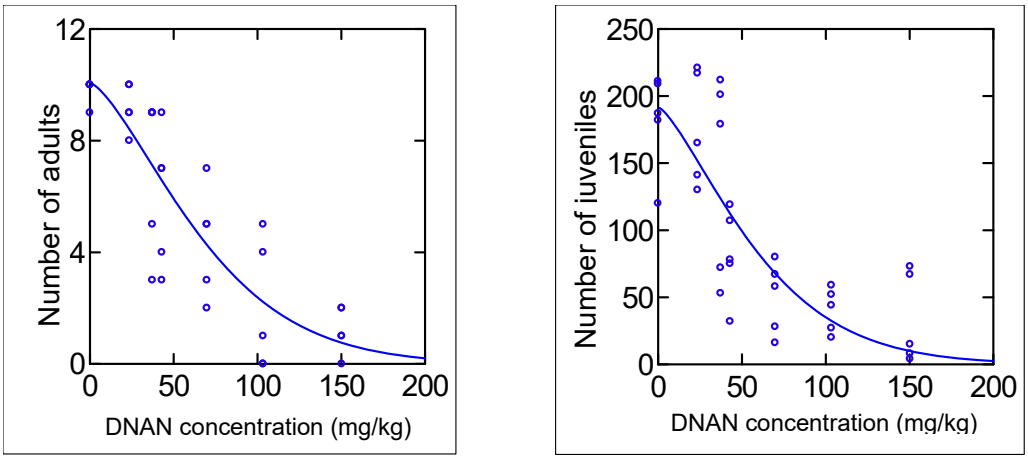

Figure 17. Effect of DNAN weathered-and-aged in WCL soil on survival of adults (left) and production of juveniles (right) by *Folsomia candida*.

3.7.5 Ecotoxicological Effects of NTO on the Collembolan *Folsomia candida* in WCL Soil

The definitive toxicity test with collembolan *F. candida* exposed to NTO weathered-and-aged in WCL soil was conducted to assess the acute (adult mortality) and chronic (juvenile production) effects of NTO on *F. candida* in WCL soil. Measurement endpoints included the number of surviving adults and the number of juveniles produced after 28 d. Exposure concentrations for definitive tests were selected to achieve bracketing of significant effects on reproduction endpoints (*i.e.*, production of juveniles).

Results of the definitive toxicity test with NTO weathered-and-aged in WCL soil complied with the validity criteria defined in the ISO 11267 (ISO, 1999) test guideline. The validity criteria for test results in negative control treatment for mean adult survival, the mean number of juveniles produced, and CV were: 100%, 141, and 16%, respectively. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the NTO treatments.

Toxicity benchmarks for NTO weathered-and-aged in WCL soil are summarized in Table 25. The bounded NOEC and LOEC values for NTO were 94 and 152 mg/kg, respectively, for the survival of adults, and 72 and 152 mg/kg, for the production of juveniles. The logistic Gompertz model had the best fit for both, the adult survival data (acute toxicity) and juvenile production data (chronic toxicity) in WCL soil (Figure 18). The EC20 and EC50 values were 141 and 211 mg/kg, respectively, for the survival of adults, and 104 and 145 mg/kg, for the production of juveniles.

Table 25. Ecotoxicological Benchmarks for NTO Weathered-And-Aged in WCL Soil Determined for Survival of Adults and Production of Juveniles by *Folsomia candida.*

Ecotoxicological Parameter	Survival (mg/kg)	Reproduction (mg/kg)
NOEC	94	72
p	1.0	0.781
LOEC	152	152
p	<0.0001	<0.0001
EC20	141	104
CI (95%)	105–177	69–140
EC50	211	145
CI (95%)	185–237	122–139
Model used	Gompertz	Gompertz
R^2	0.969	0.932

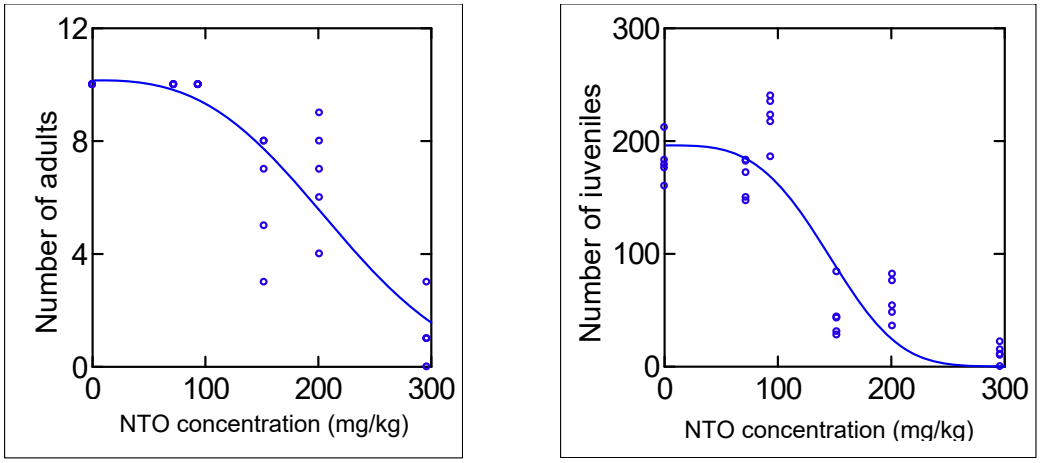

Figure 18. Effect of NTO weathered-and-aged in WCL soil on survival of adults (left) and production of juveniles (right) by *Folsomia candida*.

3.8 Toxicity and Bioaccumulation of DNAN, and NTO in Plants

Toxicity and uptake data obtained in ryegrass studies are reported in Appendix C. The definitive bioconcentration tests with ryegrass *L. perenne* consisted of an uptake exposure phase up to 42 d, as described in Rocheleau *et al.* (2014). Air-dried SSL soil was amended with DNAN at a target concentration of 50 mg/kg. For DNAN or control (0 mg/kg) treatments, dry SSL soil (1.40 kg) was spread into large rectangular containers to a depth of approximately 4 cm. DNAN was delivered dissolved in the carrier over the soil surface, followed by thoroughly mixing with a spatula. The control treatment received the carrier only. Approximately 100 mL of carrier solution was used for DNAN and the control treatment. The carrier was allowed to volatilize between the steps. Following soil amendment and mixing, the carrier was allowed to

volatize overnight under a fume hood at room temperature, in the dark. The amended soils were then placed into amber glass 2 L jars fit with air-tight lids and allowed to tumble for 18 h on a roller apparatus. After tumbling, soils were hydrated by the stepwise addition (followed by mixing) of ASTM type I water to the total volume determined to restore the soil to 95% of the WHC. The control soil was prepared in the same manner using the carrier only (500 mL).

Twenty seeds of ryegrass were sown in 10-cm wide pots each containing 200 g (dry wt.) test soil. The bottom of each pot was previously covered with a piece of cheesecloth to prevent soil loss during testing. All treatments were carried out in seven replicates. The test soil treatments included negative (no IM compound added) and carrier (solvent) controls. An unplanted (no plants added) DNAN treatment was included in the system. All exposures were conducted at 20 °C in a diurnal 16 h-light:8 h-dark photoperiod cycle. After seeds were sown, the soil was hydrated with ASTM Type 1 water every day as needed until seedlings were visible. Subsequently, the pots were watered twice per week to maintain moisture levels. To prevent nutrient deficiencies, fourteen days after the onset of the experiment, soils were hydrated with Miracle-Gro® Water Soluble All Purpose Plant Food (Scotts Miracle-Gro, Marysville, OH, USA) at approximately 1:10 dilution. At the conclusion of the experiment, both shoots and roots were harvested. The soil was washed to remove adhered debris from roots with ASTM Type I water. Excess water was blotted dry with a paper towel. The roots and shoots were oven-dried in pre-weighed weigh boats at 70 °C for 24–72 h and reweighed until a constant weight was measured. Plant tissues and soils were kept at –20 °C before lyophilization and chemical analyses. Bioconcentration factors in plants, respectively, for DNAN, as well as the extractable IM compound-degradation products, were calculated based on measured tissue and soil concentrations.

3.8.1 Data Analyses in Plant Studies

Analysis of Variance (ANOVA) and t-tests were applied and used for comparisons of DNAN or NTO data for soil, ryegrass growth, and uptake in root and shoot plant tissues using Sigma Plot 14.0. A significance level of $p \leq 0.05$ (95% confidence level) was accepted for all statistical tests. Seedling emergence after 7 d and shoot and root growth were analyzed using best-fit regression models to determine IM compound concentrations producing a 20% (EC20) or 50% (EC50) decrease in measurement endpoints compared with control using mean concentrations measured at the start of the exposure period.

The plant BCF for DNAN and its extractable transformation products were calculated by dividing the ryegrass shoot or root concentration by the initial concentration measured in the soil. Translocation factors (TF) were calculated as the ratio of chemical concentrations in shoots to those in roots.

3.8.2 DNAN Concentrations in SSL Soil Used in the Phytotoxicity Studies

DNAN concentrations were analytically determined in all treatment and control soils at the beginning and end of the testing period. Initial analytically determined soil concentrations of DNAN were within 10% of the nominal concentrations, except for the highest

concentration, for which the measured concentration was 17% lower (Table 26). DNAN concentrations decreased in the SSL soil over 21 d of exposure compared to the initial concentrations. The decrease in DNAN concentration was slightly greater in the planted treatment with ryegrass (Table 27) compared to the unplanted soil without ryegrass (Table 28). Concentrations of 2-ANAN were detected in the initial SSL soil. After 21 d, there was an increase in the final 2-ANAN soil concentrations of both the unplanted and planted SSL soils. That increase was not significantly ($p > 0.05$) different between the planted and unplanted SSL soil treatments. There was no detectable 4-ANAN in the initial soil treatments. However, 4-ANAN was detected in the SSL nominal soil treatments of 50, 100, 250, and 500 mg/kg at the test termination of the unplanted treatment (Table 28). The SumDNAN percentages decreased in the planted (Table 27) and unplanted (Table 28) treatments compared to the initial SumDNAN concentrations; the decrease was not statistically significant ($p>0.05$) between the two treatments.

Table 26. Initial Concentrations of DNAN and the Transformation Products Extracted from SSL Soil During the 21-D Range-Finding Test.

Nominal Concentration mg/kg	DNAN mg/kg (SD)	2-ANAN mg/kg (SD)	4-ANAN mg/kg(SD)	SumDNAN mg/kg (SD)
5	5.4 (0.01)	0.19 (0.23)	ND	5.6 (0.23)
10	8.6 (0.02)	0.18 (0.22)	ND	8.8 (0.21)
50	51 (0.01)	0.08 (0.001)	ND	51 (0.01)
100	102 (0.05)	0.06 (0.002)	ND	102 (0.05)
250	227 (0.7)	0.14 (0.02)	ND	227 (0.7)
500	417 (0.2)	0.14 (0.02)	ND	417 (0.2)

Numbers are means and standard deviations in parenthesis (n = 3).
ND, Not Determined; concentrations below the quantification limit of 0.025 mg/kg for soil.

Table 27. Final Concentrations of DNAN and the Transformation Products Extracted from SSL Soil at the Conclusion of the 21-D Range-Finding Test with Ryegrass

Nominal Concentration mg/kg	DNAN mg/kg (SD)	2-ANAN mg/kg (SD)	4-ANAN mg/kg (SD)	SumDNAN mg/kg (SD)	SumDNAN % Decrease
5	2.6 (0.01)	0.22 (0.01)	ND	2.8 (0.01)	49.54
10	3.6 (0.11)	0.28 (0.01)	ND	3.9 (0.09)	56.00
50	17.4 (0.03)	1.06 (0.01)	ND	18.5 (0.04)	63.96
100	37.3 (0.01)	2.95 (0.02)	ND	40.3 (0.04)	60.59
250	127 (0.02)	7.26 (0.01)	ND	134.3 (0.03)	40.77
500	366 (0.10)	3.89 (0.02)	ND	370 (0.11)	11.33

Numbers are means and standard deviations in parenthesis (n = 3).
ND, Not Determined; concentrations below the quantification limit of 0.025 mg/kg for soil.

Table 28. Final Concentrations of DNAN and the Transformation Products Extracted From SSL Soil at the Conclusion of the 21-D Range-Finding Test without Ryegrass

Nominal Concentration mg/kg	DNAN mg/kg (SD)	2-ANAN mg/kg (SD)	4-ANAN mg/kg (SD)	SumDNAN mg/kg (SD)	SumDNAN % Decrease
5	2.8 (0.15)	0.22 (0.01)	ND	3.05 (0.15)	45.05
10	3.8 (0.06)	0.24 (0.03)	ND	4.06 (0.05)	53.71
50	18 (0.01)	1.01 (0.01)	0.23 (0.004)	19.23 (0.01)	62.43
100	45.6 (0.31)	3.76 (0.01)	0.36 (0.01)	49.66 (0.31)	51.37
250	128 (0.58)	8.4 (0.01)	0.57 (0.002)	137.30 (0.58)	39.42
500	378 (0.01)	4.3 (0.004)	0.44 (0.002)	382.71 (0.01)	8.26

Numbers are means and standard deviations in parenthesis (n = 3).
ND: Not Determined; concentrations below the quantitation limit of 0.025 mg/kg for soil.

3.8.3 NTO Concentrations in SSL Soil Used in the Ryegrass Uptake Studies

The potential for NTO uptake from the soil to ryegrass was evaluated in the range-finding tests using the SSL soil amended with NTO at nominal concentrations of 0, 5, 10, 50, 100, 250, and 500 mg/kg. NTO concentrations decreased after 21 d of exposure in treatments with and without ryegrass (Table 29). The final soil NTO concentrations in the planted and unplanted treatments were not significantly different ($p > 0.05$). NTO was not detected in ryegrass roots or shoots.

Table 29. Concentrations of NTO Extracted from SSL Soil at the Start (Initial) and Conclusion (Final) of the 21-D Range-Finding Test with or without Ryegrass

Nominal Concentration (mg/kg)	NTO Initial mg/kg (SD)	NTO Final with ryegrass mg/kg (SD)	% Reduction	NTO Final without ryegrass mg/kg (SD	% Reduction
5	4.3 (0.01)	0.27 (0.02)	94	0.33 (0.02)	92
10	11 (0.55)	0.19 (0.02)	98	0.46 (0.02)	96
50	56 (0.01)	2.15 (0.02)	96	3.5 (0.02)	94
100	89 (0.56)	23.3 (0.25)	74	27.6 (0.30)	69
250	253 (0.83)	95.7 (0.69)	62	105 (0.36)	59
500	475 (0.64)	259 (0.81)	45	243 (0.06)	49

Numbers are means and standard deviations in parenthesis (n = 3)

3.8.4 Phytotoxicity of DNAN in SSL Soil

Seedling emergence was not significantly ($p>0.05$) affected at lower DNAN concentrations of 5 and 10 mg/kg compared with carrier control by day 7 (Figure 19). However, ryegrass seedling emergence was significantly inhibited ($p<0.05$) in soil DNAN concentrations ranging from 50 to 500 mg/kg as compared to seedling emergence in the carrier control. The NOEC and LOEC were 8.6 and 51 mg/kg, respectively, as measured initial DNAN concentrations.

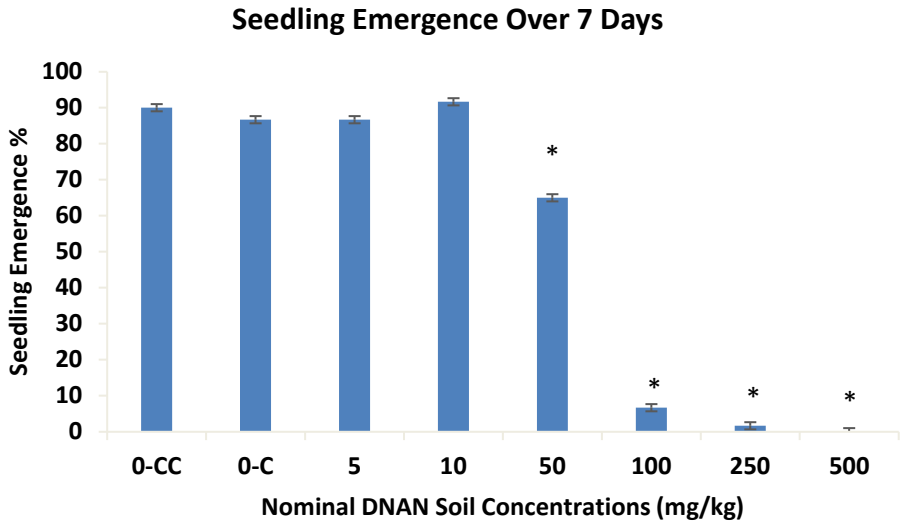

Figure 19. Seedling emergence percentages over 7 d for ryegrass exposed to DNAN in range-finding toxicity tests. Data are means ± standard deviations (n = 3). (*) indicates a significant difference ($p < 0.05$) compared to the carrier control (0-CC). 0-C = negative control.

Ryegrass shoot dry mass (SDM) was not significantly ($p > 0.05$) affected at DNAN concentrations up to and including 50 mg/kg compared with carrier control by the end of the 21-day range-finding test (Figure 20). The SDM growth was completely inhibited at DNAN soil concentrations of 250 mg/kg and 500 mg/kg. The NOEC and LOEC were 51 and 102 mg/kg, respectively, as measured initial DNAN concentrations.

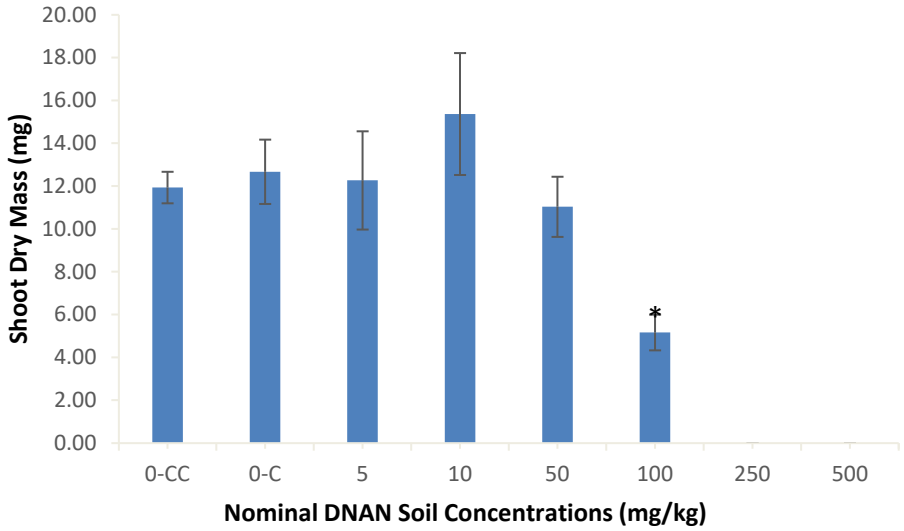

Figure 20. Ryegrass shoot dry mass at the end of the 21-d exposure to DNAN in Sassafras sandy loam soil. Data are means ± standard deviation (n = 3). (*) indicates a significant difference ($p < 0.05$) compared to the carrier control (CC). Shoot growth was inhibited at DNAN soil concentrations of 250 mg/kg and 500 mg/kg.

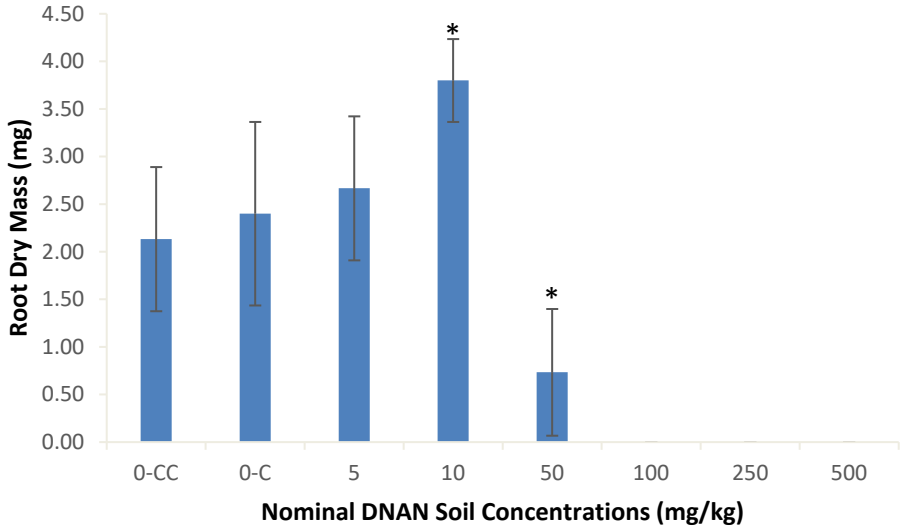

Figure 21. Ryegrass root dry mass at the end of the 21-d exposure to DNAN in Sassafras sandy loam soil. Data are means ± standard deviation (n = 3). (*) indicates a significant difference ($p < 0.05$) compared to the carrier control (CC). Root growth was inhibited at DNAN soil concentrations of 250 mg/kg and 500 mg/kg.

The ryegrass root dry mass (RDM) was significantly ($p < 0.05$) greater at 10 mg/kg compared to the carrier control (Figure 21), followed by a significant ($p < 0.05$) decrease at 50 mg/kg and complete RDM growth inhibition at 100 mg/kg and greater DNAN concentrations. The NOEC and LOEC were 8.6 and 51mg/kg, respectively, as measured initial DNAN concentrations.

Seedling emergence EC20 and EC50 values for DNAN were 46 and 66.3 (Table 30). The EC20 and EC50 values for ryegrass SDM were 47 and 85 mg/kg, respectively. The ryegrass RDM EC20 and EC50 values were 48 and 50 mg/kg, respectively. DNAN toxicity to the ryegrass growth as measured by SDM was significantly less (greater EC50 values) compared with the RDM measurement endpoint, based on the EC50 values and corresponding 95% CI (Table 30).

Table 30. Ecotoxicological Benchmarks for DNAN in SSL Soil Determined for Seedling Emergence, and Shoot and Root Growth

Ecotoxicological Parameter	DNAN (mg/kg)		
	Seedling emergence	Shoot growth	Root growth
NOEC	8.6	51	8.6
LOEC	51	102	51
EC20	46	47	48
CI (95%)	39-53	36-59	46-50
EC50	66	85	50
CI (95%)	60-73	69-100	48-52
Model used	Gompertz	Hormetic	Hormetic
R^2	0.992	0.980	0.941

3.8.5 Phytotoxicity of NTO in SSL

The lower NTO concentrations of 5 mg/kg and 10 mg/kg did not significantly reduce ($p > 0.05$) ryegrass seedling emergence percentages which were both 85% by day 7 compared to the 90% that emerged in the carrier control (Figure 22). Seedling emergence was significantly decreased ($p < 0.05$) in ryegrass grown in 50 mg/kg NTO. There was no seedling emergence observed at the higher NTO soil concentrations of 100, 250, and 500 mg/kg. The NOEC and LOEC were 11 mg/kg and 56 mg/kg, respectively, as measured initial NTO concentrations (Table 31).

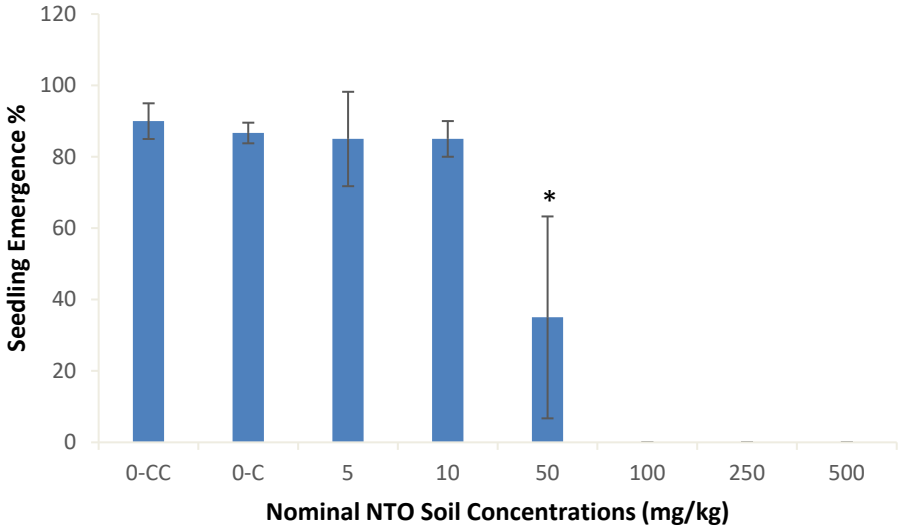

Figure 22**.** Ryegrass seedling emergence after 7 d of exposure to NTO in SSL soil. Data are means ± standard deviations (n = 3). (*) indicates significant difference ($p < 0.05$) compared to the carrier control (CC). Seedling emergence was inhibited at NTO concentrations greater than 50 mg/kg.

At the end of the 21-day range-finding test, there was no significant effect ($p > 0.05$) on ryegrass SDM growth at 5 or 10 mg/kg compared to the carrier control (Figure 23). The ryegrass SDM growth was completely inhibited at NTO soil concentrations of 100, 250, and 500 mg/kg. The NOEC and LOEC values were 11 mg/kg and 56 mg/kg, respectively, as measured initial NTO concentrations.

There was no significant effect ($p > 0.05$) on ryegrass RDM growth at 5 or 10 mg/kg compared to the carrier control (Figure 24). Inhibition of growth was observed for ryegrass RDM in the greater NTO concentrations of 100, 250, and 500 mg/kg soil. The NOEC and LOEC values were 11 and 56 mg/kg, respectively, as measured initial NTO concentrations.

Ecotoxicological benchmarks for NTO in SSL soil are summarized in Table 31. Seedling emergence EC20 and EC50 values for NTO were 28.3 and 43.8. The ryegrass RDM EC20 and EC50 values were 19.8 and 35.6, respectively. The SDM ecotoxicological parameters for NTO could not be determined within the concentration range tested in these studies.

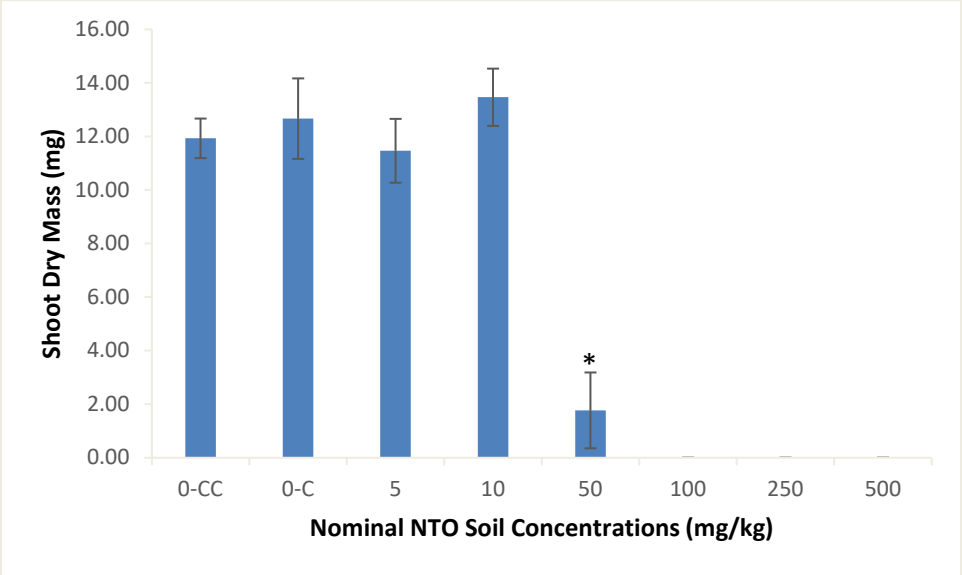

Figure 23. Shoot dry mass of ryegrass exposed to NTO over 21 d.
Data are means ± standard deviations (n = 3). (*) indicates significant difference ($p < 0.05$) compared to the carrier control (CC). Shoot growth was inhibited at NTO soil concentrations greater than 50 mg/kg.

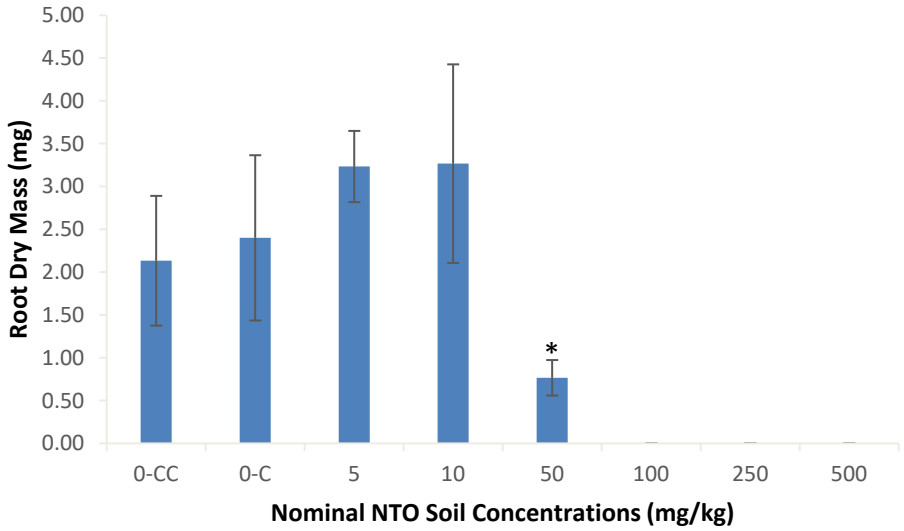

Figure 24. Root dry mass of ryegrass exposed to NTO over 21 d. Data are means ± standard deviations (n = 3). (*) Significant difference ($p < 0.05$) compared to the carrier control (CC). Root growth was inhibited at NTO soil concentrations greater than 50 mg/kg.

Table 31. Ecotoxicological Benchmarks for NTO in SSL Soil Determined for Seedling Emergence, and Shoot and Root Growth

Ecotoxicological Parameter	NTO (mg/kg)		
	Seedling emergence	Shoot growth	Root growth
NOEC	11	11	11
LOEC	56	56	56
EC20	28.3	ND	19.8
CI (95%)	0-65	ND	8-32
EC50	43.8	ND	35.6
CI (95%)	21-66	ND	13-58
Model used	Gompertz	ND	Hormetic
R^2	0.977	ND	0.936

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

3.8.6 Pilot Assessment of DNAN Bioconcentration Potential in Ryegrass

Trace amounts of DNAN, 2-ANAN, and 4-ANAN were detected in the ryegrass roots (Table 32) and shoots (Table 33) at the end of the 21-day study. Bioconcentration of DNAN and 2-ANAN in roots and shoots were all negligible to low with BCFs < 1. The TFs were < 1 for DNAN and 2-ANAN.

Table 32. Concentrations of DNAN and the Transformation Products in Ryegrass Roots at the Conclusion of the 21-D Pilot Test and the Resulting Bioconcentration Factors

Nominal Concentration (mg/kg)	Measured concentration in roots (mg/kg)			
	DNAN	2-ANAN	4-ANAN	SumDNAN
5	0.0026 (0.0003)	0.0048 (0.0062)	ND	0.0074 (0.0059)
10	0.0037 (0.0008)	0.0010 (0.0002)	ND	0.0047 (0.0009)
50	0.0431 (0.0006)	0.0064 (0.0001)	ND	0.0495 (0.0006)
100	NS	NS	NS	NS
250	NS	NS	NS	NS
500	NS	NS	NS	NS
	Bioconcentration factor			
5	0.0005 (0.00006)	0.0250 (0.03)	ND	0.0014 (0.0011)
10	0.0004 (0.0001)	0.0058 (0.0012)	ND	0.0005 (0.0001)

50	0.0008 (0.00001)	0.0800 (0.0011)	ND	0.0001 (0.00001)
100	NS	NS	NS	NS
250	NS	NS	NS	NS
500	NS	NS	NS	NS

Numbers are means and standard deviation in parentheses (n = 3).
ND, Not Detected; concentrations below the detection limit of 0.052 mg/kg for plant tissues.
NS, No sample was produced to analyze.

Table 33. Concentrations of DNAN and the Transformation Products in Ryegrass Shoots at the Conclusion of the 21-D Pilot Test and the Resulting Bioconcentration and Translocation Factors

	Determined concentrations in shoots (mg/kg)			
Nominal Concentration (mg/kg)	DNAN	2-ANAN	4-ANAN	SumDNAN
5	0.0006 (0.0002)	0.0008 (0.0001)	ND	0.0014 (0.0002)
10	0.0009 (0.0008)	0.0008 (0.0005)	ND	0.0017 (0.0013)
50	0.0111 (0.0001)	0.0029 (0.0001)	ND	0.0140 (0.0001)
100	0.0174 (0.0001)	0.0171 (0.0006)	NS	0.0345 (0.0007)
250	NS	NS	NS	NS
500	NS	NS	NS	NS
		Bioconcentration		
5	0.0001 (0.00003)	0.0039 (0.0004)	ND	0.00024 (0.00004)
10	0.0001 (0.00009)	0.0043 (0030)	ND	0.00016 (0.00015)
50	0.0002 (0.000002)	0.0363 (0.0016)	ND	0.00027 (0.000002)
100	0.0002 (0.000001)	0.2840 (0.0092)	NS	0.00035 (0.000002)
250	NS	NS	NS	NS
500	NS	NS	NS	NS
		Translocation Factors		
5	0.21 (0.04)	0.48 (0.46)	ND	0.26 (0.17)
10	0.77 (0.54)	0.69 (0.36)	ND	0.32 (0.25)
50	0.26 (0.001)	0.46 (0.01)	ND	0.28 (0.003)
100	NS	NS	NS	NS
250	NS	NS	NS	NS

500	NS	NS	NS	NS

Numbers are means and standard deviation in parentheses (n = 3).
ND, Not Detected; concentrations below the detection limit of 0.052 mg/kg for plant tissues.
NS, No sample was produced to analyze.

3.8.7 Pilot Assessment of NTO Bioconcentration Potential in Ryegrass

NTO was not detected in ryegrass roots or shoots in exposures to nominal soil concentrations ranging from 5 to 500 mg/kg. Consequently, no further evaluation of NTO bioconcentration was done.

3.8.8 Uptake of DNAN in Ryegrass

At the start of the definitive experiment, the DNAN soil concentration was 52 ± 0.6 mg/kg, therefore, close to the target concentration (Figure 25). At the end of the 42 d experiment, the DNAN concentration was 21 ± 2.4 mg/kg in the soil with ryegrass, and 32 ± 2.5 mg/kg in the soil without ryegrass. Transformation products of DNAN were also present (Figure 26). The SumDNAN (sum of DNAN, 2-ANAN, and 4-ANAN) concentration in the soil at day 42 was 22 mg/kg for planted and 33 mg/kg for unplanted, representing a statistically significant decrease ($p < 0.05$) of 58% and 38%, respectively during the 42-day experiment (Figure 25). Of that decrease in soil, DNAN comprised 93% of the total, followed by 2-ANAN, 6%, and 4-ANAN, 0.6% (Figure 26). For the decrease observed in the soil without ryegrass, fractions of DNAN, 2-ANAN, and 4-ANAN were 97.6%, 2%, and 0.4%, respectively (Figure 27).

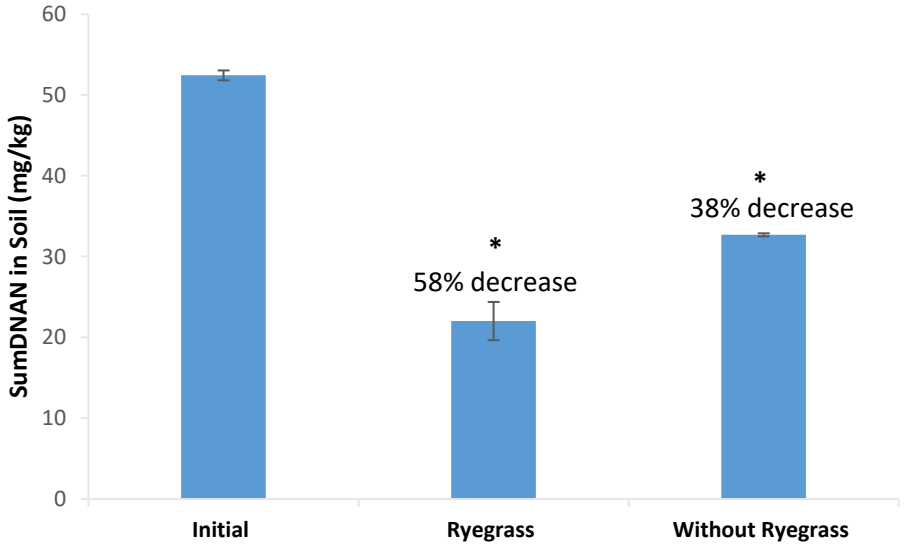

Figure 25. Concentrations of SumDNAN extracted from SSL soil at the start (initial) and conclusion (final) of the 42-d definitive test with or without ryegrass. Values are means ± standard deviation (n = 7); (*) significant difference ($p < 0.05$) compared to the initial soil concentration.

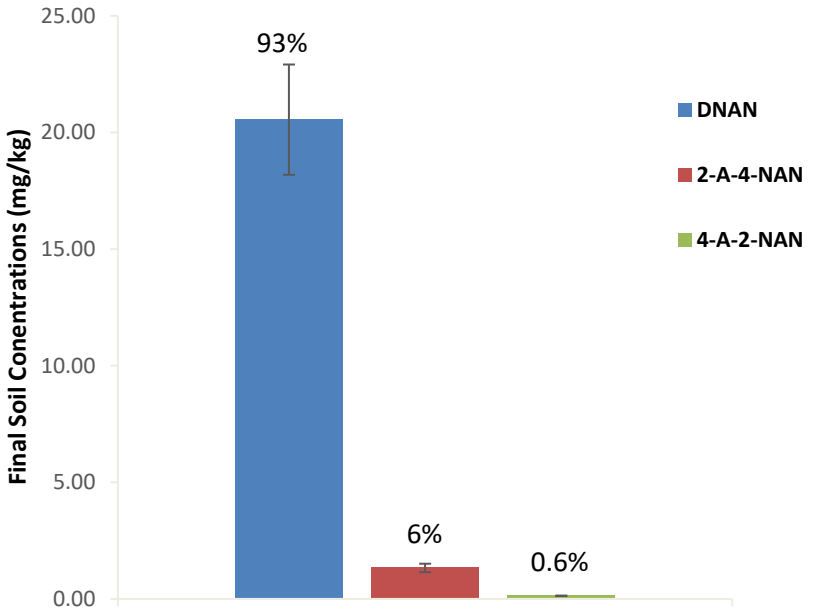

Figure 26. Concentrations of DNAN and the transformation products extracted from SSL soil at the conclusion of the 42-d definitive test with ryegrass. Values are means ± standard deviation (n = 7).

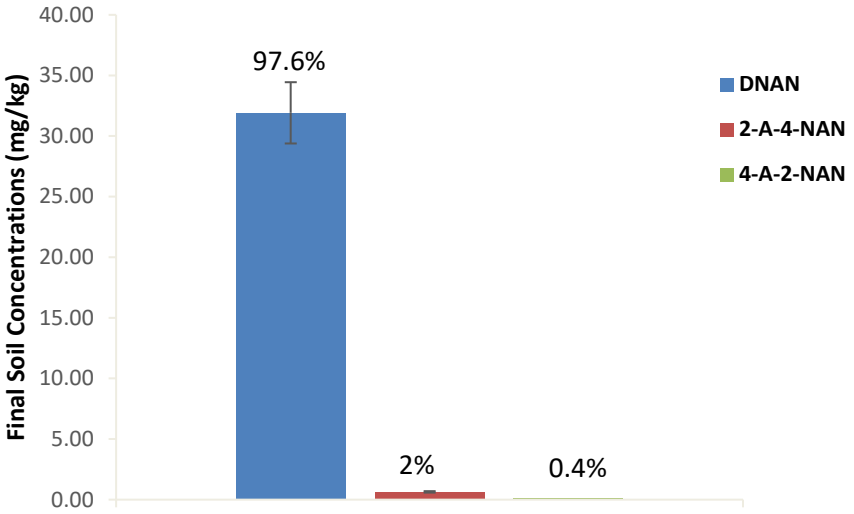

Figure 27. Concentrations of DNAN and the transformation products extracted from SSL soil at the conclusion of the 42-d definitive test without ryegrass. Values are means ± standard deviation (n = 7).

3.8.9 Effect of DNAN on Ryegrass Growth

There was a significant decrease ($p < 0.05$) in the mean SDM of 13 ± 2.9 mg compared to that in the carrier control, which was 17 ± 3.4 mg after 42 d of ryegrass exposure to the nominal DNAN concentration of 50 mg/kg in SSL soil (Figure 28). As for the ryegrass RDM, DNAN did not have a significant effect ($p > 0.05$) on growth (Figure 28), which was 3.8 ± 1.4 mg compared to 3.6 ± 0.8 mg in the carrier control. Ryegrass RDM growth increased 5x compared with RDM in the pilot test using 50 mg/kg nominal DNAN concentrations.

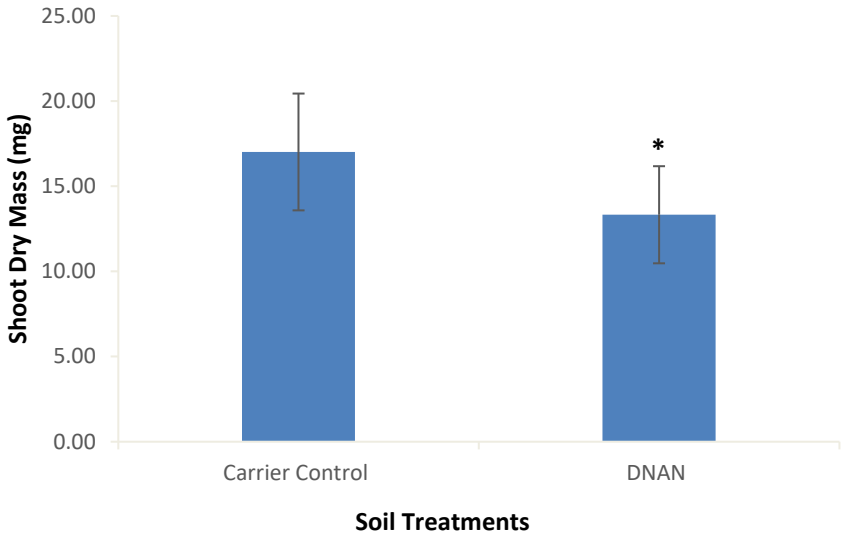

Figure 28. Shoot dry mass of ryegrass exposed to DNAN for 42 d. Values are means ± standard deviation (n = 7); *Significant difference ($p < 0.05$) compared to the carrier control.

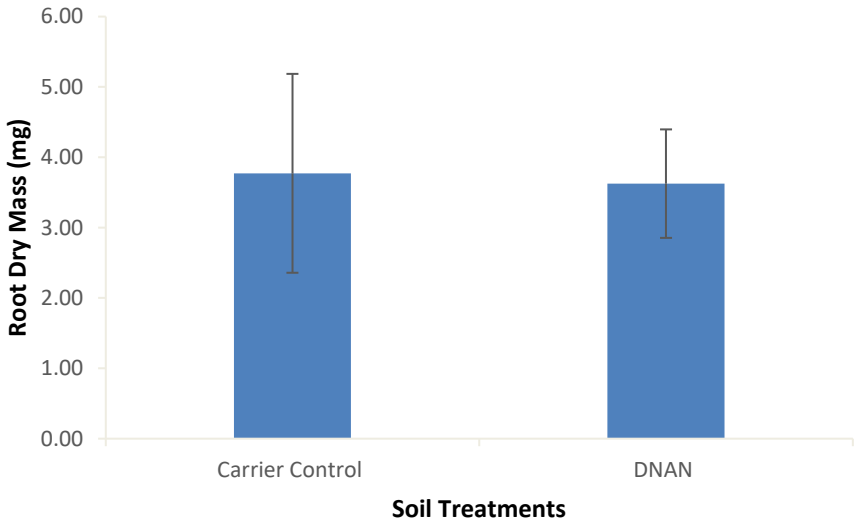

Figure 29. Root dry mass of ryegrass exposed to DNAN for 42 d. Means ± standard deviation (n = 7).

3.8.10 Uptake of DNAN in Ryegrass Roots and Shoots

Extending ryegrass exposure to DNAN for up to 42 d revealed uptake of DNAN in the shoots at 3 ± 0.7 mg/kg and in the roots at 1 ± 0.1 mg/kg (Table 34). Only one of the seven replicates in the ryegrass treatments produced the transformation product, 2-ANAN, which was detected at 0.6 mg/kg in the roots and 4.2 mg/kg in the shoots (Table 34). Because the initial soil did not contain detectable transformation products, the BCF for 2-ANAN was calculated by dividing the ryegrass tissue concentration by the concentration measured in the soil after 42 d of exposure. The mean shoot BCF for DNAN was 0.06 ± 0.01 and root BCF was 0.02 ± 0.001. The shoot BCF for 2-ANAN was 3.2 and root BCF was 0.4 (Table 34). The TF values for DNAN (3) and 2-ANAN (7.2) were established in ryegrass exposed to 52 ± 0.6 mg/kg SSL soil.

Table 34. Concentrations of DNAN and the Transformation Products in Ryegrass Shoots and Roots at the Conclusion of the 42-D Definitive Test and the Resulting Bioconcentration and Translocation Factors

Nominal Concentration (mg/kg)	DNAN	2-ANAN	4-ANAN	SumDNAN
		Concentration in shoots (mg/kg)		
50	3 (0.7)	4.2	ND	7.21
		Bioconcentration		
50	0.06 (0.01)	3.2	ND	0.14
		Translocation factor		
50	3 (0.66)	7.2	ND	4.50
		Concentration in roots (mg/kg)		
50	1 (0.1)	0.6	ND	1.601
		Bioconcentration		
50	0.02 (0.02)	0.4	ND	0.03

Numbers in parenthesis are 1 standard deviation (n = 7). ND, Not Detected

3.8.11 Individual Toxicities of DNAN and NTO in Ryegrass

Before the present studies, insufficient information was available for the phytotoxicity of DNAN compared with information available for traditional explosives RDX and TNT. In our preliminary tests, ryegrass seedling emergence and root and shoot biomass data revealed that DNAN concentrations tested can potentially affect plant development and growth. This is consistent with Dodard et al. (2013) who reported that DNAN inhibited ryegrass emergence in amended soil at concentrations > 5 mg/kg. In the present study, exposure to DNAN decreased ryegrass emergence at soil concentrations greater than 10 mg/kg. Similar to the effect on emergence rates, exposure to DNAN affected the development and growth of shoots and roots. Ryegrass shoot or root growth was inhibited at 100 mg/kg and greater concentrations. This finding is consistent with the results of other studies that investigated the effects of insensitive munitions compounds on plant growth. As summarized in Rylott and Bruce (2019), exposure to soil spiked with IMX-101 (a formulation containing DNAN, NTO, and NQ) caused toxicity to grasses. Richard and Weidhaas (2014) reported that root and shoot mass declined with increasing

IMX-101 concentrations. Exposure to IMX-101 inhibited growth in big blue stem grass (*Andropogon gerardii*), Nash Indiangrass (*Sorghastrum nutans*), and switchgrass (*Panicum virgatum*). In contrast, the present definitive study revealed that exposure to DNAN at a soil concentration of 50 mg/kg did not affect ryegrass shoot or root growth when compared with the growth in the controls. Rocheleau et al. (2014) reported a similar result for RDX effects on ryegrass growth after the 42-day exposure which showed no statistically significant effects on the shoot or root growth.

Sparse information was also available on the effects of NTO on plants. In the present studies, all measurement endpoints, including seedling emergence, and RDM and SDM were inhibited at 50 mg/kg and greater concentrations in SSL soil. The EC50 values (mg/kg) for NTO were 43.8 for ryegrass seedling emergence and 35.6 for RDM. Richard and Weidhaas (2014) investigated the effects of IMX-101 over time. That study revealed the toxic effects of that formulation after70 d following germination, further indicating the toxicity of NTO to grasses.

3.9 Development of Draft Ecological Soil Screening Level Concentrations for DNAN and NTO

Draft Eco-SSL concentrations were developed as geometric means of EC20 values for reproduction endpoints determined in weathered-and-aged treatments and are based on analytically determined DNAN or NTO concentrations in SSL soil. Soil invertebrate toxicity benchmarks used for the derivation of the soil invertebrate-based draft Eco-SSL concentrations for DNAN, and NTO are presented in Tables 35 and 36. A total of four soil invertebrate toxicity benchmarks developed in the present studies were utilized to derive draft Eco-SSL for DNAN, and three toxicity benchmarks were utilized to derive draft Eco-SSL for NTO. These toxicity benchmarks yielded the Eco-SSL values of 46 and 53 mg/kg, respectively for DNAN and NTO (Tables 35 and 36).

Table 35. Derivation of Draft Eco-SSL Values for DNAN Weathered-And-Aged in Sassafras Sandy Loam (SSL) Soil Using Reproduction Benchmarks for Earthworm *Eisenia andrei*, Potworm *Enchytraeus crypticus,* and Collembolan *Folsomia candida*.

Receptor Group	Soil	EC20 (mg/kg)	95% Confidence Internals (mg/kg)	Draft Eco-SSL (mg/kg)
Earthworm				
Cocoon production	SSL	55	43–68	
Juvenile production	SSL	42	26–58	
Potworm				46
Juvenile production	SSL	70	46–93	
Collembola				
Juvenile production	SSL	27	20–33	

Table 36. Derivation of Draft Eco-SSL Values for NTO Weathered-And-Aged in Sassafras Sandy Loam (SSL) Soil Using Reproduction Benchmarks for Earthworm *Eisenia andrei*, Potworm *Enchytraeus crypticus,* and Collembolan *Folsomia candida*.

Receptor Group	Soil	EC20 (mg/kg)	95% Confidence Internals (mg/kg)	Draft Eco-SSL (mg/kg)
Earthworm				
Cocoon production	SSL	145	77–213	53
Potworm				
Juvenile production	SSL	16	8–24	
Collembola				
Juvenile production	SSL	63	32–95	

4. DISCUSSION

4.1 Draft Ecological Soil Screening Levels

Generating toxicity data to establish benchmarks that are appropriate for use when deriving the soil invertebrate-based draft Eco-SSLs for DNAN and NTO was among the main objectives of the present studies. Ecotoxicological testing in those studies was specifically designed to meet the criteria for Eco-SSL derivation outlined in the Eco-SSL Guideline (USEPA, 2005). The draft Eco-SSL values detailed in this report were derived utilizing EC20 benchmark values for EM effects on soil invertebrate reproduction. These measurement endpoints were determined from standardized toxicity tests. The preference for reproduction benchmarks and for a low effect level (*i.e.*, EC20) was justified to ensure that Eco-SSL values would be protective of populations of the majority of ecological receptors in soil. The Eco-SSL values would also provide confidence that IM compound concentrations posing an unacceptable risk were not screened out early in the ecological risk assessment (ERA) process (*i.e*., SLERA). A review of the ecotoxicological benchmarks shows that Eco-SSL requirements, including the selection and use of reproduction effects and the EC20 response level, were well justified. Reproduction measurement endpoints were more sensitive (or not statistically different, based on the EC20 values and corresponding 95% CI) compared with adult survival in the soil invertebrate tests.

The inclusion of species from different taxonomic groups, representing a range of sensitivities, was an important consideration for selecting the test battery for Eco-SSL development because the respective sensitivities often correlated with physiologically determined modes of toxic action, and can vary among taxa. The selected species were expected to represent the spectrum of diverse ecological functions that are attributed to organisms comprising different functional groups of soil invertebrates. Test species selected for the studies were representative surrogates of species that normally inhabit a wide range of site soils and geographical areas (*i.e*., the species are ecologically relevant). The exposure focused on ingestion of IM-contaminated soil and direct-contact exposures. These exposures were considered under conditions of very high relative bioavailability of DNAN or NTO in SSL soil. The soil invertebrate species tested are sensitive to a wide range of contaminants, and represent different

routes of exposure (*e.g.*, ingestion, inhalation, dermal absorption within the soil). Finally, selected terrestrial toxicity tests with representative test species have been standardized and generate reproducible, statistically valid results. This imparts greater confidence in the data and generates less uncertainty that could be associated with the decisions and recommendations that are based on the test data. Both of these are important factors for draft Eco-SSL development.

The draft Eco-SSLs are intentionally conservative to provide confidence that potential contaminants that present an unacceptable risk are not screened out early in the SLERA process. The conservative nature of Eco-SSLs developed in this report for DNAN and NTO was achieved by

1. utilizing natural soils with properties that supported high relative bioavailability of these IMs to ecologically relevant test species
2. using reproduction measurement endpoints for benchmark derivation
3. relying on a low effect level (EC20; 20% reduction from carrier control) on respective measurement endpoints
4. using the geometric mean of the respective benchmarks to establish an Eco-SSL value (*i.e.*, more conservative than an arithmetic mean)

Conservative yet realistic soil-screening values protective of these receptor groups were derived. It was assumed that when these respective DNAN and NTO Eco-SSL values for soil invertebrates are used in conjunction with corresponding values developed for terrestrial plant, avian and mammalian wildlife, the terrestrial ecosystem will be protected from unacceptable adverse effects associated with upland aerobic soil that is contaminated with DNAN and NTO.

These draft Eco-SSLs are applicable to all sites where key soil parameters fall within a certain range of chemical and physical parameters (USEPA, 2005). They apply to upland aerobic soils where the pH is ≥ 4.0 and ≤ 8.5 and the organic matter content is ≤10%. Ecotoxicological benchmarks, utilized in this report for developing draft Eco-SSLs for DNAN and NTO, were established in toxicity studies utilizing natural soil, Sassafras sandy loam, that met the criteria for Eco-SSL development, in large part, because it is an aerobic upland soil that has characteristics (low organic matter and clay content; Table 1) supporting the very high relative bioavailability of IMs. This was necessary to ensure that the draft Eco-SSLs for soil invertebrates developed in this project were adequately conservative for a broad range of soils within the specified boundary conditions (USEPA, 2005).

Additional soil invertebrate toxicity studies were conducted using exposures in Webster clay loam. The qualitative relative bioavailability (QRB) score for organic chemicals in natural soils was considered “medium” for WCL soil, according to the Eco-SSL criteria (USEPA, 2005), thus WCL soil was hypothesized to pose a lower exposure risk for soil invertebrates compared with the risk in SSL soil. Results of the present studies showed that the EC50 values for either DNAN or NTO were greater (lower toxicity) in WCL in tests with potworms, and for DNAN in tests with earthworms, while the toxicity of either DNAN or NTO

to collembolans or of NTO to earthworms was not significantly (based on EC50 values and corresponding 95% CI) different between the two soils. Therefore, studies with WCL soil confirmed that toxicity benchmarks developed using SSL soil, which supports very high relative bioavailability of IM compounds, are sufficiently conservative for use in SLERA.

Derivation of Eco-SSL values prioritizes ecotoxicological benchmarks based on measured soil concentrations of a chemical over those based on nominal concentrations (USEPA, 2005). The exposure concentrations of DNAN and NTO in soil were analytically determined in all definitive tests from which benchmarks were determined, reported, and utilized in the derivation of draft Eco-SSL values included in this report. Furthermore, for the draft Eco-SSL development, special consideration was given to the inclusion of weathering-and-aging of contaminant explosives in soil in the assessment of the IM effects on terrestrial receptors. Consequently, ecotoxicological benchmarks for DNAN and NTO, each independently weathered-and-aged in SSL soil, more closely approximated the exposure conditions in the field, compared to benchmarks established in studies with freshly amended soil.

To ensure that draft Eco-SSL values developed in this report comply with all criteria and would obtain the highest score in each selection criteria category, experimental designs of toxicity tests used to establish the respective benchmarks for DNAN and NTO were evaluated using the literature evaluation criteria accepted by the Eco-SSL Workgroup (USEPA, 2005), summarized in Table 39. Such review will expedite the transition of the results of these investigations and the derivations of the respective DNAN and NTO draft Eco-SSL values to the USEPA Eco-SSL workgroup.

Table 37. Summary of Literature Evaluation Process for Plant and Soil Invertebrate Eco-SSLs (Modified from USEPA, 2005)

Criteria	Rationale
1: Testing is performed under conditions of high bioavailability.	The bioavailability of metals and polar organic compounds is influenced by pH and soil organic matter, cationic exchange capacity, and clay content. The scoring is intended to favor relatively high bioavailability.
2 (A) Laboratory and **(B)** field: Experimental designs for studies are documented and appropriate.	Experimental design can significantly influence the quality of a study. Higher quality studies will use an experimental design that is sufficiently robust to allow the analysis of the test variables and discriminate nontreatment effects.
3: Concentration of test substance in the soil is reported.	The concentration of the contaminant tested must be reported unambiguously.
4: Control responses are acceptable**.**	Negative controls are critical to distinguish treatment effects from nontreatment effects.
5: Chronic or life cycle test was used**.**	Chronic toxicity tests assessing long-term adverse sublethal impacts on the life-cycle phases of an organism are considered superior to acute toxicity tests.

Criteria	Rationale
6: Contaminant dosing procedure is reported and appropriate for contaminant and test.	Contaminant dosing procedure may affect the outcome of a test. The dosing procedure should include: (A) The form of the contaminant; (B) The carrier or vehicle (e.g., solvent, water, etc.); (C) How the carrier was dealt with following dosing (i.e., allowed to volatilize, controls, etc.); and (D) procedure for mixing of soil with a contaminant (homogeneity).
7: A dose-response relationship is reported or can be established from reported data.	Two methodologies that can be used to identify this benchmark concentration exist. The first method generates a no-observed effect concentration (NOEC) and a lowest-observed effect concentration (LOEC). The second method uses a statistical model to calculate a dose-response curve and estimate an effect concentration for some percentage of the population (EFCx), usually between an EC5 and an EC50.
8: The statistical tests used to calculate the benchmark and the levels of significance are described.	Statistical tests and results reported in the study should be sufficient to determine the significance of the results.
9: The origin of the test organisms is described.	The results of a toxicity test can be influenced by the condition of the test organisms. Culture conditions should be maintained so that the organisms are healthy and have had no exposure above background to contamination before testing (invertebrates) or detailed information is provided about the seed stock (plants).

Information relevant to each criterion of the evaluation processes is summarized as follows:

1. Natural soil, SSL (fine-loamy, siliceous, mesic Typic Hapludult) was used in the present studies to assess the IM compound toxicity for the chosen test species. This soil was selected for developing ecotoxicological values protective of soil biota because it is an upland aerobic soil that has physical and chemical characteristics supporting the very high relative bioavailability of DNAN and NTO (USEPA, 2005).

2. Toxicity assays were conducted to determine the effects of DNAN or NTO on soil invertebrates. Testing was designed to specifically meet the criteria required for Eco-SSL development. All the methods used were documented within the cited publications and included detailed accounts of individual studies. Studies included range finding tests to bracket DNAN or NTO concentrations for each test species and definitive tests to determine ecotoxicological benchmarks required for the development of draft Eco-SSL values.

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

4. Each toxicity test was appropriately replicated and included negative (no chemicals added), positive (reference chemical, boric acid), and carrier (ACN – in tests with DNAN or methanol – in tests with NTO) controls. Test validity criteria were met in all the definitive assays. Validity criteria for negative controls in the definitive toxicity tests with soil invertebrates specified the minimal percent adult survival, the minimal number of juveniles produced, and the boundaries for a coefficient of variation for reproduction. Toxicity tests with boric acid were conducted in SSL soil to obtain EC50 values and the corresponding 95% confidence limits (CL). All resulting EC50 values were within both the Warning Limits and the 95% CL that were established for the soil invertebrate test species cultures in tests with boric acid. These results confirmed that the condition of the test species cultures met the validity requirements of the test protocols.

5. Toxicity tests were based on the assessments of IM compound effects on reproduction. Although not utilized in the derivation of Eco-SSL values, the additional endpoints for adult survival were determined for comparison to the published reports of acute measurement endpoints.

6. Soil amendment procedures were documented to include the form of the IM compounds used, the analytical purity of each compound, procedures for the preparation of treatment concentrations using acetone or methanol carrier, the time allowed to volatilize carrier in the chemical hood, and the duration of soil mixing to ensure the homogeneity of IM compound incorporation in test soil.

7. Toxicity data were analyzed using appropriate regression models to establish concentration–response relationships for each IM compound–test species measurement endpoint pairing. SYSTAT software version 13 (Systat Inc., San Jose, CA) was used to determine the EC20 (and EC50 values) for cocoon/juvenile production in the soil invertebrate assays. The EC20 benchmark is preferred for deriving Eco-SSL values. The EC50, a commonly reported benchmark, was included to enable comparisons of the results produced in this study with results reported by other researchers.

8. The statistical tests included regression analyses and analysis of variance (ANOVA). Regression analyses were performed using SYSTAT software, version 13. 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 appropriate mathematical models. The asymptotic standard error (a.s.e.) and 95% confidence intervals (CI) associated with the point estimates were determined. Mathematically modeled concentration–response relationships are preferred for establishing benchmarks for use in Eco-SSL derivation (USEPA, 2005) and were utilized to derive the draft Eco-SSL values in this report. ANOVA was used to determine the bounded (when possible) NOEC and LOEC values. Mean separations were done using Fisher's-least-significant-difference (FLSD) pairwise comparison tests. A significance level of $p \leq 0.05$ was accepted for determining the NOEC and LOEC values.

9. Soil invertebrates used in toxicity assays came from cultures maintained by US Army DEVCOM Chemical Biological Center.

A review of the information provided for each criterion showed that the experimental design of the ecotoxicological investigations complied with all the screening criteria used by the Eco-SSL workgroup during the literature evaluation processes for selecting or developing soil invertebrate benchmarks for deriving Eco-SSL values. Benchmark data and draft Eco-SSL values developed in these studies will be submitted to the USEPA Eco-SSL workgroup for quality control review, determination of benchmarks to include in the Eco-SSL database, and for use in deriving soil invertebrate-based Eco-SSLs for DNAN and NTO, respectively. Following acceptance by the USEPA, these Eco-SSL values will be made available for use in ecological risk assessment of terrestrial habitats at military testing and training sites.

4.2 Accumulation Potential for DNAN and NTO from Soil to Plants

The present studies evaluated the potential for uptake of DNAN, and NTO into ryegrass shoots and roots using a natural soil, Sassafras sandy loam, which has low organic matter and clay contents. These soil characteristics ensured that ryegrass exposures to DNAN or NTO were conducted under conditions of very high relative bioavailability for organic chemicals (USEPA 2005). The 21-day range finding studies showed that trace amounts of DNAN, 2-ANAN, and 4-ANAN were detected in the ryegrass shoots and roots. This was consistent with findings by Dodard et al. (2013) who reported the presence of DNAN and 2-ANAN in ryegrass roots. Uptake of DNAN was also reported in the roots and shoots of a mixture of grasses, including big bluestem, Indiangrass, and switchgrass (Richard and Weidhass, 2014). The results of the preliminary studies established the BCFs and TFs < 1 for either DNAN or 2-ANAN, indicating a low potential for bioconcentration in roots and translocation from the roots into the shoots. The range finding studies showed that NTO was not detected in ryegrass roots and shoots. Similarly, Richard and Weidhaas (2014) reported that NTO was not detected in the roots and shoots. Therefore, the uptake of NTO was not evaluated further in the definitive tests.

Extending the exposures to 42 d revealed increased uptake of DNAN or 2-ANAN in the ryegrass roots and shoots, although the BCF values for the roots remained < 1. However, the BCF for 2-ANAN and the calculated TF for DNAN or 2-ANAN in the shoots were > 1. This indicates that DNAN or 2-ANAN can readily translocate from the root to shoot tissues. Therefore, DNAN and 2-ANAN can potentially accumulate in ryegrass and consequently pose a low risk of exposure to DNAN or 2-ANAN for grazers of above-ground vegetation.

4.3 Accumulation Potential for DNAN and NTO from Soil to Terrestrial Invertebrates

Generating bioaccumulation factors in compliance with the regulatory guidance for Ecological Risk Assessment (ERA) was among the main objectives of the present studies. The BAF values presented in this report were determined from standardized bioaccumulation tests. The SumDNAN (sum of DNAN, 2-ANAN, and 4-ANAN concentrations) in soil decreased during the 14-d exposure period. The SumDNAN in the earthworm body residue increased until

day 3 and decreased thereafter. Between days 3 and 14, there was a 73% decrease in tissue uptake that was greater than the 23% decrease in the soil concentration, suggesting that part of the solvent-extractable DNAN in soil was not bioavailable. By day 14, DNAN accounted for only 45% of SumDNAN indicating substantial DNAN transformation in the presence of earthworms. The highest bioaccumulation factor (BAF; the tissue to soil concentration ratio) was 6.2 kg/kg (dry wt.) on day 3 and decreased to 3.8 kg/kg by day 14. Kinetic studies indicated a BAF of 2.3 kg/kg, based on the earthworm DNAN uptake rate of 2.0 kg/kg/d, compared to the SumDNAN elimination rate of 0.87 d^{-1} (half-life = 0.79 d). Lotufo et al. (2016) reported BAF values for four different soils that when converted to dry-wt-basis were overall in the same range as the BAF values derived for SSL in the present study (Table 40). The SumDNAN BAFs from the present study were also similar to those reported for TNT (5.0 kg/kg), 2-aminodinitrotoluene (5.1 kg/kg), and 4-aminodinitrotoluene (6.4 kg/kg) (Lachance et al. 2004).

The NTO concentration in amended soil decreased by 57% from the initial target concentration (800 mg NTO/kg dry soil) during 14 d, likely due to the formation of unknown transformation products. As expected, NTO bioaccumulation was negligible (BAF = 0.018 kg/kg dry wt.) considering its low hydrophobicity. These data indicate that the studied IM compounds have a lower potential to bioaccumulate from soil compared to conventional munition compounds, and therefore pose a lower ecotoxicological risk to upper trophic level organisms.

Table 38. SumDNAN Bioaccumulation Factors Determined As the Ratio of Tissue and Soil Concentrations in Various Amended Soils Based on Earthworm Body Residues Corrected For Soil in the Gut and Soil Concentrations at Exposure Termination

Soil type	BAF	Study
Memphis	6.6	Lotufo et al. 2016
Falaya	4.0	Lotufo et al. 2016
Sunev	4.3	Lotufo et al. 2016
Ruston	14.2	Lotufo et al. 2016
SSL	3.8	Present study

5. CONCLUSIONS

This project was undertaken specifically to develop scientifically defensible soil invertebrate-based benchmarks acceptable for deriving draft Eco-SSL values for DNAN and NTO. These draft Eco-SSL values were derived using the EC20 level toxicity benchmarks for the IM compounds effects on soil invertebrate reproduction endpoints determined in standardized toxicity tests. Ecotoxicological testing was specifically designed to meet the criteria for Eco-SSL derivation outlined in the Eco-SSL Guideline (USEPA, 2005). Following acceptance by the USEPA, these Eco-SSL values will allow screening of site-soil data during the Screening Level Ecological Risk Assessment (SLERA) to identify those IM compounds that are not of potential ecological concern and do not need to be considered in the Baseline Ecological Risk Assessment (BERA), resulting in significant cost-savings during site assessments. These Eco-SSLs will also provide an indispensable tool for the installation managers to gauge the

ecotoxicological impacts of military operations that involve the use of DNAN and NTO, thus ultimately promoting the sustainable use of testing and training ranges.

This project also determined that DNAN has a relatively low potential to bioaccumulate in soil invertebrates, while uptake of NTO into earthworm tissues was negligible. Bioconcentration of NTO in ryegrass was negligible or nonexistent. These data indicate that the studied IM compounds have a lower potential to bioaccumulate or bioconcentrate from soil compared to conventional munition compounds, and therefore pose a lower ecotoxicological risk to upper trophic level organisms.

LITERATURE CITED

American Society for Testing and Materials (ASTM). 2002. Standard Guide for Conducting Terrestrial Plant Toxicity Tests. Designation E 1963-02. American Society for Testing and Materials, West Conshohocken, PA.

American Society for Testing and Materials (ASTM). 2004. Standard specification for reagent water. In: *Book of ASTM Standards, Volume 11.01*, ASTM D 1193-99e1. American Society for Testing and Materials, Philadelphia, PA, USA, pp 116−118.

Amorim MJB, Kuperman R, Römbke J. 2009. Enchytraeid Reproduction Tests. In: *Ecotoxicological Characterization of Wastes*; Moser, H. and Römbke, J., Eds; Springer Science+Business Media: New York, pp 177–182.

Amorim MJ, Römbke J, Schallnass HJ, Soares AM. 2005a. Effect of Soil Properties and Aging on the Toxicity of Copper for *Enchytraeus albidus*, *Enchytraeus luxuriosus*, and *Folsomia candida*. *Environ. Toxicol. Chem.* 24, 1875–1885.

Amorim MJ, Römbke J, Schallnass HJ, Soares AM. 2005b. Effect of different soil types on the enchytraeids *Enchytraeus albidus* and *Enchytraeus luxuriosus* using the herbicide Phenmedipham. *Chemosphere*, 61, 1102–1114.

Arthur JD, Marka NW, Taylor S, Šimunek J, Brusseau ML, Dontsova KM. 2017. Batch soil adsorption and column transport studies of 2,4-dinitroanisole (DNAN) in soils. *J. Contam. Hydrology* 199, 14-23.

Dodard SG, Sarrazin M, Hawari J, Paquet L, Ampleman G, Thiboutot S, Sunahara GI. 2013. Ecotoxicological assessment of a high energetic and insensitive munitions compound: 2,4-Dinitroanisole (DNAN). *J. Hazard. Mater.* 262, 143-150.

Dodard S, Sunahara GI, Sarrazin M, Gong P, Kuperman RG, Ampleman G, Thiboutot S, Hawari J. 2005. Survival and reproduction of enchytraeid worms (Oligochaeta) in different soil types amended with cyclic nitramine explosives. *Environ. Toxicol. Chem.* 24, 2579-2587.

Dontsova KM, Hayes C, Pennington JC, Porter B. 2009. Sorption of high explosives to water-dispersible clay: Influence of organic carbon, aluminosilicate clay, and extractable iron. *J. Environ. Qual*. 38, 1458–1465.

Environment Canada (EC). 2005. Guidance Document on Statistical Methods for Environmental Toxicity Tests. Environment Canada report EPS 1/RM/46.

Gong P, Donohue KB, Mayo AM, Wang Y, Hong H, Wilbanks MS, Barker ND, Guan X & Gust KA. 2018. Comparative toxicogenomics of three insensitive munitions constituents 2,4-dinitroanisole, nitroguanidine and nitrotriazolone in the soil nematode *Caenorhabditis elegans*. *BMC Systems Biology* 12, 92.

Gust KA, Chaitankar V, Ghosh P, Wilbanks MS, Chen X, Barker ND, Pham D, Scanlan LD, Rawat A, Talent LG. 2018. Multiple environmental stressors induce complex transcriptomic responses indicative of phenotypic outcomes in Western fence lizard. *BMC Genomics* 19, 877.

Hawari J, Sunahara GI, Perreault N, Halasz A, Paquet L, Dodard S, Sarrazin M, Savard K. 2014. Environmental Fate and Ecological Impact of Emerging Energetic Chemicals (ADN, DNAN

and its Amino-Derivatives, PETN, NTO, NQ, FOX-7, and FOX-12) and an Insensitive Formulation, DRDC-RDDC-2014-C178, National Research Council, Energy Mine and Environment, Ottawa, Ontario, Canada, 2014, https://apps.dtic.mil/dtic/tr/fulltext/u2/1017629.pdf.

International Organization for Standardization (ISO). 1998. Soil Quality – Effects of Pollutants on Earthworms *(Eisenia fetida)* – Part 2: Determination of Effects on Reproduction. ISO 11268-2. Genève, Suisse.

International Organization for Standardization (ISO). 1999. Soil Quality – Inhibition of Reproduction of Collembola (*Folsomia candida*) by Soil. ISO 11267. Genève, Suisse.

International Organization for Standardization (ISO). 2004. Soil Quality – Effects of Pollutants on Enchytraeidae (*Enchytraeus* sp.) – Determinations of Effects on Reproduction and Survival. ISO/CD 16387. Genève, Suisse.

Jaenig F. 2006. Sorption phenomena of nitroaromatic compounds in geochemical variable soils represented on the basis of column tests under in-situ conditions. *WIT Trans. Ecol. Environ*, 95, 213-222.

Kennedy AJ, Laird JG, Lounds C, Gong P, Barker ND, Brasfield SM, Russell AL, Johnson MS. 2015. Inter- and intraspecies chemical sensitivity: A case study using 2,4-dinitroanisole. *Environ. Toxicol. Chem.* 34, 402-411.

Kennedy AJ, Poda AR, Melby NL, Moores LC, Jordan SM, Gust KA, Bednar AJ. 2017. Aquatic toxicity of photo-degraded insensitive munition 101 (IMX-101) constituents. *Environ. Toxicol. Chem.* 36, 2050-2057.

Kuperman RG, Checkai RT, Simini M, Phillips CT, Kolakowski JE, Lanno R. 2013. Soil properties affect the toxicities of TNT and RDX to the enchytraeid worm, *Enchytraeus crypticus*. *Environ. Toxicol. Chem*. 32, 2648-2659.

Kuperman RG, Checkai RT, Simini M, Phillips CT, Kolakowski JE, Kurnas CW. 2006a. Toxicities of dinitrotoluenes and trinitrobenzene freshly amended or weathered and aged in a sandy loam soil to *Enchytraeus crypticus. Environ. Toxicol. Chem.* 25, 1368-1375.

Kuperman RG, Checkai RT, Simini M, Phillips CT, Anthony JS, Kolakowski JE, Davis EA. 2006b. Toxicity of emerging energetic soil contaminant CL-20 to potworm *Enchytraeus crypticus* in freshly amended or weathered and aged treatments. *Chemosphere* 62, 1282-1293.

Kuperman RG, Checkai RT, Simini M, Phillips CT, Kolakowski JE, Kurnas CW. 2005. Weathering and aging of 2,4,6-trinitrotoluene in soil increases toxicity to potworm *Enchytraeus crypticus. Environ. Toxicol. Chem.* 24, 2509-2518.

Kuperman RG, Checkai RT, Simini M, Phillips CT. 2004. Manganese toxicity in soil for *Eisenia fetida*, *Enchytraeus crypticus* (Oligochaeta), and *Folsomia candida* (Collembola). *Ecotoxicol. Environ. Saf.* 57, 48-53.

Kuperman RG, Checkai RT, Simini M, Phillips CT, Kolakowski JE, Kurnas CW, Sunahara GI. 2003. Survival and reproduction of *Enchytraeus crypticus* (Oligochaeta, Enchytraeidae) in a natural sandy loam soil amended with the nitro-heterocyclic explosives RDX and HMX. *Pedobiologia* 47, 651-656.

Kuperman R, Simini M, Phillips C, Checkai R. 1999. Comparison of malathion toxicity using enchytraeid reproduction test and earthworm toxicity test in different soil types. *Pedobiologia* 43, 630-634.

Lachance B, Renoux AY, Sarrazin M, Hawari J, Sunahara GI. 2004. Toxicity and bioaccumulation of reduced TNT metabolites in the earthworm *Eisenia andrei* exposed to amended forest soil. *Chemosphere* 55, 1339-1348.

Lotufo GR, Coleman JG, Harmon AR, Chappell MA, Bednar AJ, Russell AL, Smith JC, Brasfield SM. 2016. Accumulation of 2,4-dinitroanisole in earthworm *Eisenia fetida* from chemically spiked and aged natural soils. *Environ. Toxicol. Chem*. 35, 1835-1842.

Lotufo GR, Biedenbach JM, Sims JG, Chappell P, Stanley JK, Gust KA. 2015. Bioaccumulation kinetics of the conventional energetics TNT and RDX relative to insensitive munitions constituents DNAN and NTO in *Rana pipiens* tadpoles. *Environ. Toxicol. Chem*. 34, 880-886.

Madeira CL, Field JA, Simonich MT, Tanguay RL, Chorover J & Sierra-Alvarez R (2018) Ecotoxicity of the insensitive munitions compound 3-nitro-1,2,4-triazol-5-one (NTO) and its reduced metabolite 3-amino-1,2,4-triazol-5-one (ATO). *J. Haz. Mater*. 343, 340-346.

Mark N, Arthur J, Dontsova K, Brusseau M, Taylor S, Simunek J. 2017. Column transport studies of 3-nitro-1,2,4-triazol-5-one (NTO) in soils. *Chemosphere* 171, 427-434.

Organization for Economic Co-operation and Development guidelines (OECD). 1984. *Earthworm, acute toxicity test*, Test Guideline No. 207, Guidelines for the testing of chemicals, OECD, Paris.

Pennington JC, Brannon JM. 2002. Environmental fate of explosives. *Thermochim. Acta*, 384, 163–172.

Pillard DA, Eck WS, Johnson MS & Packard S 2017. Effects of 3-nitro-1,2,4-triazol-5-one on survival, growth and metamorphosis in the northern leopard frog, *Lithobates pipiens*. *Ecotoxicology* 26, 1170-1180.

Powell IJ (2016) Insensitive Munitions – Design principles and technology developments. *Propellants, Explosives, Pyrotechnics* 41, 409-413.

Richard T, Weidhaas J. 2014. Dissolution, sorption, and phytoremediation of IMX-101 explosive formulation constituents: 2,4-dinitroanisole (DNAN), 3-nitro-1,2,4-triazol-5-one (NTO), and nitroguanidine. *J. Hazard. Mater.* 280, 561-569.

Rocheleau S, Lachance B, Kuperman R, Hawari J, Thiboutot S, Ampleman G, Sunahara G. 2014. Toxicity and uptake of cyclic nitramine explosives in ryegrass *Lolium perenne*. *Environ. Pollut*. 156, 199-206.

Rylott EL, Bruce NC. 2019. Right on target: using plants and microbes to remediate explosives. *Int. J. Phytoremediation*. 21, 1051-1064.

Simini M, Checkai RT, Kuperman RG, Phillips CT, Kolakowski JE, Kurnas CW. 2013. Toxicities of TNT and RDX to the earthworm *Eisenia fetida* in five soils with contrasting characteristics. Technical Report No. ECBC-TR-1090, U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD.

Simini M, Checkai RT, Kuperman RG, Phillips CT, Kolakowski JE, Kurnas CW, Sunahara GI. 2003. Reproduction and survival of *Eisenia fetida* in a sandy loam soil amended with the nitro-heterocyclic explosives RDX and HMX. *Pedobiologia* 47, 657-662.

Singh N, Berns AE, Hennecke D, Hoerner J, Koerdel W, Schaeffer A. 2010. Effect of soil organic matter chemistry on sorption of trinitrotoluene and 2,4-dinitrotoluene. *J. Hazard. Mat.* 173, 343-348.

Singh N, Hennecke D, Hoerner J, Koerdel W, Schaeffer A. 2008. Sorption–desorption of trinitrotoluene in soils: effect of saturating metal cations. *Bull. Environ. Contam. Toxicol.* 80, 443-446.

Stanley JK, Lotufo GR, Biedenbach JM, Chappell P, Gust KA. 2015. Toxicity of the conventional energetics TNT and RDX relative to new insensitive munitions constituents DNAN and NTO in *Rana pipiens* tadpoles. *Environ. Toxicol. Chem*. 34, 873-879.

Taylor S, Walsh ME, Becher JB, Ringelberg DB, Mannes PZ, Gribble GW. 2017a. Photo-degradation of 2,4-dinitroanisole (DNAN): An emerging munitions compound. *Chemosphere* 167, 193-203.

Taylor S, Dontsova K, Walsh M. 2017b. Insensitive Munitions Formulations: Their dissolution and fate in soils. In Shukla MK, Boddu VM, Steevens JA, Damavarapu R, Leszczynski J. eds, *Energetic Materials: From Cradle to Grave*. Springer International, pp. 407-443.

Toghiani R, Toghiani H, Maloney S & Boddu V 2010. Prediction of physicochemical properties of energetic materials. In Boddu V, Redner P. eds, *Energetic Materials*, CRC Press, pp. 171-198.

United States Environmental Protection Agency (USEPA). 1996. Ecological Effects Test Guidelines, Early Seedling Growth Toxicity Test. EPA 712–C–96–347, OPPTS 850.4230, Public Draft; Prevention, Pesticides and Toxic Substances (7101), Washington, DC.

United States Environmental Protection Agency (USEPA). 2005. Guidance for Developing Ecological Soil Screening Levels. Directive 9285.7-55. Office of Solid Waste and Emergency Response, Washington, DC.

Viswanath DS, Ghosh TK & Boddu VM, eds. 2018. *Emerging Energetic Materials: Synthesis, Physicochemical, and Detonation Properties*. Springer Netherlands.

Blank

ACRONYMS AND ABBREVIATIONS

Abbreviation	Full name
1,3-DNB	1,3-dinitrobenzene
2-ANAN	2-Amino-4-nitroanisole
4-ANAN	4-Amino-2-nitroanisole
ACN	Acetonitrile
ACS	American Chemical Society
ANOVA	Analysis of Variance
ASTM	American Society for Testing and Materials
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
BDL	Below Detection Limit
BERA	Baseline Ecological Risk Assessment
CAS	Chemical Abstracts Service
CBC	Chemical Biological Center
CC	carrier control
CEC	Cation Exchange Capacity
CI	Confidence intervals
CL	Confidence limits
CV	Coefficient of variation
DAD	Diode Array Detection
DNAN	2,4-dinitroanisole
EC	Environment Canada
EC20	Effective concentration producing a 20% effect
EC50	The median effective concentration
Eco-SSL	Ecological Soil Screening Level
ECp	Effective concentration for a specified “p” percent effect
EM	Energetic materials
ERA	Ecological risk assessment
FA	Freshly amended treatment of soil
FLSD	Fisher’s Least Significant Difference
GC-MS	Gas chromatography/mass spectroscopy
HPLC	High-performance liquid chromatography
HPLC/MS	High-performance liquid chromatography/mass spectroscopy
IM	Insensitive munitions
IMX	Insensitive Munition eXplosive
ISO	International Organization for Standardization
K_{ow}	Octanol-water partition coefficient
L. perenne	Ryegrass *Lolium perenne*
LCS	Laboratory control sample
LOD	limit of detection
LOEC	Lowest Observed Effect Concentration
LOQ	limit of quantitation

MS	matrix spike
MSD	matrix spike duplicate
ND	Not Detected or Not Determined
NOEC	No Observed Effect Concentration
NQ	Nitroguanidine
NS	No sample produced to analyze
NTO	3-nitro-1,2,4-triazole-5-one, an insensitive munition
OECD	Organization for Economic Co-operation and Development
PAR	Photosynthetically active radiation
PTFE	Polytetrafluoroethylene
QA/QC	Quality assurance/Quality Control
QRB	Qualitative relative bioavailability
R^2	Coefficient of determination
RDM	root dry mass
RDX	Hexahydro-1,3,5-trinitro-1,3,5-triazine (Royal Demolition Explosive)
RH	Relative Humidity
rpm	revolutions per minute
SD	standard deviation
SDM	shoot dry mass
SE	standard error
SERDP	Strategic Environmental Research and Development Program
SLERA	Screening Level Ecological Risk Assessment
SSL	Sassafras sandy loam
TF	Translocation factor
TFA	trifluoroacetic acid
TNB	1,3,5-Trinitrobenzene
TNT	Trinitrotoluene
UV	Ultraviolet
USEPA	U.S. Environmental Protection Agency
W-A	Weathered-and-aged amendment of soil
WCL	Webster clay loam
WHC	Water holding capacity
wt	Weight

Blank

APPENDIX A

Accumulation of Insensitive Munitions Compounds in the Earthworm *Eisenia andrei* from Amended Soil

Environmental Toxicology and Chemistry—Volume 40, Number 6—pp. 1713–1725, 2021
Received: 30 November 2020 | Revised: 11 January 2021 | Accepted: 26 February 2021

Environmental Toxicology

Accumulation of Insensitive Munition Compounds in the Earthworm *Eisenia andrei* from Amended Soil: Methodological Considerations for Determination of Bioaccumulation Factors

G.R. Lotufo,[a,*] R.E. Boyd,[a] A.R. Harmon,[a] A.J. Bednar,[a] J.C. Smith,[a] M. Simini,[b] G.I. Sunahara,[c] J. Hawari,[d] and R.G. Kuperman[b]

[a]Environmental Laboratory, Engineer Research and Development Center, US Army, Vicksburg, Mississippi, USA
[b]US Army Chemical Biological Center, Aberdeen Proving Ground, Maryland, USA
[c]Department of Natural Resource Sciences, McGill University, Ste-Anne-de-Bellevue, QC, Canada
[d]Department of Civil, Geological and Mining Engineering, Polytechnique Montreal, Montreal QC, Canada

Abstract: The present study investigates the bioaccumulation of the insensitive munition compounds 2,4-dinitroanisole (DNAN) and 3-nitro-1,2,4-triazol-5-one (NTO), developed for future weapons systems to replace current munitions containing sensitive explosives. The earthworm *Eisenia andrei* was exposed to sublethal concentrations of DNAN or NTO amended in Sassafras sandy loam. Chemical analysis indicated that 2- and 4-amino-nitroanisole (2-ANAN and 4-ANAN, respectively) were formed in DNAN-amended soils. The SumDNAN (sum of DNAN, 2-ANAN, and 4-ANAN concentrations) in soil decreased by 40% during the 14-d exposure period. The SumDNAN in the earthworm body residue increased until day 3 and decreased thereafter. Between days 3 and 14, there was a 73% decrease in tissue uptake that was greater than the 23% decrease in the soil concentration, suggesting that the bioavailable fraction may have decreased over time. By day 14, the DNAN concentration accounted for only 45% of the SumDNAN soil concentration, indicating substantial DNAN transformation in the presence of earthworms. The highest bioaccumulation factor (BAF; the tissue-to-soil concentration ratio) was 6.2 ± 1.0 kg/kg (dry wt) on day 3 and decreased to 3.8 ± 0.8 kg/kg by day 14. Kinetic studies indicated a BAF of 2.3 kg/kg, based on the earthworm DNAN uptake rate of 2.0 ± 0.24 kg/kg/d, compared with the SumDNAN elimination rate of 0.87 d^{-1} (half-life = 0.79 d). The compound DNAN has a similar potential to bioaccumulate from soil compared with trinitrotoluene. The NTO concentration in amended soil decreased by 57% from the initial concentration (837 mg NTO/kg dry soil) during 14 d, likely due to the formation of unknown transformation products. The bioaccumulation of NTO was negligible (BAF $\leq$ 0.018 kg/kg dry wt). *Environ Toxicol Chem* 2021;40:1713–1725.

Keywords: Bioaccumulation; Contaminants of emerging concern; Earthworms; Soil ecotoxicology; Toxicokinetics; Dinitroanisole

INTRODUCTION

Unintended detonation of munitions and munition stockpiles has caused losses of human life, infrastructure, and materiel. The US Department of Defense, therefore, has a stated goal to replace traditional munitions with insensitive munitions that are chemically stable enough to withstand mechanical damage, fire, and the impact of projectiles during combat operations, yet still perform as required (Powell 2016). During live-fire training, insensitive munitions are scattered by partial detonations and may weather and dissolve once on the soil surface (Taylor et al. 2017a). Although information on concentrations of insensitive munition compounds in natural soils is not currently publicly available, and no real-world exposure information can be presently inferred, the manufacturing and use practices of the materials coupled with the chemical properties conducive to environmental transport warrant further investigation. The presence of contaminants such as insensitive munition compounds in soils may result in toxicity to soil invertebrates. In addition, assessing the bioaccumulation of contaminants from soils into soil invertebrates

This article includes online-only Supplemental Data.
* Address correspondence to Guilherme.Lotufo@usace.army.mil
Published online 1 March 2021 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.5028

is crucial to determining the potential for chemical transfer to higher trophic-level organisms and exposure risk to wildlife (Sample et al. 2014; Hoke et al. 2016).

The insensitive munition compound 2,4-dinitroanisole (DNAN) was historically used as an explosive in warheads containing Amatol 40 and is being investigated as a substitute for 2,4,6-trinitrotoluene (TNT) in insensitive munition formulations (Viswanath et al. 2018). It is a nitroaromatic compound with physicochemical properties, such as solubility in water and hydrophobicity, similar to those of TNT (Viswanath et al. 2018). The compound DNAN also has the potential to undergo photochemical transformation (Taylor et al. 2017a). It is toxic to a wide variety of aquatic species (Dodard et al. 2013; Kennedy et al. 2015; Stanley et al. 2015; Gust et al. 2018; Lotufo et al. 2018) and to soil invertebrates (Dodard et al. 2013; Gong et al. 2018). Lotufo et al. (2016) conducted bioaccumulation studies involving the uptake of DNAN by earthworms in amended soil and showed that the DNAN concentration in tissue exceeded the concentration of DNAN in soil. No published information was found on the elimination kinetics of DNAN.

The insensitive munition compound 3-nitro-1,2,4-triazol-5-one (NTO) has been used since the 1980s in multiple energetic formulations. It has excellent qualities as an explosive such as insensitivity and thermal and mechanical stability and is considered a potential insensitive replacement for hexahydro-1,3,5-triazine (RDX) in various formulations (Viswanath et al. 2018). It has relatively low toxicity to tadpoles, fish, aquatic invertebrates, and soil nematodes and was found to be considerably less toxic than TNT and DNAN in comparative studies (Stanley et al. 2015; Kennedy et al. 2017; Pillard et al. 2017; Gong et al. 2018; Gust et al. 2018; Lotufo et al. 2018; Madeira et al. 2018). The low log octanol–water partition coefficent K_{OW} values predicted for NTO (from 0.89 to −1.19; Toghiani et al. 2008) indicate a low propensity to adsorb to organic C through hydrophobic interactions. It adsorbs very weakly to soils (Mark et al. 2017), as expected for a negatively charged compound in a matrix that also possesses a net negative charge, and the organic C content in soils has been shown not to be a good predictor of NTO adsorption (Taylor et al. 2017b). Hawari et al. (2014) reported that earthworms accumulated NTO from amended soil at lower concentrations than those found in the soil. Other studies have shown that NTO was not detected in plant roots and shoots after exposure to amended soils (Richard and Weidhaas 2014).

The objective of the present study was to investigate the soil bioaccumulation potential and the uptake and elimination kinetics of sublethal concentrations of DNAN and NTO in amended natural soil using the earthworm *Eisenia andrei*. Earlier soil uptake experiments with *Eisenia fetida*, closely related and frequently misidentified as *E. andrei* and vice versa (Rombke et al. 2016), have suggested that the elimination rate of DNAN in earthworms is fast (i.e., elimination half-life as short as 9 h; Lotufo et al. 2016). Therefore, the recommended overnight (Organisation for Economic Co-operation and Development 2010) or 24-h (ASTM International 2012) duration for gut content purging of soil-exposed earthworms prior to tissue concentration determination would result in substantial loss of accumulated DNAN. Consequently, the present study investigated the influence of 24-h gut-purging on the assessment of insensitive munition compound bioaccumulation following soil exposures.

MATERIALS AND METHODS

Insensitive munition compounds and natural soil

Military-grade DNAN (CAS no. 119-27-7) and NTO (CAS no. 932-64-9) were obtained as a dry yellow powder from the Holston Army Ammunition Plant (BAE Systems; estimated purity >95%) and used without further purification. Sassafras sandy loam (SSL) soil, a fine-loamy, siliceous, semi-active, mesic Typic Hapludult (Supplemental Data, Table S1), was collected from an open grassland field in the coastal plain on the property of the US Army Aberdeen Proving Ground (MD, USA).

Culturing and handling of earthworms

Adult earthworms (*E. andrei*) obtained from the US Army Chemical Biological Center (Aberdeen Proving Ground) were used to establish the initial laboratory cultures. Earthworms were maintained in fiberglass trays (34 × 28 × 14 cm) containing a mixture of neutralized sphagnum peat moss and Miracle-Gro All Purpose Garden soil as culturing substrate. The trays were kept covered with plastic sheets to prevent drying. Moisture was monitored on a weekly basis and hydration was done with dechlorinated Vicksburg (MS, USA) municipal tap water using activated carbon as needed. The trays were maintained so that no standing water accumulated in the bottom and the surface of the bedding did not dry. The culture trays were held at 22 ± 1 (standard deviation [SD]) °C. The worms were fed dry fermented alfalfa once a week, which was sprinkled over the surface of the bedding and spritzed with dechlorinated tap water to add moisture. The amount of food needed was determined by how much food was consumed on a weekly basis; any remaining food was removed and discarded. The bedding was also turned by hand each week to inspect the general condition of the worms, remove any dead animals, and provide aeration.

Soil handling and amendments

Air-dried SSL soil was amended with DNAN or NTO at a target concentration of 50 or 800 mg/kg, respectively, following Simini et al. (2013). These concentrations were selected based on the absence of lethality in earlier exposure studies (Dodard et al. 2013; Hawari et al. 2014; Lotufo et al. 2016) and the detection limit optimization studies using tissue samples. For each insensitive munition compound or control, the appropriate mass of dry SSL soil (1.25 kg for the DNAN and NTO preliminary bioaccumulation experiment, and 7 kg for the DNAN uptake experiment) was spread into large rectangular containers to a depth of approximately 4 cm. The insensitive munition compounds were dissolved in acetone as carrier and

delivered over the soil surface, followed by thorough mixing with a spatula. The control treatment received carrier only. Although NTO is highly soluble in water, it was amended to soil dissolved in acetone so all treatments were spiked using a similar method. Moreover, the volume of water necessary to amend the soil with the appropriate mass of NTO would have taken much longer to evaporate compared with acetone. Approximately 100 and 500 mL of acetone was used to spike the DNAN and the control soil treatments for preliminary bioaccumulation and uptake experiments, respectively. Because of the poor solubility of NTO in carrier, a total of 500 mL of spiking solution was used, 100 mL at a time to spike 1.25 kg of soil for the preliminary bioaccumulation experiment. Carrier was allowed to volatilize between the steps. Following spiking and mixing, the carrier was allowed to volatize overnight under a fume hood at room temperature, in the dark. The amended soils were then placed into clear glass 4-L jars fit with air-tight lids and allowed to tumble for 18 h on a roller apparatus. After tumbling, soils were rehydrated by the stepwise addition (followed by mixing) of dechlorinated tap water to the total volume determined to restore the soil to 95% of the water-holding capacity (18% of dry SSL soil) prior to addition of test organisms. The pH remained within ±0.5 that measured for the unamended soil (Supplemental Data, Table S1). Control soil was prepared in the same manner using carrier only (500 mL). The amended soil was sampled in triplicate for chemical analysis.

Preliminary evaluation of DNAN and NTO bioaccumulation from soil and method optimization

Earthworm exposure studies using amended soil were conducted in 473-mL Mason-type glass jars with perforated lids containing 140 g of hydrated soil corresponding to 120 g of dry soil. Exposures were initiated within 24 h from hydrating the soils. In total, 6 exposure jars were set up for each soil treatment (DNAN, NTO, and control). All exposures were conducted at 22 °C under a 16:8-h light: dark photoperiod cycle. Earthworms with fully developed clitella and weighing from 0.3 to 0.6 g, depurated on moist filter paper for 24 h, were used. Postpurged earthworm weights were obtained, and 8 adult earthworms were added to each jar containing amended soil. Each jar received 2 g of dried fermented alfalfa at the start of the exposure. Earthworms were exposed to amended soils in triplicate jars for either 7 or 14 d for determining the optimal exposure duration for the uptake experiment. This exposure durations were based on apparent steady state in earthworms within 3 to 7 d in DNAN-amended soils (Lotufo et al. 2016).

Supplemental food has been used in bioaccumulation studies with energetic compounds (Lachance et al. 2004; Sarrazin et al. 2009) and is recommended for bioaccumulation studies using soil with low total organic carbon content (Organisation for Economic Co-operation and Development 2010). However, other standard guidance recommends that food not be added to test containers for tests ≤28 d in duration (ASTM International 2012). The impact of supplemental food on the bioaccumulation of insensitive munition compounds was evaluated using 3 additional replicate jars for the 14-d exposure for which supplemental food was not provided. A summary of the primary and secondary treatments used in the preliminary bioaccumulation experiment is provided in the Supplemental Data.

At each sampling time point, earthworms were removed by sorting the soils with hand forceps, and survival was recorded. Earthworms recovered from each jar were rinsed with dechlorinated tap water and blotted dry. For the DNAN and NTO treatments, worms from each replicate were handled as follows: 1) 2 earthworms were placed on moist filter paper for 24 h for emptying their gut content (see Savard et al. 2010; ASTM International 2012) and then frozen for chemical analysis; 2) 2 earthworms were immediately frozen for chemical analysis (i.e., not purged); and 3) 2 earthworms were immediately placed on preweighed aluminum pans for determination of the mass of soil remaining in the gut (Lotufo et al. 2016), for which earthworms were oven-dried at 60 °C for 24 h in preweighed pans. After this 24-h drying period, the samples were brought to room temperature in a desiccator and weighed to the nearest 0.01 mg to obtain the dry weight. The earthworms in the aluminum weigh boats were then placed in a muffle furnace at 550 °C for 4 h for calcination (loss of organic matter by ignition). The weigh boats were reweighed, and the mass of soil in the gut of earthworms was then determined by subtracting the weight of the empty pan.

The exposure soil was sampled on day 14 from each replicate jar. Tissue and soil samples were placed at −80 °C for at least 24 h, lyophilized, and stored frozen for later chemical analysis. Earthworms from each control replicate were handled as just described, with one additional handling step: 2 earthworms were placed on moist filter paper for 24 h for emptying their gut content and then oven-dried and incinerated for determination of the mass of soil remaining in the gut. A summary of the earthworm handling procedures at exposure termination is provided in the Supplemental Data.

Estimation of insensitive munition compound concentration in tissue of not-purged earthworms

The contributions of soil in the gut to the total body residue of insensitive munition compound measured in earthworm samples and the concentration of insensitive munition compound in earthworm tissue were estimated using 2 methods. The first method, hereafter termed "corrected," assumes that the concentration of insensitive munition compound in the gut soil is the same as the insensitive munition compound concentration in the exposure soil. This correction method was described by Stafford and McGrath (1986) and was used earlier in a DNAN bioaccumulation study (Lotufo et al. 2016). The second method assumes that insensitive munition compound in ingested soil is efficiently extracted into gut fluids and transferred to tissues, and therefore the insensitive munition

compound concentration in the gut soil is zero. The tissue concentration estimated using this method will be termed the "ash-free dry weight (AFDW) concentration" and was determined by subtracting the ash weight from the sample weight as follows. For each replicate, the mass of gut soil in the sample estimated using mean ash weight for the treatment was subtracted from the tissue sample dry weight for the calculation of the concentration of insensitive munition compound associated with tissues-only (C_t) in the sample using the following Equation 1:

$$C_t = \frac{\text{mass of sample} \times C_{t,\text{nonadjusted}}}{\text{mass of sample} - \text{estimated mass of gut soil in sample}} \quad (1)$$

where $C_{t,\text{nonadjusted}}$ is the insensitive munition compound concentration in the earthworm sample (mg/kg dry wt), and C_t is the estimated tissue-only insensitive munition compound concentration in earthworms (mg/kg dry wt).

Assessment of DNAN uptake kinetics

The preliminary experiment revealed negligible accumulation potential for NTO in earthworms, and is detailed in the *Results* section. Therefore, uptake kinetics were only evaluated for DNAN. Air-dried SSL soil was amended with DNAN at a target concentration of 50 mg/kg, rehydrated, and distributed into the experimental jars. The jars were kept at 22 °C for 7 d to foster DNAN partitioning to soil constituents so as to achieve more stable DNAN soil concentrations during the earthworm exposure period. A separate subset of jars was prepared and destructively sampled in triplicate after 1, 3, and 7 d for chemical analysis of soil. After the 7-d pre-exposure period, 8 earthworms were added to each jar and maintained as described for the preliminary bioaccumulation experiment. Jars were destructively sampled in triplicate after 0 h, 1 h, 6 h, 3 d, 7 d, and 14 d for chemical analyses of earthworms and soils. A separate set of jars was set up identically to the exposure jars but did not receive earthworms. This control group (soil-only jars) was included to assess the influence of the presence of earthworms on the biotransformation of DNAN in the amended soil and was sampled at the same time points as the jars containing soil and earthworms. At each sampling time point, 4 not-purged earthworms were used for the measurement of soil gut content after oven-drying and calcination, and 4 not-purged earthworms, along with a sample of the soil they were exposed to, were placed at –80 °C for at least 24 h, lyophilized, and frozen for later chemical analyses. Concentrations associated with earthworm tissues were estimated using the AFDW concentrations.

The uptake of DNAN from soil was determined using Equation 2 described in Organisation for Economic Co-operation and Development (2010):

$$C_T = (k_u \times C_S / k_e)(1 - e^{-ke \times t}) \quad (2)$$

where C_T is the compound concentration in the tissue (mg/kg dry wt), k_u is the uptake rate constant (kg dry wt soil/kg dry wt tissue/d), k_e is the elimination rate constant (d^{-1}), C_S is the compound concentration in the amended soil (mg/kg dry wt), and t is time (d).

Elimination kinetics of DNAN in clean soil experiment

Air-dried SSL soil was amended with DNAN (final nominal concentration 50 mg/kg), rehydrated, and distributed to the 18 experimental jars. The jars were stored at 22 °C for 5 d to allow for DNAN partitioning to soil constituents and to achieve more stable concentrations of DNAN products in soil prior to the elimination phase. The elimination kinetics experiment was conducted separately from the uptake experiment, and an equilibration of 5 d was used instead of the 7 d used in the uptake experiment for logistic reasons. Eight earthworms were added to each jar to initiate the 3-d DNAN uptake period. Earthworms and soil were then sampled from 3 jars for chemical analyses. For the remaining jars, earthworms were transferred to jars containing clean (i.e., nonamended) SSL soil for evaluation of the elimination kinetics of DNAN. Jars containing clean (no DNAN added) soil and DNAN-exposed earthworms were sampled in triplicates after 0 h, 1 h, 6 h, 1 d, 3 d, and 7 d. At each sampling time point, 4 not-purged earthworms were used for the measurement of soil gut content after oven-drying and calcinations, and 4 not-purged earthworms along with exposure soil samples were placed at –80 °C for at least 24 h, lyophilized, and stored frozen for chemical analysis. The AFDW concentrations were determined as described previously in the *Estimation of insensitive munition compound concentration in tissue of not-purged earthworms* section.

The elimination of DNAN from soil was determined using Equation 3, described in the test guideline of the Organisation for Economic Co-operation and Development (2010):

$$C_T = C_{T0} \times e^{-ke \times t} \quad (3)$$

where C_T is the compound concentration in the tissue (mg/kg dry wt), C_{T0} is the compound concentration in the tissues (mg/kg) at the initiation of the elimination exposure, and k_e is the elimination rate constant (d^{-1}).

Chemical analyses

Samples of DNAN-amended, NTO-amended, and control soils were frozen at –80 °C to prevent biological transformation and lyophilized in a freeze dryer for at least 24 h. Dried DNAN-amended and NTO-amended soil samples were extracted (5 mL:1 g soil) using acetonitrile and in ultrapure water, respectively. Following sonication (60 Hz) overnight (18 h), the sample vials were centrifuged at 3000 *g* for 15 min. For DNAN-amended soil samples, the supernatant was decanted from the solids and filtered through a 0.45-µm Millex Hydrophobic Fluoropore filter and diluted 50:50 (v/v) with ultrapure (18 MΩ cm) water. For NTO-amended soil samples, supernatant was

decanted from the solids and filtered through a 0.45-µm Whatman polytetrafluoroethylene membrane with a glass microfiber prefilter.

Samples of earthworms exposed to DNAN- or NTO-amended soils and to control soil were frozen at –80 °C and lyophilized in a freeze dryer for at least 72 h. Dried earthworm samples were homogenized and extracted by sonication (60 Hz) overnight (18 h) in acetonitrile using a ratio of 1 mL acetonitrile to 0.1 g of tissue for DNAN-exposed earthworms, or in 1% trifluoroacetic acid (TFA) in water using a ratio of 1 mL of 1% TFA to 0.1 g of tissue for NTO-exposed earthworms.

Soil and tissue extracts were analyzed for DNAN, 2-amino-nitroanisole (2-ANAN; also known as 2-methoxy-5-nitroaniline; CAS no. 99-59-2), and 4-amino-nitroanisole (4-ANAN; also known as 4-methoxy-3-nitroaniline; CAS no. 577-72-0) or for NTO, using a modified US Environmental Protection Agency (2006) method 8330B (Russell et al. 2014). An Agilent 1200 high-performance liquid chromatograph (LC) equipped with a Phenomenex Synergi 4 µm HydroRP (80 Å, 250- × 4.6-mm) LC column was used for these DNAN and NTO analyses. For analyte confirmation, a Restek (Pinacle II Biphenyl; 5 µm, 150- × 4.6-mm) reversed-phase column was used. Isocratic elution of analytes was accomplished using methanol:ultrapure water:acetonitrile (45:51:4, v/v/v, respectively) at 0.9 mL/min. Detection and quantification were performed at 254 nm on a diode array detector. Limits of detection for DNAN, 2-ANAN, and 4-ANAN are presented in the Supplemental Data, Table S2.

For NTO analyses, 50 µL of sample was injected into the LC column under isocratic conditions with a ratio of 76% ultrapure water, 10% methanol, 10%, TFA (1.0%), and 4% acetonitrile. The flow rate and the detection wavelength were 0.9 mL/min and 315 nm, respectively. For confirmation, a sample volume of 50 µL was injected into a Thermo Hypercarb column (5 mm, 100 × 3 mm) and eluted under isocratic conditions using 1:1 (v/v) of acetonitrile (containing 1% TFA) and ultrapure water (containing 1% TFA) with a flow rate of 0.8 mL/min under isocratic conditions. Detection and quantification were performed at 315 nm on a diode array detector. Limits of detection for NTO are presented in the Supplemental Data, Table S2. Detection and quantitation of NTO transformation products in soil and earthworm tissues were beyond the scope of the present study and were not performed.

For quality control, US Environmental Protection Agency (2003) method 8000C was used for guidance in determining acceptable calibration and quality control requirements. Briefly, each set of 20 samples of either DNAN or NTO contained a blank, laboratory control sample, a matrix spike, and a matrix spike duplicate. The laboratory control sample consisted of hydromatrix (diatomaceous earth sorbent) amended with the analytes of interest. The matrix spike consisted of an actual sample amended with the analytes of interest. The laboratory control sample, matrix spike, and matrix spike duplicate of a given set were all amended at the same concentration. Spike recoveries are reported in the Supplemental Data, Table S3.

Statistical analyses

Data were expressed as the mean ± SD, unless otherwise stated. Differences among treatments were assessed by analysis of variance (ANOVA) or Student's *t* test, with $\alpha = 0.05$ to determine whether statistically significant differences existed among or between samples, respectively. When statistical differences existed after ANOVA analysis, a post hoc test using the Tukey's procedure was conducted to further investigate the statistically different groups. All variables tested were normally distributed or log transformed to achieve a normal distribution. All statistical analyses were performed using SigmaStat1 statistical software (SPSS).

RESULTS AND DISCUSSION

Earthworm survival and behavior

Earthworm mortality was not observed, and all organisms appeared healthy at experiment termination in the negative control soil or in the insensitive munition-amended soil treatment groups for all soil 7- and 14-d experiments. Approximately 50% of the worms in each exposure jar actively avoided burrowing into the NTO-amended soil during the first 3 d of exposure, but all organisms were found burrowed after that period.

Contribution of gut content to tissue sample mass

For control earthworms exposed to soil for 7 d in the preliminary bioaccumulation experiment, gut content of the not-purged earthworms corresponded to 66 ± 1% of the tissue sample dry weight or 22 ± 1% of the sample wet weight (Figure 1). Therefore, the tissue-only represents 34 ± 1 and 78 ± 1% of the sample weight, based on the dry weight

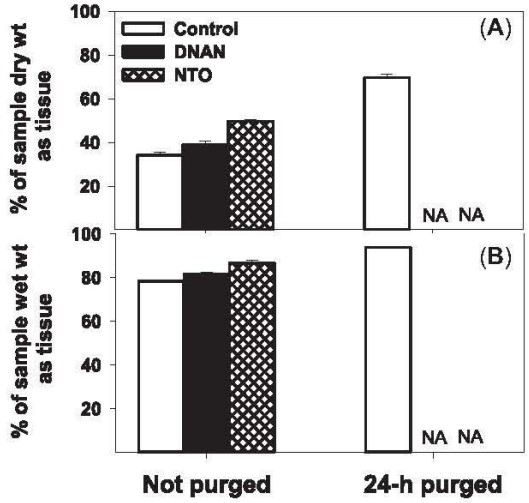

FIGURE 1: Estimated percentage (±standard deviation) of the total earthworm sample as tissue on a dry weight **(A)** and wet weight **(B)** basis for earthworms exposed to amended soil for 7 d. DNAN = 2,4-dinitroanisole; NTO = 3-nitro-1,2,4-triazol-5-one. NA = not analyzed.

(Figure 1A) and wet weight (Figure 1B), respectively. Similar values were obtained for earthworms exposed to DNAN- or NTO-amended soil for 7 d (Figure 1A and B).

Gut purging for 24 h caused most of the soil mass in the gut of control earthworms at exposure termination to egest as feces, with only 10 to 13% of the soil mass remaining. Jager et al. (2003) showed similar results and reported that 6 to 21% of the gut contents remained in the gut after a 24-h gut purging. The loss of soil during the purging period caused the fraction of the total sample mass represented by tissue to significantly increase from approximately 34 ± 1% in the not-purged earthworm tissue samples to 70 ± 2% in the 24-h purged earthworm on a dry weight basis (Figure 1A) or from 78 ± 0.5 to 94 ± 1% on a wet weight basis (Figure 1B). Therefore, 24-h gut purging is an efficient method for reducing, but not completely eliminating, the contribution of gut content in earthworms used in soil bioaccumulation studies.

Preliminary evaluation of DNAN uptake from soil by earthworms

At the start of the 14-d experiment, the measured concentration of DNAN in the soil was 52.5 ± 1.2 mg/kg, which was close to the 50 mg/kg target concentration. The low coefficient of variation (2%) indicated a uniform distribution of DNAN in the experimental soils. The sum of DNAN, 2-ANAN, and 4-ANAN (SumDNAN) concentration in the soil at day 14 was 10.4 ± 2.3 mg/kg, representing a significant decrease of 80% during the experiment (Supplemental Data, Figure S1). The soil concentration of DNAN at the end of the 14-d exposure was only 6.2 ± 2.7 mg/kg; reduced DNAN transformation products (2-ANAN and 4-ANAN) were present at lower concentrations (Supplemental Data, Figure S1), with DNAN contributing a major part (58 ± 13%) of the total, followed by 2-ANAN (37 ± 12%) and 4-ANAN (6 ± 1%). Reductive transformation of DNAN and soil concentration decrease over time are consistent with previous investigations with DNAN (Dodard et al. 2013; Richard and Weidhass 2014; Lotufo et al. 2016; Temple et al. 2018) and also with TNT, a related nitroaromatic compound (see Simini et al. 2013). The DNAN strongly adsorbs to soil and is (bio)transformed via reduction of nitro groups to amino groups to form a variety of products, including 2-ANAN, 4-ANAN, and 2,4-diaminoanisole (DAAN; Dontsova et al. 2014; Hawari et al. 2015; Lotufo et al. 2016; Olivares et al. 2016; Indest et al. 2017; Temple et al. 2018). The preferential reduction to 2-ANAN we observed further corroborates previous evidence for regioselective reduction of the *ortho*-nitro group (Hawari et al. 2015; Lotufo et al. 2016; Olivares et al. 2016; Arthur et al. 2017; Temple et al. 2018). It is unlikely that DAAN was formed in the present amended soils because this transformation process has been shown to occur under strict anaerobic conditions (Olivares et al. 2016; Indest et al. 2017). Temple et al. (2018) reported that decreases in the soil concentrations of DNAN were substantially higher for loamy soil compared with sandy soil. Hawari et al. (2015) reported strong binding of DNAN, 2-ANAN, and 4-ANAN with organic matter, including humic substances, as well as interactions between amino-nitroanisole products and clay. Therefore, the decrease in SumDNAN concentration in amended natural soils that we report was caused, at least in part, by nonextractable sorption of DNAN and its transformation products to soil components, as also reported for TNT (see Thorn et al. 2002).

The SumDNAN of DNAN and its transformation products were determined using concentrations in DNAN-amended soil and tissue residues of earthworms fed supplement food for 14 d. Figure 2 illustrates the influence of gut soil on SumDNAN bioaccumulation in 24-h purged compared with not-purged earthworms. For not-purged earthworms, concentrations were significantly higher when corrected for soil in the gut than for the whole sample (Figure 2). For purged earthworms, concentrations were not significantly different. When purged and not-purged earthworms were compared, body residues were significantly different only for AFDW concentrations (Figure 2). A similar trend was observed for earthworms sampled at day 7 and day 14 (unfed; data not shown).

The effects of gut purging and correcting for gut soil on the bioaccumulation of DNAN and its major transformation products (2-ANAN and 4-ANAN) are shown in the Supplemental Data, Figure S2. The relative contribution of DNAN and its transformation products to the SumDNAN body residue is also shown in the Supplemental Data, Figure S2. The dominant compound was DNAN (63–81%) in the tissue, followed by 2-ANAN (14–28%) and 4-ANAN (2–9%). Correcting for gut soil caused nonsignificant changes in the relative contribution of individual compounds to the SumDNAN. However, the contribution of DNAN to SumDNAN was significantly lower for 24-h purged earthworms (Supplemental Data, Figure S2A) compared with not-purged earthworms (Supplemental Data, Figure S2B). This difference was likely related to the formation of transformation products in the tissues during the 24-h purging period.

Gut purging decreases the amount of gut soil in earthworm samples generated in bioaccumulation studies (ASTM

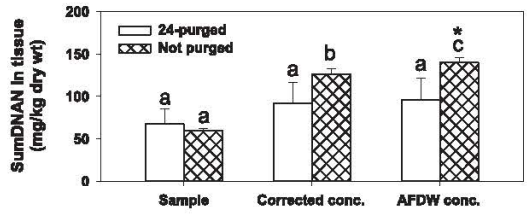

FIGURE 2: Mean concentrations (±standard deviation) of the sum of 2, 4-dinitroanisole and 2- and 4-amino-nitroanisoles (SumDNAN) in earthworm samples and estimated in tissue-only by 2 different methods in 24-h purged and not-purged earthworms exposed for 14 d and fed supplemental food. Tissue concentrations uncorrected for the presence of soil (Sample) represent the measured concentration for the earthworm samples. The concentration in the tissue-only were determined by assuming that the soil in the gut contained the same concentration of insensitive munition compound as the exposure soil (Corrected-Conc.), or by using the ash-free dry weights to calculate tissue concentrations (AFDW-Conc.). Same letters indicate no significant difference across sample correction treatments for either 24-h purged or not-purged earthworms. *indicates significant difference between 24-h purged and not-purged earthworms.

International 2012) and is therefore expected to lead to more accurate estimates of bioaccumulation. However, the present study has demonstrated that loss due to elimination of insensitive munitions during a 24-h purging period would lead to an underestimation of bioaccumulation. Based on the rate of elimination for SumDNAN estimated in Lotufo et al. (2016) from uptake experiments using *E. fetida* and experimentally determined in the present study, a reduction of approximately 60% in SumDNAN body residue is expected during a 24-h period. The body residue estimated in earthworms purged for 24 h according to standard guidance (ASTM International 2012) was approximately half the concentration for tissue-only estimated by assuming that the soil in the gut was uncontaminated; subtracting its estimated weight from the sample weight confirmed the expected reduction. The use of AFDW concentrations is likely to yield the most conservative and accurate bioaccumulation estimates for energetic compounds and other organic compounds with similar hydrophobicity amended to soil. Gut purging is not recommended for investigating the bioaccumulation of low-molecular-weight compounds in benthic oligochaetes, because these compounds may be depurated and metabolized rapidly (ASTM International 2019). Therefore, the AFDW concentrations were used for assessing the effect of exposure duration and feeding for the preliminary bioaccumulation experiment and for the uptake and elimination kinetics experiments described later in the *Earthworm uptake kinetics of DNAN in soil* section. This method assumes that insensitive munition compound in ingested soil is efficiently extracted into gut fluids and transferred to tissues and therefore the insensitive munition compound concentration in the gut soil is zero. This assumption is based on increased solubility and mobility of organic compounds in earthworm gut following their interaction with biosurfactants in gut fluid and physical stress such as abrasion (Qi and Chen 2010; Du 2018). However, for compounds with unknown elimination rates, elimination kinetics experiments should be conducted to estimate the extent of loss during the 24-h purge, to make the most informed methodological decision. For compounds with slow elimination rates, chemical analysis of worms purged for 24 h is expected to result in accurate estimates of bioaccumulation.

Bioaccumulation factors (BAFs; concentration in tissue divided by concentration in soil, dry wt basis) for SumDNAN and DNAN were calculated using tissue and soil concentration data for earthworms exposed for 14 d and fed supplementary food (Table 1). The BAFs were significantly higher for not-purged earthworms using AFDW concentrations than for sample concentrations (Table 1). For the corrected and AFDW concentrations, the BAFs of not-purged earthworms were significantly higher than the 24-h purged worms (Table 1).

For SumDNAN, body residues were significantly higher (1.5-fold) for day 7 compared with day 14 for not-purged earthworms, but no difference was observed for purged earthworms (Supplemental Data, Figure S3). For not-purged earthworms, the day 14 body residues were significantly higher (1.5-fold) in fed earthworms than in nonfed earthworms, whereas no difference was observed for purged earthworms (Supplemental Data, Figure S3). Previous studies, however, have shown that bioaccumulation of more hydrophobic contaminants, such as polycyclic aromatic hydrocarbons (PAHs) and chlorinated pesticides, is decreased when supplemental food is provided during exposure to amended soils (Ma et al. 1995; Šmídová et al. 2015). Based on Šmídová et al. (2015), the decreasing effect of food addition may not occur for low and moderate hydrophobic compounds such as DNAN.

Earthworm uptake kinetics of DNAN in soil

Because body residues were significantly higher for day 7 compared with day 14, body residue of SumDNAN was expected to have peaked before 14 d of exposure to amended soil. Therefore, 14 d was considered an adequate exposure duration for the uptake kinetics experiment. Addition of food was considered adequate for the uptake kinetics experiment because feeding enhanced the bioaccumulation of SumDNAN in earthworms exposed to amended soil. Not-purged earthworms and AFDW concentrations were used in uptake and elimination experiments with amended soils because this yielded the most conservative, and likely the most realistic, estimates of tissue-only concentrations in the preliminary bioaccumulation experiment.

TABLE 1: Mean bioaccumulation factors (±standard deviation) for 2,4-dinitroanisole (DNAN) and for the sum of DNAN and 2- and 4-amino-nitroanisole (SumDNAN) determined using earthworms exposed for 14 d and fed supplemental food and soil sampled at day 14 in the preliminary bioaccumulation experiment

Factor	Earthworms	Sample[a]	Corrected. conc.[b]	AFDW-conc.[c]
SumDNAN bioaccumulation factors	Not-purged	5.7 ± 0.3 A	12.4 ± 0.1 B	13.4 ± 0.6 C
	Purged	6.4 ± 1.7 A	8.8 ± 2.4 A	9.2 ± 2.4 A*
DNAN bioaccumulation factors	Not-purged	7.9 ± 0.3 A	17.6 ± 0.7 B	19.0 ± 0.8 B
	Purged	6.8 ± 1.5 A	9.3 ± 2.2 A*	9.7 ± 2.2 A*

[a]Tissue concentrations uncorrected for the presence of soil (Sample) representing the measured concentration for the earthworm samples.
[b]The concentration in the tissue-only determined by assuming that the soil in the gut contained the same concentration of insensitive munition compound as the exposure soil (Corrected-conc.).
[c]The ash-free dry weights used to calculate tissue concentrations (AFDW-conc.).
*Significant difference between 24-h purged and ot-purged earthworms. Same letters indicate no significant difference across sample correction treatments for either 24-h purged or not-purged earthworms.

International 2012) and is therefore expected to lead to more accurate estimates of bioaccumulation. However, the present study has demonstrated that loss due to elimination of insensitive munitions during a 24-h purging period would lead to an underestimation of bioaccumulation. Based on the rate of elimination for SumDNAN estimated in Lotufo et al. (2016) from uptake experiments using *E. fetida* and experimentally determined in the present study, a reduction of approximately 60% in SumDNAN body residue is expected during a 24-h period. The body residue estimated in earthworms purged for 24 h according to standard guidance (ASTM International 2012) was approximately half the concentration for tissue-only estimated by assuming that the soil in the gut was uncontaminated; subtracting its estimated weight from the sample weight confirmed the expected reduction. The use of AFDW concentrations is likely to yield the most conservative and accurate bioaccumulation estimates for energetic compounds and other organic compounds with similar hydrophobicity amended to soil. Gut purging is not recommended for investigating the bioaccumulation of low-molecular-weight compounds in benthic oligochaetes, because these compounds may be depurated and metabolized rapidly (ASTM International 2019). Therefore, the AFDW concentrations were used for assessing the effect of exposure duration and feeding for the preliminary bioaccumulation experiment and for the uptake and elimination kinetics experiments described later in the *Earthworm uptake kinetics of DNAN in soil* section. This method assumes that insensitive munition compound in ingested soil is efficiently extracted into gut fluids and transferred to tissues and therefore the insensitive munition compound concentration in the gut soil is zero. This assumption is based on increased solubility and mobility of organic compounds in earthworm gut following their interaction with biosurfactants in gut fluid and physical stress such as abrasion (Qi and Chen 2010; Du 2018). However, for compounds with unknown elimination rates, elimination kinetics experiments should be conducted to estimate the extent of loss during the 24-h purge, to make the most informed methodological decision. For compounds with slow elimination rates, chemical analysis of worms purged for 24 h is expected to result in accurate estimates of bioaccumulation.

Bioaccumulation factors (BAFs; concentration in tissue divided by concentration in soil, dry wt basis) for SumDNAN and DNAN were calculated using tissue and soil concentration data for earthworms exposed for 14 d and fed supplementary food (Table 1). The BAFs were significantly higher for not-purged earthworms using AFDW concentrations than for sample concentrations (Table 1). For the corrected and AFDW concentrations, the BAFs of not-purged earthworms were significantly higher than the 24-h purged worms (Table 1).

For SumDNAN, body residues were significantly higher (1.5-fold) for day 7 compared with day 14 for not-purged earthworms, but no difference was observed for purged earthworms (Supplemental Data, Figure S3). For not-purged earthworms, the day 14 body residues were significantly higher (1.5-fold) in fed earthworms than in nonfed earthworms, whereas no difference was observed for purged earthworms (Supplemental Data, Figure S3). Previous studies, however, have shown that bioaccumulation of more hydrophobic contaminants, such as polycyclic aromatic hydrocarbons (PAHs) and chlorinated pesticides, is decreased when supplemental food is provided during exposure to amended soils (Ma et al. 1995; Šmídová et al. 2015). Based on Šmídová et al. (2015), the decreasing effect of food addition may not occur for low and moderate hydrophobic compounds such as DNAN.

Earthworm uptake kinetics of DNAN in soil

Because body residues were significantly higher for day 7 compared with day 14, body residue of SumDNAN was expected to have peaked before 14 d of exposure to amended soil. Therefore, 14 d was considered an adequate exposure duration for the uptake kinetics experiment. Addition of food was considered adequate for the uptake kinetics experiment because feeding enhanced the bioaccumulation of SumDNAN in earthworms exposed to amended soil. Not-purged earthworms and AFDW concentrations were used in uptake and elimination experiments with amended soils because this yielded the most conservative, and likely the most realistic, estimates of tissue-only concentrations in the preliminary bioaccumulation experiment.

TABLE 1: Mean bioaccumulation factors (±standard deviation) for 2,4-dinitroanisole (DNAN) and for the sum of DNAN and 2- and 4-aminonitroanisole (SumDNAN) determined using earthworms exposed for 14 d and fed supplemental food and soil sampled at day 14 in the preliminary bioaccumulation experiment

Factor	Earthworms	Sample[a]	Corrected. conc.[b]	AFDW-conc.[c]
SumDNAN bioaccumulation factors	Not-purged	5.7 ± 0.3 A	12.4 ± 0.1 B	13.4 ± 0.6 C
	Purged	6.4 ± 1.7 A	8.8 ± 2.4 A	9.2 ± 2.4 A*
DNAN bioaccumulation factors	Not-purged	7.9 ± 0.3 A	17.6 ± 0.7 B	19.0 ± 0.8 B
	Purged	6.8 ± 1.5 A	9.3 ± 2.2 A*	9.7 ± 2.2 A*

[a]Tissue concentrations uncorrected for the presence of soil (Sample) representing the measured concentration for the earthworm samples.
[b]The concentration in the tissue-only determined by assuming that the soil in the gut contained the same concentration of insensitive munition compound as the exposure soil (Corrected-conc.).
[c]The ash-free dry weights used to calculate tissue concentrations (AFDW-conc.).
*Significant difference between 24-h purged and ot-purged earthworms. Same letters indicate no significant difference across sample correction treatments for either 24-h purged or not-purged earthworms.

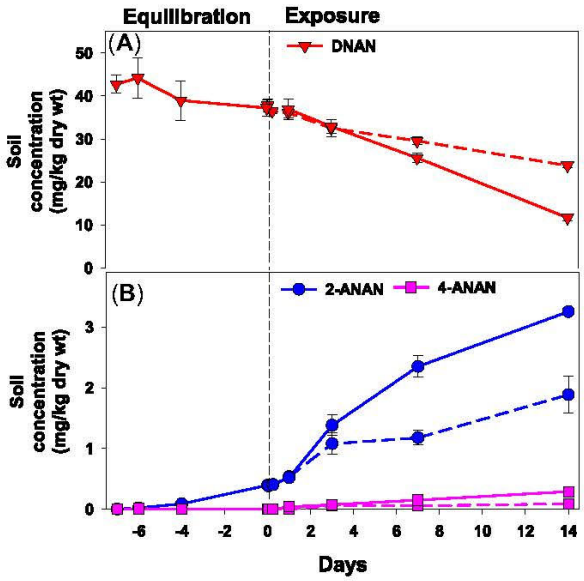

FIGURE 3: Mean concentrations (±standard deviation) of 2,4-dinitroanisole (DNAN; **A**) and of its transformation products 2-amino-nitroanisole (2-ANAN) and 4-amino-nitroanisole (4-ANAN; **B**) in amended soil in the presence or absence of earthworms, during the equilibration and exposure period of soil uptake experiment. Solid lines = with earthworms added; dashed lines = no earthworms. Some error bars are smaller than the symbol size of the data point.

The concentrations of DNAN and its transformation products during the equilibration and exposure periods (with and without earthworms) are shown in Figure 3. At experiment initiation, the mean concentration of DNAN in the soil was 42.8 ± 2.0 mg/kg and was lower than the target concentration. Following the 7-d equilibration period, the concentration of DNAN decreased by 12 ± 8%, and DNAN contributed 99.0 ± 0.1% of the SumDNAN concentration. The SumDNAN concentrations in soil at day 14 of the exposure period were significantly higher for soil without earthworms (25.8 mg/kg) than with earthworms (15.2 mg/kg; Supplemental Data, Figure S4). The presence of earthworms enhanced the disappearance of DNAN and enhanced the formation of DNAN transformation products (Figure 3), resulting in a 2-fold lower concentration of DNAN but a 2- to 3-fold higher concentration of transformation products at exposure termination relative to exposure initiation (Supplemental Data, Figure S4). This indicates that the presence of earthworms enhanced DNAN biotransformation in soil. Disappearance of DNAN in the absence of earthworms was probably largely due to soil microbial activity (biotic), with a potential contribution from photochemical degradation (abiotic). In laboratory studies similar to the present study, the presence of earthworms also enhanced the disappearance of TNT and the formation of its reduced transformation products (Renoux et al. 2000). Earthworm activity has also been shown to enhance PAH degradation in soil (Natal-da-Luz et al. 2012). The enhancement of DNAN disappearance in soil observed could be the result of either the DNAN transformation by the earthworms or a modification of the soil environment as influenced by the earthworms activities (e.g., increased aeration, microbial activation, exudation of nitrogenous compounds and waste), as hypothesized for TNT by Renoux et al. (2000). The SumDNAN concentrations in soil at day 14 of the exposure period were significantly higher for soil without earthworms (25.8 mg/kg) than with earthworms (15.2 mg/kg; Supplemental Data, Figure S4). The decrease in SumDNAN concentration during the exposure period (62%) was similar to that observed for the preliminary experiment (80%; Supplemental Data, Figure S1).

The concentrations of DNAN and its transformation products in earthworm during the 14-d exposure period are shown in Figure 4. The SumDNAN body residue increased up to day 3 and decreased by 73% between days 3 and 14. The SumDNAN body residue at day 3 was significantly higher than at day 1. Both SumDNAN (Lotufo et al. 2016) and RDX (Sarrazin et al. 2009) reached an apparent steady state in earthworms within 3 to 7 d in amended soils. A similar pattern was reported for TNT (Nurofik et al. 2015) and pyrene (Svobodová et al. 2020) in amended natural soil.

The decrease in SumDNAN concentration in tissue between days 3 and 14 (73%) was much greater than the decrease in SumDNAN concentration in soil (23%) during the same period. On day 3, DNAN was the dominant compound in tissues, representing 83% of the SumDNAN residue, but on day 14 it accounted for only 45% of the sum (Figure 5). The higher contribution of transformation products to the total body residue compared with the contribution of these products to the soil at the time of termination suggests that reductive transformation occurred in the worms. Bioaccumulation assessment in a medium where biotransformation of DNAN is kept at a minimum, such as an aqueous solution, should be conducted to confirm the suspected ability of earthworms to transform DNAN. Metabolic transformation of TNT to 2- and 4-aminodinitrotoluenes in white potworms (*Enchytraeus albidus*) exposed to TNT in aqueous medium was reported by Dodard et al. (2004).

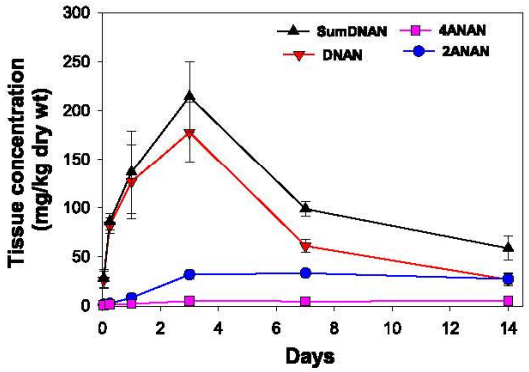

FIGURE 4: Mean tissue concentrations (±standard deviation) of 2,4-dinitroanisole (DNAN), its transformation products 2-amino-nitroanisole (2-ANAN) and 4-amino-nitroanisole (4-ANAN) and the sum of DNAN and 2- and 4-amino-nitroanisol (SumDNAN) during the uptake from soil experiment. Some error bars are smaller than the symbol size of the data point.

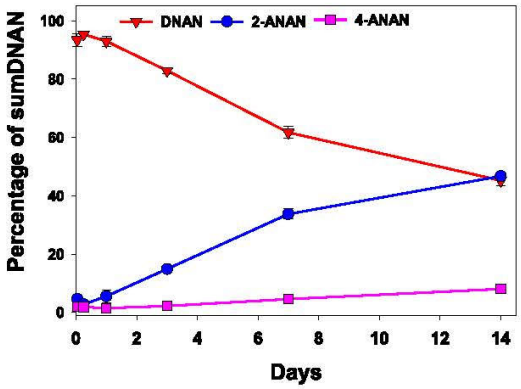

FIGURE 5: Mean percentage of SumDNA (±standard deviation) represented by 2,4-dinitroanisole (DNAN), and its transformation products 2-amino-nitroanisole (2-ANAN) and 4-amino-nitroanisole (4-ANAN) in earthworms during the exposure period of the uptake from soil experiment. Some error bars are smaller than the symbol size of the data point.

The goal of the uptake experiment was to generate the uptake rate coefficients (k_u) for DNAN. However, the decrease in DNAN concentration during the exposure period was paralleled by the increased formation of the DNAN transformation products 2-ANAN and 4-ANAN (Figures 4 and 5). The concentration of SumDNAN declined at a steady rate of 0.044 d^{-1} in soil during the 14-d exposure, and the concentration at day 14 represented only 38% of the initial concentration (Figure 6). Such a high rate of decline would preclude the use of simpler nonlinear parameter estimation methods that require a relatively constant concentration (C_S) in the source compartment (Equation 2). However, the SumDNAN concentration in the soil decreased by only 13% during the first 3 d of exposure. Therefore, a nonlinear model

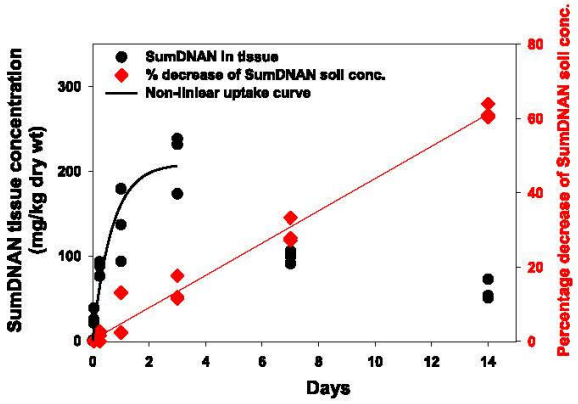

FIGURE 6: Replicate tissue concentrations of the sum of DNAN and 2- and 4-amino-nitroanisol (SumDNAN) at various time points of the uptake from soil experiment (black circles). The red line and diamond symbol represent the percentage decrease in soil SumDNAN concentration for each exposure period (ratio of replicate concentration for the exposure period and mean initial concentration). The red line represents the prediction from a linear regression ($r^2 = 0.98$). The black line represents the prediction from a first-order nonlinear model for the 0, 0.0417 (1 h), 0.25, 1, and 3-d time points only ($r^2 = 0.56$).

(Equation 2)
was used for time points 0 h, 0417 (1 h), 0.25, 1, and 3 d only (Figure 6). Using day 0 soil concentration, tissue SumDNAN concentrations at time points between days 0 and 3, and the k_e derived from the elimination experiment (see the following section, *Elimination kinetics of DNAN by earthworms in clean soil*) as a known parameter, the uptake rate coefficient, k_u, was found to be 6.12 ± 0.40 kg soil dry weight/kg tissue dry weight/d. The uptake rate for DNAN derived in the present study was lower than rates derived for *E. andrei* exposed to 2 amended natural soils (7.3 and 15.0 kg soil dry wt/kg tissue dry wt/d after conversion to dry wt basis) and was higher than the rate (1.7 kg soil dry wt/kg tissue dry wt/d) reported for *E. fetida* exposed to natural soil amended with TNT (Nurofic et al. 2015). The rates of uptake for DNAN and TNT were higher than those reported for pyrene, *p,p'*-DDT and polychlorinated biphenyl (PCB) 153, but lower than those for lindane (Svobodová et al. 2020).

Elimination kinetics of DNAN by earthworms in clean soil

Following spiking and rehydration, the mean concentration of DNAN in the soil was 51 ± 3 mg/kg, close to the target concentration. After the 5-d equilibration period, the concentration of DNAN decreased by approximately 23 ± 5%, and DNAN, 2-ANAN, and 4-ANAN represented 83 ± 4, 16 ± 6, and 1 ± 2%, respectively, of the SumDNAN concentration. The concentrations of DNAN and its transformation products in earthworms at the time they were transferred to clean soil (time 0) and at subsequent depuration periods are shown in Figure 7. At time zero, DNAN was the dominant compound (75 ± 2%) in the tissue, followed by 2-ANAN (22 ± 1%) and 4-ANAN (4 ± 0.4%). Because DNAN was likely continuously transforming to 2-ANAN and 4-ANAN in earthworm tissue during the elimination experiment, the elimination rate was determined for SumDNAN only (Figure 8). During the depuration period, SumDNAN body residue decreased by 70 and 94% of the initial residue, after 1 and 3 d, respectively. The k_e

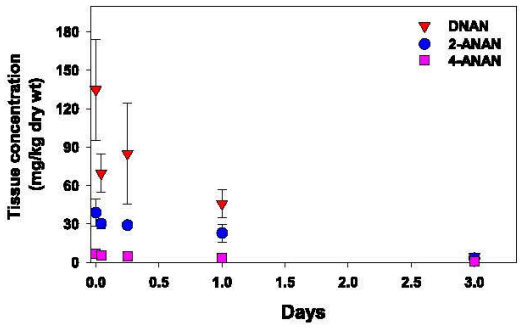

FIGURE 7: Mean tissue concentrations (±standard deviation) of 2,4-dinitroanisole (DNAN) and its transformation products 2-amino-nitroanisole (2-ANAN) and 4-amino-nitroanisole (4-ANAN), during the elimination experiment. Some error bars are smaller than the symbol size of the data point.

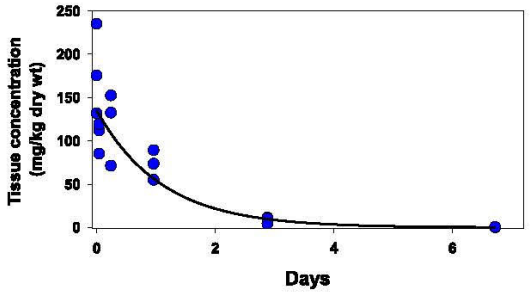

FIGURE 8: Estimated concentrations of the sum of 2,4-dinitroanisole (DNAN) and 2- and 4-amino-nitroanisol (SumDNAN) for individual replicates of the elimination experiment. The line represents prediction from a first-order decay model, $r^2 = 0.90$.

for SumDNAN was $0.87 \pm 0.14\ d^{-1}$, and its half-life was 0.79 d (or 19 h), which indicates a rapid loss of DNAN and its major transformation products from the tissue, and is consistent with the short time to achieve a steady state observed in the uptake experiment. The rate of elimination for DNAN was higher than those reported for PAHs and lindane (Svobodová et al. 2020) but much higher than those reported for pyrene, *p,p′*-DDT, PCB 153, and lindane from uptake kinetics experiments (Svobodová et al. 2020). The k_e derived in the present study is the first experimentally determined elimination rate reported for DNAN or SumDNAN.

Accumulation factor determination for DNAN in soil

The generation of BAFs has become an important parameter for regulatory agencies in the determination of whether a chemical should be deemed bioaccumulative (Hoke et al. 2016). The BAF values of SumDNAN and DNAN were generated for the uptake kinetics experiment as concentration in tissue divided by concentration in soil for different exposure periods (Table 2). For SumDNAN, the highest BAF value was 6.2 ± 1.0 kg/kg (dry wt) on day 3 and decreased to 3.8 ± 0.8 kg/kg by day 14. The highest BAF value for DNAN was 5.4 ± 0.9 kg/kg (dry wt), determined at day 3. The SumDNAN and DNAN BAF values for the uptake kinetics experiment (Table 2) were approximately 2 to 3 times lower than the 14-d results for the preliminary bioaccumulation experiment (Table 1), even though the soil, target concentration, and exposure conditions were the same. Earthworms were exposed for approximately 24 h and 7 d following hydration of amended soil for the preliminary experiment and the uptake kinetics experiment, respectively. The bioavailability of DNAN may have decreased during the additional equilibration period, resulting in lower BAF values for the uptake kinetics experiment. Measurement of porewater concentration as an indication of bioavailability (Lotufo et al. 2016) during different periods following amendment of the soil with DNAN should be conducted to elucidate the effect of aging on bioavailability. Lotufo et al. (2016) reported *E. fetida* BAF values for 4 different soils that, when converted to dry weight basis (Supplemental Data, Table S4), were overall in the same range as the BAF values derived for SSL soil in the present study (Tables 1 and 2). Following amendment with DNAN, the soils also equilibrated for 7 d in Lotufo et al. (2016). The SumDNAN BAF values determined in the present study were also similar to the BAF values reported for TNT (5.0 kg/kg dry wt), 2-aminodinitrotoluene (5.1 kg/kg dry wt), and 4-aminodinitrotoluene (6.4 kg/kg dry wt; Lachance et al. 2004). In addition to determining BAF values as the ratio of tissue and sediment concentrations, kinetically derived BAF values were determined for SumDNAN as the ratio of k_u and k_e, and resulted in a value of 7.0 kg/kg, a value similar to the mean 3-d BAF (Table 2). The steady-state BAF should preferably be calculated both as the ratio of the concentration in earthworms and in the soil at apparent steady state, and as a kinetically derived BAF, assuming first-order kinetics (Organisation for Economic Co-operation and Development 2010; Svobodová et al. 2020). The low BAF values for DNAN and TNT indicate their relatively low propensity to accumulate in soil invertebrates. The BAF values for DNT and TNT were higher than that for pyrene, but lower than that for chlorinated hydrophobic compounds (Svobodová et al. 2020).

TABLE 2: Mean bioaccumulation factors (±standard deviation) for 2,4-dinitroanisole (DNAN) and for the sum of DNAN and 2- and 4-amino-nitroanisole (SumDNAN) for each time point of the uptake experiment

Time point	DNAN	SumDNAN
1 h	0.7 ± 0.2	0.7 ± 0.2
6 h	2.1 ± 0.2	2.2 ± 0.2
1 d	3.4 ± 1.0	3.6 ± 1.1
3 d	5.4 ± 0.9	6.2 ± 1.0
7 d	2.4 ± 0.2	3.5 ± 0.3
14 d	2.3 ± 0.6	3.8 ± 0.8

Evaluation of NTO bioaccumulation from soil by earthworms

The accumulation of NTO from soil in earthworms was investigated in the preliminary bioaccumulation experiment. The soil NTO concentration significantly decreased (by 57%) from 837 ± 7 mg NTO/kg dry soil at experiment initiation to only 356 ± 3 mg NTO/kg dry soil at the termination of the 14-d experiment. The high decrease in NTO concentration in the present study agrees with earlier sorption studies on multiple soils in which the decrease in the concentration of NTO amended to soil was attributed to biodegradation (Mark et al. 2016). In addition, column studies reported a significant mass loss of NTO for loamy soil but not for sandy soil or incinerated loamy soil, suggesting a strong correlation between NTO mass loss and organic content (Temple et al. 2018). In microbially active soils subjected to localized anoxia, NTO was biotransformed to 3-amino-1, 2, 4-triazol-5-one (ATO; Krzmarzick et al. 2015). Similar to studies that also examined the biotransformation of NTO in natural soils (Indest et al. 2017; Temple et al. 2018), it is not known whether NTO was transformed to ATO in the present study. Future work should

identify and quantitate the NTO degradation products in natural soil environments.

In contrast to DNAN, the NTO concentrations were much lower in the earthworm samples than in the soil, indicating a negligible accumulation potential in earthworms. For example, at day 14, the NTO concentration of not-purged earthworm tissue sample (3.2 ± 0.6 mg/kg; Supplemental Data, Figure S5) was 119 times lower than the final concentration (356 ± 3 mg/kg) in the exposure soil. Because gut soil accounts for approximately two-thirds of the sample dry weight (Figure 1), the low NTO concentration in not-purged earthworms (Supplemental Data, Figure S5) indicates efficient clearance of NTO in ingested soil by the gut fluids followed by fast elimination from the tissues after uptake. Similar to DNAN, AFDW concentrations (Supplemental Data, Figure S5) represented more accurate estimates of the NTO accumulation in earthworms. For NTO, body residues were significantly higher (1.8-fold) at day 7 compared with day 14 for 24-h purged earthworms, but no difference was observed for not-purged earthworms (Supplemental Data, Figure S6). The day-14 body residues were significantly higher (by 2.2-fold) in fed earthworms than in nonfed earthworms for not-purged earthworms, but no difference was observed for 24-h purged earthworms (Supplemental Data, Figure S6). Therefore, the NTO data corroborate the DNAN data just presented in the *Accumulation factor determination for DNAN in soil* section showing that feeding may increase bioaccumulation of contaminants with low hydrophobicity.

The NTO concentrations in soil and in fed earthworms during the 14-d exposure were used to examine the soil bioaccumulation of NTO (Figure 9). The BAFs were low, with averages ranging from 0.0089 to 0.0182 kg/kg dry wt). The highest value (0.018 ± 0.003 kg/kg dry wt) was for not-purged earthworms using AFDW concentrations. The mean BAF value for the AFDW treatment was significantly higher than that for whole-sample BAF for not-purged earthworms, but not for 24-h purged earthworms (Figure 9). The BAF value of 0.018 kg/kg dry weight is based on the assumption that all the NTO detected in the sample was associated with earthworm tissue. The actual BAF would be even lower if NTO was present in the gut soil. The NTO BAF value of 0.018 kg/kg dry weight was 3 orders of magnitude lower compared with the highest BAF (19.0 ± 0.8 kg/kg dry wt) determined for DNAN (Table 1) in the same experiment. These results confirm those of previous studies reporting a low BAF value (0.2 kg/kg dry wt) in *E. andrei* exposed to sublethal concentrations of NTO-amended soil for 14 d (Hawari et al. 2014). The BAF values for NTO were lower than 1 and indicate lower accumulation in tissues with respect to the source compartment (i.e., soil). A negligible bioaccumulation potential of NTO would be anticipated based on its low hydrophobicity and predicted K_{OW} values. No other reports were found in the available literature on soil bioaccumulation studies of NTO using soil invertebrates. One study addressed the bioaccumulation of NTO in aquatic organisms. Lotufo et al. (2015) investigated the bioaccumulation potential of NTO using *Rana pipiens* tadpoles and reported a preliminary BCF of 0.25 L/kg, which also indicates lower

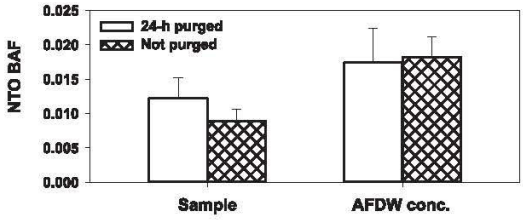

FIGURE 9: Mean 3-nitro-1,2,4-triazol-5-one (NTO) bioaccumulation factors (BAFs; ±standard deviation) of NTO for 24-h purged and not-purged day 14 (fed treatment) earthworms exposed for 14 d and fed supplemental food. Tissue concentrations uncorrected for the presence of soil (Sample) represent the measured concentration for the earthworm samples, and therefore are influenced by the presence of soil within the gut. Estimates of the concentration in the tissue-only were determined by using estimated ash-free dry weights to calculate tissue concentrations (AFDW-Conc.).

accumulation in tissues with respect to the source compartment. When the experimental results showing that the bioaccumulation potential for NTO (BAF ≤ 0.018 kg/kg) was negligible were considered, uptake and elimination kinetics experiments similar to those conducted for DNAN in the present study were not conducted for NTO-amended soil.

CONCLUSIONS

The bioaccumulation potentials of DNAN and NTO were assessed using earthworms exposed to a natural sandy loam soil individually amended with different insensitive munition compounds. Evaluation of the effect of gut content on body residue determination suggested that correction of residual gut soil provided the most conservative estimates of tissue-only concentrations. However, assessment of the combined concentration in earthworm tissue and gut soil (i.e., measurements designated as "sample" in the present study) may best represent the prey items and therefore may be more appropriate for use in the assessment of risk to invertebrate predators, because under environmental conditions, a predator eats the whole prey and is therefore exposed to contaminants associated with gut sediments (ASTM International 2019). Formation of 2-ANAN exceeded that of 4-ANAN in soil, confirming the results of previous studies indicating that DNAN is regioselectively reduced at the ortho position. The formation of 2-ANAN and 4-ANAN in soil over time did not account for the observed disappearance of DNAN in soil over time, suggesting that DNAN and its transformation products may become non-solvent–extractable. The SumDNAN concentration reached the highest levels in earthworms after approximately 3 d, indicating a fast time to steady state, which was confirmed by the experimentally measured elimination rate.

Reductive transformation of DNAN in the tissues, preferentially to 2-ANAN, was apparent but could not be confirmed because the earthworms were exposed to amino transformation products formed in the soil during the study. The measured BAF values for DNAN were generally <10 mg/kg dry weight and indicate a high potential for DNAN to accumulate in earthworms relative to PAHs but a low potential relative to

hydrophobic chlorinated compounds. In contrast, the BAFs of NTO values were <0.2 because the tissue concentrations were ≤5 times lower than the exposure soil concentration, therefore indicating that NTO poses a low risk of transfer via the food chain compared with DNAN.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.5028.

Acknowledgment—The Strategic Environmental Research and Development Program (ER-2724) funded the present study. The authors thank P. Krupa, A. Butler, and anonymous reviewers for providing us with valuable comments that helped us to improve the quality of the manuscript.

Disclaimer—The views and opinions expressed in the present study are those of the individual authors and not those of the US Army or other sponsor organizations. The use of trade, product, or firm names in this report is for descriptive purposes only and does not imply endorsement by the US Government. Permission was granted to publish this information by the US Army Chief of Engineers. The findings of the present study are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (guilherme.lotufo@usace.army.mil).

REFERENCES

Arthur JD, Mark NW, Taylor S, Simunek J, Brusseau ML, Dontsova KM. 2017. Batch soil adsorption and column transport studies of 2,4-dinitroanisole (DNAN) in soils. *J Contam Hydrol* 199:14–23.

ASTM International. 2012. Standard guide for conducting laboratory soil toxicity or bioaccumulation tests with the Lumbricid earthworm *Eisenia fetida* and the Enchytraeid potworm *Enchytraeus albidus*. E 1676-12. In *Annual Book of ASTM Standards*, Vol 11. Philadelphia, PA, USA.

ASTM International. 2019. Standard test method for measuring the toxicity of sediment-associated contaminants with freshwater invertebrates. E 1706-05 (reapproved from 2010). In *Annual Book of ASTM Standards*, Vol 11. Philadelphia, PA, USA.

Dodard SG, Powlowski J, Sunahara GI. 2004. Biotransformation of 2,4,6-trinitrotoluene (TNT) by enchytraeids (*Enchytraeus albidus*) in vivo and in vitro. *Environ Pollut* 131:263–273.

Dodard SG, Sarrazin M, Hawari J, Paquet L, Ampleman G, Thiboutot S, Sunahara GI. 2013. Ecotoxicological assessment of a high energetic and insensitive munitions compound: 2,4-dinitroanisole (DNAN). *J Hazard Mater* 262:143–150.

Dontsova K, Taylor S, Pesce-Rodriguez R, Brusseau M, Arthur J, Mark N, Walsh M, Lever J, Simunek J. 2014. Dissolution of NTO, DNAN, and insensitive munitions formulations and their fates in soils. Strategic Environmental Research and Development Program (SERDP), US Army Engineer Research and Development Center, Cold Regions Research and Engineering Laboratory (CRREL) US Army Engineer Research and Development Center, Hanover, NH, USA.

Du JH. 2018. The earthworm gastrointestinal effect on the release of organic bound residues in soils. *IOP Conf Ser Earth Environ Sci* 128:012039.

Gong P, Donohue KB, Mayo AM, Wang Y, Hong H, Wilbanks MS, Barker ND, Guan X, Gust KA. 2018. Comparative toxicogenomics of three insensitive munitions constituents 2,4-dinitroanisole, nitroguanidine and nitrotriazolone in the soil nematode *Caenorhabditis elegans*. *BMC Syst Biol* 12:92.

Gust KA, Chaitankar V, Ghosh P, Wilbanks MS, Chen X, Barker ND, Pham D, Scanlan LD, Rawat A, Talent LG. 2018. Multiple environmental stressors induce complex transcriptomic responses indicative of phenotypic outcomes in Western fence lizard. *BMC Genom* 19:877.

Hawari J, Sunahara GI, Perreault N, Halasz A, Paquet L, Dodard SG, Sarrazin M, Savard K. 2014. Environmental fate and ecological impact of emerging energetic chemicals (ADN, DNAN and its amino-derivatives, PETN, NTO, NQ, FOX-7, and FOX-12) and an insensitive formulation. Vol DRDC-RDDC-2014-C178. National Research Council, Energy Mine and Environment, Ottawa, ON, Canada.

Hawari J, Monteil-Rivera F, Perreault NN, Halasz A, Paquet L, Radovic-Hrapovic Z, Deschamps S, Thiboutot S, Ampleman G. 2015. Environmental fate of 2,4-dinitroanisole (DNAN) and its reduced products. *Chemosphere* 119:16–23.

Hoke R, Huggett D, Brasfield S, Brown B, Embry M, Fairbrother A, Kivi M, Paumen ML, Prosser R, Salvito D, Scroggins R. 2016. Review of laboratory-based terrestrial bioaccumulation assessment approaches for organic chemicals: Current status and future possibilities. *Integr Environ Assess Manag* 12:109–122.

Indest KJ, Hancock DE, Crocker FH, Eberly JO, Jung CM, Blakeney GA, Brame J, Chappell MA. 2017. Biodegradation of insensitive munition formulations IMX101 and IMX104 in surface soils. *J Ind Microbiol Biotechnol* 44:987–995.

Jager T, Fleuren RH, Roelofs W, de Groot AC. 2003. Feeding activity of the earthworm *Eisenia andrei* in artificial soil. *Soil Biol Biochem* 35:313–322.

Kennedy AJ, Laird JG, Lounds C, Gong P, Barker ND, Brasfield SM, Russell AL, Johnson MS. 2015. Inter- and intraspecies chemical sensitivity: A case study using 2,4-dinitroanisole. *Environ Toxicol Chem* 34:402–411.

Kennedy AJ, Poda AR, Melby NL, Moores LC, Jordan SM, Gust KA, Bednar AJ. 2017. Aquatic toxicity of photo-degraded insensitive munition 101 (IMX-101) constituents. *Environ Toxicol Chem* 36:2050–2057.

Krzmarzick MJ, Khatiwada R, Olivares CI, Abrell L, Sierra-Alvarez R, Chorover J, Field JA. 2015. Biotransformation and degradation of the insensitive munitions compound, 3-nitro-1,2,4-triazol-5-one, by soil bacterial communities. *Environ Sci Technol* 49:5681–5688.

Lachance B, Renoux AY, Sarrazin M, Hawari J, Sunahara GI. 2004. Toxicity and bioaccumulation of reduced TNT metabolites in the earthworm *Eisenia andrei* exposed to amended forest soil. *Chemosphere* 55:1339–1348.

Lotufo GR, Biedenbach JM, Sims JG, Chappell P, Stanley JK, Gust KA. 2015. Bioaccumulation kinetics of the conventional energetics TNT and RDX relative to insensitive munitions constituents DNAN and NTO in *Rana pipiens* tadpoles. *Environ Toxicol Chem* 34:880–886.

Lotufo GR, Coleman JG, Harmon AR, Chappell MA, Bednar AJ, Russell AL, Smith JC, Brasfield SM. 2016. Accumulation of 2,4-dinitroanisole in the earthworm *Eisenia fetida* from chemically spiked and aged natural soils. *Environ Toxicol Chem* 35:1835–1842.

Lotufo GR, Stanley JK, Chappell P, Melby NL, Wilbanks MS, Gust KA. 2018. Subchronic, chronic, lethal and sublethal toxicity of insensitive munitions mixture formulations relative to individual constituents in *Hyalella azteca*. *Chemosphere* 210:795–804.

Ma WC, Immerzeel J, Bodt J. 1995. Earthworm and food interactions on bioaccumulation and disappearance in soil of polycyclic aromatic hydrocarbons: Studies on phenanthrene and fluoranthene. *Ecotoxicol Environ Saf* 32:226–232.

Madeira CL, Field JA, Simonich MT, Tanguay RL, Chorover J, Sierra-Alvarez R. 2018. Ecotoxicity of the insensitive munitions compound 3-nitro-1,2,4-triazol-5-one (NTO) and its reduced metabolite 3-amino-1,2,4-triazol-5-one (ATO). *J Hazard Mater* 343:340–346.

Mark N, Arthur J, Dontsova K, Brusseau M, Taylor S. 2016. Adsorption and attenuation behavior of 3-nitro-1,2,4-triazol-5-one (NTO) in eleven soils. *Chemosphere* 144:1249–1255.

Mark N, Arthur J, Dontsova K, Brusseau M, Taylor S, Simunek J. 2017. Column transport studies of 3-nitro-1,2,4-triazol-5-one (NTO) in soils. *Chemosphere* 171:427–434.

Natal-da-Luz T, Lee I, Verweij RA, Morais PV, Van Velzen MJM, Sousa JP, Van Gestel CAM. 2012. Influence of earthworm activity on microbial communities related with the degradation of persistent pollutants. *Environ Toxicol Chem* 31:794–803.

Nurofik N, Choi J, Oh S, Shin WS. 2015. Toxicity and uptake of 2,4,6-trinitrotoluene (TNT) in contaminated soils to *Eisenia fetida*. *J Soil Groundw Environ* 20:46–54.

Organisation for Economic Co-operation and Development. 2010. Test No. 317: Bioaccumulation in terrestrial oligochaetes. *OECD Guidelines for the Testing of Chemicals, Section 3*. Paris, France.

Olivares CI, Abrell L, Khatiwada R, Chorover J, Sierra-Alvarez R, Field JA. 2016. (Bio)transformation of 2,4-dinitroanisole (DNAN) in soils. *J Hazard Mater* 304:214–221.

Pillard DA, Eck WS, Johnson MS, Packard S. 2017. Effects of 3-nitro-1,2,4-triazol-5-one on survival, growth and metamorphosis in the northern leopard frog, *Lithobates pipiens*. *Ecotoxicology* 26:1170–1180.

Powell IJ. 2016. Insensitive munitions—Design principles and technology developments. *Propell Explos Pyrot* 41:409–413.

Qi Y, Chen W. 2010. Comparison of earthworm bioaccumulation between readily desorbable and desorption-resistant naphthalene: Implications for biouptake routes. *Environ Sci Technol* 44:323–328.

Renoux AY, Sarrazin M, Hawari J, Sunahara GI. 2000. Transformation of 2,4,6-trinitrotoluene in soil in the presence of the earthworm *Eisenia andrei*. *Environ Toxicol Chem* 19:1473–1480.

Rombke J, Aira M, Backeljau T, Breugelmans K, Dumonguez J, Funke E, Graf N, Hajibabaei M, Perez-Losada M, Porto PB, Schmielz RM, Vierna J, Vizcaino A, Pfenniger M. 2016. DNA barcoding of earthworms (*Eisenia fetida/andrei* complex) from 28 ecotoxicological test laboratories. *Appl Soil Ecol* 104:3–11.

Richard T, Weidhaas J. 2014. Dissolution, sorption, and phytoremediation of IMX-101 explosive formulation constituents: 2,4-dinitroanisole (DNAN), 3-nitro-1,2,4-triazol-5-one (NTO), and nitroguanidine. *J Hazard Mater* 280:561–569.

Russell AL, Seiter JM, Coleman JG, Winstead B, Bednar AJ. 2014. Analysis of munitions constituents in IMX formulations by HPLC and HPLC–MS. *Talanta* 128:524–530.

Sample BE, Schlekat, C, Spurgeon DJ, Menzie C, Rauscher J, Adams B. 2014. Recommendations to improve wildlife exposure estimation for development of soil screening and cleanup values. *Integr Environ Assess Manag* 10:372–387.

Sarrazin M, Dodard SG, Savard K, Lachance B, Robidoux PY, Kuperman RG, Hawari J, Ampleman G, Thiboutot S, Sunahara GI. 2009. Accumulation of hexahydro-1,3,5-trinitro-1,3,5-triazine by the earthworm *Eisenia andrei* in a sandy loam soil. *Environ Toxicol Chem* 28:2125–2133.

Savard K, Sarrazin M, Dodard SG, Monteil-Rivera F, Kuperman RG, Hawari J, Sunahara GI. 2010. Role of soil interstitial water in the accumulation of hexahydro-1,3,5-trinitro-1,3,5-triazine in the earthworm *Eisenia andrei*. *Environ Toxicol Chem* 29:998–1005.

Simini M, Checkai RT, Kuperman RG, Philips CT, Kolakowski JE, Kurnas CW. 2013. Toxicities of TNT and RDX to the earthworm *Eisenia fetida* in five soils with contrasting characteristics. Accession No. ADA433147. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, USA.

Šmídová K, Šerá J, Bielská L, Hofman J. 2015. Influence of feeding and earthworm density on compound bioaccumulation in earthworms *Eisenia andrei*. *Environ Pollut* 207:168–175.

Stafford EA, McGrath SP. 1986. The use of acid insoluble residue to correct for the presence of soil-derived metals in the gut of earthworms used as bio-indicator organisms. *Environ Pollut A* 42:233–246.

Stanley JK, Lotufo GR, Biedenbach JM, Chappell P, Gust KA. 2015. Toxicity of the conventional energetics TNT and RDX relative to new insensitive munitions constituents DNAN and NTO in *Rana pipiens* tadpoles. *Environ Toxicol Chem* 34:873–879.

Svobodová M, Hofman J, Bielská L, Šmídová K. 2020. Uptake kinetics of four hydrophobic organic pollutants in the earthworm *Eisenia andrei* in aged laboratory-contaminated natural soils. *Ecotoxicol Environ Saf* 192:110317.

Taylor S, Walsh ME, Becher JB, Ringelberg DB, Mannes PZ, Gribble GW. 2017a. Photo-degradation of 2,4-dinitroanisole (DNAN): An emerging munitions compound. *Chemosphere* 167:193–203.

Taylor S, Dontsova K, Walsh M. 2017b. Insensitive munitions formulations: Their dissolution and fate in soils. In Shukla MK, Boddu VM, Steevens JA, Damavarapu R, Leszczynski J, eds, *Energetic Materials: From Cradle to Grave*. Springer International, New York, NY, USA, pp 407–443.

Temple T, Ladyman M, Mai N, Galante E, Ricamora M, Shirazi R, Coulon F. 2018. Investigation into the environmental fate of the combined insensitive high explosive constituents 2,4-dinitroanisole (DNAN), 1-nitroguanidine (NQ) and nitrotriazolone (NTO) in soil. *Sci Total Environ* 625:1264–1271.

Thorn KA, Pennington JC, Hayes CA. 2002. ^{15}N NMR investigation of the reduction and binding of TNT in an aerobic bench scale reactor simulating windrow composting. *Environ Sci Technol* 36: 3797–3805.

Toghiani RK, Toghiani H, Maloney SW and Boddu VM. 2008. Prediction of physicochemical properties of energetic materials. *Fluid Ph Equilib* 264:86–92.

US Environmental Protection Agency. 2003. Determinative chromatographic separations, revision 3. Washington, DC.

US Environmental Protection Agency. 2006. Method 8330B: Nitroaromatics, nitramines, and nitrate esters by high performance liquid chromatography (HPLC), revision 2. Washington, DC.

Viswanath DS, Ghosh TK, Boddu VM, eds. 2018. *Emerging Energetic Materials: Synthesis, Physicochemical, and Detonation Properties*. Springer Netherlands, Heidelberg, Germany.

Accumulation of Insensitive Munitions Compounds in the Earthworm *Eisenia andrei* from amended Soil – Methodological Considerations for Determination of Bioaccumulation Factors

G.R. Lotufo[a]; R.E. Boyd[a]; A.R. Harmon[a]; A.J. Bednar[a]; J.C. Smith[a]; M. Simini[b], G.I. Sunahara[c]; J. Hawari[d]; R.G. Kuperman[b]

[a]US Army, Engineer Research and Development Center, Environmental Laboratory, Vicksburg, MS 39180

[b]U.S. Army Chemical Biological Center, Aberdeen Proving Ground, Maryland

[c]Department of Natural Resource Sciences, McGill University, Ste-Anne-de-Bellevue, QC, Canada

[d]Department of Civil, Geological and Mining Engineering, Polytechnique Montreal, Montreal QC.

Corresponding author (*):

Guilherme.Lotufo@usace.army.mil

SUPLEMENTAL DATA

Section 1: Summary of the primary and secondary treatments, and of the handling of earthworms at exposure termination.

Soil treatments (n = 9 per treatment):

2,4-dinitroanisole-amended soil

3-nitro-1,2,4-triazol-5-one-amended soil

Control

Sub-treatments (n = 3 per soil treatment):

Supplemental food added, 7-d exposure

Supplemental food added, 14-d exposure

Supplemental food not added, 14-d exposure

Handling of earthworms for each control replicate

Two worms gut-purged for 24-h, analyzed for gut content ash-free dry wt.

Two worms not-purged, analyzed for gut content ash-free dry wt.

Handling of earthworms for each IM compound treatment replicate

Two worms gut-purged for 24-h, analyzed for bioaccumulation

Two worms not-purged, analyzed for DNAN and its amino derivatives

Two worms not-purged, analyzed for gut content ash-free dry wt.

Section 2: Tables

Table S1. Physical and chemical characteristics of Sassafras sandy loam.

Soil Parameter	
Sand (%)	55
Silt (%)	28
Clay (%)	17
Texture	Sandy loam
Cation Exchange Capacity (CEC) (cmol kg^{-1})	9.3
Organic matter (%)	2.3
pH	4.9
Water Holding Capacity (WHC) (%)	18

Table S2. Limits of detection for 2,4-dinitroanisole (DNAN), its transformation products 2-amino-nitroanisole (2-ANAN) and 4-amino-nitroanisole (4-ANAN), and 3-nitro-1,2,4-triazol-5-one (NTO) for the analytical method used in present study.

Matrix	Reporting Limit (mg/kg dry wt.)	Method detection Limit (mg/kg dry wt.)
Soil	0.1	0.006 - 0.01
Earthworm	0.1 - 0.3	0.02 - 0.06

Table S3. Percent spike recovery for 2,4-dinitroanisole (DNAN), its transformation products 2-amino-nitroanisole (2-ANAN) and 4-amino-nitroanisole (4-ANAN), and 3-nitro-1,2,4-triazol-5-one (NTO) for soil and earthworm quality control (QC) samples generated in the present study.

QC Sample Type	Analyte	Concentration mg/kg dry wt.	% Recovery
Soil samples			
LCS	DNAN	10.5	105
Matrix Spike	DNAN	61	93
Matrix Spike Dup	DNAN	58.5	68
LCS	2-ANAN	10.5	105
Matrix Spike	2-ANAN	9.95	100
Matrix Spike Dup	2-ANAN	9.68	97
LCS	4-ANAN	10.3	103
Matrix Spike	4-ANAN	6.42	64
Matrix Spike Dup	4-ANAN	6.18	62
LCS	NTO	10.4	104
Matrix Spike	NTO	10.4	104
Matrix Spike Dup	NTO	10.4	104
Earthworm samples			
LCS – 1	DNAN	10	100
LCS – 2	DNAN	8.96	90
LCS Duplicate 1	DNAN	9.05	91
LCS Duplicate 2	DNAN	9.33	93
LCS – 1	2-ANAN	10.1	101
LCS – 2	2-ANAN	9.02	90
LCS Duplicate 1	2-ANAN	9.11	91
LCS Duplicate 2	2-ANAN	9.16	92
LCS – 1	4-ANAN	10.1	101
LCS – 2	4-ANAN	11.8	118
LCS Duplicate 1	4-ANAN	9.46	95
LCS Duplicate 2	4-ANAN	10.7	107
LCS – 1	NTO	16.3	109
LCS – 2	NTO	13.8	92
LCS Duplicate 1	NTO	13.9	93
LCS Duplicate 2	NTO	15.4	103

LCS = laboratory control sample; Dup = duplicate

Table S4. Sum of 2,4-dinitroanisole and 2- and 4-amino-nitroanisole (SumDNAN) bioaccumulation factors (BAF) determined for various amended soils by Lotufo et al. (2016) using body residues estimated using correction for soil in the gut and converted to kg dry wt. in soil / kg dry wt. tissue for comparison with values presented in Tables 1 and 2.

Soil type	BAF	Study
Memphis	6.6	Lotufo et al. 2016
Falaya	4.0	Lotufo et al. 2016
Sunev	4.3	Lotufo et al. 2016
Ruston	14.2	Lotufo et al. 2016

Section 3: Figures

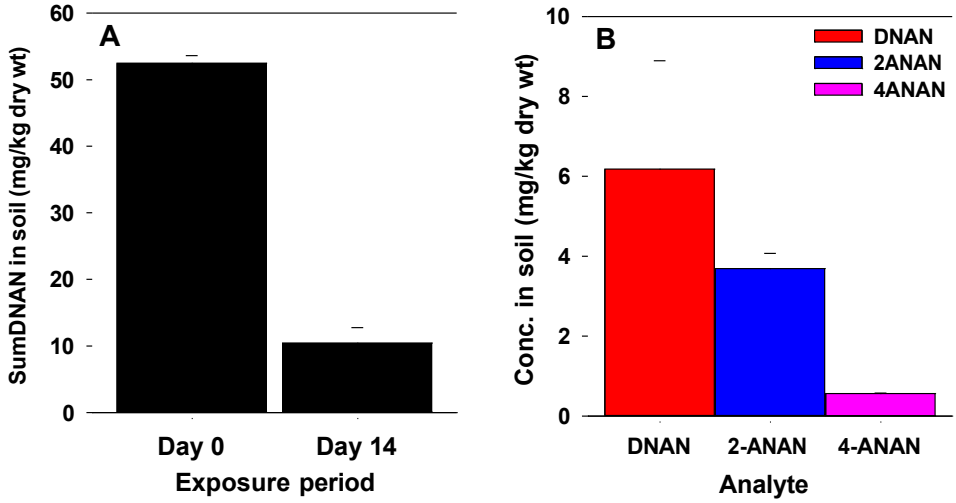

Figure S1. Mean concentrations (± SD) of the sum of 2,4-dinitroanisole (DNAN), 2-ANAN (2-ANAN) and 4-amino-nitroanisole (4-ANAN) (SumDNAN) in exposure soil initiation and termination of the preliminary uptake experiment (A), and for DNAN, 2-ANAN, and 4-ANAN in exposure soil at experiment termination (B).

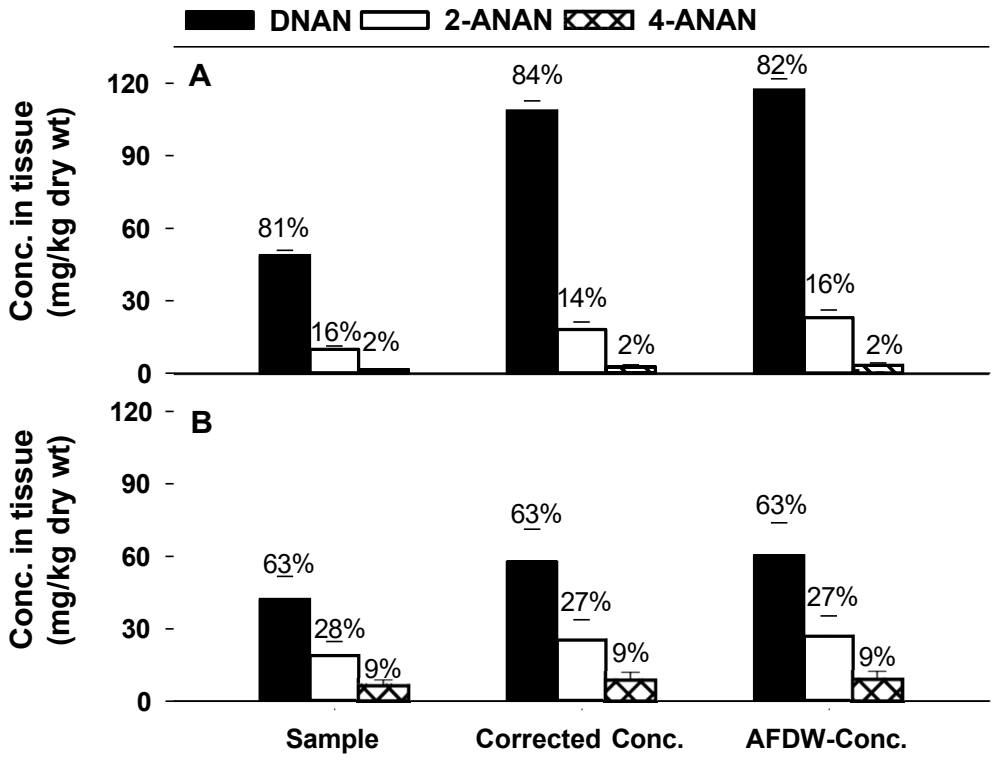

Figure S2. Mean concentrations (± SD) of 2,4-dinitroanisole (DNAN), 2- amino-nitroanisole (2-ANAN) and 4-amino-nitroanisole (4-ANAN) for not-purged (A) and 24-h purged (B) earthworms exposed for 14 d and fed supplemental food in the preliminary uptake experiment. Tissue concentrations uncorrected for the presence of soil ("Sample") represent the measured concentration for the earthworm samples. The concentration in the tissue-only were determined by assuming that the soil in the gut contained the same concentration of IM IM compound as the exposure soil ("Corrected-Conc."), or by using estimated ash-free dry weights to calculate tissue concentrations (AFDW-Conc.). The numbers above the bars indicate the contribution of the concentration of each compound to the sum concentration.

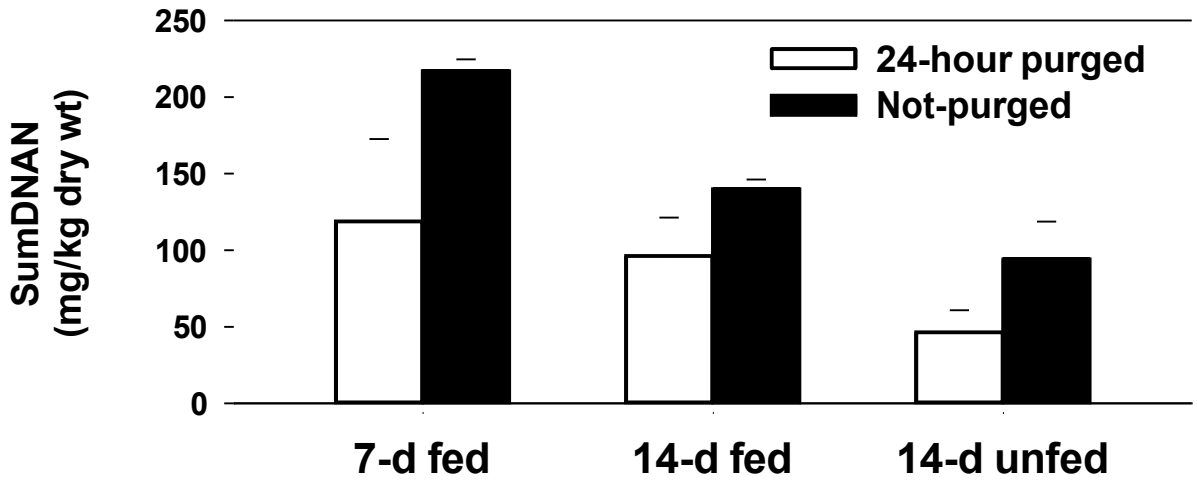

Figure S3. Mean estimated tissue-only concentrations (± SD) of the sum of 2,4-dinitroanisole and 2- and 4-amino-nitroanisole (SumDNAN) in 24-h purged and in not-purged earthworms for different treatments of the preliminary uptake experiment. For not purged earthworms, 7-d fed was significantly higher than 14-d fed, and 14-d fed was significantly higher than for 14-d unfed. For 24-h purged earthworms, no significant differences were observed between treatments.

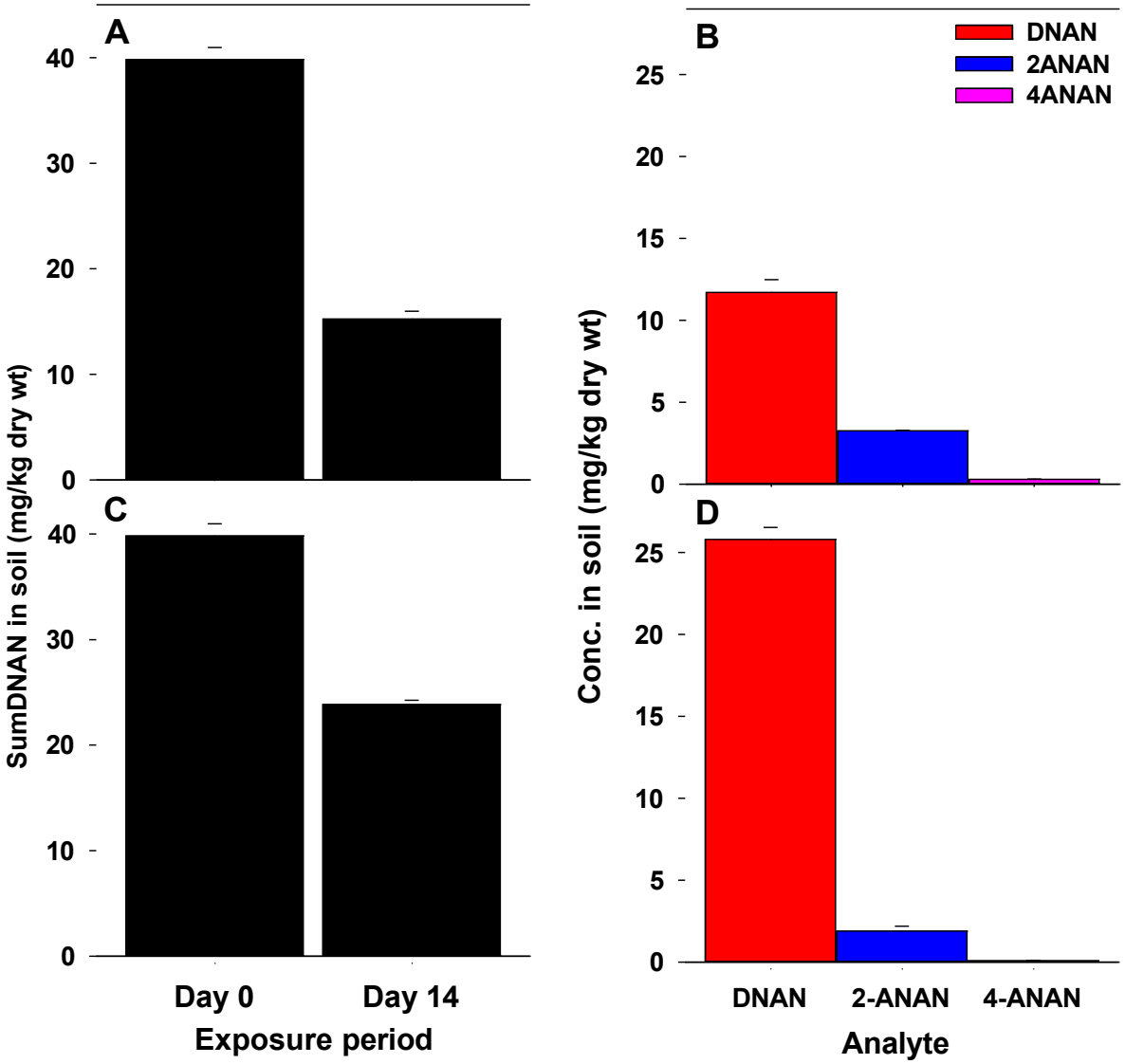

Figure S4. Mean concentrations (± SD) of the sum of 2,4-dinitroanisole (DNAN), 2-ANAN (2-ANAN) and 4-amino-nitroanisole (4-ANAN) (SumDNAN) in exposure soil at initiation and termination for jars with (A) and without (C) earthworms, and mean concentration of DNAN, 2-ANAN, and 4-ANAN in exposure soil at experiment termination of the uptake experiment with (B) and without (D) earthworms..

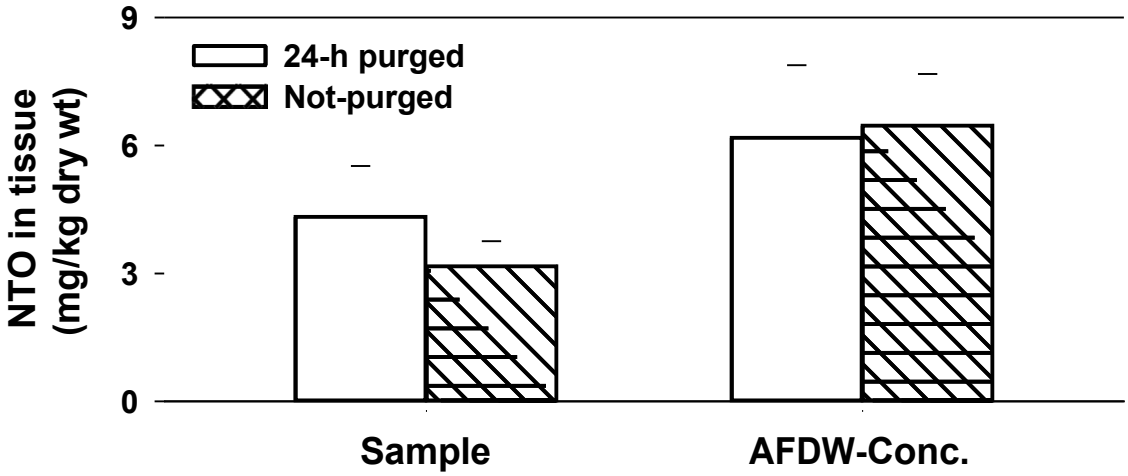

Figure S5. Mean concentrations (± SD) of 3-nitro-1,2,4-triazol-5-one (NTO) for 24-h purged and not-purged day 14 (fed treatment) earthworms exposed for 14 d and fed supplemental food. Tissue concentrations uncorrected for the presence of soil ("Sample") represent the measured concentration for the earthworm samples, and therefore are influenced by the presence of soil within the gut. Estimates of the concentration in the tissue-only were determined by using estimated ash-free dry weights to calculate tissue concentrations (AFDW-Conc.). For not-purged earthworms, the ash-wt. subtracted treatment was significantly higher. For 24-h purged earthworms, no significant differences were observed between treatments.

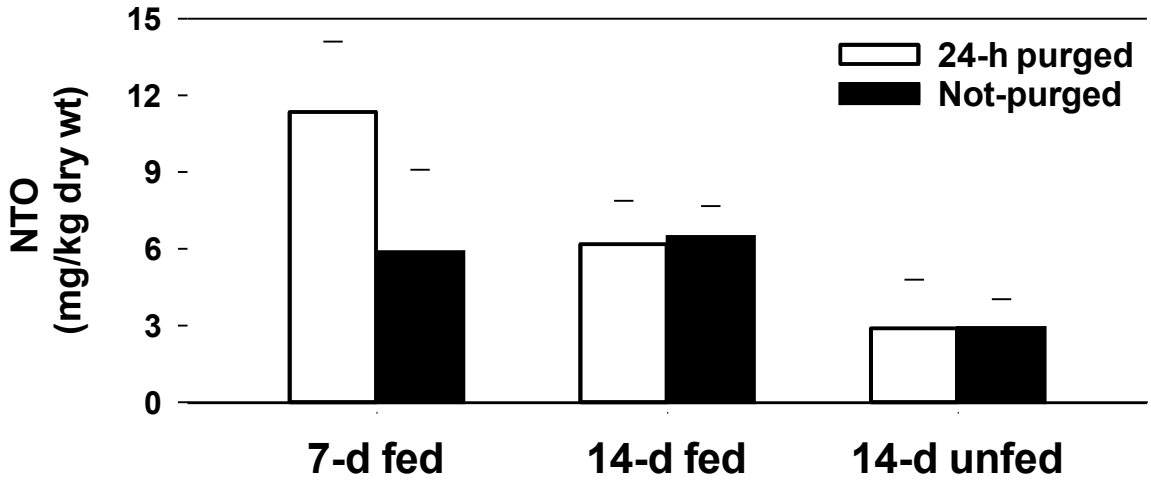

Figure S6. Mean estimated tissue-only concentrations (± SD) of 3-nitro-1,2,4-triazol-5-one (NTO) in 24-h purged and in not-purged earthworms for different treatments. For 24-h purged earthworms, 7-d fed as was significantly higher than 14-d fed and 14-d fed was not significantly different from for 14-d unfed; for not purged earthworms 7-d fed was not significantly different from 14-d fed and 14-d fed was significantly higher than 14-d unfed.

APPENDIX B
Soil Invertebrate Toxicity Tests Data

SERDP ER-2724 DNAN in SSL Definitive Freshly Amended												
Earthworm (Eisenia fetida) Chronic 56 day Toxicity Test. DNAN in Sassafras Sandy Loam Soil.												
		Nominal	28-day	28-day	Mass	28-day	56-day	56-day	56-day	56-day	56-day	56-day
	TRT	Conc.	Start Mass	End Mass	change	Adults	Total cocoons	Mean	Hatched cocoons	Mean	Juveniles	Mean
Jar #		mg/kg	g	g		survive	per jar	[S.E.]	per jar	[S.E.]		[S.E.]
1	0 (acet)	0	3.04	2.4	-0.64	5	26		16		30	
2	0 (acet)	0	2.47	2.15	-0.32	5	20		12		12	
3	0 (acet)	0	2.98	2.36	-0.62	5	23	24.25	15	14.25	5	13
4	0 (acet)	0	3.09	2.4	-0.69	5	28	1.75	14	0.85	5	5.90
5	DNAN	40	2.51	2.09	-0.42	5	16		8		3	
6	DNAN	40	2.86	2.68	-0.18	5	19		10		6	
7	DNAN	40	3.4	3.01	-0.39	5	15	17.25	9	8.5	3	3.5
8	DNAN	40	3.04	2.49	-0.55	5	19	1.03	7	0.96	2	0.87
9	DNAN	60	3.48	2.34	-1.14	5	26		9		3	
10	DNAN	60	2.56	2.22	-0.34	5	22		5		3	
11	DNAN	60	2.65	2.4	-0.25	5	20	21.25	4	6.5	1	2.25
12	DNAN	60	2.78	2.48	-0.3	5	17	1.44	8	1.29	2	0.48
13	DNAN	80	2.69	2.47	-0.22	5	23		10		11	
14	DNAN	80	2.63	2.29	-0.34	5	23		6		1	
15	DNAN	80	3.05	2.81	-0.24	5	15	19.5	8	8.5	4	5.25
16	DNAN	80	3.35	2.8	-0.55	5	17	3.16	10	2.63	5	2.10
17	DNAN	160	2.91	2.71	-0.2	5	3		0		0	
18	DNAN	160	2.9	2.86	-0.04	5	9		0		0	
19	DNAN	160	3.03	2.39	-0.64	4	4	5.25	0	0	0	
20	DNAN	160	2.92	2.3	-0.62	5	5	1.31	0		0	
21	DNAN	320	2.8	0		0	0		0		0	
22	DNAN	320	2.54	0		0	0		0		0	
23	DNAN	320	2.81	0		0	0		0	0	0	
24	DNAN	320	2.74	0		0	0	0	0		0	
25	DNAN	640	3.33	0		0	0		0		0	
26	DNAN	640	3.14	0		0	0		0		0	
27	DNAN	640	3.4	0		0	0	0	0	0	0	
28	DNAN	640	2.9	0		0	0		0		0	

SERDP ER-2724 DNAN in SSL Definitive Weathered/Aged													
Earthworm (Eisenia fetida) Chronic 56 day Toxicity Test. DNAN in Sassafras Sandy Loam Soil.													
Start 8-30-2018; End 10-25-2018													
Adult Harvest: 9-27-2018													
		Nominal											
	TRT	Conc.	28-day	28-day	Mass	28-day	56-day	56-day	56-day	56-day	56-day	56-day	56-day
Jar #		mg/kg	Start Mass	End Mass	change	Adults survive	Total cocoons	Mean	Hatched cocoons	Mean	Hatched cocoons	Juveniles	Mean
			g	g			per jar	[S.E.]	per jar	[S.E.]	% total		[S.E.]
1	0 (acet)	0	1.83	1.81	-0.02	5	17		14			30	
2	0 (acet)	0	1.51	1.35	-0.16	5	16		11			25	
3	0 (acet)	0	1.64	1.5	-0.14	5	17	17	12	12.25		35	31.75
4	0 (acet)	0	1.8	1.48	-0.32	5	18	0.41	12	0.63	72.06	37	2.69
5	DNAN	40	1.4	1.46	0.06	5	15		12			20	
6	DNAN	40	1.87	1.72	-0.15	5	19		17			36	
7	DNAN	40	1.7	1.59	-0.11	5	17	17	13	13.75		29	27.75
8	DNAN	40	1.72	1.63	-0.09	5	17	0.82	13	1.11	80.88	26	3.33
9	DNAN	60	1.82	1.53	-0.29	5	12		9			15	
10	DNAN	60	1.66	1.46	-0.2	5	15		9			25	
11	DNAN	60	1.7	1.64	-0.06	5	13	13.5	10	9.25		16	20.75
12	DNAN	60	1.6	1.64	0.04	5	14	0.65	9	0.25	68.52	27	3.07
13	DNAN	160	1.76	1.69	-0.07	5	2		0			0	
14	DNAN	160	1.7	1.72	0.02	5	4		2			0	
15	DNAN	160	1.89	1.84	-0.05	5	3	2.5	1	0.75		1	0.25
16	DNAN	160	1.7	1.65	-0.05	5	1	0.65	0	0.48	30	0	0.25
17	DNAN	320	1.55	DEAD		0	0		DEAD			0	
18	DNAN	320	1.7	DEAD		0	0		DEAD			0	
19	DNAN	320	1.7	DEAD		0	0	0	DEAD			0	0
20	DNAN	320	1.48	DEAD		0	0		DEAD			0	
21	DNAN	640	1.8	DEAD		0	0		DEAD			0	
22	DNAN	640	1.78	DEAD		0	0		DEAD			0	
23	DNAN	640	1.72	DEAD		0	0	0	DEAD	10		0	0
24	DNAN	640	2.13	DEAD		0	0		DEAD			0	
25	NegCtrl	0	1.57	1.56	-0.01	0	13		9			18	
26	NegCtrl	0	1.78	1.65	-0.13	0	15		14			15	
27	NegCtrl	0	1.55	1.4	-0.15	0	14	15.75	9	10		9	13
28	NegCtrl	0	1.97	1.59	-0.38	0	21	1.80	8	1.35	63.49	10	2.12

SERDP ER-2724 NTO in SSL Definitive								
Earthworm (Eisenia fetida) 28 day Toxicity Test. NTO freshly amended in Sassafras Sandy Loam Soil.								
		Nominal	28-day		28-day	28-day	28-day	28-day
	TRT	Conc.	Adults	Start Mass	Mean	Total cocoons	Mean	Hatched cocoons
Jar #		mg/kg	survive	g	[S.E.]	per jar	[S.E.]	per jar
1	0 (acet)	0	5	2.48		16		4
2	0 (acet)	0	5	2.24		9		1
3	0 (acet)	0	5	2.41	2.43	7	10.75	1
4	0 (acet)	0	5	2.58	0.07	11	1.93	0
5	NTO	100	5	2.5		13		1
6	NTO	100	5	2.34		17		0
7	NTO	100	5	2.11	2.31	7	13	1
8	NTO	100	5	2.28	0.08	15	2.16	0
9	NTO	200	5	2.54		15		1
10	NTO	200	5	2.48		9		1
11	NTO	200	5	2.38	2.43	7	11.50	3
12	NTO	200	5	2.31	0.05	15	2.06	1
13	NTO	400	5	2.31		4		0
14	NTO	400	5	2.64		6		0
15	NTO	400	5	2.77	2.60	13	7.50	1
16	NTO	400	5	2.67	0.10	7	1.94	0
17	NTO	800	5	2.19		3		0
18	NTO	800	5	2.43		1		0
19	NTO	800	5	2.47	2.36	2	2.25	0
20	NTO	800	5	2.33	0.06	3	0.48	0
21	NTO	1600	5	2.19		0		0
22	NTO	1600	5	2.25		0		0
23	NTO	1600	5	2.46	2.31	0	0.25	0
24	NTO	1600	5	2.33	0.06	1	0.25	0

Earthworm (Eisenia fetida) Chronic 56 day Toxicity Test. NTO Weathered/Agedin Sassafras Sandy Loam Soil.													
Start 1-30-2019; End 4-24-2019													
Adult Harvest: 2-27-2 Cocoon and juvenile harvest 4-24-2019													
		Nominal	28-day	28-day	Mass	28-day	56-day	56-day	56-day	56-day	56-day	56-day	56-day
Jar #	TRT	Conc.	Start Mass	End Mass	change	Adults	Total cocoons	Mean	Hatched cocoons	Mean	Hatched cocoons	Juveniles	Mean
		mg/kg	g	g		survive	per jar	[S.E.]	per jar	[S.E.]	% total		[S.E.]
1	NegCtrl	0	1.69	2.28	0.59	5	20		16		80.00	36	
2	NegCtrl	0	1.66	2.01	0.35	5	22		9		40.91	27	
3	NegCtrl	0	1.58	1.95	0.37	5	20	20	17	13.5	85.00	35	32
4	NegCtrl	0	1.5	2.15	0.65	5	18	0.82	12	1.85	66.67	30	2.12
5	0 (MeOH)	0	1.62	2.09	0.47	5	10		6		60.00	4	
6	0 (MeOH)	0	1.46	1.92	0.46	5	15		9		60.00	4	
7	0 (MeOH)	0	1.52	1.84	0.32	5	17	14.5	8	8.25	47.06	5	5.5
8	0 (MeOH)	0	1.73	1.84	0.11	5	16	1.55	10	0.85	62.50	9	1.19
9	NTO	178	1.72	2.24	0.52	6	14		10		71.43	7	
10	NTO	178	1.42	2.09	0.67	5	11		7		63.64	1	
11	NTO	178	1.68	2.05	0.37	5	20	15.25	14	10	70.00	14	7.25
12	NTO	178	1.24	1.71	0.47	5	16	1.89	9	1.47	56.25	7	2.66
13	NTO	300	1.49	1.91	0.42	5	14		4		28.57	1	
14	NTO	300	1.59	2.12	0.53	5	14		10		71.43	1	
15	NTO	300	1.52	2.15	0.63	5	13	14.25	8	7	61.54	8	2.75
16	NTO	300	1.63	1.93	0.3	5	16	0.63	6	1.29	37.50	1	1.75
17	NTO	400	1.6	1.8	0.2	5	9		6		66.67	5	
18	NTO	400	1.64	2.03	0.39	5	17		8		47.06	6	
19	NTO	400	1.61	1.9	0.29	5	19	14	13	8	68.42	11	5.5
20	NTO	400	1.7	2.28	0.58	5	11	2.38	5	1.78	45.45	0	2.25
21	NTO	600	1.43	2.03	0.6	5	7		1		14.29	2	
22	NTO	600	1.68	2.23	0.55	5	7		6		85.71	2	
23	NTO	600	1.63	2.24	0.61	5	7	7	5	4	71.43	4	2
24	NTO	600	1.57	1.95	0.38	5	7	0	4	1.08	57.14	0	0.82
25	NTO	800	1.89	2.18	0.29	5	6		2		33.33	0	
26	NTO	800	1.64	2.25	0.61	5	4		2		50.00	1	
27	NTO	800	1.56	1.94	0.38	5	12	7.25	3	2.75	25.00	4	1.25
28	NTO	800	1.36	1.83	0.47	6	7	1.70	4	0.48	57.14	0	0.95
29	NTO	1600	1.3	1.57	0.27	4	0		0		0	0	
30	NTO	1600	1.88	2.41	0.53	5	0		0		0	0	
31	NTO	1600	1.61	2.19	0.58	5	1	0.5	0		0	0	
32	NTO	1600	1.78	1.14	-0.64	2	1	0.29	0		0	0	
33	NTO	2400	1.2	0.91	-0.29	2	1		0		0	0	
34	NTO	2400	1.65	0.49	-1.16	1	2		0		0	0	
35	NTO	2400	1.54	2.05	0.51	5	1	1.33	0		0	0	

SERDP ER-2724 DNAN in WCL Definitive Weathered/Aged													
Earthworm (*Eisenia fetida*) Chronic 56 day Toxicity Test. DNAN in Webster clay Loam Soil.													
Start 11-13-2019; End 1-08-2019													
Adult Harvest: 12-11-2019													
		Nominal											
	TRT	Conc.	28-day	28-day		28-day	56-day	56-day	56-day	56-day	56-day	56-day	56-day
Jar #		Nominal Conc mg/kg	Adult Start Mass (g)	Adult End Mass (g)	Mass difference	Adult # survive	Total cocoons/jar	Total cocoon Mean	Hatched cocoons/ jar	Hatched cocoons Mean	Hatched cocoons % total	Juveniles	Juveniles Mean
								[S.E.]		[S.E.]			[S.E.]
1	NegCtrl	0	1.73	1.34	-0.39	5	21		17			54	
2	NegCtrl	0	1.93	1.51	-0.42	5	16		15			38	
3	NegCtrl	0	1.64	1.36	-0.28	5	18	17.8	13	14.3	80	41	39.3
4	NegCtrl	0	2.11	1.56	-0.55	5	16	1.2	12	1.1		24	6.2
5	0 (acet)	0	1.82	1.53	-0.29	5	16		8			22	
6	0 (acet)	0	1.64	1.45	-0.19	5	17		6			33	
7	0 (acet)	0	1.83	1.43	-0.4	5	10	15.5	7	7.8	50	45	35.3
8	0 (acet)	0	1.72	1.34	-0.38	5	19	1.9	10	0.9		41	5.1
9	DNAN	63	1.87	1.62	-0.25	5	17		6			42	
10	DNAN	63	1.89	1.62	-0.27	5	16		9			32	
11	DNAN	63	1.78	1.34	-0.44	5	20	17.8	9	9.5	54	58	42.0
12	DNAN	63	1.81	1.43	-0.38	5	18	0.9	14	1.7		36	5.7
13	DNAN	84	1.82	1.45	-0.37	5	15		9			44	
14	DNAN	84	1.61	1.39	-0.22	5	18		13			33	
15	DNAN	84	1.98	1.58	-0.4	5	20	17.8	10	11.8	66	19	33.8
16	DNAN	84	1.93	1.62	-0.31	5	18	1.0	15	1.4		39	5.4
17	DNAN	126	1.8	1.43	-0.37	5	21		19			32	
18	DNAN	126	1.91	1.35	-0.56	5	18		13			32	
19	DNAN	126	1.95	1.66	-0.29	5	16	17.8	11	13.0	73	32	30.5
20	DNAN	126	1.98	1.5	-0.48	5	16	1.2	9	2.2		26	1.5
21	DNAN	168	1.74	1.43	-0.31	5	18		12			35	
22	DNAN	168	2.03	1.54	-0.49	5	15		13			28	
23	DNAN	168	2.04	1.46	-0.58	5	10	14.5	5	10.5	72	19	27.8
24	DNAN	168	1.99	1.49	-0.5	5	15	1.7	12	1.8		29	3.3
25	DNAN	337	1.47	1.45	-0.02	5	6		5			6	
26	DNAN	337	1.72	1.47	-0.25	5	12		3			6	
27	DNAN	337	1.79	1.56	-0.23	5	6	7.0	3	3.3	46	3	3.8
28	DNAN	337	1.65	1.51	-0.14	5	4	1.7	2	0.6		0	1.4
29	DNAN	674	1.54	1	-0.54	4	0		0			0	
30	DNAN	674	1.97	1.56	-0.41	5	0		0			0	
31	DNAN	674	1.62	1.44	-0.18	5	0		0			0	
32	DNAN	674	1.49	1.07	-0.42	4	0		0			0	

SERDP ER-2724 NTO Weathered/Aged in Webster clay loam Soil Definitive Test										
Earthworm (Eisenia andrei) Chronic 56 day Toxicity Test.										
Start 1-07-2020; End 3-03-2020										
Adult Harvest: 2-04-2020										
		Nominal	28-day	28-day	28-day	56-day	56-day	56-day	56-day	56-day
	TRT	Conc.	Start Mass	End Mass	Adults	Total cocoons	Mean	Hatched cocoons	Juveniles	Mean
Jar #		mg/kg	g	g	survive	per jar	[S.E.]	per jar		[S.E.]
1	NegCtrl	0	1.13	1.05	5	11		8	9	
2	NegCtrl	0	1.18	1.2	5	12		8	20	
3	NegCtrl	0	1.15	1.19	5	2	9	2	3	12.25
4	NegCtrl	0	1.12	1.08	5	11	2.35	7	17	3.86
5	0 (MeOH)	0	1.14	1.09	5	2		2	2	
6	0 (MeOH)	0	1.27	1.22	5	3		3	9	
7	0 (MeOH)	0	1.34	0.84	5	16	5.75	7	8	5.25
8	0 (MeOH)	0	1.28	1.17	5	2	3.42	1	2	1.89
9	NTO	400	1.28	1.26	5	2		1	2	
10	NTO	400	1.15	1.13	5	1		1	1	
11	NTO	400	1.16	1.18	5	4	3	4	9	4.75
12	NTO	400	1.39	1.24	5	5	0.91	4	7	1.93
13	NTO	600	1.21	1.25	5	2		1	0	
14	NTO	600	1.31	1.22	5	2		1	2	
15	NTO	600	1.06	1.1	5	1	1.5	1	2	1.25
16	NTO	600	1.23	1.2	5	1	0.29	1	1	0.48
17	NTO	800	1.19	1.18	5	1		1	1	
18	NTO	800	1.24	1.29	5	1		1	1	
19	NTO	800	1.12	1.17	5	1	0.75	1	2	1
20	NTO	800	1.51	1.31	5	0	0.25	0	0	0.41
21	NTO	1200	1.35	1.24	5	5		4	6	
22	NTO	1200	1.12	1.27	5	2		1	1	
23	NTO	1200	1.54	1.13	4	2	2.25	2	1	2
24	NTO	1200	1.26	1.12	5	0	1.03	0	0	1.35
25	NTO	1600	1.29	1.24	5	0		0	0	
26	NTO	1600	1.35	1.24	5	3		2	0	
27	NTO	1600	1.16	1.22	5	1	1	1	2	0.5
28	NTO	1600	1.01	1.15	5	0	0.71	0	0	0.5

Definitive invertebrate assays						
Compound:	**DNAN freshly amended in SSL soil**					
Start Date:	**12-Apr-18**					
Invertebrate:	***Enchytraeus crypticus***					
Hydration:	**3.6 g ASTM Type I water added to 20 g dry soil. Food added.**					
	Food added.					
NOMINAL		**26-Apr-18**	**Mean**	**10-May-18**	**Mean**	**C.V.**
Concentration	**Rep**	**Adults**	**S.E.**	**Juveniles**	**S.E.**	
Negative control	1	10	10.0	392	354.5	0.209
Negative control	2	10	0.0	244	36.98	
Negative control	3	10		383		
Negative control	4	10		399		
Acetone control	1	10	9.8	415	371.75	0.161
Acetone control	2	10	0.3	344	29.86	
Acetone control	3	10		427		
Acetone control	4	9		301		**Change%**
20	1	10	9.8	339	535.75	-44.1
20	2	10	0.3	653	90.62	
20	3	10		722		
20	4	9		429		
30	1	9	9.3	647	500	-34.5
30	2	10	0.3	418	61.75	
30	3	9		380		
30	4	9		555		
40	1	9	8.8	207	235.75	36.6
40	2	10	0.6	206	20.63	
40	3	7		236		
40	4	9		294		
60	1	9	8.5	380	179.5	51.7
60	2	7	0.5	61	71.95	
60	3	9		186		
60	4	9		91		
80	1	8	7.5	16	51.5	86.1
80	2	7	0.6	27	32.97	
80	3	9		150		
80	4	6		13		
120	1	9	7.0	37	48.8	86.9
120	2	6	0.9	9	32.2	
120	3	5		143		
120	4	8		6		

Definitive invertebrate assays						
Compound:	**DNAN W/A in amended SSL soil**					
Start Date:	**30-Aug-18**					
Invertebrate:	***Enchytraeus crypticus***					
Hydration:	**7 g ASTM Type I water to 100 g w/a soil @ 60% of WHC.**					
Food added.	**2.6 g water in 15 g soil in Neg. control.**					
NOMINAL		**13-Sep-18**	**Mean**	**27-Sep-18**	**Mean**	**C.V.**
Concentration	**Rep**	**Adults**	**S.E.**	**Juveniles**	**S.E.**	
Negative control	1	10	9.3	722	776	0.171
Negative control	2	9	0.5	867	66.50	
Negative control	3	8		901		
Negative control	4	10		614		
Acetone control	1	10	10.0	886	924.0	0.102
Acetone control	2	10	0.0	810	47.1	
Acetone control	3	10		1017		
Acetone control	4	10		983		**Change%**
40	1	9	9.8	637	867.3	6.1
40	2	10	0.3	904	80.0	
40	3	10		1007		
40	4	10		921		
60	1	10	10.0	1066	841.0	9.0
60	2	10	0.0	1108	144.7	
60	3	10		530		
60	4	10		660		
80	1	10	9.0	859	780.5	15.5
80	2	8	0.6	566	71.6	
80	3	8		854		
80	4	10		843		
120	1	8	8.3	337	364.5	60.6
120	2	6	0.9	206	82.4	
120	3	10		596		
120	4	9		319		
160	1	8	7.3	221	229.0	75.2
160	2	8	0.8	291	25.5	
160	3	8		237		
160	4	5		167		
320	1	0	0.0	0	0.0	100.0
320	2	0	0.0	0	0.00	
320	3	0		0		
320	4	0		0		
640	1	0	0.0	0	0.0	100.0
640	2	0	0.0	0	0.0	
640	3	0		0		
640	4	0		0		

Definitive invertebrate assays						
Compound:	**NTO freshly amended in SSL soil**					
Start Date:	**1-Feb-19**					
Invertebrate:	***Enchytraeus crypticus***					
Hydration:	**18 g ASTM Type I water added to 100 g dry soil.**					
	Food added.					
NOMINAL		**15-Feb-19**	**Mean**	**1-Mar-19**	**Mean**	**C.V.**
Concentration	**Rep**	**Adults**	**S.E.**	**Juveniles**	**S.E.**	
Negative control	1	10	8.4	239	262	0.463
Negative control	2	9	0.3	311	42.87	
Negative control	3	9		138		
Negative control	4	10		295		
Methanol control	1	9	7.3	487	278.25	-6.2
Methanol control	2	3	1.5	112	82.91	
Methanol control	3	7		185		
Methanol control	4	10		329		**Change%**
50	1	10	9.5	332	384.25	-46.7
50	2	9	0.3	547	55.28	
50	3	9		355		
50	4	10		303		
177	1	9	9.0	322	308.75	-11.0
177	2	10	0.7	429	53.39	
177	3	7		169		
177	4	10		315		
300	1	10	9.3	215	185.8	33.2
300	2	9	0.5	228	38.38	
300	3	8		71		
300	4	10		229		
400	1	10	8.5	7	27.3	90.2
400	2	8	0.5	51	9.4	
400	3	8		32		
400	4	8		19		
600	1	6	6.3	99	119.0	57.2
600	2	6	0.3	67	38.2	
600	3	7		78		
600	4	6		232		
800	1	0	0.0	0	0.0	100.0
800	2	0	0.0	0	0.0	
800	3	0		0		
800	4	0		0		

Definitive invertebrate assays						
Compound:	**NTO W/A in amended SSL soil**					
Start Date:	**1-Mar-19**					
Invertebrate:	***Enchytraeus crypticus***					
Hydration:	**7 g DI water added to 100 g w/a soil @ 60% of WHC.**					
	Food added.					
NOMINAL		**15-Mar-19**	**Mean**	**29-Mar-19**	**Mean**	**C.V.**
Concentration	**Rep**	**Adults**	**S.E.**	**Juveniles**	**S.E.**	
Negative control	1	10	9.9	896	856.88	0.122
Negative control	2	10	0.0	828	37.09	
Negative control	3	10		852		
Negative control	4	10		793		
Methanol control	1	9	9.8	1079	871.5	0.175
Methanol control	2	10	0.3	881	76.23	
Methanol control	3	10		802		
Methanol control	4	10		724		**Change%**
50	1	10	9.5	687	610	30.0
50	2	9	0.3	449	57.45	
50	3	9		606		
50	4	10		698		
177	1	10	10.0	168	341.50	60.8
177	2	10	0.0	476	68.41	
177	3	10		421		
177	4	10		301		
300	1	9	9.5	148	136.8	84.3
300	2	10	0.3	83	32.44	
300	3	9		92		
300	4	10		224		
400	1	10	9.8	9	60.0	93.1
400	2	10	0.3	40	29.5	
400	3	10		46		
400	4	9		145		
600	1	10	10.0	8	17	98.0
600	2	10	0.0	29	5.34	
600	3	10		23		
600	4	10		8		
800	1	10	10.0	0	0.5	99.9
800	2	10	0.0	0	0.50	
800	3	10		2		
800	4	10		0		
1600	1	0	0.0	0	0.0	100.0
1600	2	0	0.0	0	0.0	
1600	3	0		0		
1600	4	0		0		
Outlier excluded from analysis						

Definitive invertebrate assays						
Compound:	**DNAN W/A in amended WCL soil**					
Start Date:	**30-Oct-19**					
Invertebrate:	***Enchytraeus crypticus***					
Hydration:	**9.2 g ASTM Type I water plus 100 g w/a soil @ 60% of WHC.**					
	Food added.					
NOMINAL		**13-Nov-19**	**Mean**	**27-Nov-19**	**Mean**	**C.V.**
Concentration	**Rep**	**Adults**	**S.E.**	**Juveniles**	**S.E.**	
Negative control	1	10	10.0	3352	3841.5	0.100
Negative control	2	10	0.0	3917	192.73	
Negative control	3	10		3809		
Negative control	4	10		4288		
Acetone control	1	10	9.8	3045	3098.3	0.095
Acetone control	2	10	0.3	3220	147.4	
Acetone control	3	9		2717		
Acetone control	4	10		3411		**Change%**
84	1	10	10.0	4272	4774.0	-54.1
84	2	10	0.0	5481	262.1	
84	3	10		4832		
84	4	10		4511		
168	1	10	10.0	3016	3226.0	-4.1
168	2	10	0.0	3950	262.9	
168	3	10		3223		
168	4	10		2715		
337	1	10	10.0	1732	1820.5	41.2
337	2	10	0.0	2337	216.08	
337	3	10		1918		
337	4	10		1295		
674	1	10	9.8	824	405.0	86.9
674	2	10	0.3	496	45.5	
674	3	9		363		
674	4	10		356		

Definitive invertebrate assays						
Compound:	**NTO W/A in amended WCL soil**					
Start Date:	**8-Jan-20**					
Invertebrate:	***Enchytraeus crypticus***					
Hydration:	**11 g DI water added to 120 g w/a soil @ 60% of WHC.**					
	Food added.					
NOMINAL		**22-Jan-20**	**Mean**	**5-Feb-20**	**Mean**	**C.V.**
Concentration	**Rep**	**Adults**	**S.E.**	**Juveniles**	**S.E.**	
Negative control	1	10	10.0	2189	2233	0.073
Negative control	2	10	0.0	2227	82	
Negative control	3	10		2062		
Negative control	4	10		2454		
Carrier control	1	10	10.0	2771	2744	0.046
Carrier control	2	10	0.0	2610	62	
Carrier control	3	10		2903		
Carrier control	4	10		2691		**Change%**
200	1	10	10.0	2710	2742	0.1
200	2	10	0.0	2836	38	
200	3	10		2657		
200	4	10		2763		
600	1	10	9.3	2109	2068	24.6
600	2	9	0.3	2223	65	
600	3	9		2024		
600	4	9		1916		
800	1	10	10.0	1975	1791	34.7
800	2	10	0.0	1642	91	
800	3	10		1918		
800	4	10		1629		

Definitive invertebrate assays						
Compound:	**DNAN weatherd and aged in amended SSL soil**					
Start Date:	**31-Aug-18**					
Invertebrate:	***Folsomia candida***					
Hydration:	**100 g aged amended soil @60% WHC + 5.04 g DI water.**					
	3.17 g water plus 20 g dry soil in Negative control per rep.					
	Food added.					
NOMINAL		**28-Sep-18**	**Mean**	**28-Sep-18**	**Mean**	**C.V.**
Concentration	**Rep**	**Adults**	**S.E.**	**Juveniles**	**S.E.**	
Negative control	1	8	7.6	85	83.4	0.149
Negative control	2	7	0.24	63	5.56	
Negative control	3	7		87		
Negative control	4	8		97		
Negative control	5	8		85		
Acetone control	1	8	8.4	80	70	0.145
Acetone control	2	7	0.51	57	4.39	
Acetone control	3	8		78		
Acetone control	4	10		72		
Acetone control	5	9		63		**Change%**
30	1	3	5	45	60.8	13.1
30	2	4	1.05	32	11.16	
30	3	4		64		
30	4	5		65		
30	5	9		98		
40	1	2	3	27	26.2	62.6
40	2	2	0.63	17	3.34	
40	3	2		21		
40	4	4		30		
40	5	5		36		
60	1	1	1.6	5	8	88.6
60	2	1	0.60	10	3.58	
60	3	4		21		
60	4	1		3		
60	5	1		1		
80	1	0	1	4	4.4	93.7
80	2	2	0.45	7	1.63	
80	3	2		9		
80	4	0		0		
80	5	1		2		
120	1	0	0.2	0	0.6	99.1
120	2	0	0.20	0	0.24	
120	3	0		1		
120	4	1		1		
120	5	0		1		
160	1	0	0	0	0	100.0
160	2	0	0.00	0	0.00	
160	3	0		0		
160	4	0		0		
160	5	0		0		

Definitive invertebrate assays						
Compound:	**NTO Feshly amended in SSL soil**					
Start Date:	**31-Jan-19**					
Invertebrate:	***Folsomia candida***					
Hydration:	**125 g freshly amended soil @88% WHC plus 19.8 g water.**					
	Food added.					
NOMINAL		**28-Feb-19**	**Mean**	**28-Feb-19**	**Mean**	**C.V.**
Concentration	**Rep**	**Adults**	**S.E.**	**Juveniles**	**S.E.**	
Negative control	1	10	9.8	38	49.8	0.298
Negative control	2	10	0.20	51	6.63	
Negative control	3	10		61		
Negative control	4	9		32		
Negative control	5	10		67		
Methanol control	1	9	9.4	49	49.2	0.290
Methanol control	2	10	0.24	60	6.37	
Methanol control	3	10		65		
Methanol control	4	9		43		
Methanol control	5	9		29		**Change%**
177	1	9	8.4	47	37.4	24.0
177	2	10	0.87	52	5.40	
177	3	9		22		
177	4	9		34		
177	5	5		32		
300	1	5	4.6	15	13.2	73.2
300	2	4	0.68	13	1.56	
300	3	7		18		
300	4	3		11		
300	5	4		9		
400	1	3	2.4	10	5.8	88.2
400	2	2	0.24	9	1.53	
400	3	2		3		
400	4	3		4		
400	5	2		3		
600	1	3	2.4	3	4.6	90.7
600	2	4	0.60	3	2.38	
600	3	3		14		
600	4	1		2		
600	5	1		1		
800	1	1	1.4	0	0	100.0
800	2	1	0.24	0	0	
800	3	2		0		
800	4	1		0		
800	5	2		0		

Definitive invertebrate assays						
Compound:	**NTO W-A in amended SSL soil**					
Start Date:	**27-Feb-19**					
Invertebrate:	***Folsomia candida***					
Hydration:	**100 g aged amended soil @60% WHC + 5.04 g DI water.**					
	Food added.					
NOMINAL		**27-Mar-19**	**Mean**	**27-Mar-19**	**Mean**	**C.V.**
Concentration	**Rep**	**Adults**	**S.E.**	**Juveniles**	**S.E.**	
Negative control	1	10	10	96	81	0.154
Negative control	2	10	0.00	77	5.58	
Negative control	3	10		73		
Negative control	4	10		92		
Negative control	5	10		67		
Methanol control	1	10	9.4	89	97.8	0.230
Methanol control	2	10	0.24	129	10.07	
Methanol control	3	9		107		
Methanol control	4	9		96		
Methanol control	5	9		68		**Change%**
177	1	9	8.8	54	91.2	6.7
177	2	10	0.58	117	13.92	
177	3	10		129		
177	4	7		83		
177	5	8		73		
300	1	4	6.8	17	60.2	38.4
300	2	9	1.24	91	14.78	
300	3	7		49		
300	4	10		96		
300	5	4		48		
400	1	2	5.2	13	42	57.1
400	2	3	1.16	27	9.55	
400	3	7		57		
400	4	6		64		
400	5	8		49		
600	1	1	2	1	7.2	92.6
600	2	4	0.55	9	2.24	
600	3	2		3		
600	4	2		13		
600	5	1		10		
800	1	1	1	0	0.6	99.4
800	2	1	0.32	0	0.40	
800	3	1		0		
800	4	2		1		
800	5	0		2		
1600	1	0	0.8	0	0	100.0
1600	2	0	0.37	0	0.00	
1600	3	2		0		
1600	4	1		0		
1600	5	1		0		

Definitive invertebrate assays						
Compound:	**DNAN weatherd and aged in amended WCL soil**					
Start Date:	**29-Oct-19**					
Invertebrate:	***Folsomia candida***					
Hydration:	**100 g aged amended soil @60% WHC + 6.44 g DI water.**					
	Food added.					
NOMINAL		**26-Nov-19**	**Mean**	**26-Nov-19**	**Mean**	**C.V.**
Concentration	**Rep**	**Adults**	**S.E.**	**Juveniles**	**S.E.**	
Negative control	1	9	9.6	72	96	0.192
Negative control	2	10	0.24	98	8.26	
Negative control	3	9		83		
Negative control	4	10		112		
Negative control	5	10		115		
Acetone control	1	10	9.8	182	181.8	0.075
Acetone control	2	10	0.20	209	16.49	
Acetone control	3	9		187		
Acetone control	4	10		211		
Acetone control	5	10		120		**Change%**
32	1	9	9.2	130	174.8	3.9
32	2	10	0.37	141	18.92	
32	3	9		165		
32	4	10		217		
32	5	8		221		
42	1	3	7	53	143.4	21.1
42	2	5	1.26	72	33.59	
42	3	9		179		
42	4	9		201		
42	5	9		212		
63	1	3	6	32	82.2	54.8
63	2	4	1.10	75	15.10	
63	3	7		119		
63	4	9		107		
63	5	7		78		
84	1	5	4.4	58	49.8	72.6
84	2	7	0.87	28	12.03	
84	3	2		16		
84	4	3		67		
84	5	5		80		
126	1	0	2	27	40.4	77.8
126	2	0	1.05	20	7.38	
126	3	5		44		
126	4	4		59		
126	5	1		52		
168	1	2	1.2	73	33.4	81.6
168	2	1	0.37	8	15.08	
168	3	1		15		
168	4	2		67		
168	5	0		4		

Definitive invertebrate assays									
Compound:	**NTO W-A in amended WCL soil**								
Start Date:	**7-Jan-20**								
Invertebrate:	***Folsomia candida***								
Hydration:	**88% of WCL WHC; 160 g W-A amended or control soil @60% WHC + 10.3 g DI water.**								
	Food added.								
NOMINAL		**4-Feb-20**	**Mean**	**4-Feb-20**	**Mean**	**C.V.**			
Concentration	**Rep**	**Adults**	**S.E.**	**Juveniles**	**S.E.**				
Negative control	1	10	10	120	141	0.164			
Negative control	2	10	0.00	114	10				
Negative control	3	10		147					
Negative control	4	10		169					
Negative control	5	10		153					
Methanol control	1	10	10	179	182	0.104			
Methanol control	2	10	0.00	212	8.46				
Methanol control	3	10		183					
Methanol control	4	10		176					
Methanol control	5	10		160		**Change%**			
300	1	10	10	150	166.8	8.4			
300	2	10	0.00	147	7.73				
300	3	10		172					
300	4	10		183					
300	5	10		182					
400	1	10	10	223	220.2	-21.0			
400	2	10	0.00	240	9.48				
400	3	10		235					
400	4	10		217					
400	5	10		186					
600	1	3	6.2	28	46	74.7			
600	2	5	0.97	44	10.01				
600	3	8		43					
600	4	8		31					
600	5	7		84					
800	1	6	6.8	82	59.2	67.5			
800	2	7	0.86	48	8.64				
800	3	8		54					
800	4	9		36					
800	5	4		76					
1200	1	1	1.2	10	11.6	93.6			
1200	2	3	0.49	11	3.59				
1200	3	0		0					
1200	4	1		15					
1200	5	1		22					

APPENDIX C

Toxicity and Uptake Data Obtained in Ryegrass Studies

Data Used for ECp Determinations in Ryegrass Toxicity Studies.

Range-finding ryegrass toxicity tests with DNAN or NTO				
	Meas. Soil Con.	Day 7	Day 21	Day 21
Treatment	(mg/kg)	# of seedlings	Dry Roots (mg)	Dry Shoots (mg)
Carrier Control	0	17	1.800	12.500
Carrier Control	0	18	3.000	12.200
Carrier Control	0	19	1.600	11.100
Negative Control 1	0	18	2.800	11.200
Negative Control 2	0	17	3.100	12.600
Negative Control 3	0	17	1.300	14.200
DNAN 5: Rep 1	5.16	17	3.200	11.200
DNAN 5: Rep 2	5.16	17	1.800	10.700
DNAN 5: Rep 3	5.16	18	3.000	14.900
DNAN 10: Rep 1	8.59	18	3.300	12.500
DNAN 10: Rep 2	8.59	19	4.000	15.400
DNAN 10: Rep 3	8.59	18	4.100	18.200
DNAN 50: Rep 1	51.1	16	1.500	9.700
DNAN 50: Rep 2	51.1	9	0.300	10.900
DNAN 50: Rep 3	51.1	14	0.400	12.500
DNAN 100: Rep 1	102	1	0.000	4.500
DNAN 100: Rep 2	102	2	0.000	4.900
DNAN 100: Rep 3	102	1	0.000	6.100
DNAN 250: Rep 1	227	1	0.000	0.000
DNAN 250: Rep 2	227	0	0.000	0.100
DNAN 250: Rep 3	227	0	0.000	1.100
DNAN 500: Rep 1	417	0	0.000	0.000
DNAN 500: Rep 2	417	0	0.000	0.000
DNAN 500: Rep 3	417	0	0.000	0.000
NTO 5: Rep 1	4.45	16	3.100	12.000
NTO 5: Rep 2	4.45	15	3.700	10.100
NTO 5: Rep 3	4.45	20	2.900	12.300
NTO 10: Rep 1	11.13	16	2.200	13.700
NTO 10: Rep 2	11.13	17	4.500	14.400
NTO 10: Rep 3	11.13	18	3.100	12.300
NTO 50: Rep 1	56.1	11	0.600	1.500
NTO 50: Rep 2	56.1	3	0.700	0.500
NTO 50: Rep 3	56.1	0	1.000	3.300
NTO 100: Rep 1	89.4	0	0	0
NTO 100: Rep 2	89.4	0	0	0
NTO 100: Rep 3	89.4	0	0	0
NTO 250: Rep 1	253	0	0	0
NTO 250: Rep 2	253	0	0	0
NTO 250: Rep 3	253	0	0	0
NTO 500: Rep 1	474	0	0	0
NTO 500: Rep 2	474	0	0	0
NTO 500: Rep 3	474	0	0	0

Initial Concentrations of DNAN and the Transformation Products in SSL soil in Range-Finding Test (Table 26).

DNAN						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	5.36	8.59	51.01	101.80	226.98	416.45
2	5.34	8.58	51.02	101.85	227.20	416.50
3	5.35	8.56	51.03	101.90	225.89	416.81
Avg	5.4	8.6	51	102	227	417
SD	0.01	0.02	0.01	0.05	0.70	0.20
2-A-4-NAN						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.46	0.06	0.08	0.06	0.15	0.14
2	0.08	0.43	0.08	0.06	0.14	0.14
3	0.08	0.04	0.08	0.06	0.14	0.15
Avg	0.21	0.18	0.08	0.06	0.14	0.14
SD	0.22	0.22	0.001	0.002	0.002	0.002
SumDNAN						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	5.82	8.65	51.09	101.86	227.13	416.35
2	5.42	9.01	51.10	101.91	227.34	416.74
3	5.43	8.60	51.11	101.96	226.03	416.47
Avg	5.6	8.8	51	102	227	417
SD	0.23	0.22	0.01	0.05	0.70	0.20

Final Concentrations of DNAN and the Transformation Products in SSL Soil in Range-Finding Test with Ryegrass (Table 27).

DNAN						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	2.58	3.58	17.40	37.30	127.00	366.00
2	2.57	3.56	17.36	37.29	126.98	365.95
3	2.59	3.76	17.35	37.28	126.96	365.80
Avg	2.6	3.6	17.4	37.3	127	366
SD	0.01	0.11	0.03	0.01	0.02	0.10
2-A-4-NAN						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.23	0.29	1.06	2.95	7.26	3.89
2	0.22	0.28	1.05	2.93	7.25	3.92
3	0.21	0.27	1.07	2.97	7.27	3.87
Avg	0.22	0.28	1.06	2.95	7.26	3.89
SD	0.01	0.01	0.01	0.02	0.01	0.03
SumDNAN						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	2.81	3.87	18.46	40.25	134.26	369.89
2	2.79	3.84	18.41	40.22	134.23	369.87
3	2.80	4.03	18.42	40.25	134.23	369.69
Avg	2.8	3.9	18	40	134	370
SD	0.01	0.10	0.03	0.02	0.02	0.11
Sum DNAN % Decrease						
	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
Initial	5.55	8.8	51.19	102.12	226.66	417.17
Final	2.8	3.86	18.45	40.25	134.25	369.9
%	49.55	56.14	63.96	60.59	40.77	11.33

Final Concentrations of DNAN and the Transformation Products in SSL Soil at the end of the 21-D Range-Finding Test without Ryegrass (Table 28).

DNAN						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	2.80	3.82	18.00	45.75	128.33	378.01
2	2.70	3.75	18.00	45.20	128.71	378.03
3	3.00	3.87	17.98	45.73	127.58	378.03
Avg	2.8	3.8	18.0	45.6	128	378
SD	0.15	0.06	0.01	0.31	0.58	0.01
2-A-4-NAN						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.21	0.24	1.01	3.77	8.44	4.32
2	0.23	0.26	1.01	3.76	8.43	4.31
3	0.22	0.21	1.02	3.76	8.45	4.32
Avg	0.22	0.24	1.01	3.76	8.4	4.3
SD	0.01	0.03	0.01	0.01	0.01	0.004
4-A-2-NAN						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	ND	ND	0.23	0.37	0.57	0.44
2	ND	ND	0.23	0.36	0.57	0.45
3	ND	ND	0.22	0.36	0.56	0.44
Avg	ND	ND	0.23	0.36	0.57	0.44
SD	ND	ND	0.004	0.01	0.002	0.002
SumDNAN						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	3.01	4.06	19.24	49.89	137.34	382.77
2	2.93	4.01	19.24	49.32	137.71	382.79
3	3.22	4.08	19.22	49.85	136.59	382.79
Avg	3.05	4.05	19.24	49.69	137.21	382.79
SD	0.15	0.04	0.01	0.32	0.57	0.01
SumDNAN % Decrease						
	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
Initial	5.55	8.8	51.19	102.12	226.66	417.17
Final	3.05	4.06	19.23	49.66	137.3	382.71
%	45.05	53.86	62.43	51.37	39.42	8.26

NTO Concentrations in SSL Soil in the 21-D Range-Finding Test with or without Ryegrass (Table 29).

NTO Initial						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	4.34	11.10	56.10	89.40	253.00	474.00
2	4.35	10.60	56.12	88.32	252.35	475.22
3	4.34	11.70	56.10	89.12	254.00	474.95
Avg	4.3	11	56	89	253	475
SD	0.01	0.55	0.01	0.56	0.83	0.64
NTO Final with Ryegrass						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.27	0.19	2.15	22.99	95.40	259.40
2	0.30	0.20	2.13	23.50	95.90	260.00
3	0.25	0.17	2.16	23.40	95.65	258.40
Avg	0.27	0.19	2.15	23.3	95.7	259
SD	0.02	0.02	0.02	0.27	0.25	0.81
% Reduction	94	98	96	74	62	45
NTO Final without Ryegrass						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.32	0.46	3.52	27.57	104.81	243.03
2	0.33	0.45	3.50	27.30	105.50	243.10
3	0.35	0.48	3.53	28.00	105.00	243.15
Avg	0.33	0.46	3.5	27.6	105	243
SD	0.02	0.02	0.02	0.35	0.36	0.06
% Reduction	92	96	94	69	58	49

DNAN and the Transformation Products in Ryegrass Roots at the Conclusion of the 21-D Pilot Test and the Resulting Bioconcentration Factors (Table 32).

DNAN						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.0028	0.0044	0.0426	NS	NS	NS
2	0.0029	0.0037	0.0429	NS	NS	NS
3	0.0022	0.0029	0.0438	NS	NS	NS
Avg	0.0026	0.0037	0.0431	NS	NS	NS
SD	0.0003	0.0008	0.0006	NS	NS	NS
2-A-4-NAN						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.0008	0.0013	0.0064	NS	NS	NS
2	0.0017	0.0009	0.0063	NS	NS	NS
3	0.0120	0.0010	0.0064	NS	NS	NS
Avg	0.0048	0.0010	0.0064	NS	NS	NS
SD	0.0062	0.0002	0.0001	NS	NS	NS
SumDNAN						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.0036	0.0057	0.0490	NS	NS	NS
2	0.0045	0.0046	0.0492	NS	NS	NS
3	0.0142	0.0039	0.0502	NS	NS	NS
Avg	0.0074	0.0047	0.0495	NS	NS	NS
SD	0.0059	0.0009	0.0006	NS	NS	NS
Bioconcentration Factor						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.0005	0.0005	0.0008	NS	NS	NS
2	0.0005	0.0004	0.0008	NS	NS	NS
3	0.0004	0.0003	0.0009	NS	NS	NS
Avg	0.0005	0.0004	0.0008	NS	NS	NS
SD	0.00006	0.0001	0.00001	NS	NS	NS

DNAN and the Transformation Products in Ryegrass Shoots at the Conclusion of the 21-D Pilot Test and the Resulting Bioconcentration and Translocation Factors (Table 33).

DNAN						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.0006	0.0015	0.0112	0.0174	NS	NS
2	0.0005	0.0003	0.0111	0.0175	NS	NS
3	0.0008	ND	0.0111	0.0174	NS	NS
Avg	0.0006	0.0009	0.0111	0.0174	NS	NS
SD	0.0002	0.0008	0.0001	0.0001	NS	NS

DNAN Bioconcentration Factor						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.0001	0.00017	0.0002	0.0002	NS	NS
2	0.0001	0.00003	0.0002	0.0002	NS	NS
3	0.0001	ND	0.0002	0.0002	NS	NS
Avg	0.0001	0.0001	0.0002	0.0002	NS	NS
SD	0.00003	0.00010	0.000001	0.000001	NS	NS

2-A-4-NAN						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.0008	0.0014	0.0029	0.0174	NS	NS
2	0.0007	0.0003	0.0028	0.0175	NS	NS
3	0.0009	0.0006	0.0029	0.0165	NS	NS
Avg	0.0008	0.0008	0.0029	0.0171	NS	NS
SD	0.0001	0.0005	0.0001	0.0006	NS	NS

2-A-4-NAN Bioconcentration Factor						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.0017	0.0230	0.0365	0.2816	NS	NS
2	0.0088	0.0008	0.0354	0.2778	NS	NS
3	0.0113	ND	0.0363	0.2750	NS	NS
Avg	0.0072	0.0119	0.0359	0.2797	NS	NS
SD	0.00493	0.0157	0.0007	0.0027	NS	NS

SumDNAN						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.0014	0.0029	0.0141	0.0348	NS	NS
2	0.0012	0.0006	0.0139	0.0350	NS	NS
3	0.0017	0.0006	0.0140	0.0339	NS	NS
Avg	0.0014	0.0014	0.0140	0.0346	NS	NS
SD	0.0003	0.0013	0.0001	0.0006	NS	NS

SumDNAN Bioconcentration						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.0019	0.0232	0.0367	0.2817	NS	NS
2	0.0088	0.0008	0.0357	0.2779	NS	NS
3	0.0114	ND	0.0365	0.2752	NS	NS
Avg	0.00736	0.01199	0.03627	0.2783	NS	NS
SD	0.00494	0.01582	0.00054	0.0033	NS	NS

DNAN Translocation Factor						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.21	0.32	0.26	NS	NS	NS
2	0.17	0.09	0.26	NS	NS	NS
3	0.36	0.21	0.25	NS	NS	NS
Avg	0.25	0.20	0.26	NS	NS	NS
SD	0.10	0.11	0.005	NS	NS	NS

2-A-4-NAN Translocation Factor						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	0.96	1.09	0.46	NS	NS	NS
2	0.42	0.38	0.44	NS	NS	NS
3	0.08	0.59	0.45	NS	NS	NS
Avg	0.49	0.69	0.45	NS	NS	NS
SD	0.45	0.36	0.01	NS	NS	NS

SumDNAN Translocation Factor						
Rep	5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	500 mg/kg
1	1.18	1.40	0.72	NS	NS	NS
2	0.59	0.47	0.70	NS	NS	NS
3	0.44	0.80	0.71	NS	NS	NS
Avg	0.74	0.89	0.71	NS	NS	NS
SD	0.39	0.47	0.01	NS	NS	NS

Concentrations of DNAN and the Transformation Products in Ryegrass Shoots and Foots at the Conclusion of the 42-D Definitive Test and the Resulting Bioconcentration and Translocation Factors (Table 34).

DNAN Shoot Concentration			
Rep	DNAN	2-A-4-NAN	4-A-2-NAN
1	3.18	4.24	ND
2	2.08	ND	ND
3	3.09	ND	ND
4	2.18	ND	ND
5	2.92	ND	ND
6	3.37	ND	ND
7	3.94	ND	ND
Avg	3	4.20	ND
SD	0.7	ND	ND
SumDNAN	7.17		

DNAN Root Concentration			
Rep	DNAN	2-A-4-NAN	4-A-2-NAN
1	0.99	0.6	ND
2	0.94	ND	ND
3	1.02	ND	ND
4	0.97	ND	ND
5	0.99	ND	ND
6	1.13	ND	ND
7	0.99	ND	ND
Avg	1	0.6	ND
SD	0.1	ND	ND
SumDNAN	1.6		

DNAN Root Concentration			
Rep	DNAN	2-A-4-NAN	4-A-2-NAN
1	0.99	0.6	ND
2	0.94	ND	ND
3	1.02	ND	ND
4	0.97	ND	ND
5	0.99	ND	ND
6	1.13	ND	ND
7	0.99	ND	ND
Avg	1	0.6	ND
SD	0.1	ND	ND
SumDNAN	1.6		

DNAN Bioconcentration			
Rep	DNAN	2-A-4-NAN	4-A-2-NAN
1	0.06	3.2	ND
2	0.04	ND	ND
3	0.06	ND	ND
4	0.04	ND	ND
5	0.06	ND	ND
6	0.06	ND	ND
7	0.08	ND	ND
Avg	0.06	3.20	ND
SD	0.01	ND	ND
SumDNAN	3.26		

DNAN Bioconcentration			
Rep	DNAN	2-A-4-NAN	4-A-2-NAN
1	0.02	0.4	ND
2	0.02	ND	ND
3	0.02	ND	ND
4	0.02	ND	ND
5	0.02	ND	ND
6	0.02	ND	ND
7	0.02	ND	ND
Avg	0.02	0.44	ND
SD	0.0012	ND	ND
SumDNAN	0.46		

DNAN Bioconcentration			
Rep	DNAN	2-A-4-NAN	4-A-2-NAN
1	0.02	0.4	ND
2	0.02	ND	ND
3	0.02	ND	ND
4	0.02	ND	ND
5	0.02	ND	ND
6	0.02	ND	ND
7	0.02	ND	ND
Avg	0.02	0.44	ND
SD	0.0012	ND	ND
SumDNAN	0.46		

DNAN Translocation Factor			
Rep	DNAN	2-A-4-NAN	4-A-2-NAN
1	3.23	7.2	ND
2	2.22	ND	ND
3	3.03	ND	ND
4	2.24	ND	ND
5	2.95	ND	ND
6	2.98	ND	ND
7	3.98	ND	ND
Avg	2.95	7.17	ND
SD	0.60	ND	ND
SumDNAN	10.12		

Seedling Emergence over 7 Days for Ryegrass Exposed to DNAN in the Range-Finding Toxicity Tests (Figure 19).

Soil Treatments	# of seedlings	Seedling Emergence %
Carrier Control : Rep 1	17	85
Carrier Control : Rep 2	18	90
Carrier Control : Rep 3	19	95
Avg	18	90
SD	1.00	5.00
Control: Rep 1	18	90
Control: Rep 2	17	85
Control: Rep 3	17	85
Average	17	87
SD	0.58	2.89
DNAN 5: Rep 1	17	85
DNAN 5: Rep 2	17	85
DNAN 5: Rep 3	18	90
Avg	17	87
SD	0.58	2.89
DNAN 10: Rep 1	18	90
DNAN 10: Rep 2	19	95
DNAN 10: Rep 3	18	90
Avg	18	92
SD	0.58	2.89
DNAN 50: Rep 1	16	80
DNAN 50: Rep 2	9	45
DNAN 50: Rep 3	14	70
Avg	13	65
SD	3.61	18.03
DNAN 100: Rep 1	1	5
DNAN 100: Rep 2	2	10
DNAN 100: Rep 3	1	5
Avg	1	7
SD	0.58	2.89
DNAN 250: Rep 1	1	5
DNAN 250: Rep 2	0	0
DNAN 250: Rep 3	0	0
Avg	0	2
SD	0.58	2.89
DNAN 500: Rep 1	0	0
DNAN 500: Rep 2	0	0
DNAN 500: Rep 3	0	0
Avg	0	0
SD	0	0

Ryegrass Shoot Dry Mass at the End of the 21-D Exposure to DNAN (Figure 20).

Soil Treatments	Shoot Dry Mass (mg)
Carrier Control : Rep 1	12.50
Carrier Control : Rep 2	12.20
Carrier Control : Rep 3	11.10
Avg	11.93
SD	0.74
Control: Rep 1	11.20
Control: Rep 2	12.60
Control: Rep 3	14.20
Avg	12.67
SD	1.50
DNAN 5: Rep 1	11.20
DNAN 5: Rep 2	10.70
DNAN 5: Rep 3	14.90
Avg	12.27
SD	2.29
DNAN 10: Rep 1	12.50
DNAN 10: Rep 2	15.40
DNAN 10: Rep 3	18.20
Avg	15.37
SD	2.85
DNAN 50: Rep 1	9.70
DNAN 50: Rep 2	10.90
DNAN 50: Rep 3	12.50
Avg	11.03
SD	1.40
DNAN 100: Rep 1	4.50
DNAN 100: Rep 2	4.90
DNAN 100: Rep 3	6.10
Avg	5.17
SD	0.83
DNAN 250: Rep 1	0
DNAN 250: Rep 2	0.10
DNAN 250: Rep 3	1.10
Avg	0
SD	0
DNAN 500: Rep 1	0
DNAN 500: Rep 2	0
DNAN 500: Rep 3	0
Avg	0
SD	0

Ryegrass Root Dry Mass at the End of the 21-D Exposure to DNAN (Figure 21).

Soil Treatments	Root Dry Mass (mg)
Carrier Control : Rep 1	1.80
Carrier Control : Rep 2	3.00
Carrier Control : Rep 3	1.60
Avg	2.13
SD	0.76
Control: Rep 1	2.80
Control: Rep 2	3.10
Control: Rep 3	1.30
Avg	2.40
SD	0.96
DNAN 5: Rep 1	3.20
DNAN 5: Rep 2	1.80
DNAN 5: Rep 3	3.00
Avg	2.67
SD	0.76
DNAN 10: Rep 1	3.30
DNAN 10: Rep 2	4.00
DNAN 10: Rep 3	4.10
Avg	3.80
SD	0.44
DNAN 50: Rep 1	1.50
DNAN 50: Rep 2	0.30
DNAN 50: Rep 3	0.40
Avg	0.73
SD	0.67
DNAN 100: Rep 1	0
DNAN 100: Rep 2	0
DNAN 100: Rep 3	0
Avg	0
SD	0
DNAN 250: Rep 1	0
DNAN 250: Rep 2	0
DNAN 250: Rep 3	0
Avg	0
SD	0
DNAN 500: Rep 1	0
DNAN 500: Rep 2	0
DNAN 500: Rep 3	0
Avg	0
SD	0

Ryegrass Seedling Emergence after 7 D of Exposure to NTO in SSL Soil (Figure 22).

Soil Treatments	# of seedlings	Seedling Emergence %
Carrier Control : Rep 1	17	85
Carrier Control : Rep 2	18	90
Carrier Control : Rep 3	19	95
Avg	18	90
SD	1.00	5.00
Control: Rep 1	18	90
Control: Rep 2	17	85
Control: Rep 3	17	85
Avg	17	87
SD	0.58	2.89
NTO 5: Rep 1	16	80
NTO 5: Rep 2	15	75
NTO 5: Rep 3	20	100
Avg	17	85
SD	2.65	13.23
NTO 10: Rep 1	16	80
NTO 10: Rep 2	17	85
NTO 10: Rep 3	18	90
Avg	17	85
SD	1	5
NTO 50: Rep 1	11	55
NTO 50: Rep 2	3	15
NTO 50: Rep 3	0	0
Avg	4.67	35
SD	5.69	28.28
NTO 100: Rep 1	0	0
NTO 100: Rep 2	0	0
NTO 100: Rep 3	0	0
Avg	0	0
SD	0	0
NTO 250: Rep 1	0	0
NTO 250: Rep 2	0	0
NTO 250: Rep 3	0	0
Avg	0	0
SD	0	0
NTO 500: Rep 1	0	0
NTO 500: Rep 2	0	0
NTO 500: Rep 3	0	0
Avg	0	0
SD	0	0

Shoot Dry Mass of Ryegrass Exposed to NTO over 21 D (Figure 23).

Soil Treatments	Shoot Dry Mass (mg)
Carrier Control : Rep 1	12.50
Carrier Control : Rep 2	12.20
Carrier Control : Rep 3	11.10
Avg	11.93
SD	0.74
Control: Rep 1	11.20
Control: Rep 2	12.60
Control: Rep 3	14.20
Avg	12.67
SD	1.50
NTO 5: Rep 1	12.00
NTO 5: Rep 2	10.10
NTO 5: Rep 3	12.30
Avg	11.47
SD	1.19
NTO 10: Rep 1	13.70
NTO 10: Rep 2	14.40
NTO 10: Rep 3	12.30
Avg	13.47
SD	1.07
NTO 50: Rep 1	1.50
NTO 50: Rep 2	0.50
NTO 50: Rep 3	3.30
Avg	1.77
SD	1.42
NTO 100: Rep 1	0
NTO 100: Rep 2	0
NTO 100: Rep 3	0
Avg	0
SD	0
NTO 250: Rep 1	0
NTO 250: Rep 2	0
NTO 250: Rep 3	0
Avg	0
SD	0
NTO 500: Rep 1	0
NTO 500: Rep 2	0
NTO 500: Rep 3	0
Avg	0
SD	0

Root Dry Mass of Ryegrass Exposed to NTO over 21 D (Figure 24).

Soil Treatments	Root Dry Mass (mg)
Carrier Control : Rep 1	1.80
Carrier Control : Rep 2	3.00
Carrier Control : Rep 3	1.60
Avg	2.13
SD	0.76
Control: Rep 1	2.80
Control: Rep 2	3.10
Control: Rep 3	1.30
Avg	2.40
SD	0.96
NTO 5: Rep 1	3.10
NTO 5: Rep 2	3.70
NTO 5: Rep 3	2.90
Avg	3.23
SD	0.42
NTO 10: Rep 1	2.20
NTO 10: Rep 2	4.50
NTO 10: Rep 3	3.10
Avg	3.27
SD	1.16
NTO 50: Rep 1	0.60
NTO 50: Rep 2	0.70
NTO 50: Rep 3	1.00
Avg	0.77
SD	0.21
NTO 100: Rep 1	0
NTO 100: Rep 2	0
NTO 100: Rep 3	0
Avg	0
SD	0
NTO 250: Rep 1	0
NTO 250: Rep 2	0
NTO 250: Rep 3	0
Avg	0
SD	0
NTO 500: Rep 1	0
NTO 500: Rep 2	0
NTO 500: Rep 3	0
Avg	0
SD	0

Initial and final Concentrations of SumDNAN Extracted from SSL Soil during the 42-D Definitive Tests with or without Ryegrass (Figure 25).

SumDNAN with Ryegrass				
Rep	Initial	DNAN	2-A-4-NAN	4-A-2-NAN
1	52.25	17.80	1.73	0.13
2	53.45	17.90	1.38	0.13
3	52.00	20.60	1.22	0.13
4	51.90	23.40	1.20	0.14
5	53.14	24.40	1.13	0.15
6	52.25	20.50	1.28	0.14
7	51.99	19.30	1.36	0.15
Avg	52.43	20.56	1.33	0.14
SD	0.61	2.55	0.20	0.01
SumDNAN without Ryegrass				
Rep	Initial	DNAN	2-A-4-NAN	4-A-2-NAN
1	52.25	30.90	0.69	0.14
2	53.45	30.90	0.59	0.13
3	52.00	33.20	0.70	0.14
4	51.90	29.90	0.69	0.14
5	53.14	31.30	0.66	0.14
6	52.25	37.10	0.62	0.15
7	51.99	30.10	0.57	0.13
Avg	52.43	31.91	0.64	0.14
SD	0.61	2.53	0.05	0.01
Sum DNAN % Decrease				
	Ryegrass	Without Ryegrass		
Initial	52.43	52.43		
SumDNAN	22.02	32.70		
%	58	38		

Concentrations of DNAN and the Transformation Products Extracted from SSL Soil at the Conclusion of the 42-D Definitive Test with Ryegrass (Figure 26).

Soil with Ryegrass			
Rep	**DNAN**	**2-A-4-NAN**	**4-A-2-NAN**
1	17.80	1.73	0.13
2	17.90	1.38	0.13
3	20.60	1.22	0.13
4	23.40	1.20	0.14
5	24.40	1.13	0.15
6	20.50	1.28	0.14
7	19.30	1.36	0.15
Avg	20.56	1.33	0.14
SD	2.55	0.20	0.01
% Decrease of Total	93	6	0.6

Concentration of DNAN and the Transformation Products Extracted from SSL Soil at the Conclusion of the 42-D Definitive Test without Ryegrass (Figure 27).

Soil without Ryegrass			
Rep	**DNAN**	**2-A-4-NAN**	**4-A-2-NAN**
1	30.90	0.69	0.14
2	30.90	0.59	0.13
3	33.20	0.70	0.14
4	29.90	0.69	0.14
5	31.30	0.66	0.14
6	37.10	0.62	0.15
7	30.10	0.57	0.13
Avg	31.91	0.64	0.14
SD	2.53	0.05	0.01
% Decrease of Total	97.6	2	0.4

Shoot Dry Mass of Ryegrass Exposed to DNAN for 42 D (Figure 28).

Soil Treatments	Shoot Dry Biomass (mg)
Carrier Control-Rep 1	17.50
Carrier Control-Rep 2	16.30
Carrier Control-Rep 3	20.30
Carrier Control-Rep 4	18.90
Carrier Control-Rep 5	10.30
Carrier Control-Rep 6	20.00
Carrier Control-Rep 7	15.80
Avg	17.01
SD	3.43
DNAN-Rep 1	13.30
DNAN-Rep 2	12.90
DNAN-Rep 3	13.70
DNAN-Rep 4	9.40
DNAN-Rep 5	10.40
DNAN-Rep 6	17.30
DNAN-Rep 7	16.30
Avg	13.33
SD	2.86

Root Dry Mass of Ryegrass Exposed to DNAN for 42 D (Figure 29).

Soil Treatments	Root Dry Biomass (mg)
Carrier Control-Rep 1	5.20
Carrier Control-Rep 2	4.60
Carrier Control-Rep 3	3.40
Carrier Control-Rep 4	2.50
Carrier Control-Rep 5	1.50
Carrier Control-Rep 6	5.30
Carrier Control-Rep 7	3.90
Avg	3.77
SD	1.41
DNAN-Rep 1	3.00
DNAN-Rep 2	2.66
DNAN-Rep 3	4.33
DNAN-Rep 4	3.26
DNAN-Rep 5	3.33
DNAN-Rep 6	4.80
DNAN-Rep 7	4.00
Avg	3.63
SD	0.77

U.S. ARMY COMBAT CAPABILITIES DEVELOPMENT COMMAND
CHEMICAL BIOLOGICAL CENTER