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Toxicity of Fire-Fighting Foams to Soil Invertebrates *Enchytraeus crypticus* and *Folsomia candida*

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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.

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Legacy aqueous film-forming foam (AFFF) formulations containing per- and polyfluoroalkyl substances (PFAS) have been linked to accumulation in soil invertebrates and terrestrial plants in previous studies. Alternative PFAS-free formulations are being developed and evaluated for their ability to meet current DoD performance requirements. The relative toxicities of PFAS-free AFFFs as compared with legacy AFFF formulations are not known. We developed ecotoxicological data for seven PFAS-free formulations and a legacy AFFF formulation by determining chronic toxicity for soil invertebrates *Enchytraeus crypticus* and *Folsomia candida.* Test species were exposed in separate studies to each formulation in a natural soil, Sassafras sandy loam. Toxicity data derived from this project will be used to develop soil ecotoxicological risk factors (SERFs) to identify which AFFF formulation would exhibit lesser environmental toxicity while meeting the current DoD performance requirements.

15. SUBJECT TERMS

Aqueous film-forming foam (AFFF)	Toxicity	*Enchytraeus crypticus*
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PREFACE

The work described in this report was authorized under project no. SERDP ER20-1506. The work was started in April 2020 and completed in July 2022.

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.

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EXECUTIVE SUMMARY

The National Defense Authorization Act of 2020 mandated the replacement of per- and polyfluoroalkyl substances (PFAS)-containing aqueous film-forming foams (AFFFs) with PFAS-free formulations by the end of US Fiscal Year 2024. Several such formulations have been developed and are being evaluated for their ability to meet current DoD performance requirements for use in fire-suppression operations. The relative toxicities of PFAS-free AFFFs as compared with legacy AFFF formulations are not known. This project was undertaken to investigate the toxicity of AFFF formulations for soil invertebrates and establish toxicity benchmarks for derivation of scientifically defensible soil invertebrate-based soil ecotoxicological risk factors (SERFs). The toxicity benchmarks detailed in this report were derived using the EC_{50} level for AFFF effects on soil invertebrate reproduction (where EC_{50} is defined as the concentration that produces a 50% decrease in reproduction compared with reproduction in negative control). Standardized toxicity tests using reproduction endpoints were performed to ensure that SERF values would be protective of populations of the majority of invertebrate ecological receptors in soil. AFFF formulations investigated in these studies are shown in Table ES-1.

Table ES-1. Formulations Used in Toxicity Assessments

Formulation	Manufacturer	ID in Report
Angus Fire JetFoam	Angus Fire Ltd; Lancaster, UK	JetFoam
Bio-Ex ECOPOL A 3% FFF	BIOEX USA; Fresno, CA	ECOPOL
Buckeye Platinum Class A	Buckeye Fire Equipment Company; Kings Mountain, NC	Buckeye
Fomtec Enviro USP FFF, 2–3%	Dafo Fomtec AB; Stockholm, Sweden	Fomtec
National Foam NFD 20-391	National Foam; Angier, NC	NFD 20-391
National Foam Avio^{F3} Green KHC 3%		Avio Green
NRL 502W siloxane-based formulation	U.S. Naval Research Laboratory; Washington, DC	NRL 502W
Solberg Re-Healing RF3 3%	Perimeter Solutions; Rancho Cucamonga, CA	Solberg

The present studies showed that for the two species tested, exposures to two PFAS-free AFFF formulations, Avio Green and JetFoam, were more or as toxic as exposure to the PFAS-containing Buckeye (legacy reference C6 AFFF) formulation. Two additional PFAS-free AFFFs, Solberg and NRL 502W, were more or as toxic for *Enchytraeus crypticus* only as compared with exposure to Buckeye. Reproduction EC_{50} values were within Category III, low toxicity (e.g., 500–5000 mg/kg) or Category IV, very low toxicity (e.g., >5000 mg/kg), in accordance with U.S. Environmental Protection Agency toxicity categories. The exception was Avio Green, which was moderately toxic (Category II; e.g., <500 mg/kg) for *Folsomia candida*.

Based on the EC_{50} values and corresponding 95% confidence intervals (CIs) of the present studies, the order of toxicity (from greatest to least) of AFFF formulations for *E. crypticus* was as follows:

JetFoam = NRL 502W = Avio Green = Solberg ≥ Buckeye > ECOPOL = Fomtec > National 20-391

The order of toxicity (from greatest to least) for *F. candida* was as follows:

Avio Green = Buckeye ≥ JetFoam = NRL 502W > Fomtec = Solberg > ECOPOL = National 20-391

AFFF formulations assessed in the present studies were mixtures of multiple constituents. The relative contributions of these constituents to the net effect of each formulation on the test species are unknown. Determination of the contribution of individual constituents to the overall effects was further complicated by inclusion of unidentified constituents (proprietary blends) in some formulations. Consequently, exposure concentrations of AFFF formulations in soil were not analytically determined, and the toxicity benchmarks determined in the present studies are based on calculated values. Additional studies would be required to determine concentrations of major constituents of AFFF formulations and better understand their contributions to the toxic effects for soil organisms.

Upon completion of this project, SERF values for soil invertebrates and terrestrial plants will provide a tool for the selection of AFFF formulations that exhibit lesser environmental toxicity while meeting the current DoD performance requirements and, ultimately, reducing the ecological impacts of fire-fighting operations at industrial and military installations.

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TOXICITY OF FIRE-FIGHTING FOAMS TO SOIL INVERTEBRATES *ENCHYTRAEUS CRYPTICUS* AND *FOLSOMIA CANDIDA*

1. INTRODUCTION

Aqueous film-forming foams (AFFFs) are used for fire suppression in airports, chemical industry, and municipal and military fire-fighting operations. They are also used in testing and training exercises, which may result in their release in the environment. Legacy AFFF formulations contain perfluorooctane sulfonate, perfluorooctanoate, and other per- and polyfluoroalkyl substances (PFAS). When released in the environment, PFAS are persistent and have contaminated terrestrial and aquatic habitats (Anderson et al., 2016; Giesy and Kannan, 2001; Guelfo and Adamson, 2018; Houtz et al, 2013; Li et al., 2020; Miner et al., 2021). PFAS have also been linked to accumulation in soil invertebrates and terrestrial plants in previous studies, with the potential for biomagnification in terrestrial food webs (Karnjanapiboonwong et al., 2018; Rich et al., 2014; Das et al., 2015; Navarro et al., 2016; Wen et al., 2015; Zhao et al., 2014).

Alternative PFAS-free AFFF formulations are being developed and evaluated for their ability to meet current DoD performance requirements for use in Class B fire-suppression operations. However, as compared with legacy AFFF formulations, the relative toxicities of PFAS-free AFFF alternatives are not known. The limited available information suggests that for 14 aquatic species, exposure to at least one PFAS-free AFFF was more or as acutely toxic as exposure to a PFAS-containing AFFF (Jones et al., 2022). Ecotoxicological data are also needed to determine relative toxicities of AFFF formulations for ecologically relevant terrestrial receptors. To address this knowledge gap, the present studies focus on developing ecotoxicological data for seven candidate PFAS-free AFFF concentrates and a legacy AFFF concentrate by determining individual chronic toxicity benchmarks for three soil invertebrate and three terrestrial plant species. Toxicity data derived from this project will be used to develop soil ecotoxicological risk factors (SERFs) to assess which PFAS-free AFFF formulations would exhibit lesser environmental toxicity while meeting the current DoD performance requirements.

This technical report addresses a portion of the overall project entitled Soil Ecotoxicity of PFAS-Free Surfactant Formulations: Soil Invertebrates and Terrestrial Plants. It includes the results of soil invertebrate toxicity studies using enchytraeid worm (potworm) *Enchytraeus crypticus* and collembolan *Folsomia candida* exposed to individual AFFF formulations amended into Sassafras sandy loam (SSL) soil. The results of the studies with earthworm *Eisenia andrei* and terrestrial plant toxicity studies will be addressed in separate reports.

2. MATERIALS AND METHODS

2.1 Test Formulations

We used standardized toxicity tests to determine the effects of one PFAS-containing formulation and seven candidate AFFF replacement formulations on reproduction of two soil invertebrate species. The PFAS-containing AFFF Buckeye Platinum Plus (Buckeye) was selected by the Strategic Environmental Research and Development Program (SERDP) as the reference formulation for all studies. Buckeye used in the present study was found to contain 3.75 g/L 6:2 fluorotelomer sulfonate, 0.332 g/L perfluorohexanoic acid, and a 6:2 fluorotelomer zwitterion surfactant identified as $C_{16}H_{23}F_{13}N_2O_6S_2$ (Jones et al., 2022). Alternative AFFF formulations selected by SERDP as potential replacement products and the corresponding abbreviations used in this report are listed in Table 1.

Table 1. Formulations Used in Toxicity Assessments

Formulation	Manufacturer	ID in Report
Angus Fire JetFoam	Angus Fire Ltd; Lancaster, UK	JetFoam
Bio-Ex ECOPOL A 3% FFF	BIOEX USA; Fresno, CA	ECOPOL
Buckeye Platinum Class A	Buckeye Fire Equipment Company; Kings Mountain, NC	Buckeye
Fomtec Enviro USP FFF, 2–3%	Dafo Fomtec AB; Stockholm, Sweden	Fomtec
National Foam NFD 20-391	National Foam; Angier, NC	NFD 20-391
National Foam Avio^{F3} Green KHC 3%		Avio Green
NRL 502W siloxane-based formulation	U.S. Naval Research Laboratory; Washington, DC	NRL 502W
Solberg Re-Healing RF3 3%	Perimeter Solutions; Rancho Cucamonga, CA	Solberg

2.2 Soil Collection and Characterization

For the purposes of ecological risk assessment, particularly for developing ecotoxicological values protective of soil biota, we used a natural soil SSL (fine-loamy, siliceous, semiactive, mesic Typic Hapludult). The qualitative relative bioavailability (QRB) scores for organic chemicals in natural soils were considered "very high" for SSL (U.S. Environmental Protection Agency [USEPA], 2005). SSL was collected from an open grassland field in a coastal plain on the property of the U.S. Army Aberdeen Proving Ground in Harford County, MD. During soil collection in the field, vegetation and the organic horizon were removed, and the top 12 cm of the A horizon were then collected. Soil was sieved through a 5 mm screen, air-dried for at least 72 h, mixed periodically to ensure uniform drying, passed through a 2 mm sieve, then stored at room temperature before use in testing. The physical and chemical characteristics of the soil were then analyzed (Table 2).

Table 2. Physical and Chemical Characteristics of SSL Soil Used in Toxicity Testing

Soil Parameter	Value
Sand	77%
Silt	13%
Clay	10%
Texture	Sandy loam
Cation exchange capacity (CEC)	4.6 cmol kg^{-1}
Organic matter	0.4%
pH	5.5
Water holding capacity (WHC)	18%
QRB*	Very high

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

The selected SSL soil had sufficiently low organic matter and clay contents to support high relative bioavailability of AFFF formulation constituents for developing conservative but realistic SERF values.

2.3 Soil Amendment Procedure for Toxicity Tests

Prepared soil was weighed separately into glass containers for each AFFF concentrate treatment. Each AFFF formulation treatment was prepared separately by combining the appropriate amount (on a weight-to-weight basis) of the concentrate and the appropriate amount of Type I water (ASTM, 2004) in a glass flask to form a dilution series for individual studies. The AFFF formulation concentrate and ASTM Type I water mixtures were allowed to regularize distribution of constituents for 24 h. The mixtures were then quantitatively transferred to the soil in the amount required to hydrate soil to either the test-specific soil moisture level for the range-finding studies, or to 60% of the water holding capacity (WHC; 18% dry weight of SSL soil) to initiate the AFFF weathering and aging procedure for the definitive tests. Treatment preparation procedures of individual formulation dilutions are reported in the Appendix. Glass containers with the amended soils were covered with plastic wrap, allowed to moisture-equilibrate for 24 h, and then mixed with a spatula to regularize distribution prior to use in the range-finding tests. Soil treatments prepared for the AFFF weathering and aging procedure were placed in the greenhouse.

2.4 Weathering and Aging of AFFF Formulation in Soils for Toxicity Studies

In assessing AFFF formulation toxicity for SERF development, special consideration was given to the inclusion of weathering and aging of concentrates in soil. The weathering and aging procedure was performed in preparation of definitive toxicity testing with soil invertebrates to closely approximate the potential exposure effects in the field. Weathering and aging of chemicals in soil may alter the exposure conditions for soil invertebrates to AFFF chemical constituents because of a variety of fate processes (e.g., photodecomposition, hydrolysis, reaction with soil constituents, immobilization, and microbial transformation) that commonly occur in soils at contaminated sites. These processes can reduce the amount of parent compound that is bioavailable, as compared to tests conducted with recently amended chemicals or performed after a short equilibration period (e.g., 24 h). Toxicity can be affected, as was demonstrated in our previous studies with a variety of organic and inorganic chemicals (e.g., Kuperman et al., 2004, 2005, 2006a, 2006b).

Weathering and aging procedures included placing amended and control soils into open glass containers, hydrating the soils to 60% of the WHC of SSL soil, placing the containers in the greenhouse at ambient temperature, and exposing the containers to alternating moistening and air-drying cycles for 21 days. During the weathering and aging procedure, each soil treatment was weighed and readjusted to its initial mass by periodic (one time each week) addition of ASTM Type I water to the soil. After 21 days, all soil treatments were readjusted to their initial masses by addition of water, covered with plastic wrap, and allowed to moisture-equilibrate for 24 h prior to use in the definitive tests.

2.5 Soil Invertebrate Toxicity Studies

2.5.1 Enchytraeid Toxicity Test

The Enchytraeid Toxicity Test was used to assess the individual effects of AFFF concentrates on the enchytraeid worm (potworm) *E. crypticus*. The test is an adaptation of an International Organization for Standardization (ISO) bioassay, *Soil Quality — Effects of Pollutants on Enchytraeidae (*Enchytraeus *sp.) — Determination of Effects on Reproduction and Survival* (ISO, 2004). This test was selected on the basis of its ability to measure chemical toxicity to ecologically relevant test species during chronic assays and its inclusion of a reproduction component among the measurement endpoints.

Potworms were bred in 4.3 L clear plastic boxes (34 × 20 × 10 cm) filled with 2 kg (dry mass) of SSL soil. The culture was kept in an environment-controlled incubator under a 16:8 h light–dark photoperiod cycle. The mean photosynthetically active radiation (PAR) light intensity was 12.8 ± 0.7 $\mu mol/m^2/s^{-1}$ (985 ± 52 lux) (standard error) and the mean temperature was 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. 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 four 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 in color, reproduced continuously, did not try to leave the soil, and exhibited a shiny outer surface with no soil particles clinging to them.

Glass jars (42 mm i.d.; 45 mm deep) were used as test containers. They were rinsed with ASTM Type I water (ASTM, 2004) before testing began. For the range-finding studies, 25 g of freshly amended or control soil hydrated to 100% of the WHC of SSL soil and 0.05 g of ground oats were added to each of the four replicate test containers. For the definitive studies with AFFF formulations or controls that were subjected to the weathering and aging procedure, 25 g of test soil hydrated to 60% of the WHC and 0.05 g of ground oats were added to each test container. Each container of hydrated soil and oats was then mixed and hydrated to 100% of the WHC of SSL soil by addition of 1.75 g of ASTM Type I water. The mass of each container with soil was recorded. After two weeks of exposure, an additional 0.05 g of ground oats was added to each test container.

Adult potworms with eggs in the clitellum region were used for testing. They were collected from culture and placed in a Petri dish filled with a small amount of ASTM Type I water for examination using a stereomicroscope. Potworms with no eggs were discarded. 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 wrap to facilitate air exchange. All containers were placed in an environment-controlled incubator under the same conditions as described above for maintenance of the potworm culture. The containers were weighed once each week, and the mass loss was replenished with the appropriate amount of ASTM Type I water.

After two weeks, soil in each test container was carefully searched, and adult potworms were removed and counted. Potworms were examined for any morphological or behavioral changes. The remaining test substrate, including any cocoons laid during the first two weeks of the test, was incubated for an additional two weeks. Ground oats (0.05 g) were added to each test container at that time. 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 at least 24 h. The contents of each test container were wet-sieved using a no. 100 mesh sieve (150 µm). Retained contents were transferred to a counting tray, where surviving adult potworms and juvenile potworms produced during the study were counted.

Range-finding studies were conducted with each AFFF concentrate freshly amended in SSL soil prior to definitive studies. The primary objective of these range-finding studies was to bracket treatment concentrations for the definitive studies to allow determination of the concentration that produced a 50% decrease (EC_{50}) in the reproduction endpoint (production of juveniles), as compared with reproduction in the negative control (no test chemicals added). When data from range-finding studies allowed us to determine concentration-response relationships, the established EC_{50} values were contrasted with those determined in the definitive studies with AFFF formulations weathered and aged in SSL soil. This allowed for the assessment of the potential effects of weathering and aging on the net toxicity of individual formulations for the test species.

Toxicity tests with the reference toxicant boric acid (the positive control) were conducted using SSL soil to assess changes in sensitivity, health, and performance of *E. crypticus* laboratory cultures maintained at the U.S. Army Combat Capabilities Development Command Chemical Biological Center (DEVCOM CBC; Aberdeen Proving Ground, MD). 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 mg/kg (the negative control) and 20, 30, 50, 80, 100, and 200 mg/kg. Nonlinear regression analyses of toxicity data from independent studies were used to establish the respective EC_{50} values and corresponding 95% confidence limits

(CLs) 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 potworms' condition and the precision within the laboratory culture. The modification included using calculations based on arithmetic (untransformed) EC_{50} values instead of logarithmic concentrations for boric acid concentrations.

Four replicates of each AFFF concentrate and controls were used in the definitive tests. Validity criteria for the negative controls in toxicity tests included the following performance parameters (ISO, 2004):

1. The adult mortality does not exceed 20% after 14 days.
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 (CV) for the mean number of juveniles is ≤50%.

2.5.2 Collembolan Toxicity Test

The *Folsomia* Toxicity Test was used to assess the individual effects of AFFF concentrates on the survival and reproduction of the collembolan *F. candida*. This test was selected on the basis of its ability to measure chemical toxicity to ecologically relevant test species during chronic assays and its inclusion of a reproduction component among the measurement endpoints. The test is an adaptation of bioassay ISO 11267, *Soil Quality — Inhibition of Reproduction of Collembola* (Folsomia candida) *by Soil* (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 AFFF concentrations that were mixed into soil. The total number of *F. candida* juveniles produced and the number that survived as adults were determined by counting the live organisms after the 28 day test duration. The reproduction and survival of *F. candida* adults exposed to AFFF concentrates were compared with those in the negative-control treatment to quantify ecotoxicological parameters.

Range-finding studies were conducted with each AFFF concentrate freshly amended in SSL soil prior to definitive studies. The primary objective of these range-finding studies was to bracket treatment concentrations for the definitive studies that would allow determination of the 50% decrease (the EC_{50}) in the reproduction endpoint (production of juveniles), compared with reproduction in the negative control (no test chemicals added). When data from range-finding studies allowed us to determine concentration–response relationships, the established EC_{50} values were contrasted with those determined in the definitive studies with AFFF formulations weathered and aged in SSL soil to assess the potential effects of weathering and aging on the net toxicity of individual formulations for the test species.

Toxicity tests with the reference toxicant boric acid (the positive control) were conducted using SSL soil to assess changes in sensitivity, health, and performance of *F. candida* maintained in DEVCOM 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 mg/kg (the negative control) and 30, 50, 80, 100, and 200 mg/kg. Nonlinear regression analyses of toxicity data from independent studies were used to establish the respective EC_{50} values and corresponding 95% CLs 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 potworms' condition and the precision within the laboratory culture. The modification included using calculations based on arithmetic (untransformed) EC_{50} values instead of logarithmic concentrations for boric acid concentrations.

Five replicates of each AFFF concentration treatment and controls were used in the definitive tests. Validity criteria for the negative controls in toxicity tests included the following performance parameters (ISO, 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 day test.
3. The CV 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 testing began. 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 by weight of each batch of hydrated treatment soil was transferred to a test container, and 0.05 g of baker's yeast was added to the soil surface. In the definitive tests with weathered and aged treatments, 20 g of test soil hydrated to 60% of the WHC and 0.05 g of baker's yeast were added to each test container. The container contents were mixed and hydrated to 88% of the WHC of SSL soil by addition of 1 g of ASTM Type I water. The mass of each container with soil was recorded so that soil moisture loss during the test could be monitored. Ten 10–12 day 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 16:8 h light–dark photoperiod cycle. The mean PAR light intensity was 12.8 ± 0.7 $\mu mol/m^2/s^{-1}$ (985 ± 52 lux), and the mean temperature was 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 the container contents were 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.

2.6 Data Analyses

Ecotoxicological data were analyzed using regression models selected from 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 (CIs). 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 models selected for data analyses in these studies were logistic (Gompertz; eq 1) or logistic hormetic (eq 2):

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

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

where *Y* is the dependent variable for a measurement endpoint (e.g., number of juveniles or adults); *a* is the *y*-axis intercept (i.e., the control response); e is the exponent of the base of the natural logarithm; *p* is the desired value for "*p*" effect (e.g., 0.50 for a 50% decrease from the control response; EC_{50}); C is the exposure concentration in test soil; *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 low-dose stimulation and high-dose inhibition (Calabrese, 2008), were fitted to the hormetic model. The ECp parameters used in these studies included the AFFF concentration that produced a 50% (EC_{50}) decrease in the measurement endpoint compared with the negative control. The 95% CIs associated with the point estimates were determined.

Analysis of variance was used to determine the bounded (when possible) no-observed-effect concentration (NOEC) and the lowest-observed-effect concentration (LOEC) values for survival, reproduction, or growth data. Mean separations were determined using Fisher's least-significant difference 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 basis of the nominal concentration of each AFFF concentrate.

3. RESULTS

3.1 Validity Criteria for Negative Controls

Results of definitive toxicity tests with all AFFF formulations weathered and aged in SSL soil complied with the validity criteria defined the respective guidelines for the Enchytraeid Toxicity Test and *Folsomia* Toxicity Test (ISO, 2004; ISO, 1999). The validity criteria (mean adult survival, mean number of juveniles produced, and CV) for test results for the negative-control treatments in the Enchytraeid and *Folsomia* Toxicity Tests are shown in Tables 3 and 4, respectively. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the AFFF formulation treatments.

Table 3. Performance Parameters (Validity Criteria) for Negative Controls in Enchytraeid Toxicity Tests with AFFF Formulations

Formulation	Mean Adult Survival	Mean Juvenile Production	CV (%)
JetFoam	10	811	11.3
ECOPOL	9.75	1,068	10.9
Buckeye	9.75	823	18.4
Fomtec	9.25	412	9.6
NFD 20-391	9.5	341	14.2
Avio Green	10	1,550	2.8
NRL 502W	9.75	816	10.9
Solberg	10	1,082	13.4

Table 4. Performance Parameter (Validity Criteria) for Negative Controls in *Folsomia* Toxicity Tests with AFFF Formulations

Formulation	Mean Adult Survival	Mean Juvenile Production	CV (%)
JetFoam	9.0	93	6.1
ECOPOL	9.6	95	11.7
Buckeye	9.6	157	23.9
Fomtec	9.6	129	25.8
NFD 20-391	9.6	100	20.4
Avio Green	9.6	143	26.7
NRL 502W	9.0	123	22.2
Solberg	10.0	186	14.1

3.2 Positive Controls

Definitive tests with boric acid (reference toxicant) were conducted in SSL soil to monitor the conditions of the *E. crypticus* and *F. candida* cultures used in the toxicity assessments of AFFF formulations. We determined the EC_{50} values and the corresponding 95% CLs in tests with *E. crypticus* and *F. candida* using nonlinear regression analyses of reproduction toxicity data established on multiple testing dates. Tests with *E. crypticus* produced the following EC_{50} 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), 55 (46–64), and 50 (31–69) mg of H_3BO_3/kg of soil. The respective values for *F. candida* cultures were 72 (68–77), 63 (53–73), 60 (53–67), 69 (61–76), 58 (39–78), and 49 (42–57) mg of H_3BO_3/kg of soil.

The EC_{50} values for each test species were plotted on the respective boric acid warning charts to monitor the condition of the laboratory cultures. All resulting EC_{50} values were within both the warning limits and the 95% CLs that were established for each test species (Figures 1 and 2). These charted results confirmed that the condition of the *E. crypticus* and *F. candida* cultures met the validity requirements of the test protocols.

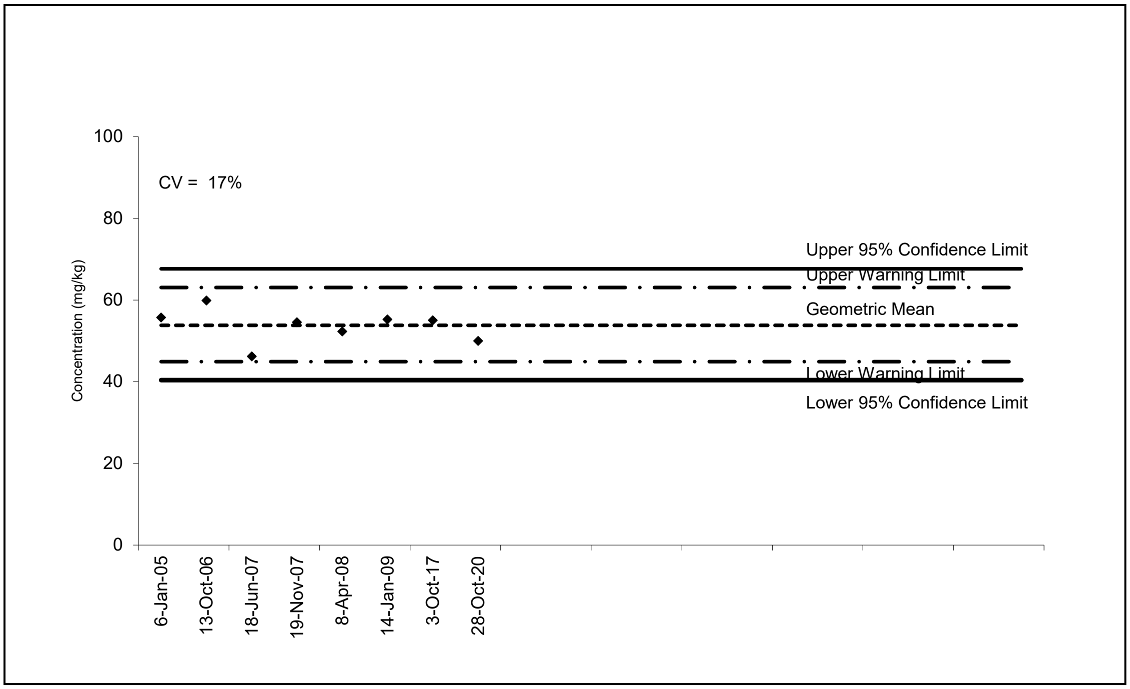


Figure 1. Warning chart for the *E. crypticus* culture showing the EC_{50} values for juvenile production established in definitive tests with the reference toxicant (boric acid) in SSL soil.

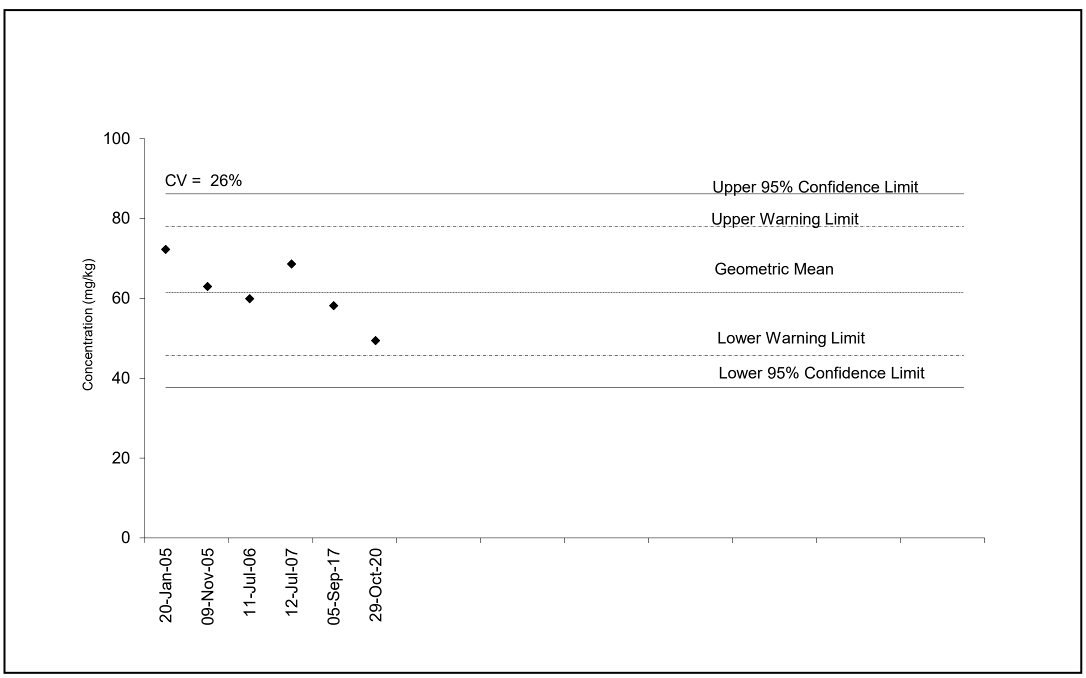


Figure 2. Warning chart for the *F. candida* culture showing the EC_{50} values for juvenile production established in definitive tests with the reference toxicant (boric acid) in SSL soil.

3.3 Effects of AFFF Formulations on the Potworm *E. crypticus*

Toxicity benchmarks for JetFoam formulation weathered and aged in SSL soil are summarized in Table 5. The bounded NOEC and LOEC values for survival of adult *E. crypticus* were 4,349 and 6,464 mg/kg, respectively. Exposure to the lowest positive JetFoam concentration resulted in a 27% decrease in the number of juveniles as compared with the negative control, resulting in an unbounded LOEC of 623 mg/kg (Table 5).

The logistic Gompertz model had the best fit for either adult survival (acute toxicity) or juvenile production (chronic toxicity) data (Figure 3) and established the EC_{50} values of 7,902 and 1,313 mg/kg, respectively. Reproduction was a more sensitive endpoint for the effects of JetFoam on *E. crypticus*, based on the EC_{50} values and corresponding 95% CIs shown in Table 5.

Table 5. Ecotoxicological Benchmarks for JetFoam Formulation Weathered and Aged in SSL Soil Determined for *E. crypticus* Adult Survival and Juvenile Production*

Ecotoxicological Parameter	**Adults**	**Juveniles**
NOEC	4,349 mg/kg	<623 mg/kg
p	0.053	ND
LOEC	6,464 mg/kg	623 mg/kg
p	0.003	<0.0001
EC_{50}	7,902 mg/kg	1,313 mg/kg
CI (95%)	7,123–8,681 mg/kg	1,125–1,500 mg/kg
Model used	Gompertz	Gompertz
R^2	0.977	0.972

*Nominal concentrations based on the dilution series of JetFoam concentrate.
ND, could not be determined within the concentration range tested.
R^2, coefficient of determination.

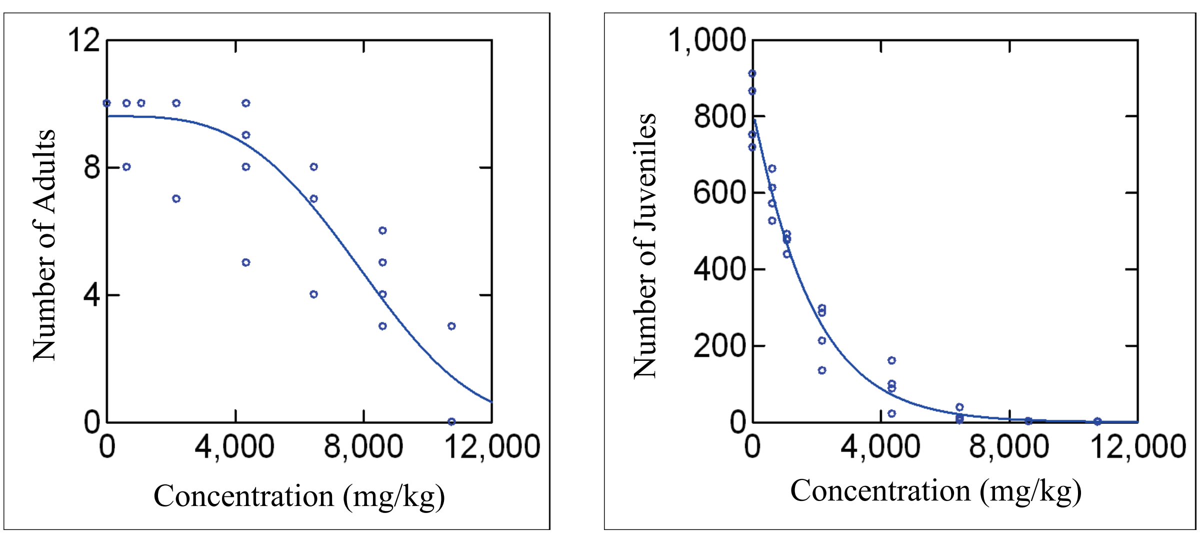


Figure 3. Effects of JetFoam formulation weathered and aged in SSL soil on *E. crypticus* survival of adults (left) and production of juveniles (right).

Results of the range-finding study with the ECOPOL formulation freshly amended in SSL soil allowed us to determine the concentration–response relationships for either survival of adults or production of juveniles by *E. crypticus*. The logistic Gompertz model had the best fit for both endpoints and established the EC_{50} values of 4,858 and 3,518 mg/kg for adults and juveniles, respectively (Table 6).

The bounded NOEC and LOEC values for the ECOPOL formulation weathered and aged in SSL soil were 6,464 and 10,788 mg/kg, respectively, for adult survival, and 2,151 and 4,346 mg/kg, respectively, for production of juveniles (Table 6). The number of surviving adults was decreased by only 40% at the greatest concentration (10,788 mg/kg) tested, which precluded determination of the EC_{50} value for this endpoint. The logistic Gompertz model had the best fit for juvenile production data (Figure 4) and established an EC_{50} value of 5,202 mg/kg. Evaluation of these data showed that weathering and aging of the ECOPOL formulation in SSL soil did not significantly (95% CI basis) affect reproduction toxicity for *E. crypticus.* Reproduction was a more sensitive endpoint for the effects of ECOPOL on *E. crypticus*, based on the bounded LOEC values shown in Table 6.

Table 6. Ecotoxicological Benchmarks for ECOPOL Formulation in SSL Soil Determined for *E. crypticus* Adult Survival and Juvenile Production*

Ecotoxicological Parameter	**Adults**	**Juveniles**
Freshly Amended Treatment		
EC_{50}	4,858 mg/kg	3,518 mg/kg
CI (95%)	842–8,875 mg/kg	1,192–5,843 mg/kg
Model used	Gompertz	Gompertz
R^2	0.961	0.990
Weathered and Aged Treatment		
NOEC	6,464 mg/kg	2,151 mg/kg
p	0.766	0.147
LOEC	10,788 mg/kg	4,346 mg/kg
p	<0.0001	<0.0001
EC_{50}	ND	5,202 mg/kg
CI (95%)	ND	3,923–6,481 mg/kg
Model used	None	Gompertz
R^2	ND	0.971

*Nominal concentrations based on the dilution series of ECOPOL concentrate.
ND, could not be determined within the concentration range tested.

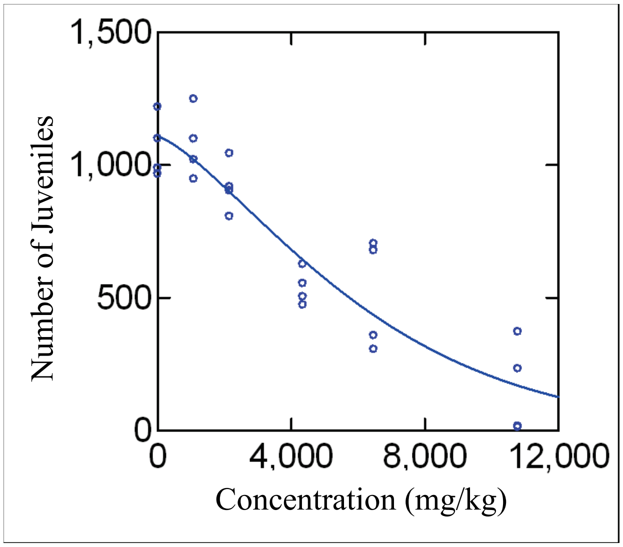


Figure 4. Effect of ECOPOL formulation weathered and aged in SSL soil on *E. crypticus* production of juveniles.

Results of the range-finding study with the Buckeye formulation freshly amended in SSL soil allowed us to determine the concentration–response relationships for either survival of adults or production of juveniles by *E. crypticus*. The logistic Gompertz model had the best fit for both endpoints and established the EC_{50} values of 3,124 and 1,628 mg/kg for adults and juveniles, respectively (Table 7).

The bounded NOEC and LOEC values for the Buckeye formulation weathered and aged in SSL soil were 2,158 and 4,331 mg/kg for adult survival or production of juveniles, respectively, by *E. crypticus* (Table 7). The logistic Gompertz model had the best fit for adult survival or juvenile production data (Figure 5) and established the EC_{50} values of 10,247 and 3,593 mg/kg for adults and juveniles, respectively. Evaluation of these data showed that weathering and aging of the Buckeye formulation in SSL soil significantly (95% CI basis) decreased both the acute (adult survival) and chronic (reproduction) toxicities of the Buckeye formulation for *E. crypticus.*

Table 7. Ecotoxicological Benchmarks for Buckeye Formulation Determined for *E. crypticus* Adult Survival and Juvenile Production*

Ecotoxicological Parameter	Adults	Juveniles
Freshly Amended Treatment		
EC_{50}	3,124 mg/kg	1,628 mg/kg
CI (95%)	1,951–4,297 mg/kg	1,281–1,974 mg/kg
Model used	Gompertz	Gompertz
R^2	0.946	0.979
Weathered and Aged Treatment		
NOEC	2,158 mg/kg	2,158 mg/kg
p	0.476	0.172
LOEC	4,331 mg/kg	4,331 mg/kg
p	<0.0001	<0.0001
EC_{50}	10,247mg/kg†	3,593 mg/kg†
CI (95%)	4,554–15,940 mg/kg	2,475–4,710 mg/kg
Model used	Gompertz	Gompertz
R^2	0.987	0.954

*Nominal concentrations based on the dilution series of Buckeye concentrate.
†Significant decrease in reproduction toxicity following weathering and aging of the formulation in SSL soil.

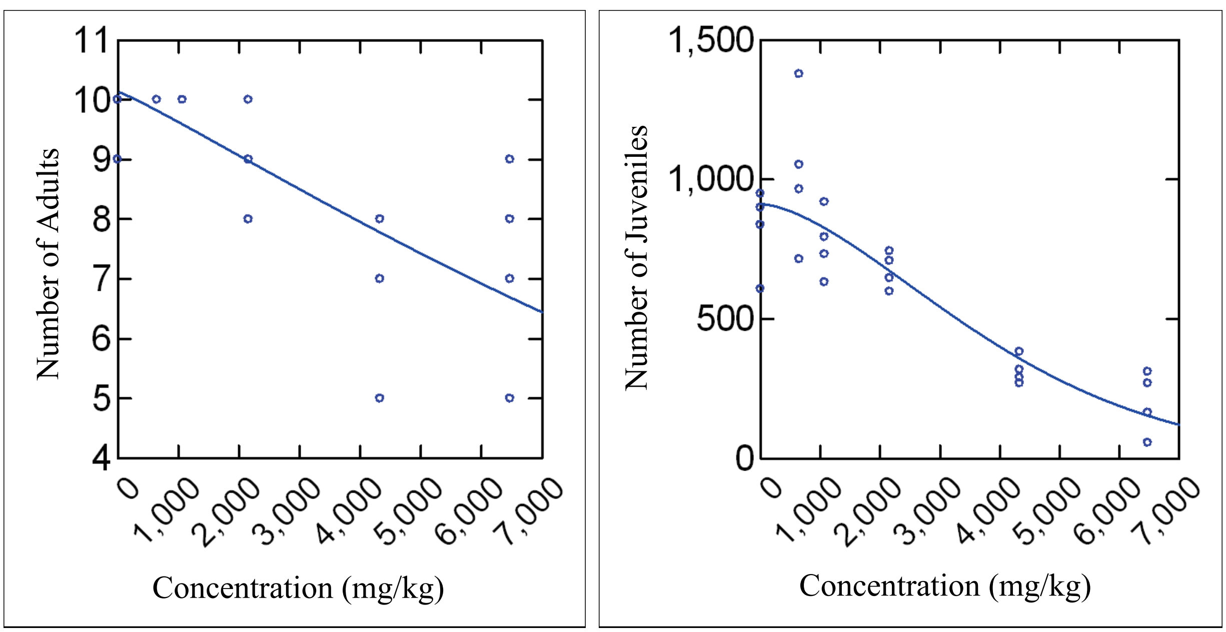


Figure 5. Effects of Buckeye formulation weathered and aged in SSL soil on *E. crypticus* survival of adults (left) and production of juveniles (right).

Toxicity benchmarks for the Fomtec formulation weathered and aged in SSL soil are summarized in Table 8. Survival of adult *E. crypticus* was not affected up to and including the greatest concentration tested, which produced a NOEC of 10,736 mg/kg and an unbounded LOEC of >10,736 mg/kg. Consequently, a concentration–response relationship could not be determined for the effects of the Fomtec formulation on the survival of *E. crypticus* as adults.

The logistic Gompertz model had the best fit for juvenile production (chronic toxicity) data (Figure 6) and established the EC_{50} value of 5,780 mg/kg. Reproduction was a more sensitive endpoint for the effects of Fomtec on *E. crypticus*, based on the NOEC values shown in Table 8.

Table 8. Ecotoxicological Benchmarks for Fomtec Formulation Weathered and Aged in SSL Soil Determined for *E. crypticus* Adult Survival and Juvenile Production*

Ecotoxicological Parameter	**Adults**	**Juveniles**
NOEC	10,736 mg/kg	2,190 mg/kg
p	0.679	0.635
LOEC	>10,736 mg/kg	4,385 mg/kg
p	ND	<0.0001
EC_{50}	ND	5,780 mg/kg
CI (95%)	ND	4,526–7,003 mg/kg
Model used	None	Gompertz
R^2	ND	0.976

*Nominal concentrations based on the dilution series of Fomtec concentrate.
ND, could not be determined within the concentration range tested.

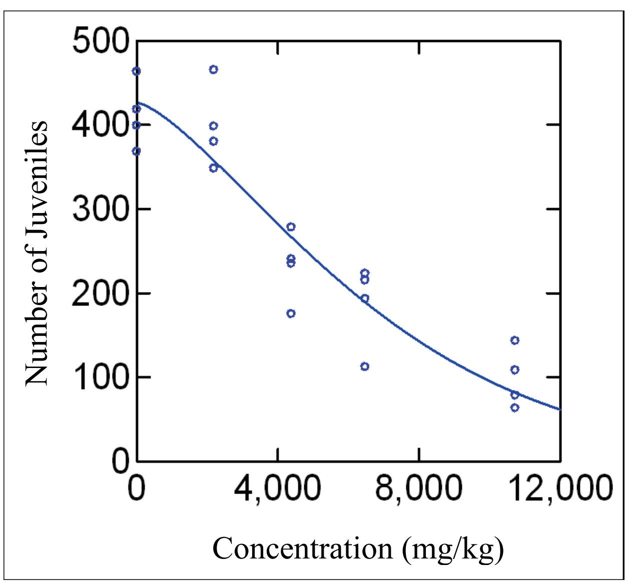


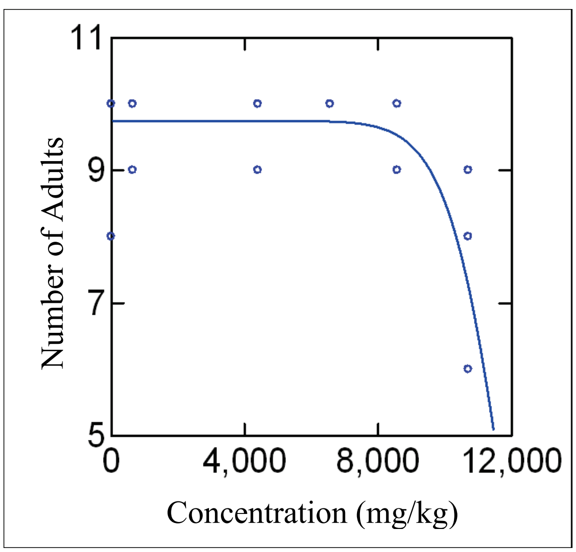
Figure 6. Effect of Fomtec formulation weathered and aged in SSL soil on *E. crypticus* production of juveniles.

Toxicity benchmarks for the NFD 20-391 formulation weathered and aged in SSL soil are summarized in Table 9. The bounded NOEC and LOEC values for survival of adult *E. crypticus* were 8,575 and 10,708 mg/kg, respectively. The logistic Gompertz model had the best fit for either adult survival (acute toxicity) or juvenile production (chronic toxicity) data (Figure 7) and established the EC_{50} values of 11,526 and 10,512 mg/kg, respectively.

Table 9. Ecotoxicological Benchmarks for NFD 20-391 Formulation Weathered and Aged in SSL Soil Determined for *E. crypticus* Adult Survival and Juvenile Production*

Ecotoxicological Parameter	Adults	Juveniles
NOEC	8,575 mg/kg	4,400 mg/kg
p	1.0	0.151
LOEC	10,708 mg/kg	6,567 mg/kg
p	0.001	0.023
EC_{50}	11,526 mg/kg	10,512 mg/kg
CI (95%)	10,109–12,943 mg/kg	7,988–13,035 mg/kg
Model used	Gompertz	Gompertz
R^2	0.994	0.967

*Nominal concentrations based on the dilution series of NFD 20-391 concentrate.



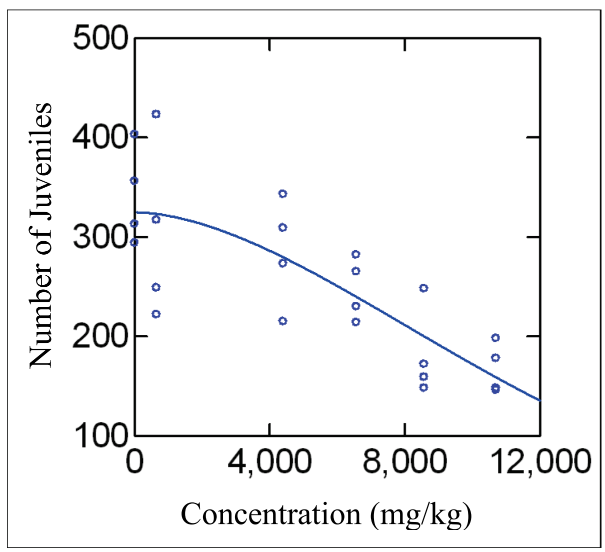


Figure 7. Effects of NFD 20-391 formulation weathered and aged in SSL soil on *E. crypticus* survival of adults (left) and production of juveniles (right).

Results of the range-finding study with Avio Green formulation freshly amended in SSL soil allowed us to determine the concentration–response relationships for *E. crypticus* survival of adults and production of juveniles. The logistic Gompertz model had the best fit for both endpoints and established the EC_{50} values of 686 and 465 mg/kg for adults and juveniles, respectively (Table 10).

Survival of adult *E. crypticus* was not affected up to the greatest tested concentration of Avio Green formulation weathered and aged in SSL soil, producing the bounded NOEC of 4,318 mg/kg and a LOEC of >4,318 mg/kg. Consequently, a concentration–response relationship could not be determined for the effects of Avio Green on *E. crypticus* adult survival.

The bounded reproduction NOEC and LOEC values for the Avio Green formulation weathered and aged in SSL soil were 424 and 623 mg/kg, respectively (Table 10). The logistic Gompertz model had the best fit for juvenile production data (Figure 8) and established an EC_{50} value of 1,657 mg/kg. Evaluation of the juvenile production data showed that weathering and aging of the Avio Green formulation in SSL soil significantly (95% CI basis) decreased chronic reproduction toxicity of this formulation for *E. crypticus*.

Table 10. Ecotoxicological Benchmarks for Avio Green Formulation in SSL Soil Determined for *E. crypticus* Adult Survival and Juvenile Production*

Ecotoxicological Parameter	**Adults**	**Juveniles**
Freshly Amended Treatment		
EC_{50}	686 mg/kg	465 mg/kg
CI (95%)	532–840 mg/kg	248–683 mg/kg
Model used	Gompertz	Gompertz
R^2	0.974	0.937
Weathered and Aged Treatment		
NOEC	4,318 mg/kg	424 mg/kg
p	0.087	0.720
LOEC	>4,318 mg/kg	623 mg/kg
p	ND	0.005
EC_{50}	ND	1,657 mg/kg†
CI (95%)	ND	1,383–1,932 mg/kg
Model used	None	Gompertz
R^2	ND	0.988

*Nominal concentrations based on the dilution series of Avio Green concentrate.
†Significant decrease in reproduction toxicity following weathering and aging of the formulation in SSL soil.
ND, could not be determined within the concentration range tested.

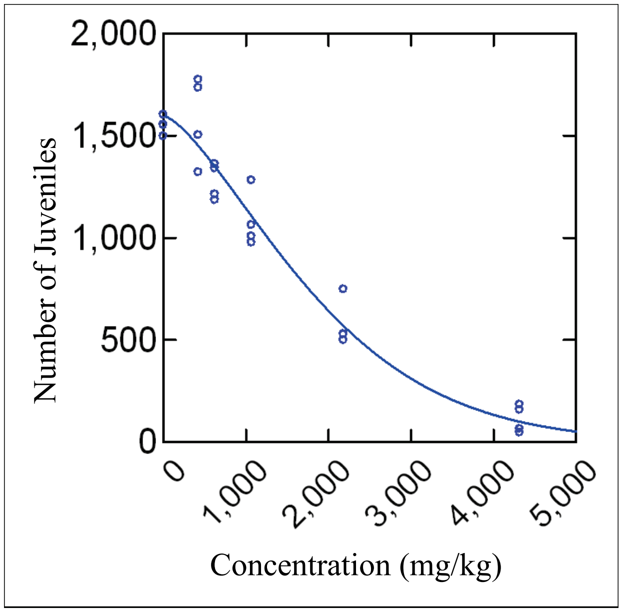


Figure 8. Effect of Avio Green formulation weathered and aged in SSL soil on *E. crypticus* production of juveniles.

Survival of adult *E. crypticus* was not affected at concentrations of up to 1,046 mg/kg (NOEC) of the NRL 502W formulation freshly amended in SSL soil. This was followed by 100% mortality at the next (and greatest) concentration of NRL 502W tested in SSL soil, which produced the bounded LOEC of 10,801 mg/kg (data not shown). Consequently, the concentration–response relationship could not be determined for the effect of NRL 502W on the survival of *E. crypticus* as adults. However, results of this range-finding study allowed us to determine the concentration–response relationships for production of juveniles by *E. crypticus*. The logistic Gompertz model had the best fit for this endpoint and established the EC_{50} value of 981 mg/kg (Table 11).

The bounded acute (adult survival) NOEC and LOEC values for the NRL 502W formulation weathered and aged in SSL soil were 2,179 and 4,380 mg/kg, respectively (Table 11). Exposure to the lowest positive NRL 502W concentration resulted in a 16.5% decrease in the number of juveniles as compared with the negative control and produced an unbounded NOEC of <434 mg/kg (Table 11). The logistic Gompertz model had the best fit for both adult survival and juvenile production data (Figure 9) and established the EC_{50} values of 7,223 and 1,348 mg/kg for adults and juveniles, respectively. Evaluation of the juvenile production data showed that weathering and aging of the NRL 502W formulation in SSL soil did not significantly (95% CI basis) affect chronic (reproduction) toxicity of this formulation for *E. crypticus.*

Table 11. Ecotoxicological Benchmarks for NRL 502W Formulation in SSL Soil Determined for *E. crypticus* Adult Survival and Juvenile Production*

Ecotoxicological Parameter	Adults	Juveniles
Freshly Amended Treatment		
EC_{50}	ND	981 mg/kg
CI (95%)	ND	768–1,195 mg/kg
Model used	None	Gompertz
R^2	ND	0.993
Weathered and Aged Treatment		
NOEC	2,179 mg/kg	<434 mg/kg
p	0.080	ND
LOEC	4,380 mg/kg	434 mg/kg
p	0.022	0.009
EC_{50}	7,223 mg/kg	1,348 mg/kg
CI (95%)	6,413–8,034 mg/kg	965–1,731 mg/kg
Model used	Gompertz	Gompertz
R^2	0.982	0.974

*Nominal concentrations based on the dilution series of NRL 502W concentrate.
ND, could not be determined within the concentration range tested.

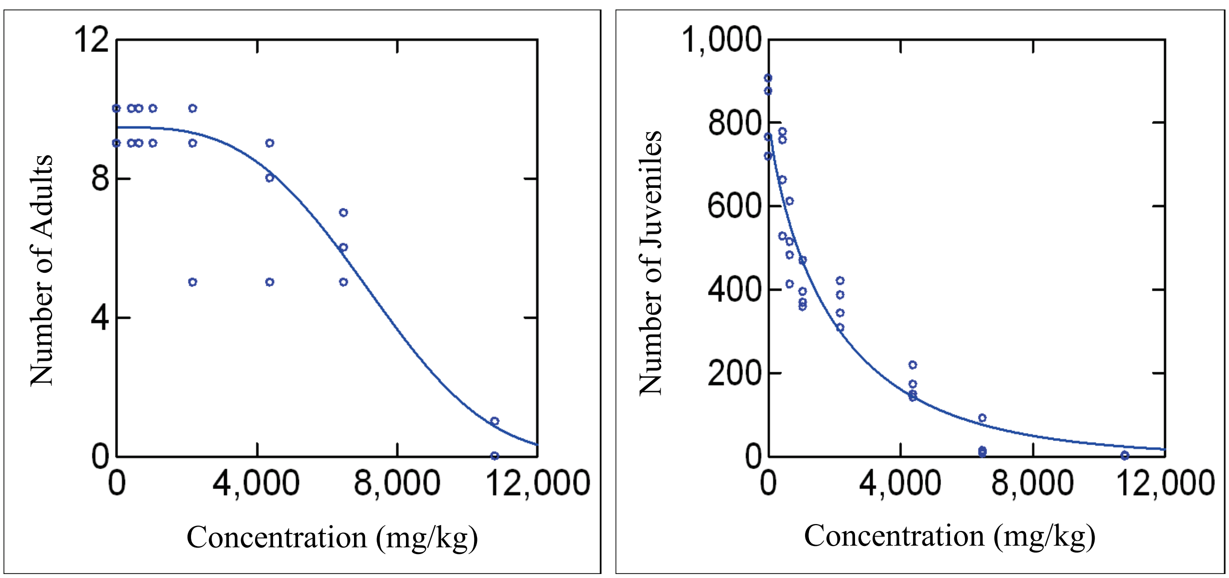


Figure 9. Effects of NRL 502W formulation weathered and aged in SSL soil on *E. crypticus* survival of adults (left) and production of juveniles (right).

Survival of adult *E. crypticus* was not affected at concentrations up to 1,100 mg/kg (NOEC) of the Solberg formulation freshly amended in SSL soil. This was followed by 100% mortality in the next (and greatest) Solberg concentration tested in SSL soil, which produced the bounded LOEC of 10,697 mg/kg (data not shown). Consequently, a concentration–response relationship could not be determined for the effect of Solberg formulation on adult survival of *E. crypticus*. However, results of this range-finding study allowed us to determine the concentration–response relationship for production of juveniles by *E. crypticus*. The logistic Gompertz model had the best fit for this endpoint and established an EC_{50} value of 2,145 mg/kg (Table 12).

The bounded acute (adult survival) NOEC and LOEC values for the Solberg formulation weathered and aged in SSL soil were 6,637 and 10,697 mg/kg, respectively (Table 12). The bounded chronic (reproduction) NOEC and LOEC values for the Solberg formulation weathered and aged in SSL soil were 448 and 643 mg/kg, respectively. The logistic Gompertz model had the best fit for both adult survival and juvenile production data (Figure 10) and established the EC_{50} values of 9,000 and 2,096 mg/kg, respectively. Evaluation of the juvenile production data showed that weathering and aging of the Solberg formulation in SSL soil did not significantly (95% CI basis) affect chronic (reproduction) toxicity of this formulation for *E. crypticus.* Reproduction was a more sensitive endpoint for the effects of the Solberg formulation on *E. crypticus* as compared with adult survival, based on the EC_{50} values and corresponding 95% CIs shown in Table 12.

Table 12. Ecotoxicological Benchmarks for Solberg Formulation Determined for *E. crypticus* Adult Survival and Juvenile Production*

Ecotoxicological Parameter	Adults	Juveniles
Freshly Amended Treatment		
EC_{50}	ND	2,145 mg/kg
CI (95%)	ND	840–3,451 mg/kg
Model used	None	Gompertz
R^2	ND	0.973
Weathered and Aged Treatment		
NOEC	6,637 mg/kg	448 mg/kg
p	0.163	0.968
LOEC	10,697 mg/kg	643 mg/kg
p	<0.0001	0.022
EC_{50}	9,000 mg/kg	2,096 mg/kg
CI (95%)	8,136–9,865 mg/kg	1,614–2,579 mg/kg
Model used	Gompertz	Gompertz
R^2	0.983	0.911

*Nominal concentrations based on the dilution series of Solberg concentrate.
ND, could not be determined within the concentration range tested.

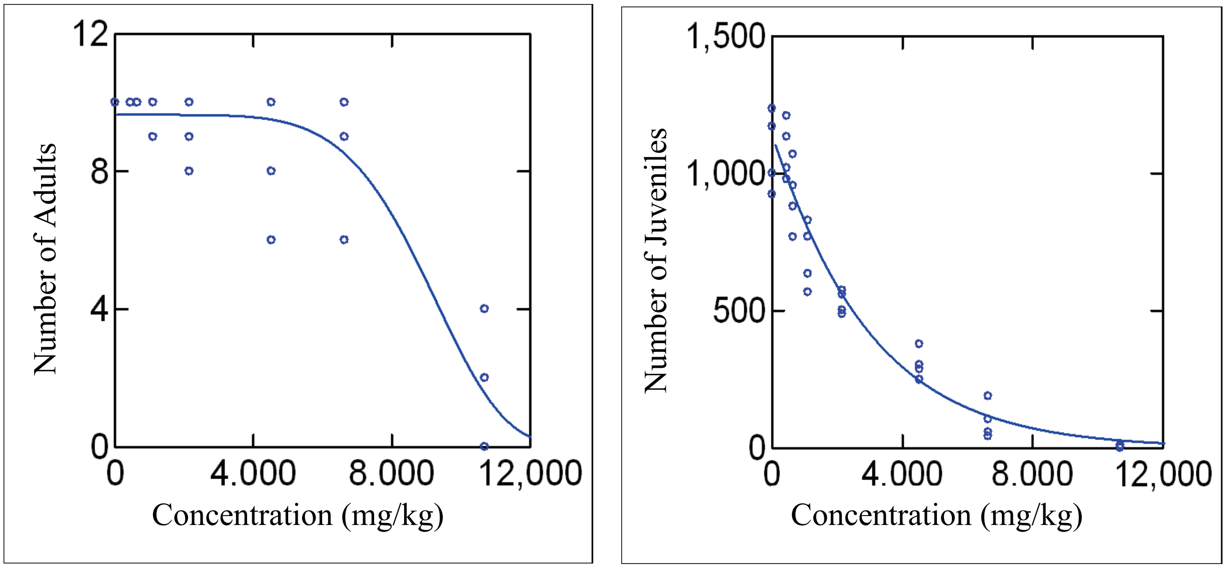


Figure 10. Effects of Solberg formulation weathered and aged in SSL soil on *E. crypticus* survival of adults (left) and production of juveniles (right).

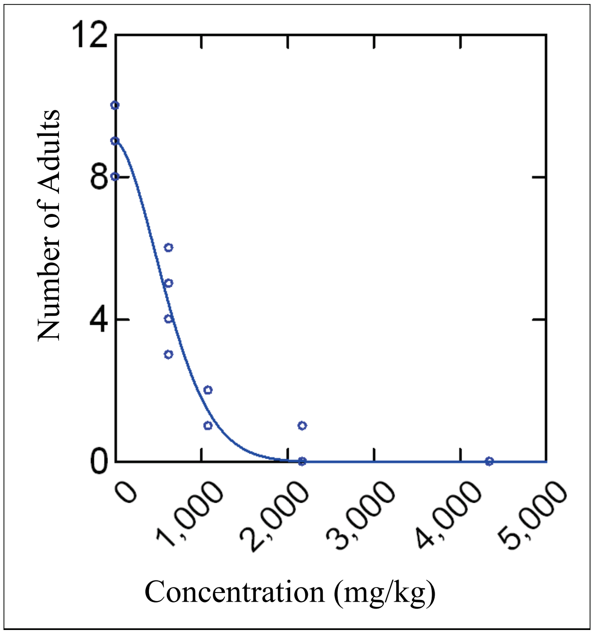
3.4 Effects of AFFF Formulations on the Collembolan *F. candida*

Toxicity benchmarks for the JetFoam formulation weathered and aged in SSL soil are summarized in Table 13. The numbers of surviving adults and juveniles produced by *F. candida* were significantly lower in the first positive concentration as compared with the negative control and produced unbounded NOEC and bounded LOEC values of 623 mg/kg for both endpoints. The logistic Gompertz model had the best fit for both adult survival and juvenile production data (Figure 11) and established the EC_{50} values of 610 and 864 mg/kg, respectively.

Table 13. Ecotoxicological Benchmarks for JetFoam Formulation Weathered and Aged in SSL Soil Determined for *F. candida* Adult Survival and Juvenile Production*

Ecotoxicological Parameter	**Adults**	**Juveniles**
NOEC	<623 mg/kg	<623 mg/kg
p	ND	ND
LOEC	623 mg/kg	623 mg/kg
p	<0.0001	0.003
EC_{50}	610 mg/kg	864 mg/kg
CI (95%)	524–697 mg/kg	773–956 mg/kg
Model used	Gompertz	Gompertz
R^2	0.978	0.972

*Nominal concentrations based on the dilution series of JetFoam concentrate.
ND, could not be determined within the concentration range tested.



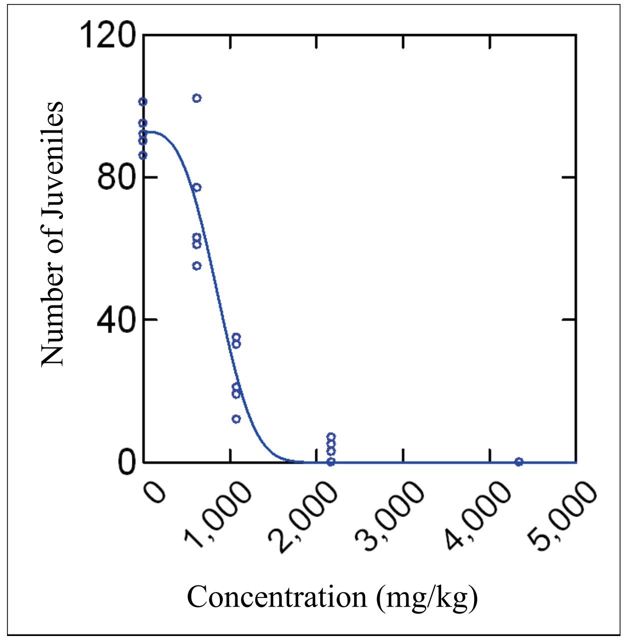


Figure 11. Effects of JetFoam formulation weathered and aged in SSL soil on *F. candida* survival of adults (left) and production of juveniles (right).

Toxicity benchmarks for the ECOPOL formulation weathered and aged in SSL soil are summarized in Table 14. The bounded NOEC values were 440 mg/kg for *F. candida* survival of adult and production of juveniles. The corresponding bounded LOEC values were 2,151 mg/kg. The logistic Gompertz model had the best fit for both adult survival and juvenile production data (Figure 12) and established the EC_{50} values of 2,116 and 5,173 mg/kg, respectively.

Table 14. Ecotoxicological Benchmarks for ECOPOL Formulation Weathered and Aged in SSL Soil Determined for *F. candida* Adult Survival and Juvenile Production*

Ecotoxicological Parameter	Adults	Juveniles
NOEC	440 mg/kg	440 mg/kg
p	0.638	0.313
LOEC	2,151 mg/kg	2,151 mg/kg
p	<0.0001	0.015
EC_{50}	2,116 mg/kg	5,173 mg/kg
CI (95%)	1,382–2,849 mg/kg	4,648–5,699 mg/kg
Model used	Gompertz	Gompertz
R^2	0.949	0.967

*Nominal concentrations based on the dilution series of ECOPOL concentrate.

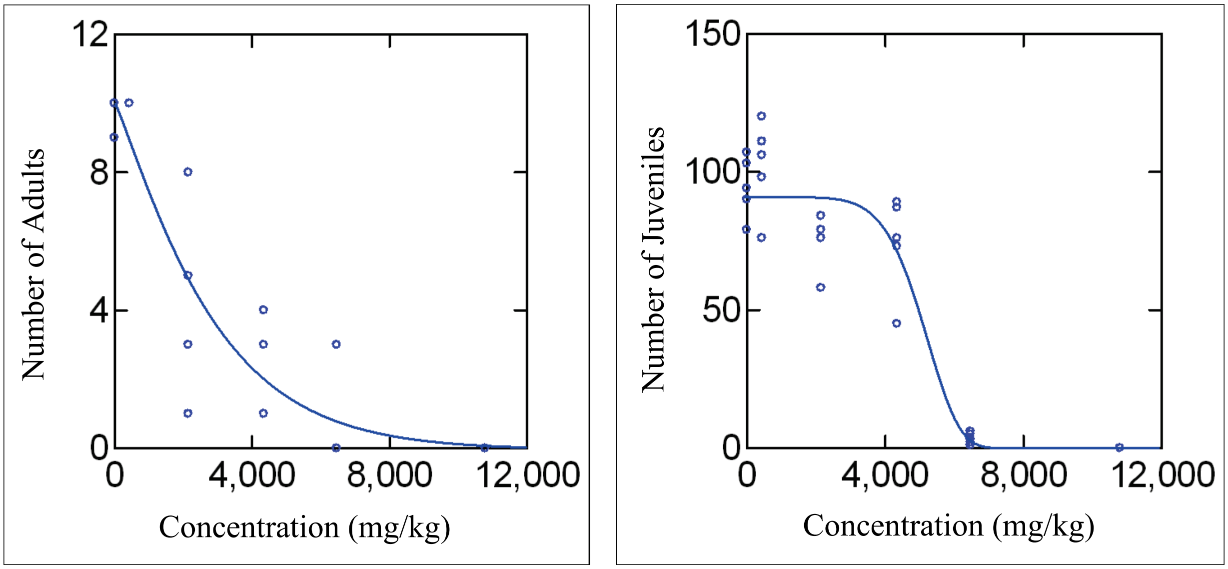


Figure 12. Effects of ECOPOL formulation weathered and aged in SSL soil on *F. candida* survival of adults (left) and production of juveniles (right).

Toxicity benchmarks for the Buckeye formulation weathered and aged in SSL soil are summarized in Table 15. The number of surviving adults was significantly ($p = 0.025$) lower in the second-lowest positive Buckeye concentration as compared with the negative control, which producing the bounded NOEC and LOEC values of 229 and 459 mg/kg, respectively. The logistic Gompertz model had the best fit for adult survival data and established the EC_{50} value of 822 mg/kg.

The logistic hormetic model had the best fit ($R^2 = 0.974$) for *F. candida* reproduction data due to stimulation of juvenile production at the lower treatment concentration of 229 mg/kg (Figure 13). The increase was statistically significant ($p \leq 0.001$) and produced an unbounded LOEC value of 229 mg/kg and bounded no-observed-adverse-effect concentration (NOAEC) and lowest-observed-adverse-effect concentration (LOAEC) values of 459 and 648 mg/kg, respectively (Table 15).

Results of the range-finding study with the Buckeye formulation freshly amended in SSL soil allowed us to determine the EC_{50} value for production of juveniles, which was 151 (14–288) mg/kg. Based on this result, weathering and aging of the Buckeye formulation in SSL soil significantly decreased reproduction toxicity for *F. candida* (Table 15).

Table 15. Ecotoxicological Benchmarks for Buckeye Formulation Determined for *F. candida* Adult Survival and Juvenile Production*

Ecotoxicological Parameter	Adults	Juveniles
Freshly Amended Treatment		
EC_{50}	102 mg/kg	151 mg/kg
CI (95%)	0–229 mg/kg	14–288 mg/kg
Model used	Gompertz	Gompertz
R^2	0.926	0.902
Weathered and Aged Treatment		
NOAEC	229 mg/kg	459 mg/kg
p	1.0	0.882
LOAEC	459 mg/kg	648 mg/kg
p	0.025	<0.0001
EC_{50}	822 mg/kg	734 mg/kg†
CI (95%)	674–971 mg/kg	638–829 mg/kg
Model used	Gompertz	Hormetic
R^2	0.938	0.974

*Nominal concentrations based on the dilution series of Buckeye concentrate.
†Significant decrease in reproduction toxicity following weathering and aging of the formulation in SSL soil.

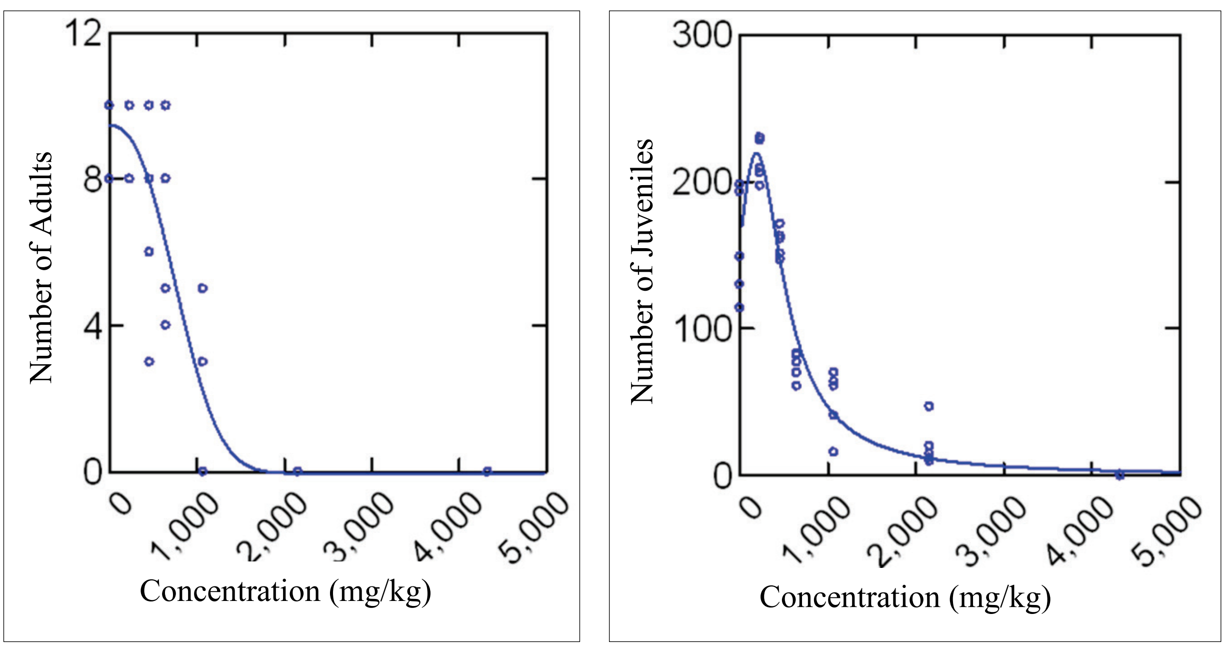


Figure 13. Effects of Buckeye formulation weathered and aged in SSL soil on *F. candida* survival of adults (left) and production of juveniles (right).

Toxicity benchmarks for the Fomtec formulation weathered and aged in SSL soil are summarized in Table 16. The number of surviving adults was significantly ($p < 0.0001$) lower in the 2,190 mg/kg treatment (LOEC) as compared with the negative control. The logistic Gompertz model had the best fit ($R^2 = 0.954$) for adult survival data and established the EC_{50} value of 1,834 mg/kg.

Reproduction NOEC and LOEC values for the Fomtec formulation are shown in Table 16. The logistic Gompertz model had the best fit for *F. candida* reproduction data (Figure 14) and established the EC_{50} value of 1,998 mg/kg.

Results of the range-finding study with the Fomtec formulation freshly amended in SSL soil allowed us to determine the EC_{50} value for production of juveniles of 530 (342–718) mg/kg. Based on this result, weathering and aging of Fomtec formulation in SSL soil significantly decreased reproduction toxicity for *F. candida* (Table 16).

Table 16. Ecotoxicological Benchmarks for Fomtec Formulation Determined for *F. candida* Adult Survival and Juvenile Production*

Ecotoxicological Parameter	Adults	Juveniles
Freshly Amended Treatment		
EC_{50}	497 mg/kg	530 mg/kg
CI (95%)	347–647 mg/kg	342–718 mg/kg
Model used	Gompertz	Gompertz
R^2	0.976	0.916
Weathered and Aged Treatment		
NOEC	1,202 mg/kg	1,202 mg/kg
p	0.111	0.680
LOEC	2,190 mg/kg	2,190 mg/kg
p	<0.0001	<0.0001
EC_{50}	1,834 mg/kg	1,998 mg/kg†
CI (95%)	1,579–2,089 mg/kg	1,659–2,336 mg/kg
Model used	Gompertz	Gompertz
R^2	0.954	0.924

*Nominal concentrations based on the dilution series of Fomtec concentrate.
†Significant decrease in reproduction toxicity following weathering and aging of the formulation in SSL soil.

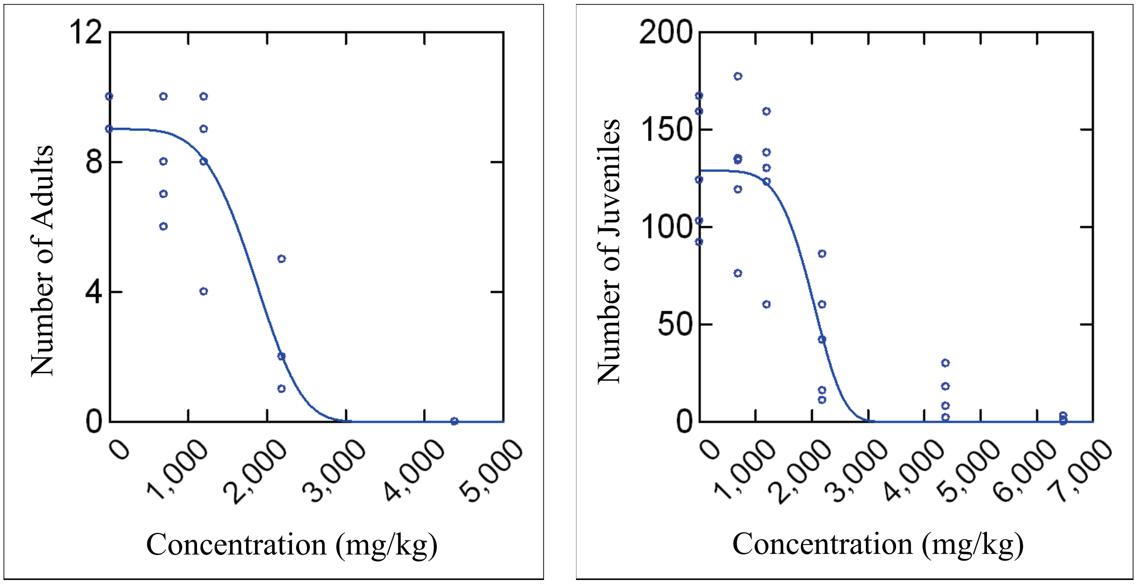


Figure 14. Effects of Fomtec formulation weathered and aged in SSL soil on *F. candida* survival of adults (left) and production of juveniles (right).

Toxicity benchmarks for the NFD 20-391 formulation weathered and aged in SSL soil are summarized in Table 17. The number of surviving adults was significantly ($p = 0.022$) decreased in the 1,077 mg/kg treatment (LOEC) as compared with the negative control. The logistic Gompertz model had the best fit ($R^2 = 0.874$) for adult survival data and established the EC_{50} value of 6,822 mg/kg.

Reproduction NOEC and LOEC values for the NFD 20-391 formulation are shown in Table 17. The logistic Gompertz model had the best fit for *F. candida* reproduction data (Figure 15) and established the EC_{50} value of 5,451 mg/kg.

Results of the range-finding study with the NFD 20-391 formulation freshly amended in SSL soil allowed us to determine the EC_{50} value for production of juveniles of 1,084 mg/kg (833–1,334 mg/kg, 95% CI). Based on this result, weathering and aging of NFD 20-391 formulation in SSL soil significantly decreased reproduction toxicity for *F. candida* (Table 17).

Table 17. Ecotoxicological Benchmarks for NFD 20-391 Formulation Determined for *F. candida* Adult Survival and Juvenile Production*

Ecotoxicological Parameter	**Adults**	**Juveniles**
Freshly Amended Treatment		
EC_{50}	904 mg/kg	1,084 mg/kg
CI (95%)	653–1,155 mg/kg	833–1,334 mg/kg
Model used	Gompertz	Gompertz
R^2	0.979	0.963
Weathered and Aged Treatment		
NOEC	<1,077 mg/kg	2,233 mg/kg
p	ND	0.901
LOEC	1,077 mg/kg	4,400 mg/kg
p	0.022	0.009
EC_{50}	6,822 mg/kg	5,451 mg/kg†
CI (95%)	0–22,869 mg/kg	3,931–6,971 mg/kg
Model used	Gompertz	Gompertz
R^2	0.874	0.921

*Nominal concentrations based on the dilution series of NFD 20-391 concentrate.
†Significant decrease in reproduction toxicity following weathering and aging of the formulation in SSL soil.
ND, could not be determined within the concentration range tested.

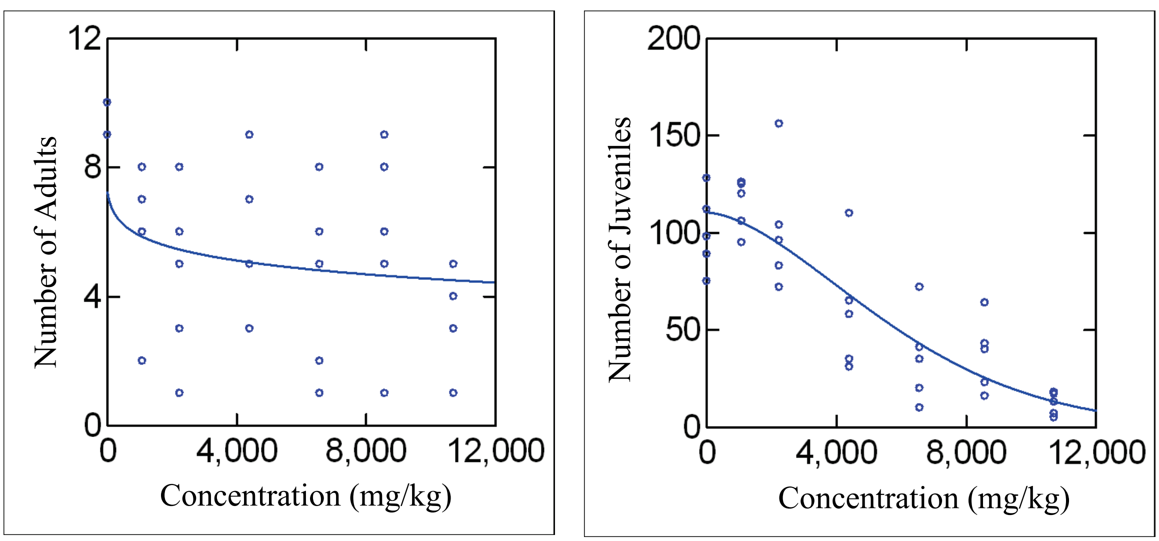


Figure 15. Effects of NFD 20-391 formulation weathered and aged in SSL soil on survival of *F. candida* adults (left) and production of juveniles (right).

Toxicity benchmarks for the Avio Green formulation weathered and aged in SSL soil are summarized in Table 18. The bounded NOEC values were 235 mg/kg for either survival of adult or production of juveniles by *F. candida*. The corresponding bounded LOEC values were 424 mg/kg. The logistic Gompertz model had the best fit for both adult survival and juvenile production data (Figure 16) and established the EC_{50} values of 376 and 493 mg/kg, respectively.

Results of the range-finding study with the Avio Green formulation freshly amended in SSL soil allowed us to determine the EC_{50} value for production of juveniles of 1,227 mg/kg (833–1,334 mg/kg, 95% CI). This value is 2.5× greater than the EC_{50} value determined in the study of Avio Green weathered and aged in SSL soil (Table 18), which suggests an increase in toxicity occurred after the weathering and aging procedure. However, based on the slight overlap in the 95% CI values, this increase was not statistically significant.

Table 18. Ecotoxicological Benchmarks for Avio Green Formulation Determined for *F. candida* Adult Survival and Juvenile Production*

Ecotoxicological Parameter	Adults	Juveniles
Freshly Amended Treatment		
EC_{50}	2,064 mg/kg	1,227 mg/kg
CI (95%)	ND	551–1,902 mg/kg
Model used	Gompertz	Gompertz
R^2	ND	0.965
Weathered and Aged Treatment		
NOEC	235 mg/kg	235 mg/kg
p	0.365	0.440
LOEC	424 mg/kg	424 mg/kg
p	<0.0001	<0.0001
EC_{50}	376 mg/kg	493 mg/kg
CI (95%)	312–440 mg/kg	401–584 mg/kg
Model used	Gompertz	Gompertz
R^2	0.923	0.944

*Nominal concentrations based on the dilution series of Avio Green concentrate.
ND, could not be determined within the concentration range tested.

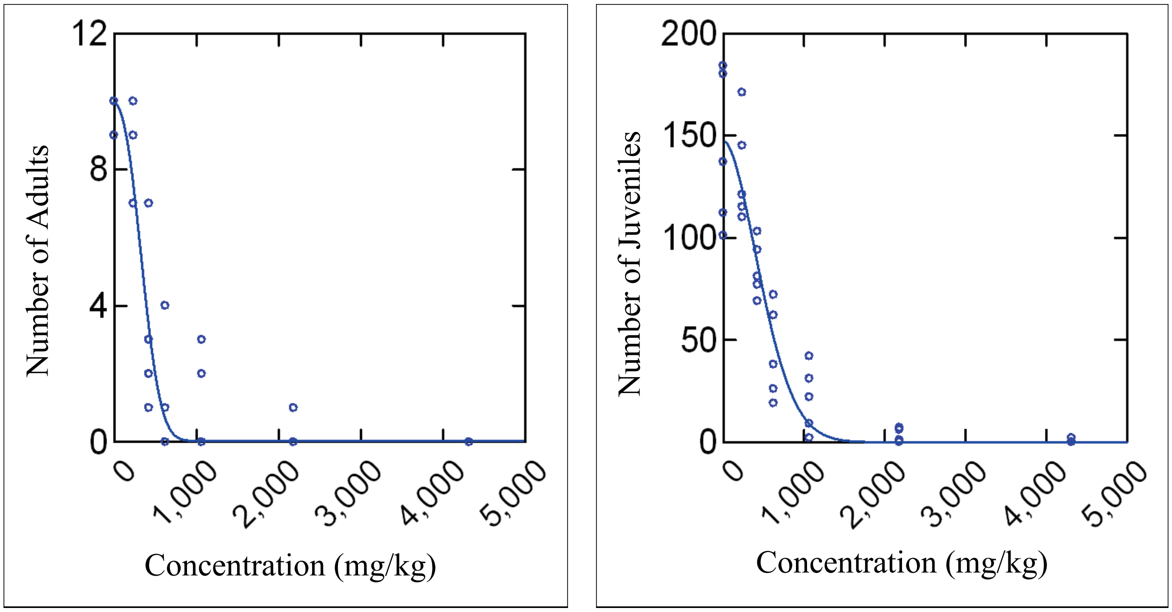


Figure 16. Effects of Avio Green formulation weathered and aged in SSL soil on *F. candida* survival of adults (left) and production of juveniles (right).

Toxicity benchmarks for NRL 502W formulation weathered and aged in SSL soil are summarized in Table 19. The bounded NOEC value was 650 mg/kg for *F. candida* survival of adults and production of juveniles. The corresponding bounded LOEC value was 1,046 mg/kg. The logistic Gompertz model had the best fit for both adult survival and juvenile production data (Figure 17) and established the EC_{50} values of 1,506 and 1,267 mg/kg, respectively.

Table 19. Ecotoxicological Benchmarks for NRL 502W Formulation Weathered and Aged in SSL Soil Determined for *F. candida* Adult Survival and Juvenile Production[*]

Ecotoxicological Parameter	**Adults**	**Juveniles**
NOEC	650 mg/kg	650 mg/kg
p	0.057	0.248
LOEC	1,046 mg/kg	1,046 mg/kg
p	0.005	<0.0001
EC_{50}	1,506 mg/kg	1,267 mg/kg
CI (95%)	1,089–1,923 mg/kg	937–1,597 mg/kg
Model used	Gompertz	Gompertz
R^2	0.947	0.938

[*]Nominal concentrations based on the dilution series of NRL 502W concentrate.

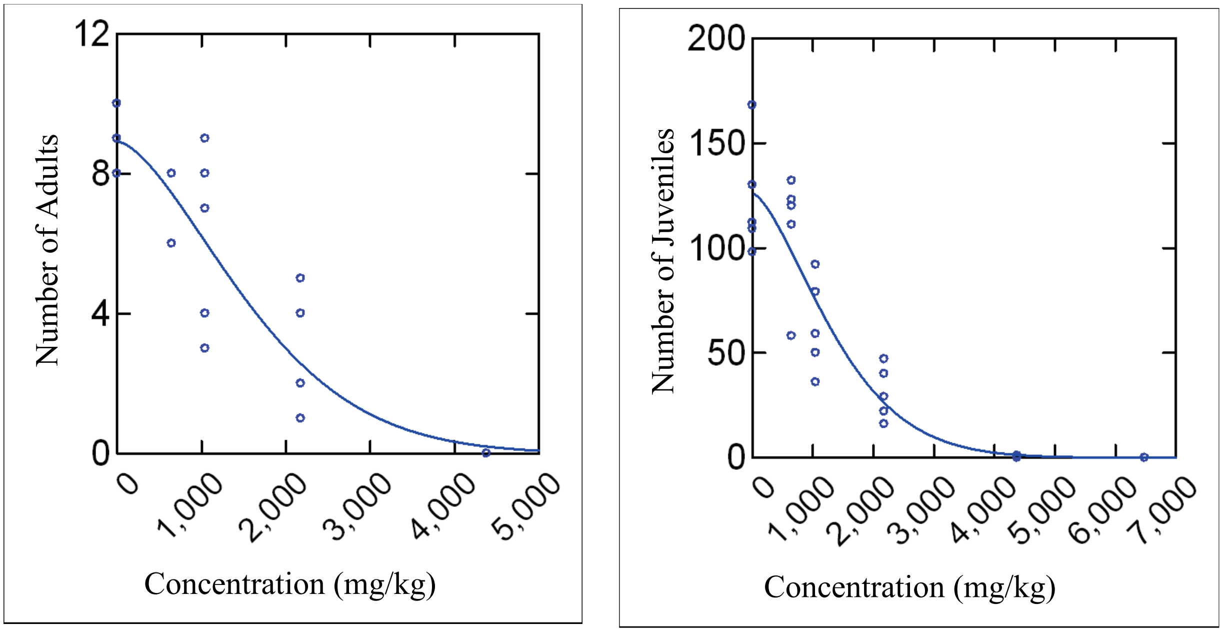


Figure 17. Effects of NRL 502W formulation weathered and aged in SSL soil on *F. candida* survival of adults (left) and production of juveniles (right).

Toxicity benchmarks for the Solberg formulation weathered and aged in SSL soil are summarized in Table 20. The number of surviving adults was significantly ($p < 0.0001$) decreased in the 4,525 mg/kg treatment (LOEC) as compared with the negative control. The concentration–response relationship for survival of *F. candida* adults could not be determined within the concentration range tested.

Reproduction NOEC and LOEC values for the Solberg formulation are shown in Table 20. The logistic Gompertz model had the best fit for *F. candida* reproduction data (Figure 18) and established the EC_{50} value of 2,100 mg/kg.

Results of the range-finding study with the Solberg formulation freshly amended in SSL soil allowed us to determine the EC_{50} value for production of juveniles of 859 mg/kg (265–1,452 mg/kg, 95% CI). Based on this result, weathering and aging of the Solberg formulation in SSL soil significantly decreased reproduction toxicity for *F. candida* (Table 20).

Table 20. Ecotoxicological Benchmarks for Solberg Formulation Determined for *F. candida* Adult Survival and Juvenile Production*

Ecotoxicological Parameter	Adults	Juveniles
Freshly Amended Treatment		
EC_{50}	1,658 mg/kg	859 mg/kg
CI (95%)	20–3,296 mg/kg	265–1,452 mg/kg
Model used	Gompertz	Hormetic
R^2	0.927	0.916
Weathered and Aged Treatment		
NOEC	2,154 mg/kg	1,100 mg/kg
p	1.0	0.439
LOEC	4,525 mg/kg	2,154 mg/kg
p	<0.0001	<0.0001
EC_{50}	ND	2,100 mg/kg†
CI (95%)	ND	1,855–2,344 mg/kg
Model used	None	Gompertz
R^2	ND	0.971

*Nominal concentrations based on the dilution series of Solberg concentrate.
†Significant decrease in reproduction toxicity following weathering and aging of the formulation in SSL soil.
ND, could not be determined within the concentration range tested.

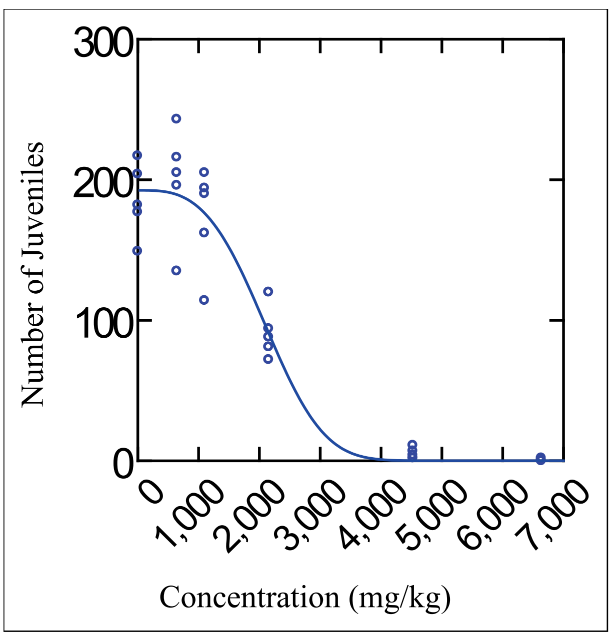


Figure 18. Effect of Solberg formulation weathered and aged in SSL soil on production of *F. candida* juveniles.

4. DISCUSSION

The National Defense Authorization Act of 2020 mandated the replacement of PFAS-containing AFFFs with PFAS-free formulations by the end of the U.S. fiscal year 2024. Several such formulations have been developed and are being evaluated for their ability to meet DoD performance requirements for use in fire-suppression operations. However, the relative toxicities of PFAS-free AFFFs, as compared with legacy AFFF formulations, are not known. We conducted the present studies to investigate the toxicity of AFFF formulations for soil invertebrates and establish toxicity benchmarks for derivation of the SERFs. Ecotoxicological testing in these studies was designed, in part, in accordance with the criteria developed for derivation of ecological soil screening levels (Eco-SSLs) outlined in the Eco-SSL Guideline (USEPA, 2005). The toxicity benchmarks detailed in this interim report were derived using EC_{50} level for AFFF effects on soil invertebrate reproduction. This measurement endpoint was determined for each formulation from standardized toxicity tests using reproduction endpoints to ensure that SERF values would be protective of populations of the majority of invertebrate ecological receptors in soil.

The present studies showed that exposures to two PFAS-free AFFF formulations, Avio Green and JetFoam, were more or as toxic for the two species tested as compared with exposure to the PFAS-containing Buckeye (legacy reference C6 AFFF) formulation. Two additional PFAS-free AFFFs, Solberg and NRL 502W, were more or as toxic for *E. crypticus* only, as compared with exposure to Buckeye. The individual EC_{50} values determined in the present studies with potworm *E. crypticus* and collembolan *F. candida* are summarized in Figure 19. Review of the toxicity data in Figure 19 shows relatively low toxicity was associated with all formulations for *E. crypticus* or *F. candida,* based on the EC_{50} values. These values were within Category III, low toxicity (e.g., 500–5000 mg/kg) or Category IV, very low toxicity (e.g., >5000 mg/kg), according to USEPA toxicity categories. The exception was Avio Green, which was moderately toxic (Category II; e.g., <500 mg/kg) for *F. candida*. Based on the results shown in Figure 19, the order of toxicity (from greatest to least, based on the EC_{50} values and the corresponding 95% CIs) of AFFF formulations for *E. crypticus* was as follows:

JetFoam = NRL 502W = Avio Green = Solberg ≥ Buckeye > ECOPOL = Fomtec > National 20-391

The order for *F. candida* was as follows:

Avio Green = Buckeye ≥ JetFoam = NRL 502W > Fomtec = Solberg > ECOPOL = National 20-391

These toxicity data comport with the results by Jones et al. (2022), who found that Avio Green was more toxic, based on median lethal concentrations (LC_{50} values), to 14 aquatic species compared with the other evaluated PFAS-free AFFF formulations that were also used in the present studies. Yu et al. (2022) assessed reproduction toxicity of the same AFFF formulations for the nematode *Caenorhabditis elegans* exposed in a similar SSL soil. The authors reported comparable toxicity (based on EC_{50} values and the corresponding 95% CIs) for Avio Green, Solberg, NRL 502W, and Fomtec, and lower toxicity for Buckeye, as compared with toxicities determined in the present studies with *E. crypticus*. Yu et al. (2022) also reported comparable findings for toxicity of NRL 502W and Fomtec to *F. candida* but lower toxicity of Buckeye, Avio Green, and Solberg for this collembolan species. Nematodes were more sensitive to ECOPOL (greater toxicity) in the study by Yu et al. (2022) than were either *E. crypticus* or *F. candida* in the present studies.

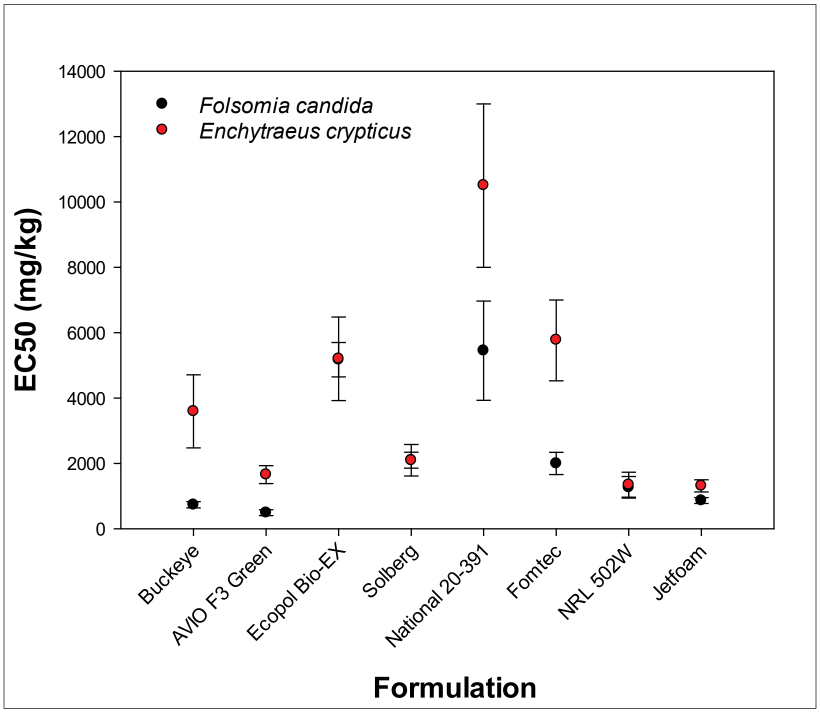


Figure 19. Calculated effect concentrations (EC_{50} values) for AFFF formulations weathered and aged in SSL soil on production of juveniles by *E. crypticus* and *F. candida*. Data points are taxonomic means ± 95% CIs.

Species sensitivity varied among AFFF formulations in the present study. There were no statistically significant differences (on a 95% CI basis) in the EC_{50} values between the two species for the ECOPOL, Solberg, and NRL 502W formulations. In contrast, toxicities of the remaining formulations were significantly greater for *F. candida* as compared with *E. crypticus*. The inclusion of species from different taxonomic groups, representing a range of sensitivities, was an important consideration for selecting the test battery for SERF development because the respective sensitivities often correlate 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 exposures focused on ingestion of AFFF-contaminated soil and direct-contact exposures. These exposures were considered under conditions of high relative bioavailability of AFFF 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, and dermal absorption within soil). Finally, selected terrestrial toxicity tests with representative test species have been standardized and have generated 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 SERF development.

AFFF formulations assessed in the present studies were mixtures of multiple constituents. The relative contributions of these constituents to the net effect of each formulation on the test species is unknown. Determination of the contribution of individual constituents to the overall effects was further complicated by inclusion of unidentified constituents (proprietary blends) in some formulations. Consequently, exposure concentrations of AFFF formulations in soil were not analytically determined, and the toxicity benchmarks determined in the present studies are based on calculated values. Additional studies are required to determine concentrations of major constituents of AFFF formulations and better understand their contributions to the toxic effects for soil organisms.

Each toxicity test was appropriately replicated and included negative (no chemicals added) and positive (reference chemical) controls. Test validity criteria were met in all of the definitive assays. Validity criteria for negative controls in the definitive toxicity tests with soil invertebrates specified the minimal percentage of adult survival, the minimal number of juveniles produced, and the boundaries for a coefficient of variation for reproduction. Toxicity tests with boric acid (reference toxicant, positive control) were conducted in SSL soil to obtain EC_{50} values and the corresponding 95% CLs. All resulting EC_{50} values were within both the warning limits and the 95% CLs 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.

Special consideration was given in the present studies to the inclusion of weathering and aging of AFFF formulations in soil in the assessment of toxic effects on terrestrial receptors to account for possible alterations in the exposure conditions for plants and soil invertebrates to AFFF chemical constituents. Ecotoxicological benchmarks for AFFF formulations, each independently weathered and aged in SSL soil, will more closely approximate the exposure conditions in the field, as compared to benchmarks established in studies with freshly amended soil. Furthermore, when range-finding studies conducted with freshly amended soils allowed us to determine EC_{50} values, we assessed the effects of weathering and aging of AFFF formulations in soil on the resulting toxicity by statistically comparing (95% CI basis) reproduction toxicity benchmarks as determined in studies with freshly amended and weathered and aged treatments. Weathering and aging of AFFF formulations in soil significantly decreased the toxicity of Buckeye and National 20-391 formulations for both species, Solberg and Fomtec formulations for *F. candida*, and Avio Green formulation for *E. crypticus*. In the present studies, no increased toxicity was identified for any of the AFFF formulations tested after weathering and aging in soil.

When developed in the final report, SERF values will be intentionally conservative to provide confidence that the formulation selection process has conveyed preference to products with the least potential ecotoxicological damage in the terrestrial environment. The conservative nature of SERF that will be developed in the final report on AFFFs will be achieved by using a natural soil with properties that support high relative bioavailability of AFFF formulation constituents to ecologically relevant test species. This will be accomplished by using reproduction measurement endpoints for toxicity benchmark derivation and by using the geometric mean of the respective benchmarks to establish a SERF value (i.e., more conservative than an arithmetic mean).

5. CONCLUSIONS

This project was undertaken to investigate the toxicity of AFFF formulations for soil invertebrates and establish toxicity benchmarks for derivation of scientifically defensible soil invertebrate-based SERFs. These SERFs are being derived using the EC_{50} level toxicity benchmarks for the AFFF effects on soil invertebrates collembolan *F. candida*, potworm *E. crypticus*, and earthworm *E. andrei*. Toxicity benchmarks were determined for each formulation from standardized toxicity tests using reproduction endpoints to ensure that SERF values would be protective of populations of the majority of invertebrate ecological receptors in soil. The toxicity data obtained in the present studies showed relatively low overall toxicity of all formulations tested with *F. candida* or *E. crypticus* based on the EC_{50} values. As compared with PFAS-containing AFFF, some PFAS-free AFFF formulations were more toxic or as toxic to the soil invertebrate species tested.

AFFF formulations assessed in these studies were mixtures of multiple constituents, and the relative contribution of each constituent to the net effect of the formulation on the test species is unknown. Additional studies are required to better understand these contributions to the toxic effects on soil organisms. Upon completion of this project, SERF values for soil invertebrates and terrestrial plants will provide a tool for the selection of AFFF formulations that exhibit lesser environmental toxicity while meeting DoD performance requirements and, ultimately, reducing the ecological impacts of fire-fighting operations at industrial and military installations.

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ACRONYMS AND ABBREVIATIONS

AFFF	aqueous film-forming foam
CEC	cation exchange capacity
CI	confidence interval
CL	confidence limit
CV	coefficient of variation
DEVCOM CBC	U.S. Army Combat Capabilities Development Command Chemical Biological Center
EC	Environment Canada
EC_{50}	concentration that produces 50% decrease in reproduction
Eco-SSL	ecological soil screening level
ECp	effective concentration for a specified percent effect
ISO	International Organization for Standardization
LOAEC	lowest-observed-adverse-effect concentration
LOEC	lowest-observed-effect concentration
ND	not determined
NOAEC	no-observed-adverse-effect concentration
NOEC	no-observed-effect concentration
PAR	photosynthetically active radiation
PFAS	per- and polyfluoroalkyl substances
QRB	qualitative relative bioavailability
R^2	coefficient of determination
SERDP	Strategic Environmental Research and Development Program
SERF	soil ecotoxicological risk factor
SSL	Sassafras sandy loam
USEPA	U.S. Environmental Protection Agency
WHC	water holding capacity

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APPENDIX:
TREATMENT PREPARATION PROCEDURES

A.1 INTRODUCTION

This research is aimed at developing ecotoxicological data for seven candidate per- and polyfluoroalkyl substance (PFAS)-free aqueous film-forming foam (AFFF) concentrates and a legacy AFFF concentrate by determining individual chronic toxicity benchmarks for soil invertebrate and plant species. Toxicity data derived from this project will be used to develop soil ecotoxicological risk factors to assess which PFAS–AFFF concentrate would exhibit less environmental toxicity while meeting the current DoD performance requirements.

A.2 TEST CONCENTRATES

We are using standardized toxicity tests to assess the effects of seven candidate AFFF replacement formulations and one reference C6 PFAS-containing formulation (Buckeye Platinum) on the reproduction of three soil invertebrate species and the growth of three plant species. These concentrate formulations and the corresponding abbreviations are listed in Table A-1.

Table A-1. Formulations Used in Toxicity Assessments

Formulation	Manufacturer	ID in Report
Angus Fire JetFoam	Angus Fire Ltd; Lancaster, UK	JetFoam
Bio-Ex ECOPOL A 3% FFF	BIOEX USA; Fresno, CA	ECOPOL
Buckeye Platinum Class A	Buckeye Fire Equipment Company; Kings Mountain, NC	Buckeye
Fomtec Enviro USP FFF, 2–3%	Dafo Fomtec AB; Stockholm, Sweden	Fomtec
National Foam NFD 20-391	National Foam; Angier, NC	NFD 20-391
National Foam Avio^{F3} Green KHC 3%		Avio Green
NRL 502W siloxane-based formulation	U.S. Naval Research Laboratory; Washington, DC	NRL 502W
Solberg Re-Healing RF3 3%	Perimeter Solutions; Rancho Cucamonga, CA	Solberg

A.3 SOIL COLLECTION AND CHARACTERIZATION

For the purposes of ecological risk assessment, particularly for developing ecotoxicological values protective of soil biota, we used a natural soil, Sassafras sandy loam (SSL; fine-loamy, siliceous, semiactive, mesic Typic Hapludult). The qualitative relative bioavailability scores for organic chemicals in natural soils were considered "very high" for

Sassafras sandy loam. SSL was collected from an open grassland field in the coastal plain on the property of Aberdeen Proving Ground in Harford County, MD. During soil collection in the field, vegetation and the organic horizon were removed, and the top 12 cm of the A horizon were then collected. Soil was sieved through a 5 mm screen, air-dried for at least 72 h, mixed periodically to ensure uniform drying, passed through a 2 mm sieve, then stored at room temperature before use in testing.

A.4 SOIL AMENDMENT PROCEDURE

Prepared soil was weighed separately into glass containers for each AFFF concentrate treatment. Each AFFF formulation treatment was prepared separately by combining the appropriate amount (weight/weight basis) of the concentrate and the appropriate amount of ASTM Type I water in a glass flask to form a dilution series selected for individual studies. The AFFF formulation concentrate and ASTM Type I water mixtures were allowed to regularize distribution of constituents for 24 h. The mixtures were then quantitatively transferred to the soil in the amounts required to hydrate soil to either test-specific soil moisture level (for the range-finding studies) or to 60% of the water holding capacity (WHC; 18% dry weight of SSL soil) to initiate the AFFF weathering and aging procedure for the definitive tests. The concentration in the soil was calculated in two primary steps:

1. Solution concentration (in grams per kilogram) for each treatment was calculated by dividing the actual weight of the AFFF concentrate by the total weight of the solution after addition of the appropriate amount of ASTM Type I water to the concentrate.
2. The final calculated concentration (in milligrams per kilogram) of the AFFF concentrate in soil was determined by first multiplying the solution concentration that was determined in step 1 by the actual weight of the solution (in kilograms) added to the soil and then dividing this value by the total weight (in kilograms) of soil used for that treatment to determine the total weight (in grams) of formulation added to soil. This value (in grams per kilogram) was then multiplied by 1000 to convert the final concentration in the soil to units of milligrams per kilogram.

The individual AFFF concentrate treatments used in definitive studies with enchytraeid worm (potworm) *Enchytraeus crypticus* and collembolan *Folsomia candida* are reported in Tables A-2 through A-9.

Glass containers with the amended soils were covered with plastic wrap and allowed to moisture-equilibrate for 24 h. The container contents were then mixed with a spatula to regularize distribution prior to use in the range-finding tests. Soil treatments prepared for the AFFF weathering and aging procedure were placed in the greenhouse.

A.5 WEATHERING AND AGING OF AFFF FORMULATION IN SOILS FOR TOXICITY STUDIES

Weathering and aging procedures included exposing the amended and control soils (which were initially hydrated to 60% of the WHC of SSL soil, in open glass containers in the greenhouse at ambient temperature) to alternating moistening and air-drying cycles for 21 days. During the weathering and aging procedure, all treated soils were weighed and readjusted to their initial weight by periodic (one time each week) addition of ASTM Type I water to the soil. After 21 days, all treated soils were readjusted to their initial weights, covered with plastic wrap, and allowed to moisture-equilibrate for 24 h prior to use in the definitive tests.

Table A-2. Serial Dilution for Preparing JetFoam Treatments in SSL Soil

Jetfoam Formulation in SSL2020 Soil; 14 February 2022											
Formulation Dilution											
		Actual		Actual	Actual		Actual				
Concentration	Formulation	Formulation	ASTM 1	Total	Solution	Solution	solution	Weight of	Weight of	Concentr	Concentr
% of formulation	Needed	weight	Water	Solution	Weight	conc.	added to soil	soil	formulation	in soil	in soil
	(g)	(g)	(g)	(g)	kg	g/kg	kg	kg	g	g/kg dw	mg/kg soil
0.6	0.48	0.4620	79.579	80.041	0.0800	5.77	0.0680	0.63	0.3924	0.6229	623
1	0.80	0.7998	79.181	79.981	0.0800	10.00	0.0680	0.63	0.6801	1.0795	1080
2	1.60	1.6133	78.397	80.011	0.0800	20.16	0.0680	0.63	1.3711	2.1764	2176
4	3.20	3.2222	76.787	80.009	0.0800	40.27	0.0680	0.63	2.7398	4.3489	4349
6	4.80	4.7957	75.272	80.067	0.0801	59.90	0.0680	0.63	4.0723	6.4640	6464
8	6.40	6.3807	73.592	79.973	0.0800	79.79	0.0680	0.63	5.4254	8.6118	8612
10	8.00	7.9752	71.988	79.963	0.0800	99.74	0.0680	0.63	6.7821	10.7652	10765

Table A-3. Serial Dilution for Preparing ECOPOL Treatments in SSL Soil

ECOPOL Formulation in SSL2020 Soil; 18 May 2021											
Formulation Dilution											
		Actual		Actual	Actual		Actual				
Concentration	Formulation	Formulation	ASTM 1	Total	Solution	Solution	solution	Weight of	Weight of	Concentr	Concentr
% of formulation	Needed	weight	Water	Solution	Weight	conc.	added to soil	soil	formulation	in soil	in soil
	(g)	(g)	(g)	(g)	kg	g/kg	kg	kg	g	g/kg dw	mg/kg soil
0.4	0.32	0.3236	79.577	79.901	0.0799	4.05	0.0685	0.63	0.2775	0.4405	440
0.6	0.48	0.4862	79.519	80.006	0.0800	6.08	0.0682	0.63	0.4145	0.6579	658
1	0.80	0.7957	79.197	79.993	0.0800	9.95	0.0682	0.63	0.6786	1.0771	1077
2	1.60	1.5971	78.551	80.148	0.0801	19.93	0.0680	0.63	1.3552	2.1511	2151
4	3.20	3.2200	76.749	79.969	0.0800	40.27	0.0680	0.63	2.7381	4.3461	4346
6	4.80	4.7875	75.147	79.934	0.0799	59.89	0.0680	0.63	4.0745	6.4675	6468
10	8.00	7.9950	72.017	80.012	0.0800	99.92	0.0680	0.63	6.7967	10.7884	10788

Table A-4. Serial Dilution for Preparing Buckeye Treatments in SSL Soil

Buckeye Formulation in SSL2020 SOIL; 20 April 2021											
Formulation Dilution											
		Actual		Actual	Actual		Actual				
Concentration	Formulation	Formulation	ASTM 1	Total	Solution	Solution	solution	Weight of	Weight of	Concentr	Concentr
% of formulation	Needed	weight	Water	Solution	Weight	conc.	added to soil	soil	formulation	in soil	in soil
	(g)	(g)	(g)	(g)	kg	g/kg	kg	kg	g	g/kg dw	mg/kg soil
0.1	0.08	0.0700	79.930	80.000	0.0800	0.88	0.0680	0.63	0.0595	0.0945	94
0.2	0.16	0.1700	79.840	80.010	0.0800	2.12	0.0680	0.63	0.1445	0.2294	229
0.4	0.32	0.3400	79.680	80.020	0.0800	4.25	0.0680	0.63	0.2891	0.4589	459
0.6	0.48	0.4800	79.520	80.000	0.0800	6.00	0.0680	0.63	0.4081	0.6478	648
1	0.80	0.7900	79.210	80.000	0.0800	9.88	0.0683	0.63	0.6747	1.0709	1071
2	1.60	1.6000	78.410	80.010	0.0800	20.00	0.0680	0.63	1.3598	2.1585	2158
4	3.20	3.2100	76.810	80.020	0.0800	40.11	0.0680	0.63	2.7286	4.3311	4331
6	4.80	4.8000	75.230	80.030	0.0800	59.98	0.0680	0.63	4.0785	6.4738	6474

Table A-5. Serial Dilution for Preparing Fomtec Treatments in SSL Soil

FOMTEC Formulation in SSL2020 SOIL; 17 August 2021											
Formulation Dilution											
		Actual		Actual	Actual		Actual				
Concentration	Formulation	Formulation	ASTM 1	Total	Solution	Solution	solution	Weight of	Weight of	Concentr	Concentr
% of formulation	Needed	weight	Water	Solution	Weight	conc.	added to soil	soil	formulation	in soil	in soil
	(g)	(g)	(g)	(g)	kg	g/kg	kg	kg	g	g/kg dw	mg/kg soil
0.6	0.48	0.5142	79.667	80.182	0.0802	6.41	0.0680	0.63	0.4360	0.6921	692
1	0.80	0.8913	79.144	80.035	0.0800	11.14	0.0680	0.63	0.7572	1.2018	1202
2	1.60	1.6194	78.419	80.038	0.0800	20.23	0.0682	0.63	1.3799	2.1903	2190
4	3.20	3.2508	76.774	80.025	0.0800	40.62	0.0680	0.63	2.7623	4.3847	4385
6	4.80	4.8091	75.310	80.119	0.0801	60.02	0.0680	0.63	4.0829	6.4808	6481
10	8.00	7.9593	72.049	80.008	0.0800	99.48	0.0680	0.63	6.7637	10.7360	10736

Table A-6. Serial Dilution for Preparing NFD 20-391 Treatments in SSL Soil

National 20-391 Formulation in SSL2020 SOIL; 5 October 2021											
Formulation Dilution											
		Actual		Actual	Actual		Actual				
Concentration	Formulation	Formulation	ASTM 1	Total	Solution	Solution	solution	Weight of	Weight of	Concentr	Concentr
% of formulation	Needed	weight	Water	Solution	Weight	conc.	added to soil	soil	formulation	in soil	in soil
	(g)	(g)	(g)	(g)	kg	g/kg	kg	kg	g	g/kg dw	mg/kg soil
0.6	0.48	0.4844	80.427	80.911	0.0809	5.99	0.0680	0.63	0.4073	0.6466	647
1	0.80	0.7981	79.181	79.979	0.0800	9.98	0.0680	0.63	0.6786	1.0771	1077
2	1.60	1.6555	78.370	80.025	0.0800	20.69	0.0680	0.63	1.4067	2.2329	2233
4	3.20	3.2638	76.794	80.058	0.0801	40.77	0.0680	0.63	2.7722	4.4003	4400
6	4.80	4.8700	75.176	80.046	0.0800	60.84	0.0680	0.63	4.1371	6.5669	6567
8	6.40	6.3565	73.651	80.008	0.0800	79.45	0.0680	0.63	5.4025	8.5754	8575
10	8.00	7.9360	72.107	80.043	0.0800	99.15	0.0680	0.63	6.7460	10.7079	10708

Table A-7. Serial Dilution for Preparing Avio Green Treatments in SSL Soil

AvioGreen Formulation in SSL2020 SOIL; 4 May 2021											
Formulation Dilution											
		Actual		Actual	Actual		Actual				
Concentration	Formulation	Formulation	ASTM 1	Total	Solution	Solution	solution	Weight of	Weight of	Concentr	Concentr
% of formulation	Needed	weight	Water	Solution	Weight	conc.	added to soil	soil	formulation	in soil	in soil
	(g)	(g)	(g)	(g)	kg	g/kg	kg	kg	g	g/kg dw	mg/kg soil
0.1	0.08	0.0868	80.010	80.097	0.0801	1.08	0.0680	0.63	0.0737	0.1170	117
0.2	0.16	0.1740	79.858	80.032	0.0800	2.17	0.0680	0.63	0.1479	0.2347	235
0.4	0.32	0.3147	79.784	80.099	0.0801	3.93	0.0680	0.63	0.2672	0.4242	424
0.6	0.48	0.4622	79.634	80.096	0.0801	5.77	0.0680	0.63	0.3925	0.6230	623
1	0.80	0.7901	79.159	79.949	0.0799	9.88	0.0681	0.63	0.6726	1.0676	1068
2	1.60	1.6176	78.326	79.944	0.0799	20.23	0.0680	0.63	1.3753	2.1831	2183
4	3.20	3.1998	76.804	80.003	0.0800	40.00	0.0680	0.63	2.7201	4.3176	4318

Table A-8. Serial Dilution for Preparing NRL 502W Treatments in SSL Soil

NRL 502W Formulation in SSL2020 Soil; 24 January 2022											
Formulation Dilution											
		Actual		Actual	Actual		Actual				
Concentration	Formulation	Formulation	ASTM 1	Total	Solution	Solution	solution	Weight of	Weight of	Concentr	Concentr
% of formulation	Needed	weight	Water	Solution	Weight	conc.	added to soil	soil	formulation	in soil	in soil
	(g)	(g)	(g)	(g)	kg	g/kg	kg	kg	g	g/kg dw	mg/kg soil
0.4	0.32	0.3218	79.673	79.995	0.0800	4.02	0.0680	0.63	0.2735	0.4341	434
0.6	0.48	0.4818	79.511	79.993	0.0800	6.02	0.0680	0.63	0.4095	0.6500	650
1	0.80	0.7755	79.222	79.998	0.0800	9.69	0.0680	0.63	0.6592	1.0463	1046
2	1.60	1.6159	78.425	80.040	0.0800	20.19	0.0680	0.63	1.3728	2.1791	2179
4	3.20	3.2278	76.717	79.945	0.0799	40.38	0.0683	0.63	2.7592	4.3798	4380
6	4.80	4.8140	75.315	80.129	0.0801	60.08	0.0680	0.63	4.0859	6.4856	6486
10	8.00	8.0118	72.055	80.067	0.0801	100.06	0.0680	0.63	6.8044	10.8006	10801

Table A-9. Serial Dilution for Preparing Solberg Treatments in SSL Soil

SOLBERG Formulation in SSL2020 SOIL; 27 July 2021											
Formulation Dilution											
Concentration % of formulation	Formulation Needed (g)	Actual Formulation weight (g)	ASTM 1 Water (g)	Actual Total Solution (g)	Actual Solution Weight kg	Solution conc. g/kg	Actual solution added to soil kg	Weight of soil kg	Weight of formulation g	Concentr in soil g/kg dw	Concentr in soil mg/kg soil
0.4	0.32	0.3321	79.674	80.006	0.0800	4.15	0.0680	0.63	0.2822	0.4480	448
0.6	0.48	0.4768	79.532	80.008	0.0800	5.96	0.0680	0.63	0.4053	0.6433	643
1	0.80	0.8149	79.191	80.006	0.0800	10.19	0.0680	0.63	0.6930	1.1000	1100
2	1.60	1.5966	78.402	79.999	0.0800	19.96	0.0680	0.63	1.3569	2.1539	2154
4	3.20	3.3533	76.653	80.006	0.0800	41.91	0.0680	0.63	2.8509	4.5253	4525
6	4.80	4.9195	75.080	79.999	0.0800	61.49	0.0680	0.63	4.1816	6.6375	6637
10	8.00	7.9287	72.073	80.002	0.0800	99.11	0.0680	0.63	6.7392	10.6972	10697

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