Diesel Spiking Study – 14-Day Earthworm Toxicity Testing Report

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Data Quality Assurance

- Nautilus Environmental is a member of the IEH Laboratories & Consulting Group. IEH acquired the San Diego aquatic toxicology group from Enthalpy Analytical on December 7, 2024.
- Nautilus Environmental is accredited in accordance with NELAP by the State of Oregon Environmental Laboratory Accreditation Program (Certificate No. 4053). It is also certified by the State of California Environmental Laboratory Accreditation Program (Certificate No. 1802) and the State of Washington Department of Ecology (Lab ID C552).
- All data have been reviewed and verified.
- All test results have met minimum test acceptability criteria under their respective EPA protocols, unless otherwise noted in this report.
- All test results have met internal Quality Assurance Program requirements.

Introduction

Nautilus Environmental, Inc. (Nautilus) was engaged by the California Department of Fish and Wildlife, Office of Spill Prevention and Response (CDFW-OSPR) to collect baseline ecological risk data for renewable diesel fuels using the earthworm *Eisenia fetida*, of the family lumbricidae, as a terrestrial model organism. The earthworm was exposed to artificially produced laboratory soils spiked with four fuels (conventional diesel, pure renewable diesel, and two blended diesel fuels) for 14 days to measure the potential toxicity to organism survival and growth. The initial rangefinder testing was conducted between August 13 and 27, 2025, and definitive testing was conducted between September 4 and 25, 2025 at the Nautilus laboratory, located in San Diego, CA.

Methods and Materials

Sample Collection and Transport

The test materials (diesel fuels, hereinafter referred to as samples) were transported on ice and delivered to Nautilus by hand on April 22, 2025, by CDFW-OSPR personnel. Appropriate chain-of-custody (COC) procedures were employed during transport. The samples arrived as clear liquids in amber glass bottles, two of which had settled precipitates. Samples were stored at 4°C in the dark until used for testing.

Sample identification, collection date and time, and receipt date and time are listed in Table 1.

Table 1. Sample, Collection, and Receipt Information.

Sample ID	Collection Date/Time	Receipt Date/Time
100% Renewable	6/21/23, 08:00	4/22/25, 09:15
100% Diesel #2	6/21/23, 10:00	
80:20 Renewable:Diesel Blend	4/11/25, 12:00	
95:5 Renewable:Diesel Blend	4/11/25, 12:00	

Artificial Soil Preparation and Handling

Artificial soil was prepared following method guidance in ASTM 2012. A moisture fraction of 35 to 40 percent was targeted (ASTM guidance suggests 35 to 45 percent) to allow for the additional volume of liquid from spiking with the diesel samples, while not oversaturating the soil beyond the level suitable for earthworms. Artificial soil was made in advance of testing,

stored in a sealed polyethylene bag, and allowed to age at test temperature for approximately two weeks prior to use.

Analysis of moisture content of the artificial soil was performed prior to test initiation preparations to confirm the percent moisture fraction was suitable. An aliquot of the soil was weighed on a pan, dried in an oven for 24 hours at 100°C, and then weighed again to determine moisture content.

Test Organism Source and Care

Cultured test organisms were purchased from Carolina Biological Supply in Burlington, NC. Upon arrival, organisms were kept in culture media consisting of peat moss moistened with deionized water (DI) and pH-controlled with calcium carbonate. Test organisms were held for at least two weeks prior to use in testing. During that time, they were kept under constant light to discourage escape and fed with DI-soaked alfalfa pellets that had aged for at least one week. They were fed three times a week and the media was monitored for moisture content. A single batch was obtained for use with all testing included in this report.

Artificial Soil Spiking Methods

Upon spiking the artificial soil with the diesel samples for both range-finding and definitive testing, a sufficient weight of artificial soil was portioned out for each sample and concentration being tested. The total volume of liquid spiked was kept constant in order to have a similar moisture fraction for each concentration after spiking and to allow for better mixing and distribution with lower concentrations of sample. This was done by mixing the target amount of diesel sample into DI so the final total volume of sample plus DI was similar for all concentrations; the exception to this process was the highest concentration tested in each spiked series which consisted of only pure sample and no DI.

To account for differences in specific gravity (i.e., density), all additions were made by weight rather than by volume. Calculation sheets were produced for each sample based on the target nominal concentration of each sample spike and the mass of artificial soil needed for testing. Each sample spike was weighed using a calibrated analytical balance. A 12-dram glass shell vial or beaker was tared on the balance prior to sample addition using a glass Pasteur pipette. After the sample mass was confirmed, half of the total DI volume was added to the shell vial, and the DI and sample were mixed by agitation. The entirety of this volume was then added to the artificial soil by dispersing the mixture evenly to aid in the homogenization process. The remaining volume of DI was then added to the shell vial to “rinse” any remaining sample that may have adhered to the vial; this second aliquot of DI was then added to the soil. The spiked soil was then mixed in a stainless-steel bucket using a stainless-steel spoon for five minutes to maximize distribution and homogenization. After the mixing process was completed, the spiked soil was immediately distributed to the test chambers.

14-day Range Finding Toxicity Test

Range-finding tests with five concentrations of each sample were conducted to assess the appropriate concentrations to include in subsequent definitive testing based on survival results. Range-finding concentrations were developed in concert with CDFW-OSPR staff and represented a 10x escalation from one concentration to the next. Range-finding was conducted with a reduced number of replicates (three replicates with 10 organisms per replicate) and the weight endpoint was not included. Nominal concentrations for range-finding tests were 10,000, 1,000, 100, 10, and 1 milligram per kilogram (mg/kg). Only the conventional diesel fuel sample (100% Diesel #2) test yielded any survival response while the remaining three samples had no survival effect at the highest tested concentration of 10,000 mg/kg.

Some qualitative behavior observations were made during the range-finding testing and at test termination. Of particular note, some observations of organism avoidance of soil were made. Avoidance is generally described as organisms actively avoiding close contact with the soil and can include climbing the test chamber walls, crawling along the surface of the soil, or remaining in small pockets of space within the visible edges of the test chamber. At test termination during organism recovery, some additional observations of organisms being “jumpy” or “writhing” versus typical crawling behaviors or consistent movements were made for the 1,000 and 10,000 mg/kg concentrations of the 80:20 Renewable:Diesel blend and the 1,000 mg/kg concentration of the 100% Diesel #2 sample.

Given that there was no effect in the 10,000 mg/kg concentration in the rangefinder for any of the renewable diesel samples (the pure renewable and the two blends), the concentrations for these three samples were increased for the definitive test. The highest concentration for these three samples was determined based on a volume of sample that would not influence the organism response due to an excessive percent moisture of the soil (i.e., the highest testable concentration of renewable diesel samples which would not result in oversaturation of the test media).

14-day Definitive Toxicity Test Protocols

The 14-day earthworm definitive toxicity exposure was conducted in accordance with procedures presented by ASTM 2012. Worms were depurated overnight on Day -1 (i.e., the day before test initiation) in jars containing DI-soaked paper towels. Depurated weights were obtained on test Day 0 (the day of initiation) as an initial weight. On Day 14 of the test, worms were recovered and again depurated overnight before obtaining a final weight the following day. One lab control was included in the test design for each sample; all concentrations of each sample were associated with their concurrent lab control spatially within the environmental chamber. Methods are summarized in Table 2.

Based on the results of the range-finding tests, the selected nominal concentrations for the definitive tests were 4,000, 2,000, 1,000, 500, and 250 mg/kg for the conventional

diesel (100% Diesel #2) sample test and 50,000, 25,000, 12,500, 6,250, and 3,125 mg/kg for the renewable diesel and two blended diesel sample tests. On the day of test setup (i.e., day before test initiation), soils were hydrated with the diesel samples diluted with enough deionized water to a target range of 40 to 50 percent moisture fraction to allow for more liquid (i.e., diesel) to be incorporated into the soil without oversaturation. Note that this moisture range is above the suggested 35-45 percent described in the test method; however, the method stipulates that increasing the moisture fraction is not detrimental to the test unless it results in oversaturation of the test soils (indicated by standing liquid visible in the test chambers). Spiked soil concentrations for the definitive test were prepared from a weight of the diesel sample added to 2,300 grams (g) of artificial soil which is later split into aliquots for individual test replicates; the spiking process was the same as that used in the range-finding tests described in the spiking procedures above. Nominal concentrations were used for all data analysis.

Due to the effort required to create the diesel-spiked soil concentrations, definitive testing was conducted in two discrete batches. A laboratory (negative) control was associated with each sample (two controls per batch) and each sample was randomized. A single batch of worms was used for both batches of samples. A single reference toxicant test (positive control) was conducted on the batch of worms used for testing. Batch 1 contained the 100% Diesel #2 and 100% Renewable Diesel samples and Batch 2 contained the 95:5 Renewable:Diesel Blend and the 80:20 Renewable:Diesel Blend.

For the definitive test batches, an additional surrogate test chamber (which did not contain worms) was set up for each spiked sample concentration to allow for collection of post-testing soil subsamples. This surrogate chamber mirrored all of the other technical parameters of the actual test replicates. At the termination of the bioassay, soil subsamples were collected from each surrogate for analysis and verification of the nominal spike concentrations. Subsamples were collected unpreserved in 8-ounce amber jars. These jars were stored at the Nautilus laboratory until they could be shipped to the CDFW-OSPR laboratory for hydrocarbon chemical analysis (see Quality Assurance section for additional details). Subsamples were shipped frozen via overnight delivery service, and all standard COC procedures were followed during the transport process.

Statistical Analyses

Mean survival and biomass loss in each sample were compared to the associated laboratory control mean survival and biomass loss. Survival data, expressed as a proportion, was arcsine square-root transformed prior to analysis to normalize the distribution of the data and satisfy statistical assumptions for analysis. The No Observed Effect Concentrations (NOEC) and Lowest Observed Effect Concentrations (LOEC) were calculated using a parametric or nonparametric analysis, as appropriate. The concentrations expected to cause an adverse effect to 25 and 50 percent of test organisms (EC25 and EC50, respectively) were calculated using linear interpolation for both endpoints. In order to calculate the NOEC, LOEC, and effect concentrations, biomass loss was converted to percent biomass retained from the weight measured at test initiation. Statistical analyses of soil test data and reference toxicant data were

performed using Comprehensive Environmental Toxicity Information System Software (CETIS™), Version 2.1.4.11. (Tidepool Scientific Software 2000-2022). Analysis for this test followed standard USEPA flow chart methods specified for this test type. Graphical representation of test data was performed using GraphPad Prism, Version 10.6.1.

Table 2. Summary of 14-day Earthworm Toxicity Testing Conditions (°C – degrees Celsius g – gram; L – Liter % - percent).

Test start date; times	Batch 1: 9/4/25; 13:30 Batch 2: 9/11/25; 14:15
Test end date; time	Batch 1: 9/18/25; 10:30 Batch 2: 9/25/25; 11:15
Test organism	*Eisenia fetida (Eisenia foetida)*
Test organism source	Carolina Biological Supply; Burlington, NC
Test organism age	Mature adults
Test duration	14 days
Test chamber	1-L glass jar with a clear lid with central vent hole
Test soil/replicate	270-g hydrated soil
Water source for hydration/misting	Deionized water
Artificial control soil	70% sand, 20% kaolin clay, 10% peat moss, 0.4% $CaCO_3$
Number of organisms/replicate	10
Number of replicates/concentration	5, plus one surrogate test chamber for measurements
Test temperature	23 ± 1°C
Illumination	Continuous lighting
Test Protocol	E1676-12 (ASTM 2012)
Test acceptance criterion	≥ 90% mean survival of control organisms
Positive control reference toxicant	2-chloroacetamide (14-day survival)
Soil measurements	Temperature (daily); pH, conductivity, and percent moisture fraction (Days 0 and 14)

Results

Testing Batch #1, September 4, 2025

Survival

Mean *Eisenia* survival in laboratory control #1 was 100 percent. This exceeds the ASTM recommended mean survival of greater than or equal to 90 percent for the laboratory control. Mean survival in all concentrations for the 100% Renewable Diesel ranged from 98 to 100 percent. There were no statistically significant effects in any concentration of the 100% Renewable Diesel, resulting in a NOEC of 50,000 mg/kg and a LOEC of greater than 50,000 mg/kg. The EC_{25} and EC_{50} values were greater than 50,000 mg/kg.

Mean *Eisenia* survival in laboratory control #2 was 98 percent. This exceeds the ASTM recommended mean survival of greater than or equal to 90 percent for the laboratory control. Mean survival in all concentrations for the 100% Diesel #2 ranged from zero to 100 percent. There were statistically significant effects to the 4,000, 2,000, and 1,000 mg/kg concentrations of the 100% Diesel #2, resulting in a NOEC of 500 mg/kg and a LOEC of 1,000 mg/kg. The EC_{25} value was calculated as 576 mg/kg and the EC_{50} value was calculated as 770 mg/kg.

Biomass Retained

Mean *Eisenia* biomass retained in laboratory control #1 was 78 percent; it should be noted that weight loss is expected to occur over the course of the 14-day test due to the lack of food media provided during the exposure. Mean biomass retained in all concentrations for the 100% Renewable Diesel ranged from 79 to 85 percent. There were no statistically significant effects in any concentration of the 100% Renewable Diesel, resulting in a NOEC of 50,000 mg/kg and a LOEC of greater than 50,000 mg/kg. The EC_{25} and EC_{50} values were greater than 50,000 mg/kg.

Mean *Eisenia* biomass retained in laboratory control #2 was 76 percent. Mean biomass retained in all concentrations for the 100% Diesel #2 ranged from zero to 75 percent. There were statistically significant effects in the 4,000, 2,000, 1,000, and 500 mg/kg concentrations of the 100% Diesel #2, resulting in a NOEC of 250 mg/kg and a LOEC of 500 mg/kg. The EC_{25} value was calculated as 550 mg/kg, and the EC_{50} value was calculated as 757 mg/kg.

Qualitative behavior and physical appearance observations were made during the definitive testing and at test termination. Some observations of soil avoidance were noted in the 100% Diesel #2 sample. The majority of these observations were made in the 500, 1,000, 2,000 and 4,000 mg/kg concentrations. At test termination during organism recovery, some additional observations of organisms being “less active” and/or having an “oily” appearance were made for the 25,000 and 50,000 mg/kg concentrations of the 100% Renewable Diesel. There were also observations of “some grouped” in a couple of replicates that did not necessarily correlate with the sample concentration. Grouping refers to the worms congregating together possibly to avoid contact with the spiked soil. For the 100% Diesel #2 there was additional observations of “lethargic”, “some grouped”, and “less movement” in the 1,000 mg/kg concentration. Lethargic and less active can be taken as equivalent observations of slower or shorter movements.

Statistical results are summarized in Table 3, detailed results for the tests are presented in Tables 4 and 5, and graphs are provided in Figures 1 and 2.

Table 3. Summary of 14-day Earthworm Statistical Results – Testing Batch #1.

Test Species and Endpoint	NOEC (mg/kg)	LOEC (mg/kg)	EC_{25} (mg/kg)	EC_{50} (mg/kg)
100% Renewable Diesel				
Survival	50,000	>50,000	>50,000	>50,000
Biomass Retained	50,000	>50,000	>50,000	>50,000
100% Diesel #2				
Survival	500	1000	576	770
Biomass Retained	250	500	550	757

NOEC = the highest concentration tested that results in no observed effect
LOEC = the lowest concentration tested that results in an observed effect.
EC_{25} = concentration at which 25 percent of the population is adversely affected
EC_{50} = concentration at which 50 percent of the population is adversely affected

Table 4. Summary of 14-day Earthworm Survival and Biomass Retained Results – 100% Renewable Diesel.

Concentration (mg/kg)	Mean Survival (%)	Standard Deviation	Mean Biomass Retained (%)	Standard Deviation
Lab Control #1	100	0.0	78	2.9
3,125	100	0.0	83	1.1
6,250	100	0.0	82	1.8
12,500	100	0.0	85	1.3
25,000	98	4.5	81	2.6
50,000	98	4.5	79	1.5

Table 5. Summary of 14-day Earthworm Survival and Biomass Retained Results – 100% Diesel #2.

Concentration (mg/kg)	Mean Survival (%)	Standard Deviation	Mean Biomass Retained (%)	Standard Deviation
Lab Control #2	98	4.5	76	2.0
250	100	0.0	75	1.5
500	84	26	62[a]	16
1,000	20[a]	20	16[a]	14
2,000	0.0[a]	0.0	0.0[a]	0.0
4,000	0.0[a]	0.0	0.0[a]	0.0

[a] Value statistically significant compared to lab control (α=0.05)

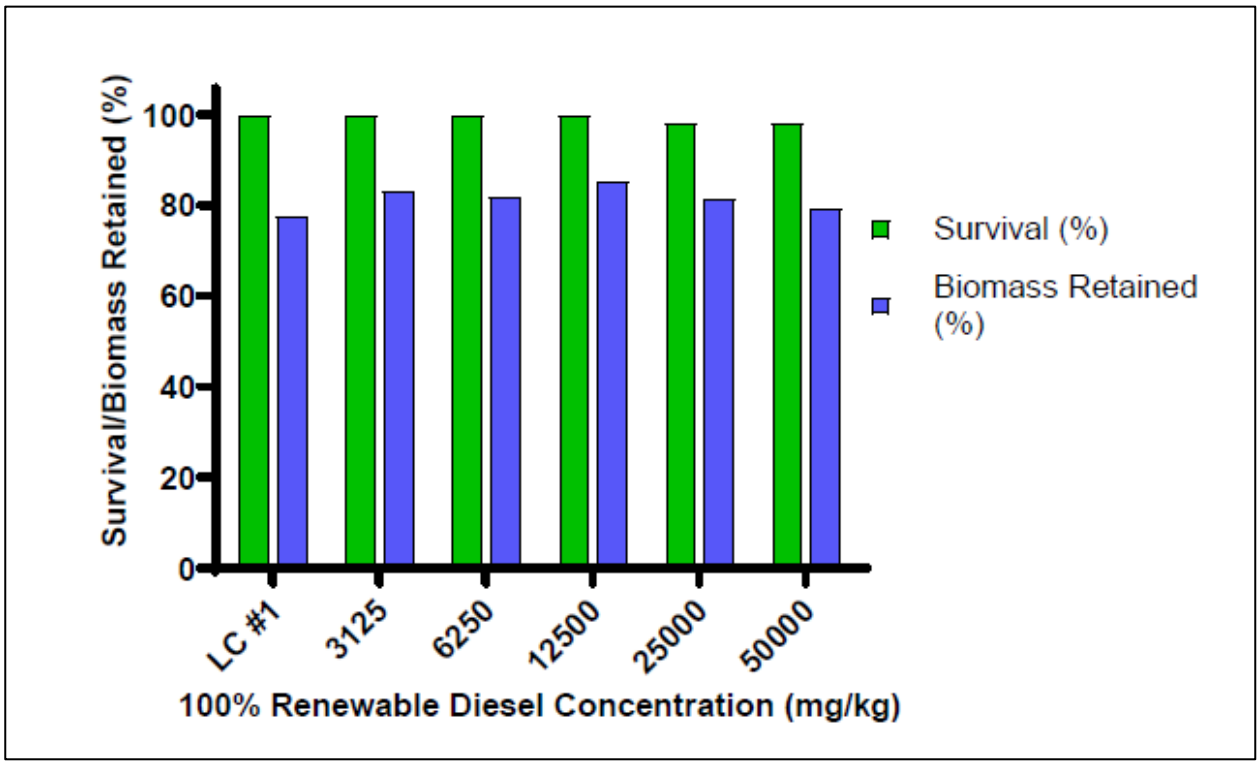

Figure 1. Summary of Eisenia Survival and Biomass Retained (mean percent; n=5).

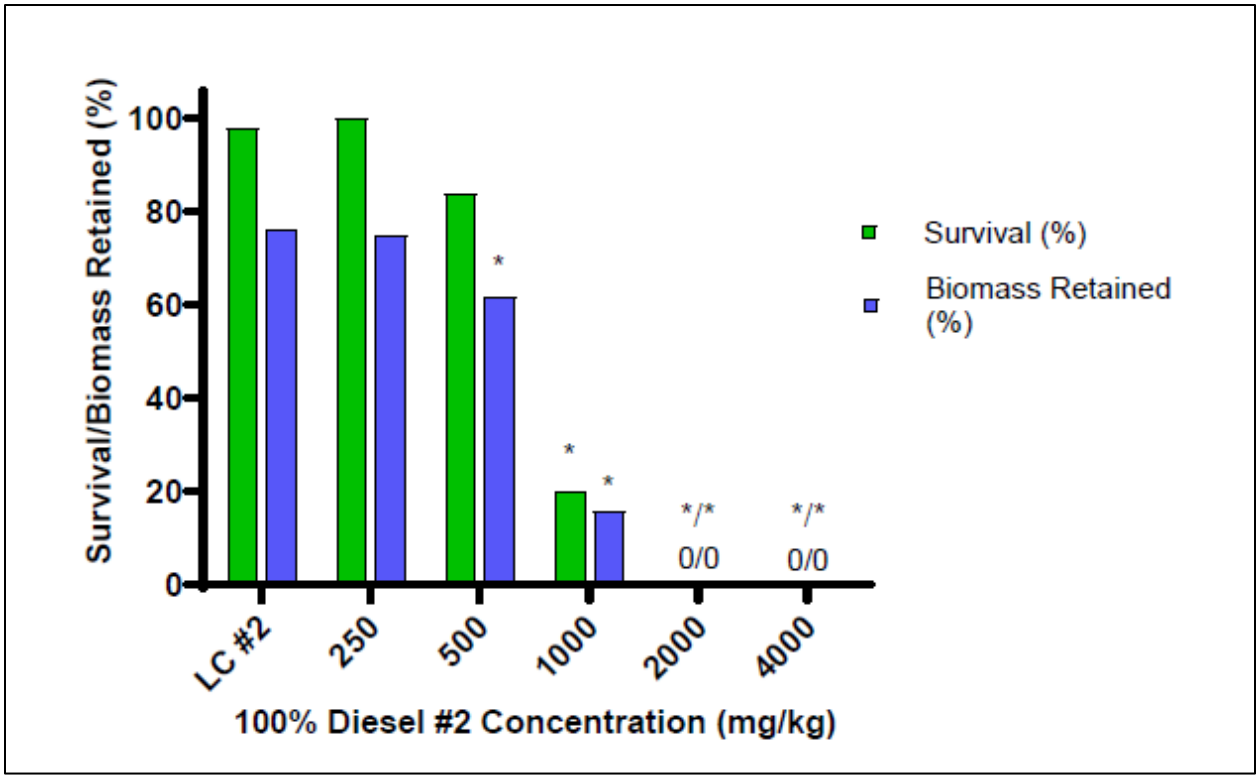

Figure 2. Summary of Eisenia Survival and Biomass Retained (mean percent; n=5). *An asterisk indicates a statistically significant difference from the Laboratory Control.

Testing Batch #2, September 11, 2025

Survival

Mean *Eisenia* survival in laboratory control #3 was 100 percent. This exceeds the ASTM recommended mean survival of greater than or equal to 90 percent for the laboratory control. Mean survival in all concentrations for the 80:20 Renewable:Diesel Blend ranged from 98 to 100 percent. There were no statistically significant effects in any concentration of the 80:20 Renewable:Diesel Blend, resulting in a NOEC of 50,000 mg/kg and a LOEC of greater than 50,000 mg/kg. The EC_{25} and EC_{50} values were greater than 50,000 mg/kg.

Mean *Eisenia* survival in laboratory control #4 was 100 percent. This exceeds the ASTM recommended mean survival of greater than or equal to 90 percent for the laboratory control. Mean survival in all concentrations for the 95:5 Renewable:Diesel Blend ranged from 98 to 100 percent. There were no statistically significant effects in any concentration of the 95:5 Renewable:Diesel Blend, resulting in a NOEC of 50,000 mg/kg and a LOEC of greater than 50,000 mg/kg. The EC_{25} and EC_{50} values were greater than 50,000 mg/kg.

Biomass Retained

Mean *Eisenia* biomass retained in laboratory control #3 was 81 percent. Mean biomass retained in all concentrations for the 80:20 Renewable:Diesel Blend ranged from 70 to 80 percent. There was a statistically significant effect in the 50,000 mg/kg concentration of the 80:20 Renewable:Diesel Blend, resulting in a NOEC of 25,000 mg/kg and a LOEC of 50,000 mg/kg. The EC_{25} and EC_{50} values were greater than 50,000 mg/kg.

Mean *Eisenia* biomass retained in laboratory control #4 was 77 percent. Mean biomass retained in all concentrations for the 95:5 Renewable:Diesel Blend ranged from 79 to 85 percent. There were no statistically significant effects in any concentration of the 95:5 Renewable:Diesel Blend, resulting in a NOEC of 50,000 mg/kg and a LOEC of greater than 50,000 mg/kg. The EC_{25} and EC_{50} values were greater than 50,000 mg/kg.

Some qualitative behavior observations were made at test termination. During organism recovery, observations of organisms being "jumpy", versus typical crawling behavior or consistent movements, were made in some replicates of all concentrations of the 80:20 Renewable:Diesel blend. For the 95:5 Renewable:Diesel Blend there were additional observations of "jumpy" and "less active" in only a few replicates throughout the test.

Statistical results are summarized in Table 6, detailed results for the tests are presented in Tables 7 and 8, and graphs are provided in Figures 3 and 4.

Table 6. Summary of 14-day Earthworm Statistical Results – Testing Batch #2

Test Species and Endpoint	NOEC (mg/kg)	LOEC (mg/kg)	EC_{25} (mg/kg)	EC_{50} (mg/kg)
80:20 Renewable:Diesel Blend				
Survival	50,000	>50,000	>50,000	>50,000
Biomass Retained	25,000	50,000	>50,000	>50,000
95:5 Renewable:Diesel Blend				
Survival	50,000	>50,000	>50,000	>50,000
Biomass Retained	50,000	>50,000	>50,000	>50,000

NOEC = the highest concentration tested that results in no observed effect
LOEC = the lowest concentration tested that results in an observed effect
EC_{25} = concentration at which 25 percent of the population is adversely affected
EC_{50} = concentration at which 50 percent of the population is adversely affected.

Table 7. Summary of 14-day Earthworm Survival and Biomass Retained Results – 80:20 Renewable:Diesel Blend.

Concentration (mg/kg)	Mean Survival (%)	Standard Deviation	Mean Biomass Retained (%)	Standard Deviation
Lab Control #3	100	0.0	81	1.9
3,125	100	0.0	80	4.5
6,250	100	0.0	79	2.5
12,500	100	0.0	79	1.0
25,000	100	0.0	78	1.7
50,000	98	4.5	70[a]	5.8

[a] Value statistically significant compared to lab control (α=0.05)

Table 8. Summary of 14-day Earthworm Survival and Biomass Retained Results – 95:5 Renewable:Diesel Blend.

Concentration (mg/kg)	Mean Survival (%)	Standard Deviation	Mean Biomass Retained (%)	Standard Deviation
Lab Control #4	100	0.0	77	3.1
3,125	100	0.0	85	9.3
6,250	100	0.0	77	2.1
12,500	100	0.0	76	5.5
25,000	98	4.5	75	5.8
50,000	100	0.0	77	3.0

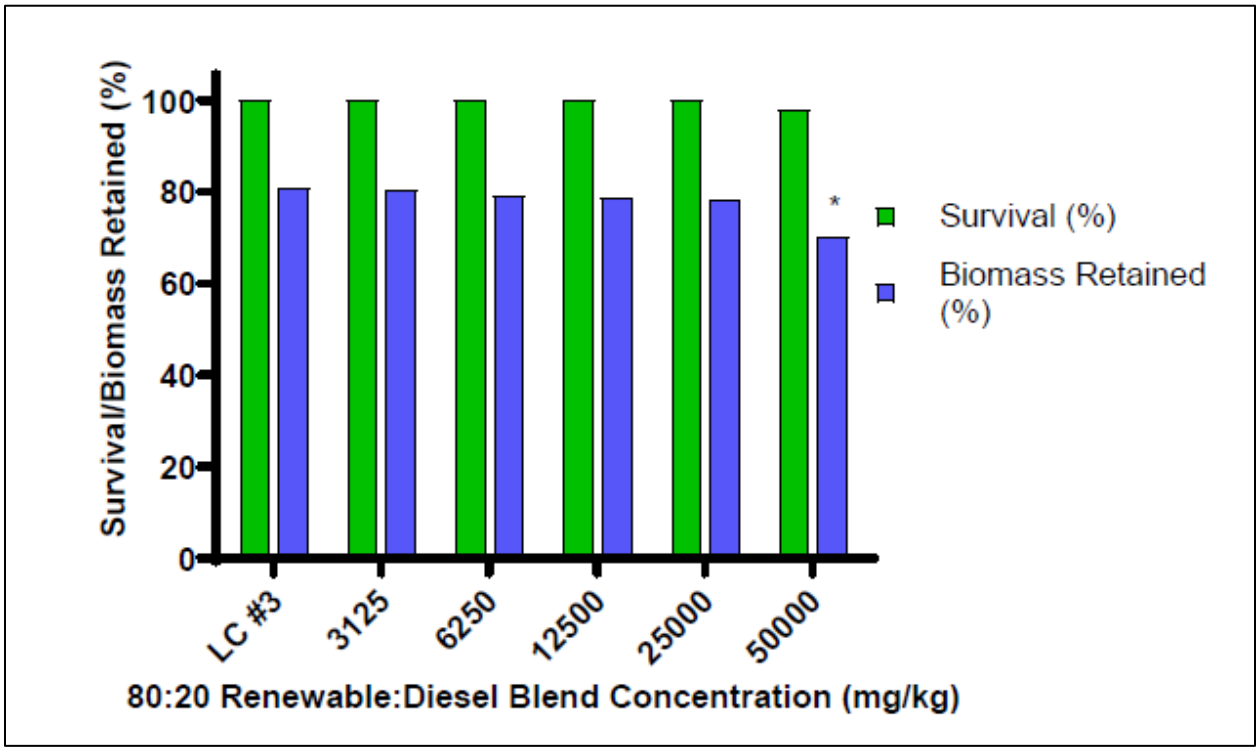

Figure 3. Summary of *Eisenia* Survival and Biomass Retained (mean percent; n=5). *An asterisk indicates a statistically significant difference from the Laboratory Control.

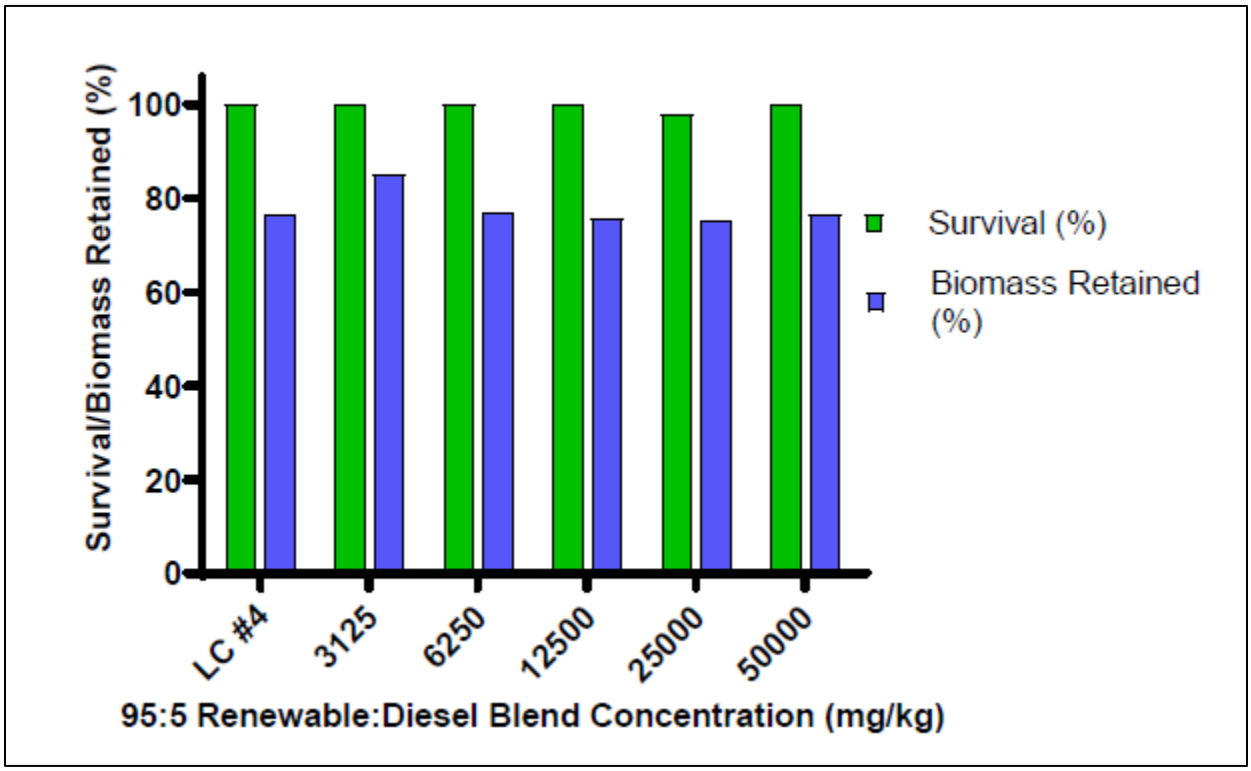

Figure 4. Summary of *Eisenia* Survival and Biomass Retained (mean percent; n=5)

Quality Assurance

All tests exceeded the minimum survival acceptability criterion for control performance. All of the data presented have been thoroughly reviewed and are deemed acceptable for reporting in accordance with our internal quality assurance program and relevant protocols. Minor deviations are noted on datasheets, with corrective actions taken when appropriate. All were determined to be minor with no likely negative impacts on the data or its final interpretation.

At the time of test termination, subsamples from each exposure concentration of all four samples were collected. Subsamples should have been immediately frozen after collection, however due to an error in communication, subsamples were placed in 4°C cold storage rather than the freezer. Upon discovering the error, subsamples were immediately moved to a -20°C freezer until they were shipped to the CDFW-OSPR lab for analysis.

Reference Toxicant Testing

The reference toxicant test met all minimum test acceptability requirements. Results of the reference toxicant test are summarized in Table 9. The calculated effect concentration for this test was within two standard deviations of the historical mean for our laboratory, indicating this batch of organisms had a typical sensitivity to 2-Chloroacetamide. The reference toxicant test showed a typical dose response curve and had acceptable control performance and was therefore deemed acceptable for the purposes of this report.

Table 9. Reference Toxicant Test Results.

Endpoint	Date Initiated	NOEC (mg/kg)	LC_{50} (mg/kg)	Historical range (mean LC_{50} ± 2 SD)	CV (%)
14d Survival	9/5/25	20	38.2	25.2 ± 15.1	30.0

NOEC = no observed effect concentration
LC50 = concentration of 2-Chloroacetamide (mg/kg) expected to cause a lethal effect to 50% of exposed organisms. SD = standard deviation
CV = coefficient of variation

References

American Society of Testing and Materials (ASTM). 2012. Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests with the Lumbricid Earthworm *Eisenia fetida* and the Enchytraeid Potworm *Enchytraeus albidus*. ASTM designation E1676-12.

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