



Toxicity testing on weathered groundwater diesel

Environmental Assessment Program (EAP)

Final Report

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SIGNATURE PAGE



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This report has been prepared by Nautilus Environmental Company Inc. based on data and/or samples provided by our client and the results of this study are for their sole benefit. Any reliance on the data by a third party is at the sole and exclusive risk of that party. The results presented here relate only to the samples tested.

1.0 INTRODUCTION

The Washington State Department of Ecology (Ecology) Environmental Assessment Program (EAP) requested Nautilus Environmental to characterize the toxicity of weathered diesel to freshwater and marine organisms. Currently, there are few aquatic toxicity studies on weathered diesel in the literature. This project was conducted to obtain toxicity information on weathered diesel for developing water quality objectives. Specifically, the goal of the project was to estimate the no-observed effects levels (NOEL) and low-observed effects levels (LOEL) of weathered diesel in contaminated groundwater on freshwater and marine organisms. These effects levels will be directly applicable to WET testing that is carried out under the Model Toxics Control Act (MTCA) as it pertains to the cleanup and investigation of hydrocarbon-contaminated sites by Ecology's Toxics Cleanup Program (TCP). TCP will use the NOEL values to establish a screening level for hydrocarbon contamination in fresh and marine waters. Previous studies had been conducted on gasoline and diesel for this purpose.

Tests were conducted on diesel contaminated groundwater DW-3, that was collected from a site in Washington State. The contaminated groundwater was analysed to determine the concentration of hydrocarbons in the diesel range and was also tested for metals, TDS, and other constituents that might affect toxicity to aquatic organisms. Samples of the groundwater were shipped by EAP to Nautilus for toxicity testing. The following toxicity tests were performed on the groundwater sample:

- Topsmelt survival and growth (EPA/600/R-95/136)
- Echinoderm fertilization (EPA/600/R-95/136)
- Fathead minnow survival and growth (EPA-821-R-02_013)
- *Ceriodaphnia dubia* survival and reproduction (EPA-821-R-02-013)

The results of these toxicity tests are presented in this report. Copies of laboratory data sheets and printouts of statistical analyses are provided in Appendices A to D; analytical chemistry is provided in Appendix E; and the chain-of-custody form is provided in Appendix F.

2.0 METHODS

Methods for the toxicity tests are summarized in Tables 1 to 4. Testing was conducted according to procedures described by the US EPA (1995, 2002) and the Washington Department of Ecology (WDOE, 2016).

Preliminary toxicity tests using *C. dubia* were conducted in December 2018 with uncontaminated groundwater to ensure that it was non-toxic to the study organisms. This was done to ensure that any observable effects could be attributed to the weathered diesel contamination. Sample MW-11 was chosen as the site control for further testing based on the results of these tests; results of these tests are presented in a separate report.

The groundwater DW-3 used for the range finding tests was collected January 29, 2019 and received on January 30, 2019. Subsamples of 100% and 50% strength dilutions were sent to Ecology's Manchester Environmental Laboratory for initial analysis of groundwater diesel prior to testing to ensure the diesel concentrations were within detection limits.

To determine an appropriate concentration series that would capture NOEC and LOEC values, range-finding tests were performed for each test species prior to initiation of the definitive tests. Subsamples of the test solutions were taken at various points of the tests to determine the concentration and stability of weathered diesel in the toxicity tests. Samples were shipped to Manchester Environmental Laboratory for analysis.

Additional groundwater sample and site control was collected March 19, received on March 20, 2019 and was homogenized with the samples from January. Subsamples were collected and sent for re-analysis of diesel prior to definitive testing to ensure concentrations were similar to those used in the range-finding tests. Measured concentrations of the refresh sample were lower than the initial measurements though were comparable.

Definitive tests using topsmelt and fathead minnow were initiated on April 23, 2019; the echinoderm test was initiated May 1, 2019; and the definitive test using *C. dubia* was initiated on May 14, 2019. Subsamples of test solutions were once again taken a various time points and sent to Manchester Environmental Laboratory for analysis. The average measured concentration of DW-3 for the duration of the range-finding tests was 5.95 mg/L diesel; the average measured concentration for the definitive tests was 4.80 mg/L diesel. Statistical analyses were performed using the site control (MW-11) as the negative control with CETIS (Tidepool Scientific Software, 2013).

Table 1. Summary of test conditions: 7-d topsmelt (*Atherinops affinis*) survival and growth test.

Test species	<i>Atherinops affinis</i>
Organism source	Commercial supplier
Organism age	9-to 15-days post-hatch
Test type	Static-renewal
Test duration	7 days
Test vessel	500-mL glass container with lid
Test volume	500 mL
Test concentrations	Six concentrations, plus laboratory and methanol control
Test replicates	5 per treatment
Number of organisms	5 per replicate
Control/dilution water	Natural seawater
Test solution renewal	Daily (80% renewal)
Test temperature	20 ± 1°C
Test salinity	30 ± 2 ppt; sample salinity adjusted by addition of H ₂ Ocean Pro+ marine salts
Feeding	Twice a day with newly hatched brine shrimp nauplii (<i>Artemia sp.</i>)
Light intensity	Ambient laboratory lighting
Photoperiod	16 hours light / 8 hours dark
Aeration	None, unless dissolved oxygen falls below 4.0 mg/L
Test measurements	Temperature, dissolved oxygen, pH and salinity measured daily; survival checked daily
Test protocol	EPA/600/R-95/136
Statistical software	CETIS Version 1.9.4
Test endpoints	Survival and biomass
Test acceptability criteria for controls	≥80% survival; ≥0.85 mg mean dry weight
Reference toxicant	Copper (added as CuCl ₂)

Table 2. Summary of test conditions: 40-min echinoderm fertilization test.

Test species	<i>Strongylocentrotus purpuratus</i>
Organism source	Commercial supplier
Organism age	< 3 hours post gamete collection
Test type	Static
Test duration	10 minutes sperm exposure; 10 minutes egg fertilization
Test vessel	30-mL glass vials with snap cap
Test volume	30 mL
Test concentrations	Six concentrations, plus laboratory and methanol control
Test replicates	4 per treatment
Number of organisms	2000 eggs per replicate
Control/dilution water	Natural seawater
Test solution renewal	None
Test temperature	12 ± 1°C
Test salinity	30 ± 2 ppt; sample salinity adjusted by addition of H ₂ Ocean Pro+ marine salts
Feeding	None
Light intensity	Ambient laboratory lighting
Photoperiod	None
Aeration	None
Test measurements	Temperature, dissolved oxygen, pH and salinity measured at test initiation
Test protocol	EPA/600/R-95/136
Statistical software	CETIS Version 1.9.4
Test endpoint	Fertilization
Test acceptability criterion for controls	≥60% and <98% mean fertilization
Reference toxicant	Copper (added as CuCl ₂)

Table 3. Summary of test conditions: 7-d fathead minnow (*Pimephales promelas*) survival and growth test.

Test species	<i>Pimephales promelas</i>
Organism source	Commercial supplier
Organism age	<24 hours post-hatch
Test type	Static-renewal
Test duration	7 days
Test vessel	375-mL glass container with lid
Test volume	375 mL
Test concentrations	Six concentrations, plus laboratory and methanol control
Test replicates	4 per treatment
Number of organisms	10 per replicate
Control/dilution water	Moderately-hard reconstituted water
Test solution renewal	Daily (80% renewal)
Test temperature	25 ± 1°C
Feeding	Twice a day with approximately 1500-2250 newly hatched brine shrimp nauplii (<i>Artemia sp.</i>) in each test container
Light intensity	100 to 500 lux
Photoperiod	16 hours light / 8 hours dark
Aeration	None, unless dissolved oxygen falls to <40% saturation
Test measurements	Temperature, dissolved oxygen, pH and conductivity measured daily; hardness and alkalinity of undiluted sample measured at test initiation; survival checked daily
Test protocol	EPA-821-R-02_013
Statistical software	CETIS Version 1.9.4
Test endpoints	Survival and biomass
Test acceptability criteria for controls	≥80% survival; ≥250 µg mean dry weight
Reference toxicant	Sodium chloride (NaCl)

Table 4. Summary of test conditions: *Ceriodaphnia dubia* survival and reproduction test.

Test species	<i>Ceriodaphnia dubia</i>
Organism source	In-house culture
Organism age	<24 hour old neonates, produced within a 8 hour window
Test type	Static-renewal
Test duration	7 ± 1 day
Test vessel	20-mL glass test tube with snap cap
Test volume	20 mL
Test concentrations	Six concentrations, plus laboratory and methanol control
Test replicates	10 per treatment
Number of organisms	1 per replicate
Control/dilution water	20% Perrier water and 80% deionized water + 5 µg/L Se and 2 µg/L vitamin B12
Test solution renewal	Daily (100% renewal)
Test temperature	25 ± 1°C
Feeding	Daily with <i>Pseudokirchneriella subcapitata</i> and YCT (3:1 ratio)
Light intensity	100 to 600 lux at water surface
Photoperiod	16 hours light / 8 hours dark
Aeration	None
Test measurements	Temperature, dissolved oxygen, pH and conductivity measured daily; hardness and alkalinity of undiluted sample measured at test initiation; survival and reproduction checked daily
Test protocol	EPA-821-R-02-013
Statistical software	CETIS Version 1.9.4
Test endpoints	Survival and reproduction
Test acceptability criteria for controls	≥80% survival; ≥15 young per surviving control producing three broods; ≥60% of controls producing three or more broods; no ephippia present
Reference toxicant	Sodium chloride (NaCl)

2.1 Subsampling Test Solutions

Subsamples of the fresh test solutions were taken during the filling of the test chambers. Subsamples of the stale test vessel solutions were collected as a composite of the test vessel replicates prior to renewal or at the end of the tests.

Test solutions were renewed daily for tests with topsmelt, fathead minnow and *C. dubia*; the 40 minute exposure echinoderm test did not require renewal of test solution. The site control MW-11 was used to calculate all test endpoints.

3.0 RESULTS

Samples of the fresh and stale test solutions were analyzed by the Manchester Environmental Laboratory. The nominal and measured concentrations for the range-finding tests are presented in Tables 5 to 7. The nominal and measured concentrations for the definitive tests are presented in Tables 8 to 10. Only the 100%, 6.3%, 0.39%, and lab (0%) and site control samples were analysed. Mean measured concentrations used for statistical analysis were calculated by plotting the measured concentrations as the dependent variable (y axis) vs. the nominal concentrations as the independent variable (x axis) for all time points in Microsoft Excel and then fitting a regression line to the data. Some data points were judged to be outliers and were not included in the regression analyses. The lab control was used in the regression lines as it was part of the test dilution series and the site control was not. The lab control concentrations were at or below detection limits. The reported analytical values were used for the lab control. The equation of the best fit regression line was then used to calculate the mean measured concentration at each time point and concentration. In all cases, an order 2 polynomial equation fit the plotted data best. The plots used to calculate the mean measured concentrations are shown in Figures 1 to 4. The calculated or mean measured concentrations are presented in Table 8.

Table 5. Results: Topsmelt and echinoderm nominal and measured test concentrations (mg diesel/L) (Range-finder).

Nominal Conc. (% v/v)	Measured Concentration (mg diesel/L)								
	t=0	t=24		t=48	t=72	t=96	t=120	t=144	t=168
	fresh	stale	fresh	fresh	fresh	fresh	fresh	fresh	stale
100	6.1	5.62	5.26	5.88	3.96	5.26	4.15	3.86	4.4
100 dupl.	6.08	--	--	--	--	--	--	--	--
25	--	--	--	--	--	--	--	--	--
6.25	0.38	0.2	0.3	0.31	0.22	0.34	0.36	0.16	0.25
6.25 dupl.	0.26	--	0.25	-	0.21	0.35	--	0.15	--
1.56	--	--	--	--	--	--	--	--	--
0.39	0.19	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.14
0.10	--	--	--	--	--	--	--	--	--
Site Control	0.31	--	--	--	--	--	--	0.26	--
Lab Control	0.15	--	--	--	--	--	--	0.15	--

t = time, Detection Limit = 0.15

Figure 1. Polynomial Regression of Test Solution Concentrations vs. Measured Concentrations – Topsmelt Range-finding Test

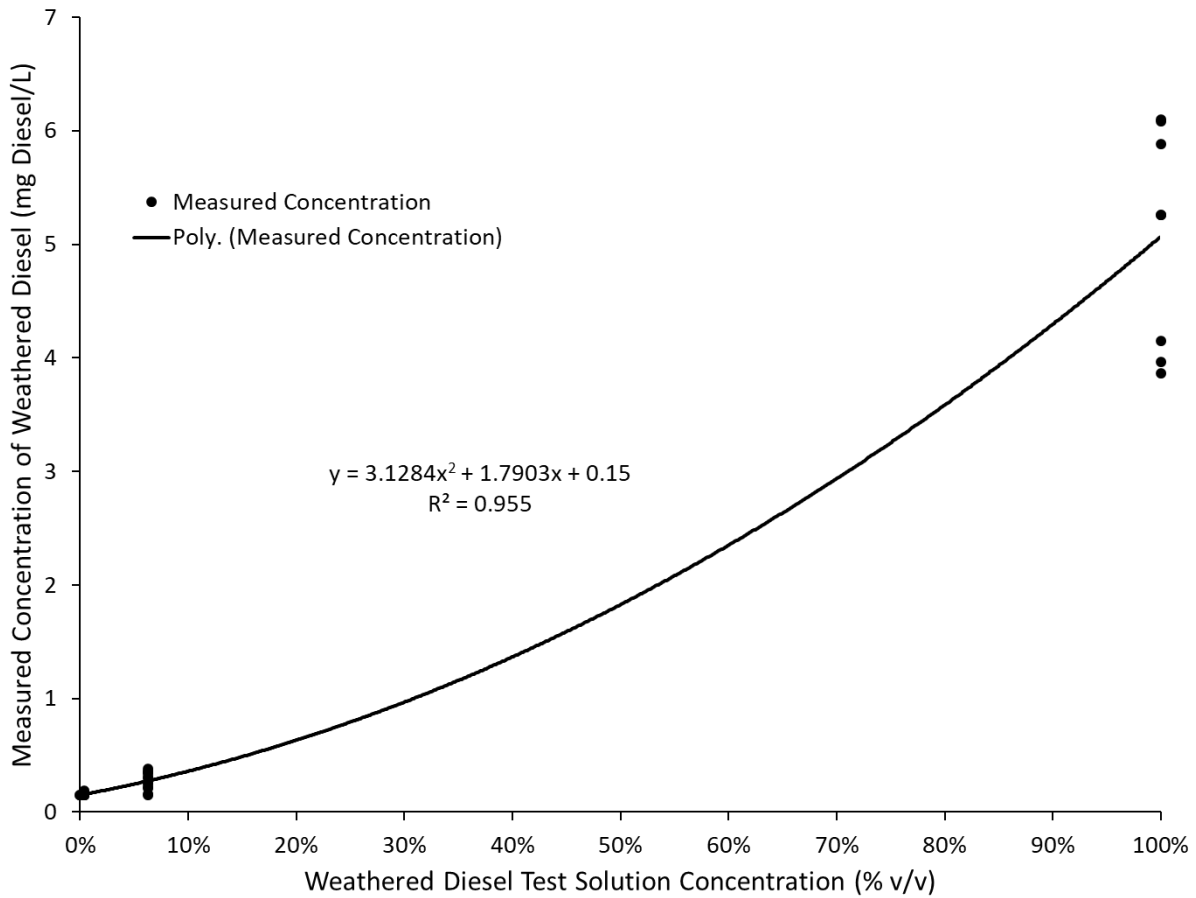


Table 6. Results: Fathead minnow nominal and measured test concentrations (mg diesel/L) (Range-finder).

Nominal Conc. (% v/v)	Measured Concentration (mg diesel/L)									
	t=0	t=24		t=48	t=72	t=96	t=120	t=144	t=168	
	fresh	stale	fresh	fresh	fresh	fresh	fresh	fresh	stale	fresh
100	6.16	5.39	6.33	6.35	7.04	0.32	4.8	6.63	5.7	6.69
100 dupl.	6.27	--	--	--	--	--	--	--	--	6.85
25	--	--	--	--	--	--	--	--	--	--
6.25	0.39	0.35	0.29	0.39	0.26	0.15	0.33	0.28	0.3	--
6.25 dupl.	0.29	--	0.3	-	0.4	0.15	--	0.28	--	--
1.56	--	--	--	--	--	--	--	--	--	--
0.39	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	--
0.10	--	--	--	--	--	--	--	--	--	--
Site Control	0.15	--	--	--	--	--	--	0.25	--	--
Lab Control	0.15	--	--	--	--	--	--	0.16	--	--

t = time, Detection Limit = 0.15

Figure 2. Polynomial Regression of Test Solution Concentrations vs. Measured Concentrations – Fathead Minnow Range-finding Test

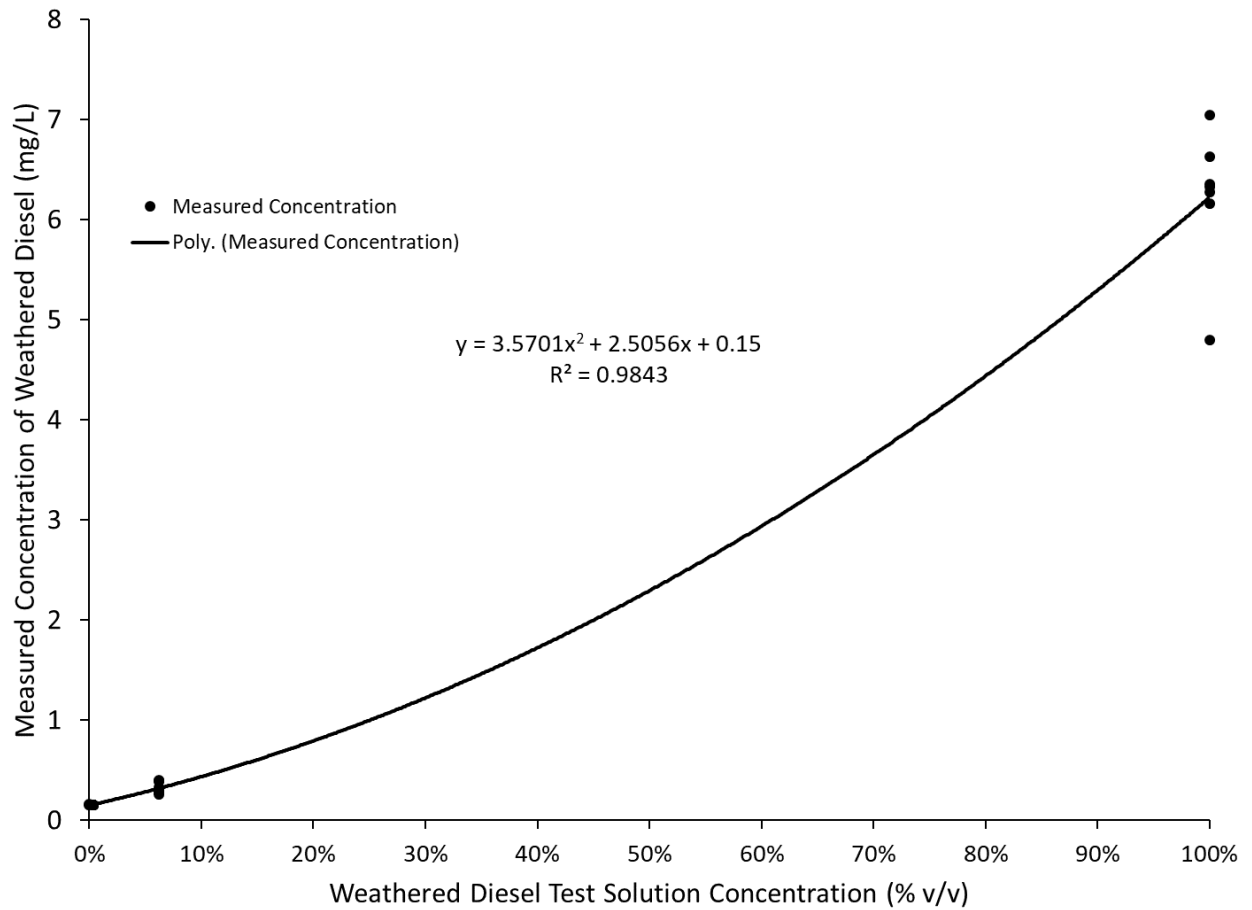


Table 7. Results: *Ceriodaphnia dubia* nominal and measured test concentrations (mg diesel/L) (Range-finder).

Nominal Conc. (% v/v)	Measured Concentration (mg diesel/L)						
	t = 0	t=24	t=48	t=72	t = 96	t = 120	t=144
	fresh	fresh	fresh	fresh	fresh	fresh	fresh
100	6.16	6.29	5.53	5.48	3.75	5.24	--
100 dupl.	6.26	--	--	--	--	--	--
25	--	--	--	--	--	--	--
6.25	0.32	0.34	0.27	0.28	0.25	0.30	--
6.25 dupl.	0.42	0.28	--	0.27	0.24	--	--
1.56	-	-	-	-	-	-	-
0.39	0.15	0.16	0.15	0.17	0.17	0.17	-
0.10	--	--	--	--	--	--	--
Site Control	0.28	--	--	--	--	--	0.23
Lab Control	0.22	--	--	--	--	--	0.16

t = time, Detection Limit = 0.16

Figure 3. Polynomial Regression of Test Solution Concentrations vs. Measured Concentrations – *C. dubia* Range-finding Test

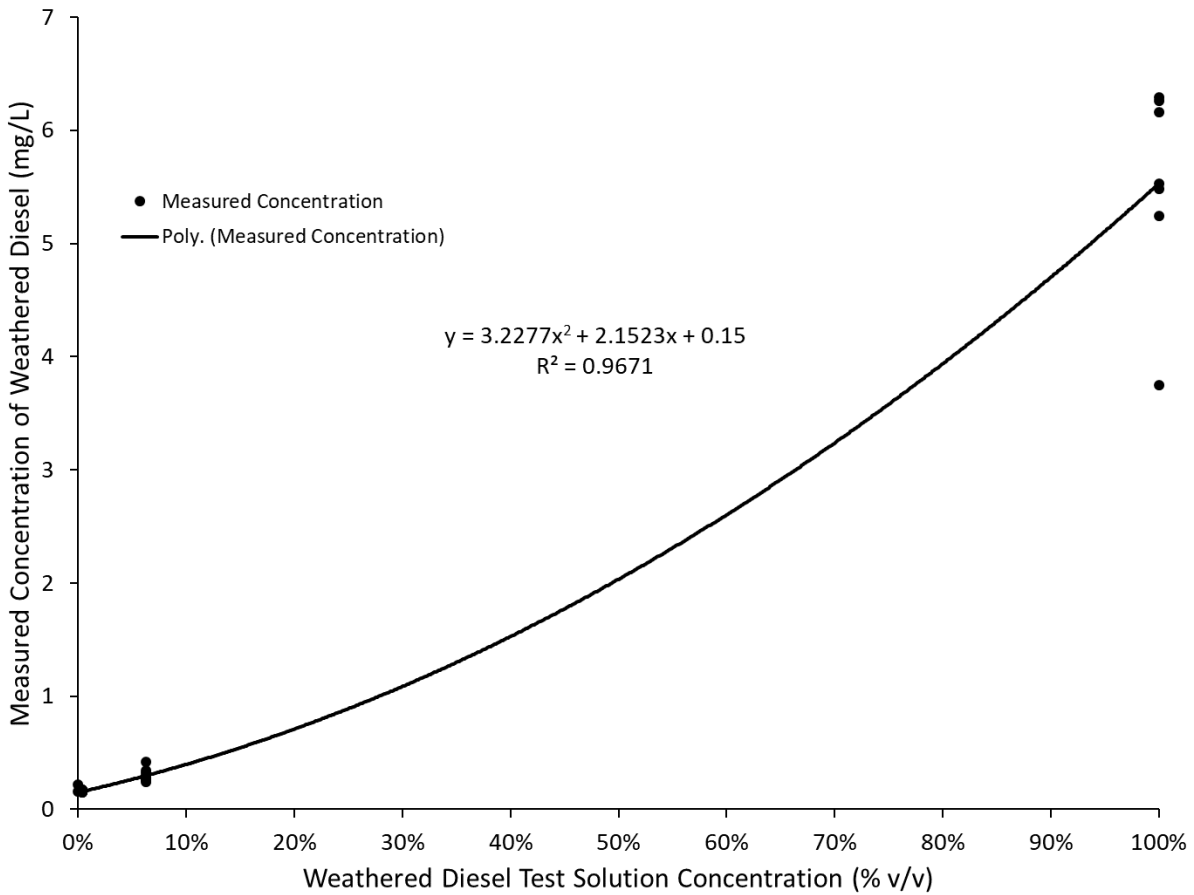


Table 8. Results: Topsmelt and echinoderm nominal and measured test concentrations (mg diesel/L) (Definitive).

Nominal Conc. (% v/v)	Measured Concentration (mg diesel/L)							
	t=0	t=24	t=48	t=72	t=96	t=120	t=144	t=168
	fresh	fresh	fresh	fresh	fresh	fresh	fresh	stale
100	3.94	2.74	3.06	3.19	4.43	2.97	2.66	2.86
100 dupl.	2.84	--	3.22	--	2.76	--	2.69	--
75	--	--	--	--	--	--	--	--
50	1.41	0.43	1.43	1.35	1.23	1.13	0.42	0.14
50 dupl.	--	--	--	--	0.27	--	1.2	--
25	0.67	0.62	0.65	0.62	0.55	0.58	0.64	0.51
12.5	--	--	--	--	--	--	--	--
Site Control	0.22	--	--	--	--	--	0.17	--
Lab Control	0.17	--	--	--	--	--	0.15	--

t = time

Table 9. Results: Echinoderm nominal and measured test concentrations (mg diesel/L) (Definitive).

Nominal Conc. (% v/v)	Measured Concentration (mg diesel/L)	Mean Value
	t=0	t=0
	fresh	fresh
100	2.83	2.71
100 dupl.	2.59	-
Site Control	0.28	0.22
Site Control dupl.	0.16	-
Lab Control dupl.	0.16	0.16
Lab Control	0.15	-

t = time

Figure 4. Polynomial Regression of Test Solution Concentrations vs. Measured Concentrations – Topsmelt Definitive Test

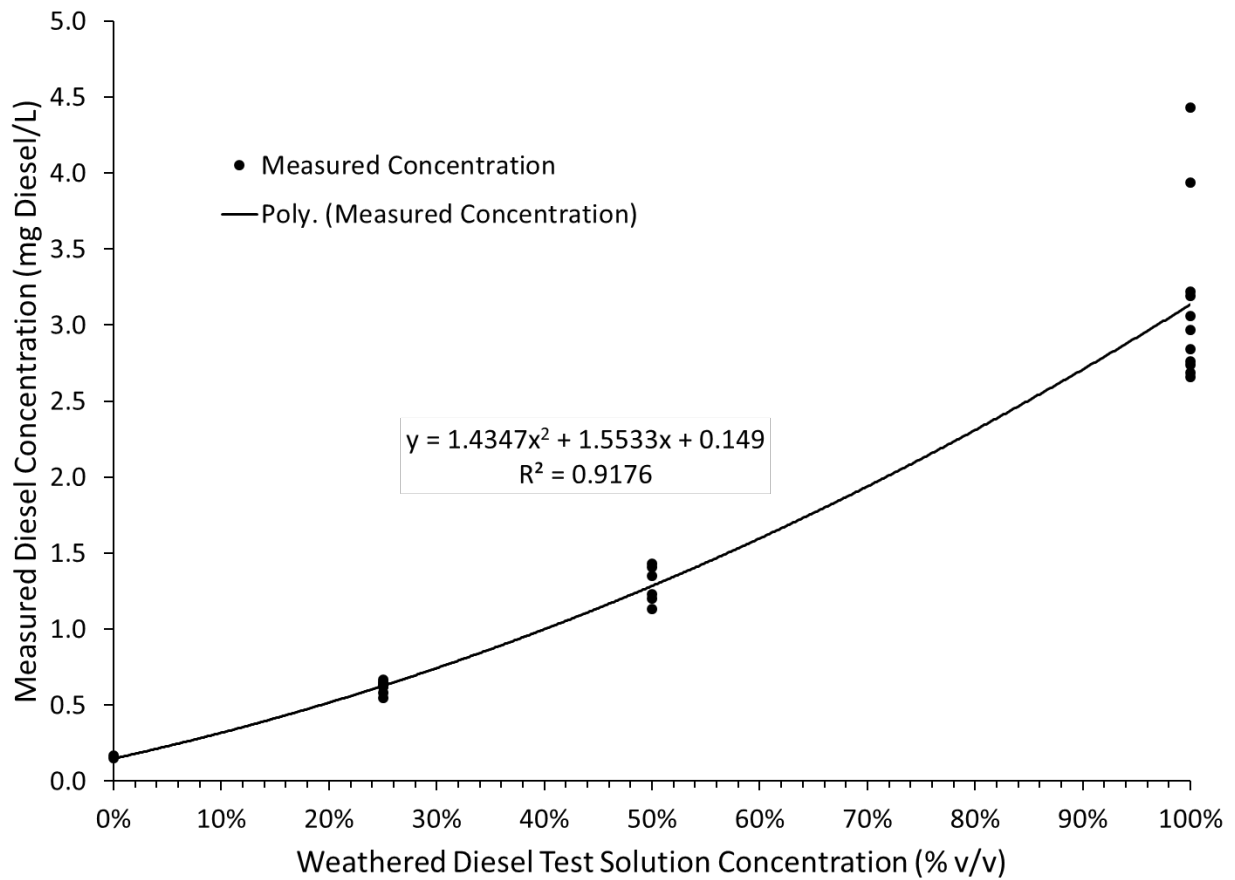


Table 10. Results: Fathead minnow nominal and measured test concentrations (mg diesel/L) (Definitive).

Nominal Conc. (% v/v)	Measured Concentration (mg diesel/L)							
	t=0	t=24	t=48	t=72	t=96	t=120	t=144	t=168
	fresh	fresh	fresh	fresh	fresh	fresh	fresh	stale
100	5.39	0.22	3.76	3.93	4	3.11	3.98	3.56
100 dupl.	5.83	--	3.98	--	4.12	--	4.02	--
75	--	--	--	--	--	--	--	--
50	2.28	0.19	1.8	1.97	1.82	2.42	1.89	1.72
50 dupl.	--	--	--	--	1.76	--	1.75	--
25	0.94	0.81	0.93	0.86	0.83	0.95	0.89	0.81
12.5	--	--	--	--	--	--	--	--
Site Control	0.2	--	--	--	--	--	0.21	--
Lab Control	0.17	--	--	--	--	--	0.17	--

t = time

Figure 5. Polynomial Regression of Test Solution Concentrations vs. Measured Concentrations – Fathead Minnow Definitive Test

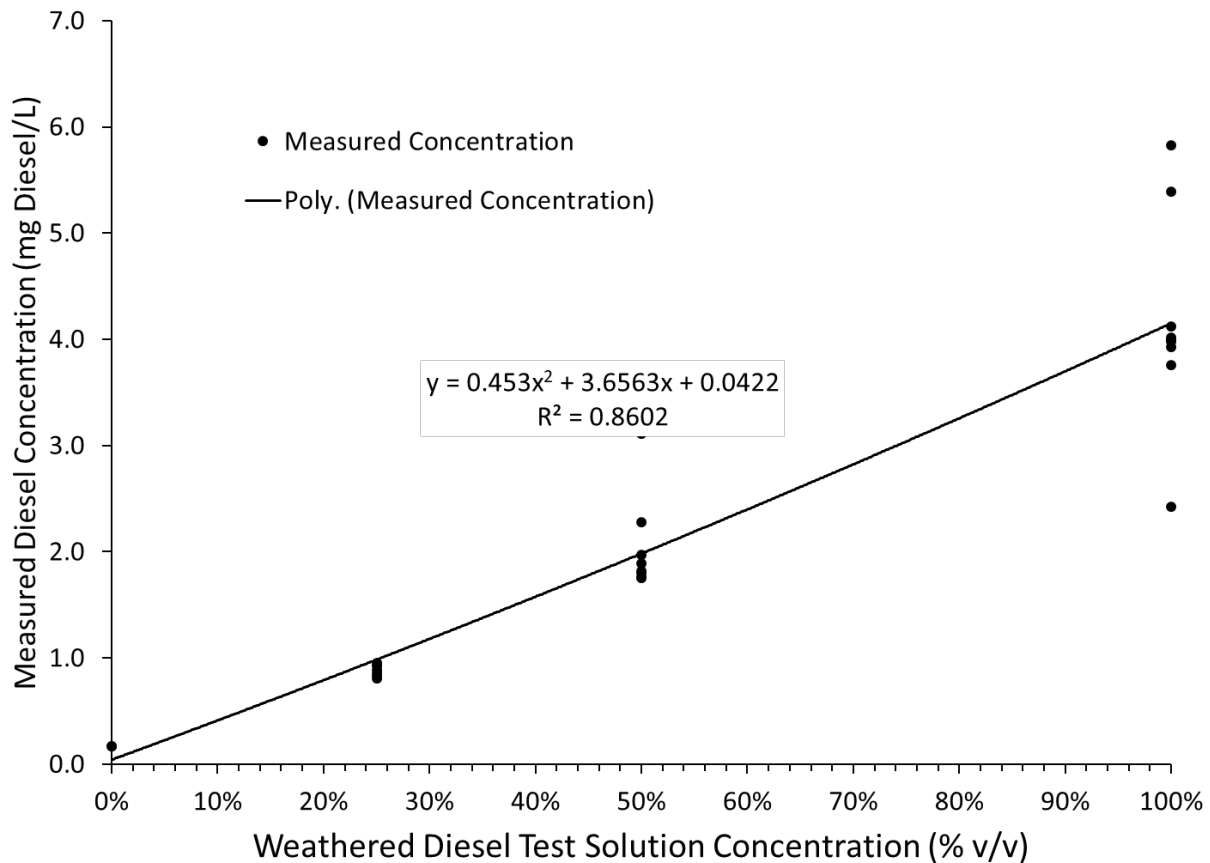


Table 11. Results: *Ceriodaphnia dubia* nominal and measured test concentrations (mg diesel/L) (Definitive).

Nominal Conc. (% v/v)	Measured Concentration (mg diesel/L)					
	t = 0	t=24	t=48	t=72	t = 96	t = 120
	fresh	fresh	fresh	fresh	fresh	fresh
100	3.76	4.02	4.13	4.24	4.02	--
100 dupl.	4.38	--	4.24	--	4.13	--
Site Control	0.22	--	--	--	--	0.28
Lab Control	0.17	--	--	--	--	0.16

t = time

3.1 Range-finding Toxicity Test Results

The results of the range-finding tests conducted in February 2019 are summarized in Tables 11 to 14. Results were compared against the laboratory control. Results of these tests were used to determine concentrations to use for the definitive tests.

Table 12. Results: Topsmelt 7-day survival and growth test (Range-finder).

Weathered Diesel Concentrations		Survival (%) (Mean ± SD)	Biomass (mg) (Mean ± SD)	Dry Weight (mg) (Mean ± SD)
Nominal (% v/v)	Mean Measured (mg diesel/L)			
Laboratory control	0.15 (DL)	100 ± 0.0	1.18 ± 0.02	1.18 ± 0.02
MW-11 (Site Control)	0.29	86.7 ± 11.6	1.04 ± 0.31	1.18 ± 0.19
0.10	<i>0.15</i>	93.3 ± 11.6	1.08 ± 0.18	1.16 ± 0.10
0.39	0.16	93.3 ± 11.6	1.16 ± 0.10	1.25 ± 0.05
1.6	<i>0.18</i>	93.3 ± 11.6	1.16 ± 0.13	1.25 ± 0.17
6.3	0.27	100 ± 0.0	1.25 ± 0.17	1.25 ± 0.17
25	<i>0.79</i>	93.3 ± 11.6	1.06 ± 0.14	1.16 ± 0.30
100	5.07	20.0 ± 20.0	0.13 ± 0.13	0.62 ± 0.07
Test endpoint (mg diesel/L) *				
	LC50 (95% CL)	2.4 (1.7 – 3.3)	--	--
	IC25 (95% CL)	--	1.3 (0.5 – 1.8)	2.1 (N/A – 2.9)
	IC50 (95% CL)	--	2.3 (1.4 – 3.2)	>5.1 (N/A)_
	NOEC	0.8	0.8	0.8
	LOEC	5.1	5.1	5.1

SD = Standard Deviation, LC = Lethal Concentration, IC = Inhibition Concentration, CL = Confidence Limits, NOEC = No Observed Effect Concentration, LOEC = Lowest Observed Effect Concentration, DL = Detection Limit (0.15 mg Diesel/L), N/A = Not Available

* = result was calculated using the laboratory control as the negative control

Calculated concentrations are represented in italics.

Table 13. Results: Echinoderm (*Strongylocentrotus purpuratus*) fertilization test (Range-finder)

Weathered Diesel Concentrations		Fertilized eggs (%) (Mean ± SD)
Nominal (% v/v)	Mean Measured (mg diesel/L)	
Laboratory control	0.15 (DL)	86.0 ± 3.8
Salt-adjusted control	-	91.2 ± 3.0
MW-11 (Site Control)	0.31	90.8 ± 2.5
0.1	--	87.2 ± 7.3
0.39	0.19	94.0 ± 3.2
1.6	--	91.2 ± 1.9
6.3	0.32	89.0 ± 2.2
25	--	90.0 ± 3.6
100	6.1	86.0 ± 3.4
Test endpoint (mg diesel/L) *		
	IC25	>6.1
	IC50	>6.1
	NOEC	6.1
	LOEC	>6.1

SD = Standard Deviation, IC = Inhibition Concentration, NOEC = No Observed Effect Concentration, LOEC = Lowest Observed Effect Concentration, DL = Detection Limit (0.15 mg Diesel/L)

(*) = Result was calculated using nominal values and the laboratory control as the negative control

Table 14. Results: Fathead minnow 7-day survival and growth test (Range-finder).

Weathered Diesel Concentrations		Survival (%) (Mean ± SD)	Biomass (mg) (Mean ± SD)	Dry Weight (mg) (Mean ± SD)
Nominal (% v/v)	Mean Measured (mg diesel/L)			
Laboratory control	0.16	100 ± 0.0	0.69 ± 0.14	0.69 ± 0.14
MW-11 (Site Control)	0.20	96.7 ± 5.8	0.80 ± 0.12	0.82 ± 0.09
0.10	<i>0.15</i>	93.3 ± 5.8	0.63 ± 0.06	0.67 ± 0.06
0.39	0.15	100 ± 0.0	0.75 ± 0.03	0.75 ± 0.03
1.6	<i>0.19</i>	100 ± 0.0	0.75 ± 0.04	0.75 ± 0.04
6.3	0.32	93.3 ± 11.6	0.66 ± 0.19	0.70 ± 0.13
25	<i>1.00</i>	96.7 ± 5.8	0.69 ± 0.03	0.71 ± 0.01
100	6.23	86.7 ± 15.3	0.35 ± 0.13	0.40 ± 0.08
Test endpoint (mg diesel /L) *				
LC50 (95% CL)		>6.28	--	--
IC25 (95% CL)		--	2.6 (N/A – 5.4)	3.1 (0.4– 4.9)
IC50 (95% CL)		--	>6.2	>6.2
NOEC		6.28	1.0	0.65
LOEC		>6.28	6.28	1.30

SD = Standard Deviation, LC = Lethal Concentration, IC = Inhibition Concentration, CL = Confidence Limits, N/A = Not Available, NOEC = No Observed Effect Concentration, LOEC = Lowest Observed Effect Concentration, DL = Detection Limit (0.15 mg Diesel/L)

* = result was calculated using the laboratory control as the negative control

Calculated concentrations are represented in italics.

Table 15. Results: *C. dubia* survival and reproduction test (Range-finder).

Weathered Diesel Concentrations		Survival (%)	Reproduction (Mean ± SD)
Nominal (% v/v)	Mean Measured (mg diesel/L)		
Laboratory control	0.19	100	18.2 ± 0.8
MW-11 (Site Control)	0.26	100	6.4 ± 1.3
0.1	0.15	100	20.6 ± 1.3
0.39	0.16	100	17.2 ± 5.8
1.6	0.19	100	20.0 ± 1.9
6.25	0.30	100	19.6 ± 1.7
25	0.89	100	19.6 ± 1.8
100	5.53	100	22.0 ± 2.2
Test endpoint (mg diesel /L) *			
	LC50	>5.53	--
	IC25 (95% CL)	--	>5.53
	IC50 (95% CL)	--	>5.53
	NOEC	5.53	5.53
	LOEC	>5.53	>5.53

SD = Standard Deviation, DL = Detection Limit (0.16 mg Diesel/L)

(*) = Result was calculated using nominal values and the laboratory control as the negative control

3.2 Definitive Toxicity Test Results

The results of the definitive toxicity tests are summarized in Tables 15 to 18. All endpoints were calculated in CETIS using the site control as the negative control. Confidence limits (95%) are shown in brackets.

The results of the 7-day Topsmelt test are shown in 15. The LC50 estimate for survival was >3.14 mg weathered diesel/L and the IC25 estimate for biomass and dry weight was 2.0 (1.4 – 2.6) and 2.4 (1.7 – 3.0) mg weathered diesel/L, respectively. The resulting LOEC estimates for survival, biomass and dry weight were >3.14, 3.14 and 3.14 mg weathered diesel/L, respectively.

The results of the Echinoderm fertilization test are shown in Table 16. No significant difference was observed in the % fertilization when the highest concentration of the weathered diesel test sample, DW-3 (100% v/v) was compared to the laboratory control.

The results of the 7-day Fathead minnow test are shown in Table 17. The lethal concentration (LC50) estimate for survival was >4.33 mg weathered diesel/L, and the 25% inhibitive concentrations (IC25) estimate for biomass and dry weight were 4.3 (3.8 – 4.6) and 4.8 (N/A – 6.0) mg weathered diesel/L, respectively.

The results of the *C. dubia* survival and reproduction test are shown in Table 19. No significant effects were observed on either survival or reproduction when the highest concentration of the weathered diesel test sample, DW-3 (100% v/v) was compared to the laboratory control.

Table 16. Results: Topsmelt 7-day survival and growth test (Definitive).

Diesel Concentrations		Survival (%) (Mean ± SD)	Biomass (mg) (Mean ± SD)	Dry Weight (mg) (Mean ± SD)
Nominal (% v/v)	Mean Measured (mg diesel/L)			
Laboratory control	0.16	100 ± 0.0	1.83 ± 0.41	1.83 ± 0.41
MW-11 (Site Control)	0.20	100 ± 0.0	2.04 ± 0.26	2.04 ± 0.26
12.5	0.37*	92.0 ± 11.0	1.71 ± 0.33	1.86 ± 0.25
25	0.62	100 ± 0.0	1.93 ± 0.31	1.93 ± 0.31
50	1.29	92.0 ± 11.0	1.65 ± 0.47	1.77 ± 0.32
75	2.12*	92.0 ± 11.0	1.30 ± 0.26	1.42 ± 0.33
100	3.14	72.0 ± 17.9	0.90 ± 0.40	1.21 ± 0.28
Test endpoint (mg diesel/L)				
LC25 (95% CL)		3.0 (2.3 – N/A)	--	--
LC50 (95% CL)		>3.14	--	--
IC25 (95% CL)		--	2.0 (1.4 – 2.6)	2.4 (1.7 – 3.0)
IC50 (95% CL)		--	3.1 (2.4 – 4.0)	>3.14
NOEC		3.14	2.12	2.12
LOEC		>3.14	3.14	3.14

SD = Standard Deviation, LC = Lethal Concentration, IC = Inhibition Concentration, CL = Confidence Limits, NOEC = No Observed Effect Concentration, LOEC = Lowest Observed Effect Concentration, N/A = Not Available, DL = Detection Limit (0.15 mg Diesel/L)

(*) Value interpolated from nominal-measured relationship; results are calculated using the laboratory control as the negative control

Table 17. Results: Echinoderm (*Strongylocentrotus purpuratus*) fertilization test (Definitive).

Sample ID	Analytical (mg diesel/L)	Fertilized eggs (%) (Mean ± SD)
Laboratory control	0.16	78.2 ± 3.5
Salt-adjusted control	-	75.2 ± 6.2
MW-11 (Site control)	0.22	78.2 ± 5.2
DW-3 (100% v/v)	2.71	79.2 ± 4.8

SD = Standard Deviation, DL = Detection Limit (0.15 mg Diesel/L)

Table 18. Results: Fathead minnow 7-day survival and growth test (Definitive).

Diesel Concentrations		Survival (%) (Mean ± SD)	Biomass (mg) (Mean ± SD)	Dry Weight (mg) (Mean ± SD)
Nominal (% v/v)	Mean Measured (mg diesel/L)			
Laboratory control	0.17	100 ± 0.0	0.63 ± 0.03	0.63 ± 0.03
MW-11 (Site Control)	0.21	95.0 ± 5.8	0.62 ± 0.05	0.65 ± 0.03
12.5	0.51*	97.5 ± 5.0	0.59 ± 0.04	0.61 ± 0.02
25	0.89	97.5 ± 5.0	0.58 ± 0.05	0.60 ± 0.03
50	1.90	87.5 ± 12.6	0.60 ± 0.09	0.69 ± 0.05
75	3.04*	95.0 ± 5.8	0.58 ± 0.05	0.61 ± 0.05
100	4.33	82.5 ± 12.6	0.44 ± 0.10	0.54 ± 0.07
Test endpoint (mg diesel/L)				
	LC50 (95% CL)	>4.33	--	--
	IC25 (95% CL)	--	4.3 (3.8 – 4.6)	>4.33
	IC50 (95% CL)	--	>4.33	>4.33
	NOEC	3.04	3.04	3.04
	LOEC	4.33	4.33	4.33

SD = Standard Deviation, LC = Lethal Concentration, IC = Inhibition Concentration, CL = Confidence Limits, N/A = Not Available, NOEC = No Observed Effect Concentration, LOEC = Lowest Observed Effect Concentration, DL = Detection Limit (0.15 mg Diesel/L)

(*) Value interpolated from nominal-measured relationship; results are calculated using the laboratory control as the negative control

Table 19. Results: *C. dubia* survival and reproduction test (Definitive).

Diesel Concentrations		Survival (%)	Reproduction (Mean ± SD)
Sample ID	Mean Measured (mg diesel/L)		
Laboratory Control	0.17	100	18.8 ± 2.4
MW-11 (Site Control)	0.25	100	15.5 ± 3.7
DW-3 (100% v/v)	4.12	100	17.4 ± 4.2

SD = Standard Deviation, DL = Detection Limit (0.15 mg Diesel/L)

4.0 QA/QC

The health history of the test organisms used in the exposures was acceptable and met the requirements of the USEPA protocol. The tests met all control acceptability criteria and water quality parameters remained within ranges specified in the protocols throughout the tests. There were no deviations from the test methodologies, aside from decreased test replicates for the range-finding tests and the sample being outside of holding time for all tests. Subsamples were analyzed each testing period to ensure the diesel in the sample remained stable for the duration of the tests. Uncertainty associated with the test is best described by the standard deviation around the mean and/or the confidence intervals around the point estimates.

Results of the reference toxicant test conducted during the testing program are summarized in Table 19. Results for this test fell within the range for organism performance of the mean and two standard deviations, based on historical results obtained by the laboratory with this test. Thus, the sensitivity of the organisms used in this test was appropriate. The reference toxicant test was performed under the same conditions as those used for the sample.

Table 20. Reference toxicant test results.

Test Species	Endpoint	Historical Mean (2SD Range)	CV (%)	Test Date
<i>A. affinis</i>	Survival (LC50): 100.1 µg/L Cu	91.3 (60.4 – 138.0)	21	February 19, 2019
	Biomass (IC50): 101.1 µg/L Cu	96.0 (65.0 – 141.6)	20	
	Survival (LC50): 104.0 µg/L Cu	95.9 (68.6 – 133.9)	17	April 23, 2019
	Biomass (IC50): 95.1 µg/L Cu	91.3 (66.1 – 126.1)	16	
<i>S. purpuratus</i>	Fertilization (IC50): 16.4 µg/L Cu	18.4 (8.4 – 40.1)	41	February 19, 2019
	Fertilization (IC50): 10.21 µg/L Cu	18.0 (8.9 – 36.5)	36	May 1, 2019
<i>P. promelas</i>	Survival (LC50): 4.0 g/L NaCl	4.7 (3.7 – 6.1)	12	February 19, 2019
	Biomass (IC50): 4.0 g/L NaCl	4.5 (3.4 – 6.1)	15	
	Survival (LC50): 4.2 g/L NaCl	4.7 (3.7 – 6.1)	13	April 23, 2019
	Biomass (IC50): 3.5 g/L NaCl	4.5 (3.4 – 6.1)	15	
<i>C. dubia</i>	Survival (LC50): 2.0 g/L NaCl	2.0 (1.8 – 2.2)	5	March 5, 2019
	Biomass (IC50): 1.8 g/L NaCl	1.5 (1.0 – 2.1)	18	
	Survival (LC50): 2.0 g/L NaCl	2.0 (1.8 – 2.2)	4	May 14, 2019
	Biomass (IC50): 1.2 g/L NaCl	1.6 (1.1 – 2.3)	20	

SD = Standard Deviation, CV = Coefficient of Variation, LC = Lethal Concentration, IC = Inhibition Concentration, EC = Effective Concentration

5.0 REFERENCES

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APPENDIX A – *Atherinops affinis* Toxicity Test Data

APPENDIX B – *Strongylocentrotus purpuratus* Toxicity Test Data

APPENDIX C – *Pimephales promelas* Toxicity Test Data

APPENDIX D – *Ceriodaphnia dubia* Toxicity Test Data

APPENDIX E – Analytical Chemistry

APPENDIX F – Chain-of-Custody Forms

END OF REPORT
