National Oil and Hazardous Substances Pollution Contingency Plan:
3.0 Revised Dispersant Toxicity Tests Report
Menidia beryllina* & *Mysidopsis bahia
FR / Vol. 59, No. 178 / 47461 - 47464

prepared for

Natural Solutions Group Corporation

Services Requested By: Marcos Gonzalez

$VirO_2Syl$. EE USA Project No.: D-006-12

Sample Received: March 6, 2012

Results:	*M. bahia* Survival		*M. beryllina* Survival	
	48-hr LC50	95% Confidence Interval	96-hr LC50	95% Confidence Interval
$VirO_2Syl$	3.95 ppm	2.66 – 5.38 ppm	94.8 ppm	72.4 – 124 ppm
No. 2 Fuel Oil	6.43 ppm	5.68 – 7.28 ppm	40.5 ppm	38.0 – 43.2 ppm
10:1 No. 2 Fuel Oil / $VirO_2Syl$	7.45 ppm	6.43 – 8.77 ppm	10.1 ppm	9.23 – 11.0 ppm
Reference Toxicant Sodium Dodecyl Sulfate (SDS)	8.68 ppm	7.49 – 10.1 ppm	2.33 ppm	2.12 – 2.56 ppm

Report Date: March 26, 2012

by

ENVIRONMENTAL ENTERPRISES USA, INC.

58485 PEARL ACRES ROAD, SUITE D
SLIDELL, LOUISIANA 70461
1-800-966-2788

This report contains ten pages plus twelve appendices, A – L.
This report must not be reproduced in part, only in whole. The results and conclusions presented in this report apply only to the sample(s) tested.
All results included in this report are from valid tests.

Veronica McNew
Effluents Testing Supervisor

03/26/12
DATE

Jennifer Griffith
QA/QC Officer

03-26-12
DATE

David L. Daniel
Laboratory Director

3/26/12
DATE

TABLE OF CONTENTS

***Menidia beryllina* ACUTE, STATIC 96-hr REVISED STANDARD DISPERSANT TOXICITY TEST, FR / Vol. 59, No. 178 / 47461 – 47464.**

TEST OVERVIEW

Four, 96-hr static definitive LC50 tests were conducted by Environmental Enterprises USA, Inc. (EE USA) using a Miscellaneous Oil Spill Control Agent ($VirO_2Syl$ supplied by Natural Solutions Group Corp., Appendix K), No. 2 Fuel Oil (obtained from Resource Technology Corporation, Lab #: D-020-10, Appendix L), a 10:1 mixture of No. 2 Fuel Oil to $VirO_2Syl$, and Sodium Dodecyl Sulfate (SDS) (obtained from Sigma-Aldrich Chemical, Lot #: 019K0085, 92.5 – 100.5% based on total alkyl sulfate content, specific gravity of 0.37 g/ml). *Menidia beryllina* was cultured at EE USA and the test organisms were 7 days old when each test was initiated. Preliminary range-finding tests were set prior to the definitive LC50 tests (Appendix A). Synthetic seawater, hw-MARINEMIX + Bio-Elements and Crystal Sea Marinemix Bioassay Laboratory Formula sea salts (80:20), was used as the laboratory performance control solution and diluent. Each definitive test included three replicates of a laboratory performance control solution and either five or six test concentrations. This document presents methods, materials, and results of this testing. The definitive tests were conducted from March 14 – March 18, 2012, at the laboratory of EE USA.

MATERIALS AND METHODS

Materials and methods for the work performed are stated in FR / Vol. 59, No.178 / 47461 – 47464: Revised Standard Dispersant Toxicity Test. Actual materials and methods are detailed below. The tests were performed with strict adherence to the method as presented in the Federal Register with the following exception(s):

1) during this test, recorded temperatures fell outside the required range by not more than 0.3°C on at least one occasion. This was a minor excursion and did not affect the results of this test.

M. beryllina were cultured and maintained at 24±1°C and 25±1ppt salinity. Several clutches from different females comprised the embryo pool from which the test organism population hatched. Prior to test initiation the test organisms were acclimated to 20±1ppt salinity. Test organisms were fed 200 µl of a standardized suspension of less than 24-hr old *Artemia* nauplii once daily. The standardized suspension is equal to 0.05 grams wet weight strained *Artemia* nauplii per ml synthetic seawater.

On March 6, 2012 $VirO_2Syl$ was received at EE USA from Natural Solutions Group Corp., (Appendix K). A 40-ml glass vial with a Teflon septum was completely filled with this sample and sealed. The remaining sample in the original product container was immediately resealed. A gas-tight syringe was used to prepare stock solutions (SSOLs) as required. The product was stored at ambient laboratory temperatures.

USEPA – API Reference Oil, No. 2 Fuel Oil, was purchased from RTC and was received at EE USA on June 29, 2010 (Appendix L). The No. 2 Fuel Oil arrived in two approximately 600 ml containers that were sealed and filled to approximately 500 ml. Twenty 40-ml glass vials with Teflon septums were completely filled with No. 2 Fuel Oil, sealed, and stored in a dark refrigerator at 0.1 – 6°C. Only one vial of No.2 Fuel Oil was used to complete these tests. A gas-tight syringe was used to deliver aliquots of No. 2 Fuel Oil.

Range-finding Tests:

On March 9, 2012 exploratory range-finding tests were initiated to estimate the LC50s of $VirO_2Syl$, No. 2 Fuel Oil, a 10:1 mixture of No. 2 Fuel Oil to $VirO_2Syl$, and SDS (Appendix A). Test chambers were labeled with test treatment, concentration, organism, replicate identification, and EE USA's project number. Test concentrations were prepared using aliquots of stock solutions (SSOLs) prepared from each neat material as received. Each SSOL and each 1-liter test concentration was prepared and mixed for five minutes using a reciprocal shaker table (EBERBACH 6010, 280 excursions/minute, 3.8 cm stroke, ID# SH1 & A43). One replicate of a concurrent laboratory performance control and at least five dilutions each of $VirO_2Syl$, No. 2 Fuel Oil, a 10:1 mixture of No. 2 Fuel Oil to $VirO_2Syl$, and SDS were prepared. One liter of each test concentration was transferred to replicate test chambers as appropriate, ten *M. beryllina* were randomly loaded, and each test chamber was put into an environmental chamber at 25 +/- 1°C.

LC50s obtained from exploratory range-finding tests were used to select a series of test concentrations for each definitive test ($VirO_2Syl$, No. 2 Fuel Oil, a 10:1 mixture of No. 2 Fuel Oil to $VirO_2Syl$, and SDS) that was expected to bracket the LC50. The SSOL concentration and volumes for each test were prepared following the examples given in the test method.

Definitive Tests:

On March 14, 2012, three replicates of a concurrent laboratory performance control and five dilutions each of $VirO_2Syl$, No. 2 Fuel Oil, a 10:1 mixture of No. 2 Fuel Oil to $VirO_2Syl$, and SDS were prepared and put into an environmental chamber at 25 +/- 1°C. Test chambers were labeled with test treatment, concentration, organism, replicate identification, and EE USA's project number. Test concentrations were prepared using aliquots of SSOLs prepared from each neat material as received. Each SSOL and each 3-liter test concentration was prepared and mixed for five minutes using a reciprocal shaker table (EBERBACH 6010, 280 excursions/minute, 3.8 cm stroke, ID# SH1 & A43). One liter of each test concentration was transferred to three replicate test chambers per treatment as appropriate. Appendices B, C, D, and E contain copies of the raw data recorded for each test.

Appendix #	Toxicant
B	$VirO_2Syl$
C	No. 2 Fuel Oil
D	10:1 No.2 Fuel Oil to $VirO_2Syl$
E	SDS, Standard Reference Toxicant

$VirO_2Syl$:

An exploratory range-finding toxicity test indicated an estimated LC50 of 88.4 ppm $VirO_2Syl$ (Appendix A). The range-finding test resulted in an LC50 which requires a stock solution greater in volume than the 1000 ppm stock solution example given in the test method. The definitive test was prepared with an 1100 ml SSOL at 1,000 ppm: 1.1 ml of $VirO_2Syl$ plus 1098.9 ml synthetic seawater. The SSOL solution was mixed on a reciprocal shaker for five minutes. Test concentrations were prepared using aliquots of the SSOL and synthetic seawater and then mixed on the reciprocal shaker for five minutes (Appendix B, page 1).

No. 2 Fuel Oil:

An exploratory range-finding toxicity test indicated an estimated LC50 of 29.5 ppm No. 2 Fuel Oil (Appendix A). The definitive test was prepared with a 550 ml SSOL at 1000 ppm: 0.55 ml No. 2 Fuel Oil plus 549.45 ml synthetic seawater. The SSOL was mixed on a reciprocal shaker for five minutes. Test concentrations were prepared using aliquots of the SSOL and synthetic seawater and then mixed on the reciprocal shaker for five minutes (Appendix C, page 1).

10:1 No. 2 Fuel Oil / $VirO_2Syl$:

An exploratory range-finding toxicity test indicated an estimated LC50 of 70.7 ppm 10:1 No. 2 Fuel Oil to $VirO_2Syl$ (Appendix A). This LC50 result was greater than 50 ppm, the highest concentration tested. The definitive test concentrations were selected using the estimated LC50 from the range-finding test, and test concentrations were selected at 62.5 and 125 ppm in order to bracket the estimated LC50 from the range-finding test. Six concentrations rather than five were tested to ensure that a definitive LC50 result was obtained. The definitive test was prepared with a stock solution greater in volume than the 1000 ppm stock solution example given in the test method, 1100 ml SSOL at 1000 ppm: 1.0 ml No. 2 Fuel Oil plus 0.1 ml $VirO_2Syl$ plus 1098.90 ml synthetic seawater. The SSOL was mixed on a reciprocal shaker for five minutes. Test concentrations were prepared using aliquots of the SSOL and synthetic seawater and then mixed on the reciprocal shaker for five minutes (Appendix D, page 1).

Standard Reference Toxicant, SDS:

Sensitivity of test organisms to a known toxicant was determined by performing a concurrent Standard Reference Toxicant (SRT) test with SDS. An exploratory range-finding toxicity test indicated an estimated LC50 of 2.30 ppm SDS (Appendix A). The definitive test was prepared with a 500 ml SSOL at 2000 ppm: 1.00 g SDS plus 497.3 ml synthetic seawater to a total volume of 500 ml. The SSOL was mixed on a reciprocal shaker for five minutes. Test concentrations were prepared using aliquots of the SSOL and synthetic seawater and then mixed on the reciprocal shaker for five minutes (Appendix E, page 1).

The initial temperature, dissolved oxygen [DO], and salinity in each treatment was measured and recorded. At the end of each 24-hour exposure period, the ending DO, temperature, salinity, and pH in each treatment was measured and recorded (Appendix #, pages 3 - 4). *M. beryllina* from the same lot of test organisms, lot#: MN-067-12, was used in each test (Appendix #, page 3). The tests were initiated from 1747 to 1934 on March 14, 2012: ten *M. beryllina* larvae were randomly distributed to each test chamber. At 24-hr intervals, the number of survivors in each replicate of each treatment was recorded (Appendix #, page 2). After 96 hours, the final survival data were recorded and these tests were terminated.

Summary of Experimental Conditions	
Test Organisms	7-day-old *Menidia beryllina* larvae
Dilution Water	Synthetic seawater, 20±1 ppt
Temperature	25 +/- 1°C
Photoperiod	16 hours light; 8 hours dark
Test Chambers	Rectangular Pyrex dish, 21cm x 11cm x 7cm
Total Chamber Volume	1.45 liters
Test Solution Volume	1000 ml
Test Solution Renewal	No
Aeration	No, DO levels remained ≥4.0 mg/L

RESULTS AND CONCLUSION

The response used in statistical analysis of survival data was the number of surviving test organisms per concentration. The 96-hr survival data were used to estimate the 96-hr LC50: a point estimate of the concentration expected to result in 50% mortality to exposed *M. beryllina* larvae after 96 hours of exposure. Survival in the concurrent laboratory performance control was 100.0%.

$VirO_2Syl$:

Definitive test concentrations tested were 6.3, 12.5, 25, 50, and 100 ppm $VirO_2Syl$. The 96-hr LC50 was 94.8 ppm with a 95% confidence interval of 72.4 to 124 ppm as determined by the Trimmed Spearman-Karber method (Appendix B, page 5a).

No. 2 Fuel Oil:

Definitive test concentrations tested were 3.8, 7.5, 15.0, 30.0, and 60.0 ppm No. 2 Fuel Oil. The 96-hr LC50 was 40.5 ppm with a 95% confidence interval of 38.0 to 43.2 ppm as determined by the Trimmed Spearman-Karber method (Appendix C, page 5a).

10:1 No. 2 Fuel Oil / $VirO_2Syl$:

Definitive test concentrations were 3.9, 7.8, 15.6, 31.3, 62.5 and 125.0 ppm 10:1 No. 2 Fuel Oil to $VirO_2Syl$. The 96-hr LC50 was 10.1 ppm with a 95% confidence interval of 9.23 to 11.0 ppm as determined by the Trimmed Spearman-Karber method (Appendix D, page 5a).

Standard Reference Toxicant, SDS:

Definitive test concentrations were 1.0, 1.7, 2.9, 4.8, and 8.0 ppm SDS. The 96-hr LC50 was 2.33 ppm with a 95% confidence interval of 2.12 to 2.56 ppm as determined by the Trimmed Spearman-Karber method (Appendix E, page 5a).

***Mysidopsis bahia* ACUTE, STATIC 48-hr REVISED STANDARD DISPERSANT TOXICITY TEST, FR / Vol. 59, No. 178 / 47461 – 47464.**

TEST OVERVIEW

Four, 48-hr static definitive LC50 tests were conducted by Environmental Enterprises USA, Inc. (EE USA) using a Miscellaneous Oil Spill Control Agent ($VirO_2Syl$ supplied by Natural Solutions Group Corp., Appendix K), No. 2 Fuel Oil (obtained from Resource Technology Corporation, Lab #: D-020-10, Appendix L), a 10:1 mixture of No. 2 Fuel Oil to $VirO_2Syl$, and Sodium Dodecyl Sulfate (SDS) (obtained from Sigma-Aldrich Chemical, Lot #: 019K0085, 92.5 – 100.5% based on total alkyl sulfate content, density of 0.37 g/ml). *Mysidopsis bahia* was cultured at EE USA and the test organisms were 5 days old when each test was initiated. Preliminary range-finding tests were set prior to the definitive LC50 tests (Appendix F). Synthetic seawater, hw-MARINEMIX + Bio-Elements and Crystal Sea Marinemix Bioassay Laboratory Formula sea salts (80:20), was used as the laboratory performance control solution and diluent. Each definitive test included three replicates of a laboratory performance control solution and five test concentrations. This document presents methods, materials, and results of this testing. The definitive tests were conducted from March 13 – March 15, 2012, at the laboratory of EE USA.

MATERIALS AND METHODS

Materials and methods for the work performed are stated in FR / Vol. 59, No.178 / 47461 - 47464: Revised Standard Dispersant Toxicity Test. Actual materials and methods are detailed below. The tests were performed with strict adherence to the method as presented in the Federal Register.

M. bahia were cultured and maintained at 24±1°C and 25±1 ppt salinity. Four days before initiating this test 12- to 24-hr-old mysids were collected from breeding cultures, moved to a holding system, and acclimated to 25±1°C. Prior to test initiation the test organisms were acclimated to 20±1 ppt salinity. Test organisms were fed 200 ul of a standardized suspension of less than 24-hr old *Artemia* nauplii once daily. The standardized suspension is equal to 0.05 grams wet weight strained *Artemia* nauplii per ml synthetic seawater.

On March 6, 2012 $VirO_2Syl$ was received at EE USA from Natural Solutions Group Corp. (Appendix K). A 40-ml glass vial with a Teflon septum was completely filled with this sample and sealed. The remaining sample in the original product container was immediately resealed. A gas-tight syringe was used to prepare stock solutions (SSOLs) as required. The product was stored at ambient laboratory temperatures.

USEPA – API Reference Oil, No. 2 Fuel Oil, was purchased from RTC and was received at EE USA on June 29, 2010 (Appendix L). The No. 2 Fuel Oil arrived in two approximately 600 ml containers that were sealed and filled to approximately 500 ml. Twenty 40-ml glass vials with Teflon septums were completely filled with No. 2 Fuel Oil, sealed, and stored in a dark refrigerator at 0.1 – 6°C. Only one glass vial of No.2 Fuel Oil was used to complete these tests. A gas-tight syringe was used to deliver aliquots of No. 2 Fuel Oil.

Range-finding Tests:

On March 9, 2012 exploratory range-finding tests were initiated to estimate the LC50s of $VirO_2Syl$, No. 2 Fuel Oil, a 10:1 mixture of No. 2 Fuel Oil to $VirO_2Syl$, and SDS (Appendix F). Test chambers were labeled with test treatment, concentration, organism, replicate identification, and EE USA's project number. Test concentrations were prepared using aliquots of stock solutions (SSOLs) prepared from each neat material as received. Each SSOL was prepared and mixed for five seconds using a blender (OSTER, Model #: MG-W00, ID# A40) at approximately 7300 to 8300 rpm. One replicate of a concurrent laboratory performance control and at least five dilutions each of $VirO_2Syl$, No. 2 Fuel Oil, a 10:1 mixture of No. 2 Fuel Oil to $VirO_2Syl$, and SDS were prepared by dispensing aliquots of each SSOL by pipet into the appropriate test chambers and adding 800 ml of dilution water into each test chamber. Ten *M. bahia* and 200 ml of dilution water were randomly distributed to each test chamber to bring the total volume of each test chamber up to 1000 ml. Test chambers were put into an environmental chamber at 25 +/- 1°C.

LC50s obtained from exploratory range-finding tests were used to select a series of test concentrations for each definitive test ($VirO_2Syl$, No. 2 Fuel Oil, a 10:1 mixture of No. 2 Fuel Oil to $VirO_2Syl$, and SDS) that was expected to bracket the LC50. The SSOL concentration and volumes for each test were prepared following the examples given in the test method.

Definitive Tests:

On March 13, 2012, three replicates of a concurrent laboratory performance control and five dilutions of each product were prepared and put into an environmental chamber. Test chambers were labeled with test treatment, concentration, organism, replicate identification, and EE USA's project number. Test concentrations were prepared using aliquots of SSOLs prepared from each neat material as received. Each SSOL was prepared and mixed for five seconds using a blender (OSTER, Model #: MG-W00, ID# A40) at approximately 7300 to 8300 rpm. Aliquots of each SSOL were dispensed directly by pipet into the appropriate test chambers and then 800 ml dilution water were poured into each test chamber. Appendices G, H, I, and J contain copies of the raw data recorded for each test.

Appendix #	Toxicant
G	$VirO_2Syl$
H	No. 2 Fuel Oil
I	10:1 No.2 Fuel Oil to $VirO_2Syl$
J	SDS, Standard Reference Toxicant

$VirO_2Syl$:

An exploratory range-finding toxicity test indicated an estimated LC50 of 6.28 ppm $VirO_2Syl$ (Appendix F). The definitive test was prepared with a 550 ml SSOL at 1,000 ppm: 0.55 ml of $VirO_2Syl$ plus 549.45 ml synthetic seawater. The SSOL was mixed with a blender for five seconds. The test solutions were mixed with aliquots of the SSOL and synthetic seawater (Appendix G, page 1).

No. 2 Fuel Oil:

An exploratory range-finding toxicity test indicated an estimated LC50 of 3.30 ppm No. 2 Fuel Oil (Appendix F). The definitive test was prepared with a 550 ml SSOL at 1000 ppm: 0.55 ml No. 2 Fuel Oil plus 549.45 ml synthetic seawater. The SSOL was mixed with a blender for five seconds. The test solutions were mixed with aliquots of the SSOL and synthetic seawater (Appendix H, page 1).

10:1 No. 2 Fuel / $VirO_2Syl$:

An exploratory range-finding toxicity test indicated an estimated LC50 of 5.12 ppm 10:1 No. 2 Fuel Oil to $VirO_2Syl$ (Appendix F). The definitive test was prepared with a 550 ml SSOL at 1000 ppm: 0.50 ml No. 2 Fuel Oil plus 0.05 ml $VirO_2Syl$ plus 549.45 ml synthetic seawater. The SSOL was mixed with a blender for five seconds. The test solutions were mixed with aliquots of the SSOL and synthetic seawater (Appendix I, page 1).

Standard Reference Toxicant, SDS:

Sensitivity of test organisms to a known toxicant was determined by performing a concurrent Standard Reference Toxicant (SRT) test with SDS. An exploratory range-finding toxicity test indicated an estimated LC50 of 8.21 ppm SDS (Appendix F). The definitive test was prepared with a 500 ml SSOL at 2000 ppm: 1.00 g or 2.7 ml SDS plus 497.3 ml synthetic seawater. The SSOL was mixed with a blender for five seconds. The test solutions were mixed with aliquots of the SSOL and synthetic seawater (Appendix J, page 1).

The initial temperature, dissolved oxygen [DO], and salinity in each treatment was measured and recorded. At the end of each 24-hour exposure period, the ending DO, temperature, salinity, and pH in each treatment was measured and recorded (Appendix #, page 3). *M. bahia* from the same lot of test organisms, lot#: MB-151-12, was used in each test (Appendix #, page 2). The tests were initiated from 1737 to 1855 on March 13, 2012: ten *M. bahia* and 200 ml of dilution water were randomly distributed to each test chamber. The 200 ml of dilution water transferred with the mysids to each test chamber brought the total volume in each up to 1000 ml. At 24-hr intervals, the number of survivors in each replicate of each treatment was recorded (Appendix #, page 2). After 48 hours, the final survival data were recorded and these tests were terminated.

Summary of Experimental Conditions	
Test Organisms	5-day-old *Mysidopsis bahia*
Dilution Water	synthetic seawater, 20±1 ppt
Temperature	25 +/-1°C
Photoperiod	16 hours light; 8 hours dark
Test Chambers	Rectangular Pyrex dish, 21cm x 11cm x 7cm
Total Chamber Volume	1.45 liters
Test Solution Volume	1000 ml
Test Solution Renewal	No
Aeration	No, DO levels remained ≥4.0 mg/L

RESULTS AND CONCLUSION

The response used in statistical analysis of survival data was the number of surviving test organisms per concentration. The 48-hr survival data were used to estimate the 48-hr LC50: a point estimate of the concentration expected to result in 50% mortality to exposed *M. bahia* after 48 hours of exposure. Survival in the concurrent laboratory performance control was 100.0%.

VirO$_2$Syl:

Definitive test concentrations were 1.6, 3.1, 6.3, 12.5, and 25.0 ppm VirO$_2$Syl. The 48-hr LC50 was 3.95 ppm with a 95% confidence interval of 2.66 to 5.38 ppm as determined by the Probit method (Appendix G, page 4a).

No. 2 Fuel Oil:

Definitive test concentrations were 0.8, 1.5, 3.0, 6.0, and 12.0 ppm No. 2 Fuel Oil. The 48-hr LC50 was 6.43 ppm with a 95% confidence interval of 5.68 to 7.28 ppm as determined by the Trimmed Spearman-Karber method (Appendix H, page 4a).

10:1 No. 2 Fuel Oil / VirO$_2$Syl:

Definitive test concentrations were 0.6, 1.3, 2.5, 5.0, and 10.0 ppm 10:1 No. 2 Fuel Oil to VirO$_2$Syl. The 48-hr LC50 was 7.45 ppm with a 95% confidence interval of 6.43 to 8.77 ppm as determined by the Probit method (Appendix I, page 4a).

Standard Reference Toxicant, SDS:

Definitive test concentrations were 0.9, 1.8, 3.5, 7.0 and 14.0 ppm SDS. The 48-hr LC50 was 8.68 ppm with a 95% confidence interval of 7.49 to 10.1 ppm as determined by the Probit method (Appendix J, page 4a).

REFERENCES

EE USA. May 2011. Quality Assurance Plan. EE USA, Slidell, LA 70461.

EE USA. March 2011. Standard Operating Procedures. EE USA, Slidell, LA 70461.

Federal Register. Thursday, September 15, 1994. Part II, Environmental Protection Agency, 40 CFR Parts 9 and 200 National Oil and Hazardous Substances Pollution Contingency Plan; Final Rule, "3.0 Revised standard dispersant toxicity test." FR / Vol. 59, No. 178 / 47461 - 47464.

Tidepool Scientific Software. 2007. ToxCalc™ Toxicity Data Analysis Software. Version 5.0.32. McKinleyville, CA.

U.S. Environmental Protection Agency. October 2002. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. EPA-821-R-02-012. 5th Edition. Office of Water (4303T). Washington, DC 20460.

Environmental Enterprises USA, Inc.

APPENDIX A