ExxonMobil BIOMEDICAL SCIENCES, INC.

EMBSI 2010-104821

Daphnia magna, Acute Immobilisation Test on Water Accommodated Fractions of a Light Catalytic Cracked Gas Oil

Final Report

Study Number: 1057642

TEST SUBSTANCE:

Light Catalytic Cracked Gas Oil
CAS No. 64741-59-9
(MRD-10-576)

PERFORMED FOR:

American Petroleum Institute
1220 L Street, NW
Washington, DC 20005-4070

PERFORMED AT:

ExxonMobil Biomedical Sciences, Inc.
1545 US Highway 22 East
Annandale, New Jersey 08801-3059

COMPLETION DATE: December 23, 2011

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APPENDICES:

APPROVAL SIGNATURES

[redacted]

23 Dec 11
Date

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1545 US Highway 22 East
Annandale, NJ 08801-3059

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22 Dec 11
Date

Environmental Toxicology & Fate
Laboratory Coordinator

[redacted]

22 Dec, 2011
Date

Section Head, Environmental Sciences

The final report was accepted by the Sponsor

[redacted]

20 Dec. 2011
Date

Sponsor Representative
American Petroleum Institute
1220 L Street, NW
Washington, DC 20005-4070

GLP COMPLIANCE STATEMENT

I hereby accept responsibility for the validity of these data and declare that to the best of my knowledge the study contained herein was performed under my supervision in compliance with the OECD Principles of Good Laboratory Practice, C(97) 186/Final, 1997 and the United States Environmental Protection Agency (USEPA) Toxic Substances Control Act, Good Laboratory Practice Standards, 40 CFR Part 792, 1989 with the exceptions listed below.

Contaminant analysis of the water was not performed in a GLP compliant manner. Accutest® laboratory is accredited by the National Environmental Laboratory Accreditation Conference (NELAC). The analyses are performed using standard US EPA methods. Accutest® has been audited by ExxonMobil Biomedical Sciences, Inc. using the ExxonMobil Quality Practices and Guidelines (QP & G v. 5.3).

The sponsor-supplied test substance analyses conducted by Intertek were not performed in a GLP compliant manner. These analyses were not conducted as part of the testing facility's protocol for this study.

As per the protocol, the range-finding and reference toxicant portions of the testing were not conducted in compliance with GLP regulations.

The check of the diurnal light range was not documented.

None of the above exceptions are believed to have an adverse effect on the study results.

[redacted]

23Dec11
Date

Study Director
ExxonMobil Biomedical Sciences, Inc.
1545 US Highway 22 East
Annandale, New Jersey 08801-3059

[redacted]

20 Dec 2011
Date

Sponsor Representative
American Petroleum Institute
1220 L Street, NW
Washington, DC 20005-4070

QUALITY ASSURANCE STATEMENT

STUDY NUMBER: 1057642

TEST SUBSTANCE: MRD-10-576

STUDY SPONSOR: American Petroleum Institute

Listed below are the inspections performed by the Quality Assurance Unit of ExxonMobil Biomedical Sciences, Inc., the date(s) of inspection, and the date(s) findings were reported to the Study Director and Management.

Study Phase Inspected	Date(s) of Inspection	Reported to Study Director	Reported to Management
Protocol	August 6, 2010	August 6, 2010	October 15, 2010 November 2, 2010
24-Hour Daphnid Observations	November 10, 2010	November 10, 2010	December 03, 2010 December13, 2010
First review of Final Report & Raw Data	April 13, 15 & April 22, 2011	April 22, 2011	November 03, 2011 November 08, 2011
Second Review of Final Report & Raw Data	October 28 & November 01, 2011	November 01, 2011	November 15, 2011 November 16, 2011
Third Review of Final Report: Appendix A only	December 06, 2011	December 06, 2011	December 16, 2011

The final report accurately reflects the methods, procedures and observations documented in the raw data.

[redacted]
Quality Assurance Unit Coordinator

19 Dec. 2011
Date

PERSONNEL

Study Director:	[redacted]
Sponsor Representative:	[redacted]
Section Head, Environmental Sciences: (until July 1, 2011)	[redacted]
Section Head, Environmental Sciences: (effective July 1, 2011)	[redacted]
Environmental Toxicology & Fate Laboratory Coordinator: (until January 1, 2011)	[redacted]
Environmental Toxicology & Fate Laboratory Coordinator: (effective January 1, 2011)	[redacted]
Environmental Chemistry Laboratory Coordinator; Principal Investigator for Characterization & Analysis of Test Solutions:	[redacted]
Quality Assurance Unit Coordinator:	[redacted]

All personnel involved in the conduct of this study, except the sponsor, are/were located at the testing facility's address. The Sponsor Representative is located at the previously cited address.

SUMMARY

This study was conducted for the Sponsor to evaluate the acute toxicity of the water accommodated fractions (WAFs) of light catalytic cracked gas oil (CAS No. 64741-59-9) to the daphnid, *Daphnia magna*. This study was performed as a 48-hour static test.

Individual treatments were prepared by adding the appropriate amount of test substance to dilution water in glass aspirator bottles and stirring on magnetic stir plates with a vortex of approximately 10% of the static liquid depth for approximately 24 hours. Approximately one hour after stirring termination, the aqueous portion of each WAF solution was removed for testing. The loading rates tested were 0 (control), 0.102, 0.256, 0.640, 1.60, and 4.00 mg/L.

Four replicate test chambers were prepared for each test substance loading rate. Each replicate test chamber contained five daphnids. Replicate chambers were 130-mL glass bottles containing approximately 130 mL of solution (no headspace) closed with PTFE-lined screw top caps. Water quality (temperature, pH, and dissolved oxygen) measurements were recorded for each batch WAF solution at the start of the test and in a composite of the replicates at termination. Water quality parameters were within acceptable limits throughout the testing period. Observations for immobilization and abnormal behavior or appearance were performed at 24 and 48 hours ± 1 hour after the beginning of the test.

Concentrations of the test substance hydrocarbon components were quantified against gas oil standards, prepared in acetone, spiked directly into water for automated static headspace gas chromatography with flame ionization detection (HS GC-FID) analysis. The total peak area for eluted hydrocarbon components from WAF headspace analysis were summed for quantification. The distribution and percentage of gas oil components measured in the WAFs differed from the parent gas oil standards owing to the differing solubilities of individual gas oil hydrocarbons. Therefore, measured concentrations do not represent all hydrocarbons constituting the test substance. Due to the complex nature of the test substance, no attempt was made to identify and quantify specific hydrocarbons solubilized in the WAFs. The mean measured hydrocarbon concentrations were ND (Not Detected; control), 0.084, 0.22, 0.58, 1.4, and 3.2 mg/L. All test solutions retained ≥80% of the initial measured hydrocarbon concentrations after the 48 hour exposure period.

Acute toxicity results are expressed as percent immobilization. The 50% Effect Loading (EL50) is the calculated loading rate of the test substance which would cause 50% immobilization in a population of test organisms over a specified exposure period. Results expressed as the 50% Effect Concentration (EC50) represent the concentration of hydrocarbons that solubilized from the test substance into each WAF at its respective loading rate.

Based on loading rate, the 48-hour EL50 value was calculated to be 0.51 mg/L with 95% confidence limits of 0.43 to 0.61 mg/L.

Based on mean measured hydrocarbon concentrations, the 48-hour EC50 value was calculated to be 0.45 mg/L with 95% confidence limits of 0.38 to 0.54 mg/L.

INTRODUCTION

Objective

This study was conducted for the Sponsor to evaluate the acute toxicity of the water-accommodated fractions (WAFs) of light catalytic cracked gas oil (CAS No. 64741-59-9) to the daphnid, *Daphnia magna*, in a 48-hour static test.

Sponsor

American Petroleum Institute
1220 L Street, NW
Washington, DC 20005-4070

Testing Facility

ExxonMobil Biomedical Sciences, Inc.
1545 US Highway 22 East
Annandale, New Jersey 08801-3059

Initial Characterization

12 July 2010

Study Initiation

13 September 2010

WAF Equilibration and Stability Trial Start (Mixing)

13 September 2010

Range-Finding Test Start (Mixing)

25 October 2010

Experimental Start (Definitive Study)

08 November 2010 (Mixing Initiation)
09 November 2010 (Test Initiation)

Experimental Termination (Definitive Study)

06 May 2011 (Batch Water Quality)

INTRODUCTION (CONT'D)

Final Characterization

26 July 2011

Compliance

The study was conducted in compliance with OECD[1] and USEPA[2] Good Laboratory Practice (GLP) standards with the exceptions outlined on page 5 and was performed in agreement with the OECD[3] and USEPA[4, 5] guidelines with the exceptions listed on page 18.

MATERIALS and METHODS

Test Substance Identification

EMBSI Identification:	MRD-10-576
Sponsor Identification:	Light catalytic cracked gas oil Distillates (Petroleum)
CAS Number	64741-59-9
Supplier:	EPL Archives, Sterling. VA
Date Received:	24 June 2010
Expiration Date:	June 2015

CAS Definition: Distillates (petroleum) light catalytic cracked. A complex combination of hydrocarbons produced by the distillation of products from a catalytic cracking process. It consists of hydrocarbons having carbon numbers predominantly in the range of C9 through C25 and boiling in the range of approximately 150 degrees C to 400 degrees C (302 degrees F to 752 degrees F). It contains a relatively large proportion of bicyclic aromatic hydrocarbons[6].

Additional test substance information supplied by the Sponsor is attached in Appendix G.

Storage Conditions: The neat test substance was stored at room temperature.

Sample Retention

A non-study specific sample of the neat test substance has been retained in the testing facility archives.

Justification of Dosing Route

Potential environmental exposure is by the test substance in water.

MATERIALS and METHODS (CONT'D)

Dilution Water

Reconstituted water[7] (Batch #219A) was prepared with UV-sterilized, deionized well water and reagent grade salts ($NaHCO_3$, $CaSO_4$, $MgSO_4$, and KCl), and was aerated prior to use. The reconstituted water contains Ca/Mg and Na/K ratios of 1.2:1 and Na/K 12.5:1, respectively. UV-sterilized, deionized well water is distributed throughtout the testing facility via PVC and stainless steel pipes. See Appendix D for the dilution water analysis.

Contaminants

There are no known contaminants in the feed or dilution water believed to be at levels high enough to interfere with this study. The algae and VitaChem are not analyzed. The algae is concentrated via settling and removing supernatant. If necessary, the algae is diluted using dilution water to achieve desired algae cell density. The deionized water is monitored annually for priority pollutants, un-ionized ammonia, total suspended solids, and for bacterial properties by Accutest®, 2235 Route 130, Dayton, NJ 08810. Contaminant analyses are not performed in a GLP compliant manner. This is not believed to affect the results of the analyses. Contaminant analysis results are maintained at the testing facility and are available upon request.

Characterization of the Test Substance

The neat test substance was characterized and the stability determined by the testing facility both prior to and after the study using the following analyses: Ultraviolet/Visible and Infrared Spectrophotometry, density, physical–state, miscibility in water, methanol and /or hexane and a GC-MS "fingerprint" of the neat test substance. The GC-MS Total Ion Chromatogram (“fingerprint”) is run against an ASTM hydrocarbon standard mixture. The ASTM D2887 standard is applied for higher boiling mixtures with compounds eluting between approximately n-octane (n-C8) and n-triacontane (n-C30). Due to the complex nature of the test substance, no reporting of specific hydrocarbon components was made. Instead, an area percent report was generated for both the pre- and post-test analysis to demonstrate stability of the test substance over the testing period. Documentation of characterization and stability assessment is maintained at the testing facility. The test substance was considered stable over the course of the testing period based on the set of analyses presented in Appendix F. The methods of synthesis, fabrication, and/or derivation of the test substance are maintained by the sponsor. The test substance, as received, was considered the "pure" substance for dosing purposes.

MATERIALS and METHODS (CONT'D)

Analysis of Test Solutions

Samples were taken from each water-accommodated fraction (WAF) and control solution on Day 0 and Day 2 (composite of replicates). The samples were taken with no headspace in 40 mL VOA vials and refrigerated pending analysis. The method of analysis was automated static headspace gas chromatography with flame ionization detection (HS GC-FID). Analysis was performed on a Perkin Elmer Autosystem XL gas chromatograph. Each concentration measurement represents the concentration of hydrocarbons in mg/L that solubilized from the test substance into each WAF at its respective loading rate. Concentrations of the test substance hydrocarbon components were quantified against gas oil standards, prepared in acetone, spiked directly into water for HS GC-FID analysis. The total peak area for eluted hydrocarbon components from WAF headspace analysis were summed for quantification. This ensured that the full range of constituent hydrocarbons that could potentially solubilize into the WAF solutions were captured and quantitated. The distribution and percentage of gas oil components measured in the WAFs differed from the parent gas oil standards owing to the differing solubilities of individual gas oil hydrocarbons. Due to the complex nature of the test substance, no attempt was made to identify and quantify specific hydrocarbons solubilized in the WAFs. The analytical method is presented in Appendix A.

Test System

Daphnia magna Straus

Justification for Selection of Test System

Daphnia magna has been used in safety evaluations and is a common test species for freshwater toxicity studies.

Supplier

Cultured at the test facility. Original culture supplied by Aquatic Biosystems, Inc., Fort Collins, Colorado. Starter culture received on 11-Apr-02.

Husbandry and Acclimation

Eight to ten daphnids are kept in 1-liter glass culture beakers with approximately 800 mL of reconstituted water (study dilution water). The culture chamber is maintained at 20 ± 2°C under a 16 hour light 8 hour dark photoperiod (10 - 20 foot/candles, 108 to 215 Lux). Two sets of Day 0 cultures are started at least five days a week. The neonates are less than 24 hours old and come from a day 12-18 culture which experienced less than an estimated 10% neonate mortality and less than or equal to 20% adult mortality.

MATERIALS and METHODS (CONT'D)

Test System (cont'd)

Husbandry and Acclimation (cont'd)

Cultures of *Daphnia magna* are fed *Pseudokirchneriella subcapitata* (approximately 4.5 - 6.0 x 10^5 cells/mL). They are also fed 25µL/L of Vita chem Fresh formula mixed on a magnetic stir plate with the reconstituted water prior to feeding with algae. The culture is fed every other day, or more frequently as needed, based on observed algal clearing. The algae feed is supplied by Aquatic Biosystems, Inc., Fort Collins, CO. The Vita chem is manufactured by Boyd Enterprises, Inc. and supplied by Foster and Smith Aquatics, Rhinelander, Wisconsin. Cultures are transferred every other day, with exceptions on holidays or weekends when staff is not present. The brood stock health is evaluated and any mortality, production of males or ephippia is documented as well as any mitigation procedures.

Number and Sex

Number: 120; Sex: Not applicable

Age at Initiation of Exposure

<24 hours old, taken from 13-day old parents.

Test System Identification

Each replicate, containing five daphnids, was labeled to show study number, loading level, replicate and randomization number.

Feed

Daphnids were not fed during the study.

Reference Toxicant Study

A reference toxicant study was run prior to the definitive study as a means of assuring that the laboratory test conditions are adequate and that the test organisms are healthy. Potassium chloride was used as the reference toxicant. The following concentrations were used for the study: 0, 375, 750 and 1500 mg/L KCl. Three replicates containing 5 daphnids each were used for the test. The test was conducted for 48 hours. A stock was prepared at 15 g/L KCl and dilutions were made to the concentrations listed above. No analysis of test solutions was conducted. This study was not subject to GLP standards. The 48-hour EC50 value was calculated to be 1061 mg KCl/L with 95% confidence limits of 750 and 1500 mg KCl/L, using the binominal method[8]. Based on literature research, this EC50 value compares to similar KCL effect concentrations (EC50) for *Daphnia magna*.

EXPERIMENTAL PROCEDURE

WAF Equilibration and Stability Trial

A WAF equilibration trial was completed prior to testing to determine the most appropriate mixing duration and to verify the analytical method for analyzing dissolved hydrocarbons. Stability of the WAF solutions also was evaluated over a period of 24 and 48 hours. Results of the equilibration trial indicated that a 24-hour mixing period was sufficient to achieve dissolution of the soluble components in the test substance in the WAF solutions. Following analytical sampling at 48 hours, the WAF solutions were determined to be relatively stable over a 48-hour period. Results of the equilibrium and stability trials are presented in Appendix B.

Range Finding Test

A 48-hour range-finding trial was performed to determine the appropriate nominal loading rate range to achieve an acceptable outcome in the definitive study. WAFs were prepared at nominal loading rates of 0.1, 1.0, 5.0 and 10 mg/L. Results of the range-finding trial are presented in Appendix C.

Definitive Test Design

GROUP	LOADING RATE* (mg/L)	NUMBER OF ORGANISMS
1 (Control)	0 (Control)	20 (5 per 4 replicates)
2	0.102	20
3	0.256	20
4	0.640	20
5	1.60	20
6	4.00	20

* Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution water.

EXPERIMENTAL PROCEDURE (CONT'D)

Preparation and Administration of Test Substance

Individual WAFs were prepared by adding the appropriate amount of test substance to 4.2, 13 or 20 L of laboratory dilution water in equivalent sized glass aspirator bottles. The test substance was added to the aspirator bottles using stainless steel and glass syringes. The loading rate was determined from the volume of test material added and converted to mass per unit volume (mg/L) based on its density. The mixing vessels were sealed with foil-covered rubber stoppers. The mixtures were stirred using a vortex ≤ 10% (of the static liquid depth) for 24±1 hour on magnetic stir plates with Teflon®-coated stir bars at 19.2 – 20.1 °C. Clear test material was noted on the surface of the clear hard recon water at both initiation and termination of mixing.

The mixtures were allowed to settle and equilibrate to test temperature (temperature generally rises a few degrees during the mixing period from heat generated by the stir plate) with ice packs for 50 minutes without stirring before removing the test solutions (aqueous portions of the WAFs) through the outlet at the bottom of the vessel. Four replicates for each treatment group were prepared by completely filling the test chambers with the test solution (no headspace). Four replicates of the control were prepared in the same manner using only laboratory dilution water.

Test Chamber / Organism Loading

The test chambers were 130-mL size glass bottles containing approximately 130 mL of solution (no headspace). The test chambers were closed with PTFE lined caps to minimize contamination, evaporation and/or volatilization.

At test initiation, approximately 26 mL of test solution was supplied to each daphnid.

Selection

Organisms were randomly assigned to intermediate chambers using a computer generated randomization scheme using (SAS 9.1)[9]. Following randomization, the organisms were transferred to their respective test chambers. The test chambers were randomly positioned within the testing location. All the randomization data is included in the raw data.

To ensure that quality organisms were used for the study, neonates were collected from parents that were 13 days old with ≤20% adult mortality. Neonates were selected from a pool of organisms larger than that needed for the study. The pool of neonates had ≤10% daily mortality on the day of test initiation. The study director determined organism suitability.

Exposure Duration

48 hours (± 1 hour)

EXPERIMENTAL PROCEDURE (CONT'D)

Exposure Conditions

The temperature in the environmental chamber ranged from 19.6°C to 19.8°C. Temperatures were taken using a mercury thermometer twice daily.

Diurnal light range: approximately 16 hours light and 8 hours dark. Daylight intensity ranged from approximately 145 - 170 lux during full daylight periods. Light intensity was measured manually using a LI-COR light meter with a photometric sensor.

Experimental Evaluation

Observations for immobilization of the daphnids were made at 24 and 48 hours (± 1 hour). Immobilization is defined as the lack of swimming ability or movement within 15 seconds after gentle agitation of the test container. In addition, observations for normal or abnormal daphnid behavior or appearance were collected.

Water quality measurements (pH, dissolved oxygen and temperature) were performed on a sub-sample of the WAFs from each treatment and control on Day 0 and on a composite of the replicates for each treatment group at termination (termination includes complete immobilization in a treatment). After completion of the study, the test organisms were discarded and monitoring of the environmental conditions was discontinued.

Calculations

Acute toxicity results are expressed as percent immobilization. The 50% Effect Loading (EL50) is the calculated loading rate of the test substance which would cause 50% immobilization in a population of test organisms over a specified exposure period. Measured concentrations do not represent all hydrocarbons constituting the test substance. Results expressed as the 50% Effect Concentration (EC50) represent the concentration of hydrocarbons that solubilized from the test substance into each WAF at its respective loading rate. The distribution and percentage of gas oil components measured in the WAFs differed from the parent gas oil standards owing to the differing solubilities of individual gas oil hydrocarbons. The Trimmed Spearman-Karber Method[10] was used to calculate the EC/EL50 values and their associated 95% confidence limits.

RESULTS AND DISCUSSION

This study met the acceptability criteria for control immobilization and dissolved oxygen concentration. In the control, no *Daphnia* were immobilized or trapped at the surface of the water. Dissolved oxygen remained above 60% (5.4 mg/L) of the air saturation value at the exposure temperature of 20°C.

The loading rates for this study were 0 (control), 0.102, 0.256, 0.640, 1.60, and 4.00 mg/L. The corresponding measured hydrocarbon concentrations at the beginning of the test (Day 0) were ND (Not Detected; control), 0.0872, 0.225, 0.580, 1.49 and 3.54 mg/L. At 48 hours, measured concentrations were ND (Not Detected; control), 0.0809, 0.214, 0.574, 1.33 and 2.83 mg/L. Each concentration measurement represents the concentration of hydrocarbons in mg/L that solubilized from the test substance into each WAF at its respective loading rate. The mean measured hydrocarbon concentrations were ND (Not Detected; control), 0.084, 0.22, 0.58, 1.4, and 3.2 mg/L. All test solutions retained ≥ 80% of the initial measured hydrocarbon concentrations after the 48 hour exposure period. The analytical results are presented in Table 1.

Water quality measurements were consistent throughout the exposure (Table 2). Dissolved oxygen concentrations for all loading rates and control remained above 7.9 mg/L. pH measurements did not differ more than 0.5 for any of the loading rates or control. The test water temperatures ranged from 19.3 to 20.7°C.

Daphnids were observed daily for immobilization, behavior and appearance during the 48 hour exposure. No observation of test substance insolubility (surface slicks, precipitates, and adherence to the test chamber) was noted during the time of organism observations. A summary of percent immobilization for each loading rate and control at 24 and 48 hours is presented in Table 3. No immobilization was observed in the control group throughout the entire exposure. At 48 hours, the percent of immobilization was 0, 0, 75, 100 and 100% for the 0.102, 0.256, 0.640, 1.60, and 4.00 mg/L loading rates, respectively. Daily observations for each replicate are presented in Tables 4 and 5 for 24 and 48 hours, respectively. The dose-response curve is presented in Figure 1.

Based on loading rate, the 48-hour EL50 value was calculated to be 0.51 mg/L with 95% confidence limits of 0.43 to 0.61 mg/L.

Based on mean measured hydrocarbon concentrations, the 48-hour EC50 value was calculated to be 0.45 mg/L with 95% confidence limits of 0.38 to 0.54 mg/L.

PROTOCOL DEVIATIONS

The protocol stated that the test chambers would be closed with ground glass stoppers. The 130mL bottles chosen for the test chambers required PTFE-lined caps for closing.

The protocol stated that the treatments would be prepared in 4L aspirator bottles. Based on the loading levels selected, larger vessels were required.

The actual mixing temperature (measured room temperature = 19.2 – 20.1° C) was lower than the room temperature prescribed in the protocol (22 ± 1° C).

The protocol incorrectly references the 20th edition of *Standard Methods for the Examination of Water and Wastewater*. The 21st edition was used. Water preparation and water quality measurements in each edition are identical.

The protocol prescribed the analysis of 24 and 48 hour stability samples following 24 hours of mixing as well as the preparation of 72 hour mixing samples for stability measurements. It was decided by the Principal Investigator of Characterization and Analysis of Test Solutions that the 48 hour mix would be the longest time frame that would be utilized during the definitive study, so the samples generated from the 48 hour mix were analyzed for 24 and 48 hour stability. Also, as the mixing for the definitive test would not exceed 48 hours, the 72 hour samples were not analyzed for stability.

Temperature and light were not recorded during the Reference Toxicant study.

None of the deviations described above are believed to have any impact on the quality or integrity of the data produced through the course of this study.

SOP DEVIATION

Final signatures were not received from the sponsor prior to initiation of the range-finding test. The sponsor was aware of this deviation and approved the initiation of the test.

The deviation described above is not believed to have any impact on the quality or integrity of the data produced through the course of this study.

GUIDELINE EXCEPTION

Due to the complex nature and relatively limited solubility of the test substances the following exceptions to the guideline apply for this study:

> Consistent with the OECD document on aquatic toxicity testing of complex substances[11], it was deemed more appropriate to prepare individual WAF treatment solutions by adding the test substance to dilution water and removing the WAF of each mixture for testing than to prepare dilutions of a stock solution.

RECORDS

All appropriate materials, methods and experimental measurements required in the protocol were recorded and documented in the raw data. Any changes, additions or revisions to the protocol were approved by the Study Director and the Sponsor Representative. These changes were documented in writing, and included the date, the signatures of the Study Director and the Sponsor Representative and the justification for the change.

The protocol, final report, raw data, a non-study specific retention sample of the test substance, computer-generated listings of raw data and supporting documentation will be maintained in the archives of the testing facility for 10 years, after which time the records will be offered to the sponsor prior to disposal.

REFERENCES

1. OECD Principles of Good Laboratory Practice (GLP), C(97)186 (Final), 1997.

2. United States Environmental Protection Agency (USEPA), Toxic Substances Control Act (TSCA) Good Laboratory Practice Standards, 40 CFR Part 792, 1989.

3. Organization for Economic Cooperation and Development (OECD). Guidelines for Testing of Chemicals. Section 2: Effects on Biotic Systems, Guideline 202: *Daphnia sp*. Acute Immobilisation Test. Adopted 13 April 2004.

4. U.S. Environmental Protection Agency, Ecological Effects Test Guidelines, OPPTS 850.1010: Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids. [Draft] April, 1996.

5. U.S. Environmental Protection Agency, TSCA, 40CFR 797.1300 Daphnid acute toxicity test. September, 1985.

6. API. Petroleum process stream terms included in the chemical substances inventory under the Toxic Substances Control Act (TSCA). American Petroleum Institute, Washington, DC. February, 1985. 40 pp.

7. American Public Health Association, American Water Works Association and Water Environment Federation. 2005. *Standard Methods for the Examination of Water and Wastewater*, 21th ed. American Public Health Association, Washington, D.C. Method 8010E (Table 8010-I).

8. Stephan, C.E., Methods for Calculating an LC50, *Aquatic Toxicology and Hazard Evaluation, ASTM STP 634*, F.L. Mayer and J.L. Hamelink, Eds., American Society for Testing and Materials, 1977, pp. 65-84.

9. SAS Version 9.1, SAS Institute, Inc., Cary, NC. 2002.

10. Hamilton, M., R. Russo, R. Thurston, 1977. Trimmed Spearman-Karber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays. *Environmental Science and Technology*, Vol. 11, No. 7, p.714-719.

11. OECD (2000). Guidance Document on Aquatic Toxicity Testing of Difficult Substances and mixtures. Environmental Health and Safety Publications. Series on Testing and Assessment, no. 23. Organisation for Economic Co-operation and Development, Paris.

Table 1. Analytical Results

Loading Rate* (mg/L)	Measured Hydrocarbon Concentration[1] (mg/L)			Retention[4] (%)
	Day 0[2]	Day 2[3]	Mean	
0 (Control)	ND	ND	--	NA
0.102	0.0872	0.0809	0.084	93
0.256	0.225	0.214	0.22	95
0.640	0.580	0.574	0.58	99
1.60	1.49	1.33	1.4	89
4.00	3.54	2.83	3.2	80

* Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution water.
[1] Duplicate analytical samples from the treatment solutions were analyzed and the two values were averaged.
[2] Samples were collected from each individual WAF on Day 0.
[3] At test termination, samples were collected from composited replicates within each treatment and control.
[4] Percent retention was determined by dividing the concentration of the old solution to the new solution concentration x 100.
ND = Non Detectable
NA = Not Applicable
PQL (Practical Quantitation Limit) = 0.014 mg/L

Table 2. Water Quality Measurements

Loading Rate* (mg/L)	Day 0			Day 2		
	Dissolved oxygen (mg/L)	pH	Temp. (°C)	Dissolved oxygen (mg/L)	pH	Temp. (°C)
Control (0)	8.11	8.01	20.7	8.38	7.91	19.3
0.102	8.38	8.39	20.4	8.39	8.38	19.6
0.256	8.35	8.34	20.2	8.41	8.32	19.5
0.640	8.30	8.43	20.1	8.44	8.89	19.7
1.60	8.51	8.47	20.0	8.39	8.46	19.7
4.00	7.86	8.47	20.7	8.61	8.50	19.6

* Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution water.

Table 3. Percent Immobilization by Loading Rate

Loading rate* (Mean measured concentration**) (mg/L)	% Immobilization 24 hrs	% Immobilization 48 hrs
0 (Control)	0	0
0.102 (0.084)	0	0
0.256 (0.22)	0	0
0.640 (0.58)	0	75
1.60 (1.4)	20	100
4.00 (3.2)	90	100

*Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution water.
** Measured concentration represents the concentration of hydrocarbons that solubilized from the test substance into each WAF at its respective loading rate.

Table 4. 24-Hour Observations

Loading Rate* (mg/L)	**Control**				**0.102**				**0.256**			
Replicate	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Daily Immobilization	0	0	0	0	0	0	0	0	0	0	0	0
Cumulative Immobilization	0	0	0	0	0	0	0	0	0	0	0	0
Lethargic	0	0	0	0	0	0	0	0	0	0	0	0
Normal	5	5	5	5	5	5	5	5	5	5	5	5
Survival	5	5	5	5	5	5	5	5	5	5	5	5
Loading Rate* (mg/L)	**0.640**				**1.60**				**4.00**			
Replicate	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Daily Immobilization	0	0	0	0	0	1	1	2	5	4	4	5
Cumulative Immobilization	0	0	0	0	0	1	1	2	5	4	4	5
Lethargic	2	2	2	2	5	4	4	3	0	1	1	0
Normal	3	3	3	3	0	0	0	0	0	0	0	0
Survival	5	5	5	5	5	4	4	3	0	1	1	0

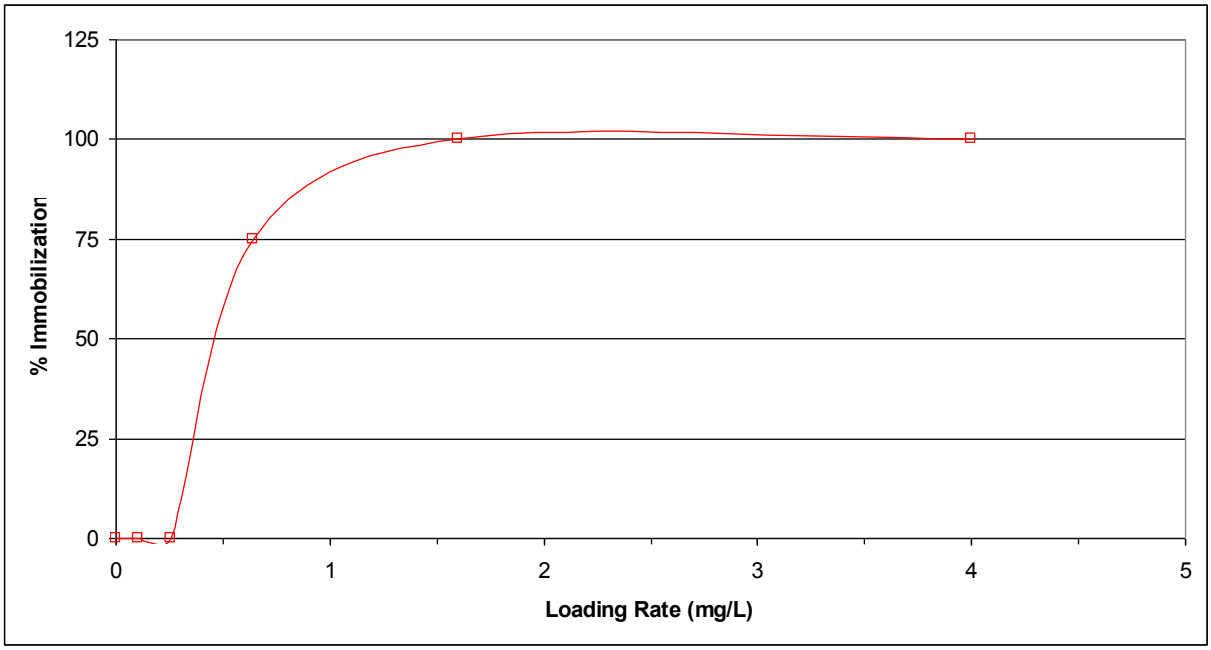
* Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution water.

Table 5. 48-Hour Observations

Loading Rate* (mg/L)	**Control**				**0.102**				**0.256**			
Replicate	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Daily Immobilization	0	0	0	0	0	0	0	0	0	0	0	0
Cumulative Immobilization	0	0	0	0	0	0	0	0	0	0	0	0
Lethargic	0	0	0	0	0	0	0	0	0	0	0	0
Normal	5	5	5	5	5	5	5	5	5	5	5	5
Survival	5	5	5	5	5	5	5	5	5	5	5	5
Loading Rate* (mg/L)	**0.640**				**1.60**				**4.00**			
Replicate	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Daily Immobilization	3	4	3	5	5	4	4	3	-	1	1	-
Cumulative Immobilization	3	4	3	5	5	5	5	5	5	5	5	5
Lethargic	2	1	2	0	0	0	0	0	0	0	0	0
Normal	0	0	0	0	0	0	0	0	0	0	0	0
Survival	2	1	2	0	0	0	0	0	0	0	0	0

* Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution water.

FIGURE 1. Dose-Response Curve



APPENDIX A - ANALYTICAL METHOD and RESULTS

Standards and samples of light catalytic cracked gas oil (CAS No. 64741-59-9) were analyzed by static headspace gas chromatography with flame ionization detection (HS GC-FID). Analysis was performed on a Perkin Elmer Autosystem XL gas chromatograph with a 30 m x 0.53 mm id, 1.5 µm film DB-5 (J&W Scientific) analytical column. The transfer line of a Perkin-Elmer TurboMatrix 40 Trap Headspace Sampler was connected directly to the analytical column. Samples and standards were equilibrated for 45 minutes at 95°C. The needle and transfer line temperatures were both 140°C, the pressurization time was 3 minutes, and the injection time was 0.15 minutes. The sampler head pressure was 28 psi. The FID was 275°C and the oven temperature was held at 50°C for 3 minutes and then ramped up to 270°C at 40°C/minute. The signal attenuation setting was -6.

Microliter aliquots of separate gas oil standard and o-xylene internal standard solutions diluted in acetone were spiked directly into the luer lock port of gas tight syringes containing 10 mL reconstituted water. The syringe contents were transferred to headspace (ca. 20 mL) sample vials containing five grams sodium sulfate. The vials were crimp sealed and shaken to solubilize the sodium sulfate prior to being placed on the headspace sampler for analysis. Gas oil standards in water were analyzed at concentrations of 13.8, 41.5, 115.0 and 345 ng/mL with a constant 27.0 ng/mL concentration of the internal standard. WAF samples were similarly prepared for analysis with 10 mL water sample aliquots transferred to gas tight syringes to which a microliter volume of the o-xylene internal standard solution in acetone was added. The syringe contents were transferred to headspace vials containing five grams sodium sulfate. As with the headspace gas oil standards, WAF sample vials were crimp sealed and shaken to solubilize the sodium sulfate prior to analysis. For higher concentration samples, aliquots of five milliliters or less were sampled in appropriate volume gas tight syringes, the internal standard added and the syringe contents transferred to headspace vials containing sodium sulfate and sufficient diluent water to yield a final volume of 10 mL.

Data were acquired and processed using Perkin Elmer TotalChrom Workstation software (version 6.3.1). Results are presented in Table A1. Standards analysis resulted in a linear response over the standard concentration range. Figure A-1 represents the gas oil standard curve.

Light catalytic cracked gas oil (MRD-10-576) eluted as a complex mixture of hydrocarbons between the approximate retention times of 3.9 and 8.1 minutes. Representative gas oil HS GC-FID chromatograms are presented in Figure A-2. The two upper plots display a low and high concentration gas oil standard. The third plot is a control sample with the fourth and fifth chromatograms from the top representing analysis of low (0.102 mg/L) and high (1.6 mg/L) sample loadings. The total area integrated for the detected hydrocarbons was used for quantification. The o-xylene internal standard eluted at about 3.0 minutes under the analytical conditions utilized. The practical quantitation limit (PQL) was approximately 14 ng/mL (0.014 µg/mL) corresponding to the lowest analyzed standard. All reported concentrations for dissolved hydrocarbons are derived from the use of the standard curve and the internal standard.

APPENDIX A - ANALYTICAL METHOD and RESULTS (CONT'D)

Table A1. Individual Analytical Results

Sample	Day 0 (new)	Day 2 (old)
Control	ND	ND
0.102 mg/L D1	0.0832	0.0819
0.102 mg/L D2	0.0911	0.0799
0.256 mg/L D1	0.233	0.220
0.256 mg/L D2	0.216	0.208
0.640 mg/L D1	0.573	0.613
0.640 mg/L D2	0.586	0.534
1.60 mg/L D1	1.61	1.39
1.60 mg/L D2	1.37	1.27
4.00 mg/L D1	3.39	2.90
4.00 mg/L D2	3.68	2.76

D1 and D2 represent duplicate analyses of a composite of each exposure solution.
ND = Not Detected.
PQL = is 0.014 µg/mL (lowest analytical standard)
Results expressed as µg/mL.

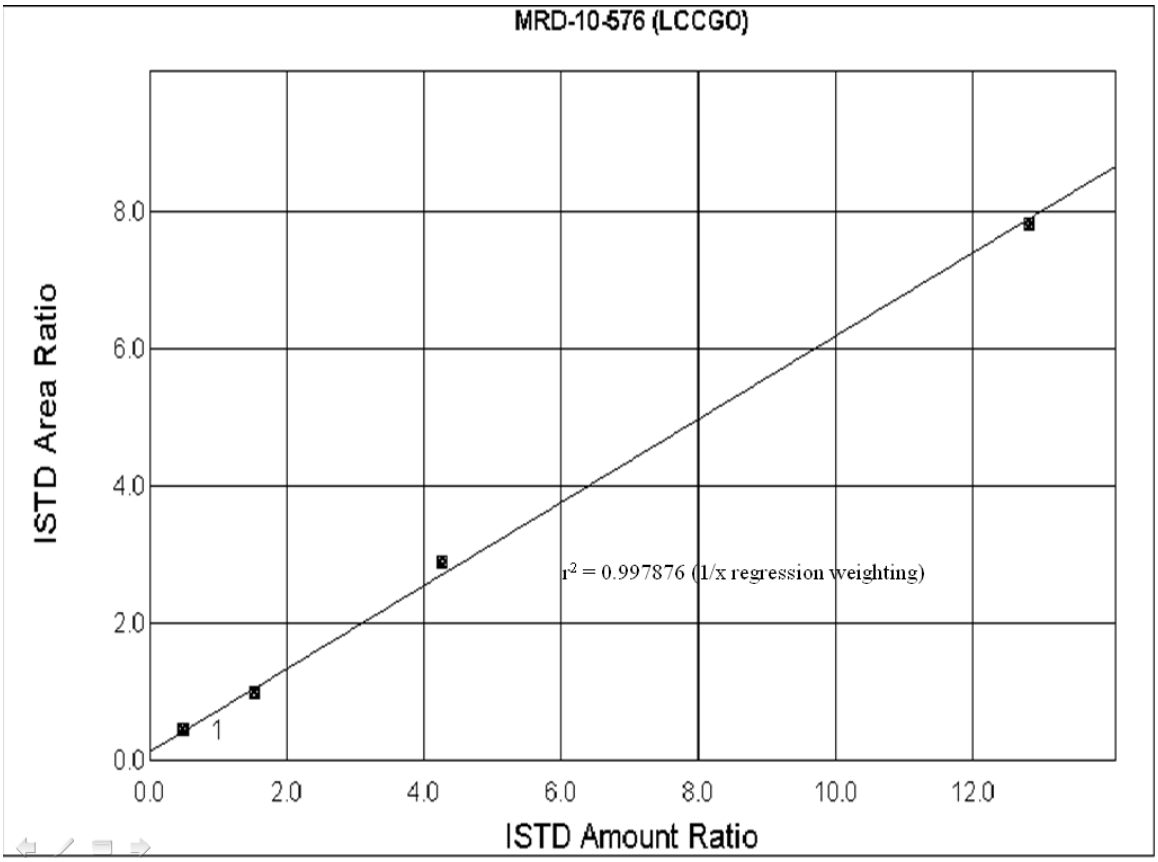
[redacted]
Laboratory Coordinator; Principal Investigator for
Characterization & Analysis of Test Solutions

19 Dec 2011
Date

APPENDIX A - ANALYTICAL METHOD and RESULTS (CONT'D)

FIGURE A-1

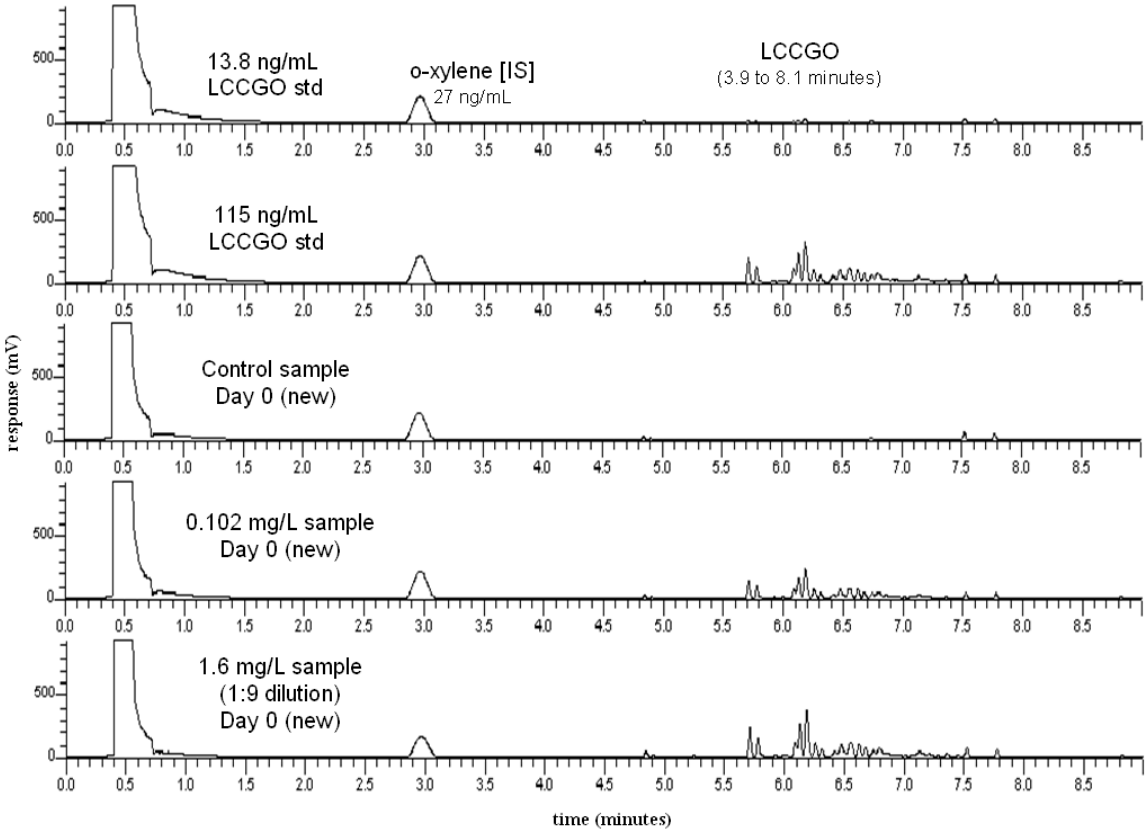
Gas Oil Standard Curve



APPENDIX A - ANALYTICAL METHOD and RESULTS (CONT'D)

FIGURE A-2

Gas Oil Standard and Sample Chromatograms



APPENDIX B - WAF EQUILIBRATION AND STABILITY TRIALS

Introduction

A WAF equilibration trial was performed prior to the range-finding and definitive testing. The purpose of the equilibration trial was to determine the optimum mixing duration to use in WAF preparation. The equilibration trial was also utilized to confirm the analytical method to be used in subsequent testing, and to evaluate the stability of the WAF solutions once they were produced. The stability information was used to establish the renewal interval for a 21-day chronic study, and to determine whether or not a renewal is needed for the definitive acute toxicity test.

Mixtures of dilution water and test substance were prepared at loading levels of 0.1, 0.5, and 5.0 mg/L, in a manner similar to the definitive test. To evaluate equilibration time and WAF stability, WAF samples were collected as described below and analyzed according to the procedures explained in the Analytical Chemistry Methodology section, Appendix A. Sufficient volumes of each WAF were available to assess equilibration time, stability, and any effects of feed (algae) in the WAFs on the stability and chemical analyses.

WAF Equilibration Testing (Assessment of Mixing Duration)

One individual WAF was prepared at each of the three loading levels. At 24, 48 and 72 hours after initiation of mixing, mixing was stopped and the solutions were allowed to settle for one hour. A sample of WAF was removed from each loading level mixture and mixing was resumed at the 24 and 48-hour time points. The concentration of hydrocarbons that had solubilized into the WAF from the test substance was measured following the analytical procedures described in Appendix A. These measurements were used to assess the time required for solubilization of constituent hydrocarbons between the aqueous phase and the un-dissolved fraction of test substance to reach steady-state equilibrium. The equilibration results are shown in Table B1.

Measured concentrations of hydrocarbons in the equilibrated WAFs represent only a portion of the hydrocarbon composition of the test substance due to the very low to negligible aqueous solubility of many of the gas oil components. Evidence of this solubility effect is apparent when comparing measured concentrations of solubilized hydrocarbons to the concentration used to prepare each WAF (i.e., loading). At WAF loadings of 0.1, 0.5 and 5.0 mg/L, measured solubilized hydrocarbon concentrations represent about 59 to 93% of the test substance loading rates at 24 hours.

As shown in Figure B1, the analytical results of the WAF Equilibration Testing indicate that in nearly every case, maximum dissolution of the gas oil was achieved after mixing for 24 hours. Further mixing time did not result in higher concentrations of solubilized hydrocarbons. It was determined that 24 hours would be a sufficient amount of time to mix for WAF generation. A 24-hour mixing duration is also a logistically convenient period for WAF generation when performing renewals.

APPENDIX B - WAF EQUILIBRATION AND STABILITY TRIALS (CONT'D)

Table B1 - WAF Equilibration Results

Loading Rate*	Measured Hydrocarbon Concentration in WAF (mg/L)					
	24 hour mix	% Solubility[2]	48 hour mix	% solubility	72 hour mix	% solubility
0.1 mg/L - 1	0.078	78	0.081	81	0.079	79
0.1 mg/L - 2	[1]	-	0.075	75	0.077	77
mean	0.078	78	0.078	78	0.078	78
0.5 mg/L - 1	0.465	93	0.439	88	0.464	93
0.5 mg/L - 2	0.415	83	0.453	91	0.425	85
mean	0.440	88	0.446	89	0.445	89
5 mg/L - 1	2.96	59	3.21	64	3.00	60
5 mg/L - 2	3.07	61	2.59	52	2.89	58
mean	3.02	60	2.90	58	2.95	59

* Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution water.
[1] Sample error – no result.
[2] Measured solubilized hydrocarbon concentration when compared to the loading rate.

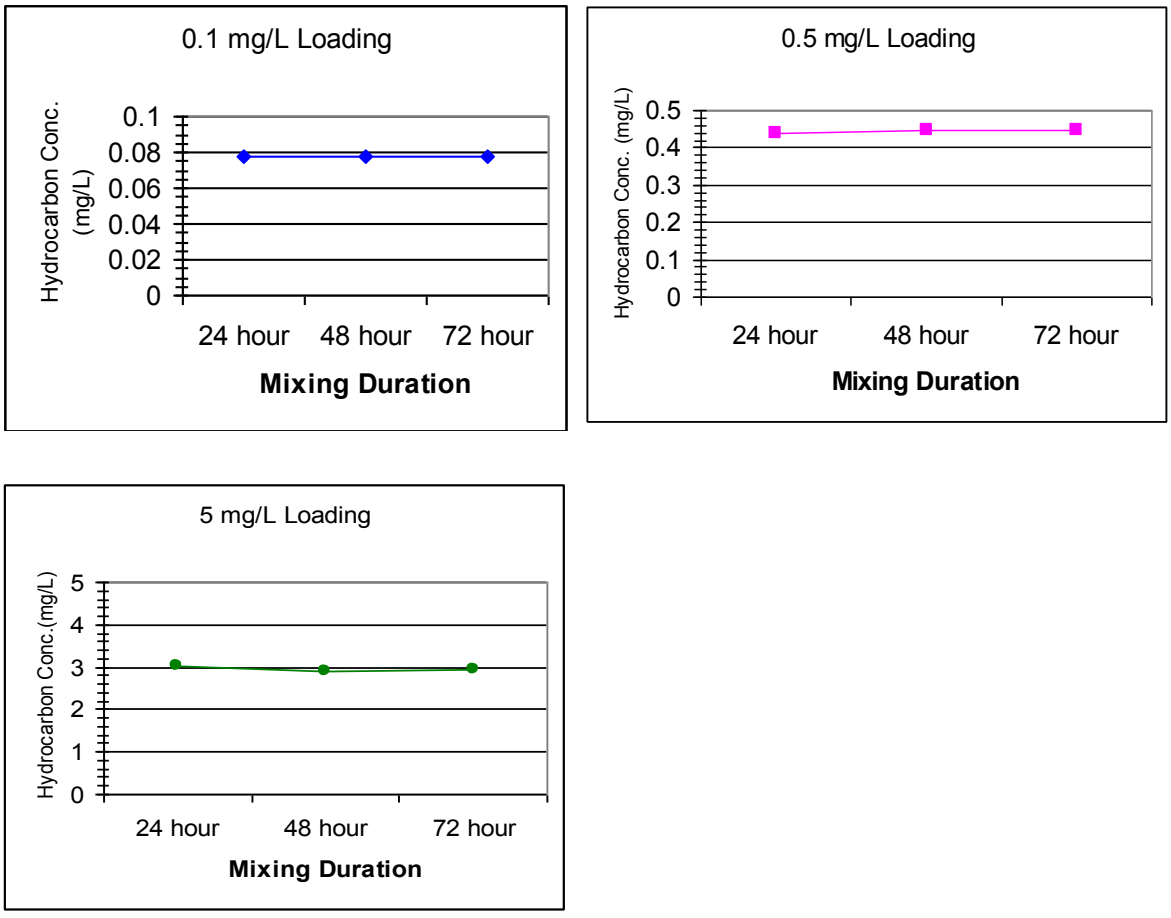


Figure B1. Concentration plots of measured hydrocarbons in WAFs at different mixing times and loading rates.

APPENDIX B - WAF EQUILIBRATION AND STABILITY TRIALS (CONT'D)

Assessment of WAF Stability

For the assessment of WAF stability, samples from the WAFs were collected after mixing for 48 hours. For WAF stability related to an acute exposure, two samples were collected at each loading level directly into screw-top sealed test chambers (130mL, no headspace) identical to those anticipated for use in the definitive acute study.

For WAF stability related to a 21-day chronic exposure, 2 L of the 0.1 and 0.5 mg/L WAF was placed into 2 L volumetric flasks. *Daphnia* chronic test feed (25ul/L Vita chem vitamin solution and 5 mL/L *P. subcapitata*) was added to the volumetric flasks. Following approximately 15 minutes of mixing, samples were taken for 24 hour and 48 hour stability assessments. The samples were placed in screw-top sealed test chambers (no headspace) identical to those anticipated for use in the definitive life cycle study.

All test chambers were set aside under environmental conditions similar to that used for testing. At 24 and again at 48 hours, test chambers were sampled and held under refrigeration pending analysis.

Dedicated samples were employed such that no repeated analysis was made on any sample (i.e., samples were destructively analyzed). The equilibration phase demonstrated good reproducibility between replicate samples; therefore, single samples were used for the stability assessment. The stability assessment results are shown below.

Table B2. WAF Stability Assessment Results

Loading Rate* (mg/L)	Measured Hydrocarbon Concentration (mg/L)				
	Initial[1]	without feed		with feed	
		24 hour stability (retention[2])	48 hour stability (retention)	24 hour stability (retention)	48 hour stability (retention)
0.1	0.078	0.076 (97%)	0.085 (109%)	0.066 (85%)	0.066 (85%)
0.5	0.446	0.472 (106%)	0.444 (100%)	0.355 (80%)	0.376 (84%)
5.0	2.90	2.96 (102%)	3.79 (131%)	not analyzed[3]	

***** Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution water.
[1] 0-hour concentration for stability assessment (48 hour mix).
[2] Percent retention was determined by dividing the concentration of the initial solution to the new solution concentration x 100.
[3] Stability determinations with feed are applicable at lower concentrations related to chronic testing.

Based on the analytical results of the WAF Stability Testing, the sponsor determined that a renewal was not necessary for the 48-hour daphnid acute testing and that a 48-hour renewal period will suffice for the chronic testing.

APPENDIX C - RANGE FINDING TEST

A 48 hour range finding test was performed to determine the loading rates of light catalytic cracked gas oil (CAS No. 64741-59-9) for the definitive *Daphnia magna* Acute Immobilisation Test.

Water-accommodated fractions (WAFs) were prepared at nominal loading rates of 0.1, 1.0, 5.0, and 10 mg/L. A control treatment consisting only of the dilution (hard reconstituted) water also was prepared. WAFs were prepared by adding the appropriate amount of test substance, via stainless steel and glass syringes, to the dilution water in glass aspirator bottles (mixing vessels) containing Teflon® coated stir bars. The mixing vessels were closed with foil covered rubber stoppers and the treatments were stirred using a ≤10% vortex (of the static liquid depth) at room temperature (approximately 22°C) on magnetic stir plates for 24 hours ± 1 hour. At stirring initiation, all treatments appeared clear with clear test substance evident on the surface. After stirring, the treatments appeared clear with clear test substance evident on the surface. The treatments were allowed to settle and equilibrate for 15 minutes in an ice/water bath at ~17° C, followed by 55 minutes at room temperature (~20° C) in the laboratory to reach test temperature.

Four replicate chambers per treatment were tested. Each replicate chamber contained five daphnids. Replicate chambers were 130 mL glass bottles containing approximately 130 mL of solution (no headspace) closed with PTFE lined plastic caps. Water quality (temperature, pH and dissolved oxygen) measurements were recorded per treatment at the start and end of the test. Observations for immobilization and abnormal behavior or appearance were performed daily. Analytical samples were collected from the individual WAFs at the initiation of the test. Composite samples of the "old" solutions from the replicate test chambers were also collected for analysis upon test termination.

A summary of the water quality measurements generated in the course of the range finding study can be found in Table C1. A summary of percent immobilization (48 hours) and mean measured concentrations are presented in Table C2. Based on the analytical results from Day 0 and Day 2 analyses, the mean measured concentrations were determined to be 0.0859, 0.888, 4.01 and 5.62 mg/L. The 48-hour EC50 value was calculated to be 0.28 mg/L with 99% confidence limits of 0.0859 and 0.888 mg/L using the Binomal method[8].

APPENDIX C - RANGE FINDING TRIAL (CONT'D)

Table C1. Summary of Water Quality Measurements

Loading Rate* (mg/L)	pH	Dissolved Oxygen (mg/L)	Temperature (°C)
0 (Control)	7.80-8.04	7.61-7.95	19.8
0.1	8.36-8.46	7.81-8.29	19.8-21.0
1.0	8.36-8.43	7.91-8.21	19.8-20.5
5	8.51-8.52	7.88-8.09	19.6-20.0
10	8.52-8.54	7.94-8.04	19.6-20.6

*Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution water.

Table C2. Percent Immobilization by Loading Rate

Loading Rate* (mg/L)	Mean Measured Concentration** (mg/L)	% Immobilization
0 (Control)	ND	0
0.1	0.0859	0
1.0	0.888	100
5* **	4.01	100
10* **	5.62	100

* Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution water.
** Measured concentration represents the concentration of hydrocarbons that solubilized from the test substance into each WAF at its respective loading rate.
***Complete immobilization occurred after 24 hours.
ND = Not Detectable

APPENDIX D - DILUTION WATER ANALYSIS

The dilution water was prepared from UV-sterilized, deionized well water that was treated and distributed throughout the testing facility via PVC and stainless-steel pipes. Batches of 500 L of this deionized water were reconstituted in the laboratory to meet aquatic toxicity testing needs, following Method 8010E of *Standard Methods for the Examination of Water and Wastewater,* 21st edition.

The following water quality data are most representative of the dilution water used during the in-life period of the study. Table D1 presents analyses performed on the reconstituted water (RW) on a batch basis. Water quality analyses were performed by Environmental Toxicology laboratory personnel. Total Organic Carbon analysis was performed by the laboratory's Environmental Fate Chemistry group. The quality of the dilution water was monitored annually for priority pollutants, un-ionized ammonia, total suspended solids, and annually for bacterial properties. Results of analyses are maintained at the testing facility.

Table D1. RESULTS OF WATER QUALITY ANALYSIS

Sample	Alkalinity as $CaCO_3$ (mg/L)[1]	Hardness as $CaCO_3$ (mg/L)[2]	pH	Temperature (°C)	Dissolved Oxygen (mg/L)	Total Organic Carbon (ppm)[3]
Batch 219A	110	178	8.44	21.2	9.48	0.1267

1 U.S. Environmental Protection Agency. 1979, Revised March 1983. *Methods for Chemical Analysis of Water and Wastes*, EPA-600/4-79-020. Office of Research and Development, Cincinnati, OH. Method 310.1, Alkalinity (Titrimetric, pH 4.5).

2 U.S. Environmental Protection Agency. 1979, Revised March 1983. *Methods for Chemical Analysis of Water and Wastes*, EPA-600/4-79-020. Office of Research and Development, Cincinnati, OH. Method 130.2, Hardness (Titrimetric, EDTA).

3 JIS K-0102: "Industrial Waste Water Testing", JIS K-0551: "Total organic carbon (TOC) testing methods for ultra-pure water", U.S. Pharmacopoceia, EPA 415.1 EPA 9060A, ASTM D2575, Standard Methods for Examination of Water and Waste Water 5301B.

APPENDIX E - STATISTICAL OUTPUT

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

DATE: 11Nov10 TEST NUMBER: 1057642 DURATION: 48 h
TOXICANT : MRD-10-576
SPECIES: D. magna

RAW DATA: ---	Concentration (mg/L)	Number Exposed	Mortalities
----	.00	20	0
	.10	20	0
	.26	20	0
	.64	20	15
	1.60	20	20
	4.00	20	20

SPEARMAN-KARBER TRIM: .00%

SPEARMAN-KARBER ESTIMATES: EC50: .51
95% LOWER CONFIDENCE: .43
95% UPPER CONFIDENCE: .61

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

DATE: 17Nov10 TEST NUMBER: 1057642 DURATION: 48 h
TOXICANT : MRD-10-576
SPECIES: D. magna

RAW DATA: ---	Concentration (mg/L)	Number Exposed	Mortalities
----	.00	20	0
	.08	20	0
	.22	20	0
	.58	20	15
	1.41	20	20
	3.19	20	20

SPEARMAN-KARBER TRIM: .00%

SPEARMAN-KARBER ESTIMATES: EC50: .45
95% LOWER CONFIDENCE: .38
95% UPPER CONFIDENCE: .54

APPENDIX F – TEST SUBSTANCE CHARACTERIZATION

TEST SUBSTANCE CHARACTERIZATION

The light catalytic cracked gas oil (CAS No. 64741-59-9) was initially characterized on July 12, 2010. Analyses included Ultraviolet-Visible (UV-VIS) spectroscopy and Fourier Transform Infrared (FT-IR) spectroscopy, density and Gas chromatography–mass spectrometry (GC-MS) analysis. Stability of the neat test substance was confirmed by repeating these same analyses on July 26, 2011 after completion of this study.

UV-VIS spectra are presented in Figures UV-VIS-1 and UV-VIS-2 representing, the initial and final spectrum at concentrations of 17.8 ppm and 13.5 ppm, respectively. UV-VIS spectra were acquired on a Hewlett-Packard 8453 diode array UV-VIS spectrophotometer using a 1 cm quartz cell, a scan time of 0.5 seconds and resolution of 2 nm.

FT-IR spectra of the neat test substance are presented in Figures FTIR-1 and FTIR-2 representing the initial and final spectra. Initial and final FT-IR spectra were acquired on a Thermo Nicolet Avatar 360 FT-IR spectrometer with a KBr plate. The spectra were obtained with the following settings: resolution of 4 cm^{-1}, gain of 1 and scan number of 32.

The test substance was also characterized by GC-MS using a Hewlett-Packard HP5890 Series II gas chromatograph with 5972 mass selective detector. For comparison of relative retention times to a series of known hydrocarbons under the analytical conditions employed, MRD-10-576 was analyzed against an ASTM D2887 calibration mixture. Figures Total IonChromatogram-1 and Total Ion Chromatogram-2 represent the initial and final GC-MS total ion chromatograms, respectively. The test substance eluted as a complex mixture with numerous chromatographic components between retention times of approximately 17 and 27 minutes. This corresponds to bracketing by standard hydrocarbons n-dodecane (n-C12) and n-eicosane (n-C20) under the analytical conditions employed.

The test substance's initial and final density was measured at 20°C with an Anton Paar DMA 4500 Density/Specific gravity/Concentration meter. The initial density was measured as 0.9576 g/mL@20°C and final density was measured as 0.9578 g/mL@20°C. The test substance was observed to be a liquid under ambient laboratory conditions and immiscible in water and methanol but miscible in hexane.

Comparison of the initial and final analyses appeared to be substantially similar indicating the neat test substance was stable over the duration of the study period.

[redacted] Principal Investigator for Characterization (located at the testing facility)

9 Nov 2011
Date

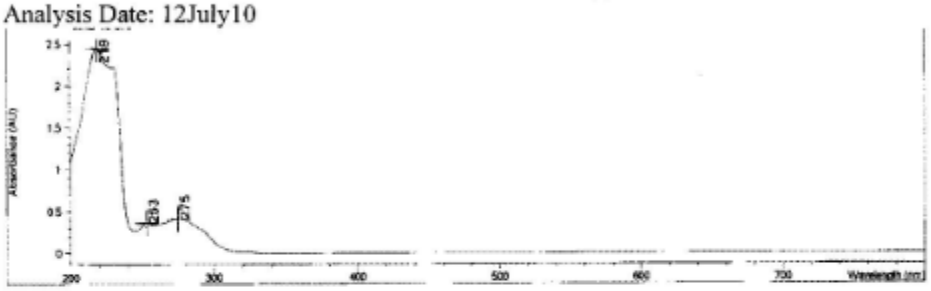
APPENDIX F – TEST SUBSTANCE CHARACTERIZATION (CONT'D)

TEST SUBSTANCE CHARACTERIZATION (CONT'D)

UV-VIS SPECTRA

Figure: UV-VIS-1
Initial

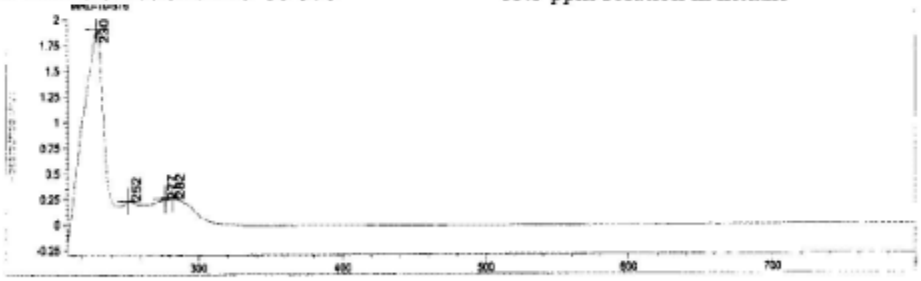
Initial Characterization MRD-10-576 17.8 ppm solution in hexane
Analysis Date: 12July10



Peak 219nm Absorbance = 2.4373
Peak 253nm Absorbance = 0.3603
Peak 275nm Absorbance = 0.408

Figure: UV-VIS-2
Final

Final Characterization MRD-10-576 13.5 ppm solution in hexane



Analysis Date: 26Jul11

Peak 230nm Absorbance = 1.90510
Peak 277nm Absorbance = 0.24967
Peak 252nm Absorbance = 0.22738
Peak 282nm Absorbance = 0.26198

APPENDIX F – TEST SUBSTANCE CHARACTERIZATION (CONT'D)

TEST SUBSTANCE CHARACTERIZATION (CONT'D)

FT-IR SPECTRA

Figure: FTIR-1
Initial

Initial Characterization MRD-10-576 Analysis Date: 12Jul10

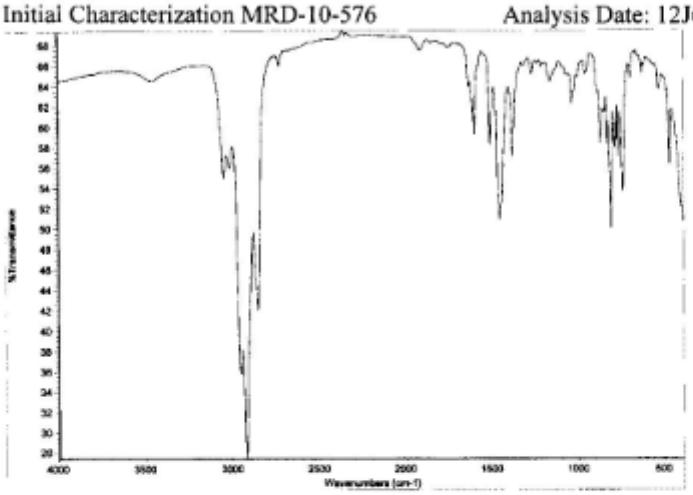
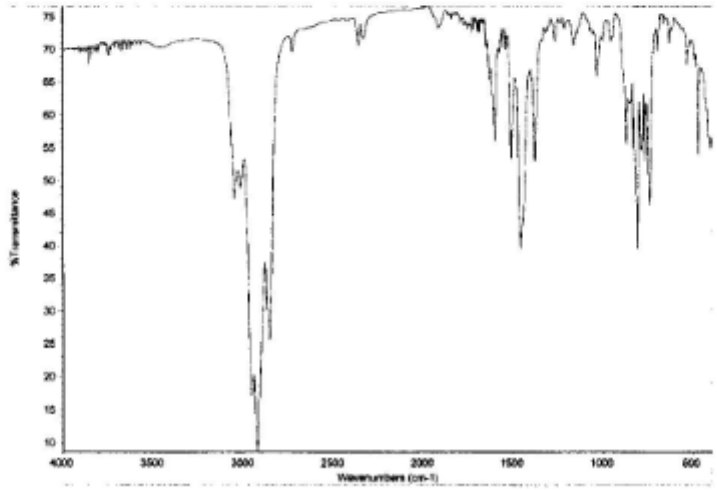


Figure: FTIR-2
Final

Final Characterization MRD-10-576 Analysis Date: 26Jul11



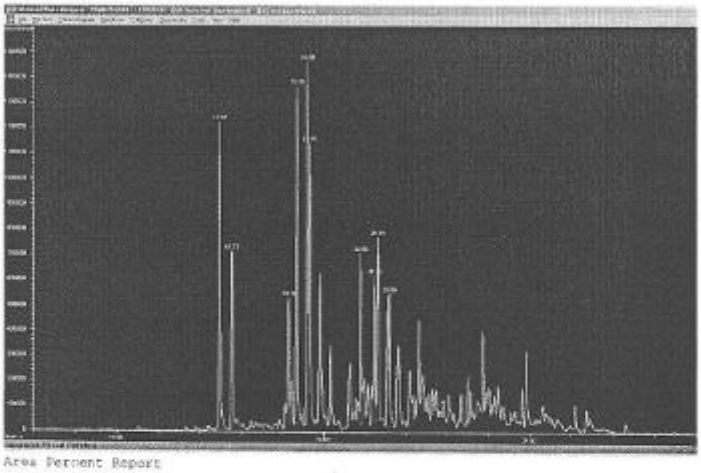
APPENDIX F – TEST SUBSTANCE CHARACTERIZATION (CONT'D)

TEST SUBSTANCE CHARACTERIZATION (CONT'D)

TOTAL ION CHROMATOGRAM

Figure: Total IonChromatogram-1

INITIAL



Area Percent Report

Data File : C:\HPCHEM\1\DATA\CBAR2010\12JUL02.D Vial: 11
Acq On : 12 Jul 2010 23:03 Operator:
Sample : MRD-10-576 (initial characterization) 10 Inst : GC/MS Ins
Misc : distillates(petroleum),light catalytic cr Multiplr: 1.00
Sample Amount: 0.00
MS Integration Params: MRD10576.E

Method : C:\HPCHEM\1\METHODS\CBAR2010.M (Chemstation Integrator)
Title :

Signal : TIC

peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	17.473	1027	1038	1055	BB	1191344	28114933	69.16%	13.215%
2	17.769	1869	1875	1886	BB	696574	15482359	38.08%	7.277%
3	19.147	2039	2047	2052	VV 2	507182	12837217	31.58%	6.034%
4	19.348	2063	2072	2084	PV	1307221	38093107	93.71%	17.908%
5	19.604	2094	2104	2107	BV	1434524	40452172	100.00%	19.109%
6	19.652	2107	2110	2126	VB	1081233	23014351	56.61%	10.818%
7	20.894	2261	2265	2270	VV	656953	14111799	34.71%	6.633%
8	21.230	2303	2307	2313	VV	564030	13289573	32.69%	6.247%
9	21.311	2313	2317	2329	VB	703810	14853731	36.54%	6.982%
10	21.599	2351	2353	2360	VB	516463	12290408	30.23%	5.777%

Sum of corrected areas: 212743651

12JUL02.D CBAR2010.M Wed Jul 14 08:12:47 2010

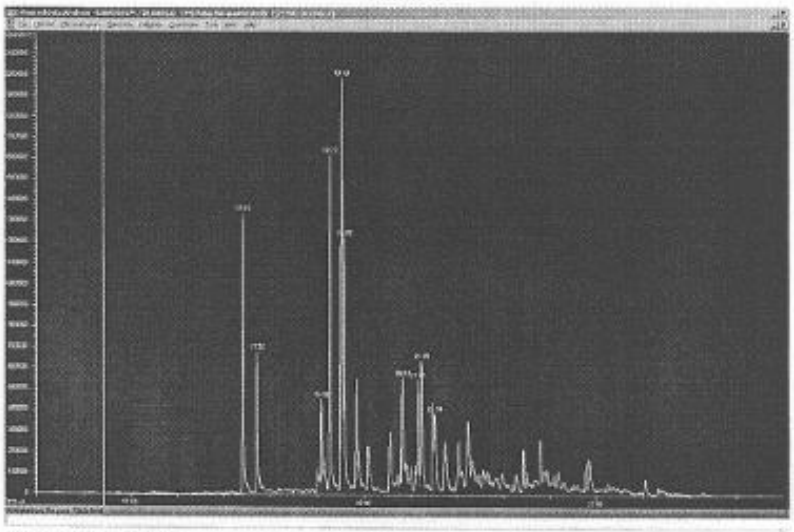
APPENDIX F – TEST SUBSTANCE CHARACTERIZATION (CONT'D)

TEST SUBSTANCE CHARACTERIZATION (CONT'D)

TOTAL ION CHROMATOGRAM

Figure: Total Ion Chromatogram-2

FINAL



Area Percent Report

Data File : C:\HPCHEM\1\DATA\CHAR2010\26JUL02.D Vial: 2
Acq On : 26 Jul 2011 9:52 Operator:
Sample : MRD-10-576(final characterization)106v/v Inst : GC/MS Ins
Misc : distillates(petroleum)light catalytic cr Multiplr: 1.00
Sample Amount: 0.00

MS Integration Params: 576.E

Method : C:\HPCHEM\1\METHODS\CHAR2010.M (Chemstation Integrator)
Title :

Signal : TIC

peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	17.400	2754	2767	2794	BB	1311520	26850418	62.74%	13.748%
2	17.702	2816	2824	2842	BV	661262	13603665	31.79%	6.966%
3	19.084	3079	3085	3092	VV 2	431463	8926702	20.86%	4.571%
4	19.280	3111	3122	3141	PV	1601489	38831581	90.73%	19.883%
5	19.529	3158	3169	3174	BV	1929536	42798102	100.00%	21.914%
6	19.582	3174	3179	3207	VB	1191563	23621237	55.19%	12.095%
7	20.831	3409	3415	3427	PV	476809	8948598	20.91%	4.582%
8	21.170	3472	3479	3487	VV	505752	10273209	24.00%	5.260%
9	21.250	3487	3494	3513	VB	586769	12203185	28.51%	6.248%
10	21.536	3544	3548	3569	VB 3	357407	9243002	21.60%	4.733%

Sum of corrected areas: 195299699

26JUL02.D CHAR2010.M Tue Jul 26 15:14:25 2011

APPENDIX G – SPONSOR SUPPLIED TEST SUBSTANCE INFORMATION

Intertek

Report of Analysis **INTERIM**

[redacted]

Sample ID: 2009-DRPK-000651-012
Drawn By: Client
Sample Designated As: Crude Oil
Representing: Site#5 Sx.#1 (As Received)

Date Taken: 15-January-2009
Date Submitted: 15-January-2009
Date Tested: 26-January-2009

Method	Test	Result	Units
ASTM D4052	Density of Liquids by Digital Density Meter		
	Relative Density @ 60/60°F	0.9618	
	API Gravity @ 60°F	15.6	°API
ASTM D2887	Boiling Range Distribution of Petroleum Fractions by GC (Simulated Distillation)		
	Boiling Point Distribution	See Attached Report	
ASTM D1319	Hydrocarbon Types (Aromatics, Olefins, Saturates) by FIA		
	Aromatics	75.3	Vol %
	Olefins	7.2	Vol %
	Saturates	17.5	Vol %
ASTM D5186	Determination of Aromatic Content and Polynuclear Aromatic Content of Diesel Fuels and Aviation Turbine Fuels by Supercritical Fluid Chromatography		
	Monoaromatics by SFC	24.0	Wt %
	Polynuclear Aromatics by SFC	> 50.0	Wt %
	Total Aromatics	83.5	Wt %

[redacted]

Page 4 of 16
61494

Information not relevant to the test sample has been blacked out.

1114 Seaco Avenue, Deer Park, Texas 77536 USA
Tel.: (713) 844-3200

05-Feb-2009 15:09
US785-0016408

APPENDIX G – SPONSOR SUPPLIED TEST SUBSTANCE INFORMATION (CONT'D)

SAMPLE:	**09-0651-12 (Site #5 Sx. #1)**	Injection Date:	)090117124109-0600
		Report Date:	1/18/09 8:07
FILE:	C:\CP32 Instruments\D2887 & D3710\Data\2009\JAN-09\09-0651-12.0007.CDF		
PROCEDURE:	C:\CP32 Instruments\D2887 & D3710\PROCEDURES\122308-D2887.prc		
EXCEL FILE:	C:\CP32 Instruments\D2887 & D3710\Reports\2009\JAN-09\09-0651-12_0007_CDF.xls		

Boiling Point Distribution Report

ASTM D2887 Simulated Distillation

%Off	BP °F	BP °C	%Off	BP °F	BP °C	%Off	BP °F	BP °C
IBP	288.8	142.7	**40%**	504.3	262.4	**80%**	573.5	300.8
1%	339.1	170.6	**41%**	508.0	264.5	**81%**	574.9	301.6
2%	398.5	203.6	**42%**	510.6	265.9	**82%**	576.5	302.5
3%	429.4	220.8	**43%**	513.2	267.3	**83%**	578.7	303.7
4%	446.0	230.0	**44%**	514.9	268.3	**84%**	581.4	305.2
5%	447.3	230.7	**45%**	516.0	268.9	**85%**	583.2	306.2
6%	448.1	231.2	**46%**	516.9	269.4	**86%**	585.1	307.3
7%	448.8	231.5	**47%**	518.0	270.0	**87%**	586.3	307.9
8%	449.6	232.0	**48%**	519.9	271.0	**88%**	588.8	309.4
9%	451.9	233.3	**49%**	521.6	272.0	**89%**	593.2	311.8
10%	453.0	233.9	**50%**	522.8	272.6	**90%**	596.7	313.7
11%	453.8	234.3	**51%**	523.6	273.1	**91%**	599.4	315.2
12%	459.1	237.3	**52%**	524.4	273.6	**92%**	602.9	317.2
13%	467.6	242.0	**53%**	525.5	274.1	**93%**	606.9	319.4
14%	475.1	246.2	**54%**	527.2	275.1	**94%**	610.4	321.3
15%	479.4	248.6	**55%**	528.3	275.7	**95%**	614.8	323.8
16%	481.0	249.4	**56%**	529.2	276.2	**96%**	619.7	326.5
17%	482.3	250.2	**57%**	530.2	276.8	**97%**	628.1	331.2
18%	483.6	250.9	**58%**	532.0	277.8	**98%**	637.1	336.2
19%	484.5	251.4	**59%**	533.3	278.5	**99%**	656.4	346.9
20%	485.2	251.8	**60%**	534.6	279.2	**FBP**	675.9	357.7
21%	485.8	252.1	**61%**	536.9	280.5			
22%	486.5	252.5	**62%**	538.5	281.4			
23%	487.3	252.9	**63%**	540.2	282.3			
24%	488.2	253.4	**64%**	541.7	283.2			
25%	489.0	253.9	**65%**	543.3	284.1			
26%	489.7	254.3	**66%**	544.7	284.8			
27%	490.2	254.5	**67%**	546.6	285.9			
28%	490.5	254.7	**68%**	549.0	287.2			
29%	491.0	255.0	**69%**	550.9	288.3			
30%	491.3	255.2	**70%**	552.5	289.2			
31%	491.6	255.4	**71%**	554.7	290.4			
32%	491.9	255.5	**72%**	556.1	291.2			
33%	492.4	255.8	**73%**	557.9	292.2			
34%	494.1	256.7	**74%**	561.2	294.0			
35%	495.1	257.3	**75%**	564.7	295.9			
36%	495.8	257.7	**76%**	567.5	297.5			
37%	496.5	258.1	**77%**	568.9	298.3			
38%	498.5	259.1	**78%**	570.1	298.9			
39%	499.9	260.0	**79%**	572.0	300.0			

Start Elution Time (mins):	0.166	**Sample Wt:**	0 **g**
End Elution Time (mins):	23.863	**Solvent Wt:**	0 **g**
		Material Balance:	100.0 **Wt%**
Blank File:	C:\CP32 Instruments\D2887 & D3710\Data\2009\JAN-09\CS2-BLANK.0009.CDF		
Calib File:	D:\CP32 Instruments\D2887 & D3710\DATA\RTMIX-060905.0006.CDF		
Resp Factor:	1.000E+00		

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS

PROTOCOL

Contract Number:	EMBSI 2010-104821
Study Title:	*Daphnia magna*, Acute Immobilisation Test on Water Accomodated Fractions of a Light Catalytic Cracked Gas Oil
EMBSI Study Number:	1057642
Test Substance:	Gas oil; CAS RN 64741-59-9, Distillates (petroleum), light catalytic cracked
EMBSI Test Substance Code:	MRD-10-576
Date:	September 13, 2010
Room Number:	LE-337/343

Proposed Key Dates:

Initial Characterization	12-Jul-10
WAF Equilibration and Stability Trial Start	13-Sep-10
Range Finding Test Start	20-Sep-10
Experimental Start	27-Sep-10
Experimental Termination	29-Sep-10
Draft Report Completion	29-Oct-10
Final Report Completion	05-Nov-10

Approved By:

[redacted]

13Sep10
Date

Study Director
ExxonMobil Biomedical Sciences, Inc.
1545 Route 22 East, P.O. Box 971
Annandale, New Jersey 08801-0971

[redacted]

13 September 2010
Date

Sponsor Representative
American Petroleum Institute
Washington DC

SAFETY FIRST

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

INTRODUCTION

Objective

This study will be conducted for the Sponsor to evaluate the acute toxicity of the water accommodated fractions (WAFs) of MRD-10-576 to the daphnid, *Daphnia magna*. This study will be performed as a 48-hour static test.

Sponsor

American Petroleum Institute
1220 L Street, NW
Washington, DC 20005-4070

Testing Facility/Test Site

ExxonMobil Biomedical Sciences Inc.
Laboratory Operations
1545 Route 22 East, P.O. Box 971
Annandale, New Jersey 08801-0971

Compliance

This test will be conducted in general agreement with the OECD[1] and EPA[2,3] guidelines, and will be conducted in compliance with OECD[4] and USEPA[5] GLP standards.

Justification for Selection of Test System

Daphnia magna has been used in safety evaluation and is a common test species for freshwater toxicity studies.

Justification of Dosing Route

Potential environmental exposure is by the test substance in water.

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

MATERIALS and METHODS

Test Substance Identification

EMBSI code	Test Substance
MRD-10-576	CAS 64741-59-9

CAS Definition: Distillates (petroleum) light catalytic cracked. A complex combination of hydrocarbons produced by the distillation of products from a catalytic cracking process. It consists of hydrocarbons having carbon numbers predominantly in the range of C9 through C25 and boiling in the range of approximately 150 degrees C to 400 degrees C (302 degrees F to 752 degrees F). It contains a relatively large proportion of bicyclic aromatic hydrocarbons[6].

Storage Conditions: The neat test substance will be stored at room temperature.

Characterization of Test Substance

Pre-test and post-test characterization and stability analysis will include the following determinations: FT-IR and UV-Vis spectra, density, physical-state, miscibility in water, methanol and/or hexane and GC-MS "fingerprint" of the neat test substance. The GC-MS fingerprint is run against an ASTM hydrocarbon standard mixture. The pretest characterizations was conducted using ASTM D2887 standard that is applied for higher boiling mixtures with compounds eluting between approximately n-octane (n-C8) and n-triacontane (n-C30). Due to the complex nature of the test substance, no reporting will be made of specific hydrocarbon components. Instead, an area percent report will be generated for both the pre- and post-test analysis to demonstrate stability of the test substance over the testing period. Documentation of characterization and stability assessment will be maintained at the testing facility and the results appended to the final report. A statement will be provided by the testing facility specifically addressing whether the test substance was stable over the course of the testing period based on the set of analyses.
The methods of synthesis, fabrication, and/or derivation of the test substance will be maintained by the sponsor. The test substance, as received, will be considered the "pure" substance.

Analysis of Mixtures

Samples will be taken from each water-accommodated fraction (WAF) and control solution on Day 0 and Day 2 (composite of replicates). If complete immobilization is observed in any treatment on Day 1, a sample will be taken (composite of replicates if applicable). The samples will be taken with no headspace and refrigerated pending analysis. Samples will be analyzed using static headspace gas chromatography with flame ionization detection (HS GC-FID). Standards of the gas oil will be prepared in reconstituted water and acetone. O-xylene will be used as an internal standard. Sample concentrations will be reported in mg/L based upon the standard curve and internal standard recovery and are representative of the total dissolved hydrocarbons of the test substance.

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

MATERIALS and METHODS (CONT'D)

Sample Retention

No retention samples (neat test substance or solutions (WAFs)) will be taken for this study.

Dilution Water

Reconstituted water[7] (the dilution water) will be prepared from UV-sterilized, deionized well water and reagent grade chemicals ($NaHCO_3$, $CaSO_4$, $MgSO_4$, and KCl), it will be aerated prior to use. The hardness will be >140 mg/L (as $CaCO_3$).

Test System

Daphnia magna Straus

Supplier

Cultured in the Environmental Toxicology Laboratory, Annandale, New Jersey. Original culture supplied by Aquatic Biosystems, Inc., Fort Collins, CO.

Husbandry and Acclimation

Eight to ten daphnids are kept in 1-liter glass culture beakers with approximately 800 mL of reconstituted water (study dilution water). The culture chamber is maintained at 20 ± 1°C under a 16 hour light 8 hour dark photoperiod (10 - 20 foot/candles, 108 - 215 Lux). Two sets of Day 0 cultures are started at least five days a week. The neonates should be less than 24 hours old and come from a day 12-18 culture which experienced less than an estimated 10% neonate mortality and less than or equal to 20% adult mortality.

Cultures of *Daphnia magna* are fed *Pseudokirchneriella subcapitata* (approximately 3.0 - 4.5 x 10^5 cells/mL). They are also fed 25µL/L of Vita chem Fresh formula mixed on a magnetic stir plate with the reconstituted water prior to feeding with algae. The culture is fed every other day or as needed based on observed algal clearing. The algae is supplied by Aquatic Biosystems, Inc., Fort Collins, CO. The Vita chem is manufactured by Boyd Enterprises, Inc. and supplied by Foster and Smith Aquatics, Rhinelander, Wisconsin.

Cultures are transferred every other day, with exceptions on holidays or weekends when staff is not present, the brood stock health is evaluated and any mortality, production of males or ephippia is documented as well as any mitigation procedures.

Number and Sex

Number: 120 Sex: Not Applicable

Age at Initiation of Exposure

<24 hours; age of parents will be noted in the final report.

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

MATERIALS and METHODS (CONT'D)

Test System Identification

Daphnids will not be individually identified. All test chambers will be labeled to show study number, loading level, replicate and randomization number.

Selection

Organisms will be randomly assigned to intermediate chambers using a computer generated randomization schedule and then transferred to their respective test chambers. The test chambers will be randomly positioned within the test area. A printout of the randomization schedule will be included in the raw data.

To ensure that quality organisms are used for the study, neonates from parents 12-18 days old (with ≤20% adult mortality) will be selected. Neonates will be selected from a pool of organisms larger than that needed for the study. The pool of neonates will have ≤10% daily mortality on the experimental start day. The study director or his designee determines organism suitability.

Feed

Daphnids are not fed during the study.

Contaminants

There are no known contaminants in the feed used in culturing the organisms or the dilution water believed to be at levels high enough to interfere with this study. The algae and Vita chem are not analyzed. The algae is prepared in dilution water with reagent grade chemicals. The dilution water is prepared from UV-sterilized, deionized well water that is treated and distributed throughout the testing facility via PVC and stainless steel pipes. The deionized water is monitored for priority pollutants, un-ionized ammonia, total suspended solids, and for bacterial properties by Accutest®, Dayton, NJ. Contaminant analyses are not performed in a GLP compliant manner. This is not believed to affect the results of the analyses. Contaminant analysis results are maintained at the testing facility.

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

EXPERIMENTAL PROCEDURE

WAF Equilibration and Stability Trial

A WAF equilibration and stability trial will be performed prior to testing. This task serves to determine the most appropriate mixing duration for WAF preparation, to confirm the analytical method, which will be used for the definitive test, and to evaluate the stability of the WAF solutions once they are produced. This information will be used to establish mixing times for the WAFs and the interval between WAF renewal times to be used in a chronic test with *D. magna,* as well as determine whether a renewal at 24 hours is necessary for the acute test with D. *magna*. The Sponsor will decide whether or not a 24-hour renewal of the test solutions is required. Specific analytical procedures, as stated in the analysis of mixtures section, will be used to detect and quantitate the soluble components of the substance.

Aqueous mixtures of the test substance and dilution water will be prepared at loading rates of 0.1 mg/L, 0.5 mg/L and 5 mg/L. Each mixing vessel will be filled to achieve minimal headspace given the constraints of the vessels and closed with foil-covered stoppers. The solutions in each vessel will be stirred at room temperature at a rate to maintain a vortex at ≤10% of the static liquid depth. At the end of 24, 48 and 72 hours, mixing will stop and the solutions will be allowed to settle for approximately 1 hour. WAF samples from each loading rate will be taken for chemical analysis. The equilibrium phase will not extend beyond 72 hours due to a potential loss of volatile components of the test substance in the mixing vessels and logistical constraints associated with a semi-static renewal type test. However, if after 72 hours the concentrations are still rising, the mixing time may be extended. Also, if soluble hydrocarbons are not found at the 5.0mg/L level, the test will be restarted with WAFs at higher levels. The WAFs used in order to measure stability and equilibrium will be documented in the raw data and included in the report.

As a measure of WAF stability, a portion of each loading rate will be stored under testing conditions in a 130 mL bottle with zero headspace. One vessel of each WAF level will be taken for analysis of dissolved hydrocarbons at 24, 48 and 72 hours.

While some non-GLP exploratory investigation may be needed to focus the conditions for a confirmatory equilibration/stability trial, the final confirmatory trial will be performed under GLP standards.

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

EXPERIMENTAL PROCEDURE (CONT'D)

Range Finding Test

A 48-hour range finding test will be performed. *D. magna* neonates will be exposed to the WAFs of three to five loading rates plus a control under static conditions; the loading rates chosen will aim to capture the 48 hour EC_{50}. The WAFs will be prepared by adding the appropriate amount of test substance to dilution water in glass vessels. The vessels will be closed using foil covered rubber/neoprene stoppers and will mix at room temperature on magnetic stirplates with Teflon® coated stirbars for the appropriate time as determined by the WAF equilibration trial (±1 hour). The vortex will be set at ≤10% of the static liquid depth and minimal headspace will be established given the constraints of the mixing vessel. The treatments will be allowed to settle and equilibrate to test temperature for 1 hour (±15 mins.) after mixing. At least five replicates containing 5 daphnids at each loading rate will be used in the range-finding test. The test chambers will be completely filled with the appropriate solution such that zero or minimal headspace exists in the test chambers. The procedures followed for the range finding study will be documented in the raw data. This phase of the study will not be subject to GLP standards.

Definitive Test Design

GROUP	LOADING LEVEL (mg/L)	NUMBER OF ORGANISMS
1 (Control)	0	20 (5 per 4 replicates)
2	TBD	20
3	TBD	20
4	TBD	20
5	TBD	20
6	TBD	20

TBD = To Be Determined

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

EXPERIMENTAL PROCEDURE (CONT'D)

Preparation and Administration of Test Substance

Individual WAFs will be prepared for each loading level by adding the appropriate amount of the test substance to 4 L of dilution water in 4 L glass aspirator bottles. The vessels will be sealed with foil covered stoppers. The solutions will be mixed with Teflon® coated stirbars on magnetic stirplates. The vortex will be set at ≤10% of the static liquid depth. The solutions will mix for the appropriate time as determined by equilibrium testing (±1 hour) at room temperature (22°±1°C). At the end of mixing, the solutions will be allowed to settle for approximately 1 hour (±15 minutes) allowing any phase separation and equilibration to test temperature. At the end of the settling period the solutions will be removed from the mixing vessels through the outlet at the bottom of the vessels and placed into four replicate chambers.

Test Chamber and Volume of Solution

The test chambers will be 130mL glass bottles containing ~130mL of solution (no headspace). Each chamber will be closed with ground glass stoppers to minimize contamination, evaporation and/or volatilization.

Exposure Duration

48 hours (±1 hour)

Environmental Conditions

Range of acceptable test water temperatures: 20°± 1°C.
Diurnal light: 16 hours light: 8 hours dark.
An environmental condition study will be activated on the laboratory computer system (Watchdog V5 monitoring system) at the start of the study to provide a record of the continuous measurements for temperature and lighting in the test area.

Experimental Evaluation

Observations for immobilization will be performed and recorded at 24 and 48 hours (±1 hour) after the beginning of the test. Additional observations may be performed. Immobilization is the lack of swimming ability or movement within 15 seconds after gentle agitation of the test container. Any abnormal behavior or appearance will also be recorded.

Observations of test substance insolubility (surface slicks, precipitates, and adherence to the test chamber) will be recorded daily at the time of organism observations.

Organisms will be discarded at termination. The monitoring of environmental conditions will be discontinued after completion of the study.

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

EXPERIMENTAL PROCEDURE (CONT'D)

Discrete Measurements

Temperature, dissolved oxygen, and pH: measured in each treatment and control at the start of the test and in a composite of replicates at termination (termination includes complete immobilization in a treatment).

Loading

At least 25 mL of test solution will be provided for each organism.

Test Acceptability

In the control, not more than 10% of the Daphnia may be immobilized or trapped at the surface of the water. Dissolved oxygen should be ≥60% of the air saturation value at the temperature tested.

Calculations

Both nominal and measured concentrations will be used in order to derive the EC/EL_{50}, defined as the concentration or loading level of the test substance estimated to immobilize 50% of the test organisms within a specified period of exposure. The statistical method used to calculate the EC/EL_{50} values and their associated 95% confidence limits will be either a maximum likelihood analysis based on D. J. Finney, 1971[8], a Trimmed Spearman-Karber Method[9], a Binomial Method[10] or a graphical method[11].

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

REPORT

After termination of the study, a final report that includes the following information will be submitted:

Test substance:

- physical nature, and where relevant, physiochemical properties
- identification data

Test *Daphnia*:

- scientific name, strain (if applicable), age, supplier, any pretreatment, breeding method (including source, kind and amount of food, feeding frequency)

Test conditions:

- test procedure used, equilibration test results
- dilution water source and chemical characteristics/composition (pH, temp. dissolved oxygen, TOC, hardness, alkalinity, latest contaminant analysis results)
- Ca/Mg ratio and Na/K ratio in the dilution water
- light quality, intensity and periodicity
- dissolved oxygen concentration, and pH values and temperature of the test solutions at study initiation and termination
- methods of preparation of test solutions
- loading levels/concentrations used
- information on concentrations of the test substance in the test solutions
- number of organisms in each test vessel
- description of the test chambers, and volume of solution

Results:

- maximum loading level/concentration causing no immobilization
- minimum loading level/concentration causing 100% immobilization
- percent of organisms that were dead per treatment
- individual daily observations, including daily and cumulative immobilization, survival and abnormal responses of the *Daphnia*
- EL_{50} or EC_{50} with 95% confidence limit at each observation interval, if possible
- statistical procedures followed
- graph of the loading level/concentration-response curve at the end of the test, if applicable
- analytical data on test solutions

Study Conduct:

- compliance statement
- quality assurance statement
- protocol with amendments appended to the report
- evidence that the quality criteria have been fulfilled
- incidents in the course of the test which may have influenced the results

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

RECORDS

All appropriate materials, methods and experimental measurements required in this protocol will be recorded and documented in the raw data. Any changes, additions or revisions of this protocol must be approved by the Study Director and the Sponsor Representative. These changes will be documented in writing, including the date the justification for the change and the signatures of the Study Director and Sponsor Representative.

The protocol, final report, raw data or computer generated listings of raw data, and supporting documentation will be maintained in the Archives of the testing facility for 10 years, after which time the records will be offered to the sponsor prior to disposal.

QUALITY ASSURANCE

The Quality Assurance Unit of ExxonMobil Biomedical Sciences, Inc. will audit the protocol, conduct study based phase inspection(s) and audit the draft final report (before sponsor review) to assure that they are in conformance with company SOPs and the appropriate guidelines and Good Laboratory Practice Regulations.

GUIDELINE EXCEPTIONS

Due to the limited solubility of the test substance the following exceptions will apply for this study:

The concentration of the test substance in solutions will not be determined prior to test initiation. Day 0 samples will be taken of the solutions at each loading level but will not necessarily be analyzed prior to test initiation. Due to the limited solubility of the test substance, it may not be possible for analytical results to demonstrate that the initial concentration of the test substance will be maintained at 80% throughout the test.

Consistent with the OECD document on aquatic toxicity testing of complex substances[12], it is deemed more appropriate to prepare individual WAF treatment solutions by adding the test substance to dilution water and removing the WAF of each mixture for testing than to prepare dilutions of a stock solution.

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

REFEREENCES

1. Organization for Economic Cooperation and Development (OECD). Guidelines for Testing of Chemicals. Section 2: Effects on Biotic Systems, Guideline 202: *Daphnia sp*. Acute Immobilisation Test. Adopted 13 April 2004.

2. U.S. Environmental Protection Agency, Ecological Effects Test Guidelines, OPPTS 850.1010: Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids. April, 1996.

3. U.S. Environmental Protection Agency, TSCA, 40CFR 797.1300 Daphnid acute toxicity test. September, 1985.

4. OECD Principles of Good Laboratory Practice (GLP), C(97)186 (Final), 1997.

5. United States Environmental Protection Agency (USEPA), Toxic Substance Control Act (TSCA) Good Laboratory Practice Standards, 40 CFR Part 792, 1989.

6. API. Petroleum process stream terms included in the chemical substances inventory under the Toxic Substances Control Act (TSCA). American Petroleum Institute, Washington, DC. February, 1985. 40 pp.

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APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

DISTRIBUTION

EMBSI - Clinton:
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- Environmental Chemistry / Principal Investigator for Characterization/Analysis of Mixtures .....
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APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD Page 1 of 3

This record must be approved by the Sponsor Representative and the Study Director for all protocol changes made subsequent to initial distribution. Upon completion, a copy of this record must be distributed to all recipients of the protocol and the original submitted to the Archivist.

Study Number: 1057642 Revision Number: 1 Date: 15-Oct-10

Page 1 / Proposed Key Dates for Completion:

Previous Statement:

Range Finding Test Start	20-Sep-10
Experimental Start	27-Sep-10
Experimental Termination	29-Sep-10
Draft Report Completion	29-Oct-10
Final Report Completion	05-Nov-10

Revised Statement:

Range Finding Test Start	22-Oct-10
Experimental Start	09-Nov-10
Experimental Termination	11-Nov-10
Draft Report Completion	17-Dec-10
Final Report Completion	14-Jan-11

Justification: Testing dates re-set

Pg. 4 / Husbandry and Acclimation:

Previous Statement:

The culture chamber is maintained at 20 ± 1°C under a 16 hour light 8 hour dark photoperiod (10 - 20 foot/candles, 108 - 215 Lux).
Cultures of *Daphnia magna* are fed *Pseudokirchneriella subcapitata* (approximately 3.0 - 4.5 x 10^5 cells/mL).

Revised Statement:

The culture chamber is maintained at 20 ± 2°C under a 16 hour light 8 hour dark photoperiod (10 - 20 foot/candles, 108 - 215 Lux).
Cultures of *Daphnia magna* are fed *Pseudokirchneriella subcapitata* (approximately 4.5 - 6.0 x 10^5 cells/mL).

Justification: corrections based on current culture method

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD Page 2 of 3

This record must be approved by the Sponsor Representative and the Study Director for all protocol changes made subsequent to initial distribution. Upon completion, a copy of this record must be distributed to all recipients of the protocol and the original submitted to the Archivist.

Study Number: 1057642 Revision Number: 1 Date: 15-Oct-10

Pg. 4 / Reference Toxicant study

Previous Statement:

none

Revised Statement:

A reference toxicant study will be run prior to the definitive study as a means of assuring that the laboratory test conditions are adequate and that the test organisms are healthy. Potassium Chloride will be used as the reference toxicant. The following concentrations will be used for the study: 0, 375 mg/L KCl, 750 mg/L KCl and 1500 mg/L KCl. Three replicates containing 5 daphnids each will be used for the tests and the test will be conducted for 48 hours using the same temperature and light conditions as the definitive study. A stock will be prepared at 15 mg/L KCl and dilutions will be made to the concentrations listed above. No analysis of mixtures will be completed. This study will not be subject to GLP standards.

Justification: addition to protocol to satisfy OECD guidelines

Pg. 10 / Report

Previous Statement:

- maximum loading level/concentration causing no immobilization
- minimum loading level/concentration causing 100% immobilization
- percent of organisms that were dead per treatment
- individual daily observations, including daily and cumulative immobilization, survival and abnormal responses of the *Daphnia*
- EL_{50} or EC_{50} with 95% confidence limit at each observation interval, if possible
- statistical procedures followed
- graph of the loading level/concentration-response curve at the end of the test, if applicable
- analytical data on test solutions

Revised Statement:

- maximum loading level/concentration causing no immobilization
- minimum loading level/concentration causing 100% immobilization
- percent of organisms that were dead per treatment
- individual daily observations, including daily and cumulative immobilization, survival and abnormal responses of the *Daphnia*
- EL_{50} or EC_{50} with 95% confidence limit at each observation interval, if possible
- statistical procedures followed
- graph of the loading level/concentration-response curve at the end of the test, if applicable
- analytical data on test solutions
- reference toxicant study results (EL_{50})

Justification: addition to protocol to satisfy OECD guidelines

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD

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- QAU

API:
- Sponsor Representative
- Sponsor's Study Monitor

Required signatures:

[redacted] 15 Oct 2010 Date
Sponsor Representative

[redacted] 15 OCT 2010 Date
Study Director

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD Page 1 of 2

This record must be approved by the Sponsor Representative and the Study Director for all protocol changes made subsequent to initial distribution. Upon completion, a copy of this record must be distributed to all recipients of the protocol and the original submitted to the Archivist.

Study Number: 1057642 Revision Number: 2 Date: 26-Oct-10

Page 7 / Range Finding Test

Previous Statement:

At least five replicates containing 5 daphnids at each loading rate will be used in the range-finding test.

Revised Statement:

At least three replicates containing 5 daphnids at each loading rate will be used in the range-finding test.

Justification: Error in protocol generation

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

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Study Technicians
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QAU

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Sponsor Representative
Sponsor's Study Monitor

Required signatures:

[redacted] 6/22/10 Date
Sponsor Representative

[redacted] 6/23/10 Date
Study Director

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD Page 1 of 3

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Study Number: 1057642 Revision Number: 3 Date: 05-Nov-10

Page 7 / Definitive Test Design

Previous Statement:

GROUP	LOADING LEVEL (mg/L)	NUMBER OF ORGANISMS
1 (Control)	0	20 (5 per 4 replicates)
2	TBD	20
3	TBD	20
4	TBD	20
5	TBD	20
6	TBD	20

TBD = To Be Determined

Revised Statement:

GROUP	LOADING LEVEL (mg/L)	NUMBER OF ORGANISMS
1 (Control)	0	20 (5 per 4 replicates)
2	0.102	20
3	0.256	20
4	0.640	20
5	1.60	20
6	4.00	20

TBD = To Be Determined

Justification: assignment of definitive concentrations

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD Page 2 of 3

This record must be approved by the Sponsor Representative and the Study Director for all protocol changes made subsequent to initial distribution. Upon completion, a copy of this record must be distributed to all recipients of the protocol and the original submitted to the Archivist.

Study Number: 1057642 Revision Number: 3 Date: 05-Nov-10

Page 8 / Environmental Conditions

Previous Statement:

An environmental condition study will be activated on the laboratory computer system (Watchdog V5 monitoring system) at the start of the study to provide a record of the continuous measurements for temperature and lighting in the test area.

Revised Statement:

An environmental condition study will be activated on the laboratory computer system (Watchdog V5 monitoring system) at the start of the study to provide a record of the continuous measurements for temperature and lighting in the test area. Should the Watchdog V5 monitoring system be unavailable, manual measurements will be taken twice a day.

Justification:

clarification

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

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Sponsor's Study Monitor

Required signatures:

[redacted]
Sponsor Representative

09 Nov 2010
Date

[redacted]
Study Director

09 NOV 10
Date

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD Page 1 of 2

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Study Numbers: 1057642 Revision Number: 4 Date: 07Nov11

Page 1 /

Previous Statement: Sponsor Representative – [redacted]

Revised Statement: Sponsor Representative – [redacted]

Justification: [redacted] has retired from American Petroleum Institute and has been replaced with [redacted]

Page 12 / PERSONNEL:

Previous Statement: Environmental Toxicology and Fate Coordinator - [redacted]

Revised Statement: Environmental Toxicology and Fate Coordinator – [redacted]

Justification: [redacted] has been replaced with [redacted] as Lab Coordinator effective January 1, 2011.

Previous Statement: Environmental Sciences, Section Head - [redacted]

Revised Statement: Environmental Sciences, Section Head – [redacted]

Justification: [redacted] has been replaced with [redacted] as Section Head effective July 1, 2011

APPENDIX H – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD Page 1 of 2

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Study Numbers: 1057642 Revision Number: 4 Date: 07Nov11

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Contract Administrator .....
QAU .....

API:
Sponsor Representative .....
.....

Required signatures:

[redacted] 29 Nov 2011 [redacted] 09Dec11

Sponsor Representative Date Study Director Date