ExxonMobil BIOMEDICAL SCIENCES, INC.

EMBSI – 2010-104821

Alga, Growth Inhibition Test on Water Accommodated Fractions of a Light Catalytic Cracked Gas Oil

Final Report

Study Number: 1057667

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, NJ 08801-3059

COMPLETION DATE: December 22, 2011

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APPENDICES:

APPROVAL SIGNATURES

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22 Dec. 2011
Date

The final report was accepted by the Sponsor

Russell White, Ph.D.
Sponsor Representative
American Petroleum Institute
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20 Dec. 2011
Date

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).

As defined in the protocol, the range-finding trial of this study was not performed in a GLP compliant manner.

Stability analysis of the test substance in the algae treatments was not conducted prior to or concomitantly with the in-life period of the study.

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.

These exceptions are not believed to have had an adverse effect on the study results.

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QUALITY ASSURANCE STATEMENT

STUDY NUMBER: 1057667

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	September 20, 2010	September 20, 2010	January 3, 2011 January 5, 2011
Light readings, Randomization of Chambers, Day 1	January 19, 2011	February 8, 2011	February 11, 2011 February 18, 2011
First review of Final Report & Raw Data	October 18-21, & October 25, 2011	October 25, 2011	November 17, 2011
Second Review of Final Report & & Raw Data	November 16, 2011	November 17, 2011	December 06, 2011 December 07, 2011
Third Review of Final Report Appendix B only	December 6, 2011	December 6, 2011	December 6, 2011 December 7, 2011

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

Robert Pristas

Robert Pristas, M.S.
Quality Assurance Unit Coordinator

19 Dec 2011
Date

PERSONNEL

Study Director:	B. A. Kelley, A.A.S.
Sponsor Representative:	Russell White, Ph.D.
Section Head, Environmental Sciences: (until July 1, 2011)	T. F. Parkerton, Ph.D.
Section Head, Environmental Sciences: (effective July 1, 2011)	R. A. Barter, Ph.D.
Environmental Toxicology & Fate Laboratory Coordinator: (until January 1, 2011)	E. J. Febbo, M.S.
Environmental Toxicology & Fate Laboratory Coordinator: (effective January 1, 2011)	G. E. Bragin, M.S.
Environmental Chemistry Laboratory Coordinator; Principal Investigator for Characterization & Analysis of Test Solutions:	D. J. Letinski, M.S.
Quality Assurance Unit Coordinator:	R. Pristas, M.S.

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 effects of the water-accommodated fractions (WAFs) of light catalytic cracked gas oil (CAS Number 64741-59-9) on the growth of the alga, *Pseudokirchneriella subcapitata*, in a 96-hour static test.

Individual treatments were prepared by adding the appropriate amount of test substance to algal nutrient media in glass aspirator bottles and stirring on magnetic stirplates using an approximately 10% (of the static liquid depth) vortex for approximately 24 hours. After approximately one hour without stirring, the aqueous portions (WAFs) were removed for testing. The loading rates were 0 (control), 0.10, 0.32, 1.02, 3.28 and 10.5 mg/L.

The test chambers were completely filled (no headspace) with the appropriate WAF and were closed with PTFE lined caps. Each chamber contained two 14-mm glass spheres to facilitate mixing. Test chambers were placed on a shaker tables and oscillated at 100 rpm to keep the algae in suspension. The study was performed under continuous light conditions with an average light intensity range from 4170 – 4345 lux and a mean test temperature of 23.7 °C. The pH in the test solutions ranged from 7.6 - 7.8 at the beginning of the test and from 7.5 - 9.2 at the end of the test. Three replicates from each loading rate were sacrificed daily for cell density determinations.

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 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 measured hydrocarbon concentrations in the WAFs at the beginning of the test were ND (Not Detected; control), 0.07, 0.27, 0.93, 2.33 and 5.54 mg/L. At 72 hours, measured hydrocarbon concentrations ranged from 4.1 to 74% of initial concentrations. Measured hydrocarbon concentrations at 96 hours ranged from 1.1 to 6.1% of initial concentrations.

Two biologically killed (i.e., abiotic) chemical control treatments were prepared at WAF loading rates of 1.02 and 10.5 mg/L to verify concentration stability without the influence of algal growth. Analytical measurements of the composite chemical control treatments on Day 3 and 4 demonstrated that the concentrations remained within 90 - 92% of the initial concentrations.

At termination, triplicate test chambers were prepared with aliquots of 3.28 mg/L test solution diluted with fresh dilution medium to 100 mL for a final concentration of approximately 0.1 mg/L. The subcultures were placed on the stir plate and incubated for ten days under similar definitive test conditions. Based on the increasing cell density, it was determined that the 3.28 mg/L treatment group produced an algistatic (reversible) effect.

SUMMARY (CONT'D)

Acute toxicity results are expressed as percent inhibition of growth derived from either the average specific growth rate (r), yield (y) or cell density relative to the control. The 50% Effect Loading (EL50) is the loading rate of the test substance in dilution medium which is calculated to result in a 50% reduction in growth in a population of test organisms over a specified exposure period. The No Observed Effect Loading Rate (NOELR) is the highest loading rate which does not exhibit a statistical difference from the control. Measured concentrations do not represent all hydrocarbons constituting the test substance. Results expressed as the 50% Effect Concentration (EC50) and the No Observed Effect Concentration (NOEC) represent the concentration of hydrocarbons that solubilized from the test substance into each WAF at its respective loading rate. The 72 and 96 hour endpoints for this study are presented in the following table.

Response Variable	72 hour		96 hour	
	Loading Rate* (mg/L)	**Day 0 Measured** (mg/L)**	**Loading Rate* (mg/L)**	**Day 0 Measured** (mg/L)**
Cell density	EL50 = 0.29 (0.25-0.33)	EC50 =0.23 (0.20-0.27)	EL50 = 0.32 (0.28-0.36)	EC50 =0.26 (0.22-0.30)
Yield	EyL50 = 0.28 (0.25-0.31) NOELR = 0.10 LOELR = 0.32	EyC50 =0.22 (0.20-0.25) NOEC < 0.07 LOEC = 0.27	EyL50 = 0.31 (0.27-0.35) NOELR < 0.10 LOELR = 0.1	EyC50 =0.25 (0.22-0.29) NOEC < 0.07 LOEC = 0.27
Growth rate	ErL50 = 0.53 (NC[1]) NOELR = 0.10 LOELR = 0.32	ErC50 = 0.49 (NC[1]) NOEC = 0.07 LOEC = 0.27	ErL50 = 0.80 (NC[1]) NOELR = 0.32 LOELR = 1.02	ErC50 =0.70 (NC[1]) NOEC = 0.27 LOEC = 0.9

* Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution medium.
**Measured concentration represents the concentration of hydrocarbons that solubilized from the test substance into each WAF at its respective loading rate.
Values in parentheses ( ) are 95% confidence intervals.
[1]NC = Not calculable

INTRODUCTION

Objective

This study was conducted for the Sponsor to evaluate the effects of the water-accommodated fractions (WAFs) of light catalytic cracked gas oil (CAS No. 64741-59-9) on the growth of the alga, *Pseudokirchneriella subcapitata*, in a 96-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, NJ 08801-3059

Initial Characterization

12 July 2010

Study Initiation Date

12 November 2010

WAF Equilibration and Stability Trial Start (Mixing)

13 September 2010

Range-Finding Test Start (Mixing)

20 November 2010

Experimental Start (In-life)

18 January 2011

In-life Termination

01 February 2011

Final Characterization

26 July 2011

INTRODUCTION (CONT'D)

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. The study was performed in general agreement with OECD[3] and USEPA[4] guidelines with the exceptions noted on page 21.

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 [5].

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.

Dilution Medium

Algal Nutrient Media[6] - filtered through a sterile 0.45 µm filter (referenced as acceptable medium in OECD 201 guideline), with 400 mg of $NaHCO_3$ per liter, added as a carbon source in a no headspace environment[7]. The algal medium meets the following limits of essential constituents: $P \leq 0.7$ mg/L, $N \leq 10$ mg/L, chelators $\leq 10^{-3}$ mmol/L and hardness $(Ca + Mg) \leq 0.6$ mmol/L. See Appendix A for composition of the algal media.

MATERIALS and METHODS (CONT'D)

Contaminants

There are no known contaminants in the nutrient medium believed to be at levels high enough to interfere with this study. The nutrient medium is prepared from reagent grade chemicals and 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®, 2235 Route 130, Dayton, NJ 08810. Contaminant analyses are not performed in a GLP compliant manner. Contaminant analysis results are maintained at the testing facility.

Characterization of the Test Substance

The neat test substance was characterized and the stability determined by the testing facility both prior to and after completion of 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 Total Ion chromatogram ("fingerprint") of the neat test substance. The GC-MS 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 and reported 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.

Analysis of Test Solutions

Samples were collected from each water-accommodated fraction (WAF) and control solution on Day 0, prior to the addition of algae. On Day 3 and 4, samples (composite of a subsample of three replicates) for each treatment group, the control and the chemical control were collected for analysis. The samples were taken in 40 mL VOA vials with no headspace 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.

MATERIALS and METHODS (CONT'D)

Analysis of Test Solutions (cont'd)

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 was 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. Stability analysis of the test substance in the algae treatments was not conducted prior to or concomitantly with the in-life period of the study as required by GLPs. The analytical method is included in Appendix B.

Test System

Pseudokirchneriella subcapitata (formerly *Selenastrum capricornutum*)

Culture date: 13 January 2011

Justification for Selection of Test System

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

Supplier

Cultured at the Environmental Toxicology Laboratory of the testing facility. Initial strain (#1648) provided by UTEX, The Culture Collection of Algae MCDB, School of Biological Sciences, The University of Texas at Austin, Austin, TX 78712. Lot # 21(slant 21 received by the laboratory on January 22, 2009).

Culture Methods

Algae are cultured in approximately 300 mL of nutrient media (same as dilution medium with the exception of additional $NaHCO_3$) prepared with deionized water and reagent grade chemicals. Cell counts are performed weekly to ensure that the cells are in log phase of growth and to verify that the culture is axenic. A new culture is started weekly using inoculum from the previous culture. Cultures of *P. subcapitata* are held at 22 - 25°C under continuous illumination (4440 to 4730 Lux) provided by cool-white fluorescent bulbs.

MATERIALS and METHODS (CONT'D)

Test System (cont'd)

Number

Initial concentration of algae was approximately 1.0 E+04 cells/mL in each replicate chamber.

Age at Initiation of Exposure

Algae were taken from 5-day old stock cultures in log phase of growth.

Test System Identification

Test organisms were not individually identified. All test chambers were labeled to show study number, loading rate, replicate, and observation day.

EXPERIMENTAL PROCEDURE

Equilibration and Stability

A WAF equilibration trial was performed prior to testing as part of the Daphnia acute immobilization study (Study number 1057642) to determine the most appropriate mixing duration and to verify the analytical method for measuring 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. Additionally, once the WAF solutions were created, they were found to be acceptably stable over a 48-hour period. Results of the equilibrium and stability studies can be found in Appendix C.

Range-Finding Trial

A 96-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, 10 and 100 mg/L. The results of the range-finding trial are presented in Appendix D. As defined in the protocol, the range-finding trial of this study was not performed in a GLP compliant manner.

EXPERIMENTAL PROCEDURE (CONT'D)

Definitive Test Design

GROUP	LOADING RATE* (mg/L)	NUMBER OF CELLS PER mL
1 (Control)	0	1.0 E+04 (12 replicates)
2	0.10	1.0 E+04 (12 replicates)
3	0.32	1.0 E+04 (12 replicates)
4	1.02	1.0 E+04 (18 replicates)
5	3.28	1.0 E+04 (12 replicates)
6	10.5	1.0 E+04 (18 replicates)

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

Preparation and Administration of Test Substance

Individual WAF treatments were prepared for each loading rate by adding the appropriate amount of test substance to algal nutrient medium in 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 substance added and converted to mass per unit volume (mg/L) based on its density. The mixing vessels were closed with foil covered rubber stoppers. The mixtures were stirred using a ≤10% (of the static liquid depth) vortex for 24 ± 1 hour on magnetic stirplates with Teflon® coated stirbars at room temperature (22.7 – 23.1°C). After stirring, mixtures were allowed to settle and equilibrate to test temperature for 60 minutes; then WAFs were removed through the outlet at the bottom of the aspirator bottles.

For the assessment of algal growth, 12 replicates were prepared for each experimental group by filling the test chambers with the appropriate WAF or control medium. For the assessment of chemical stability under abiotic conditions, six chemical control replicates were prepared with the 1.02 and 10.5 mg/L WAF solutions. Following the addition of algae, the chemical controls were "poisoned" with the addition of 50 mg/L mercuric chloride solution to eliminate biological processes and verify concentration stability without the influence of living algae in the test chambers.

EXPERIMENTAL PROCEDURE (CONT'D)

Test Chamber / Set Up

Test chambers were 125-mL size glass Erlenmeyer flasks closed with PTFE lined screw caps to prevent contamination, evaporation, and/or volatilization, each containing two 14 mm glass spheres to facilitate mixing. The chambers were filled with approximately 140-mL of the appropriate WAF (no headspace). Test chambers were conditioned with the test solutions prior to the test. The test chambers were placed on shaker tables (100 rpm) to keep the algae in suspension. Due to space limitations, the chemical control flasks were placed on the platform holding the shaker table. The chemical control flasks were not shaken during the process, but exposed to the same light and temperature as the test chambers.

Selection

Replicate chambers 1 through 12 of each loading rate were inoculated with algae and were placed on shaker tables for the duration of the study. Chamber positions were randomly assigned using a computer generated randomization schedule SAS[8] and changed daily throughout the duration of the study. Replicate chambers 13 through 18, prepared as chemical controls at the 1.02 and 10.5 mg/L loadings, were also inoculated with algae. These chambers were placed in the test area, but were not randomized among the test samples, due to space limitations.

Exposure Duration

96 hours (± 1 hour)

Exposure Conditions

Mean test temperature: 23.7°C (sd = 0.06).
Continuous light: mean daily light intensity ranged from 4170 – 4345 Lux.
Oscillation Rate: 100 rpm (verified daily).

An environmental condition study was 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. Lighting was measured twice daily at nine different locations of the shaker table, using a light meter. The sensor was positioned at the same height as the top of the solutions in the flasks.

Experimental Evaluation

Cell density was determined for each test and control chamber using a hemacytometer and microscope at 24, 48, 72 and 96 hours (± 1 hour) after the beginning of the test. Cell density determinations were performed on three replicates at each observation interval and the replicates were then discarded or sampled for concentration verification on Day 3 and 4. The pH for each treatment and control was measured at test initiation and daily after cell density determinations (composite of the three replicates).

EXPERIMENTAL PROCEDURE (CONT'D)

Experimental Evaluation (cont'd)

At test termination, the 3.28 mg/L loading rate was selected for algistatic/algicidal determination based on maximally inhibited growth of algal cells during the exposure. Test chambers in triplicate were prepared with 3.05 mL of 3.28 mg/L test solution diluted with fresh dilution medium to 100 mL for a final concentration of approximately 0.1 mg/L. The subcultures were incubated under conditions similar to the definitive test for ten days. Cell counts were made at 2, 4, 6, 8 and 10 days following initiation of incubation to determine if the growth inhibition effect observed during the 96 hour exposure would be reversible.

Calculations

Acute toxicity results are expressed as percent inhibition of growth derived from either the average specific growth rate (r), yield (y), or cell density relative to the control. The 50% Effect Loading (EL50) is the loading rate of the test substance in dilution medium which is calculated to result in a 50% reduction in growth in a population of test organisms over a specified exposure period. The No Observed Effect Loading Rate (NOELR) is the highest loading rate which does not exhibit a statistical difference from the control. Measured concentrations do not represent all hydrocarbons constituting the test substance. Results expressed as the 50% Effect Concentration (EC50) and the No Observed Effect Concentration (NOEC) 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.

Results were calculated using three approaches; average specific growth rate ($E_rL/C50$), yield ($E_yL/C50$), and cell density (EL/C50). Percent inhibition for each respective endpoint was calculated as:

$$\%I = \frac{(X_C - X_T)}{X_C} \times 100$$

where:
% I: percent inhibition;
- X_C: mean endpoint value for the control group;
- X_T: mean endpoint value for the treatment replicates.

Cell concentrations, yield, average specific growth rates and percent inhibition were calculated using Microsoft Excel®.

EXPERIMENTAL PROCEDURE (CONT'D)

Calculations (cont'd)

The section by section (e.g., each 24-hour interval) and whole test (e.g., 0 – 72 and 0 – 96 h) average specific growth rates for test validity criteria were determined from the following equation:

$$\mu_{i-j} = \frac{ln\ X_j - ln\ X_i}{t_j - t_i}\ (day^{-1})$$

where: μ_{i-j} = average specific growth rate from time i to j
X_i = biomass at time i
X_j = biomass at time j

Yield was calculated as the biomass (cell density) at the end of the test minus the starting biomass for each single vessel of controls and treatments. For each test exposure and control, mean values for yield along with variance estimates were calculated.

To determine the test substance loading rate/concentration effect relationship, the growth rate slope approach was used. The growth rate slope at loading rate / concentration (c) was determined from the regression equation of cell count over time:

$$ln\ (N_{t,c}) = \alpha_c + \mu_c - t$$

where $N_{t,c}$ = measured number of cells/mL at loading rate/concentration (c) and time t
α_c = intercept term (not used in further estimation)
μ_c = growth rate slope at loading rate/concentration (c)

The EL/EC50 values were determined based on the percent inhibition relative to the control values. For growth rate, the EL/EC50 values and confidence intervals were calculated by using a probit regression calculation based on the methods of Finney[9]. Calculations were based on the PROC PROBIT procedure and standard data manipulation methods in SAS[8]. For the cell density and yield endpoints, the statistical method used to calculate the EL/EC50 values and their associated 95% confidence limits was the Trimmed Spearman-Karber Method[10].

The No Observed Effect Loading Rate/Concentration (NOELR/NOEC) values were based on Duncan's Multiple Range test[11], and the Dunnett's test[12] determined from the GLM procedure of SAS[8] with percent inhibition of yield or growth rate slope as the dependent variable and concentration as the independent variable. The Lowest Observed Effect Loading Rate / Lowest Observed Effect Concentration (LOELR/LOEC) is the lowest loading rate or concentration which exhibits a statistical difference from the control. The Shapiro-Wilk test[13] for normality was used to test if the assumption of normality of the residuals was met; if the residuals were normally distributed the NOEC was based on the estimated values, if they were not normally distributed the NOEC was based on the ranks of the estimated values.

RESULTS AND DISCUSSION

The WAF loading rates for the definitive test were 0.0 (control), 0.10, 0.32 1.02, 3.28, and 10.5 mg/L. The corresponding measured hydrocarbon concentrations in the WAFs at the beginning of the test were ND (Not Detected; control), 0.07, 0.27, 0.93, 2.33 and 5.54 mg/L, respectively. 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. At 72 hours, measured hydrocarbon concentrations ranged from 4.1 to 74% of initial concentrations. Measured hydrocarbon concentrations at 96 hours ranged from 1.1 to 6.1% of initial concentrations. Analytical results are presented in Table 1.

Chemical controls were prepared at the 1.02 and 10.5 mg/L loadings. Measured hydrocarbon concentrations in the chemical controls at 72 and 96 hours ranged from 90 to 92% of initial concentrations. The stability of the measured concentrations in the chemical controls indicates no abiotic losses of dissolved hydrocarbons occurred via volatilization or photodecomposition in the sealed test chambers. Furthermore, the decrease in measured hydrocarbon concentrations in the course of the test in the biotic treatment chambers does not clearly correlate to any decrease in growth inhibition, as might happen with a loss of dissolved hydrocarbons. Given these circumstances, OECD Guideline 201[3] suggests it may be appropriate to base the analysis of the results on the initial nominal or measured concentrations. Therefore, the EC50 calculations were calculated using the initial measured hydrocarbon concentrations.

At WAF stirring initiation and termination, all treatments appeared clear with clear test substance floating at the surface. The pH at the beginning of the test ranged from 7.6 to 7.8. The pH increased less than 1.4 units in any treatment or the control at 72 hours, and no more than 1.5 units at 96 hours. An increase in pH is common during use of a sealed exposure system in the algal growth inhibition test. The pH measurements are presented in Table 2.

No undissolved test substance was observed in the test chambers during the study. No unusual cell shapes, color differences, differences in chloroplast morphology, flocculation, adherence of algae to test containers, or aggregation of algal cells were observed.

The mean values for cell density, overall average specific growth rate and yield for each loading concentration at 24 hour intervals are presented in Tables 3, 4 and 5, respectively. Mean values and percent inhibition for the 72 and 96 hour intervals are presented in Table 6. Individual replicate data for cell density, overall average specific growth rate and yield are presented in Appendix E.

RESULTS AND DISCUSSION (CONT'D)

The 72 and 96-hour EL/EC50 values with associated 95% confidence limits for growth rate, yield and cell density are presented below. In addition, all NOELR/NOEC and LOELR/LOEC values for growth rate and yield values are summarized below. Growth curves are depicted in Figure 1 and a graphical representation of the concentration-response relationship is presented in Figure 2.

At termination of the exposure phase, an algistatic/algicidal evaluation was performed. Based on the cell density over ten days, it was determined that the effect on the algal cells from the 96 hour exposure was algistatic and reversible at the loading rate of 3.28 mg/L. Individual and mean cell densities for the algistatic/algicidal determination are presented in Table 7.

Based on the results of the study, all guideline validity criteria were met in this study. Control cell density increased by more than a factor of 16 within 72 hours. The mean coefficient of variation for section-by-section specific growth rates in the control cultures was 22%, which is below the guideline value of 35%. The coefficient of variation of average specific growth rates during the 72-hour period in replicate control cultures was 3% and did not exceed the guideline value of 7%.

	72 hour		96 hour	
Response Variable	Loading Rate* (mg/L)	Day 0 Measured** (mg/L)	Loading Rate* (mg/L)	Day 0 Measured** (mg/L)
Cell density	EL50 = 0.29 (0.25-0.33)	EC50 = 0.23 (0.20-0.27)	EL50 = 0.32 (0.28-0.36)	EC50 = 0.26 (0.22-0.30)
Yield	EyL50 = 0.28 (0.25-0.31) NOELR <0.10 LOELR = 0.32	EyC50 = 0.22 (0.20-0.25) NOEC = 0.07 LOEC = 0.27	EyL50 = 0.31 (0.27-0.35) NOELR = 0.10 LOELR = 0.32	EyC50 = 0.25 (0.22-0.29) NOEC < 0.07 LOEC = 0.27
Growth rate	ErL50 = 0.53 (NC[1]) NOELR = 0.10 LOELR = 0.32	ErC50 = 0.49 (NC[1]) NOEC < 0.07 LOEC = 0.27	ErL50 = 0.80 (NC[1]) NOELR = 0.32 LOELR = 1.02	ErC50 = 0.70 (NC[1]) NOEC = 0.27 LOEC = 0.93

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

**Measured concentration represents the concentration of hydrocarbons that solubilized from the test substance into each WAF at its respective loading rate.

Values in parentheses ( ) are 95% confidence intervals.

[1]NC = Not calculable

PROTOCOL DEVIATIONS

During the Range Finding the light intensity was outside the required range and was not recorded on two of the days. The light intensity was measured between 4200 and 4500 Lux. The pH was not measured on Day 3 of the 100mg/l concentration.

The daily mean light intensity during the study ranged from 4170 – 4345 Lux instead of the protocol specified range of 4440 - 4730 Lux.

The labels on the test chambers did not contain chamber number since their randomization positions were changed daily.

Due to the variability in the cell counts on Day 8 for the algastatic/algicidal determination the analysis was extended to Day 10.

The above deviations are not believed to have affected the outcome or integrity of the study.

GUIDELINE EXCEPTIONS

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

> The concentration of the test substance in solution was not determined prior to use. Test substance analysis was performed on samples of the WAFs taken prior to the start of the test, at 72 and 96 hours.
>
> Consistent with the OECD document on aquatic toxicity testing of complex substances[14], 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.

During the initiation of the algistatic/algicidal determination, test chambers (triplicate) were prepared with 3.05 mL of 3.28 mg/L test solution diluted with fresh dilution medium to a volume of 100 mL. OPPTS 850.5400 guideline recommends removing 0.5 mL of test solution containing growth inhibited algae from each replicate test chamber and to combine in a new test chamber diluted with fresh nutrient media.

These exceptions are not believed to have had an adverse effect on the study results.

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 include the date, the signatures of the Study Director and the Sponsor Representative, and the justification for the change.

The protocol, final report, raw data, computer generated listings of raw data, supporting documentation and a non-study specific sample of the neat test substance 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.

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Table 1. Analytical Results

Loading Rate* (mg/L)	Measured Hydrocarbon Concentration[1] (mg/L)				
	Day 0	Day 3	Percent Retention[2]	Day 4	Percent Retention[2]
0 (Control)	ND	ND	--	ND	--
0.10	0.0716	0.005[3]	7.0	ND	--[4]
0.32	0.270	0.201	74	0.111	41
1.02	0.932[5]	0.055	5.9	0.056	6.1
1.02 w/Mercuric chloride	(0.932)[6]	0.843	90	0.853	92
3.28	2.33	0.095	4.1	0.104	4.5
10.5	5.54	0.336	6.1	0.061[7]	1.1
10.5 w/Mercuric chloride	(5.54)[5]	5.08	92	5.05	91

* Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution medium.
[1] Duplicate analytical samples from the treatment solutions were analyzed and the two values were averaged.
[2] Percent retention was determined by dividing the concentration of the old solution to the new solution concentration x 100.
[3] Three replicates were analyzed, two were detectable, but below the PQL and one was not detected.
[4] Not Calculable
[5] Average of three replicates
[6] Test solutions for the poisoned controls were collected from the corresponding WAF treatments on Day 0.
[7] Detectable, but below the PQL prior to application of the dilution factor.
ND = Not Detected
PQL (Practical Quantitation Limit) = 0.014 mg/L

Table 2. Daily pH Measurements

Loading Rate* (mg/L)	Day				
	0	**1**	**2**	**3**	**4**
Control	7.77	7.97	8.11	8.89	9.18
0.10	7.66	7.72	7.98	9.03	9.20
0.32	7.66	7.69	7.86	8.76	9.17
1.02	7.59	7.67	7.68	7.88	8.12
3.28	7.70	7.69	7.69	7.71	7.64
10.5	7.76	7.71	7.71	7.72	7.48

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

Table 3. Mean Cell Density (cells/mL)

Loading Rate* (mg/L)	Day				
	0	**1**	**2**	**3**	**4**
Control)	1.0 E+04	4.1 E+04	1.3 E+05	3.4 E+05	8.2 E+05
0.10	1.0 E+04	2.5 E+04	1.1 E+05	4.3 E+05	7.9 E+05
0.32	1.0 E+04	2.6 E+04	4.8 E+04	1.3 E+05	3.9 E+05
1.02	1.0 E+04	1.2 E+04	1.5 E+04	1.7 E+04	4.6 E+04
3.28	1.0 E+04	7.5 E+03	7.5 E+03	6.7 E+03	5.0 E+03
10.5	1.0 E+04	8.3 E+03	6.7 E+03	5.0 E+03	5.0 E+03

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

Table 4. Mean Overall[1] Average Specific Growth Rate (day^{-1})

Loading Rate* (mg/L)	Day			
	0 - 1	**0 - 2**	**0 - 3**	**0 - 4**
Control	1.40	1.27	1.18	1.10
0.10	0.91	1.21	1.25	1.09
0.32	0.94	0.78	0.84	0.92
1.02	0.20	0.20	0.17	0.38
3.28	-0.29**	-0.14	-0.13	-0.17
10.5	-0.19	-0.21	-0.23	-0.17

*Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution medium.
**Negative growth rate indicates a decline in cell density compared to the initial cell density.
[1] Overall average specific growth rate was calculated for each whole test period (e.g., 0-1, 0-2 days).

Table 5. Mean Yield (cells/mL)

Loading Rate* (mg/L)	Day			
	1	**2**	**3**	**4**
Control	3.1 E+04	1.2 E+05	3.3 E+05	8.1 E+05
0.10	1.5 E+04	1.0 E+05	4.2 E+05	7.8 E+05
0.32	1.6 E+04	3.8 E+04	1.2 E+05	3.8 E+05
1.02	2.3 E+03	5.0 E+03	7.0 E+03	3.6 E+04
3.28	-2.5 E+03**	-2.5 E+03	-3.3 E+03	-5.0 E+03
10.5	-1.7 E+03	-3.3 E+03	-5.0 E+03	-5.0 E+03

* Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution medium.
**Negative yield indicates a decline in cell density compared to the initial cell density.

Table 6. 72 & 96 Hour Mean Cell Density, Growth Rate, Yield and Percent Inhibition

Loading Rate* (mg/L)	Mean/ % Inhibition	72 hour			96 hour		
		Cell Density (cells/ml)	Yield (cells/ml)	Avg Specific Growth Rate (day^{-1})	Cell Density (cells/ml)	Yield (cells/ml)	Avg Specific Growth Rate (day^{-1})
0 (Control)	mean	3.4 E+05	3.3 E+05	1.18	8.2 E+05	8.1 E+05	1.10
0.1	mean	4.3 E+05	4.2 E+05	1.25	7.9 E+05	7.8 E+05	1.09
	% inhib.	-26%	-27%	-6%	4%	4%	1%
0.32	mean	1.3 E+05	1.2 E+05	0.84	3.9 E+05	3.8 E+05	0.92
	% inhib.	62%	64%	29%	52%	53%	16%
1.02	mean	1.7 E+04	7.0 E+03	0.17	4.6 E+04	3.6 E+04	0.38
	% inhib.	95%	98%	86%	94%	96%	65%
3.28	mean	6.7 E+03	-3.3 E+03**	-0.13**	5.0 E+03	-5.0 E+03	-0.17
	% inhib.	98%	101%	111%	99%	101%	115%
10.5	mean	5.0 E+03	-5.0 E+03	-0.23	5.0 E+03	-5.0 E+03	-0.17
	% inhib.	99%	102%	119%	99%	101%	115%

*

* Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution medium.
** Negative yield / growth rate indicates a decline in cell density compared to the initial cell density.

Table 7. Cell Density for Algistatic/Algicidal determination.

Day	Cell Density[1] (cells/mL)			
	Rep. 1	Rep. 2	Rep. 3	Mean
2	2.5 E+03	2.5 E+03	2.5 E+03	2.5 E+03
4	3.8 E+03	6.3 E+03	5.0 E+03	5.0 E+03
6	2.9 E+04	1.6 E+04	2.0 E+04	2.2 E+04
8	2.5 E+05	1.3 E+05	2.7 E+05	2.2 E+05
10	4.9 E+05	5.4 E+05	4.9 E+05	5.1 E+05

* Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution medium.
[1] Algistatic/algicidal determination was conducted on the 3.28 mg/L treatment group only.

FIGURE 1. GROWTH CURVES

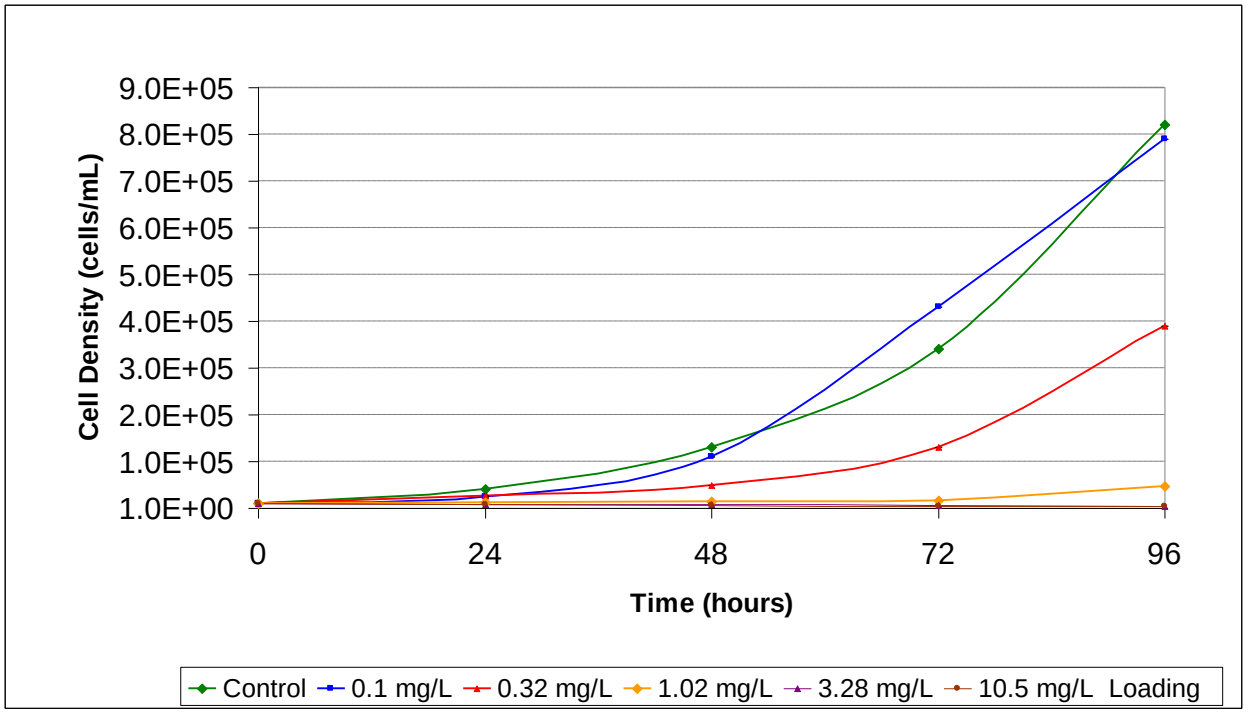
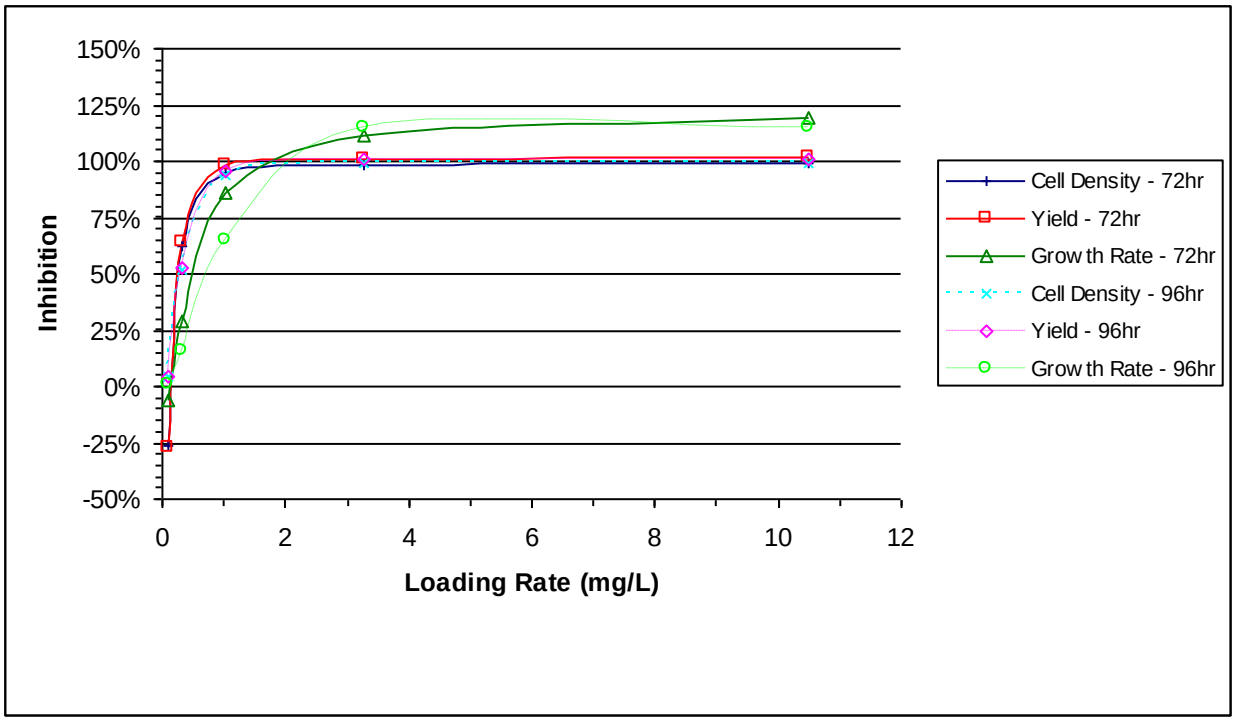


FIGURE 2. CONCENTRATION – RESPONSE CURVES



APPENDIX A – COMPOSITION OF ALGAL NUTRIENT MEDIUM

COMPOUND	CONCENTRATION (mg/L)	ELEMENT	CONCENTRATION (mg/L)
$NaNO_3$	25.500	N	4.200
$MgCl_2 \cdot 6H_2O$	12.164	Mg	2.904
$CaCl_2 \cdot 2H_2O$	4.410	Ca	1.202
$MgSO_4 \cdot 7H_2O$	14.700	S	1.911
K_2HPO_4	1.044	P	0.186
$NaHCO_3$*	15.000	Na	11.001
		K	0.469
		C	2.143

COMPOUND	CONCENTRATION (µg/L)	ELEMENT	CONCENTRATION (µg/L)
H_3BO_3	185.520	B	32.460
$MnCl_2 \cdot 4H_2O$	415.610	Mn	115.374
$ZnCl_2$	3.271	Zn	1.570
$CoCl_2 \cdot 6H_2O$	1.428	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.260	Mo	2.878
$FeCl_3 \cdot 6H_2O$	160.000	Fe	33.051
$Na_2EDTA \cdot 2H_2O$	300.00		

* An additional 400 mg of $NaHCO_3$/L, added as a carbon source in a no headspace environment.

APPENDIX B - 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, 41.45 115 and 345 ng/mL with a constant 27.0 ng/mL concentration of the o-xylene 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 three 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. Stability analysis of the test substance in the algae treatments was not conducted prior to or concomitantly with the in-life period of the study as required by GLPs.

Data were acquired and processed using Perkin Elmer TotalChrom Workstation software (version 6.3.1). Results are presented in Table B1. Standards analysis resulted in a linear response over the standard concentration range and is represented in Figure B-1.

Light catalytic cracked gas oil 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 B-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 (0.10 mg/L) and high (3.28 mg/L) sample loadings. The total area integrated for the detected hydrocarbons was used for quantification. The o-xylene internal standard eluted at about three 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 B - ANALYTICAL METHOD and RESULTS (CONT'D)

Table B1. Individual Analytical Results

Sample	Day 0	Day 3	Day 4
Control	ND	ND	ND
0.10 mg/ L D1	0.0691	0.0001 (<)	ND
0.10 mg/ L D2	0.0741	0.0103 (<)	ND
0.10 mg/ L D3	NA	ND	NA
0.32 mg/ L D1	0.276	0.204	0.112
0.32 mg/ L D2	0.264	0.197	0.110
1.02 mg/L D1	1.01	0.0553	0.0607
1.02 mg/L D2	0.822	0.0551	0.0522
1.02 mg/L D3	0.965	NA	NA
1.02 mg/L w/Mercuric chloride D1	NA	0.913	0.916
1.02 mg/L w/Mercuric chloride D2	NA	0.773	0.790
3.28 mg/L D1	2.30	0.106	0.104
3.28 mg/L D2	2.35	0.0843	0.103
10.5 mg/L D1	5.67	0.357	0.0500
10.5 mg/L D2	5.40	0.314	0.0725
10.5 mg/L w/Mercuric chloride D1	NA	5.02	5.35
10.5 mg/L w/Mercuric chloride D2	NA	5.14	4.75

D1 and D2 represent duplicate analyses of a composite of each exposure solution. D3 represents a triplicate analysis.
ND = Not Detected.
NA = Not Applicable.
PQL = is 0.014 µg/mL (lowest analytical standard)
< = detected below PQL.
Results expressed as µg/mL.

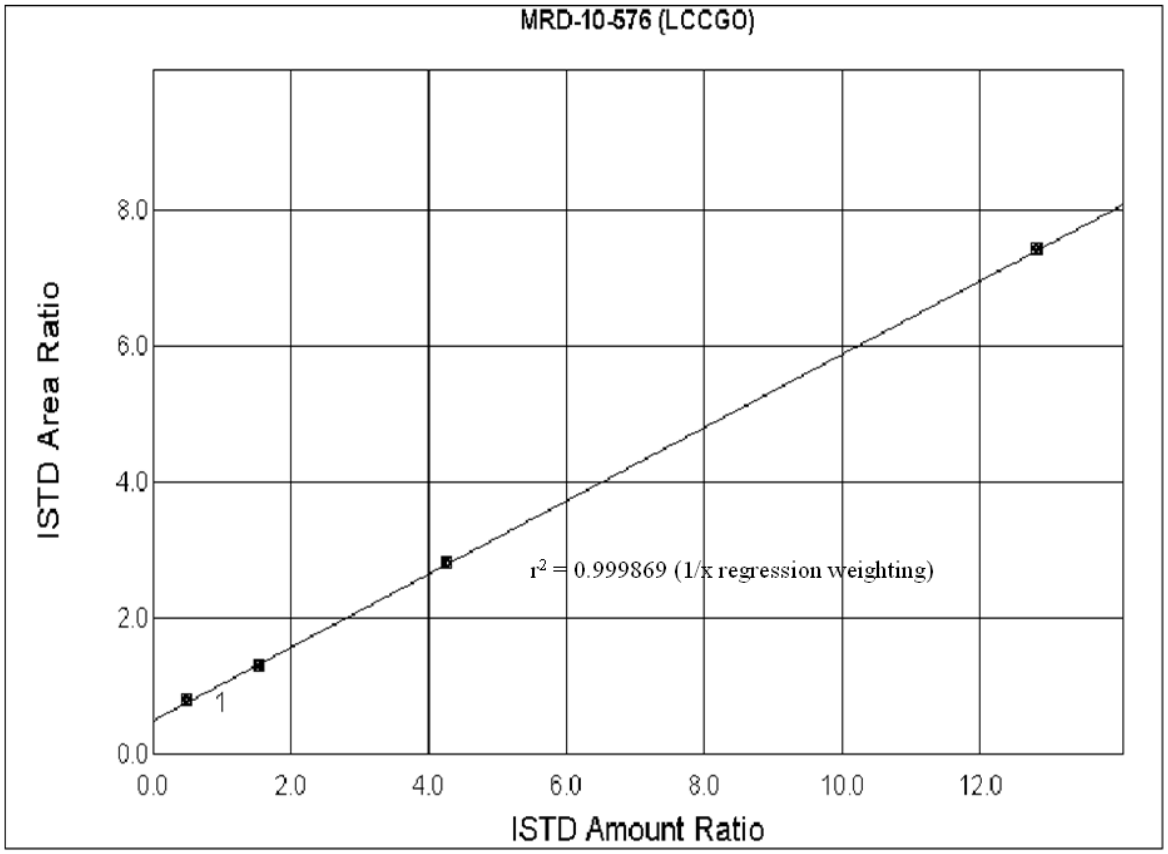
D. J. Letinski, M.S.; Environmental Chemistry
Laboratory Coordinator; Principal Investigator for
Characterization & Analysis of Test Solutions

19 Dec 2011
Date

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

Figure B-1

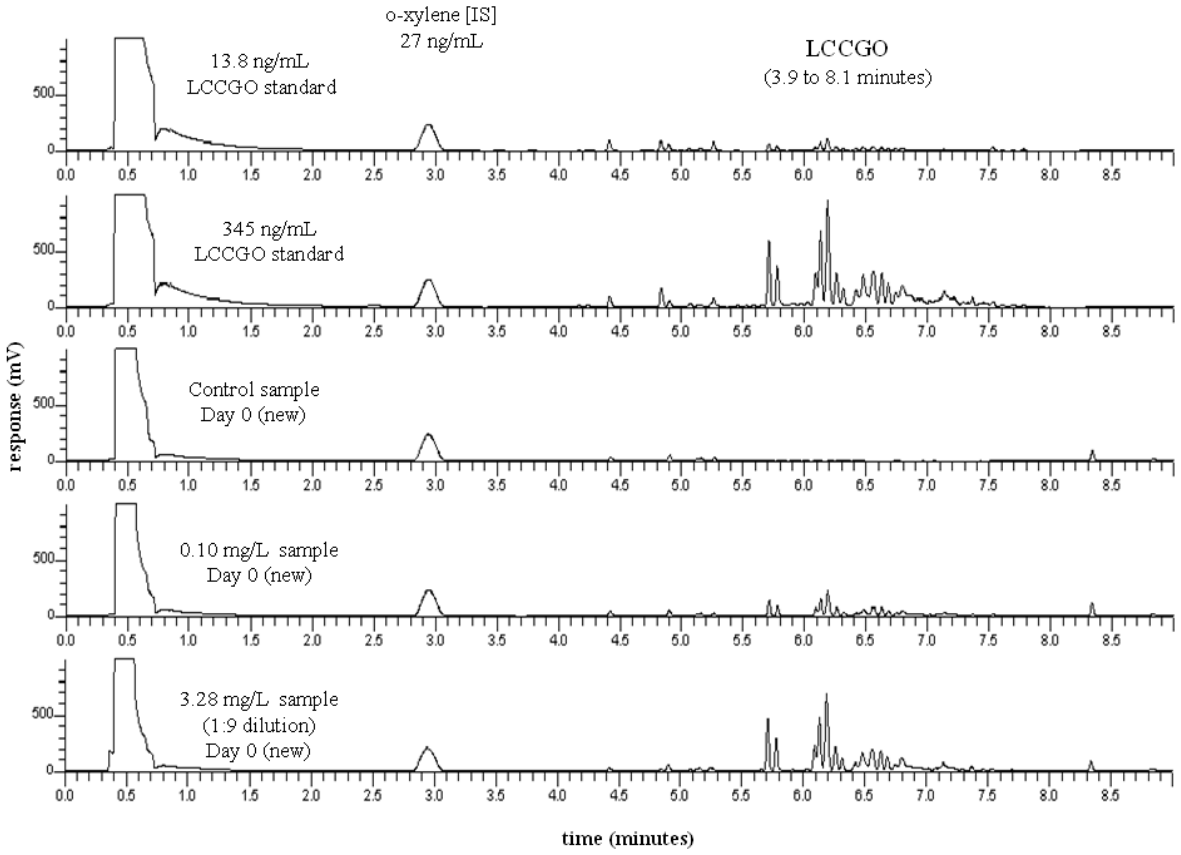
Gas Oil Standard Curve



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

Figure B-2

Gas Oil Standard and Sample Chromatogram



APPENDIX C - WAF EQUILIBRATION AND STABILITY TRIALS

Introduction

A WAF equilibration trial was performed. The purpose of the equilibration trial was to confirm the analytical method to be used in subsequent testing, to determine the optimum mixing duration to use in WAF preparation 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 chronic test with *Daphnia magna*, and to determine whether or not a renewal was needed for the acute test with *D. magna*.

Mixtures of hard reconstituted water and test substance were prepared at loading levels of 0.1, 0.5 and 5.0 mg/L. 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 sections. 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 B. 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 C1.

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.

As shown in Figure C1, the analytical results of the WAF Equilibration Testing indicate that maximum dissolution of the light catalytic cracked 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.

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

Table C1 - 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 medium.
[1] Sample error – no result.
[2] Measured solubilized hydrocarbon concentration when compared to the loading rate.

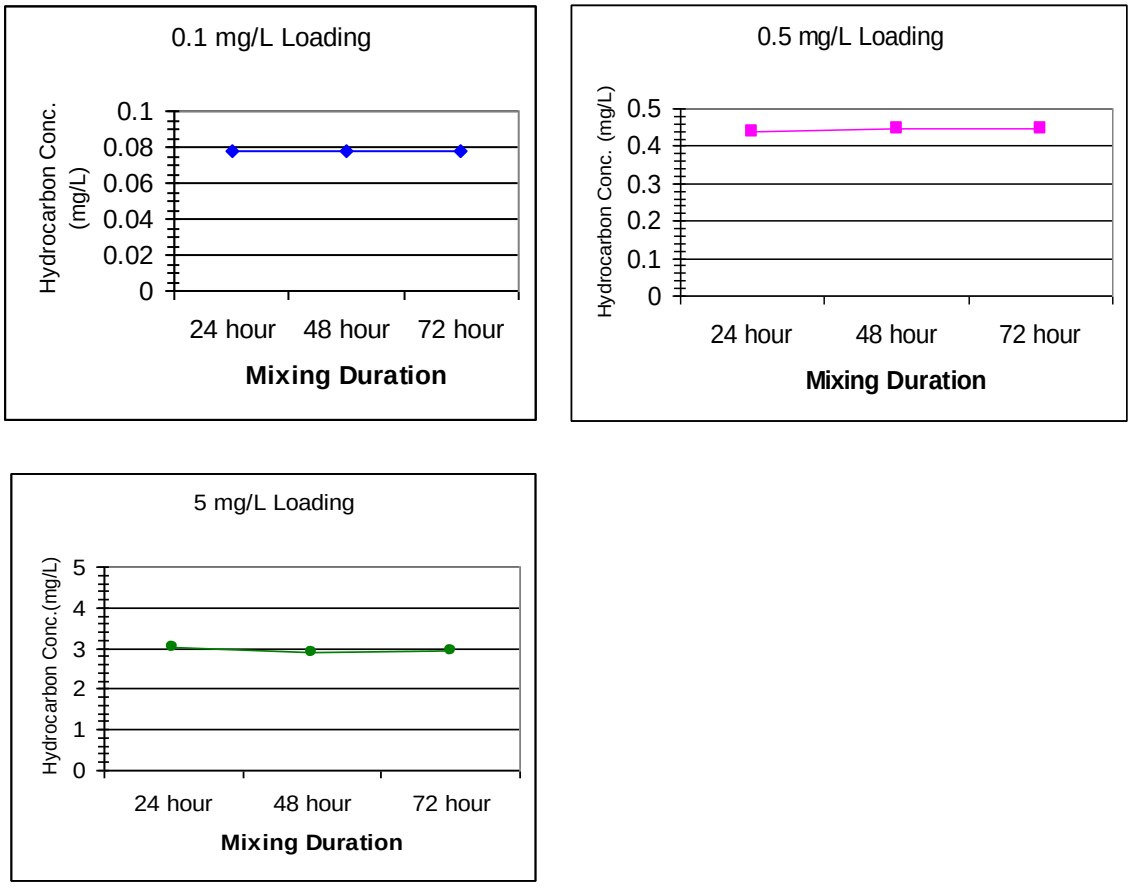


Figure C1. Concentration plots of measured hydrocarbons in WAFs at different mixing times and loading rates.

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

Assessment of WAF Stability

The WAF stability was assessed primarily to establish the renewal interval to be used in the chronic test with *Daphnia magna*, and determine whether a renewal was necessary for the acute *D. magna* test. 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, samples were collected at each loading level directly into screw-top sealed test chambers (130 mL, no headspace) identical to those anticipated for use in the definitive *D. magna* 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 *D. magna* 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 C2. 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 medium.
[1] 0-hour concentration for stability assessment.
[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, it was determined that a renewal was not necessary for the 48-hour daphnid acute testing and that a 48-hour renewal period would suffice for the chronic testing.

APPENDIX D – RANGE FINDING TEST

A 96-hour range-finding trial was performed to determine the appropriate WAF nominal loading rate range of light catalytic cracked gas oil (CAS No. 64741-59-9) on the growth of *Pseudokirchneriella subcapitata*.

Water-accommodated fractions (WAFs) were prepared at nominal loading rates of 0.1, 1.0, 10 and 100 mg/L. A control treatment consisting only of the dilution (algal media) water also was prepared. WAFs were prepared by adding the appropriate amount of test substance, via stainless steel and glass syringes and plastic syringe for the 100mg/L WAF, 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 ± 2°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 1 hour ± 15 minutes.

For the assessment of algal growth, 12 replicates were prepared for each treatment group by filling the test chambers with the appropriate WAF or control medium. Initial concentration of algae was approximately 1.0 E+04 cells/mL in each replicate chamber. Replicate chambers were 125 mL erlenmeyer flasks containing approximately 140 mL of solution (no headspace) closed with PTFE lined plastic caps. Test chambers were placed in an environmentally controlled chamber, and continuously oscillated on a shaker table at 100 rpm to keep the algae in suspension. Continuous lighting conditions, with the intensity between 4200 and 4500 Lux at a mean temperature of 24°C. The pH of the WAFs at the beginning of the test ranged from 7.8 to 8.0, and ranged from 7.5 to 9.6 at the end of the test. Cell density was determined for each test and control chamber using a hemacytometer and microscope at 24, 48, 72 and 96 hours (± 1 hour) after the beginning of the test. Cell density determinations were performed on three replicates at each observation interval and the replicates were then discarded. Analytical samples were collected from the individual WAFs at test initiation. Composite samples of the "old" solutions from the replicate test chambers were also collected for analysis on Day 3 and test termination.

Following 96 hours of exposure, the lowest treatment (0.1 mg/L) was observed to have no inhibition in growth when compared to the control. A noticeable reduction in growth (cell density) was observed at the 1.0 and 10 mg/L loadings and a complete reduction of growth occurred at 100 mg/L loadings. A summary of the cell density is presented in Table D1. Analytical results are presented in Table D2. The range finding trial of this study was not performed in a GLP compliant manner as defined in the protocol.

APPENDIX D – RANGE FINDING TEST (CONT'D)

Table D-1. Mean Cell Density (cells/mL) for the Range finding Test

Loading Rate* (mg/L)	Day				
	0	1	2	3	4 (% inhibition)
Control (ND)	1.0 E+04	1.5 E+04	8.5 E+04	3.9 E+05	8.7 E+05 (--)
0.10 (0.034)	1.0 E+04	1.8 E+04	1.1 E+05	3.7 E+05	1.1 E+06 (-26)
1.0 (0.36)	1.0 E+04	1.0 E+04	1.7 E+04	3.7 E+04	7.8 E+04 (91)
10 (2.1)	1.0 E+04	1.0 E+04	1.0 E+04	1.2 E+04	1.5 E+04 (98)
100 (4.1)	1.0 E+04	1.0 E+04	1.0 E+04	1.0 E+04	3.3 E+03 (100)

* Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution medium.
ND = Not Detected

Table D-2. Analytical Results for the Range finding Test

Loading Rate* (mg/L)	Measured Concentration**		
	Day 0	Day 3	Day 4
Control	ND	ND	ND
0.1 mg/L	0.0768	0.0150	0.0112
1.0 mg/L	0.888	0.0997	0.0953
10 mg/L	5.12	0.824	0.399
100 mg/L	10.2	1.92	0.194

* Loading rate is defined by the amount of light catalytic cracked gas oil per unit volume of dilution medium.
**Measured concentration represents the concentration of hydrocarbons that solubilized from the test substance into each WAF at its respective loading rate.
ND = Not Detected

APPENDIX E – BIOLOGICAL DATA

Cell Density by Replicate (cells/mL)

Loading Rate* (mg/L)	Initial	Rep.	Day 1	Rep.	Day 2	Rep.	Day 3	Rep.	Day 4
Control	1.0 E+04 1.0 E+04 1.0 E+04	1 2 3	4.4 E+04 3.9 E+04 3.9 E+04	4 5 6	1.3 E+05 1.2 E+05 1.3 E+05	7 8 9	3.1 E+05 3.5 E+05 3.7 E+05	10 11 12	8.0 E+05 8.6 E+05 8.0 E+05
0.10	1.0 E+04 1.0 E+04 1.0 E+04	1 2 3	2.8 E+04 2.4 E+04 2.3 E+04	4 5 6	1.2 E+05 9.9 E+04 1.2 E+05	7 8 9	4.2 E+05 4.1 E+05 4.5 E+05	10 11 12	7.6 E+05 7.9 E+05 8.3 E+05
0.32	1.0 E+04 1.0 E+04 1.0 E+04	1 2 3	2.5 E+04 2.8 E+04 2.4 E+04	4 5 6	5.4 E+04 4.3 E+04 4.6 E+04	7 8 9	1.3 E+05 1.2 E+05 1.3 E+05	10 11 12	4.3 E+05 3.8 E+05 3.6 E+05
1.02	1.0 E+04 1.0 E+04 1.0 E+04	1 2 3	1.4 E+04 1.3 E+04 1.0 E+04	4 5 6	1.5 E+04 1.6 E+04 1.4 E+04	7 8 9	1.4 E+04 1.8 E+04 1.9 E+04	10 11 12	4.5 E+04 4.0 E+04 5.4 E+04
3.28	1.0 E+04 1.0 E+04 1.0 E+04	1 2 3	7.5 E+03 7.5 E+03 7.5 E+03	4 5 6	7.5 E+03 7.5 E+03 7.5 E+03	7 8 9	7.5 E+03 6.3 E+03 6.3 E+03	10 11 12	5.0 E+03 5.0 E+03 5.0 E+03
10.5	1.0 E+04 1.0 E+04 1.0 E+04	1 2 3	1.0 E+04 7.5 E+03 7.5 E+03	4 5 6	7.5 E+03 5.0 E+03 7.5 E+03	7 8 9	3.8 E+03 5.0 E+03 6.3 E+03	10 11 12	5.0 E+03 5.0 E+03 5.0 E+03

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

APPENDIX E – BIOLOGICAL DATA (CONT'D)

Overall Average Specific Growth Rate by Replicate (day^{-1})

Loading Rate* (mg/L)	Rep.	Day 0 - 1	Rep.	Days 1 - 2	Rep.	Days 2 - 3	Rep.	Days 3 - 4
Control	1	1.48	4	1.08	7	0.87	10	0.95
	2	1.36	5	1.12	8	1.07	11	0.90
	3	1.36	6	1.20	9	1.05	12	0.77
0.10	1	1.03	4	1.46	7	1.25	10	0.59
	2	0.88	5	1.42	8	1.42	11	0.66
	3	0.83	6	1.65	9	1.32	12	0.61
0.32	1	0.92	4	0.77	7	0.88	10	1.20
	2	1.03	5	0.43	8	1.03	11	1.15
	3	0.88	6	0.65	9	1.04	12	1.02
1.02	1	0.34	4	0.07	7	-0.07	10	1.17
	2	0.26	5	0.21	8	0.12	11	0.80
	3	0	6	0.34	9	0.31	12	1.04
3.28	1	-0.29	4	0	7	0.00	10	-0.41
	2	-0.29	5	0	8	-0.17	11	-0.23
	3	-0.29	6	0	9	-0.17	12	-0.23
10.5	1	0	4	-0.29	7	-0.68	10	0.27
	2	-0.29	5	-0.41	8	0	11	0
	3	-0.29	6	0	9	-0.17	12	-0.23

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

APPENDIX E – BIOLOGICAL DATA (CONT'D)

Yield by Replicate (cells/mL)

Loading Rate* (mg/L)	Initial	Rep.	Day 1	Rep.	Day 2	Rep.	Day 3	Rep.	Day 4
Control	1.0 E+04 1.0 E+04 1.0 E+04	1 2 3	3.4 E+04 2.9 E+04 2.9 E+04	4 5 6	1.2 E+05 1.1 E+05 1.2 E+05	7 8 9	3.0 E+05 3.4 E+05 3.6 E+05	10 11 12	7.9 E+05 8.5 E+05 7.9 E+05
0.10	1.0 E+04 1.0 E+04 1.0 E+04	1 2 3	1.8 E+04 1.4 E+04 1.3 E+04	4 5 6	1.1 E+05 8.9 E+04 1.1 E+05	7 8 9	4.1 E+05 4.0 E+05 4.4 E+05	10 11 12	7.5 E+05 7.8 E+05 8.2 E+05
0.32	1.0 E+04 1.0 E+04 1.0 E+04	1 2 3	1.5 E+04 1.8 E+04 1.4 E+04	4 5 6	4.4 E+04 3.3 E+04 3.6 E+04	7 8 9	1.2 E+05 1.1 E+05 1.2 E+05	10 11 12	4.2 E+05 3.7 E+05 3.5 E+05
1.02	1.0 E+04 1.0 E+04 1.0 E+04	1 2 3	4.0 E+03 3.0 E+03 0	4 5 6	5.0 E+03 6.0 E+03 4.0 E+03	7 8 9	4.0 E+03 8.0 E+03 9.0 E+03	10 11 12	3.5 E+04 3.0 E+04 4.4 E+04
3.28	1.0 E+04 1.0 E+04 1.0 E+04	1 2 3	-2.5 E+03 -2.5 E+03 -2.5 E+03	4 5 6	-2.5 E+03 -2.5 E+03 -2.5 E+03	7 8 9	-2.5 E+03 -3.7 E+03 -3.7 E+03	10 11 12	-5.0 E+03 -5.0 E+03 -5.0 E+03
10.5	1.0 E+04 1.0 E+04 1.0 E+04	1 2 3	0 -2.5 E+03 -2.5 E+03	4 5 6	-2.5 E+03 -5.0 E+03 -2.5 E+03	7 8 9	-6.2 E+03 -5.0 E+03 -3.7 E+03	10 11 12	-5.0 E+03 -5.0 E+03 -5.0 E+03

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

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.

D. J. Letinski, M.S.; Principal Investigator for Characterization (located at the testing facility)

9 Nov 2011
Date

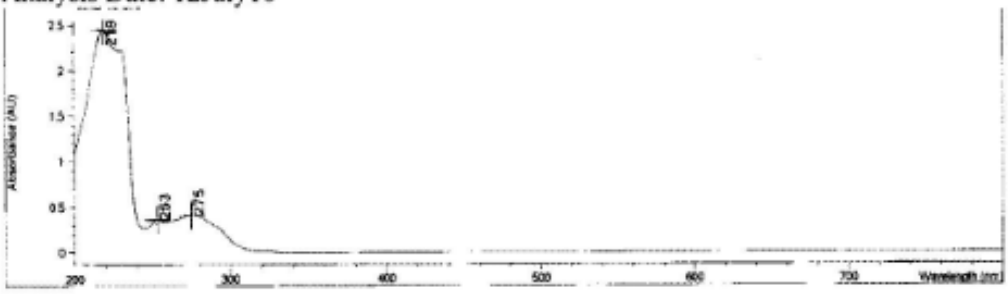
APPENDIX F - TEST SUBSTANCE CHARACTERIZATION (CONT'D)

TEST SUBSTANCE CHARACTERIZATION (CONT'D)

UV-VIS SPECTRA

Figure: UV-VIS-1
Initial

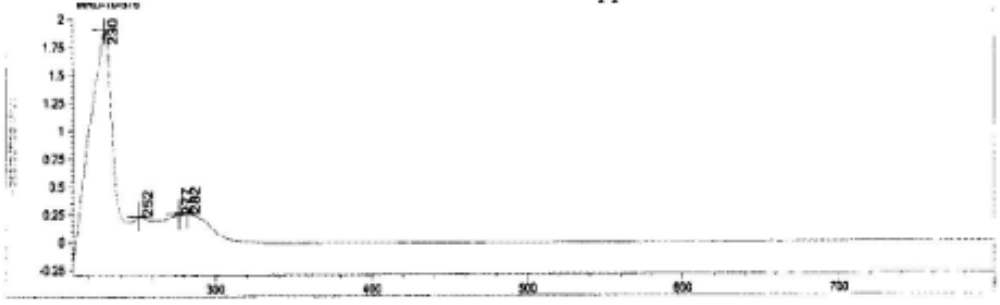
Initial Characterization MRD-10-576 17.8 ppm solution in hexane
Analysis Date: 12July10



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

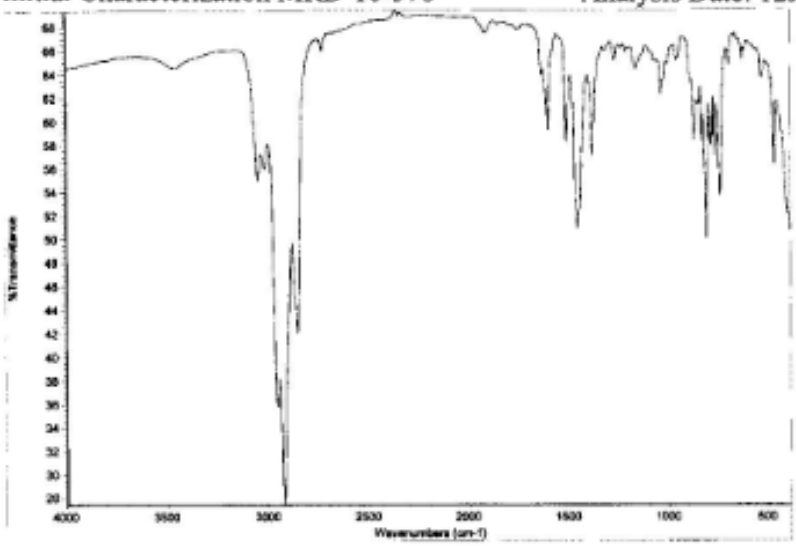
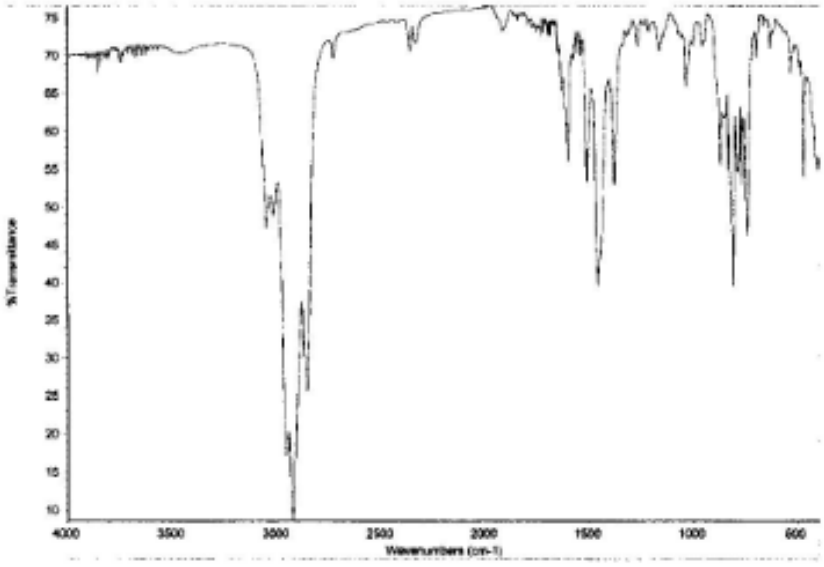


Figure: FTIR-2
Final

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



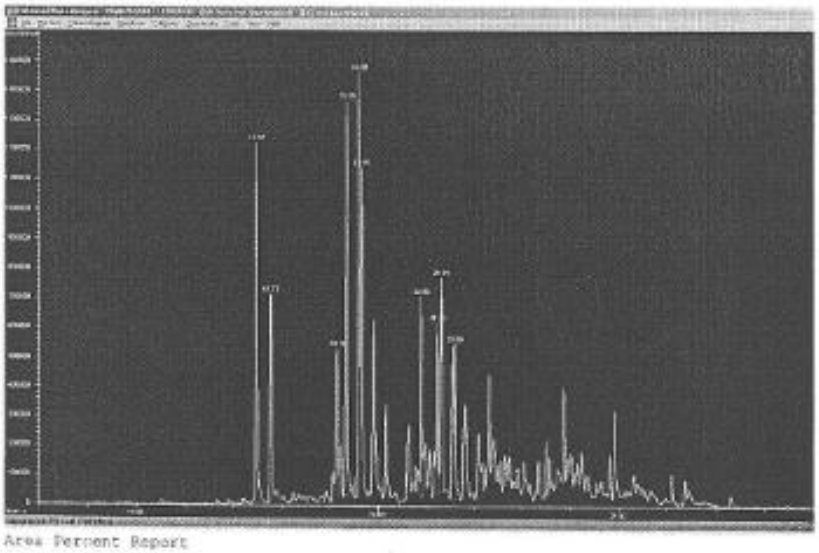
APPENDIX F - TEST SUBSTANCE CHARACTERIZATION (CONT'D)

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 characterisation) 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	1827	1838	1855	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	38097107	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	544030	13289373	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

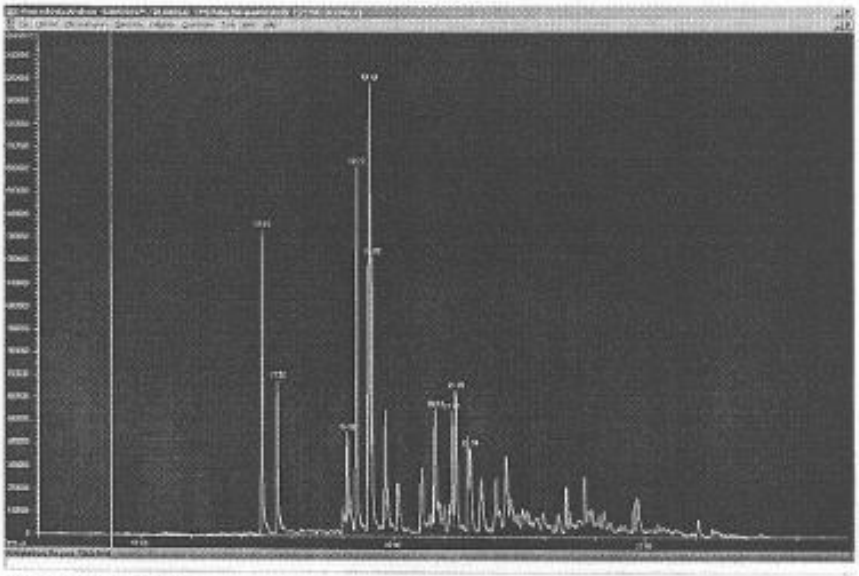
APPENDIX F - TEST SUBSTANCE CHARACTERIZATION (CONT'D)

TEST SUBSTANCE CHARACTERIZATION (CONT'D)

TOTAL ION CHROMATOGRAM

Figure: Total Ion Chromatogram-2

FINAL



Area Percent Report

Data File : C:\EPCHEM\1\DATA\CHAR2010\26JUL02.D Vial: 2
Acq On : 26 Jul 2011 9:52 Operator:
Sample : MRD-10-576(final characterization)10%v/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:\EPCHEM\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	3176	BV	1929536	42798102	100.00%	21.914%
6	19.582	3174	3179	3207	VB	1191563	23621237	55.19%	12.095%
7	20.631	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	12203165	28.51%	6.248%
10	21.536	3544	3548	3569	VB 3	357407	9263002	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 – STATISTICAL OUTPUT

The SAS System 17:55 Thursday, February 24, 2011 68

72hr ErL50 (Loading Rate) Alb 15Nov11

OECD 72 hr ErC50 CALCULATION BASED ON THE SLOPES OF THE GROWTH RATES
ErCxx 72 hr VALUES FOR DATA +/- 95 % CONFIDENCE INTERVALS

***** Confidence Intervals are for Information Only - May Not Be Appropriate Report *****

The Probit Procedure

Probit Analysis on CONC

Probability	CONC	95% Fiducial Limits	
0.01	-0.99916	.	.
0.02	-0.81981	.	.
0.03	-0.70602	.	.
0.04	-0.62042	.	.
0.05	-0.55079	.	.
0.06	-0.49152	.	.
0.07	-0.43956	.	.
0.08	-0.39303	.	.
0.09	-0.35071	.	.
0.10	-0.31176	.	.
0.15	-0.15049	.	.
0.20	-0.02232	.	.
0.25	0.08764	.	.
0.30	0.18639	.	.
0.35	0.27789	.	.
0.40	0.36472	.	.
0.45	0.44873	.	.
0.50	0.53141	.	.
0.55	0.61408	.	.
0.60	0.69809	.	.
0.65	0.78492	.	.
0.70	0.87642	.	.
0.75	0.97517	.	.
0.80	1.08513	.	.
0.85	1.21330	.	.
0.90	1.37457	.	.
0.91	1.41352	.	.
0.92	1.45584	.	.
0.93	1.50237	.	.
0.94	1.55433	.	.
0.95	1.61360	.	.
0.96	1.68323	.	.
0.97	1.76883	.	.
0.98	1.88262	.	.
0.99	2.06197	.	.

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

The SAS System 17:55 Thursday, February 24, 2011 99

96hr ErL50 (Loading Rate) MCB 15Nov11

OECD 96 hr ErC50 CALCULATION BASED ON THE SLOPES OF THE GROWTH RATES
ErCxx 96 hr VALUES FOR DATA +/- 95 % CONFIDENCE INTERVALS

***** Confidence Intervals are for Information Only - May Not Be Appropriate Report *****

The Probit Procedure

Probit Analysis on CONC

Probability	CONC	95% Fiducial Limits	
0.01	-0.85656	.	.
0.02	-0.66200	.	.
0.03	-0.53856	.	.
0.04	-0.44570	.	.
0.05	-0.37016	.	.
0.06	-0.30587	.	.
0.07	-0.24950	.	.
0.08	-0.19902	.	.
0.09	-0.15312	.	.
0.10	-0.11086	.	.
0.15	0.06409	.	.
0.20	0.20313	.	.
0.25	0.32242	.	.
0.30	0.42954	.	.
0.35	0.52880	.	.
0.40	0.62300	.	.
0.45	0.71413	.	.
0.50	0.80382	.	.
0.55	0.89351	.	.
0.60	0.98464	.	.
0.65	1.07883	.	.
0.70	1.17810	.	.
0.75	1.28522	.	.
0.80	1.40451	.	.
0.85	1.54355	.	.
0.90	1.71850	.	.
0.91	1.76075	.	.
0.92	1.80666	.	.
0.93	1.85713	.	.
0.94	1.91351	.	.
0.95	1.97780	.	.
0.96	2.05333	.	.
0.97	2.14620	.	.
0.98	2.26964	.	.
0.99	2.46420	.	.

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

The SAS System 17:55 Thursday, February 24, 2011 106

OECD NOEC 72 hr CALCULATION BASED ON THE SLOPES OF THE GROWTH RATES (72 hr ErCxx)
DUNCAN AND DUNNETT ANALYSIS FOR 72 hr NOEC DETERMINATION
COMPARE INHIB (INHIBITION) AND RKINHIB (RANKED INHIBITION) FOR USE:
USE INHIB IF ALL ASSUMPTIONS ARE MET OTHERWISE USE RKINHIB ANALYSIS

The GLM Procedure

Duncan's Multiple Range Test for INHIB

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.000663

Number of Means	2	3	4	5	6
Critical Range	.04580	.04794	.04924	.05010	.05069

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	CONC
A	0.99947	3	10.5
A			
A	0.99908	3	3.28
B	0.84916	3	1.02
C	0.29870	3	0.32
D	0.00000	3	0
E	-0.08764	3	0.1

- Inhibitory LOEC
MEB
8 Nov 11

(corresponding measured concentration
NOEC = 0.07
LOEC = 0.27)

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

The SAS System 17:55 Thursday, February 24, 2011 122

OECD NOEC 96 hr CALCULATION BASED ON THE SLOPES OF THE GROWTH RATES (96 hr ErCxx)
DUNCAN AND DUNNETT ANALYSIS FOR 96 hr NOEC DETERMINATION
COMPARE INHIB (INHIBITION) AND RKINHIB (RANKED INHIBITION) FOR USE:
USE INHIB IF ALL ASSUMPTIONS ARE MET OTHERWISE USE RKINHIB ANALYSIS

The GLM Procedure

Dunnett's t Tests for RKINHIB

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.076889
Critical Value of Dunnett's t	2.90126
Minimum Significant Difference	0.6569

Comparisons significant at the 0.05 level are indicated by ***.

CONC Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
10.5 - 0	2.0125	1.3556	2.6693	***
3.28 - 0	1.4093	0.7524	2.0661	***
1.02 - 0	0.8793	0.2224	1.5361	***
0.32 - 0	0.4615	-0.1953	1.1184	
0.1 - 0	-0.7402	-1.3970	-0.0833	***

Inhibitory LOEC BEG 8 Nov 11

The SAS System 17:55 Thursday, February 24, 2011 123

OECD NOEC 96 hr CALCULATION BASED ON THE SLOPES OF THE GROWTH RATES (96 hr ErCxx)
DUNCAN AND DUNNETT ANALYSIS FOR 96 hr NOEC DETERMINATION
COMPARE INHIB (INHIBITION) AND RKINHIB (RANKED INHIBITION) FOR USE:
USE INHIB IF ALL ASSUMPTIONS ARE MET OTHERWISE USE RKINHIB ANALYSIS

The GLM Procedure

Duncan's Multiple Range Test for RKINHIB

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.076889

Number of Means	2	3	4	5	6
Critical Range	.4933	.5163	.5303	.5395	.5459

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	CONC

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

	A	1.3421	3	10.5	
	B	0.7389	3	3.28	
	C	0.2089	3	1.02	Inhibitory LOEL
	C				
D	C	-0.2089	3	0.32	(corresponding LOEC = 0.93)
D					
D		-0.6704	3	0	MEB 15Nov11
	E	-1.4106	3	0.1	

The SAS System 17:55 Thursday, February 24, 2011 124

OECD NOEC 96 hr CALCULATION BASED ON THE SLOPES OF THE GROWTH RATES (96 hr ErCxx)
NORMALITY TEST FOR 96 hr NOEC EVALUATION

COMPARE NORMALITY FOR INHIB AND RKIHNIB FOR PARAMETRIC ASSUMPTIONS

The UNIVARIATE Procedure
Variable: RINHIB

Moments

N	18	Sum Weights	18
Mean	0	Sum Observations	0
Std Deviation	0.02008189	Variance	0.00040328
Skewness	-1.2713499	Kurtosis	3.66389691
Uncorrected SS	0.0068558	Corrected SS	0.0068558
Coeff Variation	.	Std Error Mean	0.00473335

Basic Statistical Measures

Location		Variability	
Mean	0	Std Deviation	0.02008
Median	0.000013	Variance	0.0004033
Mode	0	Range	0.09196
		Interquartile Range	0.00579

Tests for Location: Mu0=0

Test	-Statistic-		-----p Value------	
Student's t	t	0	Pr > |t|	1.0000
Sign	M	1.5	Pr >= |M|	0.6072
Signed Rank	S	12	Pr >= |S|	0.5153

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.83031	Pr < W	0.0042
Kolmogorov-Smirnov	D	0.331408	Pr > D	<0.0100
Cramer-von Mises	W-Sq	0.304526	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	1.431621	Pr > A-Sq	<0.0050

The SAS System 17:55 Thursday, February 24, 2011 125

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

The SAS System 17:55 Thursday, February 24, 2011 172

DUNCAN AND DUNNETT ANALYSIS FOR 72 hour NOEC DETERMINATION

COMPARE I72 (INHIBITION) AND RKI72 (RANKED INHIBITION) FOR USE:
USE I72 IF ALL ASSUMPTIONS ARE MET OTHERWISE USE RKINHIB ANALYSIS

The GLM Procedure

Dunnett's t Tests for RKI72

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.072273
Critical Value of Dunnett's t	2.89048
Minimum Significant Difference	0.6345

Comparisons significant at the 0.05 level are indicated by ***.

CONC Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
10.5 - 0	2.5465	1.9120	3.1810	***
3.28 - 0	1.9049	1.2704	2.5394	***
1.02 - 0	1.3101	0.6756	1.9445	***
0.32 - 0	0.7889	0.1544	1.4233	***

The SAS System 17:55 Thursday, February 24, 2011 173

DUNCAN AND DUNNETT ANALYSIS FOR 72 hour NOEC DETERMINATION

COMPARE I72 (INHIBITION) AND RKI72 (RANKED INHIBITION) FOR USE:
USE I72 IF ALL ASSUMPTIONS ARE MET OTHERWISE USE RKINHIB ANALYSIS

The GLM Procedure

Duncan's Multiple Range Test for RKI72

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.072273

Number of Means	2	3	4	5
Critical Range	.4891	.5111	.5240	.5323

Means with the same letter are not significantly different.

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

Duncan Grouping	Mean	N	CONC
A	1.2364	3	10.5
B	0.5948	3	3.28
C	-0.0000	3	1.02
D	-0.5212	3	0.32
E	-1.3101	3	0

Yield 72 hr LOEC

NOEC is 1.02 (corresponding LOEC = 0.27)

The SAS System 17:55 Thursday, February 24, 2011 174

NORMALITY TEST FOR NOEC EVALUATION

COMPARE NORMALITY OF RI72 and RRKI72 FOR PARAMETRIC ASSUMPTIONS

The UNIVARIATE Procedure
Variable: RI72

Moments

N	15	Sum Weights	15
Mean	0	Sum Observations	0
Std Deviation	0.7208206	Variance	0.51958234
Skewness	1.42723466	Kurtosis	3.88084297
Uncorrected SS	7.27415271	Corrected SS	7.27415271
Coeff Variation	.	Std Error Mean	0.18611508

Basic Statistical Measures

Location		Variability	
Mean	0	Std Deviation	0.72082
Median	1.42E-14	Variance	0.51958
Mode	1.42E-14	Range	3.00000
		Interquartile Range	0.33250

Tests for Location: Mu0=0

Test	-Statistic-		-----p Value------	
Student's t	t	0	Pr > |t|	1.0000
Sign	M	1.5	Pr >= |M|	0.6072
Signed Rank	S	-5	Pr >= |S|	0.7932

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.804941	Pr < W	0.0043
Kolmogorov-Smirnov	D	0.334484	Pr > D	<0.0100
Cramer-von Mises	W-Sq	0.269048	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	1.323681	Pr > A-Sq	<0.0050

The SAS System 17:55 Thursday, February 24, 2011 17

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

04:56 Tuesday, November 15, 2011 10

The SAS System

DUNCAN AND DUNNETT ANALYSIS FOR 96 hour NOEC DETERMINATION

COMPARE I96 (INHIBITION) AND RKI96 (RANKED INHIBITION) FOR USE:
USE I96 IF ALL ASSUMPTIONS ARE MET OTHERWISE USE RKINHIB ANALYSIS

The GLM Procedure

Duncan's Multiple Range Test for RKI96

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.09306

Number of Means	2	3	4	5	6
Critical Range	.5427	.5680	.5834	.5936	.6006

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	CONC
A	1.0405	3	3.28
A			
A	1.0405	3	10.5
B	0.2089	3	1.02
B			
B	-0.2089	3	0.32
C	-0.9955	3	0.1
C			
C	-1.0855	3	0

inh.b.tory LOEL
(corresponding LOEC=0.27)
NOEB ISNOII

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

ANALYSIS OF 96HR DATA FOR ALGAL BIOASSAYS 68
STUDY NO. 1057667 15:31 Monday, February 21, 2011

OECD 72 hr ErC50 CALCULATION BASED ON THE SLOPES OF THE GROWTH RATES
ErCxx 72 hr VALUES FOR DATA +/- 95 % CONFIDENCE INTERVALS
***** Confidence Intervals are for Information Only - May Not Be Appropriate Report *****

The Probit Procedure
Probit Analysis on CONC

Probability	CONC	95% Fiducial Limits	
0.01	-0.64263	.	.
0.02	-0.51011	.	.
0.03	-0.42604	.	.
0.04	-0.36279	.	.
0.05	-0.31134	.	.
0.06	-0.26755	.	.
0.07	-0.22915	.	.
0.08	-0.19478	.	.
0.09	-0.16351	.	.
0.10	-0.13473	.	.
0.15	-0.01557	.	.
0.20	0.07913	.	.
0.25	0.16038	.	.
0.30	0.23334	.	.
0.35	0.30095	.	.
0.40	0.36511	.	.
0.45	0.42718	.	.
0.50	0.48827	.	.
0.55	0.54935	.	.
0.60	0.61142	.	.
0.65	0.67558	.	.
0.70	0.74319	.	.
0.75	0.81615	.	.
0.80	0.89740	.	.
0.85	0.99210	.	.
0.90	1.11126	.	.
0.91	1.14004	.	.
0.92	1.17131	.	.
0.93	1.20568	.	.
0.94	1.24408	.	.
0.95	1.28787	.	.
0.96	1.33932	.	.
0.97	1.40257	.	.
0.98	1.48664	.	.
0.99	1.61916	.	.

ANALYSIS OF 96HR DATA FOR ALGAL BIOASSAYS 69
STUDY NO. 1057667 15:31 Monday, February 21, 2011

OECD 96 hr ErC50 CALCULATION BASED ON THE SLOPES OF THE GROWTH RATES

Data used to calculate 96 hr OECD ErC50

Obs	CONC	CD	REP	DAY	LNCD	TIME
1	0.00	10000	1	0	9.2103	0

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

ANALYSIS OF 96HR DATA FOR ALGAL BIOASSAYS 99
STUDY NO. 1057667 15:31 Monday, February 21, 2011

OECD 96 hr ErC50 CALCULATION BASED ON THE SLOPES OF THE GROWTH RATES
ErCxx 96 hr VALUES FOR DATA +/- 95 % CONFIDENCE INTERVALS

***** Confidence Intervals are for Information Only - May Not Be Appropriate Report *****

The Probit Procedure
Probit Analysis on CONC

Probability	CONC	95% Fiducial Limits	
0.01	-0.51721	.	.
0.02	-0.37432	.	.
0.03	-0.28366	.	.
0.04	-0.21546	.	.
0.05	-0.15898	.	.
0.06	-0.11277	.	.
0.07	-0.07136	.	.
0.08	-0.03430	.	.
0.09	-0.00058	.	.
0.10	0.03045	.	.
0.15	0.15894	.	.
0.20	0.26105	.	.
0.25	0.34866	.	.
0.30	0.42733	.	.
0.35	0.50023	.	.
0.40	0.56941	.	.
0.45	0.63634	.	.
0.50	0.70221	.	.
0.55	0.76808	.	.
0.60	0.83501	.	.
0.65	0.90419	.	.
0.70	0.97709	.	.
0.75	1.05576	.	.
0.80	1.14337	.	.
0.85	1.24548	.	.
0.90	1.37397	.	.
0.91	1.40500	.	.
0.92	1.43872	.	.
0.93	1.47578	.	.
0.94	1.51719	.	.
0.95	1.56440	.	.
0.96	1.61988	.	.
0.97	1.68808	.	.
0.98	1.77874	.	.
0.99	1.92163	.	.

ANALYSIS OF 96HR DATA FOR ALGAL BIOASSAYS 100
STUDY NO. 1057667 15:31 Monday, February 21, 2011

OECD NOEC 72 hr CALCULATION BASED ON THE SLOPES OF THE GROWTH RATES (72 hr ErCxx)
DATA FOR CALCULATION OF THE 72 hr NOEC

Obs	CONC	REP	TIME	conslop	INHIB	LNCONC	RKINHIB
1	0.00	1	0.047439	0.048906	0.00000	.	-0.67039
2	0.00	2	0.049125	0.048906	0.00000	.	-0.67039

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

```
 TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

 ENTER DATE OF TEST:
18Jan11
 ENTER TEST NUMBER:
1057667 Loading Levels - % Inhibition for cell density - EL50
 WHAT IS TO BE ESTIMATED?
 (ENTER "L" FOR LC50 AND "E" FOR EC50)
e
 ENTER TEST SPECIES NAME:
P. subcapitata
 ENTER TOXICANT NAME:
MRD-10-576
 ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT :
mg/L
 ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
100
 ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
0
 ENTER THE NUMBER OF CONCENTRATIONS
 (NOT INCLUDING THE CONTROL; MAX = 10):
4
 ENTER THE 4 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):

0.10
0.32
1.02
3.28
 ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
y
 ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:
100
 ENTER UNITS FOR DURATION OF EXPERIMENT
 (ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
h
 ENTER DURATION OF TEST:
72
 ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:

0
62
95
98
 WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
y


 DATE:  18Jan11        TEST NUMBER: 1057667    DURATION:  72 h
 TOXICANT : MRD-10-576
 SPECIES:  P. subcapitata

 RAW DATA:  Concentration     Number    Mortalities
 --- ----  (mg/L)           Exposed
             .00             100        0
             .10             100        0
             .32             100       62
            1.02             100       95
            3.28             100       98

 SPEARMAN-KARBER TRIM:          2.00%

 SPEARMAN-KARBER ESTIMATES:   EC50:          .29
          95% LOWER CONFIDENCE:              .25
          95% UPPER CONFIDENCE:              .33
 ------------------------------------------------------------------------
 WOULD YOU LIKE TO HAVE A COPY SENT TO THE PRINTER(Y/N)?
```

23Feb11
KFB

Verified by:
BKelly
18Oct 11

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

23Feb11
MEB

72-hr
Yield
EyL50

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

ENTER DATE OF TEST:
18Jan11
ENTER TEST NUMBER:
1057667yield - Loading Levels
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
E
ENTER TEST SPECIES NAME:
P. subcapitata
ENTER TOXICANT NAME:
MRD-10-576
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT :
MG/L
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
100
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
0
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL, MAX = 10):
4
ENTER THE 4 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):

0.1
0.32
1.02
3.28
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
Y
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:
100
ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
H
ENTER DURATION OF TEST:
72
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:

0
64
98
100
WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
Y

DATE: 18Jan11 TEST NUMBER: 1057667yield - Loading Levels DURATION: 72 H
TOXICANT : MRD-10-576
SPECIES: P. subcapitata

RAW DATA: Concentration (MG/L)	Number Exposed	Mortalities
.00	100	0
.10	100	0
.32	100	64
1.02	100	98
3.28	100	100

SPEARMAN-KARBER TRIM: .00%

SPEARMAN-KARBER ESTIMATES: EC50: .28
95% LOWER CONFIDENCE: .25
95% UPPER CONFIDENCE: .31

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

23 Feb 11
KEB
cell density
96 hr
EC50

ENTER DATE OF TEST:
18JAN11
ENTER TEST NUMBER:
1057667 LOADING CELL DENSITY 96HR
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
E
ENTER TEST SPECIES NAME:
P. subcapitata
ENTER TOXICANT NAME:
MRD-10-576
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT :
MG/L
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
100
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
0
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL, MAX = 10):
4
ENTER THE 4 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):

0.1
0.32
1.02
3.28
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
Y
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:
100
ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
H
ENTER DURATION OF TEST:
96
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:

4
52
94
99
WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
Y

DATE: 18JAN11 TEST NUMBER: 1057667 DURATION: 96 H
TOXICANT : MRD-10-576
SPECIES: P. subcapitata

RAW DATA: Concentration (MG/L)	Number Exposed	Mortalities
.00	100	0
.10	100	4
.32	100	52
1.02	100	94
3.28	100	99

SPEARMAN-KARBER TRIM: 4.00%

SPEARMAN-KARBER ESTIMATES: EC50: .32
95% LOWER CONFIDENCE: .28
95% UPPER CONFIDENCE: .36

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

23Feb11
MEB

96 hr
Yield
E_yL50

ENTER DATE OF TEST:
18Jan11
ENTER TEST NUMBER:
1057667yield - Loading Levels
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
E
ENTER TEST SPECIES NAME:
P. subcapitata
ENTER TOXICANT NAME:
MRD-10-576
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT:
MG/L
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
100
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
0
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL; MAX = 10):
4
ENTER THE 4 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):

0.10
0.32
1.02
3.28
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
Y
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:
100
ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
Y
ENTER DURATION OF TEST:
96
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:

4
53
96
100
WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
Y

DATE: 18Jan11 TEST NUMBER: 1057667yield - Loading Levels DURATION: 96 H
TOXICANT : MRD-10-576
SPECIES: P. subcapitata

RAW DATA: Concentration (MG/L)	Number Exposed	Mortalities
.00	100	0
.10	100	4
.32	100	53
1.02	100	96
3.28	100	100

SPEARMAN-KARBER TRIM: 4.00%

SPEARMAN-KARBER ESTIMATES: EC50: .31
95% LOWER CONFIDENCE: .27
95% UPPER CONFIDENCE: .35

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

```
TRIMMED SPEARMAN-KARBER METHOD.  VERSION 1.5

 ENTER DATE OF TEST:
18Jan11
 ENTER TEST NUMBER:
1057667  Day0 measured concnetrations  -  % Inhibition for cell density - EC50
 WHAT IS TO BE ESTIMATED?
 (ENTER "L" FOR LC50 AND "E" FOR EC50)
E
 ENTER TEST SPECIES NAME:
P. subcapitata
 ENTER TOXICANT  NAME:
MRD-10-576
 ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT :
mg/L
 ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
100
 ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
0
 ENTER THE NUMBER OF CONCENTRATIONS
 (NOT INCLUDING THE CONTROL;  MAX = 10):
4
 ENTER THE  4 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):

0.07
0.27
0.93
2.33
 ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
y
 ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:
100
 ENTER UNITS FOR DURATION OF EXPERIMENT
 (ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
h
 ENTER DURATION OF TEST:
72
 ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:

0
62
95
98
 WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
y


 DATE:  18Jan11        TEST NUMBER: 1057667     DURATION:  72 h
 TOXICANT :  MRD-10-576
 SPECIES:  P. subcapitata

 RAW DATA:  Concentration     Number     Mortalities
 --- ----  (mg/L)          Exposed
           .00             100        0
           .07             100        0
           .27             100       62
           .93             100       95
          2.33             100       98

 SPEARMAN-KARBER TRIM:          2.00%

 SPEARMAN-KARBER ESTIMATES:   EC50:          .23
          95% LOWER CONFIDENCE:          .20
          95% UPPER CONFIDENCE:          .27
-------------------------------------------------------------------------------
WOULD YOU LIKE TO HAVE A COPY SENT TO THE PRINTER(Y/N)?
```

23Feb 11
MEB

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

23Feb11
MEB
72 hr
Yield
E_yC50

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

ENTER DATE OF TEST:
18JAN11
ENTER TEST NUMBER:
1057667YIELD MEASURED CONC (DAY 0)
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
E
ENTER TEST SPECIES NAME:
P. subcapitata
ENTER TOXICANT NAME:
MRD-10-576
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT :
MG/L
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
100
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
0
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL; MAX = 10).
4
ENTER THE 4 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):

0.07
0.27
0.93
2.33
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
Y
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:
100
ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
H
ENTER DURATION OF TEST:
72
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:

0
64
98
100
WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
Y

DATE: 18JAN11 TEST NUMBER: 1057667YIELD MEASURED CONC (DAY 0)
DURATION: 72 H
TOXICANT : MRD-10-576
SPECIES: P. subcapitata

RAW DATA: Concentration (MG/L)	Number Exposed	Mortalities
.00	100	0
.07	100	0
.27	100	64
.93	100	98
2.33	100	100

SPEARMAN-KARBER TRIM: .00%

SPEARMAN-KARBER ESTIMATES: EC50: .22
95% LOWER CONFIDENCE: .20
95% UPPER CONFIDENCE: .25

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

23Feb11
AE/S
cell density
96hr
EC50

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

ENTER DATE OF TEST:
18JAN11
ENTER TEST NUMBER:
1057667 Day 0 measured concentrations
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
E
ENTER TEST SPECIES NAME:
P. subcapitata
ENTER TOXICANT NAME:
MRD-10-576
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT :
MG/L
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
100
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
0
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL, MAX = 10):
4
ENTER THE 4 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):

0.07
0.27
0.93
2.33
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
Y
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:
100
ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
H
ENTER DURATION OF TEST:
96
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:

4
52
94
99
WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
Y

DATE: 18JAN11 TEST NUMBER: 1057667 DURATION: 96 H
TOXICANT : MRD-10-576
SPECIES: P. subcapitata

RAW DATA: Concentration (MG/L)	Number Exposed	Mortalities
.00	100	0
.07	100	4
.27	100	52
.93	100	94
2.33	100	99

SPEARMAN-KARBER TRIM: 4.00%

SPEARMAN-KARBER ESTIMATES: EC50: .26
95% LOWER CONFIDENCE: .22
95% UPPER CONFIDENCE: .30

APPENDIX H – STATISTICAL OUTPUT (CONT'D)

23 Feb 11
MEB

96 hr
E_yC50

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

ENTER DATE OF TEST:
18Jan11
ENTER TEST NUMBER:
1057667yield measured conc (day 0)
WHAT IS TO BE ESTIMATED?
(ENTER "L" FOR LC50 AND "E" FOR EC50)
E
ENTER TEST SPECIES NAME:
P. subcapitata
ENTER TOXICANT NAME:
MRD-10-576
ENTER UNITS FOR EXPOSURE CONCENTRATION OF TOXICANT :
MG/L
ENTER THE NUMBER OF INDIVIDUALS IN THE CONTROL:
100
ENTER THE NUMBER OF MORTALITIES IN THE CONTROL:
0
ENTER THE NUMBER OF CONCENTRATIONS
(NOT INCLUDING THE CONTROL; MAX = 10):
4
ENTER THE 4 EXPOSURE CONCENTRATIONS (IN INCREASING ORDER):

0.07
0.27
0.93
2.33
ARE THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION EQUAL(Y/N)?
Y
ENTER THE NUMBER OF INDIVIDUALS AT EACH EXPOSURE CONCENTRATION:
100
ENTER UNITS FOR DURATION OF EXPERIMENT
(ENTER "H" FOR HOURS, "D" FOR DAYS, ETC.):
H
ENTER DURATION OF TEST:
96
ENTER THE NUMBER OF MORTALITIES AT EACH EXPOSURE CONCENTRATION:

4
53
96
100
WOULD YOU LIKE THE AUTOMATIC TRIM CALCULATION(Y/N)?
Y

DATE: 18Jan11 TEST NUMBER: 1057667yield measured conc (day 0) DURATION: 96 H
TOXICANT : MRD-10-576
SPECIES: P. subcapitata

RAW DATA: Concentration (MG/L)	Number Exposed	Mortalities
.00	100	0
.07	100	4
.27	100	53
.93	100	96
2.33	100	100

SPEARMAN-KARBER TRIM: 4.00%

SPEARMAN-KARBER ESTIMATES: EC50: .25
95% LOWER CONFIDENCE: .22
95% UPPER CONFIDENCE: .29

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS

PROTOCOL

Contract Number:	EMBSI 2010-104821
Study Title:	Alga, Growth Inhibition Test on Water Accommodated Fractions of a Light Catalytic Cracked Gas Oil
EMBSI Study Number:	1057667
Test Substance:	Gas oil; CAS RN 64741-59-9, Distillates (petroleum), light catalytic cracked
EMBSI Test Substance Code:	MRD-10-576
Date:	November 12, 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	23-Nov-10
Experimental Start	7-Dec-10
Experimental Termination	4-Dec-10
Draft Report Completion	18-Jan-11
Final Report Completion	25-Feb-11

Approved By:

E. J. Febbo, M.S.
Study Director
ExxonMobil Biomedical Sciences, Inc.
1545 US Highway 22 East
Annandale, New Jersey 08801-3059

12 NOV 10
Date

Paula Podhasky
Sponsor Representative
American Petroleum Institute
Washington DC

11 November 2010
Date

SAFETY FIRST

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

INTRODUCTION

Objective

This study will be conducted for the Sponsor to evaluate the effects of the water accommodated fractions (WAFs) of MRD-10-576 on growth of the alga, *Pseudokirchneriella subcapitata* in a 96-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 US Highway 22 East
Annandale, New Jersey 08801-3059

Compliance

This test will be conducted in general agreement with OECD 201[1] and US EPA[2] guidelines, and will be conducted in compliance with OECD[3] and USEPA[4] GLP standards.

Justification for Selection of Test System

Pseudokirchneriella subcapitata (formerly *Selenastrum capricornutum*) has been used in safety evaluations and is a common test species for freshwater toxicity studies.

Justification of Dosing Route

Potential environmental exposure is by the test substance in water.

Test Substance/Test Item 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[5].

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

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

MATERIALS and METHODS

Characterization of Test Substance

The test substance will be evaluated in several studies at the testing facility. Pre-test characterization of the test substance will be performed at the testing facility prior to its use in the first of these studies. Post-test characterization will be performed following the conclusion of the last study. The following determinations will be made: 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 prior to the addition of algae, on Day 3 (composite of the three replicates) and Day 4 (composite of the remaining three replicates). 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 or algal media (both are considered equivalent for analytical standard purposes) 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.

Sample Retention

A non-study specific retention sample of the neat test substance will be taken.

Dilution Water

Algal Nutrient Media[6] - filtered through a sterile 0.45µm filter (referenced as acceptable medium in OECD 201 guideline), with 400mg of $NaHCO_3$ per liter, added as a carbon source in a no headspace environment[7]. The algal medium meets the following limits of essential constituents: $P \leq 0.7$ mg/L, $N \leq 10$ mg/L, chelators $\leq 10^{-3}$ mmol/L and hardness (Ca + Mg) ≤ 0.6 mmol/L.

Test System

Pseudokirchneriella subcapitata (formerly *Selenastrum capricornutum*)

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

MATERIALS and METHODS (CONT'D)

Supplier

Cultured at the Environmental Toxicology Laboratory of the testing facility. Initial strain (#1648) provided by UTEX, The Culture Collection of Algae MCDB, School of Biological Sciences, The University of Texas at Austin, Austin, TX 78712.

Culture Methods

Algae are cultured in approximately 300 mL of nutrient media (same as dilution water with the exception of additional $NaHCO_3$). Cell counts are performed to ensure that the cells are in log phase of growth and to verify that the culture is axenic. A new culture is started approximately weekly using inoculum from the previous culture. Cultures of *P. subcapitata* are held at 22 - 25°C under continuous illumination (4440 to 4730 Lux) provided by cool-white fluorescent bulbs. This intensity range satisfies both the OECD and OPPTS guidelines.

Number

Initial concentration of algae will be ~1.0×10^4 cells per mL in each replicate chamber.

Age at Initiation of Exposure

Algae will be taken from stock cultures in log phase of growth (4-7 days).

Test System Identification

All test chambers will be labeled to show study number, loading level, replicate, observation day and chamber number.

Selection

Replicates 1 through 12 of each treatment will be inoculated with algae. All test flasks will then be placed on a shaker table for the duration of the study. Chamber positions on the shaker table will be randomly assigned using a computer generated randomization schedule. A printout of the randomization schedule will be included in the raw data.

Contaminants

There are no known contaminants in the dilution water (algal nutrient media) believed to be at levels high enough to interfere with this study. The media is prepared from reagent grade chemicals and 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®, 2235 Route 130, Dayton, NJ 08810. Contaminant analysis of the water is not performed in a GLP compliant manner. This is not believed to affect the results of the analysis. Contaminant analysis results are maintained at the testing facility.

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

EXPERIMENTAL PROCEDURE

Equilibration Trial

A WAF equilibration trial to determine the appropriate WAF mixing duration will not be performed specifically for this study; the mixing time periods will be based on the results of a equilibration trial run for the daphnia acute study (1057642).

Range Finding Test

A 96-hour range finding test will be performed. *P. Subcapitata* will be exposed to the WAFs of 0.10, 1.0, 10 and 100 mg/L loading rates plus a control under static conditions (the loading rates chosen were based on previous algal testing using a similar gas oil sample). Twelve replicates per loading level will be used in the range-finding test, cell counts will be performed on three replicates per day for four days. 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 the same as noted in Preparation and Administration of Test Substance, Test Chamber and Volume of Solution, and Environmental Conditions section of the protocol. This phase of the study will not be subject to GLP standards.

Definitive Test Design

GROUP	LOADING LEVEL (mg/L)	NUMBER OF CELLS PER mL
1 (Control)	0	~1.0×10^4 (per 12 replicates)
2	TBD	~1.0×10^4
3	TBD	~1.0×10^4
4	TBD	~1.0×10^4
5	TBD	~1.0×10^4
6	TBD	~1.0×10^4

TBD = To Be Determined

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 algal nutrient media in glass aspirator bottles. The vessels will be closed using foil covered neoprene 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 24 hours (±1 hour) at room temperature (22°±2°C). At the end of mixing, the solutions will be allowed to settle and equilibrate to test temperature for 1 hour (±15 minutes). 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. Test flasks will be conditioned by rinsing with the appropriate solution. Twelve replicates at each loading will be prepared. Each test flask will be inoculated with ~1.0×10^4 cells per mL.

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

EXPERIMENTAL PROCEDURE (CONT'D)

Test Chamber and Volume of Solution

Test chambers will be glass 125mL Erlenmeyer flasks closed with screw caps to prevent contamination, evaporation and/or volatilization. Each chamber will contain ~140mL of test solution (no headspace) and two 14mm glass spheres to facilitate mixing.

Exposure Duration

96 hours (±1 hour)

Environmental Conditions

Range of acceptable test temperatures: 22° to 25°C.
Continuous light at 4440 to 4730 Lux; provided by cool-white fluorescent bulbs. The sensor will be located on the shaker table with the photometric cell at the same height as the top of the solution in the flasks.
The OECD guideline states that the pH of the medium should not increase by more than 1.5 units; this is not applicable for the sealed test design to be used in this study.
Oscillation Rate: 100rpm ± 10%.

Oscillation rate will be verified daily. The pH of each treatment and control will be measured on Day 0 and daily after cell density determinations (composite of three replicates).

Environmental conditions (light and temperature) will be monitored using the laboratory computer system (Watchdog V5 monitoring system) to provide a record of the continuous measurements for temperature and lighting in the test area, in the event that Watchdog is not functioning, manual measurements will be recorded twice daily.

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

EXPERIMENTAL PROCEDURE (CONT'D)

Experimental Evaluation

Cell density is determined for each test and control chamber using a hemacytometer and microscope at 24, 48, 72, and 96 hours (± 1 hour) after the beginning of the test. Cell density determinations will be performed on three replicates at each observation interval and the replicates will then be discarded. Any unusual cell shapes, color differences, differences in chloroplast morphology, flocculations, adherence of algae to test containers, or aggregation of algal cells (clumping) and any test substance insolubility (surface slicks, precipitates) will be documented at the time of cell density determinations and will be reported. Following in-life termination, it will be determined whether the altered growth response between controls and test algae (in highest test chemical concentration(s)) was due to a change in relative cell numbers, cell sizes, or both. These observations are qualitative and descriptive, and are not used in end-point calculations. In test concentration(s) where growth is maximally inhibited, algistatic effects may be differentiated from algicidal effects by the following method. Aliquots of test solution from the replicate chambers having the lowest loading level/concentration which completely inhibited algal growth or the highest loading level/concentration which inhibited algal growth will be combined into a new test container with a sufficient volume of fresh nutrient medium to dilute to a loading level/concentration which does not affect growth. The subculture will be incubated under the environmental conditions used in the definitive test for a period of up to 9 days, and observed periodically (e.g. every other day) for algal growth to determine if the algistatic effect noted after the 96–h test is reversible. This subculture will be discontinued as soon as growth occurs.

Test Acceptability

A test may not be acceptable if cell density in the Control does not increase by a factor of $\geq$ 16 within three days.

The mean coefficient of variation for section-by-section specific growth rates in the control cultures must not exceed 35%. This criterion applies to the mean value of coefficients of variation calculated for replicate control cultures.

The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7% in tests with *Pseudokirchneriella subcapitata*.

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

EXPERIMENTAL PROCEDURE (CONT'D)

Calculations

Effect Loading/Concentration 50 (EL/EC_{50}) values will be determined. This is defined as the loading level/concentration of the test substance which results in a 50% reduction in growth, as determined by average specific growth rate and yield (relative to the Control) for the specified time of exposure. The 72- and 96-hour values will be calculated where appropriate. However, if the 72 or 96-h measured values are outside the range +/-20% of the initial measured concentration, then results will be based on the geometric mean concentration during the exposure.

Results will be calculated with average specific growth rate (E_rL/C_{50}) and yield (E_YL/C_{50}).

The specific growth rates for each treatment are determined by calculating the slope of the regression line of the ln (cell density) versus time using the PROC REGRESSION procedure from SAS[8].

Yield is calculated as the biomass at the end of the test minus the starting biomass for each single vessel of controls and treatments. For each test concentration and control, a mean value for yield along with variance estimates will be calculated. The percent inhibition in yield (%Iy) will be calculated for each treatment replicate as follows:

$$\%I_y = \frac{(Y_C - Y_T)}{Y_C} \text{ X } 100$$

where:
% Iy: percent inhibition of yield;
- Y_C: mean value for yield in the control group;
- Y_T: value for yield for the treatment replicate.

The EL/EC_{50} value will be determined based on the percent inhibition relative to the control values. The EL_{50} values will be calculated by using the inverse interpolation method of Snedecor and Cochran[9], the Trimmed Spearman-Karber Method[10], or another appropriate method to be documented in the raw data and report.

The No Observed Effect Loading/Concentration (NOEC/NOEL) will also be determined. An analysis of variance ANOVA procedure[11] of SAS will be used to determine loadings/concentrations which are statistically inhibited based on the Control treatment.

APPENDIX I – 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 algae: origin, lab culture, strain, method of cultivation

Test conditions:

- date of the start and end of the test
- test procedure used
- composition of the medium
- temperature and pH values of the test solutions at the start and end of the test
- methods of preparation of test solutions
- loading levels/concentrations used
- information on concentrations of the test substance in the test solutions
- light intensity
- description of the test chambers, volume of solution
- culturing apparatus

Results

- cell density for each flask at each measuring point and method for measuring cell density
- mean values of cell density
- growth curves, if applicable
- EC and EL values and method of calculation
- NOEC and NOEL
- other observed effects
- statistical output from endpoint determinations
- deviations from experimental design

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, supporting documentation, and a non-study specific sample of the neat test substance will be maintained in the Archives of the testing facility.

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

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. As stated in the Calculations section; if measured values are outside the range +/-20% of the initial measured concentration, then results will be based on the geometric mean concentration during the exposure.

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.

None of these planned guideline exceptions are believed to affect the outcome, integrity or quality of the study.

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

REFERENCES

1. Organization for Economic Cooperation and Development (OECD). Guidelines for Testing of Chemicals, Section 2: Effects on Biotic Systems, Guideline 201: Alga, Growth Inhibition Test. 23 March 2006.

2 U.S. Environmental Protection Agency, Ecological Effects Test Guidelines, OPPTS 850.5400: Algal Toxicity, Tiers I and II.

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

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

5. 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.

6. USEPA. The *Selenastrum capricornutum* Printz Algal Assay Bottle Test. EPA-600/9-78-018. July 1978.

7. International Organisation for Standardisation (1998). ISO/DIS 14442. Water quality – Guidelines for algal growth inhibition tests with poorly soluble materials, volatile compounds, metals and wastewater

8. *SAS User's Guide: Statistics*, Version 5.18 Edition. SAS Institute, Inc., Cary, NC. 1985.

9. Snedecor, G.W. and W.G. Cochran, *Statistical Methods*, 8th Edition, 1989, Iowa State University Press / Ames.

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. Duncan, D.B. (1975), *t-Tests and Intervals for Comparisons Suggested by the Data,* Biometrics, 31, 339-359.

12. 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.

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

DISTRIBUTION

EMBSI - Clinton:

Study Director, Environmental Toxicology and Fate Coordinator	E. J. Febbo
Environmental Sciences, Section Head	T. F. Parkerton
Environmental Chemistry / Contributing Scientist for Characterization/Analysis of Mixtures	D. J. Letinski
Study Technicians	J. D. Butler
	M. J. Connelly
	T. M. Knarr
	E. M. Gallagher
	B. A. Kelley
	R. G. Manning
Contract Administrator	B. J. Foster
QAU	D.M. McDougall

API:

Sponsor Representative	Paula Podhasky
Sponsor's Study Monitor	Jim Swigert

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD Page 1 of 5

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: 1057667 Revision Number: 1 Date: 10-Jan-11

Administrative Change:

B. A. Kelley will replace E. J. Febbo as Study Director

Justification:
E. J. Febbo will be taking a long-term assignment for the Upstream Research Company.

Page 1 / Proposed Key Dates for Completion:

Previous Statement:

Experimental Start	7-Dec-10
Experimental Termination	4-Dec-10
Draft Report Completion	18-Jan-11
Final Report Completion	25-Feb-11

Revised Statement:

Experimental Start	18-Jan-11
Experimental Termination	22-Jan-11
Draft Report Completion	25-Feb-11
Final Report Completion	8-Apr-11

Justification:
Revised dates, based on range finder completion and method development to provide more consistent light levels for definitive study, also acknowledge error for experimental termination date in original protocol.
Note: The light intensity will not change, additional bulbs were added and the test chambers will be placed further away from the bulbs, this provides more consistent light levels to all test chambers.

Page 3 / *Analysis of Mixtures*

Previous Statement:
Samples will be taken from each water-accommodated fraction (WAF) and control solution on Day 0 prior to the addition of algae, on Day 3 (composite of the three replicates) and Day 4 (composite of the remaining three replicates).

Revised Statement:
Samples will be taken from each water-accommodated fraction (WAF) and control solution on Day 0 prior to the addition of algae, on Day 3 (composite of a subsample from the three replicates) and Day 4 (composite of a subsample from the three replicates).

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD Page 2 of 5

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: 1057667 Revision Number: 1 Date: 10-Jan-11

Additional Statement

Chemical control replicates will be prepared at the mid (1.02 mg/L) and high loading levels (10.5 mg/L), (six replicates at both levels), mercuric chloride will added to eliminate biological growth, composite of a subsample from the three replicates will be analyzed after 72 and 96 hours along with the normal test samples. These samples will be taken and held in the same manner as the test samples. The concentration of mercuric chloride used will be documented in the raw data and reported.

Justification:

Sampling clarification for addition of "subsample". Significant chemical loss was observed in the Range Finding test, these "poisoned" chemical control samples will eliminate biological processes and verify test chamber integrity for concentration stability.

Page 4 / *Selection*:

Previous Statement:

Replicates 1 through 12 of each treatment will be inoculated with algae. All test flasks will then be placed on a shaker table for the duration of the study. Chamber positions on the shaker table will be randomly assigned using a computer generated randomization schedule. A printout of the randomization schedule will be included in the raw data.

Revised Statement:

Replicates 1 through 18 of each treatment will be inoculated with algae. All test flasks will then be placed on a shaker table for the duration of the study. Chamber positions on the shaker table will be randomly assigned using a computer generated randomization schedule. A printout of the randomization schedule will be included in the raw data. The chemical control samples will not be randomized among the test samples, they will be placed near the shaker table, exposed to the same light levels as the test replicates.

Justification:

Addition of the chemical control samples.

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD Page 3 of 5

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: 1057667 Revision Number: 1 Date: 10-Jan-11

Page 5 / *Definitive Test Design*

Previous Statement:

GROUP	LOADING LEVEL (mg/L)	NUMBER OF CELLS PER mL
1 (Control)	0	~1.0 x 10^4 (per 12 replicates)
2	TBD	~1.0 x 10^4
3	TBD	~1.0 x 10^4
4	TBD	~1.0 x 10^4
5	TBD	~1.0 x 10^4
6	TBD	~1.0 x 10^4

TBD = To Be Determined

Revised Statement:

GROUP	LOADING LEVEL (mg/L)	NUMBER OF CELLS PER mL
1 (Control)	0	~1.0 x 10^4 (per 12 replicates)
2	0.10	~1.0 x 10^4 (per 12 replicates)
3	0.32	~1.0 x 10^4 (per 12 replicates)
4	1.02	~1.0 x 10^4 (per 18 replicates)
5	3.28	~1.0 x 10^4 (per 12 replicates)
6	10.5	~1.0 x 10^4 (per 18 replicates)

Justification: addition of definitive loading levels and chemical control replicates

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD Page 4 of 5

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: 1057667 Revision Number: 1 Date: 10-Jan-11

Page 5 / *Preparation and Administration of Test Substance*:

Previous Statement:
Twelve replicates at each loading will be prepared.

Revised Statement:
Twelve replicates at each loading will be prepared; plus six additional replicates at the 1.02 and 10.5 mg/L loading levels.

Justification:
Addition of the chemical control replicates.

Page 12 / *Distribution*

Previous Statement:
Study Director,
Environmental Toxicology and Fate Coordinator..... E. J. Febbo

Revised Statement:
Study Director..... B. A. Kelley
Environmental Toxicology and Fate Coordinator..... G. E. Bragin

Justification:
E. J. Febbo will be taking a long-term assignment for the Upstream Research Company.

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD

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: 1057667 Revision Number: 1 Date: 7-Jan-11

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Environmental Toxicology and Fate Coordinator	G. E. Bragin
Environmental Sciences, Section Head	T. F. Parkerton
Environmental Chemistry / Contributing Scientist for Characterization/Analysis of Mixtures	D. J. Letinski
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	E. M. Gallagher
	B. A. Kelley
	R. G. Manning
Contract Administrator	B. J. Foster
QAU	D.M. McDougall

API:

Sponsor Representative	Paula Podhasky
Sponsor's Study Monitor	Jim Swigert

Required signatures:

RD White for Paula Podhasky 1-18-2011
Paula Podhasky Date
Sponsor Representative

B. A. Kelley 31 Jan 11
B. A. Kelley Date
Study Director

G. E. Bragin 31 Jan 11
G. E. Bragin Date
Environmental Toxicology and Fate Coordinator

APPENDIX I – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD Page 1 of 1

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 Numbers: 1057667 Revision Number: 2 Date: 27 October 2011

Page 1 / Sponsor Representative:

Paula Podhasky

Revised:
Russell White

Justification: Paula Podhasky has retired from American Petroleum Institute and has been replaced with Russell White.

Page 12 / PERSONNEL:

Section Head, Env. Sciences.....T. F. Parkerton

Revised:
Section Head, Env. Sciences.....R. A. Barter

Justification: T. F. Parkerton has been replaced with R.A. Barter as Section Head effective July 1, 2011

DISTRIBUTION

Study Director.....B. A. Kelley
Sponsor Representative.....Russell White
Sponsor's Study Monitor.....Jim Swigert
Section Head, Env. Sciences.....R. A. Barter
Lab Coordinator, Env. Toxicology and Fate.....G. E. Bragin
Lab Coordinator, Environmental Chemistry,
Principal Investigator Characterization (EMBSI) ...D. J. Letinski
Study Technicians.....J. D. Butler
.....R. G. Manning
.....T.M. Knarr
.....M.J. Connelly
Quality Assurance Unit.....D.M. McDougall
Contract Administrator.....B. J. Foster

Required signatures:

Russell White — 28 Nov 2011 — B. A. Kelley — 01 Dec 2011

Russell White
Sponsor Representative

Date

B. A. Kelley
Study Director

Date