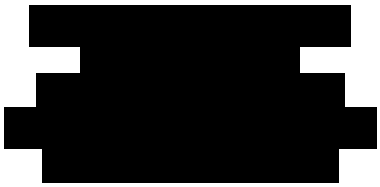
PETROLEUM COKE:
A 96-HOUR TOXICITY TEST WITH THE
FRESHWATER ALGA (*Selenastrum capricornutum*)

AMENDED FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 472A-114

OECD GUIDELINE 201
EU DIRECTIVE 92/69/EEC, METHOD C.3.
U.S. EPA OPPTS NUMBER 850.5400

AUTHORS:



STUDY INITIATION DATE: April 28, 2004

STUDY COMPLETION DATE: June 22, 2006

AMENDED REPORT DATE: April 10, 2007

SUBMITTED TO

American Petroleum Institute
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AMENDED

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: American Petroleum Institute

TITLE: Petroleum Coke: A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)

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This study was conducted in compliance with Good Laboratory Practice Standards as published by TSCA Good Laboratory Practice Standards (40 CFR Part 792) (1) and OECD Principles of Good Laboratory Practice, (ENV/MC/CHEM (98) 17) (2), with the following exceptions:

The characterization of the test and reference substances, and the stability of the substances under conditions of storage at the test site, were not determined in compliance with Good Laboratory Practice Standards.

Periodic analyses of well water for potential contaminants were performed using a certified laboratory and standard U.S. EPA analytical methods, but not under Good Laboratory Practice Standards.

STUDY DIRECTOR:

[redacted]

4/10/07
Date

SPONSOR:
American Petroleum Institute, by:

[redacted]

4/26/2007
Date

QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by TSCA Good Laboratory Practice Standards (40 CFR Part 792) (1) and OECD Principles of Good Laboratory Practice, (ENV/MC/CHEM (98)17) (2). The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

		DATE REPORTED TO:	
ACTIVITY:	DATE CONDUCTED:	STUDY DIRECTOR:	MANAGEMENT:
Protocol	April 20, 2004	April 20, 2004	April 29, 2004
Test Substance Preparation	Mach 23, 2005	March 23, 2005	March 28, 2005
Matrix Fortification	April 4, 2005	April 4, 2005	April 5, 2005
Biological Data and Draft Report	May 5 & 6, 2005	May 6, 2005	May 12, 2005
Analytical Data and Draft Report	May 6, 9-11, 2005	May 11, 2005	May 12, 2005
Final Report	June 19, 2006	June 19, 2006	June 22, 2006
Amended Report	April 9, 2007	April 9, 2007	April 9, 2007

All inspections were study-based unless otherwise noted.

[redacted]

4/10/2007
Date

AMENDED REPORT APPROVAL

SPONSOR: American Petroleum Institute

TITLE: Petroleum Coke: A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 472A-114

This report was reviewed by the individuals involved in the conduct and management of the study, and was found to be an accurate reflection of the methods used, data collected and results of the study.

STUDY DIRECTOR:

[redacted]

4/10/07
Date

4-10-07
Date

April 10, 2007
Date

WILDLIFE INTERNATIONAL, LTD. MANAGEMENT:

[redacted]

10 April 07
Date

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SUMMARY

SPONSOR: American Petroleum Institute

TITLE: Petroleum Coke: A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 472A-114

TEST SUBSTANCE: Petroleum Coke

GUIDELINES: OECD Guidelines for Testing of Chemicals, 201: *Algal, Growth Inhibition Test*; Official Journal of the European Communities No. L383 C.3.: *Algal Inhibition Test*; U.S. EPA Series 850 – Ecological Effects Test Guidelines OPPTS Number 850.5400: *Algal Toxicity Tiers I and II*

TEST DATES:	Study Initiation:	April 28, 2004
	Experimental Start (OECD):	March 23, 2005
	Experimental Start (EPA):	March 24, 2005
	Biological Termination:	March 28, 2005
	Experimental Termination:	April 5, 2005
LENGTH OF EXPOSURE:	96 Hours	
TEST ORGANISMS:	Freshwater Alga (*Selenastrum capricornutum*)	
SOURCE OF TEST ORGANISMS:	Wildlife International, Ltd. Cultures Easton, Maryland 21601	

TEST CONCENTRATIONS:	Nominal WAF Loading Rate
Negative	Control
1000	mg/L

SUMMARY (Continued)

RESULTS: Based on nominal WAF loading rate:

Cell Density:

72-hour EL50	>1000 mg/L WAF
95% confidence limits:	Not Calculable
96-hour EL50:	>1000 mg/L WAF
95% confidence limits:	Not Calculable
72-hour NOELR:	<1000 mg/L WAF
96-hour NOELR:	<1000 mg/L WAF

RESULTS: Based on nominal WAF loading rate:

Area Under the Growth Curve:

72-hour E_bL50:	>1000 mg/L WAF
95% confidence limits:	Not Calculable
96-hour E_bL50:	>1000 mg/L WAF
95% confidence limits:	Not Calculable
72-hour NOELR:	<1000 mg/L WAF
96-hour NOELR:	<1000 mg/L WAF

RESULTS: Based on nominal WAF loading rate:

Growth Rate

72-hour E_rL50:	>1000 mg/L WAF
95% confidence limits:	Not Calculable
96-hour E_rL50:	>1000 mg/L WAF
95% confidence limits:	Not Calculable
72-hour NOELR:	<1000 mg/L WAF
96-Hour NOELR:	<1000 mg/L WAF

INTRODUCTION

Wildlife International, Ltd conducted an algal toxicity test to determine the effects of a water accommodated fraction of petroleum coke on the freshwater alga *Selenastrum capricornutum*, for American Petroleum Institute at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. Petroleum coke is defined as a the product formed by subjecting the heavy tar-like residue remaining following oil refining to high temperatures and pressures. It consists of primarily elemental carbon with considerably smaller amounts of hydrocarbons, sulfur and trace amounts of heavy metals. The in-life exposure phase of the test was conducted from March 24 to March 28, 2005. A copy of the final report and all original raw data generated at Wildlife International, Ltd. are filed under Project Number 472A-114 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of the study was to evaluate the toxicity of a water accommodated fraction of petroleum coke to the freshwater alga, *Selenastrum capricornutum*, during a 96-hour exposure period in a sealed exposure system.

EXPERIMENTAL DESIGN

The freshwater alga, *Selenastrum capricornutum*, was exposed to a water accommodated fraction (WAF) of petroleum coke prepared at a loading rate of 1000 mg/L and a negative control for 96 hours. For this test, the term loading rate means the total amount of test substance added to dilution water volume (mg/L) to achieve the respective WAF solution. Because petroleum coke is a multi-component substance not fully soluble in water, WAFs are an acceptable means of creating exposure solutions for ecotoxicity tests (3). The nominal test concentration was selected in consultation with the Sponsor and was based upon the results of a range-finding test (Appendix 1). Twenty-four closed bottle replicates were maintained for the treatment and control group. Water samples were collected at test initiation, on Day 3 and test termination for analysis for selected constituents of petroleum coke. Because petroleum coke is a complex mixture of elemental carbon and low levels of hydrocarbons and metals, several poly aromatic hydrocarbons and metals were selected to be monitored in the test solutions during the algal test. The components selected for measurement were those that are either of ecological concern or were known to occur in petroleum coke in amounts that might be measured in a WAF solution. Those constituents of interest included the following:

PAH	Metals and Sulfur
Acenaphthene	Nickel
Acenaphthylene	Vanadium
Anthracene	Iron
Benzo(a)anthracene	Copper
Benzo(a)pyrene	Selenium
Benzo(b)fluoranthene	Arsenic
Benzo(g,h,i)perylene	Sulfur
Benzo(k)fluoranthene	
Chrysene	
Dibenzo(a,e)pyrene	
Dibenz(a,h)anthracene	
Fluoranthene	
Fluorene	
Indeno(1,2,3-cd)pyrene	
Naphthalene	
Phenanthrene	
Pyrene	
1-Methylnaphthalene	
2-Methylnaphthalene	

At test initiation, an inoculum of the algal cells was prepared at a concentration of approximately 1.5 X 10^6 cells/mL. The concentration of algal cells in the inoculum was verified and 1.0 mL was added to each test chamber to achieve a nominal concentration of approximately 5,000 cells/mL. Samples were collected from six replicate test chambers at each 24 hour interval to determine cell densities (i.e, replicates A, B, C, D, E and F at 24 hours, replicates G, H, I, J, K and L at 48 hours, replicates M, N, O, P, Q and R at 72 hours, and replicates S, T, U, V, W and X at 96 hours). Cell densities were measured for each replicate and were used to calculate area under the growth curve and growth rates. Percent inhibition values relative to the control were calculated for each parameter at 24, 48, 72 and 96 hours. EL50 values were estimated based upon percent inhibition measured in the biological parameters for each sampling interval. The no-observed-effect-loading rate (NOELR) was determined at 72 and 96 hours based upon evaluation of the concentration-response pattern and the results of statistical analyses.

MATERIALS AND METHODS

The study was conducted in accordance to the procedures outlined in the protocol, "Petroleum Coke: A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)" (Appendix 2). The protocol was based on procedures outlined in OECD Guidelines for Testing of Chemicals, 201 *Algal, Growth Inhibition Test* (4); Official Journal of the European Communities No. L383 C.3. *Algal Inhibition Test* (5); and the U.S. Environmental Protection Agency Series 850 – Ecological Effects Test Guidelines OPPTS Number 850.5400: *Algal Toxicity Tiers I and II* (draft) (6).

Test Substance

The test substance was green petroleum coke (CAS Number 64741-79-3). The test substance was received from Experimental Pathology Laboratories, Herndon, VA for API on October 7, 2003. It was assigned Wildlife International, Ltd. identification number 6485 upon receipt and was stored under ambient conditions. The test substance, black pellets, was identified as 2 mm particle size Petroleum Coke (aka Milled Pellets).

The identity, strength, purity, composition (Appendix 4), storage stability, and method of synthesis, fabrication and/or derivation (Appendix 3) of each batch of the test substance and the maintenance of these records were the responsibility of the Sponsor.

Reference Substances

Purified polyaromatic hydrocarbons (PAH) were made up of components received from three manufacturers. The following reference standards were received from AccuStandard Inc. and were stored under ambient conditions:

Component	Test Substance Number	Lot/Batch	CAS Number	Date Received	Expiration Date	Description
Benzo(a)pyrene	6705	052803MT-AC	50-32-8	6/07/04	6/03/07	green powder
Anthracene	6706	A33783	120-12-7	6/07/04	6/03/07	white powder
Benz(a)anthracene	6707	19587	56-55-3	6/07/04	6/03/07	colorless plates
Acenaphthylene	6708	011504MS-AC	208-96-8	6/07/04	6/03/07	yellow powder
Acenaphthene	6709	01915EQ	83-32-9	6/07/04	6/03/07	white crystal
Benzo(b)fluoranthene	6710	020402AG-AC	205-99-2	6/07/04	6/03/07	white flakes
Benzo(g,h,i)perylene	6711	122 500MT-AC	191-24-2	6/07/04	6/03/07	green powder
Benzo(k)fluoranthene	6712	112603AG-AC	207-08-9	6/08/04	6/03/07	yellow powder
Chrysene	6713	13103	218-01-9	6/08/04	6/03/07	white powder
Dibenz(a,h)anthracene	6714	13246	53-70-3	6/08/04	6/03/07	green powder
Fluoranthene	6715	19762	206-44-0	6/08/04	6/03/07	white powder
Fluorene	6716	19675	86-73-7	6/08/04	6/03/07	white powder
Indeno(1,2,3-cd)pyrene	6717	19641	193-39-5	6/08/04	6/03/07	yellow powder
Naphthalene	6718	167A-A	91-20-3	6/08/04	6/03/07	white flakes
Phenanthrene	6719	090903AG-AC-1	85-01-8	6/08/04	6/03/07	white powder
Pyrene	6720	09617LR	129-00-0	6/08/04	6/03/07	green crystal

The following reference standard was received from Cambridge-Isotope Labs and was stored under ambient conditions:

Component	Test Substance Number	Lot/Batch	CAS Number	Date Received	Expiration Date	Description
Dibenzo(a,e)pyrene	6518	I1-7628	192-65-4	10/22/03	Not given	Solids

The following standards were received from ChemService and were stored under ambient conditions:

Component	Test Substance Number	Lot/Batch Number	CAS Number	Date Received	Expiration Date	Description
2-Methylnaphthalene	6765	310-43C	91-57-6	8/03/04	9/01/08	Solid
1-Methylnaphthalene	6766	325-31A	90-12-0	8/03/04	5/01/09	Liquid

Analytical standards for each of the seven elements of interest were received from Spex Industries (Metuchen, N.J. 08840) and were stored under ambient conditions. All of the materials were 1,000 mg/L Spex CertiPrep® plasma standards in 2% HNO_3, with the exception of the sulfur standard which was a 10,000 mg/L preparation in water. The following tabulation summarizes pertinent data for each analytical standard:

Component	Test Substance Number	Lot/Batch	CAS Number	Date Received	Expiration Date	Description
Arsenic (As)	6543	10-06AS	7440-38-2	11/06/03	11/15/05	Clear liquid
Copper (Cu)	6544	9-183CU	7440-50-8	11/06/03	11/15/05	Blue liquid
Iron (Fe)	6545	9-184FE	7439-89-6	11/06/03	11/15/05	Clear Liquid
Nickel (Ni)	6546	10-29NI	7440-02-0	11/06/03	11/15/05	Blue liquid
Selenium (Se)	6547	10-31SE	7782-49-2	11/06/03	11/15/05	Clear Liquid
Sulfur (S)	6890	S9-51S	7704-34-9	10/18/04	10/15/05	Liquid
Vanadium (V)	6549	10-88V	7440-62-2	11/06/03	11/15/05	Yellow Liquid

Test Organism

The freshwater green alga, *Selenastrum capricornutum* Printz (UTCC 37), was selected as the test species for this study . The species is represen tative of an im portant group of freshwater algae, and was selected for use in the test based upon a past history of use, and ease of culturing in the laboratory. Original algal cultures were obtained from the University of Toronto Culture Collection, and had been m aintained in culture m edium at Wildlife International, Ltd., Easton, Mary land. Algal cells used in this test were obtained from Wildlife International, Ltd. cultures that had been actively growing in culture m edium for at least two weeks prior to test initiation. The culture was last transferred to fresh m edium three day s prior to t est initiation. The negative control organism s were expected to exhibit exponential growth over the 96-hour exposure period. Exponential growth phase, defined as the period of growth where the algal cells are dividing at a constant rate, is indicated by the linear section of the growth curve (Figure 1).

Culture Medium

The algal cells were tested in freshwater algal medium with supplemental sodium bicarbonate (6, 7). Stock nutrient solutions were prepared by adding reagent-grade chem icals to purified well water (NANOpure®). The test medium was prepared by adding the appropriate volum es of stock nutrient solutions to NANOpure® water (Appendix 6). The pH of the m edium was adjusted to 7.5 ± 0.1 using 10% HCl and 0.1N NaOH as needed and the m edium was sterilized by filtration (0.22 µm) prior to use. Analy ses perform ed at least once annually to determ ine the concentrations of selected organic and inorganic constituents in the well water. The results of analy ses performed to measure the concentrations of selected contaminants in well water used by Wildlife International, Ltd. are presented in Appendix 7.

Test Apparatus

Test chambers were sterile, 300-mL glass BOD bottles with glass stoppers and were completely filled with medium. Each test chamber contained two glass marbles to facilitate mixing. The test chambers were labeled with the project number, loading rate and replicate, and were indiscriminately positioned daily on a mechanical shaker in an environmental chamber designed to maintain the desired test temperature throughout the test. The test chambers were shaken continuously at 100 rpm. The shaker tables were checked daily during the test to ensure the proper setting.

Environmental Conditions

Test flasks were held in an environmental chamber at a temperature of 24 ± 2° C. The temperature of a container of water adjacent to the test flasks in the environmental chamber was recorded twice daily during the test using a liquid-in-glass thermometer.

The algae were held under continuous cool-white fluorescent lighting throughout the test. The target light intensity was 4300 ± 430 lux. Light intensity was measured at five locations on each shaker table daily during the test. Light intensity was measured using an SPER Scientific Model 840006C light meter.

The pH of the medium prepared for the treatment and control group was measured at test initiation, and at approximately 24 hour intervals thereafter using a Fisher Accumet Model 525 pH meter. Samples for pH measurement at test initiation were collected from the individual batches of test solution prepared for the treatment and control group. At 24, 48, 72 and 96 hours, samples of test solution were collected from pooled replicates sacrificed at each time interval.

Preparation of Test Concentrations

The single, limit test concentration was a WAF prepared at a petroleum coke loading rate of 1000 mg/L. The test substance was added to 9 L of algal growth medium in a 9.5-L glass vessel and stirred for 24 hours by means of a Teflon® coated stir bar on a magnetic stir plate. Stirring speed was adjusted to produce a vortex depth of approximately 30% of the test solution height. The duration of the mixing period was established during an exploratory WAF equilibration test (Appendix 8). After mixing, the WAFs were allowed to settle for approximately 1 hour.

Analytical Sampling

Samples of the test solutions were collected at approximately 0, 72 and 96 hours to measure concentrations of soluble components of the test substance. Samples at test initiation were collected at mid-depth from the individual batches of test solution prepared for the treatment and control group prior to addition of the algae. The 72-hour samples were collected from pooled replicates M, N, O, P, Q and R of the treatment and control group. At test termination, samples were collected from pooled replicates S, T, U, V, W and X of the treatment and control group. All samples were stored until the end of the test. Samples collected of organic analysis were preserved by storing refrigerated with zero headspace. Samples collected for inorganic analysis were preserved by the addition of sufficient nitric acid to achieve a final acid concentration of 2%.

Analytical Method by HPLC

The method used for the analysis of the method verification samples was based upon methodology developed by Wildlife International, Ltd. (8). The analytical method consisted of diluting the samples in freshwater algal medium, as necessary, and analyzing by direct injection high performance liquid chromatography (HPLC) with either UV detection at 220 nm or fluorescence detection at 340 nm to 425 nm.

Concentrations of each PAH compound in the fortified samples were determined using an Agilent Model 1100 High Performance Liquid Chromatograph, equipped with either an Agilent Series 1100 Variable Wavelength Detector or a Jasco Model FP-1520 Fluorescence Detector. Chromatographic separations were achieved using a YMC Pack ODS-AM column (150 mm x 4.6 mm, 3 µm particle size). A method flow chart is provided in Appendix 9.1 and instrumental parameters for the analysis of PAH components are summarized in Appendix 9.2.

Five calibration standards of PAH, ranging in concentration from 5.00 to 50.0 µg/L, were prepared prior to the test using a stock solution of PAH analytical standards in methanol (Appendix 9.3). The calibration standards were analyzed with each sample set. Linear regression equations were generated using the peak area responses versus the respective concentrations of the calibration standards. The concentration of PAH in the samples was determined by substituting the peak area responses of the samples into the applicable linear regression equation.

The method limit of quantitation (LOQ) was defined as 5.00 µg/L, calculated as the product of the concentration of the lowest calibration standard (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00). One matrix blank sample was analyzed with each sample set to determine possible interferences. No interferences were observed at or above the LOQ during the sample analyses.

Samples of freshwater algal medium were fortified at 10.0, 40.0 and 100 µg/L using a stock solution of PAH analytical standard in methanol (Appendix 9.3), and were analyzed concurrently with the test samples. The measured concentrations for the matrix fortification samples ranged from 86.3 to 109% of fortified concentrations (Tables 1 through 19).

Representative calibration curves are presented in Appendices 9.4 through 9.22. Representative chromatograms of low and high-level calibration standards are presented in Appendices 9.23 and 9.24, respectively. Representative chromatograms of a freshwater algal medium matrix blank sample and a matrix fortification sample are presented in Appendices 9.25 and 9.26, respectively. A representative chromatogram of a test sample is presented in Appendix 9.27.

Analytical Method by ICP-AES

The analytical method used for the analysis of As, Se, Fe, Ni, Se, V and S in the WAF samples was based upon methodology developed by Wildlife International, Ltd. (9). The analytical method consisted of acidifying the samples 2% by volume with concentrated nitric acid and direct injection into the ICP-AES system. Concentrations of As, Cu, Fe, Ni, Se, S and V in the samples were determined using a Perkin-Elmer Optima 3000 DV ICP-AES configured in axial view mode and equipped with a Cetac U-5000AT $^{+}$ Ultrasonic Nebulizer (sample introduction). Simultaneous measurements were made for six of the seven elements (As, Cu, Fe, Ni, Se and V). For sulfur, a single element method was employed due to the need for higher concentration-level calibration standards. A method flowchart is provided in Appendix 10.1 and instrumental parameters for the analysis of the seven elements are summarized in Appendix 10.2.

Multi-element calibration standards were analyzed with the WAF samples. Preparations of stock and calibration standard solutions are detailed in Appendix 10.3. The calibration standard series was injected at the beginning and end of each analytical run. In addition, a standard was injected following a maximum of five sample analyses. For a given injection of a sample (including standards), the ICP-AES instrument integrated the steady-state emission signal at designated emission wavelengths for a method-specified period (read time). The net integrated intensity was then automatically corrected by subtraction of the mean corrected intensity of the calibration blank (determined at sequence initiation). The measurement cycle was automatically repeated two additional times during the sample injection (read replicates). The mean of the three measurements produced a mean corrected intensity for each monitored element in the sample. Linear regression equations for each monitored element were generated using mean corrected intensities versus the respective concentrations of the element in the calibration standards. Representative calibration curves for As, Cu, Fe, Ni, Se, S and V are presented in Appendices 10.4 – 10.10. The concentrations of each of the seven elements in the WAF samples were calculated by substituting their mean corrected intensities into the applicable linear regression equation, and applying the appropriate dilution and unit conversion factors. Representative emission spectra of low- and high-level calibration standards are presented in Appendices 10.11 – 10.13. An example calculation for a study sample is provided in Appendix 10.14.

A matrix blank was analyzed for each component concurrent with the study samples to determine possible interferences. No interferences were observed at or above the limit of quantitation (LOQ) during the sample analyses (Appendices 10.15 – 10.17). A sample of freshwater algal media was fortified at 2X (S) or 2.5X (As, Cu, Fe, Ni, Se and V) the method LOQ for each element and analyzed for each component concurrent with the study samples using the combined stock and the sulfur reference standard. Results are presented in Tables 20 through 26. Emission spectra of freshwater algal media matrix blank and matrix fortification samples are presented in Appendices 10.15 – 10.17. The measured concentrations for the matrix fortification samples ranged from 87.3 to 124% of fortified concentrations (Tables 20 through 26). Representative emission spectra of a WAF test sample are presented in Appendices 10.18 – 10.20.

Inoculation of Test Chambers

Prior to test initiation, an inoculum of the algal cells was prepared in freshwater algal medium at a concentration of approximately 1.5 X 10^6 cells/mL. The concentration of algal cells was verified using a hemacytometer and microscope, and 1.0 mL of the inoculum was added to each test chamber to achieve a nominal concentration of approximately 5,000 cells/mL at test initiation.

Algal Growth Measurements and Observations

Test medium samples were collected from six replicates sacrificed at each sampling interval (i.e., replicates A, B, C, D, E and F at 24 hours, replicates G, H, I, J, K and L at 48 hours, replicates M, N, O, P, Q and R at 72 hours, and replicates S, T, U, V, W and X at 96 hours) for determination of algal cell densities. Samples were collected at approximately 24-hour intervals during the 96-hour exposure and were held for a maximum of nine days under refrigerated conditions sufficient to inhibit growth until cell counts could be performed. Cell counts were performed using a hemacytometer and microscope. The samples then were diluted using an electrolyte solution (Isoton®), as needed, to maintain counting accuracy. A small amount of each sample was loaded onto a hemacytometer and 10 grids were counted. The mean number of cells per grid was calculated and this value was used to calculate the cell density of the sample. Using this technique, the minimum quantifiable cell density was 1,000 cells/mL.

Recovery Phase

After 96 hours of exposure, the treatment group exhibited visual evidence of algal growth (i.e., color in the test solution). Therefore, a recovery phase was not initiated.

Conditions for the Validity of the Test

1. The mean cell density in the control flasks had increased by a factor of at least 16 within three days.
2. While the coefficient of variance (CV) for the cell density in the control flasks at 96 hours was 22%, this is a criteria for an open bottle system. The growth of a closed bottle system is limited by the lack of gas exchange causing the density between replicates to be variable. Since the CV of the closed bottle system was close to the requirement of an open bottle system (20%), it is not considered to invalidate the study.

Statistical Analyses

The calculation of cell densities, areas under the growth curve, growth rates and percent inhibition values, as well as all statistical analyses, were conducted using "The SAS System for Windows", Version 8.02 (10). Area under the growth curve was calculated for each replicate of the treatment and control group at each 24-hour exposure interval using the following formula:

$$A = ((N_1-N_o)/2)(t_1)+((N_1+N_2-2N_o)/2)(t_2-t_1) +...+((N_{n-1}+N_n-2N_o)/2)(t_n-t_{n-1})$$

where:
- A = Area under the growth curve
- N_o = Mean nominal number of cells/mL at t_o
- N_1 = Mean measured number of cells/mL at t_1
- N_2 = Mean measured number of cells/mL at t_2
- N_n = Mean measured number of cells/mL at t_n
- t_1 = Time of first measurement after beginning of test (hours)
- t_2 = Time of second measurement after beginning of test (hours)
- t_n = Time of n^{th} measurement after beginning of test (hours)

Growth rate was calculated for each replicate of the control and treatment group using the following formula:

$$\mu = \frac{\ln N_n - \ln N_o}{t_n - t_o}$$

where:
- μ = Average specific growth rate
- N_o = Nominal cell density (cells/mL) at t_o
- N_n = Measured cell density (cells/mL) at t_n
- t_o = Time of beginning of test (hours)
- t_n = Time after beginning of test (hours)

Inhibition values were calculated for the 1000 mg/L WAF treatment group as the percent reduction in cell density, area under the growth curve (biomass) and growth rate relative to the negative control replicates. The following formula was used:

$$\text{Percent Inhibition} = \frac{\text{Mean Negative Control Response - Mean Treatment Response}}{\text{Mean Negative Control Response}} \times 100$$

The percent inhibition observed in the treatment group was used to determine whether the EL50 values were greater or less than the 1000 mg/L WAF loading rate. The data were evaluated for normality and homogeneity of variance (p=0.05) using the Shapiro-Wilk's and Levene's tests, respectively. The treatment group then was compared to the negative control using Dunnett's test (p=0.05). The results of the statistical analyses, as well as an evaluation of the concentration-response pattern, were used to determine the NOELR relative to each parameter at 72 and 96 hours.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Results of analyses to measure polyaromatic hydrocarbon (PAH) and metals in the WAF and control solutions are presented in Tables 1-27 and the analytical chemistry reports (Appendix 9 & 10). All measurements of PAH's and metals in control and WAF solutions were below detection limits for the methods. Therefore estimates of the EL50 and NOELR values were based on the nominal WAF loading rate of 1000 mg/L used in the test.

Observations and Measurements

At stirring initiation, the test solution appeared clear with test material floating. After stirring, solutions appeared to have test material floating on the surface and settling at the bottom. The test solutions were siphoned from the bottom of the mixing vessels directly into the test chambers. At each sampling interval no surface slicks were noted, however, particles were observed at the bottom of the test chambers.

Measurements of temperature, pH and light intensity are presented in Tables 27, 28 and 29, respectively. Temperatures ranged from 23.0 to 23.6°C and were within the 24 ± 2°C range established for the test. Measurements of pH in both control and WAF solutions were 7.9 on Day 0 and Day 1. On Day 2 pH measurements were 8.6 in the control and 8.3 in the 1000 mg/L WAF. On Days 3 and 4, pH measurements in the control and 1000 mg/L WAF were 8.9 and 8.5, and 10.6 and 9.8, respectively. The pH tended to increase relative to increases in algal densities, which is typical for tests conducted with *Selenastrum capricornutum*. The light intensity ranged from 3870 to 4670 lux, which was within the desired range of 4300 ± 430 lux. The shaker tables maintained their speed of 100 rpm.

The toxicity of the petroleum coke WAF to *Selenastrum capricornutum* was determined by evaluating changes in cell density over a 96-hour exposure period. Cell densities were used to calculate areas under the growth curve (biomass) and growth rates for each 24-hour interval of exposure. Mean cell densities, areas under the growth curve and growth rates with the corresponding percent inhibition values are presented in Tables 30, 31, and 32, respectively. Estimates of the EL50, E_bL50 and E_rL50 are presented in Table 33. Individual replicate data for each growth parameter are presented in Appendices 11, 12 and 13, while mean cell densities are illustrated graphically in Figure 1. Changes in mean cell density in the negative control replicates over the 96-hour exposure period indicated that exponential growth of cells occurred in those replicates (Figure 1).

After 72 hours of exposure the percent inhibition values for cell density, area under the growth curve and growth rate in the 1000 mg/L WAF treatment group were 34, 26 and 12%, respectively, relative to the negative control. Dunnett's test indicated that cell density, biomass and growth rate were significantly reduced ($p<0.05$) in the 1000 mg/L WAF treatment group. Consequently, the 72-hour NOELR for cell density, biomass and growth rate was <1000 mg/L.

After 96 hours of exposure, the percent inhibition values for cell density, area under the growth curve and growth rate in the 1000 mg/L WAF treatment group were 27, 28 and 7.1%, respectively, relative to the negative control. Dunnett's test indicated that cell density, biomass and growth rate were significantly reduced ($p<0.05$) in the 1000 mg/L WAF treatment group. Consequently, the 96-hour NOELR for cell density, biomass and growth rate was <1000 mg/L.

After 96 hours of exposure, there were no signs of adherence of cells to the test chambers or aggregation/flocculation of algae in the controls or in the treatment group. There were no noticeable changes in cell morphology in the tested concentration when compared to the control.

CONCLUSIONS

Selenastrum capricornutum were exposed to a 1000 mg/L water accommodated fraction (WAF) loading rate of petroleum coke and evaluated for effects on cell density, area under the growth curve and growth rate. The 72 and 96-hour EL50 values, based on cell density (EL50), biomass (EL_b50) and growth rate (EL_r50), were >1000 mg/L. The 72 and 96 hour NOELR, based on cell density, biomass and growth rate was <1000 mg/L.

REFERENCES

1 **Title 40 of the Code of Federal Regulations, Part 792.** 1989. *Toxic Substances Control Act (TSCA) Good laboratory Practice standards*.

2 **OECD.** 1998. *OECD Principle of Good Laboratory Practice* ENV/MC/CHEM (98) 17.

3 **Organization for Economic Cooperation and Development.** 2000. *Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures.* ENV/JM/MONO (2000) 6. OECD Environmental Health and Safety Publication Series on Testing and Assessment No. 23. OECD, Paris, September 2000.

4 **Organization of Economic Cooperation and Development.** 1984. *Algal, Growth Inhibition Test*. OECD Guideline for Testing of Chemicals. Guideline 201. Paris.

5 **Official Journal of the European Communities.** 1992. No. L383. Method C.3.: *Algal Inhibition Test.*

6 **U.S. Environmental Protection Agency.** 1996. Series 850 – Ecological Effects Test Guidelines (draft), OPPTS Number 850.5400: *Algal Toxicity, Tiers I and II*.

7 **ASTM Standard Guide 1218-90E** *, Standard Guide for Conducting Static 96-Hour Toxicity Tests with Microalgae*. August 1990.

8 **Timothy Z. Kendall and Willard B. Nixon.** 2005. Analytical Method Verification for the Determination of Water Soluble Components of Petroleum Coke in Freshwater Using Gas Chromatography (GC) or High Performance Liquid Chromatography (HPLC). Wildlife International, Ltd. Project Number 472C-104. Unpublished.

9 **Raymond L. Van Hoven and Willard B. Nixon.** 2005. Analytical Method Verification for the Determination of Water Soluble Components of Petroleum Coke in Freshwater Using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). Wildlife International, Ltd. Project Number 472C-105. Unpublished.

10 **The SAS System for Windows**. 1996. Version 8.02. SAS Institute Inc., Cary, North Carolina.

Table 1

Measured Concentrations of Naphthalene Analyzed by HPLC/UV

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	9.96	99.6
40.0	MAS-2	--	39.9	99.9
100	MAS-3	--	97.8	97.8
			Mean =	99.1
			Standard Deviation =	1.14
			CV =	1.15%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 2

Measured Concentrations of Acenaphthylene Analyzed by HPLC/UV

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	9.88	98.8
40.0	MAS-2	--	40.2	101
100	MAS-3	--	100	100
			Mean =	99.9
			Standard Deviation =	1.10
			CV =	1.10%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 3

Measured Concentrations of 1-Methylnaphthalene Analyzed by HPLC/UV

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	10.0	100
40.0	MAS-2	--	40.1	100
100	MAS-3	--	98.3	98.3
			Mean =	99.4
			Standard Deviation =	0.98
			CV =	0.99%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 4

Measured Concentrations of 2-Methylnaphthalene Analyzed by HPLC/UV

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	10.1	101
40.0	MAS-2	--	40.1	100
100	MAS-3	--	98.2	98.2
			Mean =	99.7
			Standard Deviation =	1.42
			CV =	1.42%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 5

Measured Concentrations of Fluorene Analyzed by HPLC/UV

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	10.9	109
40.0	MAS-2	--	39.6	98.9
100	MAS-3	--	99.3	99.3
			Mean =	102
			Standard Deviation =	5.72
			CV =	5.61%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 6

Measured Concentrations of Acenaphthene Analyzed by HPLC/UV

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	9.84	98.4
40.0	MAS-2	--	40.5	101
100	MAS-3	--	100	100
			Mean =	99.8
			Standard Deviation =	1.31
			CV =	1.31%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 7

Measured Concentrations of Phenanthrene Analyzed by HPLC/UV

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	9.90	99.0
40.0	MAS-2	--	40.6	101
100	MAS-3	--	101	101
			Mean =	100
			Standard Deviation =	1.15
			CV =	1.15%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 8

Measured Concentrations of Anthracene Analyzed by HPLC with Fluorescence Detection

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	10.7	107
40.0	MAS-2	--	41.6	104
100	MAS-3	--	103	103
			Mean =	105
			Standard Deviation =	2.08
			CV =	1.98%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 9

Measured Concentrations of Fluoranthrene Analyzed by HPLC with Fluorescence Detection

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	9.89	98.9
40.0	MAS-2	--	41.4	103
100	MAS-3	--	99.9	99.9
			Mean =	101
			Standard Deviation =	2.14
			CV =	2.12%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 10

Measured Concentrations of Pyrene Analyzed by HPLC with Fluorescence Detection

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	9.89	98.9
40.0	MAS-2	--	40.9	102
100	MAS-3	--	99.4	99.4
			Mean =	99.8
			Standard Deviation =	1.66
			CV =	1.66%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 11

Measured Concentrations of Chrysene Analyzed by HPLC/UV

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	8.75	87.5
40.0	MAS-2	--	39.2	98.0
100	MAS-3	--	99.1	99.1
			Mean =	94.9
			Standard Deviation =	6.40
			CV =	6.75%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 12

Measured Concentrations of Benzo(a)anthracene Analyzed by HPLC with Fluorescence Detection

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	9.81	98.1
40.0	MAS-2	--	40.9	102
100	MAS-3	--	101	101
			Mean =	100
			Standard Deviation =	2.03
			CV =	2.03%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 13

Measured Concentrations of Benzo(b)fluoranthene Analyzed by HPLC with Fluorescence Detection

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	9.03	90.3
40.0	MAS-2	--	39.2	97.9
100	MAS-3	--	98.1	98.1
			Mean =	95.4
			Standard Deviation =	4.45
			CV =	4.66%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 14

Measured Concentrations of Benzo(k)fluoranthene Analyzed by HPLC with Fluorescence Detection

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	8.98	89.8
40.0	MAS-2	--	38.9	97.2
100	MAS-3	--	99.0	99.0
			Mean =	95.3
			Standard Deviation =	4.88
			CV =	5.12%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 15

Measured Concentrations of Benzo(a)pyrene Analyzed by HPLC/UV

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	9.30	93.0
40.0	MAS-2	--	39.5	98.7
100	MAS-3	--	99.7	99.7
			Mean =	97.1
			Standard Deviation =	3.61
			CV =	3.72%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 16

Measured Concentrations of Dibenz(a,h)anthracene Analyzed by HPLC with Fluorescence Detection

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	9.07	90.7
40.0	MAS-2	--	39.1	97.9
100	MAS-3	--	99.9	99.9
			Mean =	96.2
			Standard Deviation =	4.84
			CV =	5.03%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 17

Measured Concentrations of Indeno(1,2,3-cd)pyrene Analyzed by HPLC/UV

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	8.63	86.3
40.0	MAS-2	--	39.4	98.6
100	MAS-3	--	99.7	99.7
			Mean =	94.9
			Standard Deviation =	7.44
			CV =	7.84%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 18

Measured Concentrations of Benzo(g,h,i)perylene Analyzed by HPLC with Fluorescence Detection

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	9.43	94.3
40.0	MAS-2	--	40.1	100
100	MAS-3	--	99.7	99.7
			Mean =	98.0
			Standard Deviation =	3.21
			CV =	3.27%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 19

Measured Concentrations of Dibenzo(a,e)pyrene Analyzed by HPLC with Fluorescence Detection

Nominal Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]
Negative Control	3	0	< LOQ	--
1000	4	0	< LOQ	--
Negative Control	7	72	< LOQ	--
1000	8	72	< LOQ	--
Negative Control	11	96	< LOQ	--
1000	12	96	< LOQ	--

Matrix Blank and Fortification Samples

Nominal Concentration (µg/L)	Sample Identification (472A-114-)	Sampling Interval (Hour)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]
0.0	MAB-1	--	< LOQ	--
10.0	MAS-1	--	9.89	98.9
40.0	MAS-2	--	39.8	99.5
100	MAS-3	--	102	102
			Mean =	100
			Standard Deviation =	1.64
			CV =	1.64%

[1] The limit of quantitation (LOQ) was 5.00 µg/L, calculated as the product of the lowest standard concentration (5.00 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Table 20

Measured Concentrations of Arsenic Analyzed by ICP-AES

Nominal Petroleum Coke Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hours)	Measured Arsenic Concentration (µg/L) [1,2]
Negative Control	1	0	< LOQ
1000	2	0	< LOQ
Negative Control	5	72	< LOQ
1000	6	72	< LOQ
Negative Control	9	96	< LOQ
1000	10	96	< LOQ

Matrix Blank and Fortification Samples

Nominal Arsenic Concentration (µg/L)	Sample Identification (472A-114-)	Measured Arsenic Concentration (µg/L) [1]	Percent of Nominal [1]
0.0	MAB-1	< LOQ	--
50.0	MAS-2	45.4	90.7

[1] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

[2] The limit of quantitation (LOQ) for these analyses was set at 20 µg/L (7).

Table 21

Measured Concentrations of Copper Analyzed by ICP-AES

Nominal Petroleum Coke Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hours)	Measured Copper Concentration (µg/L) [1,2]
Negative Control	1	0	< LOQ
1000	2	0	< LOQ
Negative Control	5	72	< LOQ
1000	6	72	< LOQ
Negative Control	9	96	< LOQ
1000	10	96	< LOQ

Matrix Blank and Fortification Samples

Nominal Copper Concentration (µg/L)	Sample Identification (472A-114-)	Measured Copper Concentration (µg/L) [1]	Percent of Nominal [1]
0.0	MAB-1	< LOQ	--
50.0	MAS-2	43.6	87.3

[1] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

[2] The limit of quantitation (LOQ) for these analyses was set at 20 µg/L (7).

Table 22

Measured Concentrations of Iron Analyzed by ICP-AES

Nominal Petroleum Coke Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hours)	Measured Iron Concentration (µg/L) [1,2]
Negative Control	1	0	< LOQ
1000	2	0	< LOQ
Negative Control	5	72	< LOQ
1000	6	72	< LOQ
Negative Control	9	96	< LOQ
1000	10	96	< LOQ

Matrix Blank and Fortification Samples

Nominal Iron Concentration (µg/L)	Sample Identification (472A-114-)	Measured Iron Concentration (µg/L) [1]	Percent of Nominal [1]
0.0	MAB-1	< LOQ	--
25.0	MAS-1	27.5	110

[1] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.
[2] The limit of quantitation (LOQ) for these analyses was set at 10 µg/L (7).

Table 23

Measured Concentrations of Nickel Analyzed by ICP-AES

Nominal Petroleum Coke Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hours)	Measured Nickel Concentration (µg/L) [1,2]
Negative Control	1	0	< LOQ
1000	2	0	< LOQ
Negative Control	5	72	< LOQ
1000	6	72	< LOQ
Negative Control	9	96	< LOQ
1000	10	96	< LOQ

Matrix Blank and Fortification Samples

Nominal Nickel Concentration (µg/L)	Sample Identification (472A-114-)	Measured Nickel Concentration (µg/L) [1]	Percent of Nominal [1]
0.0	MAB-1	< LOQ	--
25.0	MAS-1	27.0	108

[1] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.
[2] The limit of quantitation (LOQ) for these analyses was set at 10 µg/L (7).

Table 24

Measured Concentrations of Selenium Analyzed by ICP-AES

Nominal Petroleum Coke Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hours)	Measured Selenium Concentration (µg/L) [1,2]
Negative Control	1	0	< LOQ
1000	2	0	< LOQ
Negative Control	5	72	< LOQ
1000	6	72	< LOQ
Negative Control	9	96	< LOQ
1000	10	96	< LOQ

Matrix Blank and Fortification Samples

Nominal Selenium Concentration (µg/L)	Sample Identification (472A-114-)	Measured Selenium Concentration (µg/L) [1]	Percent of Nominal [1]
0.0	MAB-1	< LOQ	--
500	MAS-2	459	91.9

[1] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

[2] The limit of quantitation (LOQ) for these analyses was set at 200 µg/L (7).

Table 25

Measured Concentrations of Vanadium Analyzed by ICP-AES

Nominal Petroleum Coke Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hours)	Measured Vanadium Concentration (µg/L) [1,2]
Negative Control	1	0	< LOQ
1000	2	0	< LOQ
Negative Control	5	72	< LOQ
1000	6	72	< LOQ
Negative Control	9	96	< LOQ
1000	10	96	< LOQ

Matrix Blank and Fortification Samples

Nominal Vanadium Concentration (µg/L)	Sample Identification (472A-114-)	Measured Vanadium Concentration (µg/L) [1]	Percent of Nominal [1]
0.0	MAB-1	< LOQ	--
1.00	MAS-1	1.13	113

[1] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

[2] The limit of quantitation (LOQ) for these analyses was set at 0.40 µg/L (7).

Table 26

Measured Concentrations of Sulfur Analyzed by ICP-AES

Nominal Petroleum Coke Concentration (mg/L)	Sample Identification (472A-114-)	Sampling Interval (Hours)	Measured Sulfur Concentration (mg/L) [1,2]
Negative Control	1	0	< LOQ
1000	2	0	< LOQ
Negative Control	5	72	< LOQ
1000	6	72	< LOQ
Negative Control	9	96	< LOQ
1000	10	96	< LOQ

Matrix Blank and Fortification Samples

Nominal Sulfur Concentration (mg/L)	Sample Identification (472A-114-)	Measured Sulfur Concentration (mg/L) [1]	Percent of Nominal [1]
0.0	MAB-1	< LOQ	--
20.0	MAS-1	24.9	124

[1] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.
[2] The limit of quantitation (LOQ) for these analyses was set at 10 mg/L (7).

Table 27

Temperature Measurements

Time (Days)	Temperature (°C)	
	Measurement 1	Measurement 2[1]
0	23.1	23.5
1	23.6	23.4
2	23.6	23.0
3	23.6	23.0
4	23.1	23.0

[1] Temperature Measurement 2 was typically taken at least 4 hours after Measurement 1.

Table 28

pH Measurements

Nominal WAF Concentration (mg/L)	pH Measurements				
	Day 0[1] Day	1[2] Day	2[3] Day	3[4] Day	4[5]
Negative Control	7.9 7.9 8.6			8.9	10.6
1000	7.9 7.9 8.3			8.5	9.8

[1] Day 0 samples were collected from the individual batches of test solution prepared for each treatment and control group at test initiation.
[2] 24-hour samples were collected from the pooled A, B, C, D, E and F replicates.
[3] 48-hour samples were collected from the pooled G, H, I, J, K and L replicates.
[4] 72-hour samples were collected from the pooled M, N, O, P Q and R replicates.
[5] 96-hour samples were collected from the pooled S, T, U, V, W and X replicates.

Table 29

Light Intensity Measurements

Test Day	Light Intensity (lux)[1]				
	Measurement No. 1	Measurement No. 2	Measurement No. 3	Measurement No. 4	Measurement No. 5
0 3980		3880	4520	4620	4140
1 4370		3990	3970	4040	4580
2 4000		4200	4520	4370	3880
3 4080		4090	4480	4210	3910
4 4020		3870	4280	4240	3910

[1] Measurements taken on shaker table # 1 at five locations surrounding the test flasks at test solution level.

Test Day	Light Intensity (lux)[1]				
	Measurement No. 1	Measurement No. 2	Measurement No. 3	Measurement No. 4	Measurement No. 5
0 4010		4380	4520	3910	4090
1 4120		4450	3920	4470	4340
2 4130		4360	4670	4040	3920
3 4180		4280	4570	4020	3980
4 3980		4190	4060	3960	4620

[1] Measurements taken on shaker table # 7 at five locations surrounding the test flasks at test solution level.

Table 30

Mean Cell Densities and Percent Inhibition

Nominal WAF Concentration (mg/L)	24 Hours		48 Hours		72 Hours		96 Hours	
	Cell Density[1]	Percent Inhibition[1,2]	Cell Density[1]	Percent Inhibition[1,2]	Cell Density[1]	Percent Inhibition[1,2]	Cell Density[1]	Percent Inhibition[1,2]
Negative Control	24,000	--	68,500	--	181,000	--	413,333	--
1000	20,667	14	58,833	14	119,333*	34	301,667*	27

[1] Calculations were performed using SAS version 8.02. Manual calculations may differ slightly due to rounding.
[2] Percent inhibition was calculated relative to the control replicates.
* There were statistically significant differences ($p \leq 0.05$) at 72 and 96 hours from the control replicates using the Dunnett's test.

Table 31

Mean Areas Under the Growth Curve (Biomass) and Percent Inhibition

Nominal WAF Concentration (mg/L)	0 - 24 Hours		0 - 48 Hours		0 - 72 Hours		0 - 96 Hours	
	Mean Area [1]	Percent Inhibition [1,2]	Mean Area [1]	Percent Inhibition [1,2]	Mean Area [1]	Percent Inhibition [1,2]	Mean Area [1]	Percent Inhibition [1,2]
Negative Control	228,000	--	1,218,000	--	4,092,000	--	1,1104,000	--
1000	188,000	18	1,022,000	16	3,040,000*	26	7,972,000*	28

[1] Calculations were performed using SAS version 8.02. Manual calculations may differ slightly due to rounding.
[2] Percent inhibition was calculated relative to the control replicates.
* There were statistically significant differences ($p \leq 0.05$) at 72 and 96 hours from the control replicates using the Dunnett's test.

Table 32

Mean Growth Rates and Percent Inhibition

Nominal WAF Concentration (mg/L)	0 - 24 Hours		0 - 48 Hours		0 - 72 Hours		0 - 96 Hours	
	Mean Growth Rate [1]	Percent Inhibition [1,2]	Mean Growth Rate [1]	Percent Inhibition [1,2]	Mean Growth Rate [1]	Percent Inhibition [1,2]	Mean Growth Rate [1]	Percent Inhibition [1,2]
Negative Control	0.0645	--	0.0542	--	0.0497	--	0.0457	--
1000	0.0586	9.0	0.0507	6.4	0.0438*	12	0.0425*	7.1

[1] Calculations were performed using SAS version 8.02. Manual calculations may differ slightly due to rounding.
[2] Percent inhibition was calculated relative to the control replicates.
* There were statistically significant differences ($p \leq 0.05$) at 72 and 96 hours from the control replicates using the Dunnett's test.

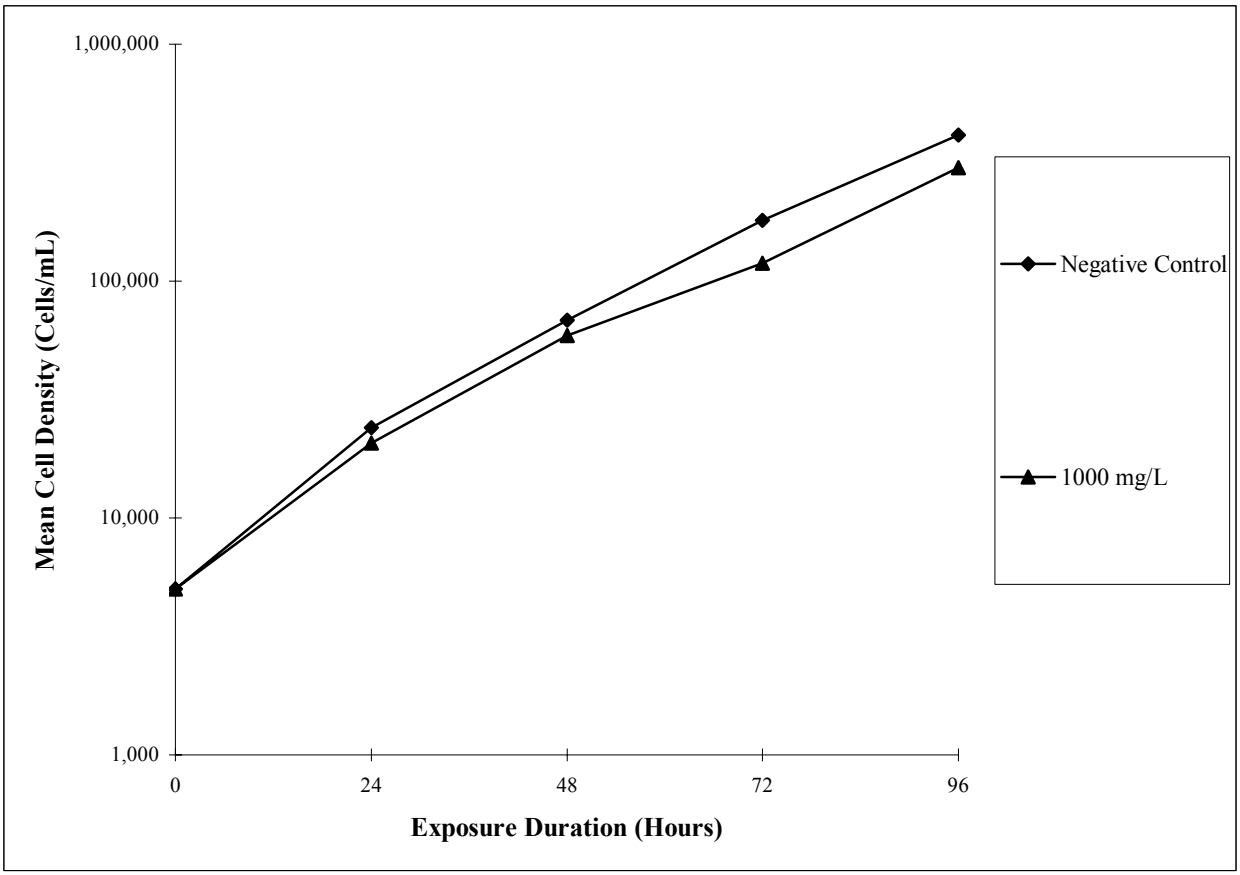
Table 33

Estimates of the EL50, E_bL50 and E_rL50 Values Over the 96-Hour Exposure Period

Time	Cell Density		Area Under the Growth Curve (Biomass)		Growth Rate	
	EL50 (mg/L)	95% Confidence Interval (mg/L)	E_bL50 (mg/L)	95% Confidence Interval (mg/L)	E_rL50 (mg/L)	95% Confidence Interval (mg/L)
24 Hours	>1000	--[1]	>1000	--[1]	>1000	--[1]
48 Hours	>1000	--[1]	>1000	--[1]	>1000	--[1]
72 Hours	>1000	--[1]	>1000	--[1]	>1000	--[1]
96 Hours	>1000	--[1]	>1000	--[1]	>1000	--[1]

[1] 95% confidence limits could not be calculated with the data obtained.

Figure 1. Concentration-response curve for *Selenastrum capricornutum* exposed to a 1000 m g/L WAF solution of Petroleum Coke for 96 hours, expressed as cell density.



Appendix 1

Exploratory Non-GLP Rangefinding Toxicity Test with Petroleum Coke

Wildlife International, Ltd.
ECOTOXICOLOGY & ANALYTICAL TESTING SERVICES

EXPLORATORY NON-GLP RANGEFINDING TOXICITY TEST WITH PETROLEUM COKE

96-Hour Toxicity Rangefinding Test with *Selenastrum capricornutum*

Project Number 472A-114

Introduction

An exploratory non-GLP rangefinding test was conducted from December 8 to 12, 2004 in the Wildlife International, Ltd. aquatic toxicology laboratory. The test was conducted under static conditions for 96 hours, with no renewal of test solutions.

Methods and Materials

Test solutions were prepared as water accomodated fractions (WAF) at nominal loading rates of 10, 100 and 1000 mg test substance/L. An untreated control group was maintained concurrently. For each WAF, a calculated amount of test substance was mixed with 4 L of algal media in a 4 L Pyrex aspirator bottle with tubulation. The solution was stirred for approximately 24 hours on a magnetic stir plate, with a vortex maintained at approximately 30% of the solution height. After mixing, each solution was allowed to settle for approximately one hour, and the solution was decanted into one test chamber per concentration. Test chambers were 300 mL BOD bottles with glass stoppers, filled completely with test solution to minimize headspace.

One test chamber per treatment and control group was initiated with 5,000 *Selenastrum* cells/mL of test solution. The test chambers were placed in a temperature-controlled environmental chamber set to maintain the target temperature of 24 ± 2°C and target light intensity of 4300±430 lux. Cell counts were preformed using a hemacytometer and microscope after 96 hours of incubation.

Results

The results of the rangefinding test are included in the attached table. Based on the nominal WAF concentrations, the 96-hour EL50 value for the rangefinding test was estimated to be greater than 1000 mg/L, the highest loading rate tested. No effects were seen at the highest loading rate used in the test (no observed effect level = 1000 mg/L loading rate).

Wildlife International, Ltd.

PETROLEUM COKE

RESULTS OF A 96-HOUR RANGEFINDING TEST WITH *Selenastrum capricornutum*

(Preliminary results not audited by Quality Assurance)

STUDY: Petroleum Coke: A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)
SPONSOR: American Petroleum Institute
PROJECT NO.: 472A-114

Nominal Loading rate (mg/L)	96-Hour Cell Density (cells/mL)	Percent Inhibition
Negative Control	4.25×10^5	--
10	4.30×10^5	-1.2
100	4.65×10^5	-9.4
1000[1]	4.35×10^5	-2.4

[1]Particulates noted at bottom of test chamber

Appendix 2

Protocol, Protocol Amendments and Deviations

PROTOCOL

PETROLEUM COKE: A 96-HOUR TOXICITY TEST
WITH THE FRESHWATER ALGA (*Selenastrum capricornutum*)

Guidelines:

OECD Guideline 201

EU Directive 92/69/EEC, Method C.3.

U.S. EPA OPPTS Number 850.5400

Submitted to

American Petroleum Institute
1220 L Street, N.W.
Washington, DC 20005

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

March 30, 2004

Wildlife International, Ltd.

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PETROLEUM COKE: A 96-HOUR TOXICITY TEST
WITH THE FRESHWATER ALGA (*Selenastrum capricornutum*)

SPONSOR: American Petroleum Institute
1220 L Street, N.W.
Washington, DC 20005

SPONSOR'S REPRESENTATIVE: [redacted]

SPONSOR'S TECHNICAL STUDY MONITOR: [redacted]

TESTING FACILITY: Wildlife International, Ltd.
8598 Commerce Drive
Easton, Maryland 21601

STUDY DIRECTOR: [redacted]
Wildlife International Ltd.

LABORATORY MANAGEMENT: [redacted]

FOR LABORATORY USE ONLY

Proposed Dates:	
Experimental Start Date: _____	Experimental Termination Date: _____
Project No.: 472A-114	
Test Concentrations: _____	
Test Substance No.: 6485 Reference Substance No. (if applicable): _____	

PROTOCOL APPROVAL

[redacted]

28 April 2004
DATE

28 April 2004
DATE

01 April, 2004
DATE

PROTOCOL NO.: 472/033004/SEL-OECD/SUB472

Wildlife International, Ltd.

INTRODUCTION

Wildlife International, Ltd. will conduct an algal toxicity test to determine the effects of water soluble components of petroleum coke on the freshwater alga, *Selenastrum capricornutum*, for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. Petroleum coke is defined as the product formed by subjecting the heavy tar-like residue remaining following oil refining to high temperatures and pressures. It consists of primarily elemental carbon with considerably smaller amounts of hydrocarbons, sulfur and trace amounts of heavy metals. The study will be performed based on procedures outlined in OECD Guideline for Testing of Chemicals 201: *Alga, Growth Inhibition Test* (1); Official Journal of the European Communities No. L383 C.3. *Algal Inhibition Test* (2); and U.S. Environmental Protection Agency Series 850 - Ecological Effects Test Guidelines OPPTS Number 850.5400 (3), when possible. Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site or at an alternative location to be specified in the final report.

OBJECTIVE

The objective of this study is to determine the toxicity of water soluble components of petroleum coke to the freshwater green alga, *Selenastrum capricornutum*.

EXPERIMENTAL DESIGN

The green alga, *Selenastrum capricornutum*, will be exposed to a geometric series of at least five water accommodated fraction (WAF) loading rates and a negative (culture medium) control for 96 hours. WAF loading rates will be selected in consultation with the Sponsor and will be based on information such as a results of rangefinding toxicity data, known toxicity data, physical/chemical properties of the test substance or other relevant information. Target loading rates will not exceed 1000 mg/L. For this test, the term loading rate means the total amount of test substance added to the dilution water volume (mg/L) to achieve the respective WAF solution. Generally, each concentration of test material used in the definitive test will be at least 50% of the next higher treatment, unless information concerning the concentration-effect curve indicates that a different dilution factor is more appropriate. Water samples will be collected at specified intervals for analysis of selected constituents of petroleum coke.

Test solutions will be inoculated at the beginning of the test with approximately 5,000 *Selenastrum* cells/mL. Twelve replicates in the control and treatment groups will be tested. In order to control bias, the

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position of the flasks will be determined by indiscriminate draw at the beginning of the experiment and daily during the exposure period. No other potential sources of bias are expected to affect the results of the study.

The response of the algae will be measured in terms of biomass expressed as cell density, area under the growth curve and growth rate. EL50 values (i.e., the theoretical loading rate that produces a 50% reduction in the measured parameter) will be calculated, if possible, for each 24-hour interval of the test. The no observed adverse effect level (NOAEL), which is the highest WAF loading rate that has no adverse inhibitory effect on algal growth, will be determined relative to each parameter at 72 and 96 hours based on evaluation of the statistical results and the dose-response pattern. At the end of the 96-hour exposure, algistatic effects will be differentiated from algicidal effects in those treatments which are maximally inhibited.

MATERIALS AND METHODS

Test Substance

The test substance is green coke (CAS Number 64741-79-3) sieved to approximately 2 mm particle size. Information on the characterization of test, control or reference substances is required by OECD Principles of Good Laboratory Practice (3) and TSCA Good Laboratory Practice Standards (40 CFR Part 792) (4). The Sponsor is responsible for providing Wildlife International, Ltd. written verification that the test substance has been characterized according to GLP prior to its use in the study. If written verification of GLP test substance characterization is not provided to Wildlife International, Ltd., it will be noted in the compliance statement of the final report.

The Sponsor is responsible for all information related to the test substance and agrees to accept any unused test substance and/or test substance containers remaining at the end of the study.

Preparation of Water Accommodated Fraction Solutions

The test substance will be mixed directly with culture medium on a weight:volume basis. Each WAF will be prepared individually in a 4 L Pyrex® aspirator bottle with tubulation by mixing an amount of the test substance in approximately 4 L of dilution water using a Teflon®-coated stir bar on a magnetic stir plate. Care will be taken to maintain a vortex depth of approximately 30% of the test solution height.

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The length of the mixing time in the definitive test will be determined based on results of the WAF equilibration trial. Following the mixing period, the WAF solutions will then be allowed to settle for approximately 1 hour and the test solution will be decanted off the top (or bottom, as appropriate).

Justification for Route of Exposure

The test substance will be administered to the test organism in algal medium in the form of water accommodated fractions. The route of exposure is justified because it is the primary exposure pathway of algae to chemicals.

Test Organism

The freshwater green alga, *Selenastrum capricornutum*, will be used in this test. This species is representative of an important group of algae and was selected for use in the test based upon past use and ease of handling in the laboratory. Stock cultures, obtained from the Culture Collection of Algae at the University of Texas at Austin or another supplier, will be maintained in culture medium at Wildlife International, Ltd. for a minimum of two weeks prior to use in a toxicity test. Algae used in toxicity tests will be in exponential growth phase, which is defined as the period of growth when algal cells are dividing at a constant rate.

Just prior to beginning the test, an inoculum of the stock culture will be prepared so that each milliliter of inoculum contains enough cells to provide an initial cell density of approximately 5,000 cells/mL in each replicate.

Culture Medium

Culture medium prepared according to Wildlife International, Ltd. Standard Operating Procedures will be used as dilution water. The concentrations of the components in the medium are presented in Table 1 (1, 6). In addition to the medium components presented in Table 1, the medium will also contain supplemental sodium bicarbonate (35 mg/L). The total concentration of sodium bicarbonate in the medium will be 50 mg/L. Stock nutrient solutions will be prepared by adding reagent-grade chemicals to purified Wildlife International, Ltd. well water (e.g., NANOpure®). Appropriate volumes of the stock nutrient solutions will then be diluted with purified well water to prepare the medium. The medium will be filter sterilized (0.22 μm) or autoclaved prior to use. The final pH of the medium should be 7.5 ± 0.1. Analyses will be performed at least once annually to determine the concentrations of selected organics and

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inorganic constituents of the well water and the results of the most recent GLP-compliant analyses will be summarized in the final report.

Specifications for acceptable levels of contaminants have not been established for culture medium. However, there are no levels of contaminants reasonably expected to be present in the medium that are considered to interfere with the purpose or conduct of the study.

Test Apparatus

Test chambers will be sterile, 300-mL glass BOD bottles with glass stoppers. The bottles will be completely filled with test solution to avoid headspace. Each test chamber will contain two glass marbles to promote mixing. Test chambers will be indiscriminately positioned on a mechanical shaker table in an environmental chamber and will be shaken continuously at approximately 100 rpm. The shaker will be checked daily during the test to ensure the proper setting. Test chambers will be labeled with the project number, test loading rate, and replicate.

Environmental Conditions

Test flasks will be held at 24 ± 2°C under continuous fluorescent lighting (e.g., cool white light tubes) at an intensity of approximately 4300 ± 430 lux. Light intensity will be measured at five locations on the shaker table daily during the test. Temperature will be measured twice daily in the environmental chamber using a liquid-in-glass thermometer.

The pH of each treatment and the control will be measured at test initiation, at each 24-hour sampling interval and at termination using a Fisher Accumet Model 915 pH meter or equivalent. Samples will be collected from the batch of test solution prepared at test initiation for each treatment and control group for the measurement of pH. At 24, 48, 72 and 96 hours, pH will be measured in pooled test solution samples collected from the three replicates sacrificed from each treatment and the control group. At test termination, pH will be measured in pooled test solution samples collected from the remaining three replicates of each treatment and control group. An explanation will be provided if pH deviations of more than one unit are observed.

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Observations of test substance solubility (e.g., surface slicks, precipitates, etc.) also will be recorded.

Biological Measurements

Cell densities will be monitored during the test by conducting cell counts using either a hemacytometer and a microscope or an electronic particle counter (Coulter Electronics, Hialeah, Florida). One sample will be collected from three replicates of the treatment and control groups at 24, 48, 72 and 96 hours. At each measurement period, the three replicates will be destructively sampled (i.e., samples taken and then replicates removed from the test). The samples will be evaluated immediately or will be held in the dark under refrigeration until cell counts can be performed. Each sample will be diluted using an electrolyte solution (Isoton®), as needed, to maintain counting accuracy. If counts are performed with a hemacytometer, a small amount of each sample will be loaded onto the hemacytometer and the total number of cells in 10 grids will be counted. The cell density of the sample will be calculated based on the mean number of cells per grid. If an electronic particle counter is used, the samples will be diluted as necessary, using Isoton®, and three, 0.5-mL volumes of the sample will be counted. In this case, the cell density of each sample will be the mean of the three counts. Exponential growth in the negative control will be confirmed at 96 hours. The test will not be considered valid if exponential growth at 96 hours cannot be determined.

Samples of test solutions will be collected from each of the three replicate per treatment and control group at the end of the test. These samples will be pooled within their respective treatments, and subsamples will be removed and examined microscopically for atypical cell morphology (e.g., changes in shape, size or color). Aggregations or flocculations of cells and adherence of the cells to the test chamber will be documented if observed during the test.

At the end of the test, algistatic effects, which result in the inhibition of cell growth, will be differentiated from algicidal effects, which result in the death of cells. Aliquots (0.5 mL) of test solutions will be taken from each replicate test chamber where growth is maximally inhibited. Maximally inhibited treatments are those in which it cannot be visually determined if live algal cells are present (e.g. absence of color to the test solution). These aliquots will be combined and diluted with algal medium to a concentration of the test substance that theoretically should not affect growth. A negative control will be

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prepared by diluting a 0.5-mL aliquot from one negative control replicate to 100 mL with algal medium. Growth of these subcultures will be monitored for up to 9 days to determine whether inhibition of growth observed during the test is reversible. Samples will be collected for cell counts at recovery phase initiation, termination and at approximately 3-day intervals between initiation and termination. The recovery phase may be terminated once algal growth is sufficient to indicate that the algal cells have fully recovered from effects due to the test substance. This will be determined through visual examination of the recovery test solutions.

Sampling for Analytical Measurements

Test solution samples will be decanted from each WAF preparation vessel at the beginning of the test, and collected from the pooled replicates of each treatment level after 72 hours and at the end of the test to determine concentrations of the constituents of interest in petroleum coke. Samples will be collected and analyzed immediately or placed in a glass container with minimal head space and stored under refrigeration until analyzed. Samples will be analyzed for the components of petroleum coke listed in Table 2. The sample scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES

Experimental Group	Day 0	Day 3	Day 4
Control	1	1	1
Level 1-Low Concentration	1	1	1
Level 2	1	1	1
Level 3	1	1	1
Level 4	1	1	1
Level 5-High Concentration	1	1	1
Totals	6	6	6

Total Number of Verification Samples = 18

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study. At the discretion of the Study Director, samples from one or more appropriate test chambers will be collected and analyzed if an error in sampling or analysis is suspected.

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The reason for the additional samples will be documented in the raw data and summarized in the final report.

Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International, Ltd. The analytical method used will be based upon chromatographic methodology and/or ICP analysis for metals. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Conditions for the Validity of the Test

In order for the test to be considered valid, the test must have demonstrated that:

1. the mean cell density in the control flasks must have increased by a factor of at least 16 within 3 days or at least 100 within 4 days; and
2. the coefficient of variability for the cell density in the control flasks at 96 hours is less than 20%.

Data Analysis

Area under the growth curve will be calculated for the treatment and control groups using the following formula:

$$A = ((N_1-N_o)/2)(t_1)+((N_1+N_2-2N_o)/2)(t_2-t_1)+...+((N_{n-1}+N_n-2N_o)/2)(t_n-t_{n-1})$$

Where:

A = Area under the growth curve
N_o = Nominal number of cells/mL at t_o (beginning of test)
N_1 = Measured number of cells/mL at t_1
N_2 = Measured number of cells/mL at t_2
N_n = Measured number of cells/mL at t_n
t_1 = Time of first measurement after beginning of test (hours)
t_2 = Time of second measurement after beginning of test (hours)
t_n = Time of nth measurement after beginning of test (hours)

Growth rates will be calculated for the treatment and control groups using the following formula:

$$\text{Growth Rate} = \frac{\ln N_n - \ln N_1}{t_n - t_1}$$

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where:

N_n = Measured number of cells/mL at t_n
N_1 = Measured number of cells/ml at t_1
t_n = time of n^{th} measurement after beginning of test (hours)
t_1 = time of first measurement after beginning of test (hours)

Percent inhibition will be calculated for each treatment group using the following formula:

$$\text{Percent Inhibition} = \frac{\text{Mean Control Response} - \text{Mean Treatment Response}}{\text{Mean Control Response}} \text{X} 100$$

EL50, E_bL50 values and E_rL50 values will be determined, when possible, using linear interpolation (7) or other suitable techniques with treatment response (cell density, biomass and growth rate) and WAF loading rate data. All values will be based on nominal loading rates used in the test.

A no-observed-adverse-effect level (NOAEL) will be selected at 72 and 96 hours based on an evaluation of the concentration-response pattern and the results of the statistical analyses of the cell density, biomass and growth rate data at 72 and 96 hours. Statistically significant differences between the control and the treatment groups will be identified using analysis of variance (ANOVA) and a test to compare treatment mean responses to the control response (e.g., Dunnett's test or Bonferroni's t-test). Data will be assessed for normality and homogeneity of variances prior to performing the ANOVA. Transformations will be used to correct any condition of non-normality or unequal error variances, when possible. Additional analysis of data may be conducted if deemed appropriate by the Study Director. The results of the analysis will be documented in the raw data and summarized in the final report. Statistical comparisons for the ANOVA and the comparison of mean responses will be carried out using SAS System for Windows software (8) or equivalent.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated at Wildlife International, Ltd. will include, but not be limited to:

1. Copy of signed protocol.
2. Identification and characterization of the test substance, if provided by Sponsor.

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3. Dates of initiation and termination of the test.
4. Source of algae.
5. Culture conditions.
6. Growth measurements.
7. Calculation and preparation of test concentrations.
8. Observations.
9. The methods used to analyze test substance concentrations and the results of analytical measurements.
10. Statistical calculations.
11. Test conditions and physical/chemical measurements.
12. Copy of final report.

FINAL REPORT

A final report of the results of the study will be prepared by Wildlife International, Ltd. The report will include, but not be limited to the following, when applicable:

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures, as stated in the approved protocol, including a copy of the final protocol, and all amendments and deviations to the protocol.
5. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
6. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
7. A description of the methods used to conduct the test.
8. A description of the test organisms, including the source, scientific name, age or life stage, light intensity and photoperiod.
9. A description of the preparation of the test solutions.

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10. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and chambers per treatment, the duration of the test, and environmental conditions during the test.
11. A description of circumstances that may have affected the quality or integrity of the data.
12. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
13. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses. A graph plotting the concentration response curve, if possible.
14. Statistical methods used to evaluate the data, and copies of the output from the statistics programs.
15. The signed and dated reports of each of the individual scientists or other professionals involved in the study.
16. The location where raw data and final report are to be stored.
17. A statement prepared by the Quality Assurance Unit listing the dates that study inspections were made and the dates of any findings reported to the Study Director and Management.
18. If it is necessary to make corrections or additions to a final report after it has been accepted, such changes will be made in the form of an amendment issued by the Study Director. The amendment will clearly identify the part of the final report that is being amended and the reasons for the amendment, and will be signed by the Study Director.
19. Any statistical program output.

CHANGES TO PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and approved by the Sponsor's Representative. Amendments will be considered as part of the protocol and will be attached to the final protocol. Any other changes will be in the form of written deviations signed by the Study Director and filed with the raw data. All changes to and deviation from the protocol will be indicated in the final report.

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17) (3) and TSCA Good Laboratory Practice Standards (40 CFR Part 792) (4). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International,

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Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site or at an alternative location to be specified in the final report.

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REFERENCES

1 **Organisation for Economic Cooperation and Development.** 1984. OECD Guideline for Testing of Chemicals, Guideline 201: *Alga, Growth Inhibition Test.*

2 **Official Journal of the European Communities.** 1992. No. L383. Method C.3. *Algal Inhibition Test.*

3. **U.S. Environmental Protection Agency.** 1996. Series 850- Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.5400: *Algal Toxicity, Tiers I and II.*

4 **OECD.** 1998. *OECD Principles of Good Laboratory Practice* ENV/MC/CHEM (98) 17.

5 **Title 40 of the Code of Federal Regulations, Part 792.** 1989. *Toxic Substances Control Act (TSCA) Good Laboratory Practice Standards.*

6 **ASTM Standard Guide 1218-90E.** 1990. *Standard Guide for Conducting Static 96-Hour Toxicity Tests with Microalgae.* American Society for Testing and Materials. Philadelphia, Pennsylvania.

7 **Norberg-King, T.J.** 1993. *A Linear Interpolation Method for Sublethal Toxicity: The Inhibition Concentration (ICp) Approach.* Version 2.0. U.S. Environmental Protection Agency. National Effluent Toxicity Assessment Center. Duluth, Minnesota. Technical Report 03-93.

8 **The SAS System for Windows**. 2001. Version 8.02. SAS Institute Inc., Cary, North Carolina.

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TABLE 1

FRESHWATER ALGAL MEDIUM CONSTITUENTS

Component	Nominal Concentration
$MgCl_2 \cdot 6H_2O$	12.164 mg/L
$CaCl_2 \cdot 2H_2O$	4.410 mg/L
H_3BO_3	0.1855 mg/L
$MnCl_2 \cdot 4H_2O$	0.4154 mg/L
$ZnCl_2$	3.27 µg/L
$FeCl_3 \cdot 6H_2O$	0.1598 mg/L
$CoCl_2 \cdot 6H_2O$	1.428 µg/L
$Na_2MoO_4 \cdot 2H_2O$	7.26 µg/L
$CuCl_2 \cdot 2H_2O$	0.012 µg/L
$Na_2EDTA \cdot 2H_2O$	0.300 mg/L
$NaNO_3$	25.50 mg/L
$MgSO_4 \cdot 7H_2O$	14.70 mg/L
K_2HPO_4	1.044 mg/L
$NaHCO_3$	15.00 mg/L

The pH of the medium will be adjusted, as necessary, to 7.5 ± 0.1 using 0.1 N NaOH or 10% HCl.

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Table 2.

Analytes of Interest in Petroleum Coke

PAH	Metals and Sulfur
Acenaphthene	Nickel
Acenaphthylene	Vanadium
Anthracene	Iron
Benzo(a)anthracene	Copper
Benzo(a)pyrene	Selenium
Benzo(b)fluoranthene	Arsenic
Benzo(g,h,i)perylene	Sulfur
Benzo(k)fluoranthene	
Chrysene	
Dibenzo(a,e)pyrene	
Dibenz(a,h)anthracene	
Fluoranthene	
Fluorene	
Indeno(1,2,3-cd)pyrene	
Naphthalene	
Perylene	
Phenanthrene	
Pyrene	

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Petroleum Coke: A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)

PROTOCOL NO.: 472/033004/SEL-OECD/SUB472 **AMENDMENT NO.:** 1

SPONSOR: American Petroleum Institute **PROJECT NO.:** 472A-114

EFFECTIVE DATE: March 8, 2005

AMENDMENT: Page 2:

ADD: Proposed Dates: Experimental Start Date (OECD): March 24, 2005
Experimental Start Date (EPA): March 25, 2005
Experimental Termination Date: March 29, 2005
Test Concentrations: 1000 mg/L

REASON: This information was not available at the time the protocol was signed by the Study Director.

AMENDMENT: Experimental Design, Page 3, 1st Paragraph:

DELETE: Generally, each concentration of the test material used in the definitive test will be at least 50% of the next higher treatment, unless information concerning the concentration-effect curve indicates that a different dilution factor is more appropriate.

REASON: Because the test will be run at a single limit concentration, discussion of dilution factors becomes irrelevant.

AMENDMENT: Experimental Design, Pages 3 and 4:

CHANGE: The green algae, *Selenastrum capricornutum* will be exposed to a geometric series of at least five water accommodated fraction (WAF) loading rates and a negative (dilution water) control for 96 hours.

TO: The green algae, *Selenastrum capricornutum* will be exposed to a single accommodated fraction (WAF) loading rate and a negative (dilution water) control for 96 hours.

CHANGE: Twelve replicates in the control and treatment groups will be tested.

TO: Twenty-four replicates in the control and treatment group will be tested.

REASON: A limit test will be conducted.

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AMENDMENT: Experimental Design, Page 4, 2nd Paragraph:

CHANGE: EL50 values (i.e., the theoretical loading rate that produces a 50% reduction in the measured parameter) will be calculated, if possible, for each 24-hour interval of the test.

TO: Percent inhibition measured in the biological parameters will be used to determine whether the EL50 values at each time interval are greater than or less than the 1000 mg/L WAF loading rate used in the test.

REASON: EL50 values cannot be calculated in a limit test. Therefore, the response observed in the treatment group will provide a relative indication of where the EL50 might lie.

AMENDMENT: Preparation of Water Accommodated Fraction Solutions, Page 5:

CHANGE: The length of the mixing time in the definitive test will be determined based on results of the WAF equilibration trial.

TO: The WAF mixing time will be approximately 24 hours.

REASON: The WAF equilibration trial showed no effect of mixing time on concentrations of the analytes of interest. Therefore, it is not necessary to stir the WAF mixture beyond 24 hours.

AMENDMENT: Environmental Conditions, Page 6:

CHANGE: At 24, 48, 72 and 96 hours, pH will be measured in pooled test solution samples collected from the three replicates sacrificed from each treatment and control group. At test termination, pH will be measured in pooled test solution samples collected from the remaining three replicates of each treatment and control group.

TO: At 24, 48, 72 and 96 hours, pH will be measured in pooled test solution samples collected from the six replicates sacrificed from each treatment and control group.

REASON: Six replicates will be sacrificed at each sampling interval.

AMENDMENT: Biological Measurements, Page 7:

CHANGE: One sample will be collected from three replicates of the treatment and control group at 24, 48, 72 and 96 hours. At each measurement period, the three replicates will be destructively sampled (i.e. samples taken and then replicates removed from test).

TO: One sample will be collected from six replicates of the treatment and control group at 24, 48, 72 and 96 hours. At each measurement period, the six replicates will be destructively samples (i.e. samples taken and then replicates removed from test).

CHANGE: Samples of test solution will be collected from each of the three replicates per treatment and control group at the end of the test.

TO: Samples of test solution will be collected from each of the six replicates per treatment and control group at the end of the test.

REASON: A limit test will be conducted with six replicates per sampling interval.

AMENDMENT: Sampling for Analytical Measurements, Page 8:

CHANGE: Replace the entire section with the following.

TO: Water samples will be decanted from each WAF preparation vessel at the beginning of the test, and pooled from each replicate test chamber after 72 and 96 hours of the test to determine concentrations of the compounds and elements of interest in petroleum coke (see Table 1). One set of samples will be collected and analyzed for selected organic compounds, and a second set of samples will be collected and analyzed for selected inorganic elements. Samples will be collected at mid-depth and stored with zero headspace until the end of the test. Samples for organic analyses should be preserved by storage at 4°C. Samples for inorganic analyses should be preserved by treating the sample with nitric acid (HNO_3) to a pH of <2. The sample scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES

Experimental Group	0-Hours [1]	72-Hours [1] (old)	96-Hours [1] (old)
Control	2	2	2
Treatment (1000 mg/L loading)	2	2	2
	4	4	4

[1] At each sampling interval, one set of samples will be collected and analyzed for selected organic compounds, and a second set of samples will be collected and analyzed for selected inorganic elements.

Total Number of Verification Samples = 12

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study. At the discretion of the Study Director, water samples from one or more appropriate test chambers will be collected and analyzed if an analytical error in sampling or analysis is suspected. The reason for the additional samples will be documented in the raw data and summarized in the final report.

REASON: The change to a limit test necessitated a revision in the numbers of samples collected for analytical verification.

AMENDMENT: Data Analysis, Page 10:

CHANGE: EL50, E_bL50 values and E_rL50 values will be determined, when possible, using linear interpolation (7) or other suitable techniques with treatment response (cell density, biomass and growth rate) and WAF loading rate data. All values will be based on nominal loading rates used in the test.

A no-observed-adverse-effect level (NOAEL) will be selected at 72 and 96 hours based on an evaluation of the concentration-response pattern and the results of the statistical analyses of the cell density, biomass and growth rate data at 72 and 96 hours. Statistically significant differences between the control and the treatment groups will be identified using analysis of variance (ANOVA) and a test to compare treatment mean responses to the control response (e.g., Dunnett's test or Bonferroni's t-test). Data will be assessed for normality and homogeneity of variances prior to performing the ANOVA. Transformations will be used to correct any condition of non-normality or unequal error variances, when possible. Additional analysis of data may be conducted if deemed appropriate by the Study Director. The results of the analysis will be documented in the raw data and summarized in the final report. Statistical comparisons for the ANOVA and the comparison of mean responses will be carried out using SAS System for Windows software (8) or equivalent.

TO: In the definitive limit test, the percent inhibition obtained at the limit concentration will be used to determine if the 72 and 96-hour EL50 concentrations are greater than or less than the nominal limit WAF loading rate.

REASON: Because the test will be run at a single limit concentration, statistically defined EL50 concentrations cannot be determined.

AMENDMENT: Table 12, Page 16:

ADD: 1-methylnaphthalene
2-methylnaphthalene

REASON: The Sponsor requested the addition of these two compounds to the list of analytes of interest.

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[redacted]

3-24-05
DATE

3/24/05
DATE

3/18/05
DATE

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AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Petroleum Coke: A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)

PROTOCOL NO.: 472/033004/SEL-OECD/SUB472 **AMENDMENT NO.:** 2

SPONSOR: American Petroleum Institute **PROJECT NO.:** 472A-114

EFFECTIVE DATE: March 18, 2005

AMENDMENT: Sampling for Analytical Measurements, Page 8 (as amended in Amendment #1):

CHANGE: Samples will be collected at mid-depth and stored with zero headspace until the end of the test. Samples for organic analyses should be preserved by storage at 4°C. Samples for inorganic analyses should be preserved by treating the sample with nitric acid (HNO_3) to a pH of <2.

TO: Samples will be collected at mid-depth and stored until the end of the test. Samples for organic analyses should be preserved by storage at 4°C with zero headspace. Samples for inorganic analyses should be preserved by the addition of sufficient nitric acid (HNO_3) to achieve a final acid concentration of 2%.

REASON: The procedures specified in Amendment #1 did not accurately reflect the procedures to be used for the preservation of samples collected for inorganic analyses.

[redacted]

3-24-05
DATE

3/24/05
DATE

March 24, 2005
DATE

Wildlife International, Ltd.

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Petroleum Coke: A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)

PROTOCOL NO.: 472/033004/SEL-OECD/SUB472 **AMENDMENT NO.:** 3

SPONSOR: American Petroleum Institute **PROJECT NO.:** 472A-114

EFFECTIVE DATE: February 2, 2007

AMENDMENT: Page 2:

Change Study Director to: Anne B. Sindermann, Senior Biologist

REASON: Study Director responsibilities have been reassigned by Wildlife International, Ltd. management.

[redacted]

3/26/07
DATE

3/26/07
DATE

3/11/07
DATE

Wildlife International, Ltd.

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: Petroleum Coke: A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)

PROTOCOL NO.: 472/033004/SEL-OECD/SUB472 **DEVIATION NO.:** 1

SPONSOR: American Petroleum Institute **PROJECT NO.:** 472A-114

DATE OF DEVIATION: March 23, 2005

DEVIATION: Individual WAF solutions were prepared in 9.5L glass bottles instead of 4L aspirator bottles with tubulation. The test solutions were siphoned from the bottom of the vessel at approximately the same level as an aspirator bottle. The media volume used for each WAF was 9L instead of 4L.

REASON: To achieve the volumes needed for the replications of a limit test a 9.5L vessel was required. The vessel available for the 9L volume was not an aspirator bottle with tubulation. Since the intent of the aspirator bottle with tubulation was to siphon the test solutions at a certain depth of the test solutions, a siphon was placed in the 9.5L vessel at the same depth as the tubulation of the aspirator bottle. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.

[redacted]

5-3-05
DATE

5/3/05
DATE

AMENDED

Wildlife International, Ltd.

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: Petroleum Coke: A 96-Hour Static-Renewal Acute Toxicity Test with the Freshwater Algae (*Selenastrum capricornutum*)

PROTOCOL NO.: 472/033004/SEL-OECD/SUB472 **DEVIATION NO.:** 2

SPONSOR: American Petroleum Institute **PROJECT NO.:** 472A-114

DATE OF DEVIATION: March 31, 2005

DEVIATION: Water samples were not analyzed to determine the concentration of perylene, one analyte of interest in petroleum coke listed in Table 1 of the protocol.

REASON: It was determined in method development work that the analyte co-eluted with another analyte of interest, and the concentration in the water samples would not be evaluated during the definitive test. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.

[redacted]

8-3-05
DATE

8/3/05
DATE

Wildlife International, Ltd.

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: Petroleum Coke: A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)

PROTOCOL NO.: 472/033004/SEL-OECD/SUB472 **DEVIATION NO.:** 3

SPONSOR: American Petroleum Institute **PROJECT NO.:** 472A-114

DATE OF DEVIATION: September 12, 2005

DEVIATION: The summary of the water analysis for selected organic and inorganic constituents that was included in the study report was from the most recent water analysis, but was not from the most recent GLP-compliant analysis, as indicated in the protocol.

REASON: The periodic water analyses are no longer conducted under GLP Standards. The most recent GLP-compliant analysis was conducted in 2002. The most recent non-GLP analysis conducted in 2004 was included in the report since it was considered to be more representative of the dilution water used in the test. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.

[redacted]

6-9-06
DATE

09 June 06
DATE

AMENDED

Appendix 3

Test Article Selection

THE PACE CONSULTANTS INC.
Post Office Box 53473 Houston, Texas 77052 853/351-7800 Fax 853/351-7887
A Member of Jacobs Engineering Group

February 22, 2001

[redacted]
American Petroleum Institute
1220 L Street, NW
Washington, D.C. 20005-4070

Attached is Pace's report covering Task 1 and 2 entitled "U.S. Delayed Coker Petroleum Coke Quality Survey 1998-1999."

We would be pleased to answer any questions concerning this work for API. Please contact me at 832/351-7811 or email [redacted]

For PACE

[redacted]

[redacted]
Attachment

U.S. DELAYED COKER PETROLEUM COKE QUALITY SURVEY 1998-1999

INTRODUCTION

In 1998 the United States Environmental Protection Agency (EPA) challenged chemical producers and importers to provide voluntarily basic toxicity information on their high production volume (HPV) chemicals, defined as those chemicals which are produced in or imported to the U.S. in amounts greater than 1 million pounds per year. The goal of the HPV Challenge Program is to ensure that the American public has access to basic information about the hazards associated with chemicals manufactured and used in the greatest quantities in the United States. It is designed to generate the complete hazard screening data for HPV commercial chemicals.

The American Petroleum Institute (API) serves as administrator of the Petroleum HPV Testing Group, a consortium made up of 72 member companies from API, the National Petrochemical & Refiners Association (NPRA), the Gas Producers Association (GPA) and the Asphalt Institute. These companies represent 92% of the nation's refinery capacity. The Petroleum HPV Testing Group has sponsored 396 substances produced and used by the nation's petroleum industry to meet the EPA's HPV challenge.

Pace was retained by the API HPV Testing Group to assist in identifying potential sources of U.S. petroleum coke samples that could be used in the HPV testing program. As the first step in this process, Pace undertook a review of its quarterly petroleum coke production data to help characterize current U.S. petroleum coke production qualities. Pace has now completed the review of its 1998 and 1999 quarterly petroleum coke production data for all U.S.-based delayed cokers. The results of this review are discussed below.

METHODOLOGY

Pace's petroleum coke production database was used to determine quality characteristics of petroleum coke produced by U.S. refineries. Pace has conducted a survey of U.S. petroleum coker production on a quarterly basis since the second quarter of 1983. Refineries provide the bulk of the data, but some data are also gathered from other market participants. These data are maintained in a database from which the 1998 and 1999 quarterly data were extracted for this study. It was decided that data analysis would concentrate on delayed cokers (excluding needle cokers) since for 1999 our delayed coker data set includes 92+% of all the petroleum coke produced in the United States. Accordingly, fluid and Flexicokers[1] were removed from the data set.

Needle cokers were removed from the delayed coker database because needle cokers represent a special subset of delayed coking production. Needle coke differences include:

[1] Flexicoke is a proprietary coking process developed by Exxon. It involves partially gasifying fluid coke.

1. Needle coke quality is much higher than other delayed coke
2. Needle coke is produced using different feedstock & coking operational procedures because it is a product, not a by-product like other delayed cokes
3. The quantity of needle coke produced is very small
4. Needle coke is handled very carefully due to its high price (typically > $350/metric ton)

SUMMARY AND DATA ANALYSIS

These data were analyzed to determine the ton-weighted average petroleum coke qualities of sulfur (wt%), nickel (ppm), vanadium (ppm), and volatile material (wt%). All data are presented on a dry basis. The results are presented in Table 1 below.

TABLE 1

U.S. DELAYED PETROLEUM COKE QUALITY SUMMARY
TON-WEIGHTED QUARTERLY AVERAGES

	Sulfur, Wt%		Nickel, ppm		Vanadium, ppm		Vol. Mat., Wt%	
Quarter	1998	1999	1998	1999	1998	1999	1998	1999
1Q	4.15	4.11	286	275	758	801	10.9	10.5
2Q	4.22	4.22	277	283	811	821	10.8	11.0
3Q	4.21	4.21	277	282	811	857	10.9	10.9
4Q	4.21	4.22	282	276	854	852	10.7	10.9
Ton-Wt Avg	4.20	4.19	280	279	809	833	10.8	10.8

Ton-weighted average qualities for each quarter were calculated in the following manner:

$$\frac{\sum (\text{quality value})_{\text{delayed coker}} * (\text{quarterly production})_{\text{delayed coker}}}{\text{Total quarterly production}}$$

Where:

quality value = sulfur, vanadium, nickel or volatile content of petroleum coke produced by each delayed coker

quarterly production = petroleum coke produced by that delayed coker

Pace next reviewed the data to determine a ton-weighted frequency distribution for each of the qualities listed. The results of this analysis are presented in Table 2 and in Figures 1 through 4.

TABLE 2

U.S. DELAYED PETROLEUM COKE QUALITY SUMMARY BY PRODUCTION QUARTILE

Cumulative Production	Sulfur, Wt%		Nickel, ppm		Vanadium, ppm		Vol, Wt%	
	1998	1999	1998	1999	1998	1999	1998	1999
min.	0.90	0.50	50	5	45	45	7.0	4.0
25%	3.20	3.10	180	185	400	445	10.0	10.0
50%	4.45	4.60	250	250	650	675	10.7	10.7
75%	5.34	5.30	360	400	1205	1200	12.0	12.0
100%	6.90	6.30	568	568	1900	2000	14.0	14.0

Quality quartiles for each year were calculated in the following manner:

> Annual data were sorted according to each specific quality value (e.g., sulfur, vanadium, nickel, and volatile content) and the cumulative production of petroleum coke by delayed coker was calculated. Quartiles were then calculated for the annual production total, and the quality value at the cumulative total that equaled each quartile was used to determine the quality for that quartile.

TRENDS

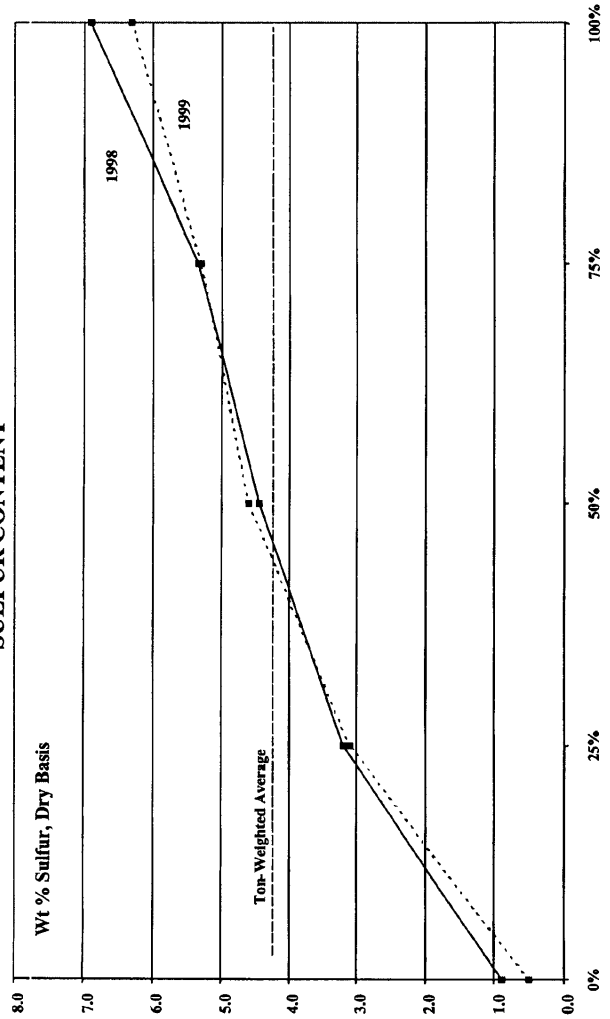
Comparing the ton-weighted averages to the 50% production quartile (i.e., the median) reveals the following trends:

- The weighted average nickel and vanadium content of U.S. delayed petroleum coke is higher than the median. This is a direct result of the increasing amount of heavy crudes, particularly Mexican and Venezuelan crudes, processed by U.S. refineries. Because these crudes produce petroleum cokes with nickel and vanadium contents that are significantly above the median, they skew the weighted average away from the median.

- Ton-weighted sulfur content is slightly below the median because some cokers produce petroleum cokes that are well below the median sulfur content (i.e., anode-grade coke which is calcined and primarily used to make anodes for the aluminum smelting industry).

FIGURE 1

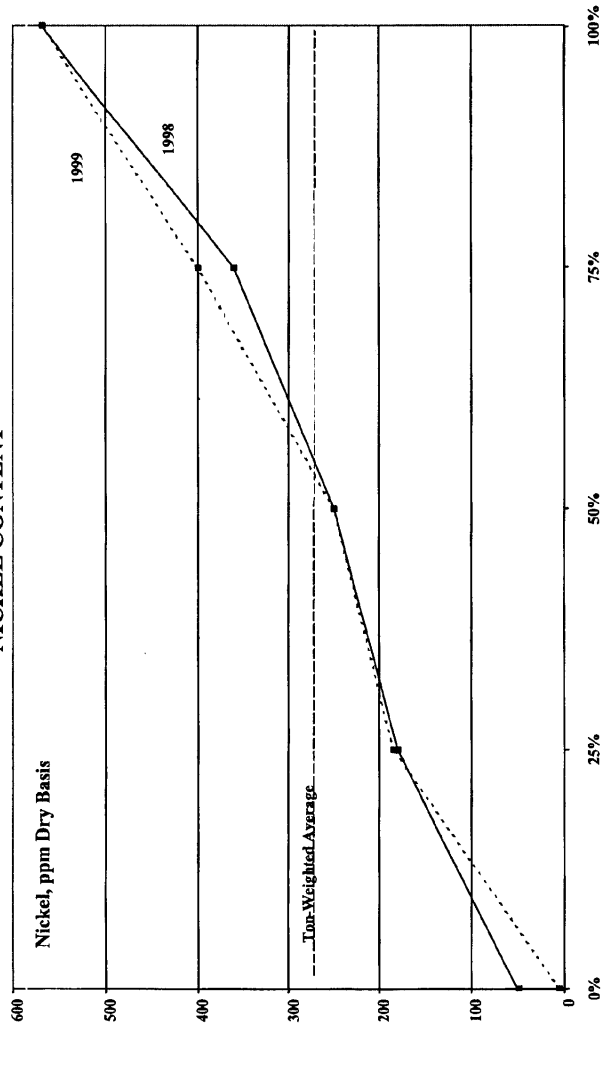
U.S. DELAYED PETROLEUM COKE
SULFUR CONTENT



THE PACE CONSULTANTS INC.

FIGURE 2

U.S. DELAYED PETROLEUM COKE
NICKEL CONTENT



THE PACE CONSULTANTS INC.

FIGURE 3

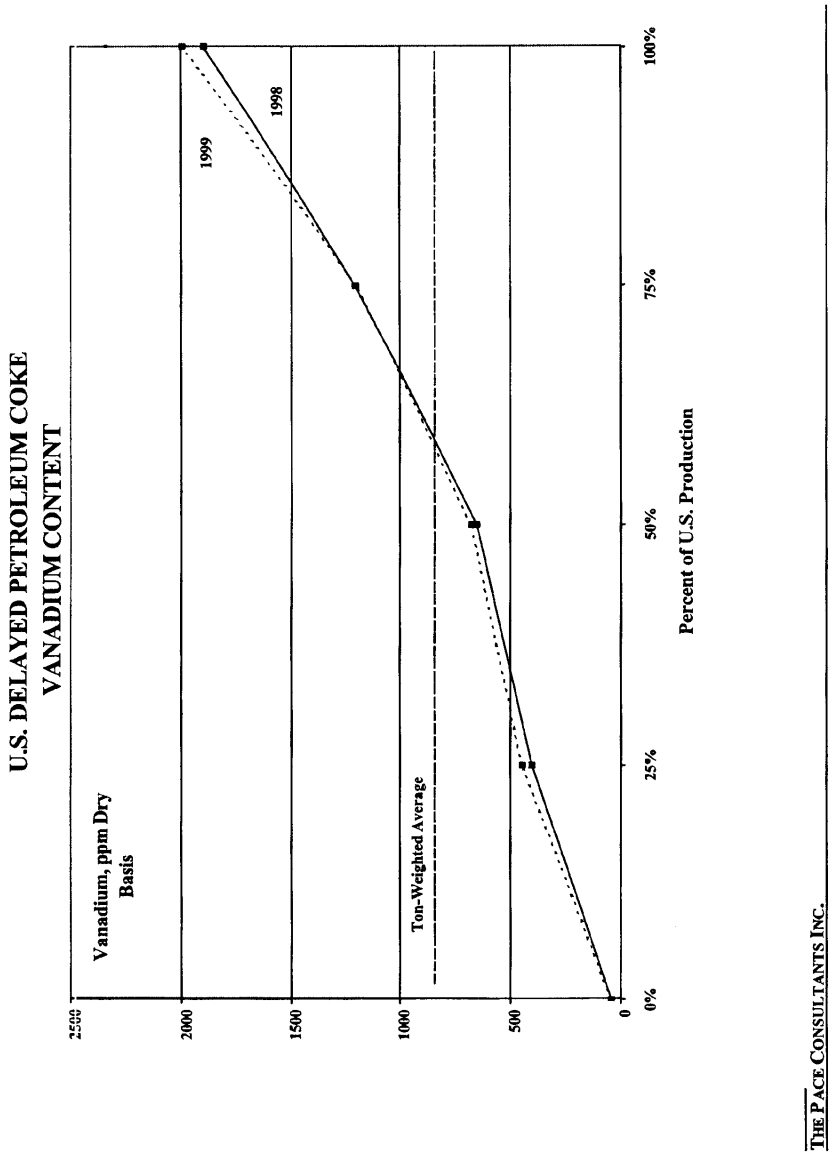
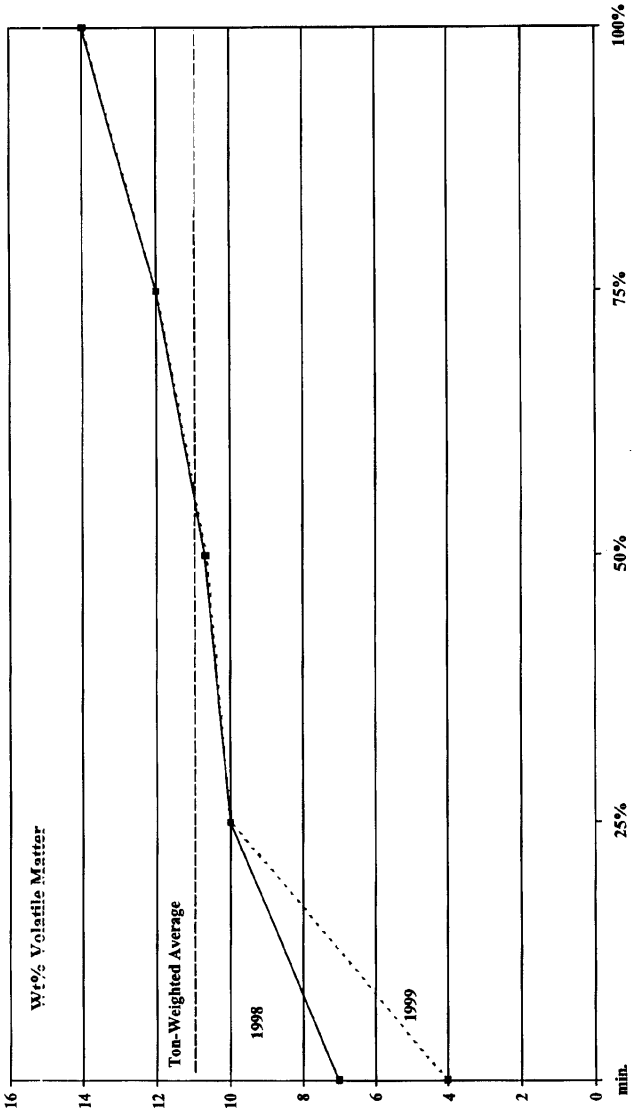

U.S. DELAYED PETROLEUM COKE
VANADIUM CONTENT
Vanadium, ppm Dry Basis
2500
2000
1500
1000
500
0
1999
1998
Ton-Weighted Average
0%
25%
50%
75%
100%
Percent of U.S. Production
THE PACE CONSULTANTS INC.

FIGURE 4
U.S. DELAYED PETROLEUM COKE VOLATILE MATTER CONTENT



THE PACE CONSULTANTS INC.

- The sulfur content at the upper and lower ends of the quality spectrum was better in 1999 than in 1998. We believe the lower sulfur content in 1999 was a result of crude production cut-backs by OPEC (Organization of Petroleum Exporting Countries) and other crude oil producers. These producers preferentially reduced the production of their lower quality crude oils in order to minimize the production reductions of their higher quality (i.e. higher priced) crude oils. We see 1999 as an aberration in the general trend of increasing sulfur content in U.S. petroleum cokes.

- We expect the metals content and sulfur content of U.S. petroleum coke will deteriorate beginning in 2001 as new U.S. cokers scheduled to begin operations in the 2000-2002 time frame start up.

- The average volatile matter content is essentially equal to the median.

RECOMMENDATIONS

Pace identified candidate refineries for sampling based on the quality data from the third quarter of 2000, which is the most recent quarter for which data are available. It should be noted that these data may vary slightly from the 1998-1999 averages as increasing amounts of heavy crude are processed. Based on these data, Pace recommends the following candidates for sampling in support of the Petroleum HPV Testing Program:

PETROLEUM HPV TESTING PROGRAM
DELAYED PETROLEUM COKE SAMPLE CANDIDATES

	Candidate A		Candidate B		Candidate C	
	Value	Percentile	Value	Percentile	Value	Percentile
Sulfur, Wt%	6.00	93	5.75	86	5.50	80
Nickel, ppm	500	90	300	58	250	50
Vanadium, ppm	1,500	84	1,200	75	1,000	65
Volatiles, Wt%	10.00	25	12.00	75	13.00	88

PETROLEUM HPV TESTING PROGRAM
DELAYED PETROLEUM COKE SAMPLE CANDIDATES

	Candidate D			Candidate E	
	Value	Percentile		Value	Percentile
Sulfur, Wt%	4.20	43		5.50	80
Nickel, ppm	250	50		350	67
Vanadium, ppm	1,500	84		1,100	70
Volatiles, Wt%	15.00	100		10.00	25

Our analysis indicates that some compromises will have to be made in obtaining a sample for the HPV program since no refinery's petroleum coke is in the upper 75th percentile in all four quality parameters we have evaluated. Additionally, we have spent some time and effort trying to find petroleum cokes which are sampled with automatic sampling equipment that has been bias tested and is operated by an independent laboratory. Unfortunately, we have found that the locations with the best sampling systems have petroleum cokes of generally better quality. Therefore, we do not believe that we will be able to find a "perfect" candidate petroleum coke.

While the sampling at the candidate refineries may not be ideal, the sampling and analysis data have been used for commercial transactions. Substantial quantities of petroleum coke from each of the candidate refineries have been sold in the petroleum coke market. Commercial transactions have relied on the laboratory results for determining quality bonus and penalties and conformance with contract quality specifications. Thus, the samples taken for the HPV study would conform to generally accepted industry sampling practice.

The sampling plan would be to have the sample analyzed for the quality parameters used in this screening analysis (i.e. sulfur, vanadium, nickel, volatile matter) as well as four other commonly tested quality parameters—gross calorific value (Btu/lb), moisture (%), ash (%), and Hardgrove Grindability Index (HGI)–to verify that the sample obtained is similar to the anticipated quality characteristics. This plan would assure that the sample submitted for detailed HPV testing conforms to our quality expectations.

We may not be able to receive authorization from a refinery to use a sample of their petroleum coke for the HPV test. Our present plan would be to approach Refineries B and C regarding obtaining a sample. In the event that these two refineries choose not to participate, then the choice would be either refinery A or E, which have high sulfur and metals but low volatile content or refinery D, which has high vanadium and volatile matter but low sulfur content. (note: each of the five candidate refineries has a different corporate owner).

Pace requests that the HPV Committee confirm Pace's recommended plan to approach refineries B and C regarding obtaining an HPV sample. It is not necessary for the HPV committee to decide now on the preferred refinery to contact in the event that refineries B and C do not wish to participate in the program. However, we would suggest that the committee begin to think about this issue so that decisions can be made expeditiously in the event that refineries B and C choose not participate.

Appendix 4

AVEKA, Inc. Particle Processing Report

AVEKA, Inc.
PARTICLE PROCESSING & CUSTOM RESEARCH

Date: May 29, 2003

Make Order #: 5369

Company Name: API

Contact Person: [redacted]

Material: Green Petroleum Coke

Objective: Task 1: Hammermill, Ball-mill and Classify Petroleum Coke to a mean particle size less than 3.6 microns. Task 2: Crush and Classify petroleum coke to a mean particle size of 2 mm.

Equipment: Homoloid JT Hammermill (SN # JT-694) with 0.0093 screen
5 Gallon Ball-mill with 0.25 inch alumina media
Majac A-12 classifier
Horiba LA-910 Laser Light Scattering Particle Sizer
Marcy 4"x 6" Jaw Crusher
Gilson Sonic Sieve

Receipt: Approximately 80 lbs. of material was received 3-19-03 from Federal Express. Confirmation of receipt (EPL Project Identification 1203-001) was returned upon delivery.

Storage: Petroleum coke was stored at room temperature in sealed polyethylene bags when the material was not being processed.

Processing Procedure:

The green petroleum coke showed high moisture content upon inspection. The high moisture content was indicated by condensation on the inside of the received petroleum coke bags. After consulting with Deborah Herron and Jacobs Consultancy, the material was dried according to ASTM D 3302-00 (Standard Test Method for Total Moisture in Coal).

Task 1

All processes were run at room temperature. The dried petroleum coke was then run through a Homoloid JT Hammermill (SN # JT-694) equipped with a 0.0093 screen.

The resulting hammermilled powder was loaded into 5-gallon ball mills loaded with 0.25 inch ceramic (alumina) media. The loading level in the ball mill was 27 lbs. of media with 5.5 lbs. of petroleum coke.

651-730-1729 ■ 2045 Wooddale Drive, Woodbury, MN 55125 ■ FAX 651-730-1826

AVEKA, INC.
PARTICLE PROCESSING & CUSTOM RESEARCH

The mills were rotated at 36 rpm for 17.25 hours. The resulting powder had a mean particle size of 9.56 microns (Attch 1) when tested with the Horiba LA-910 in water.

The oversized petroleum coke material was removed using a Majac A-12 Classifier. The Majac was run at 1800 RPM and 8.5 cfm. The resulting particle size of the petroleum coke was a 3.3 micron mean (Attch. 2) when tested with the Horiba LA-910 in water. The Horiba LA-910 test method for the petroleum coke samples is outlined in Attch. 3.

The final yield of product was 10.5 kg of powder.

Task 2

All processes were run at room temperature. An 18" Sweco Screener was set-up with a 7 mesh (2.8 mm) top-screen and a 14 mesh (1.4 mm) bottom-screen. Petroleum coke was fed through the screener and 2-mm material was collected from between the top and bottom screen. Oversized petroleum coke was jaw crushed with a Marcy 4"x 6" Jaw Crusher and rescreened. A Gilson Sonic Sieve particle size analysis (Attch. 4) was run on the screened petroleum coke and the results showed 99.4 % of the material between 1.4 mm – 2.8 mm. Final yield was 3.3 kg of 2 mm Petroleum Coke.

Shipping

All samples were shipped UPS Ground. The following is a summary of the sample disposition.

Sample/Amount	Address	Person
200 grams of 2-3 micron particle size sample	ChevronTexaco Energy Research and Technology Corp. 100 Chevron Way Richmond, CA 94802 Tel: 510-242-7037	Richard Dutta

AVEKA, Inc.

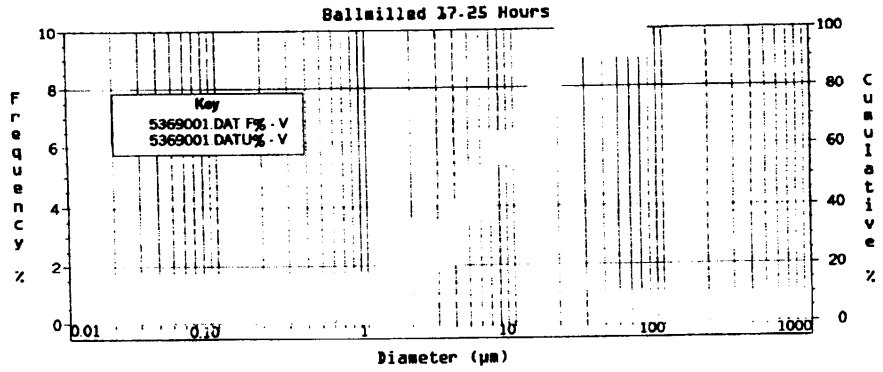
PARTICLE PROCESSING & CUSTOM RESEARCH

200 grams of 2 mm particle sample	ChevronTexaco Energy Research and Technology Corp. 100 Chevron Way Richmond, CA 94802 Tel: 510-242-7037	Richard Dutta
10.5 kg of 2-3 micron particle size sample	FPL Archives, Inc. 45610 Terminal Drive Sterling, Virginia 20166 703/435-8780 ext 201	Sam Busey
Remainder (slightly less than 3 kg) of 2 mm particle size sample)	FPL Archives, Inc. 45610 Terminal Drive Sterling, Virginia 20166 703/435-8780 ext 201	Sam Busey
Leftover petroleum coke material, i.e., that material not used in samples	FPL Archives, Inc. 45610 Terminal Drive Sterling, Virginia 20166 703/435-8780 ext 201	Sam Busey

651-730-1729 ■ 2045 Wooddale Drive, Woodbury, MN 55125 ■ FAX 651-730-1826

Attch 1

HORIBA LA-910
PARTICLE SIZE DISTRIBUTION DATA TABLE Standard 04/23/03
File Name: 5369001.DAT Sample Name: Ballmilled 17.25 Hours ID No: **/04/23-350
Dist. Form: STANDARD R.R. Index: co.rrj□
Dist. Mode: VOLUME Laser: 65.128 % Lamp: 61.185 %
Circulation: 2 Agitation: 7 U.Sonic ** (min)
Material: Petroleum Coke Source: American Petroleum
Lot No: MO5369 Test No: 5369001

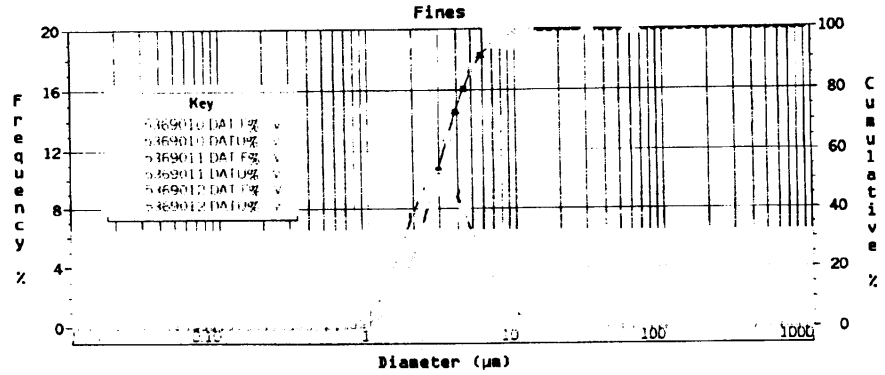


	SIZE(µm)	FREQ%	UNDR%	No.	SIZE(µm)	FREQ%	UNDR%
(1)	0.020	0.0	0.0	(28)	0.766	0.5	0.8	(55)	29.907	1.5	95.4
(2)	0.022	0.0	0.0	(29)	0.877	0.8	1.6	(56)	34.255	1.2	96.6
(3)	0.026	0.0	0.0	(30)	1.005	1.2	2.8	(57)	39.234	0.9	97.5
(4)	0.029	0.0	0.0	(31)	1.151	1.6	4.4	(58)	44.938	0.7	98.2
(5)	0.034	0.0	0.0	(32)	1.318	2.0	6.3	(59)	51.471	0.5	98.8
(6)	0.039	0.0	0.0	(33)	1.510	2.3	8.7	(60)	58.953	0.4	99.2
(7)	0.044	0.0	0.0	(34)	1.729	2.6	11.3	(61)	67.523	0.3	99.5
(8)	0.051	0.0	0.0	(35)	1.981	2.9	14.2	(62)	77.340	0.2	99.7
(9)	0.058	0.0	0.0	(36)	2.269	3.0	17.2	(63)	88.582	0.2	99.9
(10)	0.067	0.0	0.0	(37)	2.599	3.2	20.4	(64)	101.460	0.1	100.0
(11)	0.076	0.0	0.0	(38)	2.976	3.5	23.9	(65)	116.210	0.0	100.0
(12)	0.087	0.0	0.0	(39)	3.409	3.7	27.7	(66)	133.103	0.0	100.0
(13)	0.100	0.0	0.0	(40)	3.905	4.0	31.6	(67)	152.453	0.0	100.0
(14)	0.115	0.0	0.0	(41)	4.472	4.3	35.9	(68)	174.616	0.0	100.0
(15)	0.131	0.0	0.0	(42)	5.122	4.6	40.6	(69)	200.000	0.0	100.0
(16)	0.150	0.0	0.0	(43)	5.867	5.1	45.6	(70)	229.075	0.0	100.0
(17)	0.172	0.0	0.0	(44)	6.720	5.5	51.2	(71)	262.376	0.0	100.0
(18)	0.197	0.0	0.0	(45)	7.697	5.9	57.0	(72)	300.518	0.0	100.0
(19)	0.226	0.0	0.0	(46)	8.816	6.0	63.0	(73)	344.205	0.0	100.0
(20)	0.259	0.0	0.0	(47)	10.097	5.8	68.8	(74)	394.244	0.0	100.0
(21)	0.296	0.0	0.0	(48)	11.565	5.4	74.2	(75)	451.556	0.0	100.0
(22)	0.339	0.0	0.0	(49)	13.246	4.8	79.1	(76)	517.200	0.0	100.0
(23)	0.389	0.0	0.0	(50)	15.172	4.2	83.3	(77)	592.387	0.0	100.0
(24)	0.445	0.0	0.0	(51)	17.377	3.5	86.8	(78)	678.504	0.0	100.0
(25)	0.510	0.0	0.0	(52)	19.904	2.9	89.7	(79)	777.141	0.0	100.0
(26)	0.584	0.1	0.1	(53)	22.797	2.3	92.0	(80)	890.116	0.0	100.0
(27)	0.669	0.2	0.3	(54)	26.111	1.9	93.9	(81)	1019.510	0.0	100.0

Median : 6.533 (µm) Mean: 9.561 (µm) Mode: 8.237 (µm)
Std. Dev.: 10.531 (µm) Span: 10.623
Coef. Var: 110.14% Spec. Area: 15308 (cm2/cm3)

Attch. 2

HORIBA LA-910
PARTICLE SIZE DISTRIBUTION DATA TABLE Standard 05/15/03
File Name: 5369011.DAT Sample Name: Fines ID No: **/04/30-566
Dist. Form: STANDARD R.R. Index: co.rnj□
Dist. Mode: VOLUME Laser: 85.118 % Lamp: 86.338 %
Circulation: 3 Agitation: 7 U.Sonic OFF (min)
Material: Petroleum Coke Source: American Petroleum
Lot No: MO5369 Test No: 5369004



No.	SIZE(μm)	FREQ%	UNDR%	No.	SIZE(μm)	FREQ%	UNDR%	No.	SIZE(μm)	FREQ%	UNDR%
(1)	0.020	0.0	0.0	(28)	0.766	0.2	0.2	(55)	29.907	0.0	100.0
(2)	0.022	0.0	0.0	(29)	0.877	0.5	0.7	(56)	34.255	0.0	100.0
(3)	0.026	0.0	0.0	(30)	1.005	1.0	1.7	(57)	39.234	0.0	100.0
(4)	0.029	0.0	0.0	(31)	1.151	1.7	3.5	(58)	44.938	0.0	100.0
(5)	0.034	0.0	0.0	(32)	1.318	2.8	6.3	(59)	51.471	0.0	100.0
(6)	0.039	0.0	0.0	(33)	1.510	4.3	10.6	(60)	58.953	0.0	100.0
(7)	0.044	0.0	0.0	(34)	1.729	5.9	16.5	(61)	67.523	0.0	100.0
(8)	0.051	0.0	0.0	(35)	1.981	7.6	24.1	(62)	77.340	0.0	100.0
(9)	0.058	0.0	0.0	(36)	2.269	9.0	33.0	(63)	88.582	0.0	100.0
(10)	0.067	0.0	0.0	(37)	2.599	10.1	43.1	(64)	101.460	0.0	100.0
(11)	0.076	0.0	0.0	(38)	2.976	10.6	53.7	(65)	116.210	0.0	100.0
(12)	0.087	0.0	0.0	(39)	3.409	10.2	63.8	(66)	133.103	0.0	100.0
(13)	0.100	0.0	0.0	(40)	3.905	9.0	72.9	(67)	152.453	0.0	100.0
(14)	0.115	0.0	0.0	(41)	4.472	7.6	80.4	(68)	174.616	0.0	100.0
(15)	0.131	0.0	0.0	(42)	5.122	6.0	86.5	(69)	200.000	0.0	100.0
(16)	0.150	0.0	0.0	(43)	5.867	4.6	91.1	(70)	229.075	0.0	100.0
(17)	0.172	0.0	0.0	(44)	6.720	3.4	94.5	(71)	262.376	0.0	100.0
(18)	0.197	0.0	0.0	(45)	7.697	2.3	96.8	(72)	300.518	0.0	100.0
(19)	0.226	0.0	0.0	(46)	8.816	1.5	98.3	(73)	344.205	0.0	100.0
(20)	0.259	0.0	0.0	(47)	10.097	0.9	99.1	(74)	394.244	0.0	100.0
(21)	0.296	0.0	0.0	(48)	11.565	0.5	99.6	(75)	451.556	0.0	100.0
(22)	0.339	0.0	0.0	(49)	13.246	0.2	99.9	(76)	517.200	0.0	100.0
(23)	0.389	0.0	0.0	(50)	15.172	0.1	100.0	(77)	592.387	0.0	100.0
(24)	0.445	0.0	0.0	(51)	17.377	0.0	100.0	(78)	678.504	0.0	100.0
(25)	0.510	0.0	0.0	(52)	19.904	0.0	100.0	(79)	777.141	0.0	100.0
(26)	0.584	0.0	0.0	(53)	22.797	0.0	100.0	(80)	890.116	0.0	100.0
(27)	0.669	0.0	0.0	(54)	26.111	0.0	100.0	(81)	1019.510	0.0	100.0

Median : 2.840 (μm) Mean: 3.289 (μm) Mode: 2.781 (μm)
Std. Dev.: 1.844 (μm) Span: 2.780
Coef. Var: 56.08% Spec. Area: 23790 (cm2/cm3)

Attch. 3

TEST METHOD FOR API PETROLEUM COKE

Sample Preparation **May 15, 2003**

Mix 0.15 – 0.2 grams of petroleum coke with 5-6 grams distilled water. Add TX-100 surfactant to aid dispersion. Mix thoroughly until no large concentrations of sample are evident.

LA-910 Preparation

Fill the test chamber to capacity with 140 ml distilled water. Add 3-4 drops of TX-100 surfactant from a 10% concentrate source, resulting in approximately a .1% diluted total. Select the relative refractive index appropriate for this material (1.61-3.02i). Circulate the solvent using a pump speed of 2-3, subtract the background. Add the sample drop by drop until the laser transmission falls into the acceptable range (70 – 95)% transmittance. Activate the sonicator to aid dispersion, cease sonication when sample is completely dispersed.

Sample Test

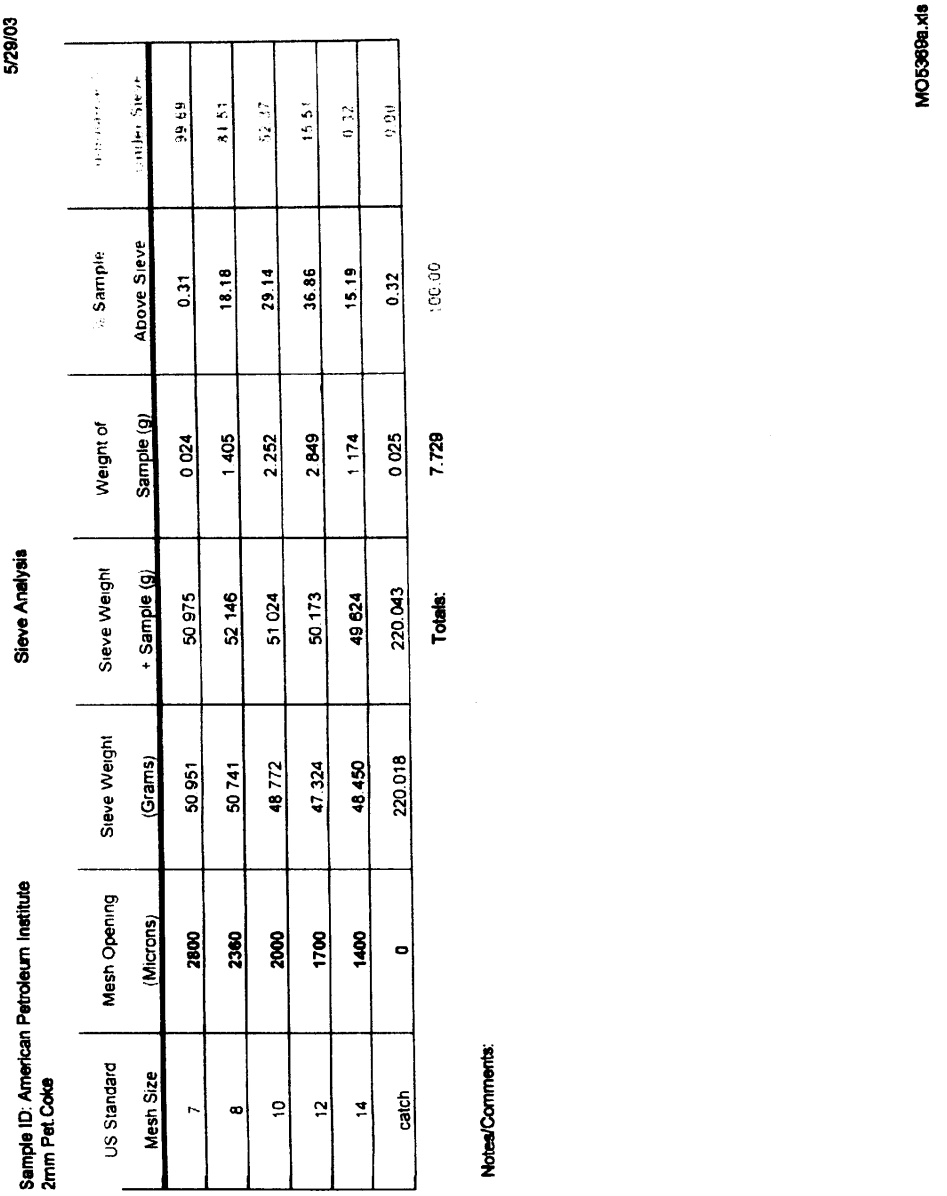
Measure the sample three times. Save each measurement. Overlay the three measurements on a graph. If they appear stable, the test is complete. If not, investigate. A steady increase in the laser transmission rate indicates more particles are present from pass to pass. That indicates the sample was not completely dispersed yet. A steady decrease in the laser transmission rate indicates the sample is agglomerating, settling, or dissolving.

Report

Using the Display module, graph the three test runs over one another. A stable test will appear as one line, an unstable condition will clearly show all three runs, indicating instability. If stable, select a run (typically the middle run) and print the complete data table along with the graph.

Author: [redacted]
Aveka, Inc.
(651) 714-4293 ext 208

Attch. 4



Sample ID: American Petroleum Institute 2mm Pet.Coke

Sieve Analysis

5/29/03

US Standard Mesh Size	Mesh Opening (Microns)	Sieve Weight (Grams)	Sieve Weight + Sample (g)	Weight of Sample (g)	% Sample Above Sieve	[illegible]
7	2800	50.951	50.975	0.024	0.31	99.69
8	2360	50.741	52.146	1.405	18.18	81.51
10	2000	48.772	51.024	2.252	29.14	52.37
12	1700	47.324	50.173	2.849	36.86	15.51
14	1400	48.450	49.624	1.174	15.19	0.32
catch	0	220.018	220.043	0.025	0.32	0.00

Totals: 7.729 100.00

Notes/Comments:

MO5369a.xls

AMENDED

Appendix 5

Laboratory Characterization of 2 mm Particle Size Petroleum Coke

[redacted] Analysis Report

Lancaster Laboratories

ANALYTICAL RESULTS

Prepared for:

Chevron Products Company
940 Hensley St. Bldg. 210

Richmond CA 94801
510-242-8191

Prepared by:

Lancaster Laboratories
2425 New Holland Pike
Lancaster, PA 17605-2425

SAMPLE GROUP

The sample group for this submittal is 857532. Samples arrived at the laboratory on Friday, June 27, 2003. The PO# for this group is 99011184 and the release number is BEATTY.

Client Description	Lancaster Labs Number
Pet Coke 2mm Solid Sample	4073301
Pet Coke Micronized Solid Sample	4073302

1 COPY TO Lancaster Laboratories
1 COPY TO Chevron CRTC [redacted]

Questions? Contact your Client Services Representative
Alison M O'Connor at (717) 656-2300.

Respectfully Submitted,

[redacted]

A4200

3.66

MEMBER [redacted] Lancaster Laboratories, Inc.
2425 New Holland Pike
PO Box 12425

AMENDED

Analysis Report

Lancaster Laboratories

Lancaster Laboratories Sample No. SW 4073301

Collected:06/26/2003 00:00

Account Number: 10863

Submitted: 06/27/2003 10:40
Reported: 07/09/2003 at 11:42
Discard: 08/09/2003
Pet Coke 2mm Solid Sample
Cost Center# ENG-4066
HPV Petroleum Cake

Chevron Products Company
940 Hensley St. Bldg. 210

Richmond CA 94801

2MMPC

CAT No.	Analysis Name	CAS Number	As Received Result		As Received Method Detection Limit	Units	Dilution Factor
07804	PAHs in Soil by GC/MS						
01191	Acenaphthene	83-32-9	N.D.		330.	ug/kg	10
01195	Pyrene	129-00-0	1,300.	J	330.	ug/kg	10
02751	1-Methylnaphthalene	90-12-0	2,700.	J	330.	ug/kg	10
03761	Naphthalene	91-20-3	3,600.		330.	ug/kg	10
03765	Acenaphthylene	208-96-8	N.D.		330.	ug/kg	10
03768	Fluorene	86-73-7	340.	J	330.	ug/kg	10
03775	Phenanthrene	85-01-8	690.	J	330.	ug/kg	10
03776	Anthracene	120-12-7	N.D.		330.	ug/kg	10
03778	Fluoranthene	206-44-0	N.D.		330.	ug/kg	10
03781	Benzo(a)anthracene	56-55-3	580.	J	330.	ug/kg	10
03782	Chrysene	218-01-9	880.	J	330.	ug/kg	10
03786	Benzo(b)fluoranthene	205-99-2	520.	J	330.	ug/kg	10
03787	Benzo(k)fluoranthene	207-08-9	N.D.		330.	ug/kg	10
03788	Benzo(a)pyrene	50-32-8	1,800.	J	330.	ug/kg	10
03789	Indeno(1,2,3-cd)pyrene	193-39-5	340.	J	330.	ug/kg	10
03790	Dibenz(a,h)anthracene	53-70-3	490.	J	330.	ug/kg	10
03791	Benzo(g,h,i)perylene	191-24-2	1,100.	J	330.	ug/kg	10
04694	2-Methylnaphthalene	91-57-6	11,000.		330.	ug/kg	10

Due to the sample matrix an initial dilution was necessary to perform the analysis. Therefore, the reporting limits for the GC/MS somivolatile compounds were raised.

State of California Lab Certification No. 2116

Laboratory Chronicle

CAT No.	Analysis Name	Method	Trial#	Analysis Date and Time	Analyst	Dilution Factor
07804	PAHs in Soil by GC/MS	SW-846 8270C	1	07/02/2003 15:41	Susan L Schoening	10
07806	DNA Soil Extraction	SW-846 3550B	1	06/30/2003 20:00	Sally L Appleyard	1

MEMBER

Lancaster Laboratories, Inc.
2425 New Holland Pike
PO Box 12425

Analysis Report

Lancaster Laboratories

Quality Control Summary

Client Name: Chevron Products Company Group Number: 857532
Reported: 07/09/03 at 11:42 AM

Laboratory Compliance Quality Control

Analysis Name	Blank Result	Blank MDL	Report Units	LCS %REC	LCSD %REC	LCS/LCSD Limits	RPD	RPD Max
Batch number: 031815LA026	Sample number(s): 4073301-4073302							
Acenaphthene	N.D.	33.	ug/kg	91		76-109		
Pyrene	N.D.	33.	ug/kg	89		71-110		
1-Methylnaphthalene	N.D.	33.	ug/kg	87		76-101		
Naphthalene	N.D.	33.	ug/kg	87		73-103		
Acenaphthylene	N.D.	33.	ug/kg	94		73-106		
Fluorene	N.D.	33.	ug/kg	93		66-115		
Phenanthrene	N.D.	33.	ug/kg	88		70-107		
Anthracene	N.D.	33.	ug/kg	86		71-107		
Fluoranthene	N.D.	33.	ug/kg	90		69-107		
Benzo(a)anthracene	N.D.	33.	ug/kg	93		74-107		
Chrysene	N.D.	33.	ug/kg	89		72-109		
Benzo(b)fluoranthene	N.D.	33.	ug/kg	95		71-113		
Benzo(k)fluoranthene	N.D.	33.	ug/kg	97		75-112		
Benzo(a)pyrene	N.D.	33.	ug/kg	94		79-111		
Indeno(1,2,3-cd)pyrene	N.D.	33.	ug/kg	88		74-113		
Dibenz(a,h)anthracene	N.D.	33.	ug/kg	95		81-118		
Benzo(g,h,i)perylene	N.D.	33.	ug/kg	92		74-114		
2-Methylnaphthalene	N.D.	33.	ug/kg	90		70-102		

Sample Matrix Quality Control

Analysis Name	MS %REC	MSD %REC	MS/MSD Limits	RPD	RPD MAX	BKG Conc	DUP Conc	DUP RPD	Dup RPD Max
Batch number: 031815LA026	Sample number(s): 4073301-4073302								
Acenaphthene	107	93	48-132	14	30				
Pyrene	82	68	28-144	12	30				
1-Methylnaphthalene	75	67*	72-100	5	30				
Naphthalene	77	61	38-132	9	30				
Acenaphthylene	108	91	46-128	18	30				
Fluorene	88	75	39-137	14	30				
Phenanthrene	88	74	29-143	13	30				
Anthracene	101	85	35-138	17	30				
Fluoranthene	81	72	19-145	11	30				
Benzo(a)anthracene	89	75	26-144	14	30				
Chrysene	101	90	29-150	9	30				
Benzo(b)fluoranthene	90	74	32-140	16	30				
Benzo(k)fluoranthene	103	88	36-143	16	30				
Benzo(a)pyrene	90	72	23-154	13	30				
Indeno(1,2,3-cd)pyrene	92	78	13-155	15	30				
Dibenz(a,h)anthracene	110	86	19-163	19	30				
Benzo(g,h,i)perylene	99	83	17-152	13	30				
2-Methylnaphthalene	38	19*	32-133	6	30				

*- Outside of specification
(1) The result for one or both determinations was less than five times the LOQ.
(2) The background result was more than four times the spike added.

MEMBER
Lancaster Laboratories, Inc.
2425 New Holland Pike
PO Box 12425
Lancaster PA 17605-2425

Analysis Report

Lancaster Laboratories

Quality Control Summary

Client Name: Chevron Products Company Group Number: 857532
Reported: 07/09/03 at 11:42 AM

Sample Matrix Quality Control

Analysis Name	MS %REC	MSD %REC	MS/MSD Limits	RPD	RPD MAX	DUP Conc	DUP Conc	DUP RPD	Dup RPD Max

Surrogate Quality Control

Analysis Name: PAHs in Soil by GC/MS
Batch number: 031618LA026

	Nitrobenzene-d5	2-Fluorobiphenyl	Terphenyl-d14
4073301	101	108	92
4073302	101	99	84
Blank	87	85	83
LCS	94	92	93
MS	105	107	88
MSD	90	90	78
Limits:	47-128	55-123	39-128

*- Outside of specification
(1) The result for one or both determinations was less than five times the LOQ.
(2) The background result was more than four times the spike added.

MEMBER

Lancaster Laboratories, Inc.
2425 New Holland Pike
PO Box 12425

[redacted]

From: [redacted]
Sent:
To:
Cc:
Subject: Found your results.

micronized

3030999 PETROLEUM COKE 2NM YCJ58009 REGULAR SERVICE
REPORTED 06/13/2003 Marked-up: 06/12/2003 by [redacted] at 50-1118
[redacted] (474/0) Prj Id: GLOBETECH

Test code	Test Name/Element/Result	Test Status	Analyst	Status date	Test Cost
30258	MICROWAVE DIGST/ICP PLUS	REPORTED	[redacted]	06/13/2003	$200.00

AL	300.200 PPM	AS	<29.61 PPM	B	<29.61 PPM
BA	<29.61 PPM	BE	<14.805 PPM	BI	<29.61 PPM
CA	121.600 PPM	CD	<14.805 PPM	CO	<14.805 PPM
CR	<14.805 PPM	CU	<17.766 PPM	FE	247.000 PPM
K	<44.414 PPM	LI	<14.805 PPM	MG	60.850 PPM
MN	<29.61 PPM	MO	<29.61 PPM	NA	114.600 PPM
NI	351.700 PPM	P	30.300 PPM	PB	<29.61 PPM
S	58060.000 PPM	SB	<74.024 PPM	SE	<29.61 PPM
SI	554.600 PPM	SN	<44.414 PPM	TI	<14.805 PPM
V	1805.000 PPM	ZN	<14.805 PPM		

2mm

3030251 PETROLEUM COKE YCJ58009 REGULAR SERVICE
REPORTED 06/09/2003 Marked-up: 06/09/2003 by [redacted] at 50-1118
[redacted] (474/0) Prj Id: [redacted]

Test code	Test Name/Element/Result	Test Status	Analyst	Status date	Test Cost
30258	MICROWAVE DIGST/ICP PLUS	REPORTED	[redacted]	06/09/2003	$200.00

AL	321.000 PPM	AS	<19.279 PPM	B	<19.279 PPM
BA	<19.279 PPM	BE	<9.639 PPM	BI	<19.279 PPM
CA	178.000 PPM	CD	<9.639 PPM	CO	<9.639 PPM
CR	<9.639 PPM	CU	<11.567 PPM	FE	310.000 PPM
K	<28.918 PPM	LI	<9.639 PPM	MG	77.370 PPM
MN	<19.279 PPM	MO	<19.279 PPM	NA	133.000 PPM
NI	367.100 PPM	P	<19.279 PPM	PB	<19.279 PPM
S	73920 PPM	SB	<48.197 PPM	SE	<19.279 PPM
SI	743.200 PPM	SN	<28.918 PPM	TI	12.910 PPM
V	1938.000 PPM	ZN	12.010 PPM		

-----Original Message-----
From: [redacted]

1

Appendix 6

Freshwater Algal Medium with Supplemental Sodium Bicarbonate

Compound	Nominal Concentration	
$MgCl_2•6H_2O$	12.164	mg/L
$CaCl_2•2H_2O$	4.410	mg/L
H_3BO_3	0.1855	mg/L
$MnCl_2•4H_2O$	0.4154	mg/L
$ZnCl_2$	3.27	µg/L
$FeCl_3•6H_2O$	0.1598	mg/L
$CoCl_2•6H_2O$	1.428	µg/L
$Na_2MoO_4•2H_2O$	7.26	µg/L
$CuCl_2•2H_2O$	0.012	µg/L
$Na_2EDTA•2H_2O$	0.300	mg/L
$NaNO_3$	25.50	mg/L
$MgSO_4•7H_2O$	14.70	mg/L
K_2HPO_4	1.044	mg/L
$NaHCO_3$	50.0	mg/L

[1] The pH was adjusted to 7.5 ± 0.1, as necessary using 10% HCl and 0.1N NaOH, as needed.

Appendix 7

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water[1]

Pesticides and Organics			
Component	Measured Concentration (µg/L)	Component	Measured Concentration (µg/L)
Aldrin	< 0.0099	Heptachlor Epoxide	< 0.0099
Alpha BHC	< 0.0099	Malathion	< 2.0
Beta BHC	< 0.040	Merphos	< 2.0
Bolstar	< 2.0	Methoxychlor	< 0.099
Chlordane	< 0.50	Methyl Parathion	< 2.0
Coumaphos	< 3.0	Mevinphos	< 2.0
Delta BHC	< 0.0099	Mirex	< 0.050
Demeton-O	< 2.0	Naled	< 3.0
Demeton-S	< 2.0	o,p-DDD	< 0.020
Diazinon	< 2.0	o,p-DDE	< 0.020
Dichlorvos	< 2.0	o,p-DDT	< 0.020
Dieldrin	< 0.020	p,p-DDD	< 0.020
Disulfoton	< 2.0	p,p-DDE	< 0.020
Dursban (Chlorpyrifos)	< 2.0	p,p-DDT	< 0.025
Endosulfan I	< 0.0099	PCB-1016	< 0.50
Endosulfan II	< 0.042	PCB-1221	< 1.2
Endosulfan Sulfate	< 0.020	PCB-1232	< 0.89
Endrin	< 0.020	PCB-1242	< 0.50
EPN	< 4.0	PCB-1248	< 0.50
Ethion	< 2.0	PCB-1254	< 0.50
Ethoprop	< 2.0	PCB-1260	< 0.50
Ethyl Parathion	< 2.0	Phorate	< 2.0
Famphur	< 2.0	Ronnel	< 2.0
Fensulfothion	< 4.0	Stirophos	< 2.0
Fenthion	< 2.0	Telodrin	< 0.0099
Gamma BHC – Lindane	< 0.0099	Tokuthion	< 2.0
Guthion (Azinphos-methyl)	< 4.0	Toxaphene	< 0.99
HCB	< 0.099	Trichloronate	< 2.0
Heptachlor	< 0.0099	Trithion	< 2.0

[1]Analyses performed by Lancaster Laboratories on samples collected on December 22, 2004.

Appendix 7 (continued)

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water[1]

Metals			
Component	Measured Concentration (mg/L)	Component	Measured Concentration (mg/L)
Aluminum <	0.200	Magnesium	12.7
Antimony	< 0.0200	Manganese	< 0.0050
Arsenic	< 0.0100	Mercury	< 0.00020
Barium	< 0.0050	Nickel	< 0.0100
Beryllium	< 0.0050	Nitrate Nitrogen	< 0.50
Bromide	< 2.5	Nitrite Nitrogen	< 0.50
Cadmium <	0.0050	Potassium	6.64
Calcium 31.1		Selenium	< 0.0100
Chloride 6.9		Silver	< 0.0050
Chromium <	0.0050	Sodium	19.7
Cobalt <	0.0050	Sulfate	5.5
Copper	< 0.0100	Thallium	< 0.0200
Fluoride	< 0.50	Vanadium	< 0.0050
Iron	< 0.200	Zinc	< 0.0200
Lead <	0.0200		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 22, 2004.

Appendix 8

Results of WAF Equilibration Trial

Polyaromatic Hydrocarbon Analysis

Water accommodated fractions (WAFs) were analyzed for the presence of the 19 polyaromatic hydrocarbons (PAHs) after mixing for 24, 48, 72 and 96 hours. No PAHs were detected in any WAF samples. Results of PAH analyses in WAFs are presented in Wildlife International, Ltd. Project Number 472C-104.

Metals Analysis

Water accommodated fractions (WAFs) were analyzed for the presence of the six metals and sulfur after mixing for 24, 48, 72 and 96 hours. Except for a trace of iron contamination in one test vessel, no metals were detected in any WAF samples. Sulfur was not detected above the background level in the freshwater used. Results of the metals analyses in WAFs are presented in Wildlife International, Ltd. Project Number 472C-105.

Appendix 9

The Analysis of Organic Constituents in Petroleum Coke in Freshwater Algal Medium

Appendix 9.1

Flowchart for the Analysis of PAH Components of Petroleum Coke
in Freshwater Algal Medium

FLOWCHART FOR THE ANALYSIS OF WATER SOLUBLE COMPONENTS OF PETROLEUM COKE IN FRESHWATER ALGAL MEDIUM

Prepare samples in freshwater algal medium using volumetric flasks, volumetric pipettes, gas tight syringes and culture tubes. Freshwater algal medium served as the matrix blanks.

↓

Dilute samples, as necessary, with freshwater algal medium such that the final sample concentrations fall within the calibration standard range.

↓

Transfer samples and standards to autosampler vials for analysis by either HPLC/UV or fluorescence detection.

Figure 1. Analytical method flowchart for the analysis of PAH components of petroleum coke in freshwater algal medium by HPLC.

Appendix 9.2

Typical HPLC Operational Parameters

INSTRUMENT:	Agilent Model 1100 High Perform ance Liquid Chromatograph (HPLC) with a either an Agilent Series 1100 Variable Wavelength Detector or a Jasco Model FP-1520 Fluorescence Detector
ANALYTICAL COLUMN:	YMC-Pack ODS AM (150 mm x 4.6 mm, 3 μm particle size)
STOP TIME:	35 minutes
FLOW RATE:	1.00 mL/minute
OVEN TEMPERATURE:	40°C
MOBILE PHASE:	SOLVENT A: 0.1% H_3PO_4 SOLVENT B: CH_3CN

GRADIENT:

Time (min)	%A	%B (m	Flow L/min)
0.01	40.0	60.0	1.000
1.00	40.0	60.0	1.000
30.00	0.0	100.0	1.000
30.10	40.0	60.0	1.000
35.00	40.0	60.0	1.000

INJECTION VOLUME:	100 μL	
APPROXIMATE RETENTION TIMES:	Naphthalene = 6.8 min. Acenaphthylene = 7.8 min. 1-Methylnaphthalene = 8.9 min. 2-Methylnaphthalene = 9.2 min. Fluorene = 9.8 min. Acenaphthene = 9.9 min. Phenanthrene = 10.7 min. Anthracene = 11.4 min. Fluoranthene = 13.0 min. Pyrene = 13.8 min.	Chrysene = 16.2 min. Benz(a)anthracene = 16.4 min. Benzo(b)fluoranthene = 19.2 min. Benzo(k)fluoranthene = 19.5 min. Benzo(a)pyrene = 20.2 min. Dibenz(a,h,)anthracene = 21.8 min. Indeno(1,2,3-cd)pyrene = 23.1 min. Benzo(g,h,i)perylene = 23.4 min. Dibenzo(a,e)pyrene = 25.2 min.
PRIMARY ANALYTICAL WAVELENGTHS	UV = 220 nm; Fluorescence = 340 nm to 425 nm	

Appendix 9.3

Analytical Stocks and Standards Preparation

For all compounds received from AccuStandard, with the exception of Benzo(g,h,i)perylene, Benzo(k)fluoranthene and Fluoranthene, the mass received was quantitatively transferred to a 100-mL class A volumetric flask using methanol. These primary stock solution concentrations were 0.100 mg/mL. Benzo(g,h,i)perylene, Benzo(k)fluoranthene and Fluoranthene were quantitatively transferred to a 200-mL class A volumetric flask using methanol. These primary stock solution concentrations were 0.05 mg/mL.

A stock of Dibenzo(a.e)pyrene (received from Cambridge Isotope Labs) was prepared by weighing 0.01000 g on an analytical balance, transferred to a 100-mL class A volumetric flask and brought to volume using tetrahydrofuran. This primary stock solution concentration was 0.100 mg/mL.

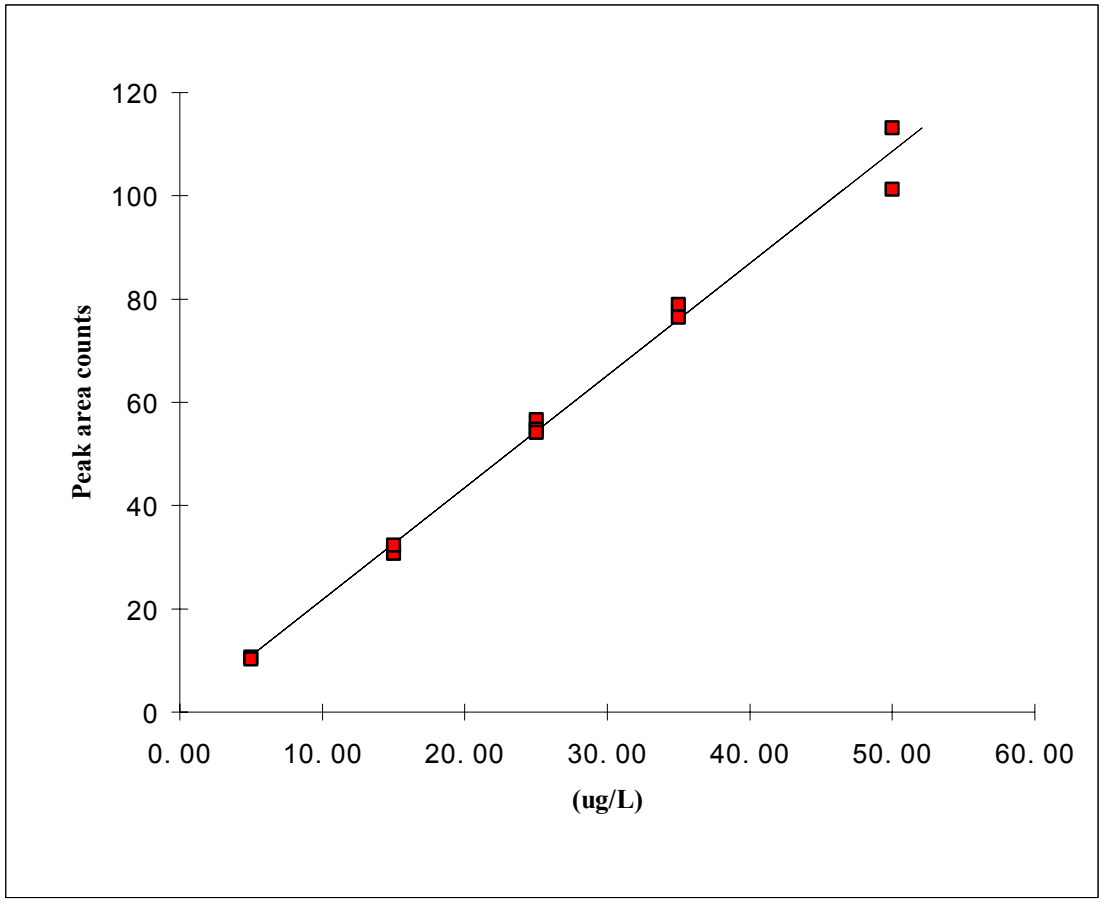
Stocks of 2-methylnaphthalene and 1-methynaphthalene (received from ChemService) were prepared by weighing 0.1004 g and 0.01003 g, respectively, on an analytical balance. The test materials were transferred to 100-mL class A volumetric flasks and brought to volume using methanol. These primary stock solutions contained 1.00 mg/mL of the test material and were diluted in methanol to prepare 0.100 mg/mL stock solutions.

Aliquots (1.00 mL) of the 0.100 mg/mL primary stocks and 2.00 mL of the 0.05 mg/L, were added to a 100-mL class A volumetric flask and brought to volume with methanol. The following shows the dilution scheme for the set of calibration standards:

Stock Concentration mg/L	Aliquot (µL)	Final Volume (mL)	Standard Concentration (µg/L)
1.00	50.0	10.0	5.00
1.00	150	10.0	15.0
1.00	250	10.0	25.0
1.00	350	10.0	35.0
1.00	500	10.0	50.0

Appendix 9.4

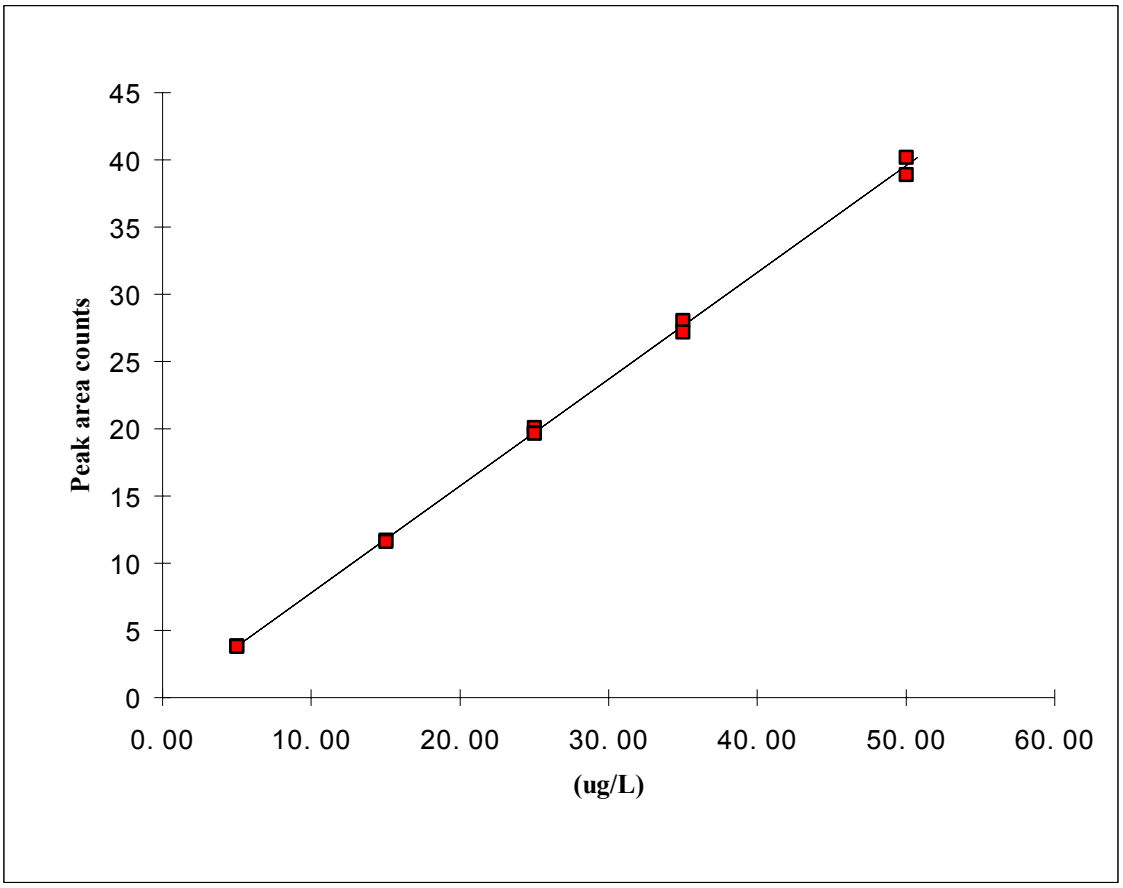
Calibration Curve for Naphthalene Analyzed by HPLC/UV



Slope = 2.1714; Y-intercept = 0.0560; R^2 = 0.9920

Appendix 9.5

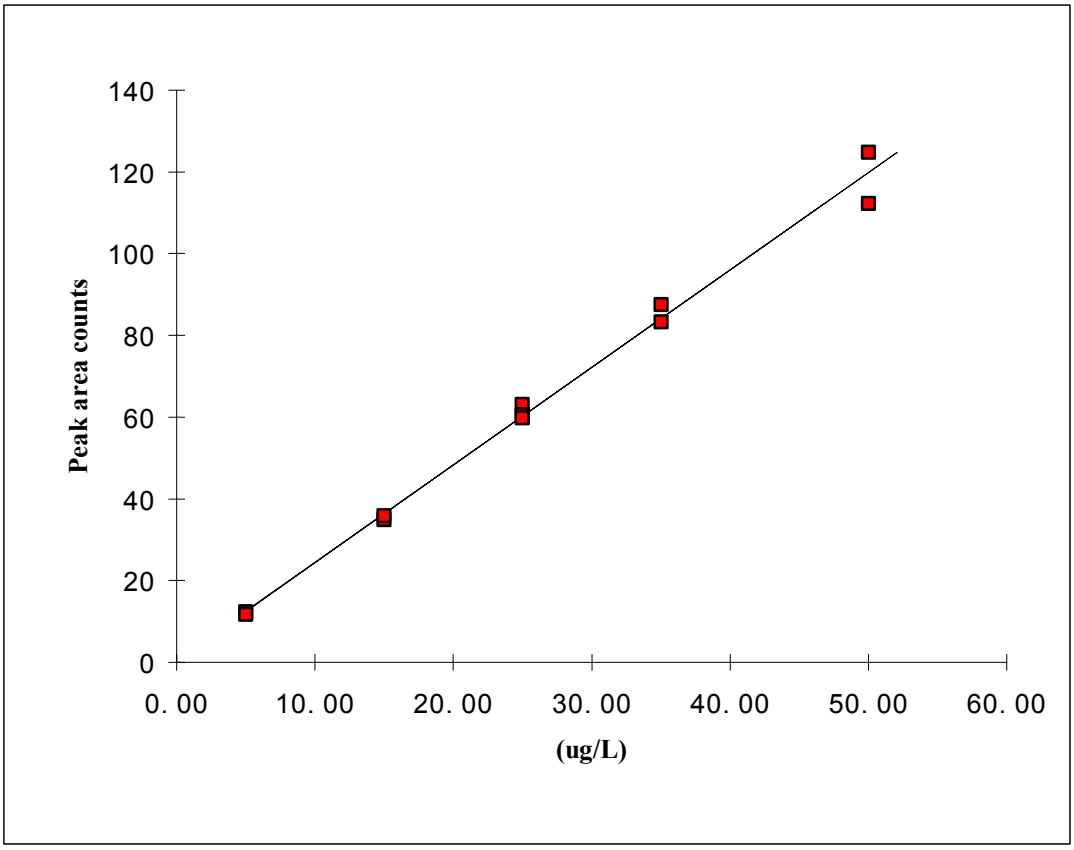
Calibration Curve for Acenaphthylene Analyzed by HPLC/UV



Slope = 0.7938; Y-intercept = -0.1343 ; R^2 = 0.9991

Appendix 9.6

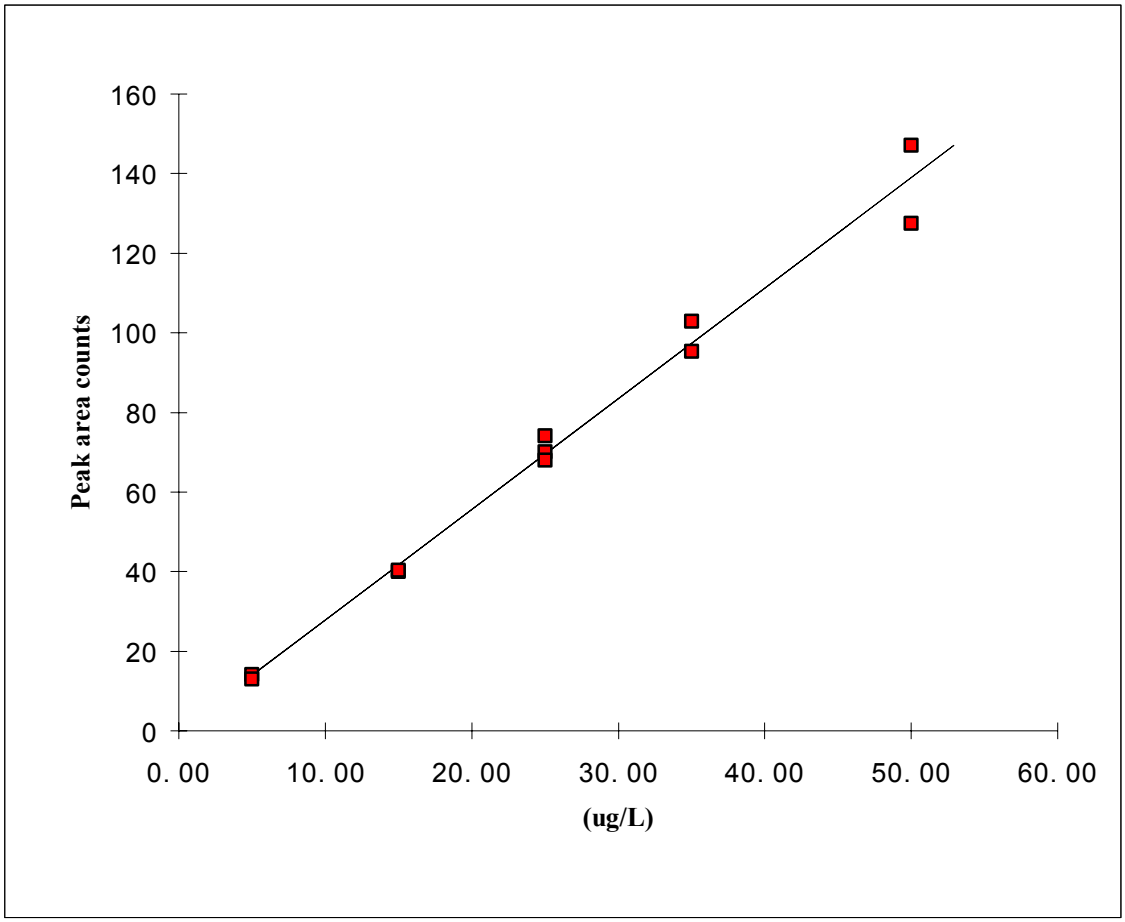
Calibration Curve for 1-Methylnaphthalene Analyzed by HPLC/UV



Slope = 2.3862; Y-intercept = 0.5760; R^2 = 0.9924

Appendix 9.7

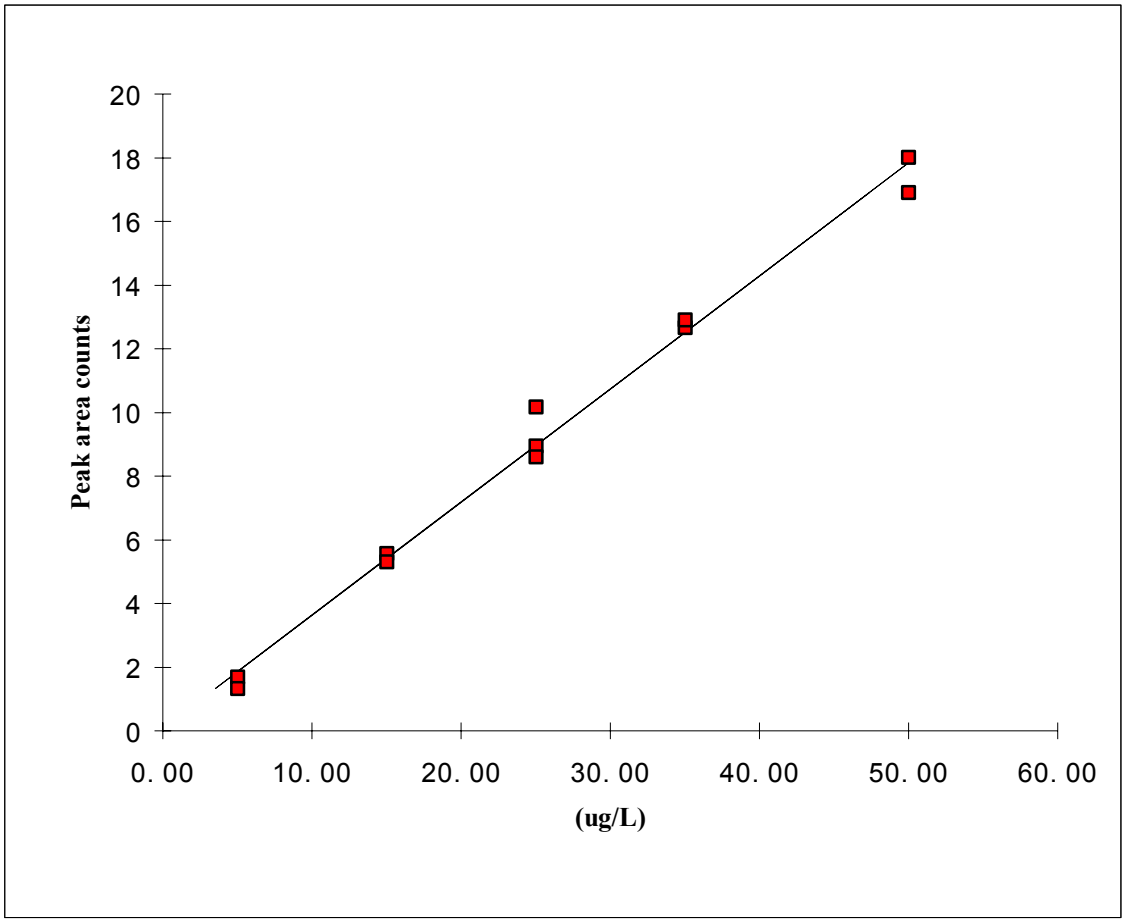
Calibration Curve for 2-Methylnaphthalene Analyzed by HPLC/UV



Slope = 2.7779; Y-intercept = 0.0779; R^2 = 0.9864

Appendix 9.8

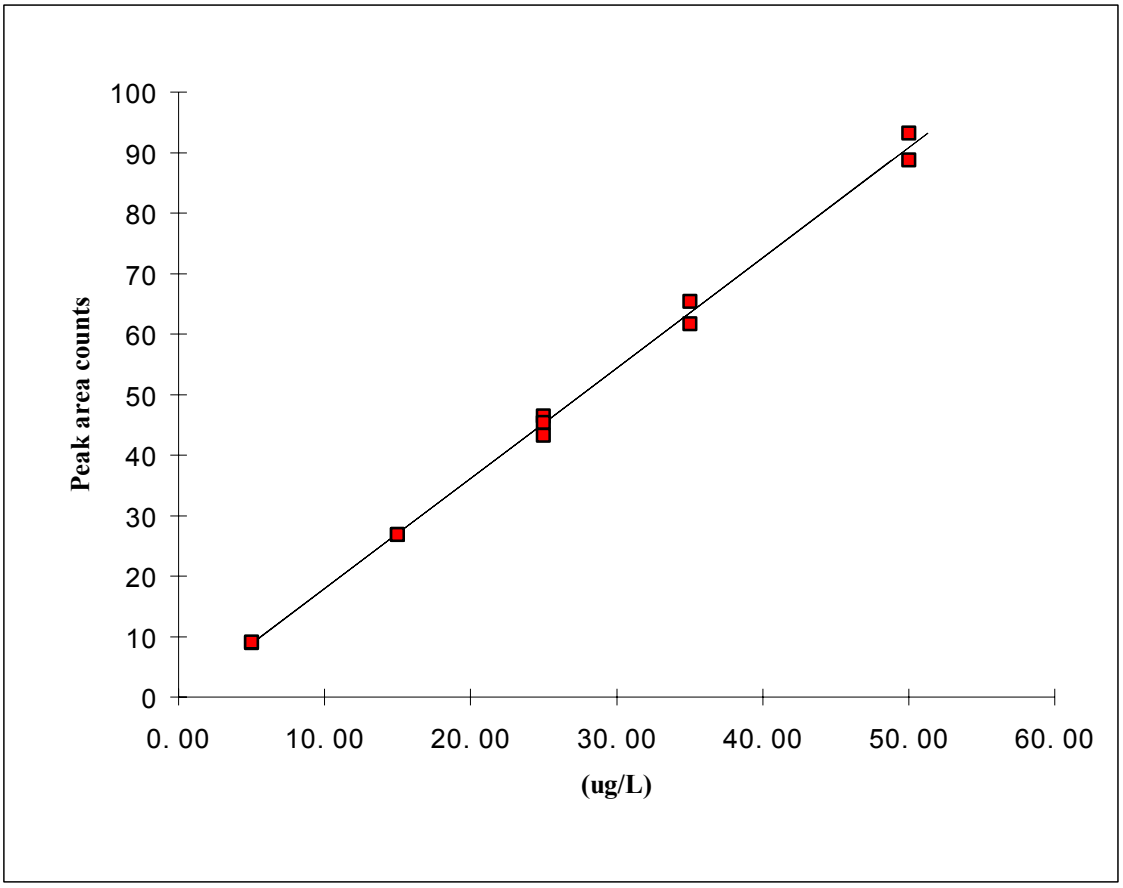
Calibration Curve for Fluorene Analyzed by HPLC/UV



Slope = 0.3551; Y-intercept = 0.0798; R^2 = 0.9904

Appendix 9.9

Calibration Curve for Acenaphthene Analyzed by HPLC/UV

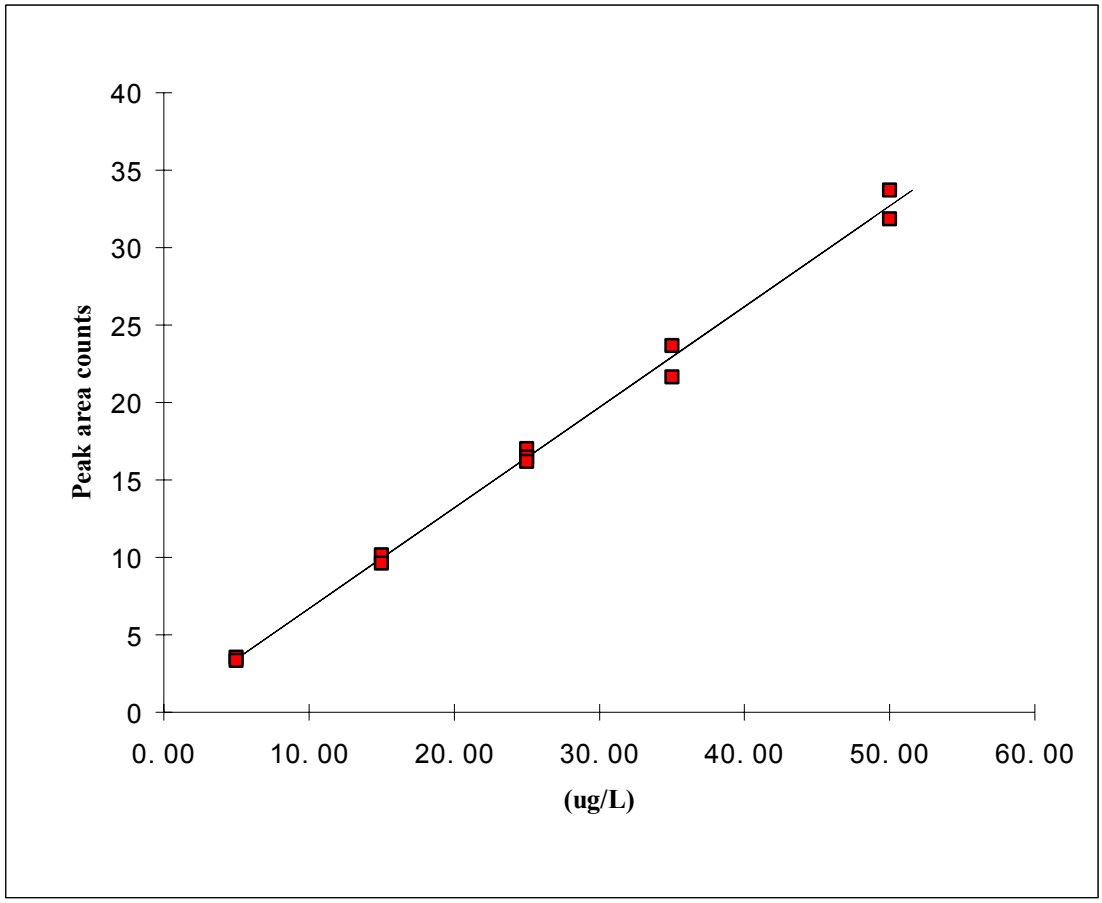


Slope = 1.8240; Y-intercept = -0.3335; R^2 = 0.9972

- 128 -

Appendix 9.10

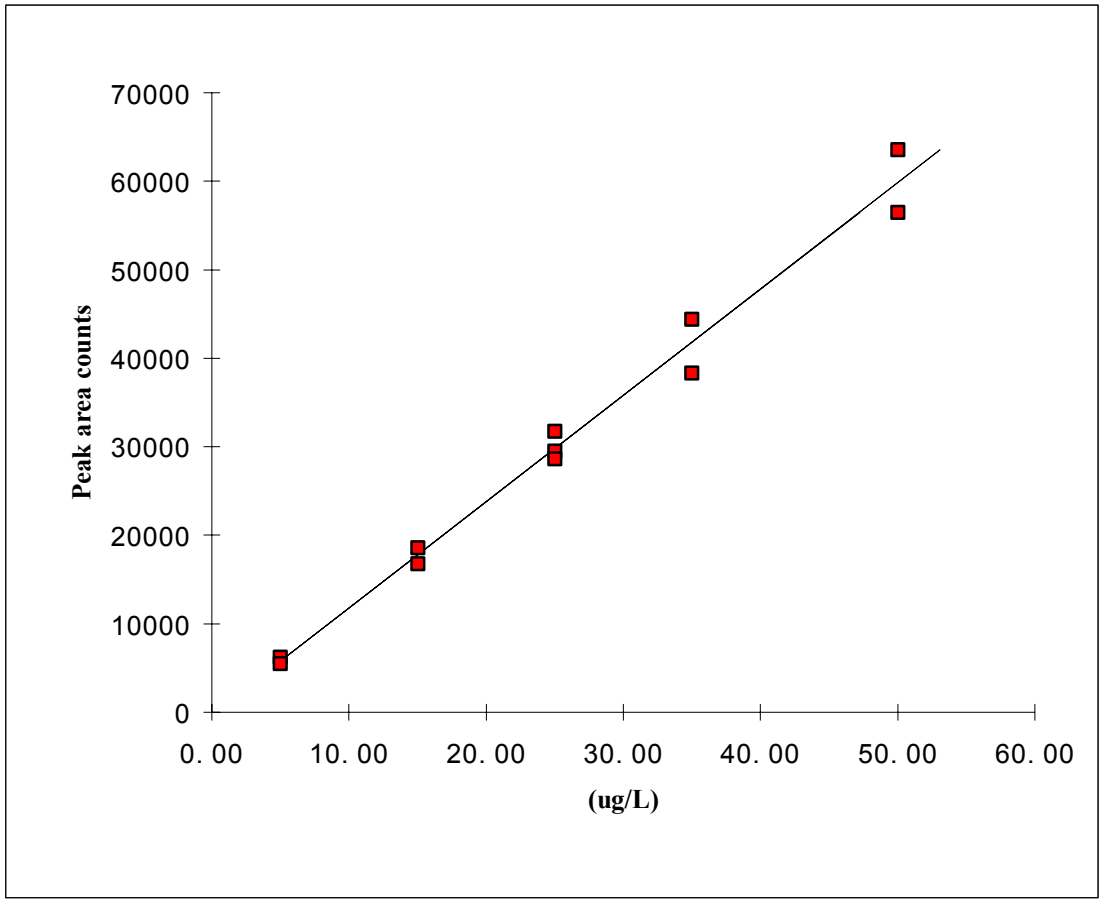
Calibration Curve for Phenanthrene Analyzed by HPLC/UV



Slope = 0.6500; Y-intercept = 0.1881; R^2 = 0.9957

Appendix 9.11

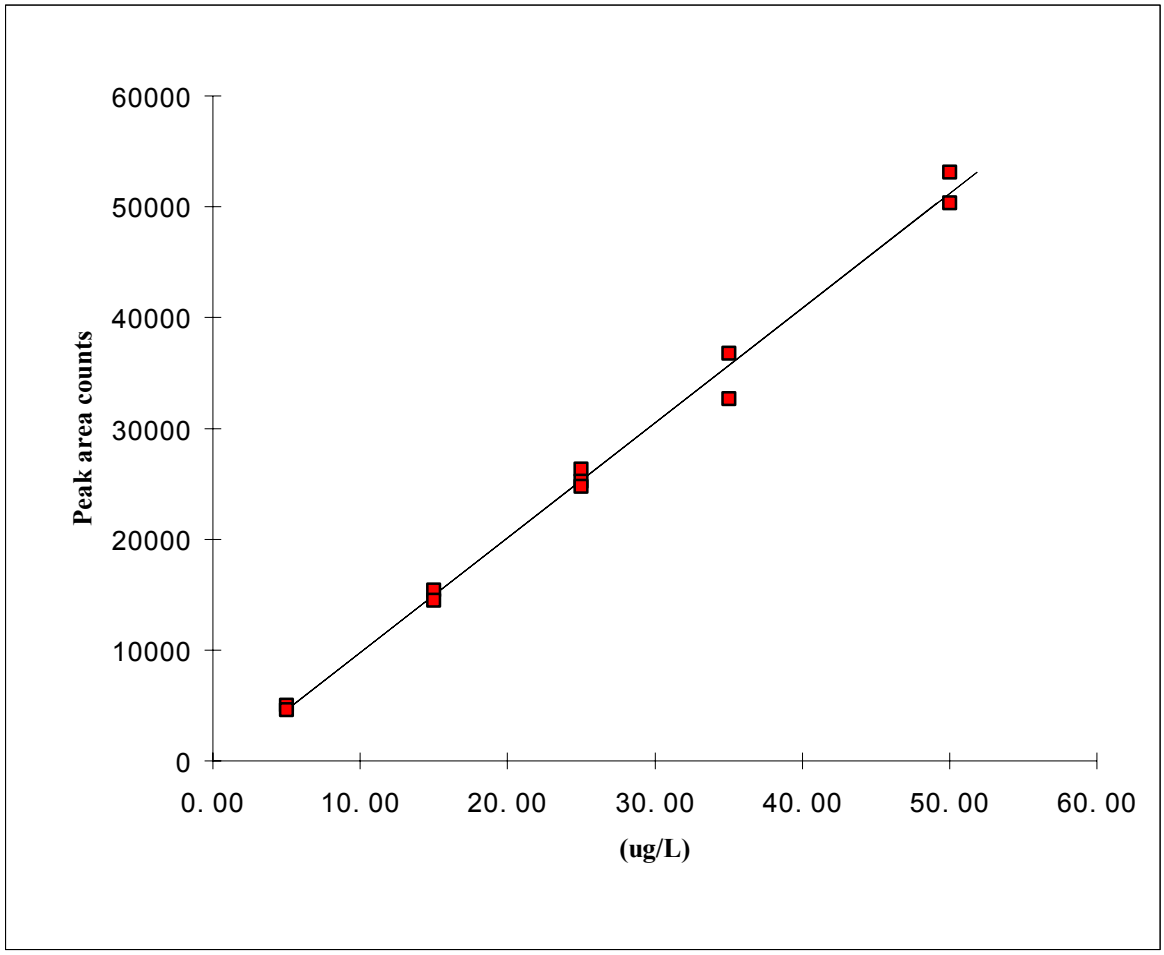
Calibration Curve for Anthracene Analyzed by HPLC with Fluorescence Detection



Slope = 1201.8184; Y-intercept = -266.6260; R^2 = 0.9857

Appendix 9.12

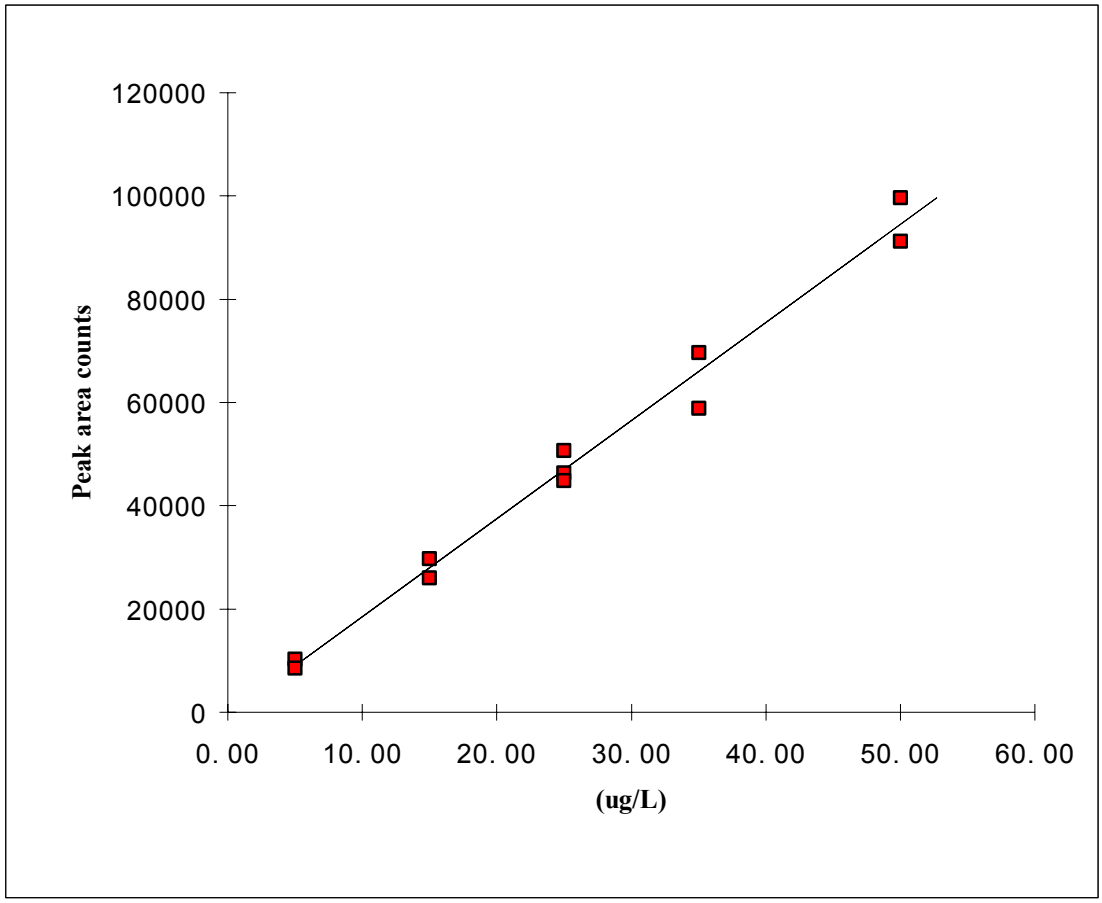
Calibration Curve for Fluoranthene Analyzed by HPLC with Fluorescence Detection



Slope = 1035.2596; Y-intercept = -558.9382; R^2 = 0.9936

Appendix 9.13

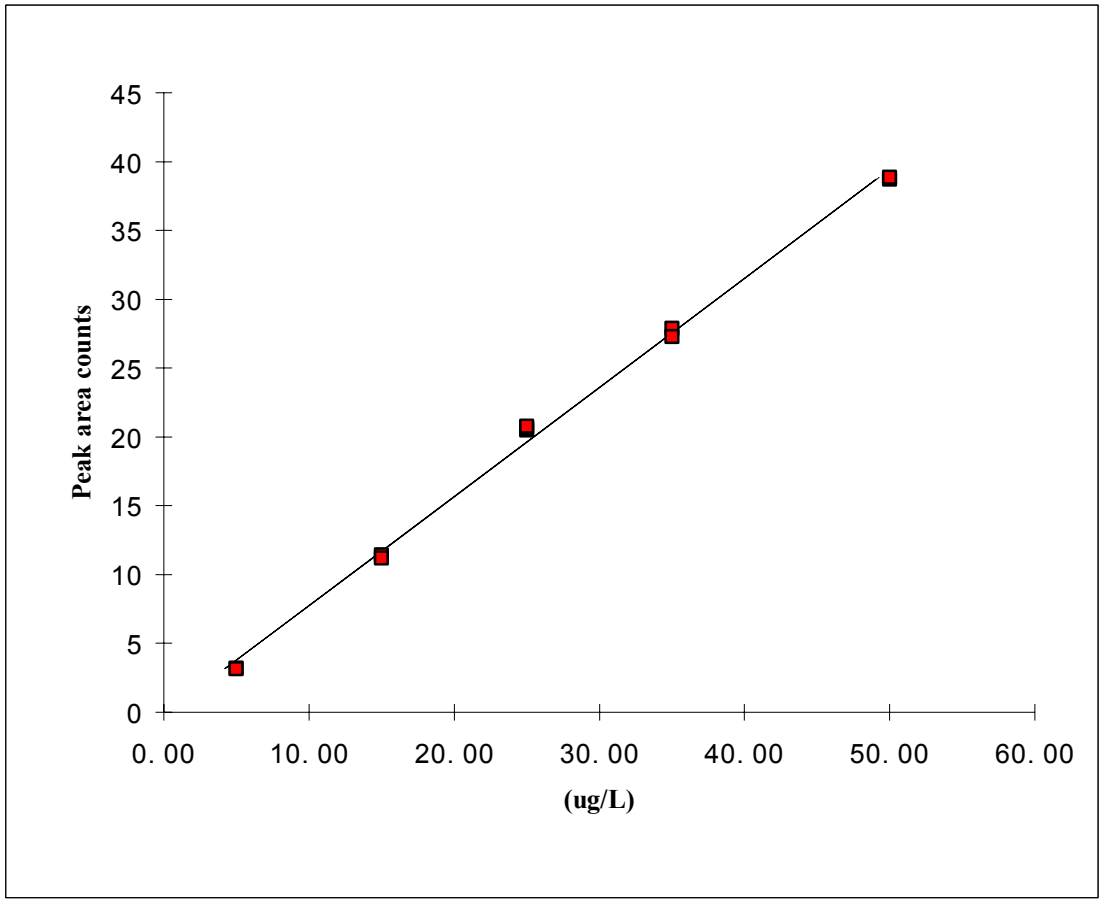
Calibration Curve for Pyrene Analyzed by HPLC with Fluorescence Detection



Slope = 1900.5190; Y-intercept = -501.3931; R^2 = 0.9857

Appendix 9.14

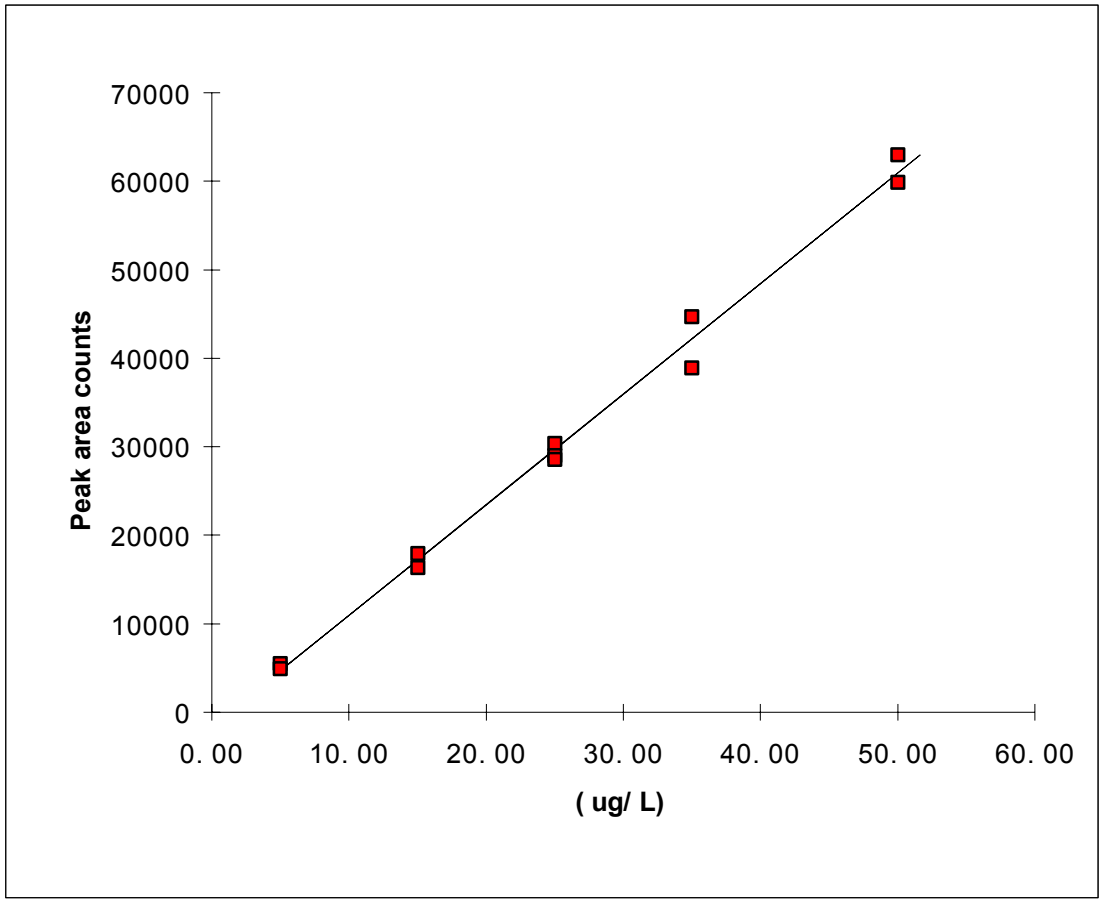
Calibration Curve for Chrysene Analyzed by HPLC/UV



Slope = 0.7922; Y-intercept = -0.1830; R^2 = 0.9966

Appendix 9.15

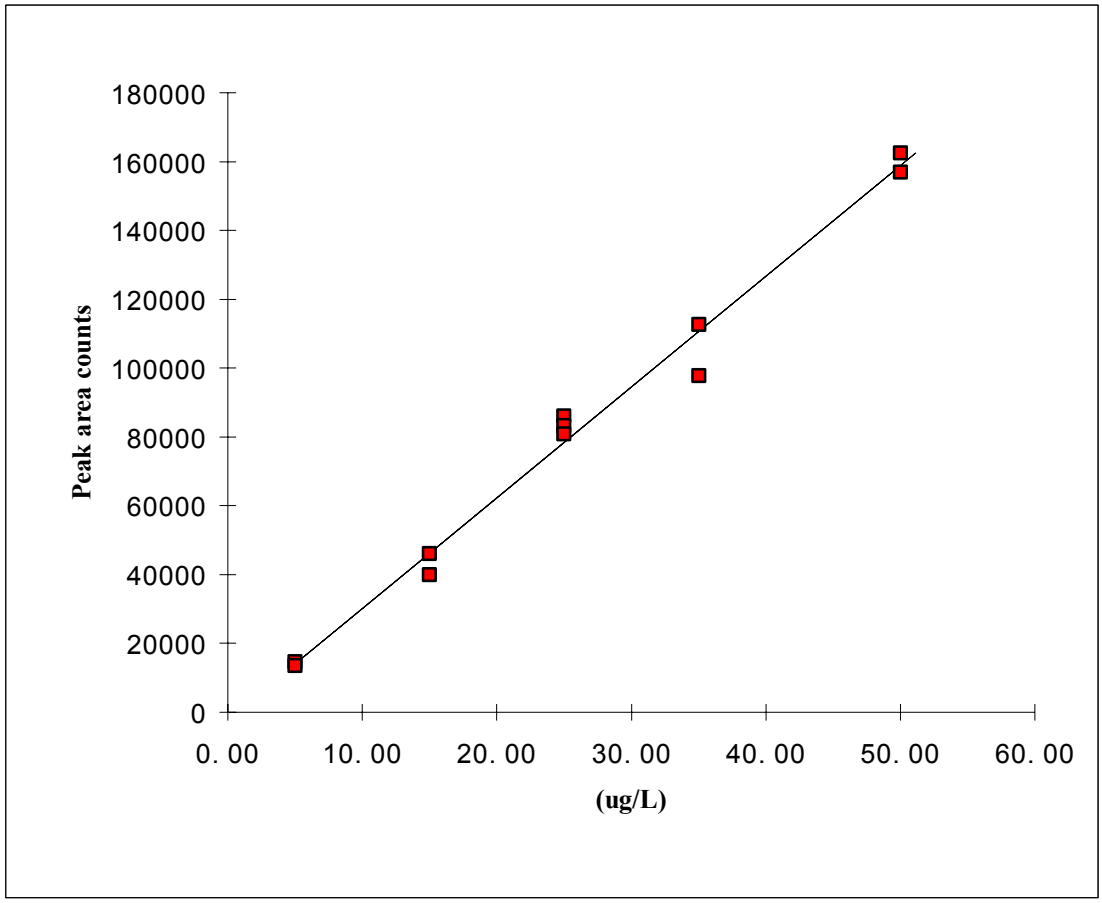
Calibration Curve for Benz(a)anthracene Analyzed by HPLC with Fluorescence Detection



Slope = 1249.0398; Y-intercept = -1535.9726 R^2 = 0.9932

Appendix 9.16

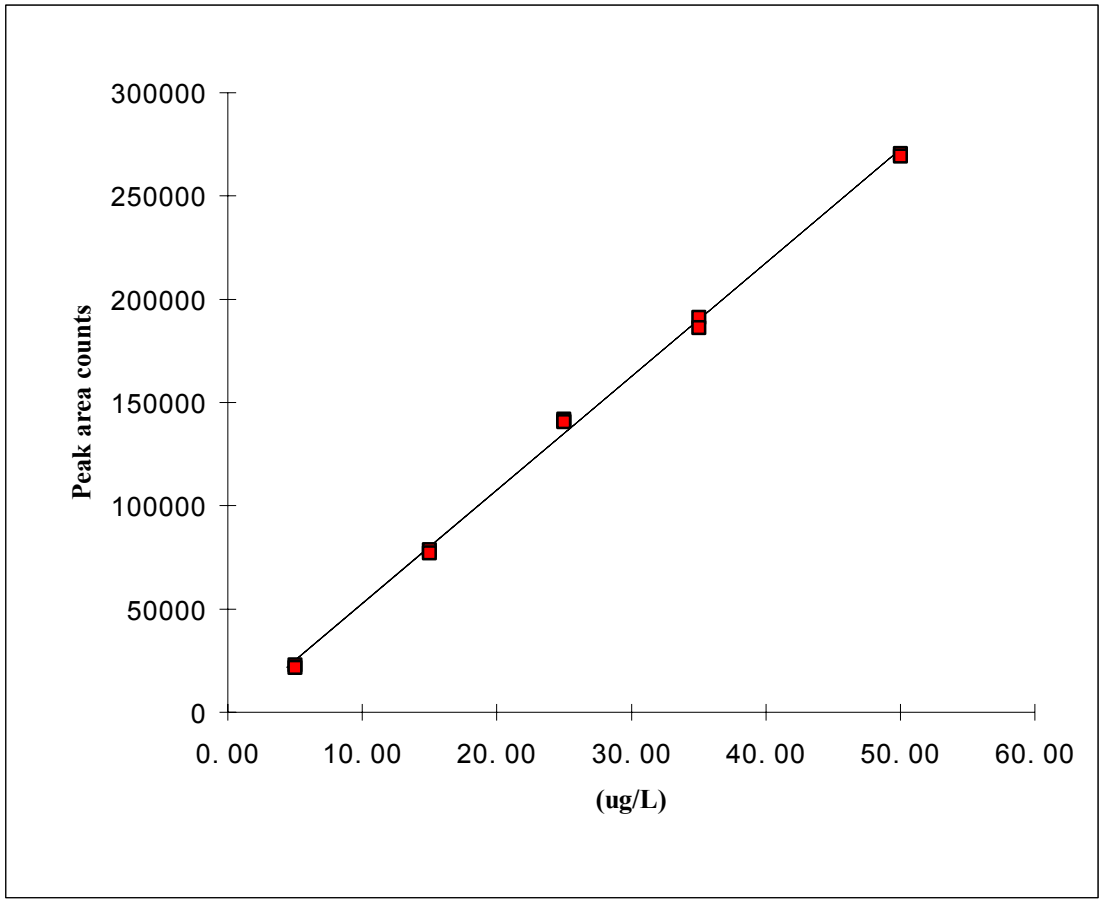
Calibration Curve for Benzo(b)fluoranthene Analyzed by HPLC with Fluorescence Detection



Slope = 3216.3001; Y-intercept = -2008.2201; R^2 = 0.9877

Appendix 9.17

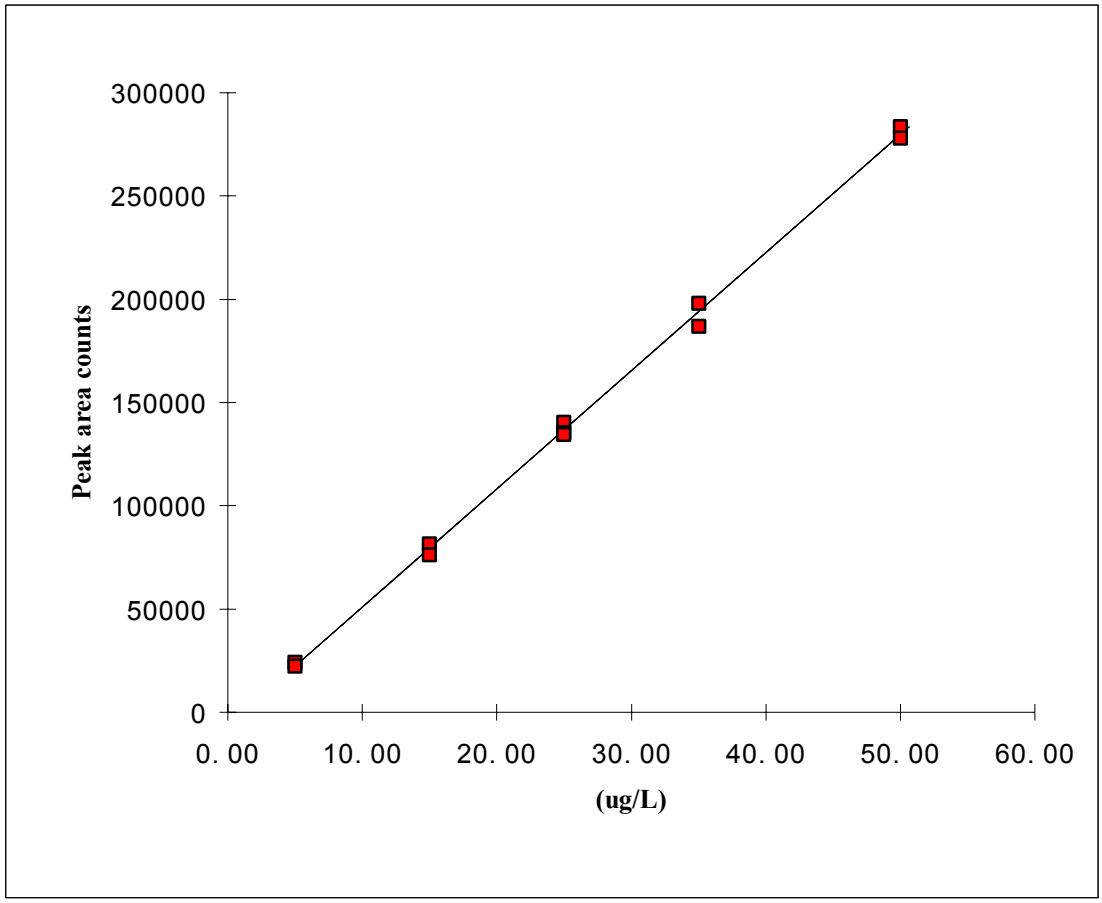
Calibration Curve for Benzo(k)fluoroanthene Analyzed by HPLC with Fluorescence Detection



Slope = 5498.7539; Y-intercept = -2357.1609; R^2 = 0.9976

Appendix 9.18

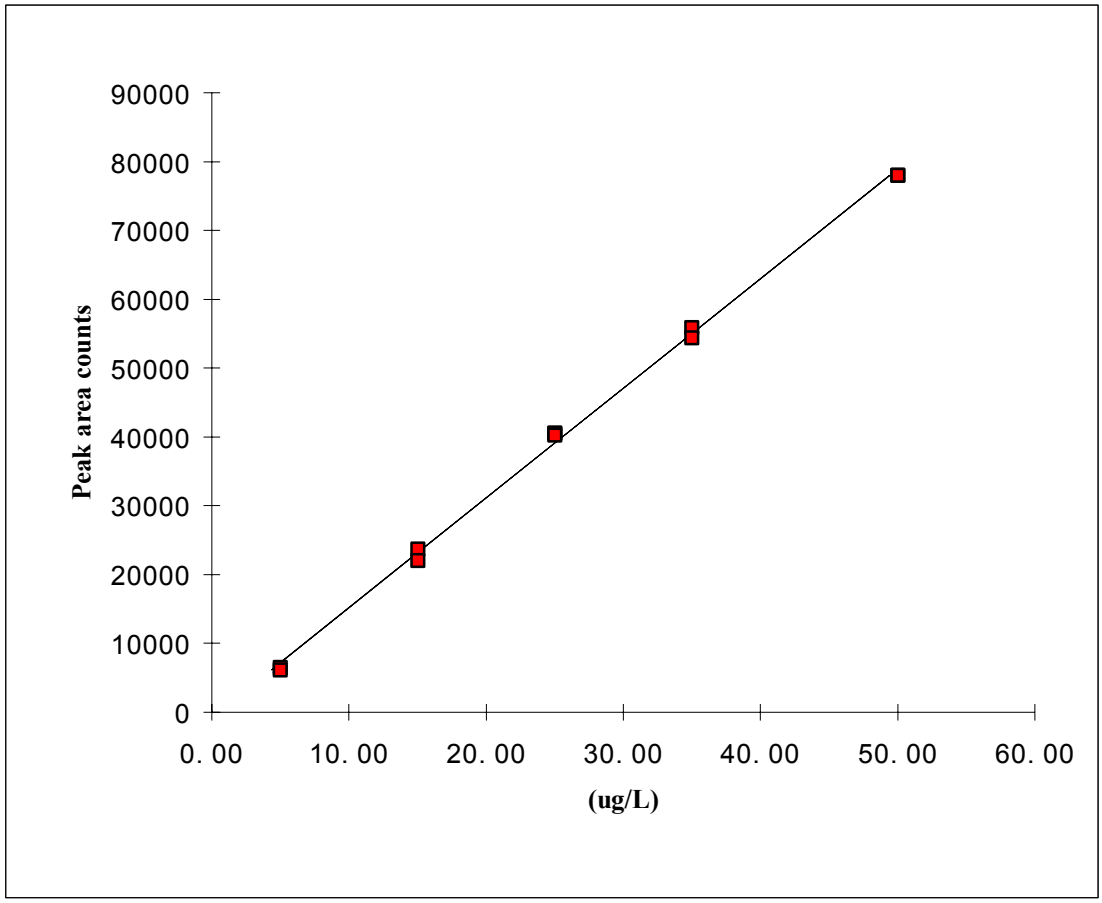
Calibration Curve for Benzo(a)pyrene Analyzed by HPLC with Fluorescence Detection



Slope = 5719.5557; Y-intercept = -6253.9436; R^2 = 0.9984

Appendix 9.19

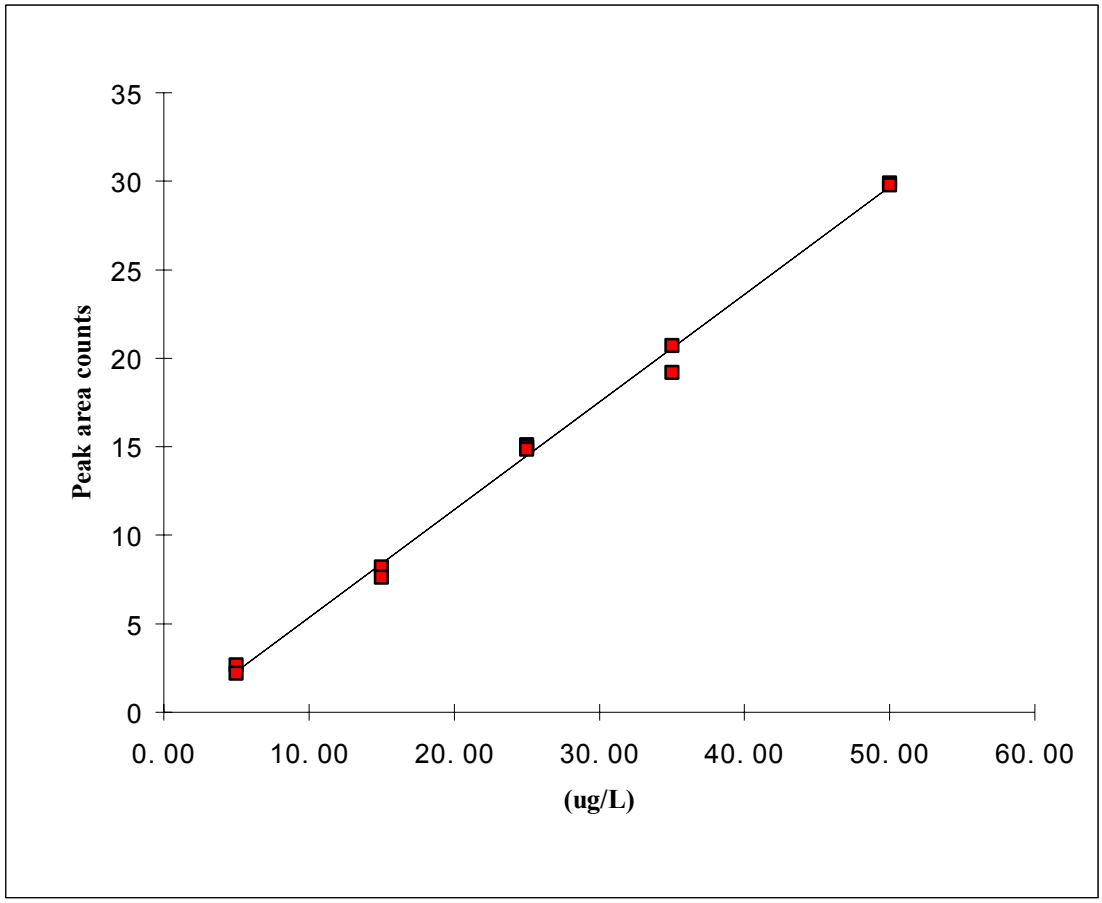
Calibration Curve for Dibenz(a,h)anthracene Analyzed by HPLC with Fluorescence Detection



Slope = 1592.8616; Y-intercept = -724.3072; R^2 = 0.9982

Appendix 9.20

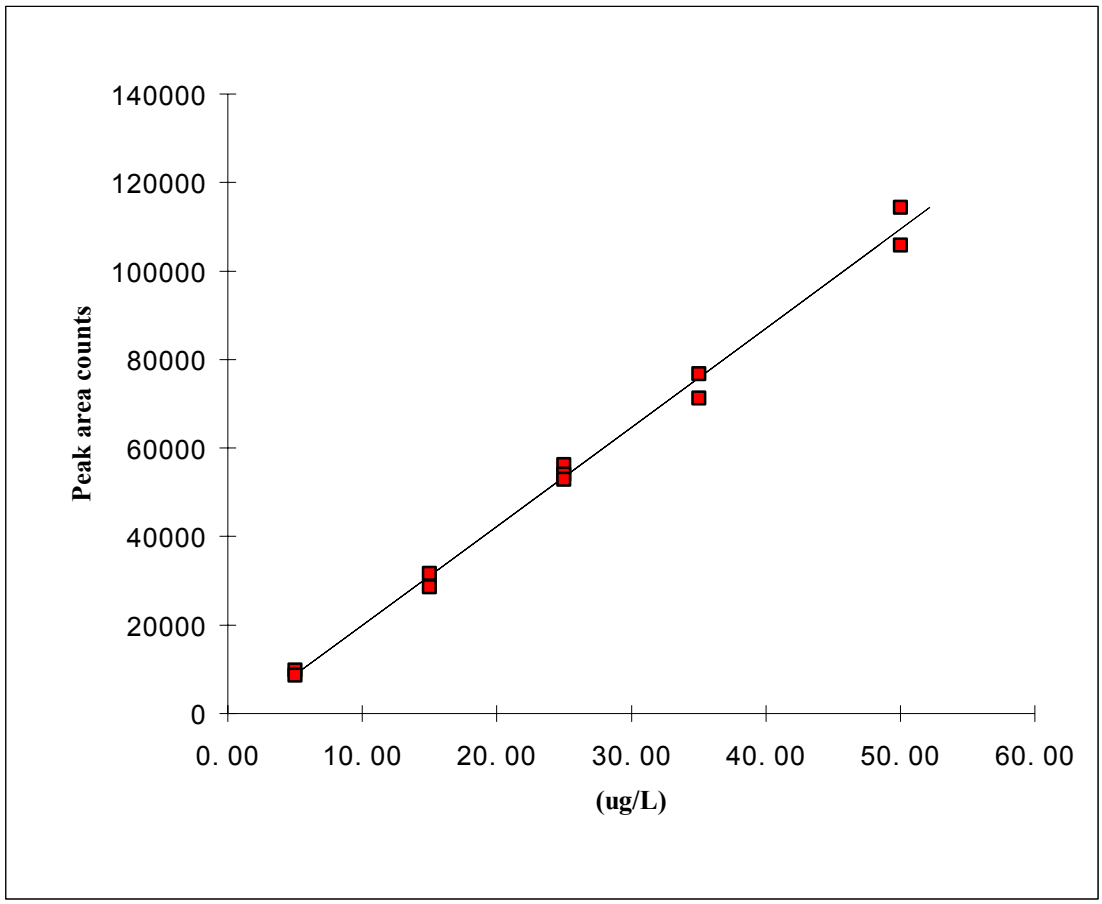
Calibration Curve for Indeno(1,2,3-cd)pyrene Analyzed by HPLC/UV



Slope = 0.6085; Y-intercept = -0.7359; R^2 = 0.9962

Appendix 9.21

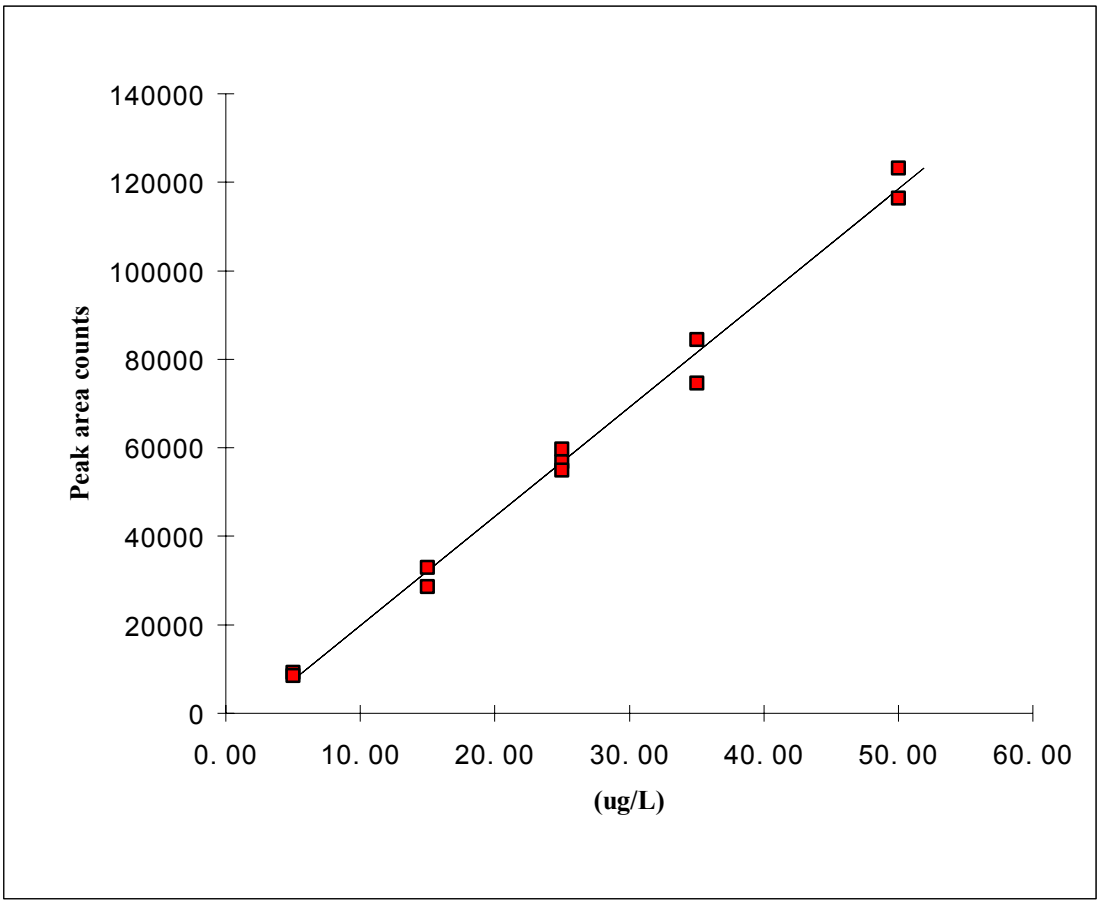
Calibration Curve for Benzo(g,h,i)perylene Analyzed by HPLC with Fluorescence Detection



Slope = 2236.0634; Y-intercept = -2425.5273; R^2 = 0.9939

Appendix 9.22

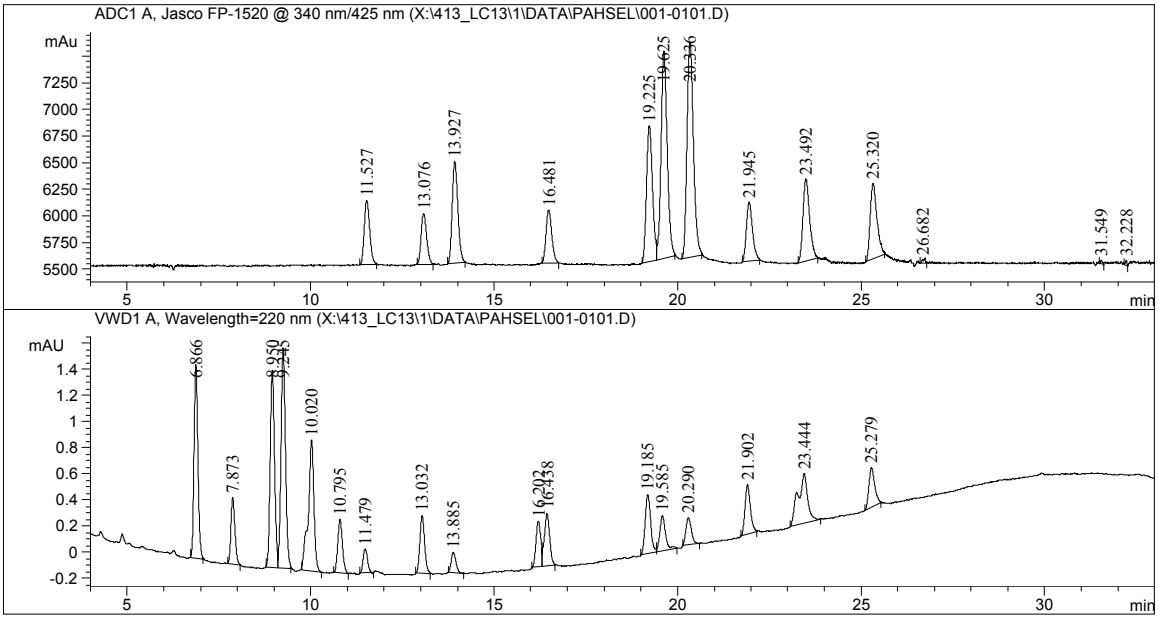
Calibration Curve for Dibenzo(a,e)pyrene Analyzed by HPLC with Fluorescence Detection



Slope = 2466.8520; Y-intercept = -4840.6442; R^2 = 0.9926

Appendix 9.23

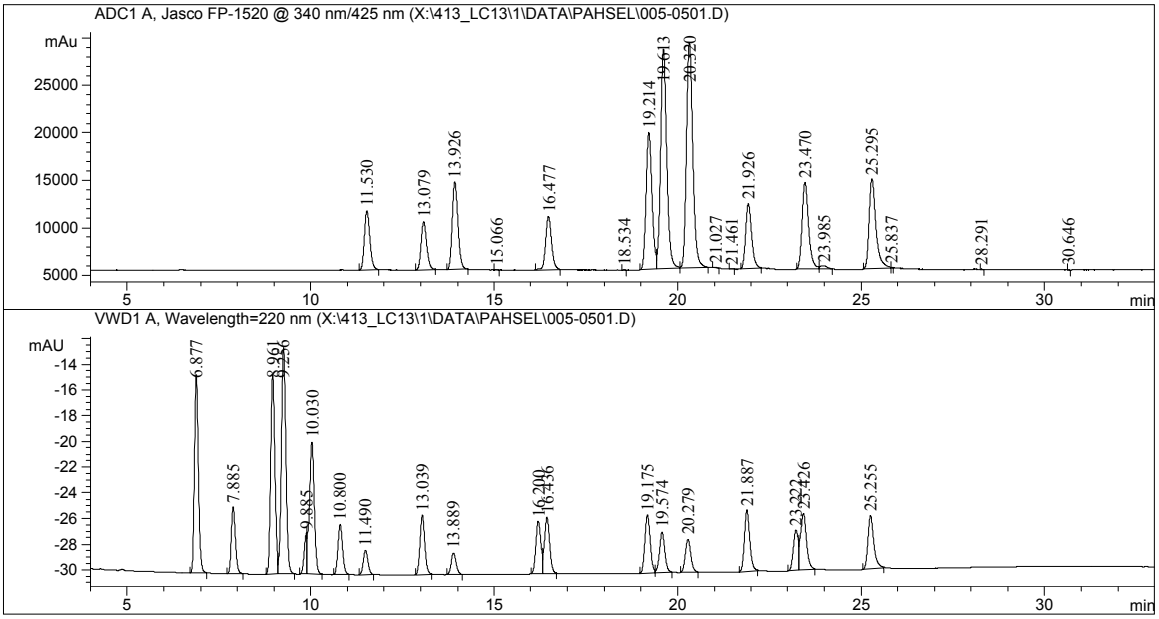
Representative Chromatograms of a Low-level Calibration Standard
Analyzed by HPLC/UV and Fluorescence Detection.



Nominal Concentration: 5.00 µg/L

Appendix 9.24

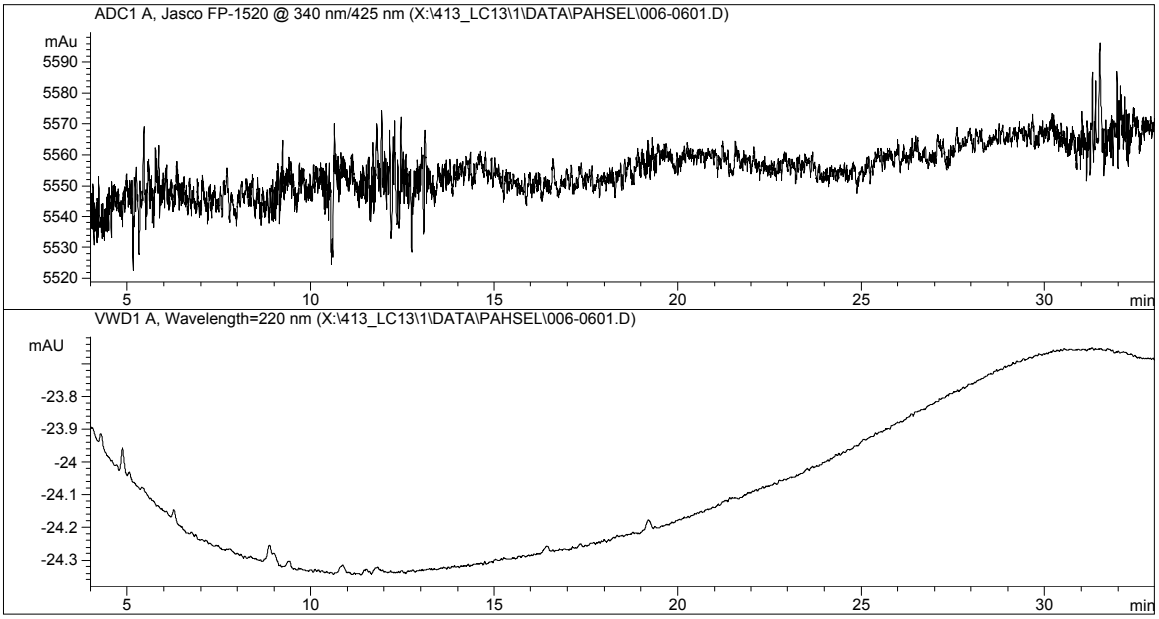
Representative Chromatograms of a High-level Calibration Standards
Analyzed by HPLC/UV and Fluorescence Detection



Nominal Concentration: 50.0 µg/L

Appendix 9.25

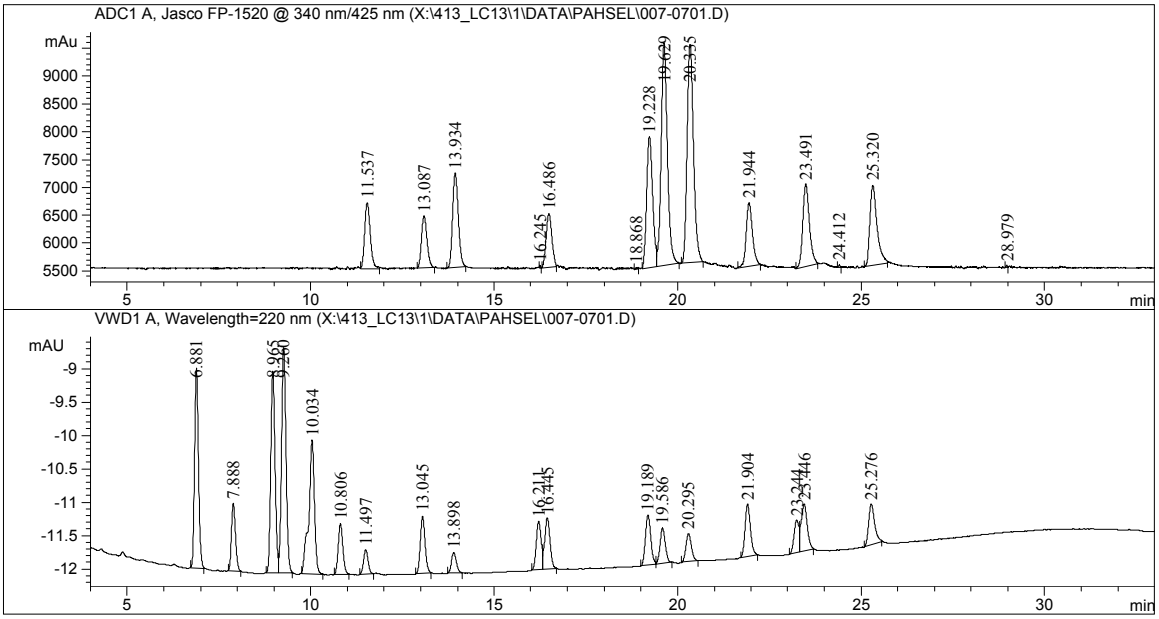
Representative Chromatograms of a Matrix Blank Sample
Analyzed by HPLC/UV and Fluorescence Detection



Sample Number 472A-114-MAB-1.

Appendix 9.26

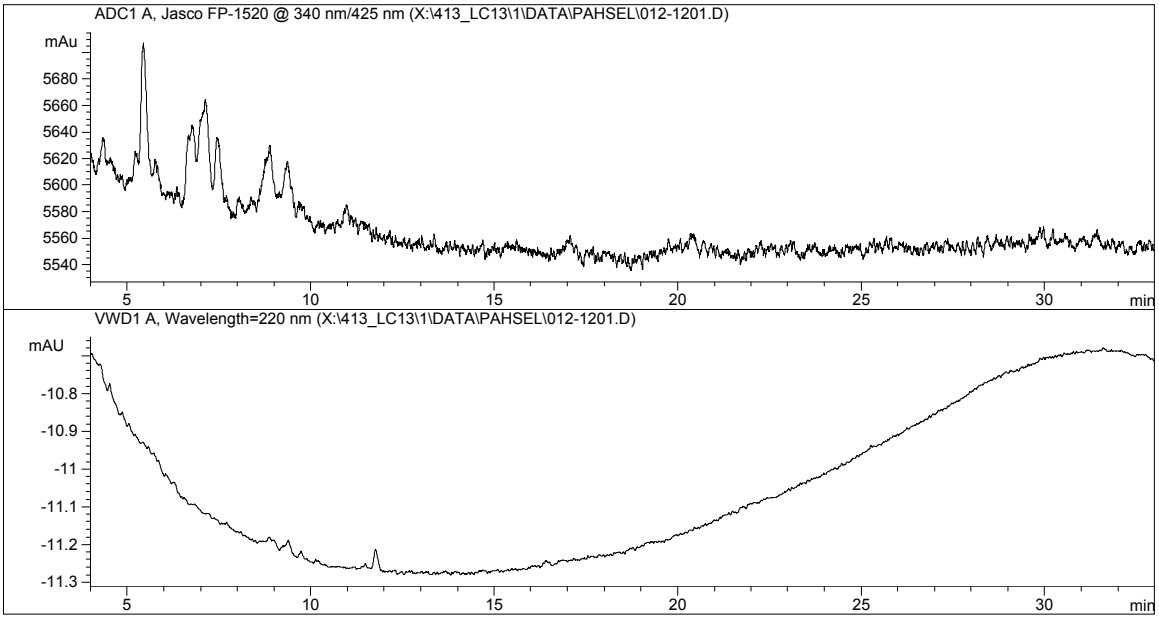
Representative Chromatograms of a Matrix Fortification Sample
Analyzed by HPLC/UV and Fluorescence Detection



Sample Number 472A-114-MAS-1. Nominal Concentration: 10.0 µg/L

Appendix 9.27

Representative Chromatograms of a Test Sample
Analyzed by HPLC/UV and Fluorescence Detection



Sample Number 472A-114-4. Nominal Concentration: 1000 mg/L WAF.

Appendix 10

The Analysis of Inorganic Constituents in Petroleum Coke in Freshwater Algal Medium

Appendix 10.1

Analytical Method Flowchart for the Analysis of As, Cu, Fe, Ni, Se, V and S in Freshwater Algal Media Analyzed by ICP-AES

Analytical Method Flowchart for the Analysis of As, Cu, Fe, Ni, Se, V and S in Freshwater Algal Media Analyzed by ICP-AES

Prepare quality control (QC) samples concurrent with the analyses of test samples as follows: Prepare matrix fortification samples by spiking the requisite volume(s) of the appropriate combined and/or individual element stock solution(s) directly into freshwater algal media. Perform fortifications with calibrated micropipettors and graduated centrifuge tubes. Bring to final volume with freshwater algal media. The matrix blank consists of unfortified freshwater algal media.

↓

Partially fill a graduated centrifuge tube with each sample. Using a calibrated micropipettor, fortify each QC and test sample with 200 µL of concentrated nitric acid. Bring to final volume with the sample. For samples not requiring further dilution into the calibration range of the ICP-AES methodology, submit for ICP-AES analysis.

↓

For those samples requiring dilution into the calibration range of the ICP-AES methodology, perform dilutions using graduated centrifuge tubes, calibrated micropipettor(s), and 2% (v/v) nitric acid in freshwater algal media solution. Mix dilutions well and transfer into separate, labeled, 15-mL centrifuge tubes. Submit for ICP-AES analysis.

Appendix 10.2

Typical ICP-AES Operational Parameters for the Analysis of
As, Cu, Fe, Ni, Se, V and S in Freshwater Algal Media

Instrument:	Perkin-Elmer Optima 3000 DV Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES)	
Sample Introduction System:	Cetac U-5000AT^{+} Ultrasonic Nebulizer	
Analytical Wavelengths:	As 188.979 nm Cu 224.700 nm Fe 239.562 nm Ni 231.604 nm Se 196.026 nm S 180.669 nm V 292.402 nm	
Plasma:	Plasma Gas Flow: Auxiliary Gas Flow: Nebulizer Gas Flow: RF Power:	15 L/min Ar 0.5 L/min Ar 0.7 L/min Ar 1300 W
Pump:	Sample Flow Rate: Sample Flush Time: Wash Rate: Wash Time: Wash Frequency:	2.00 mL/min 15 sec 2.00 mL/min 60 sec Between Samples
Spectrometer: View	Mode: Read Delay: Read Time: Read Replicates: Peak Algorithm: Points/Peak: Background Correction:	Axial 60 sec Min: 10.000 sec Max: 20.000 sec 3 Peak Area 3 2-Point

Appendix 10.3

Analytical Stocks and Standards Preparation

A combined stock solution containing the seven elements of interest was prepared either directly from the procured Spex primary standards, or from single element secondary stock preparations. The combined stock solutions were used to prepare external calibration standards and matrix fortification samples. Volumetric flasks and calibrated micropipettors were used for all preparations. Preparation details for the stocks were as follows:

A single component secondary stock for vanadium was first prepared from a 10x dilution of the 1.00 mg/mL V primary standard using 2% (v/v) nitric acid in reagent grade water (2:98 HNO_3: H_2O) dilution solvent. The nominal concentration of the resultant vanadium stock was 0.100 mg V/mL. A combined secondary stock in 2:98 HNO_3: H_2O dilution solvent was then prepared as follows:

Element	Primary Stock Concentration mg/mL	Aliquot (mL)	Final Volume (mL)	Secondary Stock Concentration (mg/L)
As	1.00	1.00		10.0
Cu	1.00	1.00		10.0
Fe	1.00	0.500	100	5.00
Ni	1.00	0.500		5.00
Se	1.00	10.0		100
V	0.100	0.200		0.200

This combined secondary stock solution was used to prepare a set of calibration standards, each in 2% (v/v) nitric acid in Wildlife International, Ltd. freshwater algal media (2:98 HNO_3: media) dilution solvent and diluted to a 100-mL final volume, using the following dilution scheme:

Appendix 10.3 (continued)

Analytical Stocks and Standards Preparation

Stock Aliquot:	0.100 mL	0.250 mL	0.500 mL	0.750 mL	1.00 mL
Element	Standard Concentration µg/L	Standard Concentration µg/L	Standard Concentration µg/L	Standard Concentration µg/L	Standard Concentration µg/L
As	10.0	25.0	50.0	75.0	100
Cu	10.0	25.0	50.0	75.0	100
Fe	5.00	12.5	25.0	37.5	50.0
Ni	5.00	12.5	25.0	37.5	50.0
Se	100	250	500	750	1000
V	0.200	0.500	1.00	1.50	2.00

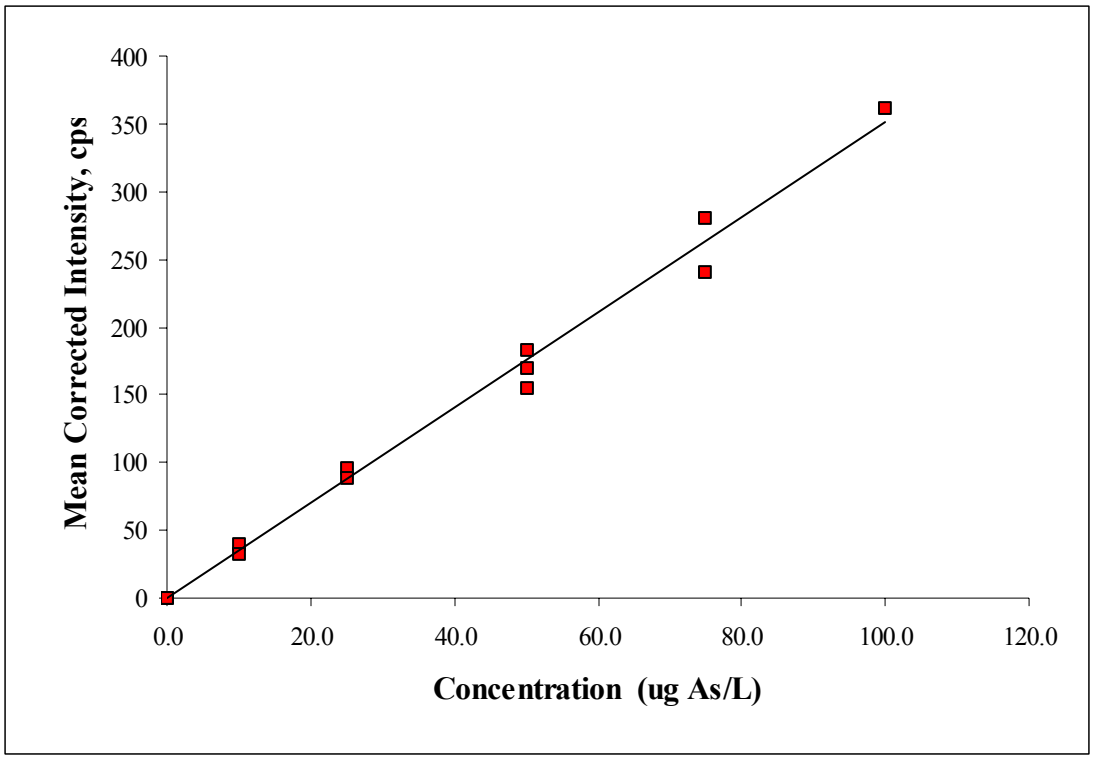
A separate set of sulfur calibration standards, each in 2:98 HNO_3: media dilution solvent, were prepared from the 10.0-mg S/mL primary standard using the following dilution scheme:

Stock Concentration mg S/mL	Aliquot (µL)	Final Volume (mL)	Standard Concentration (mg S/L)
10.0	25.0	50.0	5.00
10.0	50.0	50.0	10.0
10.0	125	50.0	25.0
10.0	175	50.0	35.0
10.0	250	50.0	50.0

The 2:98 HNO_3: media dilution solvent, prepared concurrently with these calibration standards was also utilized as a calibration/reagent blank.

Appendix 10.4

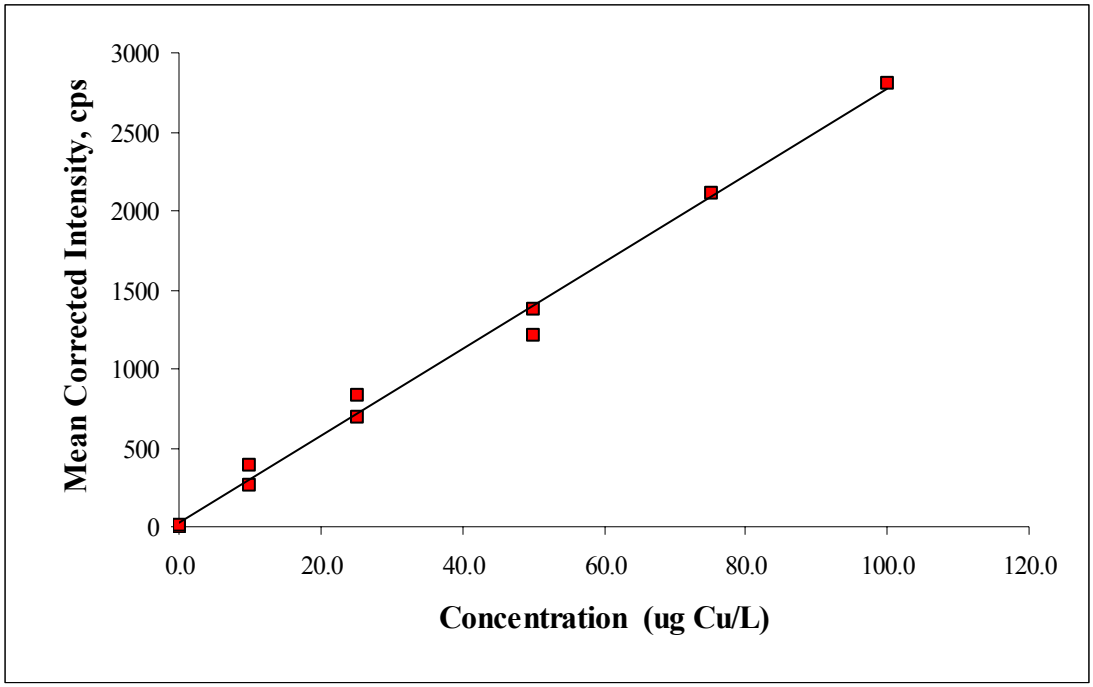
Calibration Curve for Arsenic Analyzed by ICP-AES



Slope = 3.5184; Y-intercept = -0.41106; R^2 = 0.98945

Appendix 10.5

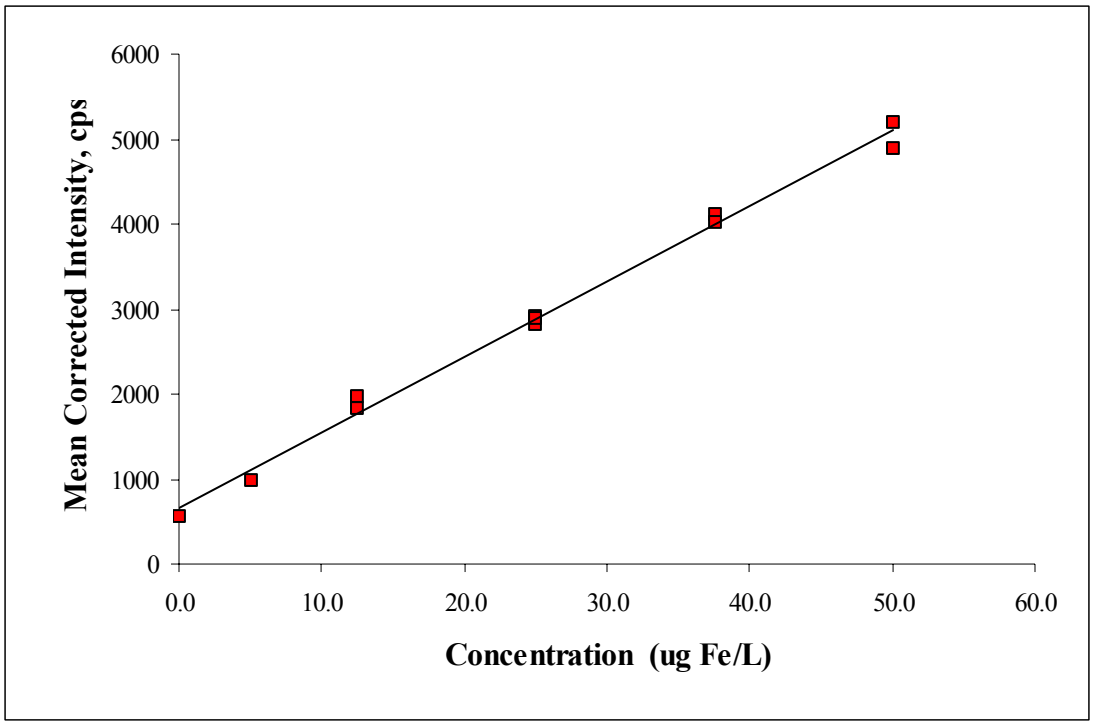
Calibration Curve for Copper Analyzed by ICP-AES



Slope = 27.460; Y-intercept = 24.417; R^2 = 0.9916

Appendix 10.6

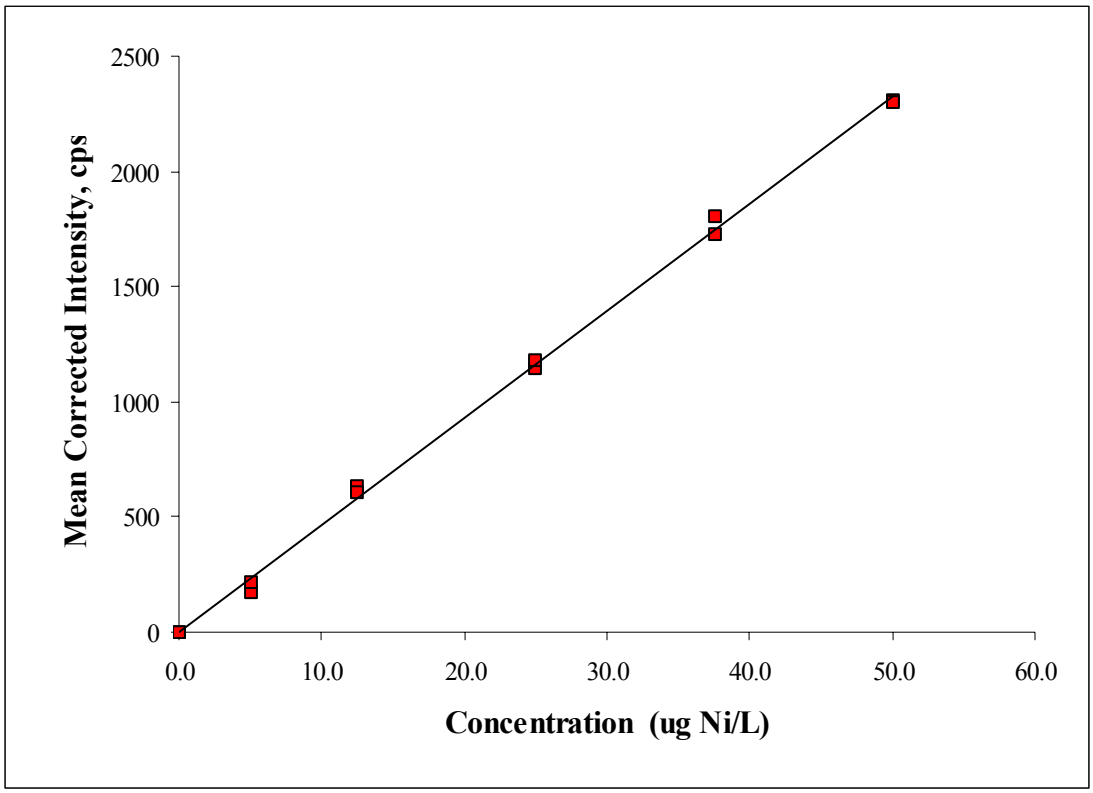
Calibration Curve for Iron Analyzed by ICP-AES



Slope = 88.909; Y-intercept = 664.867; R^2 = 0.9939

Appendix 10.7

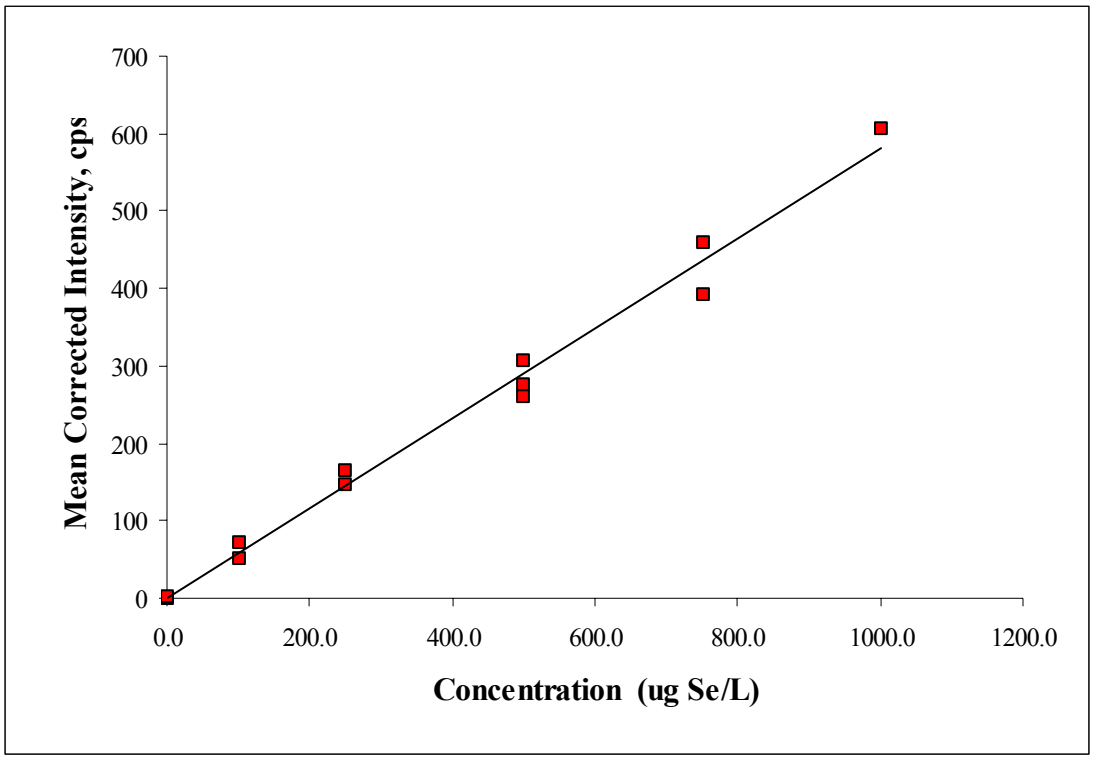
Calibration Curve for Nickel Analyzed by ICP-AES



Slope = 46.521; Y-intercept = -0.1733; R^2 = 0.9985

Appendix 10.8

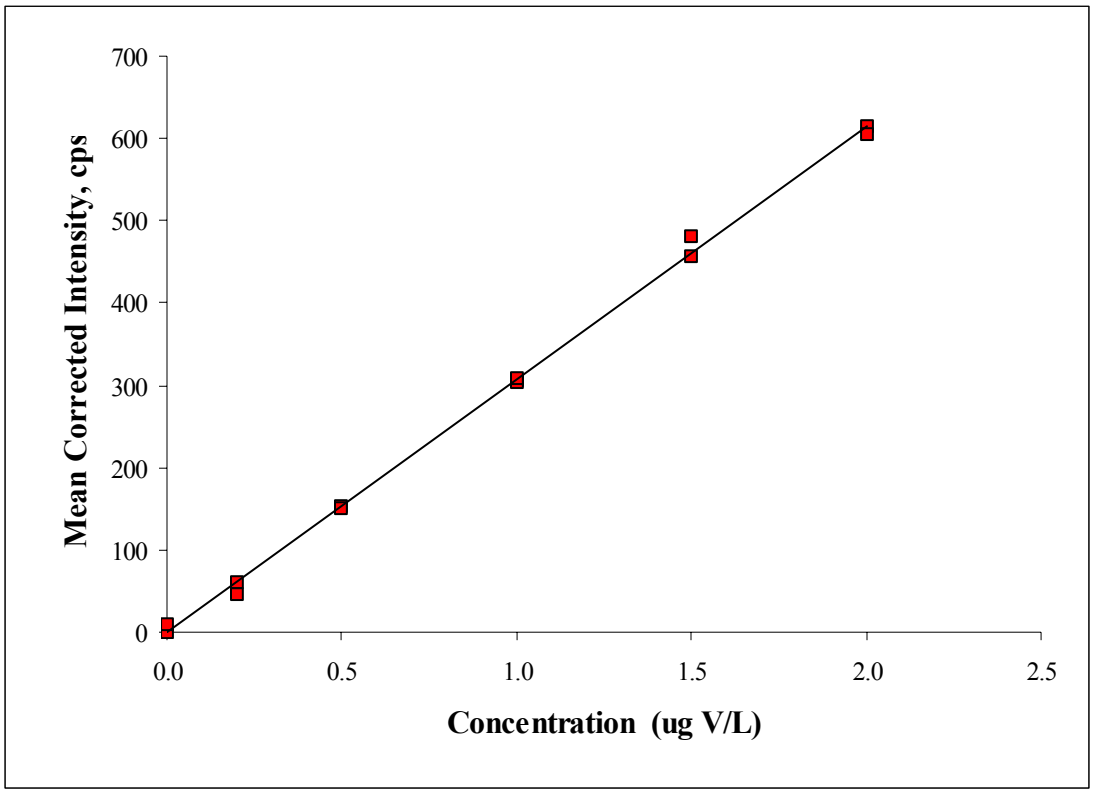
Calibration Curve for Selenium Analyzed by ICP-AES



Slope = 0.5804; Y-intercept = 0.75145; R^2 = 0.9876

Appendix 10.9

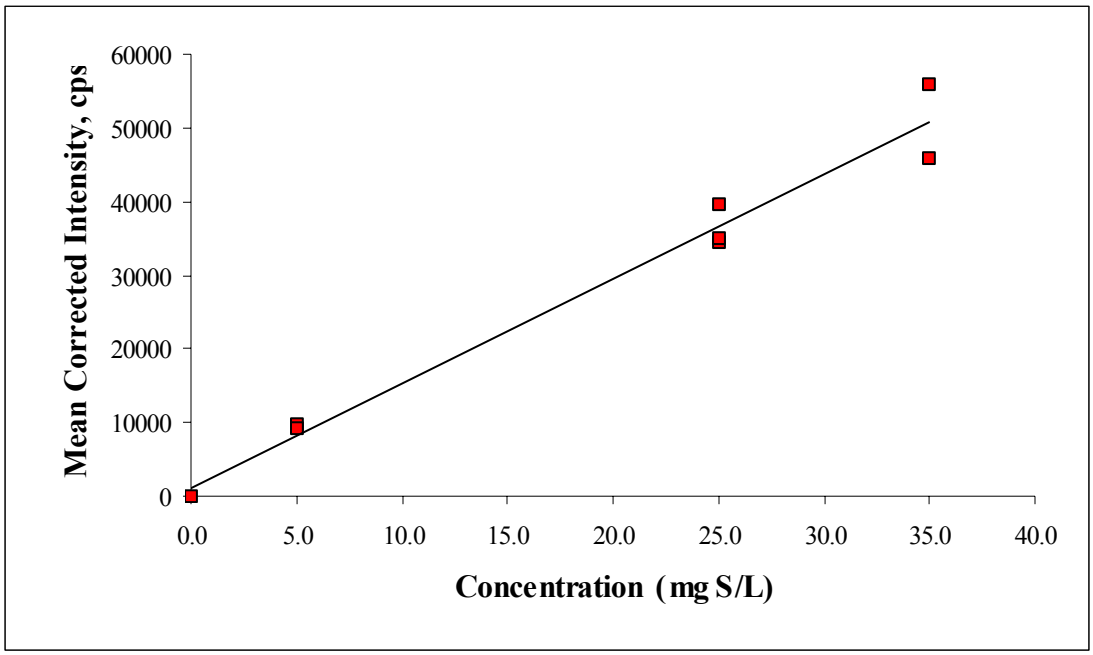
Calibration Curve for Vanadium Analyzed by ICP-AES



Slope = 307.93; Y-intercept = -0.8174; R^2 = 0.9985

Appendix 10.10

Calibration Curve for Sulfur Analyzed by ICP-AES



Slope = 1425.6; Y-intercept = 977.0; R^2 = 0.9789

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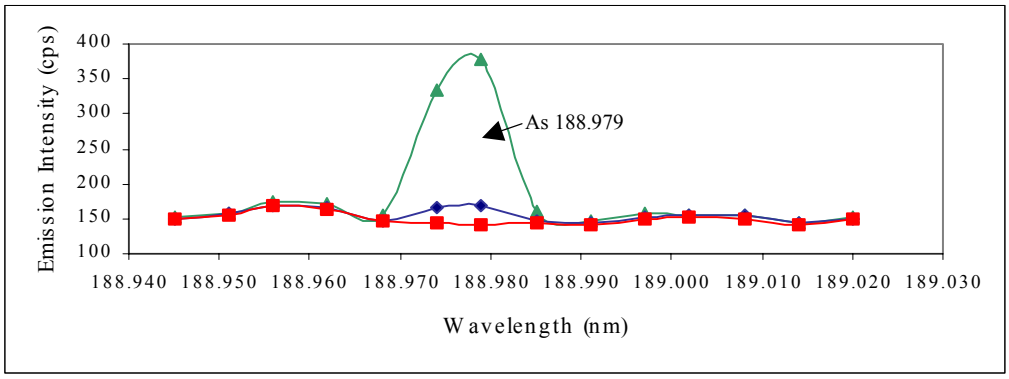
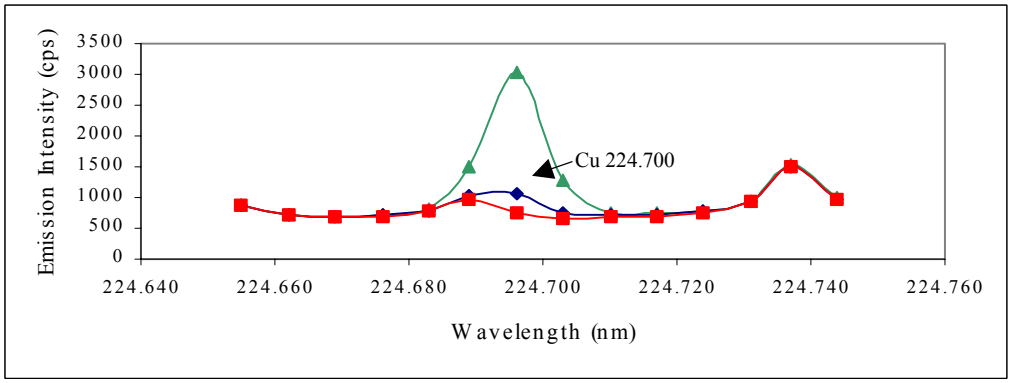
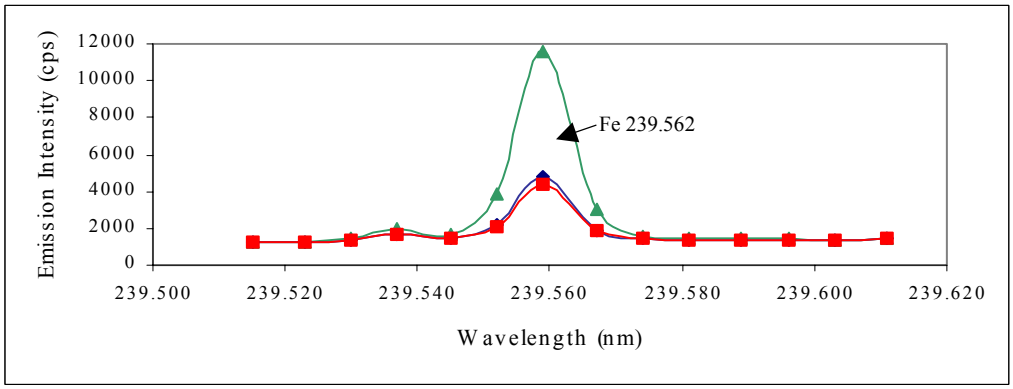
Appendix 10.11

Representative Emission Spectra for Arsenic (top), Copper (middle) and Iron (bottom) in Low- and High-Level Calibration Standards Prepared in Freshwater Algal Media and Analyzed by ICP-AES







Squares = freshwater algal media calibration blank (I.D.: 472A-114-M-0); Diamonds = low-level standard (I.D.: 472A-114-M-1); Triangles = high-level standard (I.D.: 472A-114-M-5). Nominal concentrations for As, Cu and Fe = 10.0, 10.0 and 5.00 µg/L and 100, 100 and 50.0 µg/L, in the low- and high-level standards, respectively.

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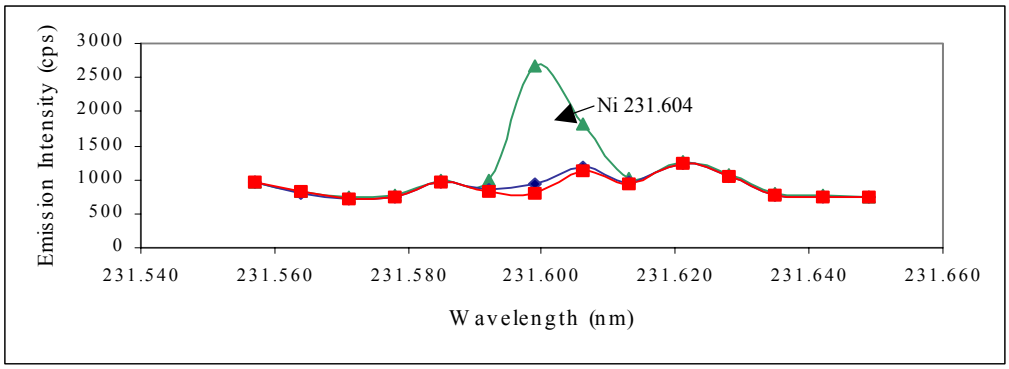
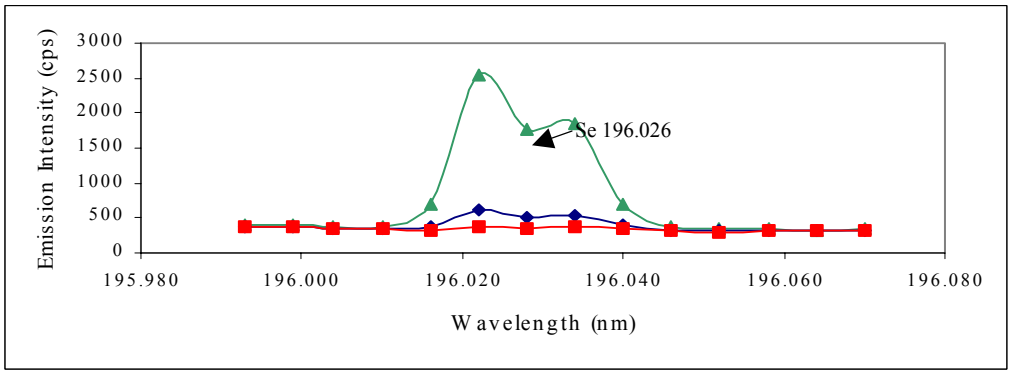
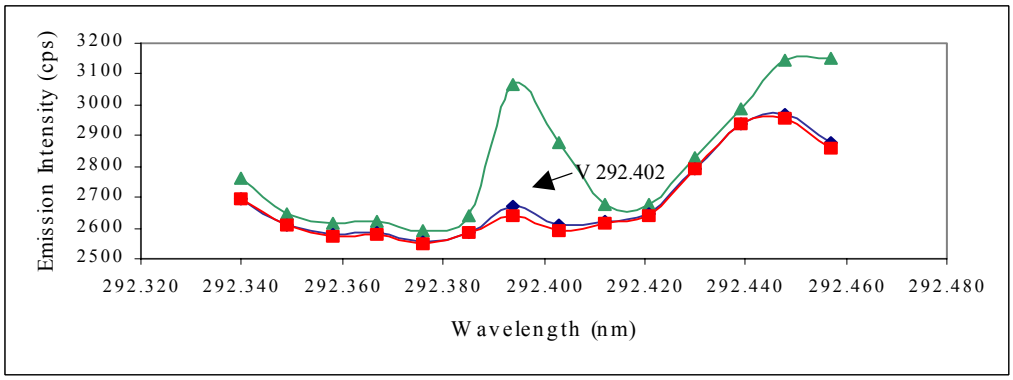
Appendix 10.12

Representative Emission Spectra for Nickel (top), Selenium (middle) and Vanadium (bottom) in Low- and High-Level Calibration Standards Prepared in Freshwater Algal Media and Analyzed by ICP-AES



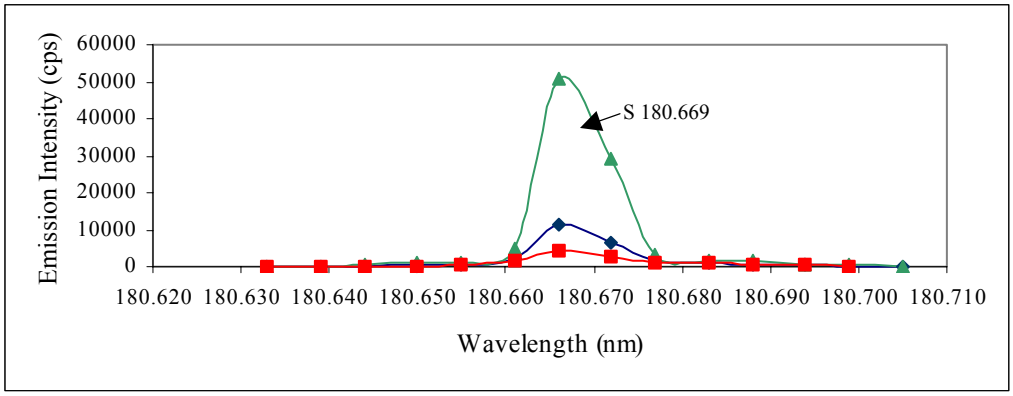




Squares = freshwater algal media calibration blank (I.D.: 472A-114-M-0); Diamonds = low-level standard (I.D.: 472A-114-M-1); Triangles = high-level standard (I.D.: 472A-114-M-5). Nominal concentrations for Ni, Se and V = 5.00, 100 and 0.200 µg/L and 50.0, 1000 and 2.00 µg/L, in the low- and high-level standards, respectively.

Appendix 10.13

Representative Emission Spectra for Sulfur in Low- and High-Level Calibration Standards Prepared in Freshwater Algal Media and Analyzed by ICP-AES



Squares = freshwater algal media calibration blank (I.D.: 472A-114-S-0); Diamonds = low-level standard (I.D.: 472A-114-S-1); Triangles = high-level standard (I.D.: 472A-114-S-5). Nominal concentrations for S = 5.00 and 50.0 mg/L in the low- and high-level standards, respectively.

Appendix 10.14

Example Calculations

The analytical result and percent recovery for sample number 472A-114-MAS-1 for vanadium, nominal concentration of 1.00 µg/L in freshwater algal m edia , were calculated using the following equations:

$$\text{Vanadium (µg/L) in sample} = \frac{\text{Mean Corrected Intensity - (Y-intercept)}}{\text{Slope}} \text{ X Dilution factor}$$

Mean Corrected Intensity = 340.9
Y-intercept = -0.8174
Slope = 307.93
Dilution Factor = 1.02

$$\text{Concentration of Vanadium (µg/L) in sample} = \frac{340.9 - (-0.8174)}{307.93} \text{ X } 1.02$$

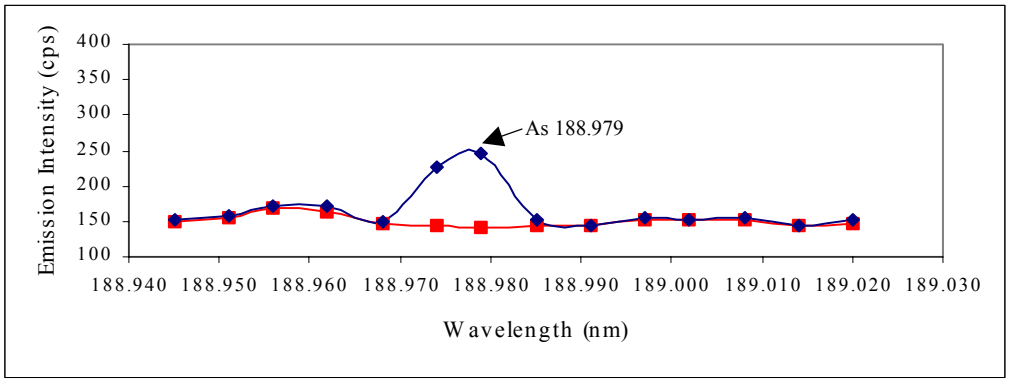
Concentration of Vanadium in sample (µg/L) = 1.13

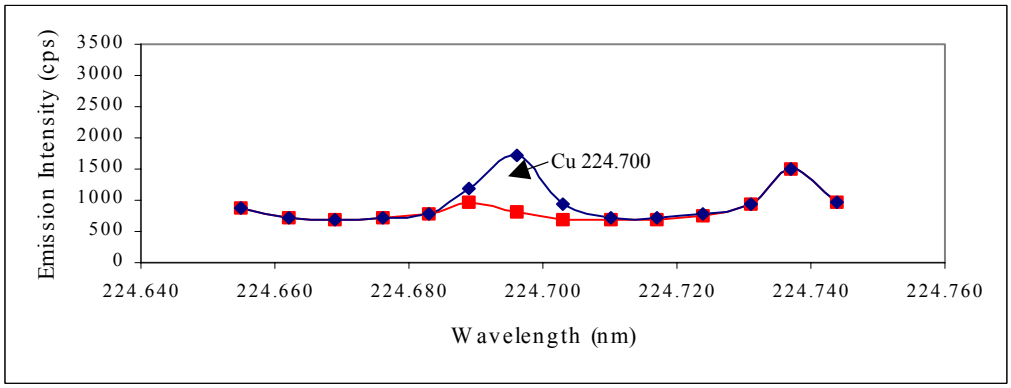
$$\text{Percent of nominal concentration} = \frac{1.13 \text{ (µg/L)}}{1.00 \text{ (µg/L)}} \text{ X } 100$$

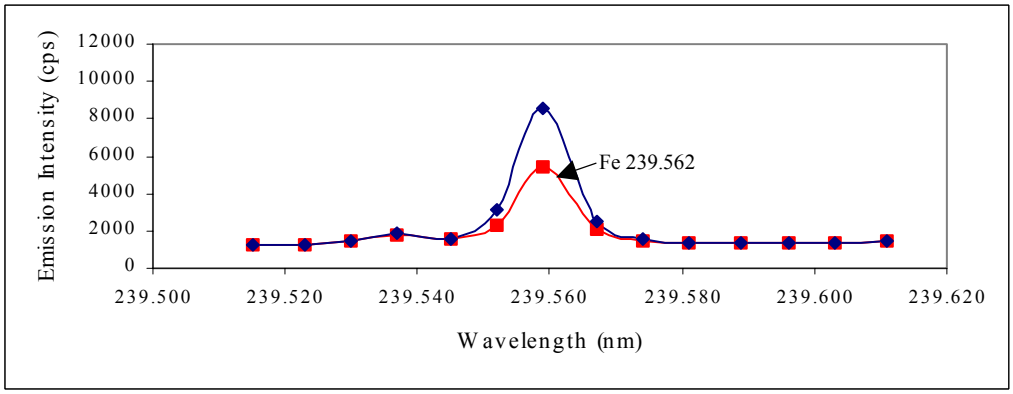
Percent of nominal concentration = 113%

Appendix 10.15

Emission Spectra for Arsenic (top), Copper (middle) and Iron (bottom)
in Matrix Blank and Matrix Fortification Samples Prepared in Freshwater Algal Media
and Analyzed by ICP-AES



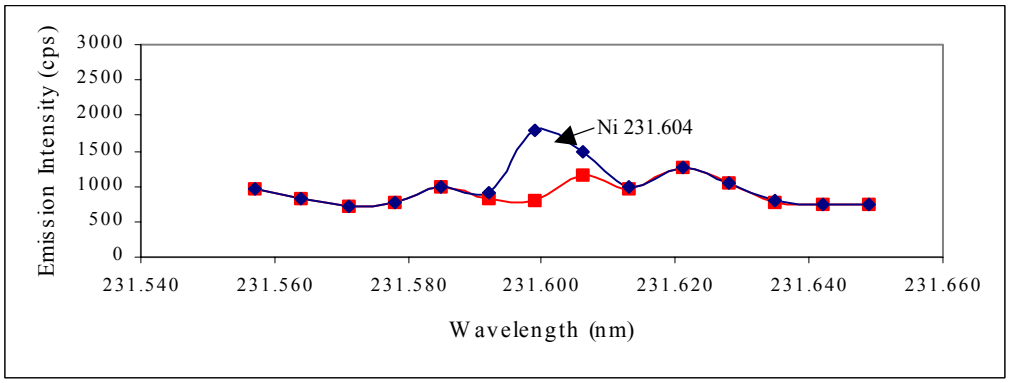


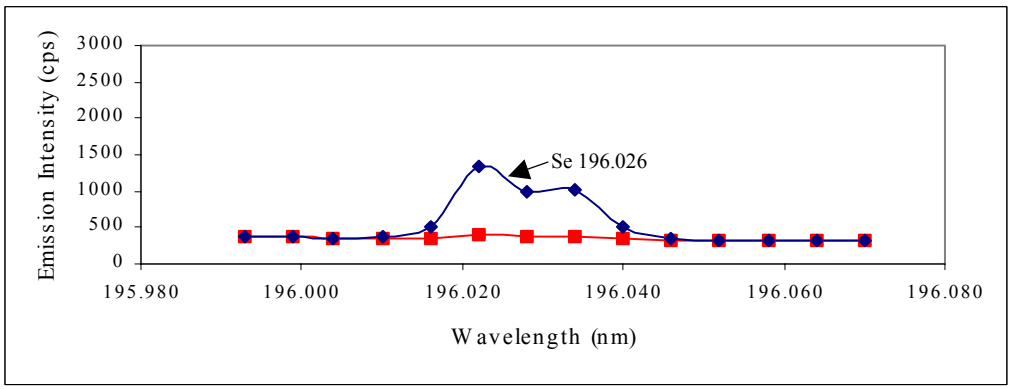


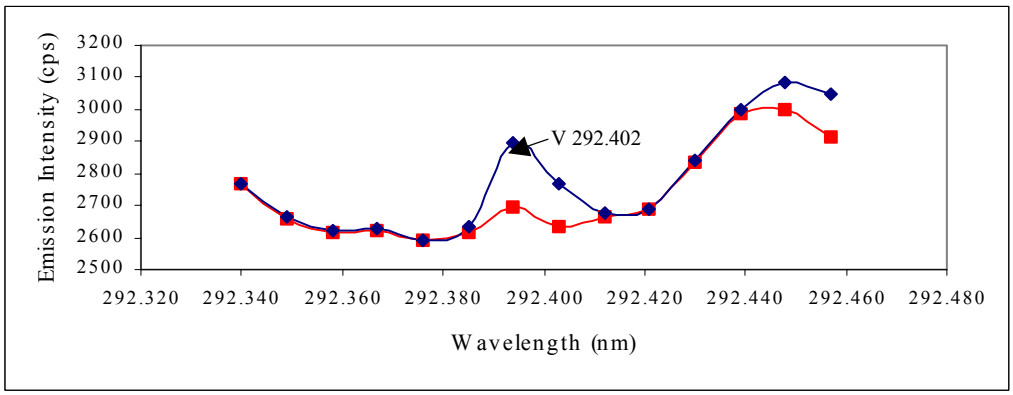
Squares = freshwater algal media matrix blank (I.D.: 472A-114-M AB-1, D_f = 1.02x); Diamonds = matrix fortification (I.D.: 472A-114-M AS-1, D_f = 1.02x). Nominal concentrations for As, Cu and Fe in matrix fortification sample = 50.0, 50.0 and 25.0 µg/L, respectively.

Appendix 10.16

Emission Spectra for Nickel (top), Selenium (middle) and Vanadium (bottom)
in Matrix Blank and Matrix Fortification Samples Prepared in Freshwater Algal Media
and Analyzed by ICP-AES



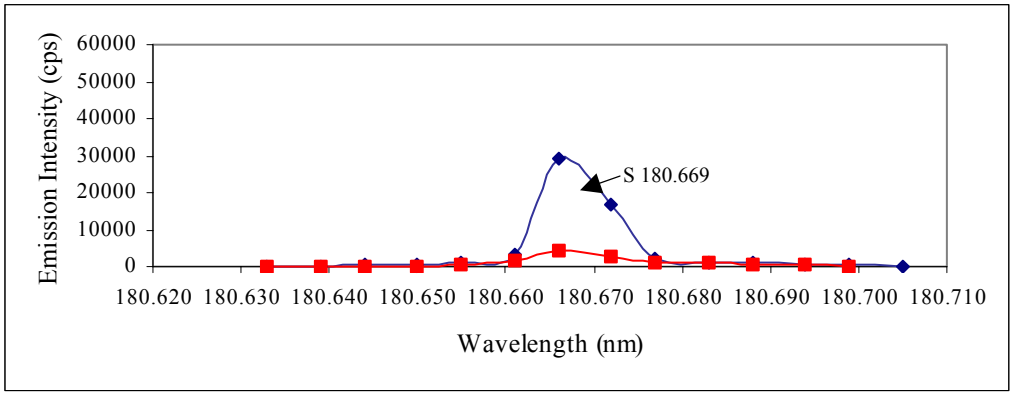




Squares = freshwater algal media matrix blank (I.D.: 472A-114-M AB-1, D_f = 1.02x); Diamonds = matrix fortification (I.D.: 472A-114-M AS-1, D_f = 1.02x). Nominal concentrations for Ni, Se and V in matrix fortification sample = 25.0, 500 and 1.00 µg/L, respectively.

Appendix 10.17

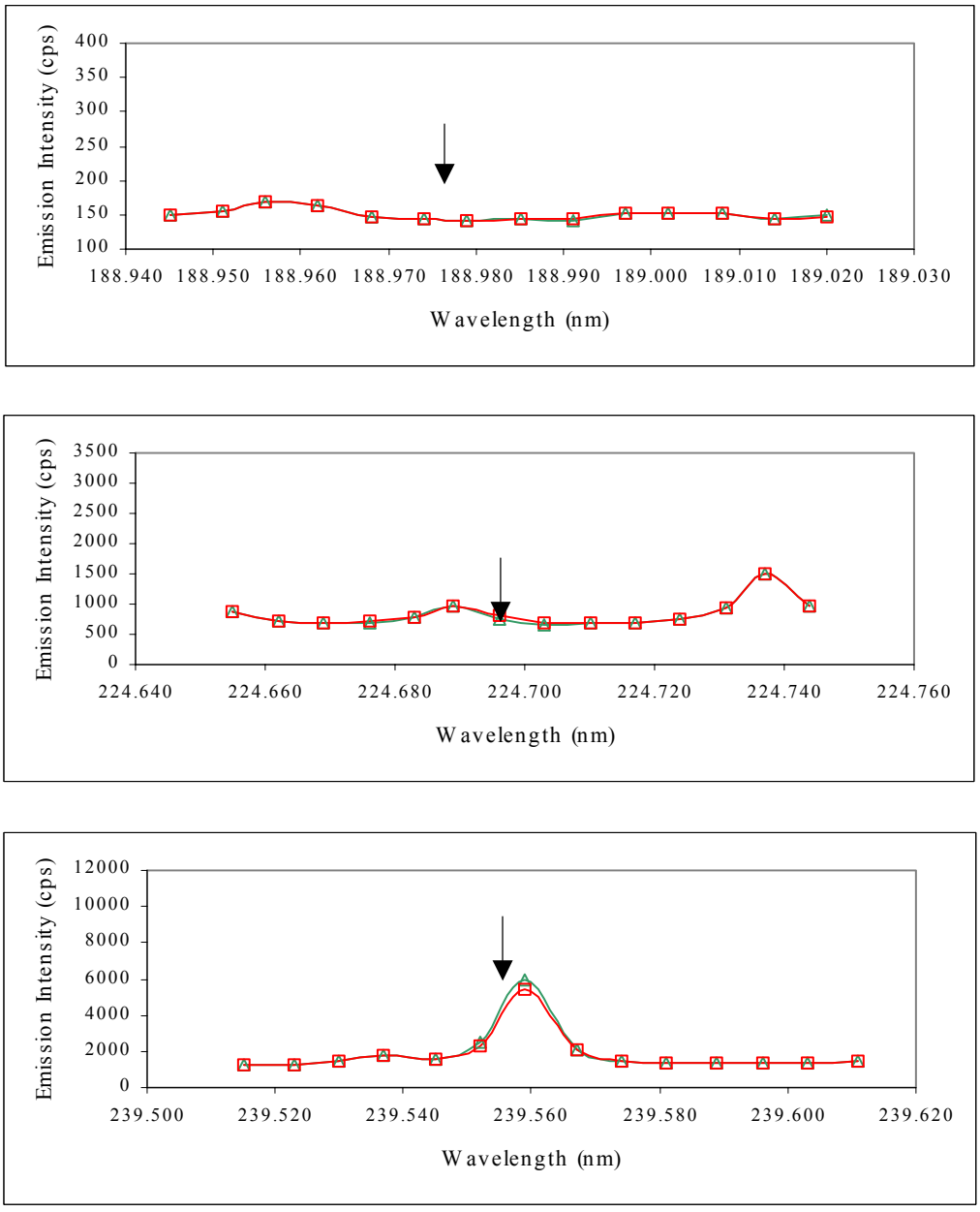
Emission Spectra for Sulfur in Matrix Blank and Matrix Fortification Samples
Prepared in Freshwater Algal Media and Analyzed by ICP-AES



Squares = freshwater algal media matrix blank (I.D.: 472A-114-MAB-1, D_f = 1.02x); Diamonds = matrix fortification (I.D.: 472A-114-MAS-1, D_f = 1.02x). Nominal concentrations for S in matrix fortification sample = 20.0 mg/L.

Appendix 10.18

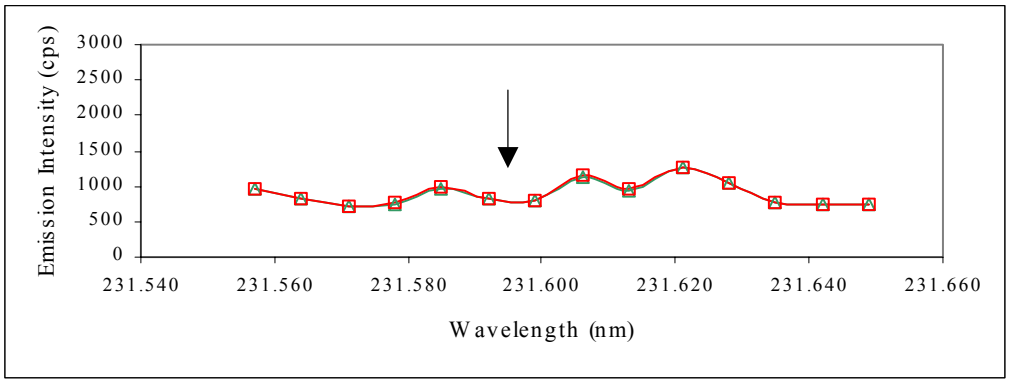
Representative Emission Spectra of Arsenic (top), Copper (middle) and Iron (bottom) in a Test Sample Analyzed by ICP-AES

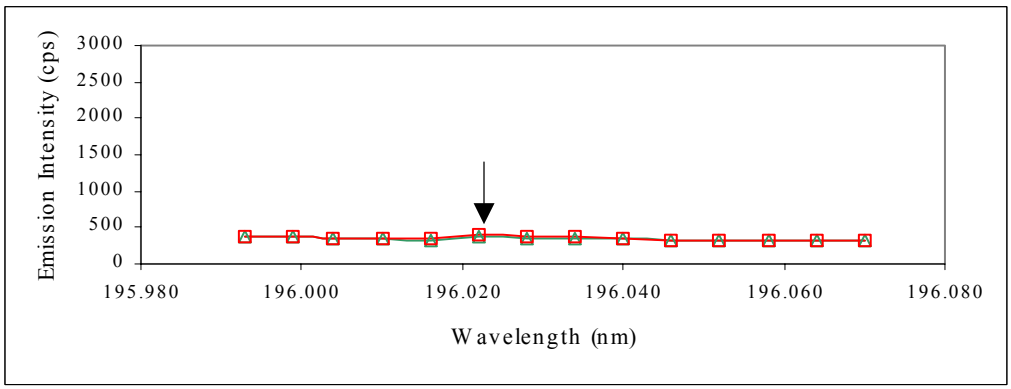


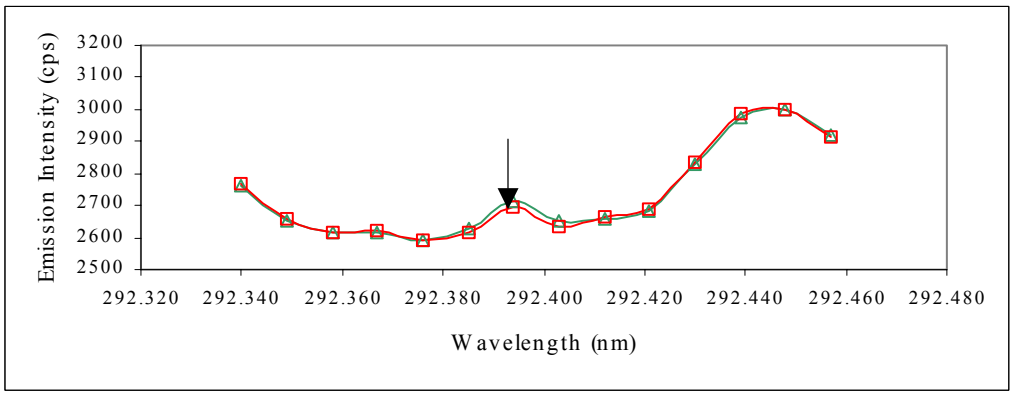
Squares = freshwater algal media calibration blank (I.D.: 472A-114-M-0); Triangles = 0 hour WAF test sample (472A-114-2, 1000 mg/L petroleum coke nominal concentration). The arrows indicate expected wavelength for each element response.

Appendix 10.19

Representative Emission Spectra of Nickel (top), Selenium (middle) and Vanadium (bottom) in a Test Sample Analyzed by ICP-AES



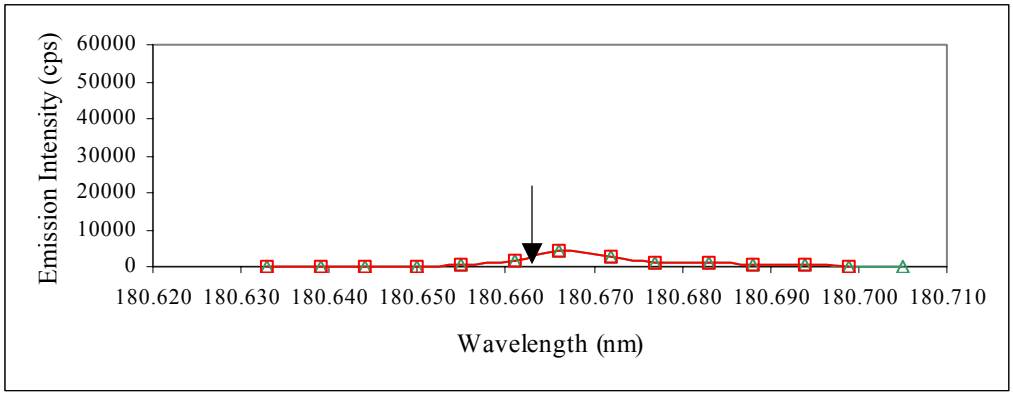




Squares = freshwater algal media calibration blank (I.D.: 472A-114-M-0); Triangles = 0 hour WAF test sample (472A-114-2, 1000 mg/L petroleum coke nominal concentration). The arrows indicate expected wavelength for each element response.

Appendix 10.20

Representative Emission Spectra of Sulfur in a Test Sample Analyzed by ICP-AES



Squares = freshwater algal media matrix blank (I.D.: 472A-114-MAB-1, D_f = 1.02x); Triangles = 0 hour WAF test sample (472A-114-2, 1000 mg/L petroleum coke nominal concentration). The arrow indicates the expected wavelength for sulfur response above background levels.

Appendix 11

Cell Density By Replicate Over the 96-Hour Exposure Period

Nominal WAF Concentration (mg/L)	Rep.	Cell Density (cells/mL) 24 Hours [1]	Rep.	48 Hours	Rep.	72 Hours	Rep.	96 Hours
Negative Control	A	29,000	G	73,000	M	209,000	S	490,000
	B	27,000	H	49,000	N	163,000	T	465,000
	C	28,000	I	59,000	O	133,000	U	250,000
	D	17,000	J	90,000	P	184,000	V	400,000
	E	25,000	K	71,000	G	180,000	W	385,000
	F	18,000	L	69,000	R	217,000	X	490,000
1000	A	19,000	G	35,000	M	117,000	S	420,000
	B	28,000	H	48,000	N	135,000	T	305,000
	C	21,000	I	73,000	O	73,000	U	300,000
	D	19,000	J	74,000	P	117,000	V	215,000
	E	18,000	K	64,000	G	144,000	W	285,000
	F	19,000	L	59,000	R	130,000	X	285,000

[1] The initial cell density of the stock culture was determined and an inoculum volume was administered to each test chamber to yield a cell density of approximately 5,000 cells/mL at test initiation (0 hours).

Appendix 12

Area Under the Growth Curve (Biomass) By Replicate Over the 96-Hour Exposure Period

Nominal WAF Concentration (mg/L)	Cumulative Area Under the Growth Curve							
	Rep.	0 - 24 Hours	Rep.	0 - 48 Hours	Rep.	0 – 72 Hours	Rep.	0 – 96 Hours
Negative Control	A	288,000 G		1,392,000	M	4,656,000	S	12,924,000
B		264,000	H	1,056,000	N	3,480,000	T	10,896,000
C		276,000	I	1,200,000	O	3,384,000	U	7,860,000
D		144,000	J	1,308,000	P	4,476,000	V	11,364,000
E		240,000	K	1,272,000	G	4,164,000	W	10,824,000
F		156,000	L	1,080,000	R	4,392,000	X	12,756,000
1000	A	168,000 G		696,000	M	2,400,000	S	8,724,000
B		276,000	H	1,068,000	N	3,144,000	T	8,304,000
C		192,000	I	1,200,000	O	2,832,000	U	7,188,000
D		168,000	J	1,164,000	P	3,336,000	V	7,200,000
	E	156,000	K	1,020,000 G		3,396,000	W	8,424,000
F		168,000	L	984,000	R	3,132,000	X	7,992,000

Appendix 13

Growth Rate By Replicate Over the 96-Hour Exposure Period

Nominal WAF Concentration (mg/L)	Rep.	Growth Rate 0 - 24 Hours	Rep.	0 - 48 Hours	Rep.	0 – 72 Hours	Rep.	0 – 96 Hours
Negative Control	A	0.0732	G	0.0559	M	0.0518	S	0.0478
	B	0.0703	H	0.0476	N	0.0484	T	0.0472
C		0.0718	I	0.0514	O	0.0456	U	0.0408
D		0.0510	J	0.0602	P	0.0501	V	0.0456
E		0.0671	K	0.0553	G	0.0498	W	0.0452
	F	0.0534	L	0.0547	R	0.0524	X	0.0478
1000 A		0.0556	G	0.0405	M	0.0438	S	0.0462
	B	0.0718	H	0.0471	N	0.0458	T	0.0428
C		0.0598	I	0.0559	O	0.0372	U	0.0426
D		0.0556	J	0.0561	P	0.0438	V	0.0392
E		0.0534	K	0.0531	G	0.0467	W	0.0421
	F	0.0556	L	0.0514	R	0.0453	X	0.0421

Appendix 14

Personnel Involved in the Study

The following key Wildlife International, Ltd. personnel were involved in the conduct or management of this study:

1. [redacted]
2. [redacted]
3. [redacted]
3. [redacted]
4. [redacted]
5. [redacted]
6. [redacted]

Appendix 15

Report Amendment

1.	Original Report:	Title Page
	Amended Report:	The amended report date was added and the modified study director was changed. The total number of pages was changed from 159 to 173.
	Reason:	To indicate that the report was amended, study director change and note change in pagination.
2.	Original Report:	Page 2
	Amended Report:	The amended report date was added and new signatures and dates were added.
	Reason:	To show the amended report date and to provide new signatures and dates for the amended report.
3.	Original Report:	Page 3
	Amended Report:	The audit dates for the amended report were added and a new signature and date were added.
	Reason:	To show the amended report audit dates and to provide a new signature and date for the amended report.
4.	Original Report:	Page 4
	Amended Report:	New signatures and dates were added.
	Reason:	To provide new signatures and dates for the amended report.
5.	Original Report:	Page 8
	Amended Report:	The Table of Contents was updated to show the addition of Appendix 3. A protocol amendment was added to Appendix 2 to reflect the study director change so renumbering all appendices from Appendix 3 throughout the end of the report was needed and to add the Report Amendment appendix (Appendix 15).
	Reason:	The Sponsor requested that Appendix 3 be added to the final report and the protocol amendment was required to change the study director from Debbie Desjardins to Anne B. Sindermann.
6.	Original Report:	Pages 15-19 and 22-23
	Amended Report:	The appendix reference numbers were modified.
	Reason:	The Sponsor requested that Appendix 3 be added to the final report, therefore, all appendices thereafter had to be renumbered.

Appendix 15
(continued)

Report Amendment

7.	Original Report:	Page 85
	Amended Report:	Protocol amendment no. 3 was added to the report to reflect the study director change.
	Reason:	The Sponsor requested that Appendix 3 be added to the final report. In order to amend the final report the study director had to be modified, therefore a protocol amendment was required.
8.	Original Report:	Pages 88-159
	Amended Report:	Appendix 3 was added to the report.
	Reason:	The Sponsor requested that Appendix 3 be added to the final report.
8.	Original Report:	Page 159
	Amended Report:	Anne B. Sindermann, M.S. was added to Appendix 14.
	Reason:	The Sponsor requested that Appendix 3 be added to the final report. In order to amend the final report the study director had to be changed.

AMENDMENT SIGNATURES:

[redacted]

4/10/07
Date

10 April 07
Date

4/20/2007
Date

4/10/2007
Date