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89940000240

Dear Sir:

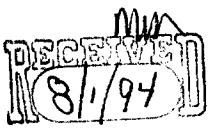
SUBJECT: ACUTE TOXICITY OF DAN 216 TO THE FATHEAD MINNOW AND DAPHNIA MAGNA

The subject TSCA 8(e) submission was filed by Shell Oil Company on November 15, 1993 (8EHQ-1193-12772) and preliminary data transmissions were provided. The complete reports (attached) are now available and are provided as supplemental information to the original TSCA 8(e).

This supplemental report is filed to provide information EPA may find useful. In no way is it intended as a waiver of any rights or privileges belonging to Shell Oil Company as the reporting corporation, its agents or employees. The reporting corporation, its agents and employees, reserve the right to object to this report's use or admissibility in any subsequent judicial or administrative proceeding against the corporation, its agents or employees.

This report has been compiled based on information available as of the date of filing. The corporation, its agents and employees reserve the right to supplement the data contained in this report, and to revise and amend any conclusions drawn therefrom.

This report contains no confidential business information.



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The following person should be contacted if you have questions or a need for discussion.

J. C. Willett
Manager, Product Safety and Compliance
Shell Oil Company
P.O. Box 4320
Houston, TX 77210
Telephone No. 713-241-6958
Fax. 713-241-3325

Very truly yours,

R. N. Shulman, General Manager
Health, Safety, and Environment
Shell Oil Company

THG/sjh

Attachments

DAN 216:
A 48-HOUR STATIC-RENEWAL ACUTE TOXICITY TEST WITH THE CLADOCERAN (*Daphnia magna*)

FINAL REPORT

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 109A-107

TITLE 40 OF THE CODE OF FEDERAL REGULATIONS
PART 797.1300

AUTHORS:

Kurt R. Drottar
James P. Swigert, Ph.D.

STUDY INITIATION DATE: December 15, 1993

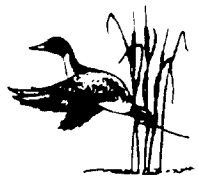
STUDY COMPLETION DATE: April 8, 1994

Submitted to

Shell Development Company
Westhollow Research Center
3333 Highway Six South
Houston, Texas 77082

WILDLIFE INTERNATIONAL LTD.

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



GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Shell Development Company

TITLE: DAN 216: A 48-Hour Static-Renewal Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 109A-107

STUDY COMPLETION DATE: April 8, 1994

This study was conducted to conform with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Part 792, 17 August 1989.

STUDY DIRECTOR:

Kurt R. Drottar
Senior Aquatic Biologist

DATE: 4/8/94

REPORT APPROVED BY:

James P. Swigert, Ph.D.
Manager, Aquatic Toxicology Laboratory

DATE: 4/8/94

Sponsor

DATE:

Applicant/Submitter

DATE:

QUALITY ASSURANCE STATEMENT

This study was examined for conformance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Part 792, 17 August 1989. The dates of all inspection and audits and the dates that any findings were reported to the Study Director/Laboratory Management were as follows:

ACTIVITY	DATE CONDUCTED	DATE REPORTED TO STUDY DIRECTOR/MANAGEMENT:
Protocol	January 3, 1994	January 3, 1994
Aquatic Toxicology Laboratory		
Procedure (Test Substance Preparation)	January 5, 1994	January 5, 1994
Procedures (Test Initiation)	January 5, 1994	January 6, 1994
Data and Draft Report	February 7, 1994	February 8, 1994
Second Draft Report	March 29, 1994	March 29, 1994
Final Report	April 8, 1994	April 8, 1994

Lisa Read DATE 4/12/94

Lisa Read
Quality Assurance Representative

TABLE OF CONTENTS

Title/Cover Page..... 1
Good Laboratory Practice Compliance Statement..... 2
Quality Assurance Statement..... 3
Table of Contents..... 4
Summary..... 6
Introduction..... 7
Objective..... 7
Experimental Design..... 7
Materials and Methods..... 8
Results and Discussion..... 12
Conclusion..... 13
References..... 14

TABLES

Table 1. Summary of Analytical Chemistry Data..... 15
Table 2. Temperature of Water in the Test Chambers..... 16
Table 3. Dissolved Oxygen of Water in the Test Chambers..... 17
Table 4. pH of Water in the Test Chambers..... 18
Table 5. Cumulative Mortality/Immobility and Treatment-Related Effects..... 19
Table 6. EC50 Values..... 21

TABLE OF CONTENTS

PAGE 2

APPENDICES

Appendix	I	Conductivity, Hardness, Alkalinity and pH of Well Water Measured During the 4-Week Period Immediately Preceding the Test.....	22
Appendix	II	Pesticides, Organics, Metals and other Inorganics Analyzed in Wildlife International Ltd. Well Water.....	23
Appendix	III	Measurement of DAN 216 in Dilute Aqueous Samples in Support of Acute Aquatic Toxicity Testing with the Cladoceran (*Daphnia magna*).....	25
Appendix	IV	EC50 Calculations.....	45
Appendix	V	Changes in Protocol.....	48
Appendix	VI	Personnel Involved in the Study.....	49

SUMMARY

SPONSOR:	Shell Development Company
CONTACT:	Ms. Diana C. L. Wong
LOCATION OF STUDY, RAW DATA AND FINAL REPORT:	Wildlife International Ltd. Easton, Maryland 21601

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER:	109A-107
TEST SUBSTANCE:	DAN 216
STUDY:	DAN 216: A 48-Hour Static-Renewal Acute Toxicity Test with the Cladoceran (*Daphnia magna*)
NOMINAL TEST CONCENTRATIONS:	Negative Control 0.39, 0.65, 1.1, 1.8 and 3.0 mg DAN 216/L
MEAN MEASURED TEST CONCENTRATIONS:	<LOD, 0.33, 0.53, 0.93, 1.5 and 1.7 mg DAN 216/L
TEST DATES:	Experimental Start - January 5, 1994 Biological Termination - January 7, 1994 Experimental Termination - March 10, 1994
LENGTH OF TEST:	48 Hours

48-HOUR EC50:	1.2 mg DAN 216/L
95% CONFIDENCE LIMITS:	1.1 and 1.4 mg DAN 216/L
NO MORTALITY/IMMOBILITY CONCENTRATION:	0.53 mg DAN 216/L

TEST ORGANISM:	Neonate Cladocerans (*Daphnia magna*)
SOURCE OF TEST ORGANISMS:	Wildlife International Ltd. 8651 Brooks Drive, Easton, MD 21601
AGE OF TEST ORGANISMS:	<24 hours at test initiation

INTRODUCTION

This study was conducted by Wildlife International Ltd. for Shell Development Co., at the Wildlife International Ltd. aquatic toxicology facility in Easton, Maryland. The test was conducted from January 5, 1994 to January 7, 1994. Raw data generated by Wildlife International Ltd. and an authenticated copy of the final report are filed under Project Number 109A-107 in archives located on the Wildlife International Ltd. site.

OBJECTIVE

The objective of this study was to evaluate the acute toxicity of DAN 216 to the Cladoceran (*Daphnia magna*) during a 48-hour exposure period under static-renewal test conditions.

EXPERIMENTAL DESIGN

Daphnia magna were exposed to a geometric series of five test concentrations and a negative (well water) control under static-renewal test conditions for 48 hours. Four replicate test chambers were maintained in each treatment and control group, with 5 daphnids in each test chamber. Nominal test concentrations were selected in consultation with the Sponsor, and were based upon results of a range finding test. Nominal test concentrations selected were 0.39, 0.65, 1.1, 1.8, and 3.0 mg DAN 216/L.

Immediately prior to initiating the test, daphnids were impartially removed from the culture beakers in groups of one or two and distributed to 25-mL plastic cups until each contained 5 daphnids. The daphnids were then transferred into the test chambers. At approximately 24 hours, new test solutions were prepared and daphnids were transferred to the new solutions. Observations of mortality, immobility and other clinical signs of toxicity were made at approximately 8, 24, and 48 hours after test

initiation. Cumulative percent mortality and immobility values in each treatment group were used to calculate an EC50 value and 95% confidence limits at 48 hours. The no mortality/no immobilization concentration was determined by visually examining the mortality and immobilization data.

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, DAN 216: A 48-Hour Static-Renewal Acute Toxicity Test with the Cladoceran (*Daphnia magna*). The protocol was based on procedures outlined in TSCA Title 40 of the Code of Federal Regulations, Part 797.1300 (1); and ASTM Standard E-729-88, *Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (2).

Test Substance

The test substance was received from Shell Development Company on September 28, 1993. The test substance was a white powder identified as: Shell Development Company; WRC TOX SAMPLE NO. 1232, PSIS 1232-2, STORAGE CONDITIONS Ambient; COMPOSITION DAN 216 Secondary alcohol sulfate, sodium salt (100g); HAZARDS low toxicity, irritant, DATE DISPENSED 24 Sept. 93; RESPONSIBLE INDIVIDUAL H.C. Smith/EC-644; EXPIR. DATE Sept. 1994. The test substance was assigned Wildlife International Ltd. Identification Number WIL-2737 upon receipt. Information provided by the Sponsor indicated a purity of 98%.

Preparation of Test Concentrations

All glassware used to make the stock solutions was serially rinsed with reverse osmosis (RO) water, 70% isopropanol/30% water, and RO water prior to use. The primary stock was prepared by adding 0.1009 g of test substance to a 200-mL volumetric flask. The volumetric flask was then brought to volume with RO water, heated to approximately 60°C and inverted to aid in solubilization of the test substance. A new primary stock solution was freshly prepared on Day 1 of the test for renewal of test

solutions. The test solutions were prepared by adding the appropriate amount of primary stock and dilution water to a 2-L beaker and stirring with a glass rod for approximately 1 minute. The test solutions were then distributed to the test chambers.

Test Organism

The cladoceran, (*Daphnia magna*) was selected as the test species for this study. Daphnids are representative of an important group of aquatic invertebrates and were selected for use in the test based upon past history of use and ease of culturing in the laboratory. Daphnid neonates used in the test were less than 24-hours old and were obtained from cultures maintained by Wildlife International Ltd., Easton, Maryland.

The daphnids were cultured and held in water from the same source and at approximately the same temperature as used during the test. The adult daphnids were held for 22 days prior to collection of the juveniles for testing. The adults showed no signs of disease or stress during the holding period. Neonate daphnids were obtained for testing from individual adult daphnids which were observed to have no neonates present less than 24 hours prior to test initiation. The progeny from five adults were used in the test. At test initiation, the juvenile daphnids were carefully collected from the cultures using wide-bore disposable pipettes, and transferred to 25-mL plastic cups. The daphnids were then transferred from the cups to the test chambers using wide-bore pipettes and released beneath the air/water interface. Daphnids in the cultures were fed a mixture of yeast, Cerophyll®, and trout chow, as well as a suspension of the freshwater green alga, *Selenastrum capricornutum*. The adults were fed prior to test initiation, but neonates were not fed during the test.

Test Apparatus

The test chambers were 250-mL beakers containing 200 mL of test solution. The test chambers were rinsed with RO water, 70% isopropanol/30% water, and RO water prior to use. The test chambers were placed

in a temperature-controlled water bath designed to maintain a temperature of 20±1°C and loosely covered with plastic wrap. The test chambers were labeled with the project number, test concentration, and replicate.

Dilution Water

The water used for culture, holding and testing was freshwater obtained from a well 45 meters deep located on the Wildlife International Ltd. site. The well water is characterized as medium-hard water. The conductivity, hardness, alkalinity, and pH of the well water during the four-week period immediately preceding the test are presented in Appendix I.

The well water was passed through a sand filter to remove particles greater than approximately 25 μm, and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to delivery to the diluter system, the water again was filtered to remove microorganisms and particles. The results of analyses performed to measure the concentrations of pesticides and metals in well water used by Wildlife International Ltd. are presented in Appendix II. Well water used in culture of *Daphnia magna* was supplemented with selenium (Dilution water used in the test was not supplemented with selenium).

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during holding, and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (e.g., Chroma 50). A photoperiod of 16 hours of light and 8 hours of darkness was controlled with an automatic timer. A 30-minute transition period of low light intensity was provided when lights went on and off to avoid sudden changes in lighting. Light intensity during the test was approximately 807 lux at the surface of the water.

Temperature was measured in both the old and new test solutions of all replicate test chambers at approximately 24-hour intervals using a calibrated hand-held thermometer. Temperature also was measured continuously in one negative control replicate using a Fulscope ER/C Recorder. The target test temperature during the study was 20±1°C. The pH and dissolved oxygen content of the water in both the old and new test solutions of all replicate test chambers were measured at approximately 24-hour intervals. Hardness, alkalinity, total organic carbon (TOC), particulate matter and conductivity were also measured in the dilution water.

Measurements of pH were made using a Fisher Accument Model 915 pH meter, and dissolved oxygen was measured using a Yellow Springs Instrument Model 51B dissolved oxygen meter. Total organic carbon was measured with a Shimadzu Model 5000 TOC analyzer. Conductivity was measured using a Yellow Springs Instruments Model 33 Salinity-Conductivity-Temperature meter. Hardness, particulate matter and alkalinity measurements were made in accordance with *Standard Methods for the Examination of Water and Wastewater* (3).

Observations

All organisms were observed at appoximately 8, 24 and 48 hours to evaluate the number of mortalities and the number of individuals exhibiting clinical signs of toxicity and abnormal behavior. Dead organisms were removed from the test chambers during observations.

Statistical Analyses

When the dose response pattern allows for the calculation of an EC50 value, the data are analyzed using the computer program of C. E. Stephan (4). The program was designed to calculate the EC50 value and the 95% confidence interval by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (5, 6, 7). In this study, the moving average method was used to evaluate mortality at 48

hours. The no mortality/no immobility concentration was determined by inspection of the mortality and immobility data.

Analytical Chemistry

Water samples were collected in each treatment and control: 1) at test initiation; 2) on Day 1 after approximately 24 hours of exposure; 3) on Day 1 in new solutions; and 4) at test termination. Water samples were collected from all replicates of each treatment and control group and composited for analysis. Samples were collected in glass containers with Teflon®-lined caps. Sample containers were rinsed with RO water, 70% isopropanol/30% water, and RO water prior to use. Samples of each primary stock were also collected. All samples were preserved with 1 percent formalin, pH adjusted to approximately 8.0 using NaOH and shipped to Shell Development Company, Houston, TX for analysis (Appendix III).

RESULTS AND DISCUSSION

Measurements and Test Concentrations

Results of analyses to measure concentrations of DAN 216 in water samples collected during the test are presented in Table 1 and in the analytical chemistry report (Appendix III). Nominal concentrations selected for use in this study were 0.39, 0.65, 1.1, 1.8 and 3.0 mg DAN 216/L. Samples collected on Day 0 had measured values that ranged from 60 to 89% of nominal values. After approximately 24 hours of exposure (old solutions), measured values ranged from 50 to 105% of nominal. One of the water samples sent to Shell Development Company for analysis was broken upon receipt. Consequently, this sample was not included in the calculation of the mean measured concentrations. When intact samples collected at test initiation, on Day 1 (old and new) and at test termination were averaged, the mean measured concentrations for the study were 0.33, 0.53, 0.93, 1.5 and 1.7 mg DAN 216/L. Mean measured concentrations were used in the calculations of EC50 values. Results of analyses

to measure concentrations of DAN 216 in primary stock solutions are presented in Appendix III.

Observations and Measurements

Measurements of temperature, dissolved oxygen, and pH are presented in Tables 2, 3 and 4, respectively. Temperatures were within the limits of the range established for the test, or 20±1°C. Dissolved oxygen concentrations exceeded 60% of saturation throughout the test. Measurements of pH during the test ranged from 8.3 to 8.6.

Daily observations of mortality, immobility and other signs of toxicity used to evaluate the effects of the test substance on *Daphnia magna* are shown in Table 5. Test organisms in the control, 0.33 and 0.53 mg DAN 216/L treatment groups appeared normal and healthy throughout the test. In the 0.93 mg DAN 216/L treatment group, two organisms were immobile and two organisms were lethargic at test termination. In the 1.5 and 1.7 mg DAN 216/L treatment groups, mortality/immobility was 45 and 100% at test termination, respectively. EC50 values and 95% confidence limits at 24 and 48 hours were calculated from the mortality/immobility data (Table 6 and Appendix IV).

CONCLUSION

The 48-hour EC50 value for *Daphnia magna* exposed to DAN 216 was 1.2 mg DAN 216/L. The lower and upper 95% confidence limits were 1.1 and 1.4 mg DAN 216/L, respectively. Based on visual interpretation of the data, the no mortality/no immobility concentration was 0.53 mg DAN 216/L.

REFERENCES

1 Title 40 of the Code of Federal Regulations, Part 797, Section 1300, *Daphnid Acute Toxicity Test*. July 1992.

2 American Society for Testing and Materials. 1988 *Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians*, ASTM E 729-88. Philadelphia, PA.

3 APHA, AWWA, WPCF, *Standard Methods for the Examination of Water and Wastewater*. 16th Edition, 1985. American Public Health Association, Washington, D.C.

4 Stephan, C.E. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. 1978.

5 Finney, D.J. *Statistical Methods in Biological Assay*, second edition. Griffin Press, London. 1971.

6 Thompson, W.R. *Bacteriological Reviews*, Vol. II, No. 2. June, 1947. Pp. 115-145.

7 Stephan, C.E. "Methods for Calculating an LC50", Pages 65-81, *in Aquatic Toxicology and Hazard Evaluations*, American Society for Testing and Materials. Publication Number STP 634 (1977), pp 65-84. Philadelphia, PA.

Table 1

Summary of Analytical Chemistry Data

Sponsor:	Shell Development Company
Test Substance:	DAN 216
Test Organism:	Cladoceran *(Daphnia magna)*
Dilution Water:	Well Water

Nominal Concentration (mg DAN 216/L)	Measured Concentration (mg DAN 216/L)				Grand Mean (mg DAN 216/L)
	Day 0 (New)	Day 1 (Old)	Day 1 (New)	Day 2 (Old)	
Control	<LOD[1]	<LOD	<LOD	<LOD	<LOD
0.39	0.32	0.41	0.33	0.24	0.33
0.65	0.55	0.65	0.43	0.49	0.53
1.1	0.94	1.10	0.80	0.86	0.93
1.8	1.60	--[2]	1.44	1.31	1.5
3.0	1.81	1.51	1.66	1.69	1.7

[1] The limit of detection (LOD) was 0.050 mg DAN 216/L.
[2] Sample broken during shipment.

Table 2
Temperature of Water in the Test Chambers

Sponsor: Shell Development Co.
Test Substance: DAN 216
Test Organism: Cladoceran *(Daphnia magna)*
Dilution Water: Well Water

Mean Measured Concentration (mg DAN 216/L)	Replicate	Temperature (°C) Day 0[1] (New)	Day 1 (Old)	Day 1 (New)	Day 2 (Old)
Control	A[2]	20.0	20.0	20.1	20.1
	B	20.0	20.0	20.1	20.0
	C	20.0	20.0	20.1	20.0
	D	20.0	20.0	20.1	20.0
0.33	A	20.0	20.1	20.2	20.1
	B	20.0	20.0	20.2	20.1
	C	20.0	20.1	20.2	20.0
	D	20.0	20.1	20.2	20.0
0.53	A	20.0	20.2	20.2	20.1
	B	20.0	20.2	20.2	20.1
	C	20.0	20.2	20.2	20.1
	D	20.0	20.2	20.2	20.1
0.93	A	20.0	20.1	20.2	20.1
	B	20.0	20.1	20.2	20.1
	C	20.0	20.1	20.1	20.1
	D	20.0	20.1	20.1	20.1
1.5	A	20.0	20.0	20.2	20.1
	B	20.0	20.0	20.2	20.1
	C	20.0	20.0	20.2	20.1
	D	20.0	20.0	20.2	20.1
1.7	A	20.0	20.1	20.2	20.1
	B	20.0	20.1	20.2	20.1
	C	20.0	20.1	20.2	20.1
	D	20.0	20.1	20.2	20.2

[1] The 0-hour dilution water measurements for hardness, alkalinity, conductivity, total organic carbon and particulate matter were 136 mg/L as $CaCO_3$, 188 mg/L as $CaCO_3$, 310 µmhos/cm, 0.7 mg/L and 2.3 mg/L.

[2] Temperature measured continuously during the test ranged from approximately 19.5 to 20.5°C.

Table 3
Dissolved Oxygen[1] of Water in the Test Chambers

Sponsor: Shell Development Co.
Test Substance: DAN 216
Test Organism: Cladoceran *(Daphnia magna)*
Dilution Water: Well water

Mean Measured Concentration (mg DAN 216/L)	Replicate	Dissolved Oxygen (mg/L) Day 0 (New)	Day 1 (Old)	Day 1 (New)	Day 2 (Old)
Control[1]	A	8.8	8.8	8.9	8.8
	B	8.8	8.8	8.9	8.8
	C	8.8	8.8	8.9	8.7
	D	8.7	8.8	8.9	8.8
0.33	A	8.8	8.8	8.8	8.8
	B	8.8	8.8	8.8	8.7
	C	8.7	8.8	8.9	8.7
	D	8.8	8.8	8.9	8.8
0.53	A	8.8	8.8	8.9	8.8
	B	8.8	8.8	8.8	8.8
	C	8.8	8.8	8.9	8.8
	D	8.7	8.8	8.8	8.8
0.93	A	8.7	8.7	8.8	8.8
	B	8.7	8.8	8.8	8.8
	C	8.7	8.7	8.9	8.8
	D	8.7	8.8	8.9	8.8
1.5	A	8.8	8.8	8.8	8.8
	B	8.8	8.8	8.8	8.7
	C	8.7	8.5	8.9	8.8
	D	8.8	8.7	8.9	8.8
1.7	A	8.7	8.7	8.8	8.8
	B	8.8	8.8	8.8	8.8
	C	8.8	8.8	8.9	8.9
	D	8.8	8.8	8.8	8.8

[1] A dissolved oxygen concentration of 5.4 mg/L represents 60% saturation at 20°C in fresh water.

Table 4

pH of Water in the Test Chambers

Sponsor: Shell Development Co.
Test Substance: DAN 216
Test Organism: Cladoceran *(Daphnia magna)*
Dilution Water: Well water

Mean Measured Concentration (mg DAN 216/L)	Replicate	pH			
		Day 0 (New)	Day 1 (Old)	Day 1 (New)	Day 2 (Old)
Control	A	8.3	8.3	8.3	8.5
	B	8.3	8.4	8.3	8.5
	C	8.3	8.4	8.3	8.5
	D	8.3	8.5	8.3	8.5
0.33	A	8.3	8.5	8.3	8.5
	B	8.3	8.5	8.4	8.5
	C	8.3	8.5	8.3	8.5
	D	8.3	8.5	8.3	8.5
0.53	A	8.3	8.5	8.4	8.6
	B	8.3	8.5	8.4	8.6
	C	8.3	8.5	8.4	8.5
	D	8.4	8.5	8.3	8.5
0.93	A	8.3	8.5	8.4	8.6
	B	8.3	8.5	8.4	8.6
	C	8.3	8.5	8.4	8.5
	D	8.3	8.5	8.4	8.5
1.5	A	8.3	8.5	8.4	8.6
	B	8.3	8.5	8.5	8.6
	C	8.3	8.5	8.4	8.6
	D	8.3	8.5	8.4	8.6
1.7	A	8.4	8.5	8.4	8.6
	B	8.4	8.5	8.4	8.6
	C	8.4	8.6	8.4	8.6
	D	8.4	8.6	8.4	8.6

Table 5
Cumulative Percent Mortality/Immobility and Treatment-Related Effects[1]

Sponsor: Shell Development Co.
Test Substance: DAN 216
Test Organism: Cladoceran *(Daphnia magna)*
Dilution Water: Well Water

Mean Measured Concentration (mg DAN 216/L)	Replicate	Daphnia/ Replicate	8 Hours			24 Hours			48 Hours			Percent Immobile and Dead
			Effects	Number Immobile	Number Dead	Effects	Number Immobile	Number Dead	Effects	Number Immobile	Number Dead	
Negative Control	A	5	5 AN	0	0	5 AN	0	0	5 AN	0	0	0%
	B	5	5 AN	0	0	5 AN	0	0	5 AN	0	0	
	C	5	5 AN	0	0	5 AN	0	0	5 AN	0	0	
	D	5	5 AN	0	0	5 AN	0	0	5 AN	0	0	
0.33	A	5	5 AN	0	0	5 AN	0	0	5 AN	0	0	0%
	B	5	5 AN	0	0	5 AN	0	0	5 AN	0	0	
	C	5	5 AN	0	0	5 AN	0	0	5 AN	0	0	
	D	5	5 AN	0	0	5 AN	0	0	5 AN	0	0	
0.53	A	5	5 AN	0	0	5 AN	0	0	5 AN	0	0	0%
	B	5	5 AN	0	0	5 AN	0	0	5 AN	0	0	
	C	5	5 AN	0	0	5 AN	0	0	5 AN	0	0	
	D	5	5 AN	0	0	5 AN	0	0	5 AN	0	0	

[1] Observed Effects: AN = Appears Normal; C = Lethargic

Table 5 (Continued)
Cumulative Percent Mortality and Treatment-Related Effects[1]

Sponsor: Shell Development Co.
Test Substance: DAN 216
Test Organism: Cladoceran *(Daphnia magna)*
Dilution Water: Well Water

Mean Measured Concentration (mg DAN 216/L)	Replicate	Daphnia/ Replicate	8 Hours			24 Hours			48 Hours			Percent Immobile and Dead
			Effects	Number Immobile	Number Dead	Effects	Number Immobile	Number Dead	Effects	Number Immobile	Number Dead	
0.93	A	5	5 AN	0	0	5 AN	0	0	3AN;1C	1	0	10%
	B	5	5 AN	0	0	5 AN	0	0	3AN;1C	1	0	
	C	5	5 AN	0	0	5 AN	0	0	5 AN	0	0	
	D	5	5 AN	0	0	5 AN	0	0	5 AN	0	0	
1.5	A	5	5 AN	0	0	5 AN	0	0	3 AN; 1C	1	0	45%
	B	5	5 AN	0	0	5 AN	0	0	2 AN	3	0	
	C	5	5 AN	0	0	5 AN	0	0	4 AN	1	0	
	D	5	5 AN	0	0	5 AN	0	0	1 AN	4	0	
1.7	A	5	5 AN	0	0	1 AN	4	0	--	0	5	100%
	B	5	5 AN	0	0	2 AN	2	1	--	1	4	
	C	5	5 AN	0	0	2C	3	0	--	0	5	
	D	5	5 AN	0	0	3C	2	0	--	0	5	

[1] Observed Effects: AN = Appears Normal; C = Lethargic

Table 6

EC50 Values

Sponsor: Shell Development Company
Test Substance: DAN 216
Test Organism: Cladoceran *(Daphnia magna)*
Dilution Water: Well Water

Time	EC50 (mg DAN 216/L)	Lower 95% Confidence Limits (mg DAN 216/L)	Upper 95% Confidence Limits (mg DAN 216/L)	Statistical Method
24 Hours	1.7	> 1.5	N/A	Binomial
48 Hours	1.2	1.1	1.4	Moving Average

APPENDIX I

Conductivity, Hardness, Alkalinity, and pH of Well Water Measured During the 4-Week Period Immediately Preceding the Test

Sponsor: Shell Development Company
Test Substance: DAN 216
Test Organism: Cladoceran *(Daphnia magna)*
Dilution Water: Well Water

	Mean	Range
Conductivity (μmhos/cm)	313 (n = 4)	310 - 320
Hardness (mg/L as $CaCO_3$)	133 (n = 4)	132 - 136
Alkalinity (mg/L as $CaCO_3$)	181 (n = 4)	172 - 186
pH	8.1 (n = 4)	8.0 - 8.2

APPENDIX II

Pesticides, Organics, Metals and Other Inorganics Analyzed in Wildlife International Ltd. Well Water[1]

Sponsor: Shell Development
Test Substance: DAN 216
Test Organism: Cladoceran *(Daphnia magna)*
Dilution Water: Well Water

ANALYSIS	MEASURED CONCENTRATION
Organophosphorus & Organonitrogen Pesticides	
Azodrin (Monochrotophos)	< 0.250 µg/L
Bolstar	< 0.250 µg/L
Chlorpyrifos	< 0.250 µg/L
Coumaphos	< 0.250 µg/L
Demeton	< 0.250 µg/L
Diazinon	< 0.250 µg/L
Dichlorvos	< 0.250 µg/L
Dimethoate	< 0.250 µg/L
Disulfoton	< 0.250 µg/L
EPN	< 0.250 µg/L
Ethoprop	< 0.250 µg/L
Fenthion	< 0.250 µg/L
Fensulfothion	< 0.250 µg/L
Guthion (Methyl Azinphos)	< 0.250 µg/L
Malathion	< 0.250 µg/L
Merphos	< 0.250 µg/L
Mevinphos	< 0.250 µg/L
Naled	< 0.250 µg/L
Methylparathion	< 0.249 µg/L
Parathion	< 0.250 µg/L
Phorate	< 0.250 µg/L
Ronnel	< 0.250 µg/L
Stirofos	< 0.250 µg/L
Sulfotepp	< 0.250 µg/L
Tepp	< 0.250 µg/L
Tokuthion	< 0.250 µg/L
Trichloronate	
Metals and Other Inorganics	
Aluminum	< 40.0 µg/L
Arsenic	< 2.5 µg/L
Beryllium	< 5.0 µg/L
Cadmium	< 5.0 µg/L
Calcium	35800 µg/L
Chromium	< 10.0 µg/L
Copper	< 5.0 µg/L
Iron	< 45.0 µg/L
Lead	< 2.0 µg/L
Magnesium	13.7 mg/L
Manganese	< 5.0 µg/L
Mercury	< 0.20 µg/L
Nickel	< 15.0 µg/L
Potassium	6160 µg/L
Selenium	< 2.5 µg/L
Silver	< 5.0 µg/L
Sodium	22000 µg/L
Zinc	< 20.0 µg/L
Molybdenum	< 10.0 µg/L

[1] Analyses performed by Environmental Science & Engineering, Inc., Gainesville, Florida for samples collected on November 11, 1993.

APPENDIX II (Continued)

Pesticides, Organics, Metals, and Other Inorganics Analyzed in Wildlife International Ltd. Well Water[1]

Sponsor: Shell Development Company
Test Substance: DAN 216
Test Organism: Cladoceran *(Daphnia magna)*
Dilution Water: Well Water

ANALYSIS	MEASURED CONCENTRATION
Miscellaneous Measurements	
Total Dissolved Solids	267 mg/L
Ammonia Nitrogen	< 0.050 mg/L
Total Organic Carbon[2]	1.7 mg/L
Total Cyanide	< 0.003 mg/L
Organochlorines and PCBs	
Aldrin	< 0.005 µg/L
Alpha BHC	< 0.005 µg/L
Beta BHC	< 0.005 µg/L
Delta BHC	< 0.005 µg/L
Gamma BHC (Lindane)	< 0.005 µg/L
Chlordane	< 0.025 µg/L
DDD, pp'	< 0.005 µg/L
DDE, pp'	< 0.005 µg/L
DDT, pp'	< 0.005 µg/L
Dieldrin	< 0.005 µg/L
Endosulfan, A	< 0.005 µg/L
Endosulfan, B	< 0.005 µg/L
Endosulfan Sulfate	< 0.005 µg/L
Endrin	< 0.005 µg/L
Endrin Aldehyde	< 0.005 µg/L
Heptachlor	< 0.005 µg/L
Methoxychlor	< 0.005 µg/L
Heptachlor Epoxide	< 0.500 µg/L
Toxaphene	< 0.100 µg/L
PCB-1016	< 0.100 µg/L
PCB-1221	< 0.100 µg/L
PCB-1232	< 0.100 µg/L
PCB-1242	< 0.100 µg/L
PCB-1248	< 0.100 µg/L
PCB-1254	< 0.100 µg/L
PCB-1260	
Chlorophenoxy Acid Herbicides	
2,4-D, Total	< 0.025 µg/L
2,4-DB	< 0.028 µg/L
2,4,5-T Water	< 0.025 µg/L
2,4,5-TP/Silvex	< 0.021 µg/L
Dalapon	< 0.025 µg/L
Dicamba (Banvel)	< 0.023 µg/L
Dichloroprop	< 0.020 µg/L
Dinoseb	< 0.021 µg/L
MCPA	< 0.540 µg/L
MCPP	< 0.440 µg/L

[1] Analyses performed by Environmental Science & Engineering, Inc., Gainesville, Florida for samples collected on November 11, 1993.
[2] Analyses performed by Wildlife International Ltd. for samples collected on February 3, 1994.

APPENDIX III

Measurement of DAN 216 in Dilute Aqueous Samples in Support of Acute Aquatic Toxicity Testing with the Cladoceran (*Daphnia magna*)

Data Report

Measurement of DAN 216 in Dilute Aqueous Samples in Support of Acute Aquatic Toxicity Testing with The Cladoceran (Daphnia magna)

Performed at

Shell Development Company
Westhollow Research Center
3333 Highway 6 South
Houston, Texas 77082

in Accordance with United States Environmental Protection Agency (EPA) Toxic Substances Control Act (TSCA), Good Laboratory Practice Standards (40 CFR, Part 792) in support of the aquatic toxicity test with the Cladoceran conducted at:

Wildlife International, Ltd.
8598 Commerce Drive
Easton, Maryland 21601

WRC Tox Study No. 1232

Compliance Statement

Title: Measurement of DAN 216 Dilute Aqueous Samples in Support of Acute Aquatic Toxicity Testing with The Cladoceran

Report: WRC Tox Sample No. 1232

The study named above was reviewed according to the Good Laboratory Practices regulations outlined by the Environmental Protection Agency (EPA) in 40 CFR Part 792 under the authority of the Toxic Substances Control Act. Based upon the Quality Assurance inspection reports provided, the practices described in the report were found to be in compliance with the above regulations, with the exception of the following:

1. No expiration dates were found on the reagents used during the course of the phase-study. Expiration dates have been added to the labels on the reagents.

2. Milli Q maintenance records were not completely documented.

3. The DAN samples were stored in the shipping cooler received from the contract laboratory. This adequately separates the samples from potential contamination in the laboratory.

4. The Milli-Q water system filters are changed every six months. This routine maintenance has not been documented in the past.

5. The sample lines of the AutoTrace Workstation were flushed out using H_2O on the first run and 50:50 Methanol:IPA on the second run rather than the 50:50 IPA:H_2O documented in the method.

The above exceptions to the requirements of the Good Laboratory Practice regulations have been investigated and are judged not to impact the quality or integrity of the effected study.

H. C. Smith
Analytical Study Director
Analytical Chemistry R&D - WRC
Shell Development Company

Date

Quality Assurance Statement

Report: Measurement of DAN 216 in Dilute Aqueous Samples in Support of Acute Aquatic Toxicity Testing with The Cladoceran (Daphnia magna)

In accordance with Good Laboratory Practice Standards (40 CFR, Part 792), this study has been monitored by the Westhollow Research Center's (WRC) Quality Assurance Unit. The quality assurance audit was made and reported to the Analytical Study Director and Management as noted below:

DATE INSPECTED	DATE REPORTED
February 1, 1994	February 15, 1994

The final report accurately describes the methods and procedures used in the study and, to the best of my knowledge, accurately reflects the raw data obtained.

Donald L. Box

D. L. Box
Good Laboratory Practice Coordinator
Analytical Chemistry R&D - WRC
Shell Development Company

3/02/94
Date

INTRODUCTION

The chemical analytical work in this report is a component of the aquatic toxicological testing performed at Wildlife International. Chemical analyses of aqueous samples and primary stock solutions, for the determination of concentrations of DAN 216, were performed using the Standard Operating Procedure (SOP) 00014, Determination of Trace Amounts of Secondary Alkyl Sulfate (SAS) in Aqueous Samples. This procedure was approved by Analytical Management on October 20, 1993 and by the GLP Coordinator on October 10, 1993.

Experimental Initiation Date: February 1994
Experimental Completion: March 1994

The Test Substance Identification

Test Substance:	DAN 216
Test Substance PSIS #:	1232-3
Test Substance Tox #:	1232
Test Substance Batch Reference #:	22056-62
Test Substance Shell Toxicology Protocol #:	SETP-93-007
Test Substance Purity:	98%
Test Substance Analysis:	

The percent purity is based on process knowledge and analytical characterization performed prior to dispensing of the test substance. A concentration of 99% total active matter was obtained by direct measurement while a concentration of 98% was obtained by subtracting the concentrations of sodium sulfate, olefinic material and secondary alcohol from 100%. These concentrations lie within the experimental error of the various techniques. The concentration of 98% was used as the actual test substance purity.

METHOD DESCRIPTION

Method Abstract

This method is used to determine DAN 216 in aqueous solutions at the parts per billion level using solid phase extraction (SPE) to concentrate the sample for analysis by HPLC using an evaporative light scattering detector (ELSD). For a more specific description of the procedure used during this analysis, please refer to SOP 00014-93 and the deviations to the analytical method found as an attachment to this report. This method quantifies the total DAN 216 content and does not distinguish various carbon chain distributions. No extraction step was needed to analyze the stock solution.

Quality Control

The water samples were concentrated using solid phase extraction in batches of approximately six samples. Primary stock solutions were prepared for analysis by making weight /volume dilutions. A seven point multi-level calibration was generated for each sequence of samples analyzed using the HPLC/ELSD. The seven point multi-level calibration was regenerated after any suspected computer or instrument error. At the start of each HPLC run, a correlation coefficient exceeding 0.99 was generated for all sample sequences using the seven-point multi-level instrument calibration. Test samples followed the initial calibration. A spike, prepared from a 98 % pure DAN 216 standard, followed a sequence of approximately 25 samples.

Calculations

Instrument response, and thus sample concentrations, were determined by calibration with multi-level calibration standards with quadratic curve fit, the x and y axes of the calibration curve being converted to the natural logarithms of the values, and by using the equations found in the "Calculations" section of the attached SOP. Standards were prepared so that the calibration curve, generated at the initiation of each sample run, bracketed the expected sample concentration range. Original aqueous sample concentrations were then determined using the extraction and pre-injection volumes.

$$RF(y) = AREA\ (y) / AMOUNT\ (y)$$

where,

RF is the absolute response factor for peak y.

AREA is the peak area of peak y in the standard.

AMOUNT is the amount, in µg/ml, of y in the standard.

The amount of y in the unknown is calculated using the following equation:

$$ppm(y) = [(AREA(y) \times SPE\ PREP\ VOL \times 0.01) / (RF(y) \times SX\ WT \times TOTAL\ MLS\ SAMPLE) \times 100]$$

Limit of Detection

A water sample equivalent LOD of 50 ppb has been established for this method. The analyte provided a detector response of greater than 3:1 s/n to be considered detected. The water sample equivalent LOD was found using the above equation.

Westhollow Research Center Tox Sx. #: 1232

The following are deviations to the Analytical Method (SOP 00014-93)

1. Change: A secondary alkyl sulfate (SAS) concentration of 98 %, rather than the 99% purity stated in the method, was used to prepare the standard.

   Reason: Although a concentration of 99% was obtained by direct measurement, 98% was obtained by subtraction of all known impurities from 100%. The additive errors of the methods used to measure the impurities are believed to be less than that of the method used to directly measure active matter.

2. Change: A pH meter and Rainin Microman 250µl adjustable positive displacement pipet with stainless steel plungers were used during the analyses described by this method.

   Reason: The two pieces of equipment, omitted from the apparatus list found in the method, are necessary to obtain optimal recovery of the test substance.

3. Change: During sample preparation a pH adjustment of 8 is made to the samples.

   Reason: Experimentation has proven that the pH adjustment results in better recovery of the test substance dilutions.

4. Change: Using the automated system, the sample lines of the AutoTrace Workstation are cleaned using a Milli-Q water wash followed by 50:50 IPA:Methanol then again by Milli-Q water.

   Reason: The additional and final Milli-Q washing step was included to provide greater assurance that the sample lines are clean and contamination is avoided.

5. Change: After samples are extracted, sonicated, and vortexed, 100 µl of the sample is diluted with 100 µl of Milli-Q water. Calibration standards are also diluted.

   Reason: The dilution step is performed to assure that the signal remains within the dynamic range of the instrument.

6. Change: A Phenomenex HPLC column was used , rather than a Supelco LC-18 (see

Attachment).

Reason: The smaller internal diameter of the column avoids solvent waste.

7. Change: The scope of SOP 00014-93 states that the method is intended for determination of C_{14} SAS in aqueous sample matrices.

Reason: Further testing has proven that the method found in SOP 00014-93 is applicable to analyses of C_{16} SAS compounds as well as C_{14} compounds.

Approved:

Harriet C. Smith

March 10, 1994

H. C. Smith
Analytical Study Director
Analytical Chemistry R&D - WRC
Shell Development Company

Date

Sheila Dubey
Sr. Research Chemist
Analytical Chemistry - Organic and Detergent Analysis
Shell Development Company

Date

SPONSOR REPORT APPROVAL

Report: Measurement of DAN 216 in Dilute Aqueous Samples in Support of Acute Aquatic Toxicity Testing with The Cladoceran

Shell Toxicology Protocol No.: SETP 93-007

This report has been reviewed and found to be satisfactory.

Diana C. Wong

D. C. L. Wong
Sponsor Representative
Environmental RD&T - WRC
Shell Development Company

3/22/94
Date

DAN 216 Daphnia magna acute toxicity test

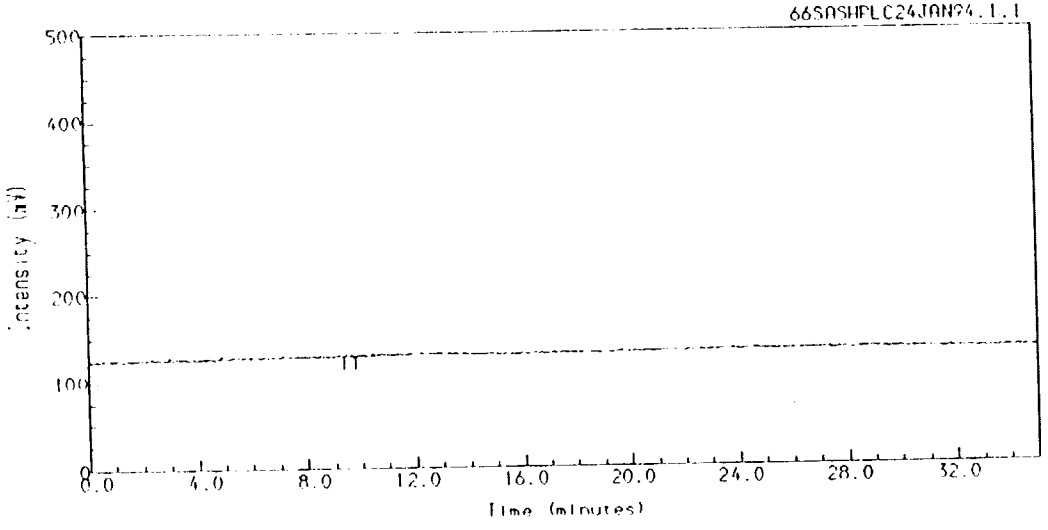
Compound	Sample ID	Sample #	Nominal Conc (ppm)	Measured Conc (ppm)
DAN 216	Day 0	109A-107-1	0.00	ND
	Day 0	109A-107-2	0.39	0.32
	Day 0	109A-107-3	0.65	0.55
	Day 0	109A-107-4	1.10	0.94
	Day 0	109A-107-5	1.80	1.60
	Day 0	109A-107-6	3.00	1.81
	Day 1 old	109A-107-7	0.00	ND
	Day 1 old	109A-107-8	0.39	0.41
	Day 1 old	109A-107-9	0.65	0.65
	Day 1 old	109A-107-10	1.10	1.10
	Day 1 old	109A-107-11	1.80	broken
	Day 1 old	109A-107-12	3.00	1.51
	Day 1 new	109A-107-13	0.00	ND
	Day 1 new	109A-107-14	0.39	0.33
	Day 1 new	109A-107-15	0.65	0.43
	Day 1 new	109A-107-16	1.10	0.80
	Day 1 new	109A-107-17	1.80	1.44
	Day 1 new	109A-107-18	3.00	1.66
	Day 2	109A-107-19	0.00	ND
	Day 2	109A-107-20	0.39	0.24
	Day 2	109A-107-21	0.65	0.49
	Day 2	109A-107-22	1.10	0.86
	Day 2	109A-107-23	1.80	1.31
	Day 2	109A-107-24	3.00	1.69
	Day 0, stock	109A-107-49	500	422
	Day 1, stock	109A-107-50	500	453

ND = not detected, <LOD

February 3,1994

SASHPLC24JAN94.1.1
LR Sample Prep

BLANK
sx amount : 1.000000
PREP VOL 1.00000E+00 (cup1) FUDGE FACTOR - 1.00000E 02 (cup2) ORIG VOL OF SX 1.00000E+00 (cup3)



66 SASHPLC24JAN94.1.1

RT area counts PPM (UGFEAK NAME

TOTAL AMOUNT 4.4502

Column: IB-Sil 5u ODS (C18)
250 x 3.2 mm ID
Phenomenex #00G-0082-R0
100ul Injection size
Column temp 40 C
Precolumn filter Upchurch #A-318 with 5um frit
Detectors:
1. ACS MODEL 750/14 MASS DETECTOR (ELSD)
NEBULIZER TEMP 65oC
NEBULIZING GAS = N2 @ 15 PSI
(Primary regulator set to 20 PSI
Secondary regulator set to 15 PSI)
TIME CONSTANT = 10 SEC
PHOTOMULTIPLIER SENSITIVITY = 6

Gradient:

time	B	C	flow
0	80	20	0.35
3	80	20	
15	20	80	B = H2O
20	0	100	C = IPA
26	80	20	
40	80	20	

Samples diluted with Milli-Q water before injection (100 ul sx + 100 ul Milli-Q water)

METHOD NAME: M66SAS
CALIBRATION NAME: C66SAS14
VG ARTIST NAME: M66SAS
RUN SEQUENCE FILE: R66SAS14
useraccess routine: [illegible]
Date Reported:
24-JAN 1994 08:24
Date Acquired:
24-Jan 1994 07:48

COMPLETED

SHELL DEVELOPMENT COMPANY
WESTHOLLOW RESEARCH CENTER
ANALYTICAL CHEMISTRY R&D

SOP NUMBER: SOP 00014-93
REVISION: 0

TITLE: Determination of Trace Amounts of Secondary Alkyl Sulfate (SAS) in Aqueous Samples.

AUTHORS: TL Bruin, ST Dubey

SCOPE: This method is intended for the determination of parts per million (ppm) of C_{14} SAS in aqueous sample matrixes using solid phase extraction (SPE) to concentrate the sample for analysis by HPLC using an evaporative light scattering detector (ELSD).

PRINCIPAL AUTHOR: Terri Bruin 10/15/93
Date

QUALITY ASSURANCE REVIEW: Darryl K Box 10/19/93
D.L. Box
G.L.P. Coordinator
Analytical Chemistry R&D
Date

MANAGEMENT APPROVAL: M D Baker 20 Oct '93
M.D. Baker
Research Manager
Date

DISTRIBUTION: Original: Analytical Methods Repository
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Reviewers
Analytical Study Directors
Chemical Products Analysis Manager

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Shell Development Company

REAGENTS:

(a) ISOPROPANOL : HPLC grade

(b) WATER : Milli-Q Plus system with ion-exchange cartridge replaced when water resistivity falls below 18.2 Megohm-cm

(c) SECONDARY ALCOHOL SULFATE STANDARD : C_{14} SAS with purity of 99+%

(d) FORMALIN SOLUTION : ~35% formaldehyde in water, reagent grade

APPARATUS:

(a) Volumetric Pipets : 1 ml, 2 ml, 3 ml, 4 ml, 9 ml, 10 ml class A.

(b) Volumetric Flasks : 10 ml, class A.

(c) Graduated Cylinder : 1000 ml, stoppered, class A.

(d) Glass Vials : 2 dram, 4 dram, 6 dram, 8 dram, borosilicate glass.

(e) Glass Culture Tubes : borosilicate glass, 16 x 100 mm

(f) Parafilm "M" Laboratory Film

(g) Analytical Balance : Mettler AE 163, or equivalent.

(h) Syringe : 500 µl Hamilton high precision, glass.

(i) Varian Star HPLC system, equipped with a 9010 pump and a Shimadzu Sil-9A Auto Injector equipped with a 100 µl sample loop. Equivalent equipment may be substituted.

(j) ACS Evaporative Light Scattering Detector, model 750/14 (ELSD).

(k) VG Multichrom data collection system and software. Substitutes are acceptable.

(l) Genie Vortex mixer, or equivalent.

(m) Branson 1200 sonicator, or equivalent.

(n) SPE Cartridge : Octyl, 1000 mg, 3 ml reservoir

(o) 12 port solid phase extraction manifold or an AutoTrace SPE Workstation (Zymark)

(p) Pierce Reacti-Therm heating/stirring module

(q) Pierce Reacti-Vap evaporation module : 9 port

(r) Pierce Reacti-Block : Model B-1

(s) HPLC Column : Supelco LC-18, 25 cm x 4.6 mm ID, 5 micron, Cat# 5-8298

CALIBRATION STANDARDS PREPARATION:

(a) 0.1 gram C_{14} SAS is weighed, to the nearest 0.1 milligram, into a 10 ml volumetric flask. The flask is then filled to the mark with a

solution of 50 parts Milli-Q water (with pH adjusted to ~8) and 50 parts isopropanol. The solution is sonicated to help dissolve the SAS. This results in a solution of 10,000 µg/ml SAS.

(b) 2.0 mls of the 10,000 µg/ml solution is pipeted into an 8 dram vial. 18.0 mls of the 50:50 IPA:Water solution is then added. This results in a solution of 1000 µg/ml concentration.

(c) 3.0 mls of the 1000 µg/ml solution is pipeted into a 2 dram vial. 1.0 ml of the 50:50 IPA:Water solution is then added. This results in a solution of 750µg/ml concentration.

(d) 2.0 mls of the 1000 µg/ml solution is pipeted into a 2 dram vial. 2.0 mls of the 50:50 IPA:Water solution is then added. This results in a solution of 500 µg/ml concentration.

(e) 1.0 ml of the 1000 µg/ml solution is pipeted into a 2 dram vial. 4.0 mls of the 50:50 IPA:Water solution is then added. This results in a solution of 200 µg/ml concentration.

(f) 1.0 ml of the 1000 µg/ml solution is pipeted into a 6 dram vial. 9.0 mls of the 50:50 IPA:Water solution is then added. This results in a solution of 100 µg/ml concentration.

(g) 2.0 mls of the 100 µg/ml solution is pipeted into a 2 dram vial. 2.0 mls of the 50:50 IPA:Water solution is then added. This results in a solution of 50 µg/ml concentration.

(h) 1.0 ml of the 100 µg/ml solution is pipeted into a 6 dram vial. 9.0 mls of the 50:50 IPA:Water solution is then added. This results in a solution of 200 µg/ml concentration.

SAMPLE PREPARATION:

(a) <u>SPE/Extraction of SAS using Manual System</u>:

1. Cleaning glassware: All glassware should be free of surfactant. No detergent should be used for cleaning glassware. The recommended cleaning procedure involves washing with hot tap water followed by reagent grade IPA, MeOH, then Milli-Q water, followed by drying in an oven.

2. Condition SPE cartridge: Octyl SPE cartridge is conditioned with 10 mls IPA followed by 10 mls of water at a flow of approximately 5 mls/min. ***IMPORTANT***: the cartridge is NOT allowed to dry between the conditioning step and the application of the sample.

3. An appropriate volume of aqueous sample is then loaded onto the column at a flow rate of approximately 5 mls/min. The volume of sample loaded onto the column depends on the concentration of SAS in the sample. The lowest desired amount of SAS to be loaded on the SPE cartridge should be 15 µg. Approximately 300 mls is needed for a sample with a SAS concentration of 0.05 ppm.

4. The SPE cartridge is rinsed with 10 mls of water, then allowed to dry under vacuum for 10 minutes.

5. SAS is eluted from the cartridge into an appropriate size vial with 12 mls IPA.

6. The IPA is evaporated off using a heating block at 60°C with a gentle nitrogen stream. The walls of the vial are washed down 2-3 times with IPA and evaporated to dryness.

7. The residue is redissolved in 250 µl 50:50 IPA:Water using a 500 µl Hamilton high precision syringe. The sample is sonicated for several minutes to help dissolve, then vortexed to mix. This solution is then transferred to a autosampler microvial for injection onto the HPLC.

(b) SPE/Extraction of SAS using Automated System:

1. The solvent syringe of the Zymark AutoTrace SPE Workstation is purged using the purge program located on floppy.

2. The sample lines of the AutoTrace Workstation are cleaned using the clean sample lines program located on floppy. Samples lines are cleaned using Milli-Q water followed by 50:50 IPA:Water.

3. The sample is loaded onto the SPE cartridge using the program listed in figure 1. Test tubes of 16 x 100mm size are loaded to collect sample. Prior to loading sample, ensure that the waste container has sufficient space for collecting waste.

4. After the sample program has completed, the test tubes containing sample are transferred to a heating block set at 60°C and the solvent evaporated under a gentle nitrogen stream. The walls of the test tube are rinsed 2-3 times with IPA and allowed to evaporate to dryness.

5. The residue is redissolved in 250 µl 50:50 IPA:Water using a Hamilton 500 µl high precision syringe. The test tube is capped using parafilm, and the sample is sonicated for several minutes to help dissolve, then vortexed to mix. This solution is then transferred to a autosampler microvial for injection onto the HPLC.

SAVE solvent
3.2 mm

- HPLC CONDITIONS:

Column: Supelco LC-18, 25cm x 4.6mm ID, 5 micron
Column Temperature: 40°C
Injection size: 100 µl
Detector: ELSD
Nebulizer temperature: 70°C
Nebulizing gas: Nitrogen at 18 psig
Time Constant: 10 seconds
Photomultiplier Sensitivity: 6

diluted
Sx 1 2
M.11.Q

Gradient:

Time	Water	IPA	Flow
0	80	20	0.8 ml/min
3	80	20	
15	20	80	
20	0	100	
25	0	100	
26	80	20	
35	80	20	

0.35

Zymark AutoTrace Extraction WorkStation 1.20

AutoTrace Extraction Procedure : SAS
Estimated Time for samples : 212.2 minutes
Date : 1 Sep 93

Step 1 : Process 4 samples using the following procedure:
Step 2 : Condition column with 10 ml of IPA into AQUEOUS WASTE
Step 3 : Condition column with 10 ml of H2O into AQUEOUS WASTE
Step 4 : Load 300 ml of sample onto column
Step 5 : Rinse column with 10 ml of H2O into AQUEOUS WASTE
Step 6 : Dry Column with gas for 10 minutes
Step 7 : Soak and Collect 8 ml fraction using IPA
Step 8 : Soak and Collect 3.5 ml fraction using IPA
Step 9 : Collect 0.5 ml fraction into sample tube using IPA
Step 10 : Wash syringe with 5 ml of H2O
Step 11 : END

SETUP PARAMETERS

AutoTrace Extraction Workstation

FLOW RATES			SPE PARAMETERS		
Cond Flow:	5.0	ml/min	Push Delay:	5	sec
Load Flow:	5.0	ml/min	Air Factor:	1.0	
Rinse Flow:	10.0	ml/min	Autowash Vol:	1.00	ml
Elute Flow:	1.00	ml/min			
Cond Air Push:	10.0	ml/min	WORKSTATION PARAMETERS		
Rinse Air Push:	10.0	ml/min	Max. Elution Vol:	12.0	ml
Elute Air Push:	3.00	ml/min	Exhaust Fan on:	Y	Y=YES N=NO
			Beeper on:	Y	Y=YES N=NO

Zymark AutoTrace Extraction WorkStation 1.20

AutoTrace Extraction Procedure : SAS
Date : 1 Sep 93

NAME SOLVENTS

Solvent 1 : MEOH
Solvent 2 : H2O
Solvent 3 : HCL/H2O
Solvent 4 : MEOH/H2O
Solvent 5 : IPA

Figure 1

A typical chromatogram of a 0.05 ppm aqueous solution of C_{14} SAS prepared as described above is shown in figure 2.

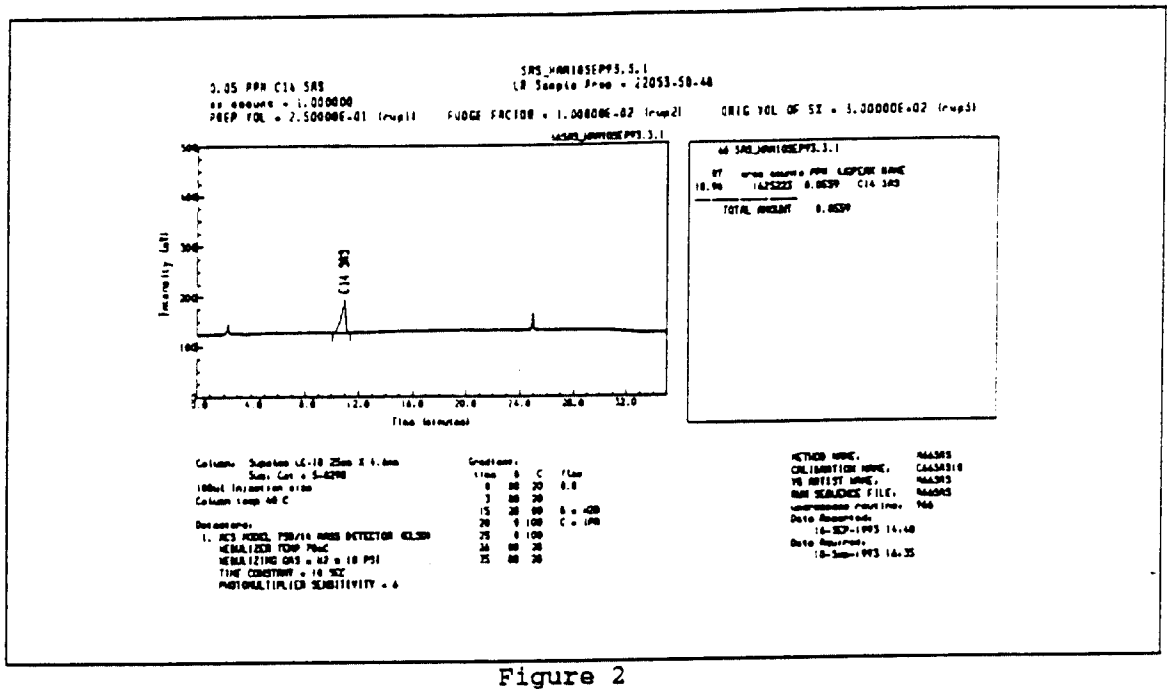


Figure 2

CALCULATIONS:

Calculations are performed using the VG Multichrom External Standard Percent calibration.

The response is determined by calibration with multilevel calibration standards, with quadratic curvefit (with a natural log X and Y transform) and using the following equation (a typical calibration curve is shown in figure 3):

$$RF(y) = AREA(y) / AMOUNT(y)$$

where,

RF is the absolute response factor for peak y.

AREA is the peak area of peak y in the standard.

AMOUNT is the amount, in µg/ml, of y in the standard.

The amount of y in the unknown is calculated using the following equation:

$$ppm(y) = \frac{AREA(y) \times SPE\ PREP\ VOL \times 0.01}{RF(y) \times SX\ WT \times TOTAL\ MLS\ SAMPLE} \times 100$$

where,

ppm(y) is the amount of y, in parts per million (µg/ml), in the sample

AREA is the peak area of component y.

SPE PREP VOL is the volume the SPE residue is redissolved in after sample concentration and solvent evaporation, typically 0.25 mls.

0.01 is a user parameter used to counteract the multiplier of 100 that Multichrom uses in the final calculation when using an External Standard % calculation.

RF is the absolute response factor for peak y.

SX WT is assumed to be 1 (the density of water)

TOTAL MLS SAMPLE is the amount of sample, in mls, passed through the SPE cartridge.

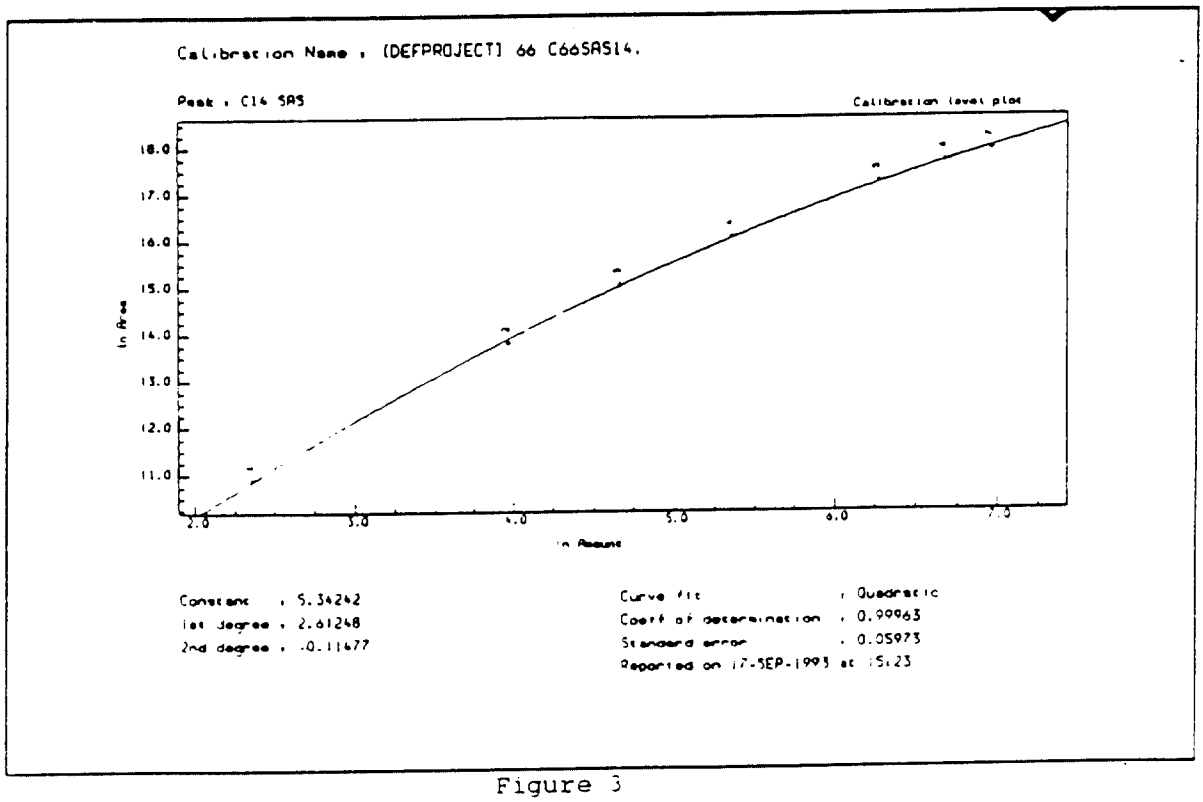


Figure 3

PRECISION AND ACCURACY:

Precision, accuracy, and recovery of C_{14} SAS was determined as follows: 10 mls formalin are added to a 1 liter stoppered graduated

cylinder. The cylinder is then filled to the 1000 ml mark with Milli-Q water. This solution is then spiked with 50 µg SAS (0.1 ml of the 500 µg/ml SAS calibration solution is used for the spike using a hamilton high precision syringe). The solution is mixed well. The Zymark AutoTrace Workstation is used to sample 300 mls of this solution in triplicate onto separate octyl SPE cartridges, and preparation continued as described in the SAMPLE PREPARATION section above. A total of 3 samples were analyzed by HPLC, and the results are as follows:

C_{14} SAS	amount of spike	obtained result	% recovery
	0.0529 ppm	0.0583 ppm	110.2
		0.0559 ppm	105.7
		0.0569 ppm	107.6
	average	0.0570 ppm	107.8
	SD	0.0012 ppm	2.2
	%SD	2.11%	2.06%

APPENDIX IV

EC50 Calculations

APPENDIX IV

A(N) 48-H LC50 (24-H DATA) STUDY
WITH DAPHNIA MAGNA

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 109A-107

SPONSOR NAME: Shell Development Company

TEST MATERIAL: DAN 216

****

CONC. mg/L	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROBABILITY (PERCENT)
1.7	20	12	60.00001	25.17218
1.5	20	0	0	LESS THAN 0.001
.93	20	0	0	LESS THAN 0.001
.53	20	0	0	LESS THAN 0.001
.33	20	0	0	LESS THAN 0.001

AT A CONFIDENCE LEVEL OF 95 PERCENT THE BINOMIAL TEST SHOWS THAT THE LC50 IS ABOVE 1.5

THE USEFULNESS OF ANY LC50 CALCULATED FROM THIS SET OF DATA IS QUESTIONABLE BECAUSE A CONCENTRATION-EFFECT RELATIONSHIP HAS NOT BEEN DEMONSTRATED OVER A REASONABLE RANGE (E.G. <37 TO >63) OF PERCENT DEAD

AN APPROXIMATE LC50 OF 1.67374 IS OBTAINED BY NONLINEAR INTERPOLATION BETWEEN 1.5 AND 1.7

WHEN THERE ARE LESS THAN TWO CONCENTRATIONS AT WHICH THE PERCENT DEAD IS BETWEEN 0 AND 100, NEITHER THE MOVING AVERAGE METHOD NOR THE PROBIT METHOD CAN GIVE ANY STATISTICALLY SOUND RESULTS.

COMPARE RESULTS WITH ORIGINAL DATA TO SEE IF THEY ARE REASONABLE

****

NOTE: This program automatically reports LC50 values. In this case, the values reported are actually EC50 values. The number dead reflect daphnia which were dead and/or immobile.

APPENDIX IV

A(N) 48-H LC50 (48-H DATA) STUDY
WITH DAPHNIA MAGNA

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 109A-107

SPONSOR NAME: Shell Development Company

TEST MATERIAL: DAN 216

CONC. mg/L	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROBABILITY (PERCENT)
1.7	20	20	100	LESS THAN 0.001
1.5	20	9	45	41.19008
.93	20	2	10	2.012252E-02
.53	20	0	0	LESS THAN 0.001
.33	20	0	0	LESS THAN 0.001

THE BINOMIAL TEST SHOWS THAT .93 AND 1.7 CAN BE USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT CONFIDENCE LIMITS BECAUSE THE ACTUAL CONFIDENCE LEVEL ASSOCIATED WITH THESE LIMITS IS GREATER THAN 95 PERCENT.

AN APPROXIMATE LC50 OF 1.512447 IS OBTAINED BY NONLINEAR INTERPOLATION BETWEEN 1.5 AND 1.7

--------RESULTS CALCULATED USING THE MOVING AVERAGE METHOD----------

SPAN	G	LC50	95 PERCENT CONFIDENCE LIMITS	
4	3.662191E-02	1.183539	1.05581	1.353582
3	4.495283E-02	1.238334	1.134148	1.36374
2	.0760278	1.346524	1.247011	1.449267

AN LC50 CALCUALTED USING THE MOVING AVERAGE METHOD MAY NOT BE A VERY GOOD ESTIMATE IF THE SPAN IS MUCH LESS THAN THE NUMBER OF CONCENTRATIONS

**********RESULTS CALCULATED USING THE PROBIT METHOD**********

ITERATIONS	G	H	CHI-SQUARE	PROBABILITY
6	1.39648	3.028702	9.086106	2.816695E-02

BECAUSE THE PROBABILITY IS LESS THAN 0.05, RESULTS CALCULATED USING THE PROBIT METHOD PROBABLY SHOULD NOT BE USED.

SLOPE = 9.584216
95 PERCENT CONFIDENCE LIMITS =-1.741717 AND 20.91015

LC50 = 1.343348
95 PERCENT CONFIDENCE LIMITS = 0 AND +INFINITY

COMPARE RESULTS WITH ORIGINAL DATA TO SEE IF THEY ARE REASONABLE

****

NOTE: This program automatically reports LC50 values. In this case, the values reported are actually EC50 values. The number dead reflect daphnia which were dead and/or immobile.

APPENDIX V

CHANGES IN PROTOCOL

The study was conducted in accordance with the approved Protocol with the following changes:

1. The proposed dates, study room, test concentrations, test substance number and receipt date were amended to the protocol.
2. The protocol was amended to change sample preparation and shipment.
3. Water and stock samples for analytical confirmation were collected in sample containers composed of flint glass.

In the opinion of the Study Director, the above changes in the approved protocol did not adversely affect the results of this study.

APPENDIX VI
PERSONNEL INVOLVED IN THE STUDY

The following key personnel were involved in the conduct or management of this study:

Aquatic Toxicology Laboratory

1. James P. Swigert, Ph.D., Manager, Aquatic Toxicology
2. Kurt R. Drottar, Senior Aquatic Biologist
3. Barry P. Conner, Aquatic Biologist
4. Gordon R. Pepper, Aquatic Biologist
5. Cynthia A. Roberts, Senior Aquatic Biologist
6. Edward C. Schaefer, Manager, Biodegradation

Analytical Chemistry Laboratory

1. Sheila Dubey, Chemist, Shell Development Company

DAN 216:
A 96-HOUR STATIC-RENEWAL ACUTE TOXICITY TEST WITH THE FATHEAD MINNOW (*Pimephales promelas*)

FINAL REPORT

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 109A-108

TITLE 40 OF THE CODE OF FEDERAL REGULATIONS
PART 797.1400

AUTHORS:

Kurt R. Drottar
James P. Swigert, Ph.D.

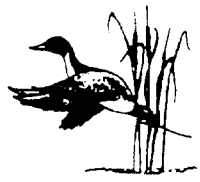
STUDY INITIATION DATE: December 15, 1993

STUDY COMPLETION DATE: April 8, 1994

Submitted to

Shell Development Company
Westhollow Research Center
3333 Highway Six South
Houston, Texas 77082

WILDLIFE INTERNATIONAL LTD.
8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600



GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Shell Development Company

TITLE: DAN 216: A 96-Hour Static-Renewal Acute Toxicity Test with the Fathead Minnow (*Pimephales promelas*)

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 109A-108

STUDY COMPLETION DATE: April 8, 1994

This study was conducted to conform with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Part 792, 17 August 1989.

STUDY DIRECTOR:

_____ DATE: 4/8/94
Kurt R. Drottar
Senior Aquatic Biologist

REPORT APPROVED BY:

_____ DATE: 4/8/94
James P. Swigert, Ph.D.
Manager, Aquatic Toxicology Laboratory

_____ DATE: _____
Sponsor

_____ DATE: _____
Applicant/Submitter

QUALITY ASSURANCE STATEMENT

This study was examined for conformance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Part 792, 17 August 1989. The dates of all inspections and audits and the dates that any findings were reported to the Study Director/Laboratory Management were as follows:

ACTIVITY	DATE CONDUCTED	DATE REPORTED TO STUDY DIRECTOR/MANAGEMENT:
Protocol	January 4, 1994	January 4, 1994
Aquatic Toxicology Laboratory		
Procedure (Test substance preparation, temperature measurements, analytical sampling)	January 3, 1994	January 3, 1994
Data and Draft Report	February 7-9, 1994	February 9, 1994
Second Draft Report	March 29, 1994	March 30, 1994
Final Report	April 8, 1994	April 8, 1994

Lisa Read DATE 4/8/94

Lisa Read
Quality Assurance Representative

TABLE OF CONTENTS

Title/Cover Page..... 1

Good Laboratory Practice Compliance Statement..... 2

Quality Assurance Statement..... 3

Table of Contents..... 4

Summary..... 6

Introduction..... 7

Objective..... 7

Experimental Design..... 7

Materials and Methods..... 8

Results and Discussion..... 12

Conclusion..... 13

References..... 14

TABLES

Table 1. Summary of Analytical Chemistry Data..... 15

Table 2. Temperature of Water in the Test Chambers..... 16

Table 3. Dissolved Oxygen of Water in the Test Chambers..... 17

Table 4. pH of Water in the Test Chambers..... 18

Table 5. Cumulative Percent Mortality and Treatment-Related Effects..... 19

Table 6. LC50 Values..... 20

TABLE OF CONTENTS

PAGE 2

APPENDICES

Appendix I Conductivity, Hardness, Alkalinity and pH of Well Water Measured During the 4-Week Period Immediately Preceding the Test..... 21

Appendix II Pesticides, Organics, Metals and Other Inorganics Analyzed in Wildlife International Ltd. Well Water..... 22

Appendix III Measurement of DAN 216 in Dilute Aqueous Samples in Support of Acute Aquatic Toxicity Testing with the Fathead Minnow (*Pimephales promelas*)..... 24

Appendix IV LC50 Calculations..... 44

Appendix V Changes to Protocol..... 49

Appendix VI Personnel Involved in the Study..... 50

SUMMARY

SPONSOR:	Shell Development Company
CONTACT:	Ms. Diana C. L. Wong
LOCATION OF STUDY, RAW DATA AND FINAL REPORT:	Wildlife International Ltd. Easton, Maryland 21601

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER:	109A-108
TEST SUBSTANCE:	DAN 216
STUDY:	DAN 216: A 96-Hour Static-Renewal Acute Toxicity Test with the Fathead Minnow (*Pimephales promelas*)
NOMINAL TEST CONCENTRATIONS:	Negative Control, 0.19, 0.32, 0.54, 0.90 and 1.5 mg DAN 216/L
MEAN MEASURED TEST CONCENTRATIONS:	<LOD, 0.12, 0.21, 0.39, 0.65 and 1.1 mg DAN 216/L
TEST DATES:	Experimental Start - January 3, 1994 Biological Termination - January 7, 1994 Experimental Termination - March 10, 1994
LENGTH OF TEST:	96 Hours

96-HOUR LC50:	0.50 mg DAN 216/L
95% CONFIDENCE LIMITS:	0.39 and 0.65 mg DAN 216/L
NO MORTALITY CONCENTRATION:	0.39 mg DAN 216/L

TEST ORGANISM:	Fathead Minnow (*Pimephales promelas*)
SOURCE OF TEST ORGANISMS:	Wildlife International Ltd. 8651 Brooks Drive, Easton, MD 21601
AGE:	Juveniles
WET WEIGHT: STANDARD LENGTH:	Mean=0.36 g; Range=0.22 g to 0.58 g (10 Controls) Mean=34 mm; Range=29 mm to 40 mm (10 Controls)

INTRODUCTION

This study was conducted by Wildlife International Ltd. for Shell Development Company, at the Wildlife International Ltd. aquatic toxicology facility in Easton, Maryland. The test was conducted from January 3, 1994 to January 7, 1994. Raw data generated by Wildlife International Ltd. and an authenticated copy of the final report are filed under Project Number 109A-108 in archives located on the Wildlife International Ltd. site.

OBJECTIVE

The objective of this study was to evaluate the acute toxicity of DAN 216 to the fathead minnow (*Pimephales promelas*) during a 96-hour exposure period under static-renewal test conditions.

EXPERIMENTAL DESIGN

Fathead minnows were exposed to a geometric series of five test concentrations and a negative (well water) control under static-renewal test conditions for 96 hours. Two replicate test chambers were maintained in each treatment and control group, with 10 fish in each test chamber. Nominal test concentrations were selected in consultation with the Sponsor, and were based upon the results of a range finding test. Nominal test concentrations selected were 0.19, 0.32, 0.54, 0.90, and 1.5 mg DAN 216/L.

Immediately prior to initiating the test, fish were impartially removed from holding tanks in groups of two and distributed among the test chambers until each contained 10 fish. At approximately 24, 48 and 72 hours, new test solutions were prepared and fish were transferred to the new solutions. Observations of mortality and other clinical signs of toxicity were made at approximately 5, 24, 48, 72, and 96 hours after test initiation. Cumulative percent mortality values in each treatment group were used to calculate LC50 values and 95% confidence limits at 24, 48, 72, and 96 hours. The no mortality concentration was determined by visually examining the mortality data.

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, DAN 216: A 96-Hour Static-Renewal Acute Toxicity Test with the Fathead Minnow (*Pimephales promelas*). The protocol was based on procedures outlined in TSCA Title 40 of the Code of Federal Regulations, Part 797.1400 (1); and ASTM Standard E-729-88, *Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (2).

Test Substance

The test substance was received from Shell Development Company on September 28, 1993. The test substance was a white powder identified as: Shell Development Company; WRC TOX SAMPLE No. 1232, PSIS 1232-2; STORAGE CONDITIONS Ambient; COMPOSITION DAN 216 Secondary alcohol sulfate, sodium salt (100g); HAZARDS low toxicity, irritant; DATE DISPENSED 24 Sept. 93; RESPONSIBLE INDIVIDUAL H.C. Smith/EC-644; EXPIR. DATE Sept. 1994. The test substance was assigned Wildlife International Ltd. Identification Number WIL-2737 upon receipt. Information provided by the Sponsor indicated a purity of 98%.

Preparation of Test Concentrations

All glassware used to make the stock solutions was serially rinsed with reverse osmosis (RO) water, 70% isopropanol/30% water, and RO water prior to use. The primary stocks were prepared by adding the appropriate amount of test substance to RO water to achieve a concentration of 500 mg DAN 216/L. The stocks were heated to approximately 60°C and inverted to aid in solubilization of the test substance. New primary stock solutions were freshly prepared on Days 1, 2 and 3 of the test for renewal of test solutions. The test solutions were prepared by adding the appropriate amount of primary stock to dilution water in the test chambers. The test solutions were mixed by stirring with a glass rod for approximately 1 minute.

Test Organism

The fathead minnow (*Pimephales promelas*) was selected as the test species for this study. The fathead minnow was considered representative of an important group of aquatic vertebrates and was selected for use in this test based upon past history of use, and ease of culturing in the laboratory. Fish used in the test were juveniles obtained from cultures maintained by Wildlife International Ltd., Easton, Maryland.

The juvenile fathead minnows were held in cultures for at least 14 days prior to testing. The water used during holding was obtained from the same source as water used during the test. The test fish were acclimated to test conditions for approximately 55 hours prior to test initiation. Two mortalities occurred during acclimation, however, the remaining fish showed no signs of disease or stress.

All fish used in the test were from the same source and year class. The lengths and weights of 10 control fish were measured at the end of the test, and it was determined that the standard length of the longest fish measured was no more than twice the standard length of the shortest. The average length was 34 mm with a range of 29 mm to 40 mm, while the average weight was 0.36 g with a range of 0.22 g to 0.58 g. Instantaneous loading in the test chambers was calculated to be 0.24 grams of fish per liter of test water.

During holding, the fish were fed flaked fish food (Zeigler Brothers, Inc., Gardners, PA 17324) live artemia nauplii (Artemia Inc., Newark, CA 94560) and frozen brine shrimp (Kordon, Hayward, CA 94545). Feed was withheld during acclimation and the test.

Test Apparatus

The test chambers were Teflon® lined 25-L polyethylene tanks containing 15 L of test solution. The test chambers were impartially positioned in an environmentally controlled room designed to maintain a temperature of 22±1°C. The test chambers were labeled with the project number, test concentration, and replicate.

Dilution Water

The water used for holding, acclimation, and testing was freshwater obtained from a well 45 meters deep located on the Wildlife International Ltd. site. The well water is characterized as medium-hard water. The conductivity, hardness, alkalinity, and pH of the well water during the four-week period immediately preceding the test are presented in Appendix I.

The well water was passed through a sand filter to remove particles greater than approximately 25 μm, and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to delivery to the diluter system, the water again was filtered to remove microorganisms and particles. The results of analyses performed to measure the concentrations of pesticides and metals in well water used by Wildlife International Ltd. are presented in Appendix II. The dilution water was aerated overnight prior to use in the test.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during holding, acclimation, and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (e.g., Chroma 50). A photoperiod of 16 hours of light and 8 hours of darkness was controlled with an automatic timer. A 30-minute transition period of low light intensity was provided when lights went on and off to avoid sudden changes in lighting. Light intensity during the test was approximately 398 lux at the surface of the water.

Temperature was measured in both old and new solutions of all replicate test chambers at approximately 24-hour intervals using a calibrated hand-held thermometer. Temperature also was measured continuously in one negative control replicate using a Fulscope ER/C Recorder. The target test temperature during the study was 22±1°C. The pH and dissolved oxygen content of the water in both the old and new test solutions of all replicate test chambers were measured at approximately 24-hour intervals. Hardness, alkalinity, acidity, total organic carbon

(TOC), particulate matter and conductivity were also measured in the dilution water.

Measurements of pH were made using a Fisher Accument Model 915 pH meter, and dissolved oxygen was measured using a Yellow Springs Instrument Model 51B dissolved oxygen meter. Total organic carbon was measured with a Shimadzu Model 5000 TOC analyzer. Conductivity was measured using a Yellow Springs Instruments Model 33 Salinity-Conductivity-Temperature meter. Hardness, acidity, particulate matter and alkalinity measurements were made in accordance with *Standard Methods for the Examination of Water and Wastewater* (3).

Observations

All organisms were observed at approximately 5, 24, 48, 72 and 96 hours to evaluate the number of mortalities and the number of individuals exhibiting clinical signs of toxicity and abnormal behavior. The test organisms were transferred from the holding tanks into the test solutions and from old test solutions to new test solutions with an aquarium net. Dead organisms were removed from the test chambers every 24 hours. At test termination, 10 control fish were retained for measurements of length and weight.

Statistical Analyses

The data were analyzed using the computer program of C. E. Stephan (4). The program was designed to calculate the LC50 value and the 95% confidence interval by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (5, 6, 7). In this study, the binomial method was used to evaluate mortality at 24, 48, 72 and 96 hours. The no mortality concentration was determined by visual inspection of the mortality data.

Analytical Chemistry

Water samples were collected in each treatment and control: 1) at test initiation; 2) on Days 1, 2 and 3 in old and new solutions and 3) at test termination. If 100 percent mortality occurred in any treatment, then collection of water samples was terminated after the next sampling

interval. All water samples were collected from both replicates of each treatment and control group and composited for analysis. Samples were collected in glass containers with Teflon®-lined caps. Sample containers were rinsed with RO water, 70% isopropanol/30% water, and RO water prior to use. Samples of each primary stock were also collected. All samples were preserved with 1 percent formalin , pH adjusted to approximately 8.0 using NaOH and shipped to Shell Development Company, Houston, TX for analysis (Appendix III).

RESULTS AND DISCUSSION

Measurements and Test Concentrations

Results of analyses to measure concentrations of DAN 216 in water samples collected during the test are presented in Table 1 and in the analytical chemistry report (Appendix III). Nominal concentrations selected for use in this study were 0.19, 0.32, 0.54, 0.90 and 1.5 mg DAN 216/L. Samples collected on Day 0 had measured values that ranged from 76 to 103 percent of nominal values. After approximately 24 hours of exposure (old solutions), measured values ranged from 34 to 68 percent of nominal. Two of the water samples sent to Shell Development Company for analysis were broken upon receipt. Consequently, these samples were not included in the calculation of mean measured concentrations. When intact samples collected at test initiation, on Day 1, 2 and 3 (old and new), and at test termination were averaged, the mean measured concentrations for the study were 0.12, 0.21, 0.39, 0.65 and 1.1 mg DAN 216/L. Mean measured concentrations were used in the calculations of LC50 values. Results of analyses to measure concentrations of DAN 216 in primary stock solutions are presented in Appendix III.

Observations and Measurements

Measurements of temperature, dissolved oxygen, and pH are presented in Tables 2, 3 and 4, respectively. Temperatures were within the limits of the range established for the test, or 22±1°C. Dissolved oxygen concentrations exceeded 60% of saturation throughout the test. Measurements of pH during the test ranged from 8.2 to 8.5.

Daily observations of mortality and other signs of toxicity used to evaluate the effects of the test substance on fathead minnows are shown in Table 5. Test organisms in the control, 0.12, 0.21 and 0.39 mg DAN 216/L treatment groups appeared normal and healthy during the test. In the 0.65 mg DAN 216/L treatment group, mortality was 100% at 24 hours. In the 1.1 mg DAN 216/L treatment group, mortality was 95% at 5 hours and 100% at 24 hours. LC50 values and 95% confidence limits for the 24, 48, 72 and 96-hour time intervals were calculated from the mortality data (Table 6 and Appendix IV).

CONCLUSION

The 96-hour LC50 value for fathead minnows exposed to DAN 216 was 0.50 mg DAN 216/L. The lower and upper 95% confidence limits were 0.39 and 0.65 mg DAN 216/L, respectively. The no mortality concentration was 0.39 mg DAN 216/L.

REFERENCES

1 Title 40 of the Code of Federal Regulations, Part 797, Section 1400, *Fish Acute Toxicity Test*. July 1992.

2 American Society for Testing and Materials. 1988 *Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians*, ASTM E 729-88. Philadelphia, PA.

3 APHA, AWWA, WPCF, *Standard Methods for the Examination of Water and Wastewater*. 16th Edition, 1985. American Public Health Association, Washington, D.C.

4 Stephan, C.E. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. 1978.

5 Finney, D.J. *Statistical Methods in Biological Assay*, second edition. Griffin Press, London. 1971.

6 Thompson, W.R. *Bacteriological Reviews*, Vol. II, No. 2. June, 1947. Pp. 115-145.

7 Stephan, C.E. "Methods for Calculating an LC50", Pages 65-81, in *Aquatic Toxicology and Hazard Evaluations*, American Society for Testing and Materials. Publication Number STP 634 (1977), pp 65-84. Philadelphia, PA.

Table 1

Summary of Analytical Chemistry Data

Sponsor: Shell Development Company
Test Substance: DAN 216
Test Organism: Fathead Minnow *(Pimephales promelas)*
Dilution Water: Well Water

Nominal Concentration (mg DAN 216/L)	Measured Concentrations (mg DAN 216/L)								Grand Mean (mg DAN 216/L)
	Day 0 (New)	Day 1 (Old)	Day 1 (New)	Day 2 (Old)	Day 2 (New)	Day 3 (Old)	Day 3 (New)	Day 4 (Old)	
Control	<LOD[1]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
0.19	0.17	0.09	0.16	0.11	0.13	0.11	0.16	0.03	0.12
0.32	0.33	0.11	0.24	0.22	0.20	0.24	0.29	0.03	0.21
0.54	0.42	--[2]	0.45	0.41	0.37	0.35	0.50	0.24	0.39
0.90	0.69	0.61	--[3]	--[3]	--[3]	--[3]	--[3]	--[3]	0.65
1.5	1.14	--[2]	--[3]	--[3]	--[3]	--[3]	--[3]	--[3]	1.1

[1] The limit of detection (LOD) was 0.050 mg DAN 216/L.
[2] Sample broken during shipment.
[3] Sample not collected due to 100% mortality in this treatment group.

Table 2

Temperature of Water in the Test Chambers

Sponsor: Shell Development Co.
Test Substance: DAN 216
Test Organism: Fathead Minnow (*Pimephales promelas*)
Dilution Water: Well Water

Mean Measured Concentration (mg DAN 216/L)	Replicate	Temperature (°C)							
		Day 0[1] (New)	Day 1 (Old)	Day 1 (New)	Day 2 (Old)	Day 2 (New)	Day 3 (Old)	Day 3 (New)	Day 4 (Old)
Control	A[2]	22.3	21.9	21.9	21.7	21.9	21.8	21.8	21.9
	B	22.3	21.9	21.9	21.7	21.8	21.7	21.8	21.8
0.12	A	22.3	21.9	21.8	21.7	21.9	22.0	21.6	21.8
	B	22.3	22.0	21.8	21.8	21.9	21.9	21.6	21.8
0.21	A	22.3	21.9	21.7	21.8	21.8	21.7	21.7	21.8
	B	22.4	22.0	21.8	21.7	21.9	22.0	21.7	21.8
0.39	A	22.2	21.9	21.6	21.7	22.0	21.9	21.5	21.7
	B	22.1	21.9	21.5	21.7	22.0	22.0	21.4	21.6
0.65	A	21.2	21.4	--[3]	--	--	--	--	--
	B	21.5	21.7	--	--	--	--	--	--
1.1	A	22.2	21.9	--	--	--	--	--	--
	B	22.3	22.0	--	--	--	--	--	--

[1] The 0-hour dilution water measurements for hardness, alkalinity, conductivity, acidity, total organic carbon, and particulate matter were 124 mg/L as $CaCO_3$, 184 mg/L as $CaCO_3$, 320 μmhos/cm, 24 mg/L phenolphthalein acidity as $CaCO_3$, 1.0 mg/L and 0.67 mg/L, respectively.

[2] Temperature measured continuously during the test ranged from approximately 21.5 to 22.5°C.

[3] No temperature measurements were made due to 100% mortality in the replicate.

Table 3

Dissolved Oxygen[1] of Water in the Test Chambers

Sponsor: Shell Development Co.
Test Substance: DAN 216
Test Organism: Fathead Minnow (*Pimephales promelas*)
Dilution Water: Well Water

Mean Measured Concentration (mg DAN 216/L)	Replicate	Dissolved Oxygen (mg/L)							
		Day 0 (New)	Day 1 (Old)	Day 1 (New)	Day 2 (Old)	Day 2 (New)	Day 3 (Old)	Day 3 (New)	Day 4 (Old)
Control	A	8.5	8.0	8.5	7.6	8.6	7.8	8.7	8.4
	B	8.5	8.0	8.5	7.6	8.6	7.8	8.7	8.4
0.12	A	8.6	8.0	8.6	7.7	8.6	7.9	8.6	8.2
	B	8.6	7.8	8.6	7.8	8.6	7.9	8.6	8.2
0.21	A	8.6	7.8	8.6	7.7	8.6	7.8	8.6	8.1
	B	8.6	7.8	8.6	7.8	8.7	7.8	8.7	8.2
0.39	A	8.6	7.8	8.6	7.8	8.6	7.7	8.6	8.2
	B	8.6	7.8	8.6	7.8	8.6	7.7	8.6	8.0
0.65	A	8.6	7.7	--[2]	--	--	--	--	--
	B	8.6	7.9	--	--	--	--	--	--
1.1	A	8.6	8.1	--	--	--	--	--	--
	B	8.6	8.2	--	--	--	--	--	--

[1] A dissolved oxygen concentration of 5.2 mg/L represents 60% saturation at 22°C in freshwater.
[2] No DO measurements were made due to 100% mortality in the replicate.

Table 4

pH of Water in the Test Chambers

Sponsor: Shell Development Co.
Test Substance: DAN 216
Test Organism: Fathead Minnow (*Pimephales promelas*)
Dilution Water: Well Water

Mean Measured Concentration (mg DAN 216/L)	Replicate	pH							
		Day 0 (New)	Day 1 (Old)	Day 1 (New)	Day 2 (Old)	Day 2 (New)	Day 3 (Old)	Day 3 (New)	Day 4 (Old)
Control	A	8.5	8.2	8.4	8.3	8.4	8.4	8.4	8.3
	B	8.5	8.3	8.4	8.3	8.4	8.4	8.4	8.4
0.12	A	8.5	8.3	8.4	8.3	8.4	8.4	8.5	8.4
	B	8.5	8.3	8.5	8.3	8.5	8.4	8.5	8.4
0.21	A	8.5	8.3	8.5	8.3	8.5	8.4	8.5	8.4
	B	8.5	8.3	8.5	8.3	8.5	8.4	8.5	8.4
0.39	A	8.5	8.4	8.5	8.3	8.5	8.4	8.5	8.4
	B	8.5	8.4	8.4	8.3	8.5	8.4	8.5	8.4
0.65	A	8.5	8.3	--[1]	--	--	--	--	--
	B	8.5	8.4	--	--	--	--	--	--
1.1	A	8.5	8.4	--	--	--	--	--	--
	B	8.5	8.4	--	--	--	--	--	--

[1]No pH measurements were made due to 100% mortality in the replicate.

Table 5
Cumulative Percent Mortality and Treatment-Related Effects[1]

Sponsor: Shell Development Company
Test Substance: DAN 216
Test Organism: Fathead Minnow *(Pimephales promelas)*
Dilution Water: Well Water

Mean Measured Concentration (mg DAN 216/L)	Rep.	No Exposed	5 Hours		24 Hours		48 Hours		72 Hours		96 Hours		Cumulative Percent Mortality
			No. Dead	Effects	No. Dead	Effects	No. Dead	Effects	No. Dead	Effects	No. Dead	Effects	
Negative Control	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0%
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
0.12	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0%
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
0.21	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0%
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
0.39	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0%
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
0.65	A	10	0	10 AN	10	--	10	--	10	--	10	--	100%
	B	10	0	10 AN	10	--	10	--	10	--	10	--	
1.1	A	10	9	1 AN	10	--	10	--	10	--	10	--	100%
	B	10	10	--	10	--	10	--	10	--	10	--	

[1] Observed Effects: AN = Appears Normal; - Not applicable due to total mortality.

Table 6
LC50 Values

Sponsor: Shell Development Company
Test Substance: DAN 216
Test Organism: Fathead Minnow *(Pimephales promelas)*
Dilution Water: Well Water

Time	LC50 (mg DAN 216/L)	Lower 95% Confidence Limits (mg DAN 216/L)	Upper 95% Confidence Limits (mg DAN 216/L)	Statistical Method
24 Hours	0.50	0.39	0.65	Binomial
48 Hours	0.50	0.39	0.65	Binomial
72 Hours	0.50	0.39	0.65	Binomial
96 Hours	0.50	0.39	0.65	Binomial

APPENDIX I

Conductivity, Hardness, Alkalinity, and pH of Well Water Measured During the 4-Week Period Immediately Preceding the Test

Sponsor: Shell Development Company
Test Substance: DAN 216
Test Organism: Fathead Minnow *(Pimephales promelas)*
Dilution Water: Well Water

	Mean	Range
Conductivity (μmhos/cm)	313 (n = 4)	310 - 320
Hardness (mg/L as $CaCO_3$)	134 (n = 4)	132 - 136
Alkalinity (mg/L as $CaCO_3$)	177 (n = 4)	168 - 186
pH	8.1 (n = 4)	8.0 - 8.2

APPENDIX II

Pesticides, Organics, Metals and Other Inorganics Analyzed in Wildlife International Ltd. Well Water[1]

Sponsor: Shell Development Company
Test Substance: DAN 216
Test Organism: Fathead Minnow *(Pimephales promelas)*
Dilution Water: Well Water

ANALYSIS	MEASURED CONCENTRATION
Organophosphorus & Organonitrogen Pesticides	
Azodrin (Monochrotophos)	< 0.250 µg/L
Bolstar	< 0.250 µg/L
Chlorpyrifos	< 0.250 µg/L
Coumaphos	< 0.250 µg/L
Demeton	< 0.250 µg/L
Diazinon	< 0.250 µg/L
Dichlorvos	< 0.250 µg/L
Dimethoate	< 0.250 µg/L
Disulfoton	< 0.250 µg/L
EPN	< 0.250 µg/L
Ethoprop	< 0.250 µg/L
Fenthion	< 0.250 µg/L
Fensulfothion	< 0.250 µg/L
Guthion (Methyl Azinphos)	< 0.250 µg/L
Malathion	< 0.250 µg/L
Merphos	< 0.250 µg/L
Mevinphos	< 0.250 µg/L
Naled	< 0.250 µg/L
Methylparathion	< 0.249 µg/L
Parathion	< 0.250 µg/L
Phorate	< 0.250 µg/L
Ronnel	< 0.250 µg/L
Stirofos	< 0.250 µg/L
Sulfotepp	< 0.250 µg/L
Tepp	< 0.250 µg/L
Tokuthion	< 0.250 µg/L
Trichloronate	
Metals and Other Inorganics	
Aluminum	< 40.0 µg/L
Arsenic	< 2.5 µg/L
Beryllium	< 5.0 µg/L
Cadmium	< 5.0 µg/L
Calcium	35800 µg/L
Chromium	< 10.0 µg/L
Copper	< 5.0 µg/L
Iron	< 45.0 µg/L
Lead	< 2.0 µg/L
Magnesium	13.7 mg/L
Manganese	< 5.0 µg/L
Mercury	< 0.20 µg/L
Nickel	< 15.0 µg/L
Potassium	6160 µg/L
Selenium	< 2.5 µg/L
Silver	< 5.0 µg/L
Sodium	22000 µg/L
Zinc	< 20.0 µg/L
Molybdenum	< 10.0 µg/L

[1] Analyses performed by Environmental Science & Engineering, Inc., Gainesville, Florida for samples collected on November 11, 1993.

APPENDIX II (Continued)

Pesticides, Organics, Metals, and Other Inorganics Analyzed in Wildlife International Ltd. Well Water[1]

Sponsor: Shell Development Company
Test Substance: DAN 216
Test Organism: Fathead Minnow *(Pimephales promelas)*
Dilution Water: Well Water

ANALYSIS	MEASURED CONCENTRATION
Miscellaneous Measurements	
Total Dissolved Solids	267 mg/L
Ammonia Nitrogen	< 0.050 mg/L
Total Organic Carbon[2]	1.7 mg/L
Total Cyanide	< 0.003 mg/L
Organochlorines and PCBs	
Aldrin	< 0.005 µg/L
Alpha BHC	< 0.005 µg/L
Beta BHC	< 0.005 µg/L
Delta BHC	< 0.005 µg/L
Gamma BHC (Lindane)	< 0.005 µg/L
Chlordane	< 0.025 µg/L
DDD, pp'	< 0.005 µg/L
DDE, pp'	< 0.005 µg/L
DDT, pp'	< 0.005 µg/L
Dieldrin	< 0.005 µg/L
Endosulfan, A	< 0.005 µg/L
Endosulfan, B	< 0.005 µg/L
Endosulfan Sulfate	< 0.005 µg/L
Endrin	< 0.005 µg/L
Endrin Aldehyde	< 0.005 µg/L
Heptachlor	< 0.005 µg/L
Methoxychlor	< 0.005 µg/L
Heptachlor Epoxide	< 0.500 µg/L
Toxaphene	< 0.100 µg/L
PCB-1016	< 0.100 µg/L
PCB-1221	< 0.100 µg/L
PCB-1232	< 0.100 µg/L
PCB-1242	< 0.100 µg/L
PCB-1248	< 0.100 µg/L
PCB-1254	< 0.100 µg/L
PCB-1260	
Chlorophenoxy Acid Herbicides	
2,4-D, Total	< 0.025 µg/L
2,4-DB	< 0.028 µg/L
2,4,5-T Water	< 0.025 µg/L
2,4,5-TP/Silvex	< 0.021 µg/L
Dalapon	< 0.025 µg/L
Dicamba (Banvel)	< 0.023 µg/L
Dichloroprop	< 0.020 µg/L
Dinoseb	< 0.021 µg/L
MCPA	< 0.540 µg/L
MCPP	< 0.440 µg/L

[1] Analyses performed by Environmental Science & Engineering, Inc., Gainesville, Florida for samples collected on November 11, 1993.
[2] Analyses performed by Wildlife International Ltd. for samples collected on February 3, 1994.

APPENDIX III

Measurement of DAN 216 in Dilute Aqueous Samples in Support of Acute Aquatic Toxicity Testing with the Fathead Minnow (*Pimephales promelas*)

Data Report

Measurement of DAN 216 in Dilute Aqueous Samples in Support of Acute Aquatic Toxicity Testing with The Fathead Minnow (Pimephales promelas)

Performed at

Shell Development Company
Westhollow Research Center
3333 Highway 6 South
Houston, Texas 77082

in Accordance with United States Environmental Protection Agency (EPA) Toxic Substances Control Act (TSCA), Good Laboratory Practice Standards (40 CFR, Part 792) in support of the aquatic toxicity test with the Fathead Minnow conducted at:

Wildlife International, Ltd.
8598 Commerce Drive
Easton, Maryland 21601

WRC Tox Study No. 1232

Compliance Statement

Title: Measurement of DAN 216 Dilute Aqueous Samples in Support of Acute Aquatic Toxicity Testing with The Fathead Minnow

Report: WRC Tox Sample No. 1232

The study named above was reviewed according to the Good Laboratory Practices regulations outlined by the Environmental Protection Agency (EPA) in 40 CFR Part 792 under the authority of the Toxic Substances Control Act. Based upon the Quality Assurance inspection reports provided, the practices described in the report were found to be in compliance with the above regulations, with the exception of the following:

1. No expiration dates were found on the reagents used during the course of the phase-study. Expiration dates have been added to the labels on the reagents.

2. Milli Q maintenance records were not completely documented.

3. The DAN samples were stored in the shipping cooler received from the contract laboratory. This adequately separates the samples from potential contamination in the laboratory.

4. The Milli-Q water system filters are changed every six months. This routine maintenance has not been documented in the past.

5. The sample lines of the AutoTrace Workstation were flushed out using H_2O on the first run and 50:50 Methanol:IPA on the second run rather than the 50:50 IPA:H_2O documented in the method.

The above exceptions to the requirements of the Good Laboratory Practice regulations have been investigated and are judged not to impact the quality or integrity of the effected study.

H. C. Smith
Analytical Study Director
Analytical Chemistry R&D - WRC
Shell Development Company

Date

Quality Assurance Statement

Report: Measurement of DAN 216 in Dilute Aqueous Samples in Support of Acute Aquatic Toxicity Testing with The Fathead Minnow (Pimephales promelas)

In accordance with Good Laboratory Practice Standards (40 CFR, Part 792), this study has been monitored by the Westhollow Research Center's (WRC) Quality Assurance Unit. Quality assurance audits were made and reported to the Analytical Study Director and Management as noted below:

DATE INSPECTED	DATE REPORTED
February 1, 1994	February 15, 1994
February 9, 1994	March 2, 1994

The final report accurately describes the methods and procedures used in the study and, to the best of my knowledge, accurately reflects the raw data obtained.

Darryl L Box

D. L. Box
Good Laboratory Practice Coordinator
Analytical Chemistry R&D - WRC
Shell Development Company

March 21, 1994
Date

INTRODUCTION

The chemical analytical work in this report is a component of the aquatic toxicological testing performed at Wildlife International. Chemical analyses of aqueous samples and primary stock solutions, for the determination of concentrations of DAN 216, were performed using the Standard Operating Procedure (SOP) 00014, Determination of Trace Amounts of Secondary Alkyl Sulfate (SAS) in Aqueous Samples. This procedure was approved by Analytical Management on October 20, 1993 and by the GLP Coordinator on October 10, 1993.

Experimental Initiation Date: February 1994
Experimental Completion: March 1994

The Test Substance Identification

Test Substance:	DAN 216
Test Substance PSIS #:	1232-3
Test Substance Tox #:	1232
Test Substance Batch Reference #:	22056-62
Test Substance Shell Toxicology Protocol #:	SETP-93-007
Test Substance Purity:	98%
Test Substance Analysis:	

The percent purity is based on process knowledge and analytical characterization performed prior to dispensing of the test substance. A concentration of 99% total active matter was obtained by direct measurement while a concentration of 98% was obtained by subtracting the concentrations of sodium sulfate, olefinic material and secondary alcohol from 100%. These concentrations lie within the experimental error of the various techniques. The concentration of 98% was used as the actual test substance purity.

METHOD DESCRIPTION

Method Abstract

This method is used to determine DAN 216 in aqueous solutions at the parts per billion level using solid phase extraction (SPE) to concentrate the sample for analysis by HPLC using an evaporative light scattering detector (ELSD). For a more specific description of the procedure used during this analysis, please refer to SOP 00014-93 and the deviations to the analytical method found as an attachment to this report. This method quantifies the total DAN 216 content and does not distinguish various carbon chain distributions. No extraction step was needed to analyze the stock solution.

Quality Control

The water samples were concentrated using solid phase extraction in batches of approximately six samples. Primary stock solutions were prepared for analysis by making weight /volume dilutions. A seven point multi-level calibration was generated for each sequence of samples analyzed using the HPLC/ELSD. The seven point multi-level calibration was regenerated after any suspected computer or instrument error. At the start of each HPLC run, a correlation coefficient exceeding 0.99 was generated for all sample sequences using the seven-point multi-level instrument calibration.

A 1.7 µg/ml spike was prepared from a 98% pure DAN 216 standard. Upon running the spike, poor recovery was observed. The spike was rerun and a value of 1.3 µg/ml was obtained, resulting in a recovery of 76 %.

Calculations

Instrument response, and thus sample concentrations, were determined by calibration with multi-level calibration standards with quadratic curve fit, the x and y axes of the calibration curve being converted to the natural logarithms of the values, and by using the equations found in the "Calculations" section of the attached SOP. Standards were prepared so that the calibration curve, generated at the initiation of each sample run, bracketed the expected sample concentration range. Original aqueous sample concentrations were then determined using the extraction and pre-injection volumes.

$$RF(y) = AREA\ (y) / AMOUNT\ (y)$$

where,

RF is the absolute response factor for peak y.

AREA is the peak area of peak y in the standard.

AMOUNT is the amount, in µg/ml, of y in the standard.

The amount of y in the unknown is calculated using the following equation:

$$ppm(y) = [(AREA(y) \times SPE\ PREP\ VOL \times 0.01)/ (RF(y) \times SX\ WT \times TOTAL\ MLS\ SAMPLE) \times 100]$$

Limit of Detection

A water sample equivalent LOD of 50 ppb has been established for this method. The analyte provided a detector response of greater than 3:1 s/n to be considered detected. The water sample equivalent LOD was found using the above equation.

ANALYTICAL RESULTS

Test Samples

Analytical results can be found in the attached Table. A record of the condition of these samples, upon receipt from Wildlife International, can be found in LR 22053. Two of the 40 samples shipped were found to be broken. The sample bottles were also filled in a manner making opening the bottles without spilling a portion of the contents difficult. Wildlife International has been informed not to fill the sample bottles as full during subsequent shipments.

Archiving

Study records will be maintained by Westhollow Research Center in the WRC Analytical Special Collection of Files-Technical Information Center according to SOP P018. Study records will include, but are not limited to:

- Verified copies of all generated raw data and documentation records
- Verified copy of signed and approved Analytical Chemistry Method and subsequent amendments and deviations
- All correspondence, memos, or notes pertaining to the study
- Verified copy of the final data report
- Test substance records, including receipt and inventory, and physical and chemical characterization data

As sponsor of this study, Shell Development Company is additionally responsible for archiving an aliquot of the test substance.

Westhollow Research Center Tox Sx. #: 1232

The following are deviations to the Analytical Method (SOP 00014-93)

1. Change: A secondary alkyl sulfate (SAS) concentration of 98 %, rather than the 99% purity stated in the method, was used to prepare the standard.

   Reason: Although a concentration of 99% was obtained by direct measurement, 98% was obtained by subtraction of all known impurities from 100%. The additive errors of the methods used to measure the impurities are believed to be less than that of the method used to directly measure active matter.

2. Change: A pH meter and Rainin Microman 250µl adjustable positive displacement pipet with stainless steel plungers were used during the analyses described by this method.

   Reason: The two pieces of equipment, omitted from the apparatus list found in the method, are necessary to obtain optimal recovery of the test substance.

3. Change: During sample preparation a pH adjustment of 8 is made to the samples.

   Reason: Experimentation has proven that the pH adjustment results in better recovery of the test substance dilutions.

4. Change: Using the automated system, the sample lines of the AutoTrace Workstation are cleaned using a Milli-Q water wash followed by 50:50 IPA:Methanol then again by Milli-Q water.

   Reason: The additional and final Milli-Q washing step was included to provide greater assurance that the sample lines are clean and contamination is avoided.

5. Change: After samples are extracted, sonicated, and vortexed, 100 µl of the sample is diluted with 100 µl of Milli-Q water. Calibration standards are also diluted.

   Reason: The dilution step is performed to assure that the signal remains within the dynamic range of the instrument.

6. Change: A Phenomenex HPLC column was used , rather than a Supelco LC-18 (see Attachment).

   Reason: The smaller internal diameter of the column avoids solvent waste.

7. Change: The scope of SOP 00014-93 states that the method is intended for determination of C_{14} SAS in aqueous sample matrices.

   Reason: Further testing has proven that the method found in SOP 00014-93 is applicable to analyses of C_{16} SAS compounds as well as C_{14} compounds.

Approved:

Harriet Smith

H. C. Smith
Analytical Study Director
Analytical Chemistry R&D - WRC
Shell Development Company

March 10 1994
Date

Sheila Dubey

Sheila Dubey
Sr. Research Chemist
Analytical Chemistry - Organic and Detergent Analysis
Shell Development Company

3/10/94
Date

SPONSOR REPORT APPROVAL

Report: Measurement of DAN 216 in Dilute Aqueous Samples in Support of Acute Aquatic Toxicity Testing with The Fathead Minnow

Shell Toxicology Protocol No.: SETP 93-007

This report has been reviewed and found to be satisfactory.

D. C. L. Wong
Sponsor Representative
Environmental RD&T - WRC
Shell Development Company

3/21/94
Date

DAN 216 Fathead minnow acute toxicity test

Compound	Sample ID	Sample #	Nominal Conc (ppm)	Measured Conc (ppm)
DAN 216	Day 0	109A-108-1	0.00	ND
	Day 0	109A-108-2	0.19	0.17
	Day 0	109A-108-3	0.32	0.33
	Day 0	109A-108-4	0.54	0.42
	Day 0	109A-108-5	0.90	0.69
	Day 0	109A-108-6	1.50	1.14
	Day 1 old	109A-108-7	0.00	ND
	Day 1 old	109A-108-8	0.19	0.09
	Day 1 old	109A-108-9	0.32	0.11
	Day 1 old	109A-108-10	0.54	broken
	Day 1 old	109A-108-11	0.90	0.61
	Day 1 old	109A-108-12	1.50	broken
	Day 1 new	109A-108-13	0.00	ND
	Day 1 new	109A-108-14	0.19	0.16
	Day 1 new	109A-108-15	0.32	0.24
	Day 1 new	109A-108-16	0.54	0.45
	Day 2 old	109A-108-19	0.00	ND
	Day 2 old	109A-108-20	0.19	0.11
	Day 2 old	109A-108-21	0.32	0.22
	Day 2 old	109A-108-22	0.54	0.41
	Day 2 new	109A-108-25	0.00	ND
	Day 2 new	109A-108-26	0.19	0.13
	Day 2 new	109A-108-27	0.32	0.20
	Day 2 new	109A-108-28	0.54	0.37
	Day 3 old	109A-108-29	0.00	ND
	Day 3 old	109A-108-30	0.19	0.11
	Day 3 old	109A-108-31	0.32	0.24
	Day 3 old	109A-108-32	0.54	0.35
	Day 3 new	109A-108-33	0.00	ND
	Day 3 new	109A-108-34	0.19	0.16
	Day 3 new	109A-108-35	0.32	0.29
	Day 3 new	109A-108-36	0.54	0.50
	Day 4	109A-108-37	0.00	ND
	Day 4	109A-108-38	0.19	0.03
	Day 4	109A-108-39	0.32	0.03
	Day 4	109A-108-40	0.54	0.24
	Day 0 stock	109A-108-97	500	436
	Day 1 stock	109A-108-98	500	463
	Day 2 stock	109A-108-99	500	441
	Day 3 stock	109A-108-100	500	*

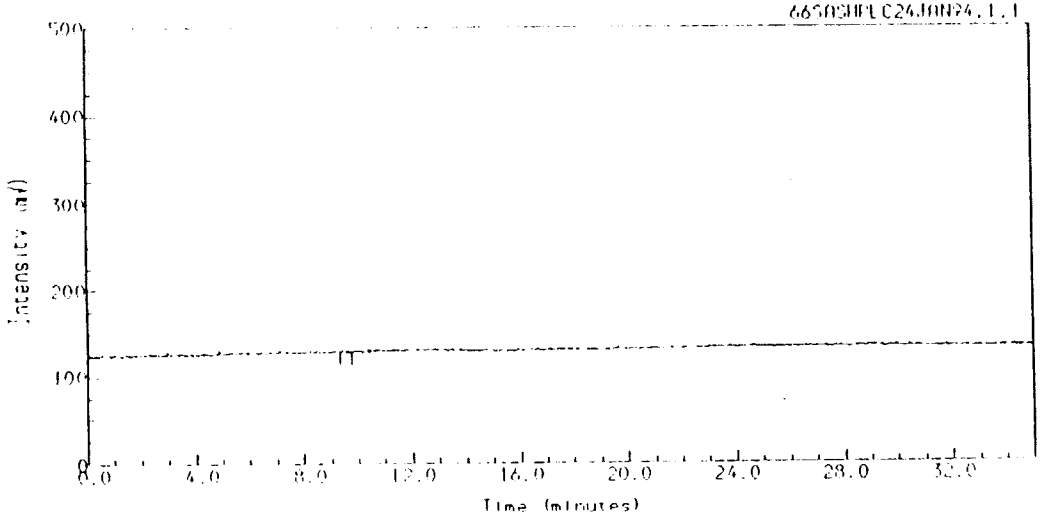
ND = not detected, <LOD

* sample not analyzed RE Diane Wong's instructions
Sheila Bailey
3/10/94

February 22,1994

SASHPLC24JAN94,1.1
LR Sample Prep =

BLANK
sx amount = 1.000000
PREP VOL = 1.00000E+00 (rup1) FUDGE FACTOR = 1.00000E-02 (rup2) ORIG VOL OF SX = 1.00000E+00 (rup3)



66 SASHPLC24JAN94,1.1

RT area counts PPM (UNREAL NAME

TOTAL AMOUNT 4.4502

Column: IB-Sil 5u ODS (C18)
250 x 3.2 mm ID
Phenomenex #00G-0082-R0
100ul Injection size
Column temp 40 C
Precolumn filter Upchurch #A-318 with 5um frit
Detectors:
1. ACS MODEL 750/14 MASS DETECTOR (ELSD)
NEBULIZER TEMP 65oC
NEBULIZING GAS = N2 @ 15 PSI
(Primary regulator set to 20 PSI
Secondary regulator set to 15 PSI)
TIME CONSTANT = 10 SEC
PHOTOMULTIPLIER SENSITIVITY = 6

Gradient:

time	B	C	flow
0	80	20	0.35
3	80	20	
15	20	80	B = H2O
20	0	100	C = IPA
26	80	20	
40	80	20	

Samples diluted with Milli-Q water before injection (100 ul sx + 100 ul Milli-Q water)

METHOD NAME: M66SAS
CALIBRATION NAME: C66SAS14
VG ARTIST NAME: M66SAS
RUN SEQUENCE FILE: R66SAS14
useraccess routine: 964
Date Reported:
24-JAN-1994 08:24
Date Aquired:
24-Jan-1994 07:48

COMPLETED

SHELL DEVELOPMENT COMPANY
WESTHOLLOW RESEARCH CENTER
ANALYTICAL CHEMISTRY R&D

SOP NUMBER: SOP 00014-93
REVISION: 0

TITLE: Determination of Trace Amounts of Secondary Alkyl Sulfate (SAS) in Aqueous Samples.

AUTHORS: TL Bruin, ST Dubey

SCOPE: This method is intended for the determination of parts per million (ppm) of C_{14} SAS in aqueous sample matrixes using solid phase extraction (SPE) to concentrate the sample for analysis by HPLC using an evaporative light scattering detector (ELSD).

PRINCIPAL AUTHOR: Terri Bruin 10/15/93
Date

QUALITY ASSURANCE REVIEW: Donald L Box 10/19/93
D.L. Box
G.L.P. Coordinator
Analytical Chemistry R&D
Date

MANAGEMENT APPROVAL: M Baker 20 Oct '93
M.D. Baker
Research Manager
Date

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Copies: Author(s)
Reviewers
Analytical Study Directors
Chemical Products Analysis Manager

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REAGENTS:

(a) ISOPROPANOL : HPLC grade

(b) WATER : Milli-Q Plus system with ion-exchange cartridge replaced when water resistivity falls below 18.2 Megohm-cm

(c) SECONDARY ALCOHOL SULFATE STANDARD : C_{14} SAS with purity of 99+%

(d) FORMALIN SOLUTION : ~35% formaldehyde in water, reagent grade

APPARATUS:

(a) Volumetric Pipets : 1 ml, 2 ml, 3 ml, 4 ml, 9 ml, 10 ml class A.

(b) Volumetric Flasks : 10 ml, class A.

(c) Graduated Cylinder : 1000 ml, stoppered, class A.

(d) Glass Vials : 2 dram, 4 dram, 6 dram, 8 dram, borosilicate glass.

(e) Glass Culture Tubes : borosilicate glass, 16 x 100 mm

(f) Parafilm "M" Laboratory Film

(g) Analytical Balance : Mettler AE 163, or equivalent.

(h) Syringe : 500 µl Hamilton high precision, glass.

(i) Varian Star HPLC system, equipped with a 9010 pump and a Shimadzu Sil-9A Auto Injector equipped with a 100 µl sample loop. Equivalent equipment may be substituted.

(j) ACS Evaporative Light Scattering Detector, model 750/14 (ELSD).

(k) VG Multichrom data collection system and software. Substitutes are acceptable.

(l) Genie Vortex mixer, or equivalent.

(m) Branson 1200 sonicator, or equivalent.

(n) SPE Cartridge : Octyl, 1000 mg, 8 ml reservoir

(o) 12 port solid phase extraction manifold or an AutoTrace SPE Workstation (Zymark)

(p) Pierce Reacti-Therm heating/stirring module

(q) Pierce Reacti-Vap evaporation module : 9 port

(r) Pierce Reacti-Block : Model B-1

(s) HPLC Column : Supelco LC-18, 25 cm x 4.6 mm ID, 5 micron, Cat# 5-8298

CALIBRATION STANDARDS PREPARATION:

(a) 0.1 gram C_{14} SAS is weighed, to the nearest 0.1 milligram, into a 10 ml volumetric flask. The flask is then filled to the mark with a

solution of 50 parts Milli-Q water (with pH adjusted to ~8) and 50 parts isopropanol. The solution is sonicated to help dissolve the SAS. This results in a solution of 10,000 µg/ml SAS.

(b) 2.0 mls of the 10,000 µg/ml solution is pipeted into an 8 dram vial. 18.0 mls of the 50:50 IPA:Water solution is then added. This results in a solution of 1000 µg/ml concentration.

(c) 3.0 mls of the 1000 µg/ml solution is pipeted into a 2 dram vial. 1.0 ml of the 50:50 IPA:Water solution is then added. This results in a solution of 750µg/ml concentration.

(d) 2.0 mls of the 1000 µg/ml solution is pipeted into a 2 dram vial. 2.0 mls of the 50:50 IPA:Water solution is then added. This results in a solution of 500 µg/ml concentration.

(e) 1.0 ml of the 1000 µg/ml solution is pipeted into a 2 dram vial. 4.0 mls of the 50:50 IPA:Water solution is then added. This results in a solution of 200 µg/ml concentration.

(f) 1.0 ml of the 1000 µg/ml solution is pipeted into a 6 dram vial. 9.0 mls of the 50:50 IPA:Water solution is then added. This results in a solution of 100 µg/ml concentration.

(g) 2.0 mls of the 100 µg/ml solution is pipeted into a 2 dram vial. 2.0 mls of the 50:50 IPA:Water solution is then added. This results in a solution of 50 µg/ml concentration.

(h) 1.0 ml of the 100 µg/ml solution is pipeted into a 6 dram vial. 9.0 mls of the 50:50 IPA:Water solution is then added. This results in a solution of 200 µg/ml concentration.

SAMPLE PREPARATION:

(a) <u>SPE/Extraction of SAS using Manual System</u>:

1. Cleaning glassware: All glassware should be free of surfactant. No detergent should be used for cleaning glassware. The recommended cleaning procedure involves washing with hot tap water followed by reagent grade IPA, MeOH, then Milli-Q water, followed by drying in an oven.

2. Condition SPE cartridge: Octyl SPE cartridge is conditioned with 10 mls IPA followed by 10 mls of water at a flow of approximately 5 mls/min. ***IMPORTANT***: the cartridge is NOT allowed to dry between the conditioning step and the application of the sample.

3. An appropriate volume of aqueous sample is then loaded onto the column at a flow rate of approximately 5 mls/min. The volume of sample loaded onto the column depends on the concentration of SAS in the sample. The lowest desired amount of SAS to be loaded on the SPE cartridge should be 15 ug. Approximately 300 mls is needed for a sample with a SAS concentration of 0.05 ppm.

4. The SPE cartridge is rinsed with 10 mls of water, then allowed to dry under vacuum for 10 minutes.

5. SAS is eluted from the cartridge into an appropriate size vial with 12 mls IPA.

6. The IPA is evaporated off using a heating block at 60°C with a gentle nitrogen stream. The walls of the vial are washed down 2-3 times with IPA and evaporated to dryness.

7. The residue is redissolved in 250 µl 50:50 IPA:Water using a 500 µl Hamilton high precision syringe. The sample is sonicated for several minutes to help dissolve, then vortexed to mix. This solution is then transferred to a autosampler microvial for injection onto the HPLC.

(b) SPE/Extraction of SAS using Automated System:

1. The solvent syringe of the Zymark AutoTrace SPE Workstation is purged using the purge program located on floppy.

2. The sample lines of the AutoTrace Workstation are cleaned using the clean sample lines program located on floppy. Samples lines are cleaned using Milli-Q water followed by 50:50 IPA:Water.

3. The sample is loaded onto the SPE cartridge using the program listed in figure 1. Test tubes of 16 x 100mm size are loaded to collect sample. Prior to loading sample, ensure that the waste container has sufficient space for collecting waste.

4. After the sample program has completed, the test tubes containing sample are transferred to a heating block set at 60°C and the solvent evaporated under a gentle nitrogen stream. The walls of the test tube are rinsed 2-3 times with IPA and allowed to evaporate to dryness.

5. The residue is redissolved in 250 µl 50:50 IPA:Water using a Hamilton 500 µl high precision syringe. The test tube is capped using parafilm, and the sample is sonicated for several minutes to help dissolve, then vortexed to mix. This solution is then transferred to a autosampler microvial for injection onto the HPLC.

HPLC CONDITIONS:

Column: Supelco LC-18, 25cm x 4.6mm ID, 5 micron
Column Temperature: 40°C
Injection size: 100 µl
Detector: ELSD
Nebulizer temperature: 70°C
Nebulizing gas: Nitrogen at 18 psig
Time Constant: 10 seconds
Photomultiplier Sensitivity: 6

Gradient:

Time	Water	IPA	Flow
0	80	20	0.8 ml/min
3	80	20	
15	20	80	
20	0	100	
25	0	100	
26	80	20	
35	80	20	

Zymark AutoTrace Extraction WorkStation 1.20

AutoTrace Extraction Procedure : SA8
Estimated Time for samples : 213.2 minutes
Date : 1 Sep 93

Step 1 : Process 6 samples using the following procedure:
Step 2 : Condition column with 10 ml of IPA into AQUEOUS WASTE
Step 3 : Condition column with 10 ml of H2O into AQUEOUS WASTE
Step 4 : Load 300 ml of sample onto column
Step 5 : Rinse column with 10 ml of H2O into AQUEOUS WASTE
Step 6 : Dry Column with gas for 10 minutes
Step 7 : Soak and Collect 8 ml fraction using IPA
Step 8 : Soak and Collect 3.5 ml fraction using IPA
Step 9 : Collect 0.5 ml fraction into sample tube using IPA
Step 10 : Wash syringe with 5 ml of H2O
Step 11 : END

SETUP PARAMETERS

AutoTrace Extraction Workstation

FLOW RATES

Cond Flow:	5.0	ml/min
Load Flow:	5.0	ml/min
Rinse Flow:	10.0	ml/min
Elute Flow:	1.00	ml/min
Cond Air Push:	10.0	ml/min
Rinse Air Push:	10.0	ml/min
Elute Air Push:	3.00	ml/min

SPE PARAMETERS

Push Delay:	5	sec
Air Factor:	1.0	
Autovasm Vol:	1.00	ml

WORKSTATION PARAMETERS

Max. Elution Vol:	12.0	ml
Exhaust Fan on:	Y	Y=YES N=NO
Beeper on:	Y	Y=YES N=NO

Zymark AutoTrace Extraction WorkStation 1.20

AutoTrace Extraction Procedure : SA8
Date : 1 Sep 93

NAME SOLVENTS

Solvent 1 : MEOH
Solvent 2 : H2O
Solvent 3 : HCL/H2O
Solvent 4 : MEOH/H2O
Solvent 5 : IPA

Figure 1

A typical chromatogram of a 0.05 ppm aqueous solution of C_{16} SAS prepared as described above is shown in figure 2.

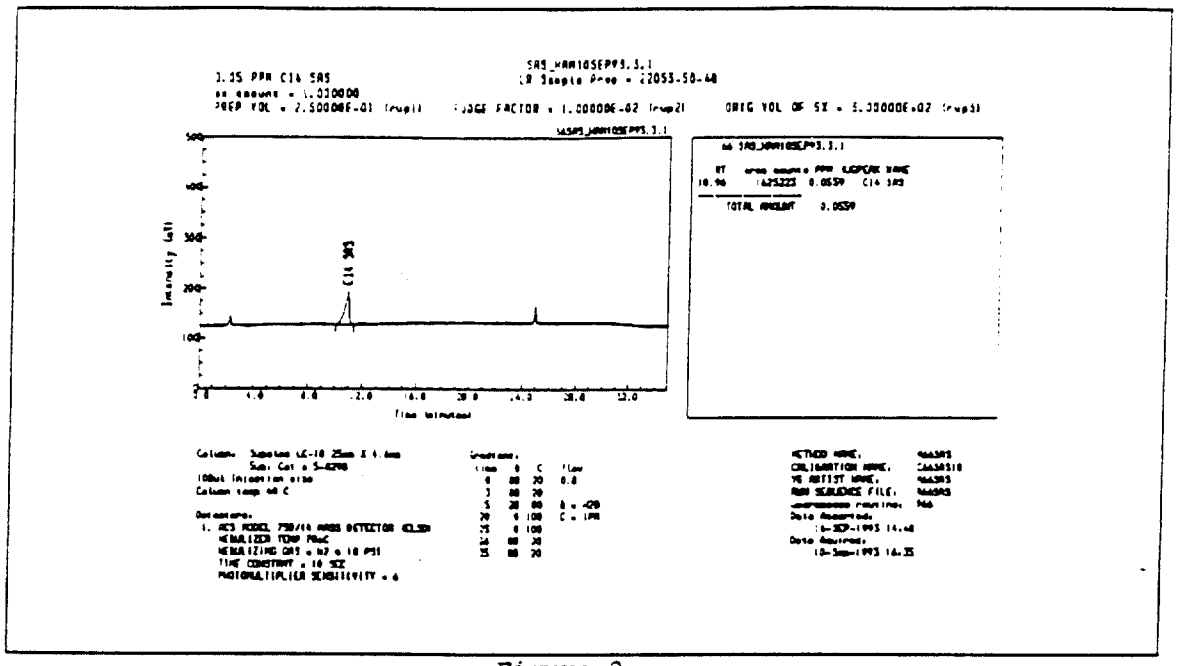

Figure 2

CALCULATIONS:

Calculations are performed using the VG Multichrom External Standard Percent calibration.

The response is determined by calibration with multilevel calibration standards, with quadratic curvefit (with a natural log X and Y transform) and using the following equation (a typical calibration curve is shown in figure 3):

$$RF(y) = AREA(y) \quad AMOUNT(y)$$

where,

RF is the absolute response factor for peak y.

AREA is the peak area of peak y in the standard.

AMOUNT is the amount, in µg/ml, of y in the standard.

The amount of y in the unknown is calculated using the following equation:

$$ppm(y) = \frac{AREA(y) \times SPE\ PREP\ VOL \times 0.01}{RF(y) \times SX\ WT \times TOTAL\ MLS\ SAMPLE} \times 100$$

where,

ppm(y) is the amount of y, in parts per million (µg/ml), in the sample

AREA is the peak area of component y.

SPE PREP VOL is the volume the SPE residue is redissolved in after sample concentration and solvent evaporation, typically 0.25 mls.

0.01 is a user parameter used to counteract the multiplier of 100 that Multichrom uses in the final calculation when using an External Standard % calculation.

RF is the absolute response factor for peak y.

SX WT is assumed to be 1 (the density of water)

TOTAL MLS SAMPLE is the amount of sample, in mls, passed through the SPE cartridge.

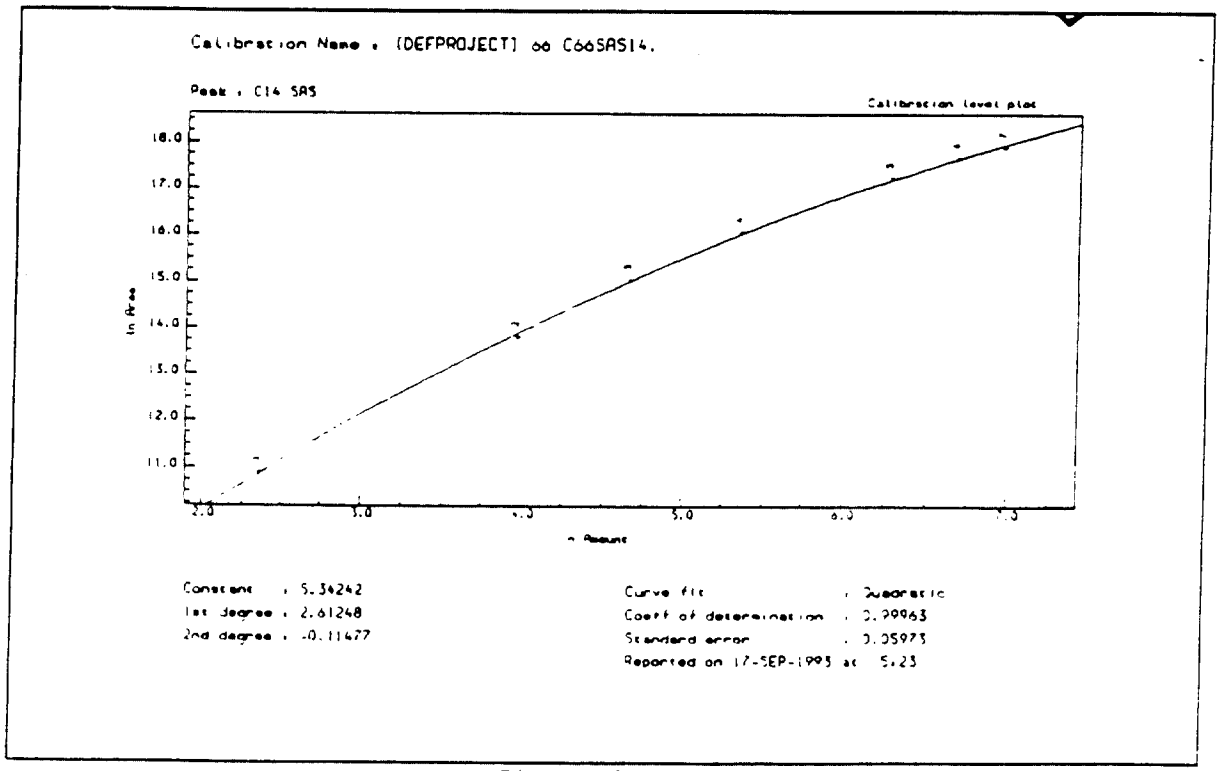

Figure 3

PRECISION AND ACCURACY:

Precision, accuracy, and recovery of C_{14} SAS was determined as follows: 10 mls formalin are added to a 1 liter stoppered graduated

cylinder. The cylinder is then filled to the 1000 ml mark with Milli-Q water. This solution is then spiked with 50 µg SAS (0.1 ml of the 500 µg/ml SAS calibration solution is used for the spike using a hamilton high precision syringe). The solution is mixed well. The Zymark AutoTrace Workstation is used to sample 300 mls of this solution in triplicate onto separate octyl SPE cartridges, and preparation continued as described in the SAMPLE PREPARATION section above. A total of 3 samples were analyzed by HPLC, and the results are as follows:

C_{14} SAS	amount of spike	obtained result	% recovery
	0.0529 ppm	0.0583 ppm	110.2
		0.0559 ppm	105.7
		0.0569 ppm	107.6
	average	0.0570 ppm	107.8
	SD	0.0012 ppm	2.2
	%SD	2.11%	2.06%

APPENDIX IV

LC50 Calculations

APPENDIX IV

A(N) 96-H LC50 (24-H DATA) STUDY
WITH FATHEAD MINNOW

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 109A-108

SPONSOR NAME: Shell Development Company

TEST MATERIAL: DAN 216

CONC. mg/L	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROBABILITY (PERCENT)
1.1	20	20	100	LESS THAN 0.001
.65	20	20	100	LESS THAN 0.001
.39	20	0	0	LESS THAN 0.001
.21	20	0	0	LESS THAN 0.001
.12	20	0	0	LESS THAN 0.001

THE BINOMIAL TEST SHOWS THAT .39 AND .65 CAN BE USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT CONFIDENCE LIMITS BECAUSE THE ACTUAL CONFIDENCE LEVEL ASSOCIATED WITH THESE LIMITS IS GREATER THAN 95 PERCENT.

AN APPROXIMATE LC50 OF .5034878 IS OBTAINED BY NONLINEAR INTERPOLATION BETWEEN .39 AND .65

WHEN THERE ARE LESS THAN TWO CONCENTRATIONS AT WHICH THE PERCENT DEAD IS BETWEEN 0 AND 100, NEITHER THE MOVING AVERAGE METHOD NOR THE PROBIT METHOD CAN GIVE ANY STATISTICALLY SOUND RESULTS.

COMPARE RESULTS WITH ORIGINAL DATA TO SEE IF THEY ARE REASONABLE

APPENDIX IV

A(N) 96-H LC50 (48-H DATA) STUDY
WITH FATHEAD MINNOW

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 109A-108

SPONSOR NAME: Shell Development Company

TEST MATERIAL: DAN 216

CONC. mg/L	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROBABILITY (PERCENT)
1.1	20	20	100	LESS THAN 0.001
.65	20	20	100	LESS THAN 0.001
.39	20	0	0	LESS THAN 0.001
.21	20	0	0	LESS THAN 0.001
.12	20	0	0	LESS THAN 0.001

THE BINOMIAL TEST SHOWS THAT .39 AND .65 CAN BE USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT CONFIDENCE LIMITS BECAUSE THE ACTUAL CONFIDENCE LEVEL ASSOCIATED WITH THESE LIMITS IS GREATER THAN 95 PERCENT.

AN APPROXIMATE LC50 OF .5034878 IS OBTAINED BY NONLINEAR INTERPOLATION BETWEEN .39 AND .65

WHEN THERE ARE LESS THAN TWO CONCENTRATIONS AT WHICH THE PERCENT DEAD IS BETWEEN 0 AND 100, NEITHER THE MOVING AVERAGE METHOD NOR THE PROBIT METHOD CAN GIVE ANY STATISTICALLY SOUND RESULTS.

COMPARE RESULTS WITH ORIGINAL DATA TO SEE IF THEY ARE REASONABLE

APPENDIX IV

A(N) 96-H LC50 (72-H DATA) STUDY
WITH FATHEAD MINNOW

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 109A-108

SPONSOR NAME: Shell Development Company

TEST MATERIAL: DAN 216

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CONC. mg/L	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROBABILITY (PERCENT)
1.1	20	20	100	LESS THAN 0.001
.65	20	20	100	LESS THAN 0.001
.39	20	0	0	LESS THAN 0.001
.21	20	0	0	LESS THAN 0.001
.12	20	0	0	LESS THAN 0.001

THE BINOMIAL TEST SHOWS THAT .39 AND .65 CAN BE USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT CONFIDENCE LIMITS BECAUSE THE ACTUAL CONFIDENCE LEVEL ASSOCIATED WITH THESE LIMITS IS GREATER THAN 95 PERCENT.

AN APPROXIMATE LC50 OF .5034878 IS OBTAINED BY NONLINEAR INTERPOLATION BETWEEN .39 AND .65

WHEN THERE ARE LESS THAN TWO CONCENTRATIONS AT WHICH THE PERCENT DEAD IS BETWEEN 0 AND 100, NEITHER THE MOVING AVERAGE METHOD NOR THE PROBIT METHOD CAN GIVE ANY STATISTICALLY SOUND RESULTS.

COMPARE RESULTS WITH ORIGINAL DATA TO SEE IF THEY ARE REASONABLE

****

APPENDIX IV

A(N) 96-H LC50 (96-H DATA) STUDY
WITH FATHEAD MINNOW

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 109A-108

SPONSOR NAME: Shell Development Company

TEST MATERIAL: DAN 216

CONC. mg/L	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROBABILITY (PERCENT)
1.1	20	20	100	LESS THAN 0.001
.65	20	20	100	LESS THAN 0.001
.39	20	0	0	LESS THAN 0.001
.21	20	0	0	LESS THAN 0.001
.12	20	0	0	LESS THAN 0.001

THE BINOMIAL TEST SHOWS THAT .39 AND .65 CAN BE USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT CONFIDENCE LIMITS BECAUSE THE ACTUAL CONFIDENCE LEVEL ASSOCIATED WITH THESE LIMITS IS GREATER THAN 95 PERCENT.

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COMPARE RESULTS WITH ORIGINAL DATA TO SEE IF THEY ARE REASONABLE

APPENDIX V

CHANGES TO PROTOCOL

The study was conducted in accordance with the approved Protocol with the following changes:

1. The proposed dates, study room, test concentrations, test substance number and receipt date were amended to the protocol.

2. The protocol was amended to change the sample preparation and shipment.

3. Temperature, dissolved oxygen and pH measurements were discontinued when mortality reached 100%.

4. Water and stock samples for analytical confirmation were collected in sample containers composed of flint glass.

In the opinion of the Study Director, the above changes in the approved protocol did not adversely affect the results of this study.

APPENDIX VI
PERSONNEL INVOLVED IN THE STUDY

The following key personnel were involved in the conduct or management of this study:

Aquatic Toxicology Laboratory

1. James P. Swigert, Ph.D., Manager, Aquatic Toxicology
2. Kurt R. Drottar, Senior Aquatic Biologist
3. Barry P. Conner, Aquatic Biologist
4. Gordon R. Pepper, Aquatic Biologist
5. Cynthia A. Roberts, Senior Aquatic Biologist
6. Edward C. Schaefer, Manager, Biodegradation

Analytical Chemistry Laboratory

1. Sheila Dubey, Chemist, Shell Development Company