EASTMAN

Eastman Chemical Company
P. O. Box 511
Kingsport, Tennessee 37662

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October 4, 2006

Document Control Office (DCO 7407M)
Office of Pollution Prevention and Toxics
Environmental Protection Agency
1200 Pennsylvania Ave. NW
Washington, DC 20460-0001
ATTN: 8(d) Health and Safety Reporting Rule (Notification/Reporting)

CONTAIN NO CBI

Dear Administrator,

The Eastman Chemical Company has reviewed their files in response to requests by the Environmental Protection Agency to report unpublished health and safety data pursuant to Section 8(d) of the Toxic Substances Control Act (71 Fed. Reg. 47,130, August 16, 2006; 71 Fed. Reg. 57,439, September 29, 2006).

The table below lists those chemicals for which Eastman possessed health and safety data. Copies of this data are organized by the chemical's CAS number on the enclosed compact disc. Please contact Dr. James A. Deyo at 423-229-5208 should you have any problems extracting the information from the disc.

CAS No.	Chemical Name
110-18-9	1,2-Ethanediamine, N,N,N',N'-tetramethyl-
110-33-8	Hexanedioic acid, dihexyl ester
111-85-3	Octane, 1-chloro-
124-63-0	Methanesulfonyl chloride
131-57-7	Methanone, (2-hydroxy-4-methoxyphenyl)phenyl-
25646-71-3	Methanesulfonamide, N-[2-[(4-amino-3-methylphenyl)ethylamino]ethyl]-, sulfate (2:3)
4170-30-3	2-Butenal
645-62-5	2-Hexenal, 2-ethyl-
68987-66-6	Ethene, hydrated, by-products from
7795-95-1	1-Octanesulfonyl chloride
81-07-2	1,2-Benzisothiazol-3(2H)-one, 1,1-dioxide
94-96-2	1,3-Hexanediol, 2-ethyl-
97-00-7	Benzene, 1-chloro-2,4-dinitro-

Regards,

Marc G. Schurger
Director, Product Safety & Health

8 6 0 7 0 0 0 0 0 1

Enclosure: Compact Disc labeled "Eastman TSCA 8(d) Submission; October 4, 2006

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Eastman Attachments (MR-29312)

81-07-2

94-96-2

97-00-7

110-18-9

110-33-8

111-85-3

124-63-0

131-57-7

4170-30-3

7795-95-1

25646-71-3

68987-66-6

901878

HAZLETON
LABORATORIES AMERICA INC
1200 B PICCARD DRIVE, ROCKVILLE, MARYLAND 20850

ABSTRACT

Crotonaldehyde is a representative alkyl-substituted vinyl aldehyde. It is one of seven aldehydes nominated by NTP for a variety of in-depth toxicity tests. This compound is considered to have great potential for exposure to humans. Oral ingestion and inhalation are considered the major routes of potential human exposure.

There is virtually no information in the literature on the toxicity of Crotonaldehyde by oral administration in rats and mice. There is suspicion of carcinogenic potential based on increased cancer incidence of workers exposed at an aldehyde manufacturing plant. Based on this background information, Crotonaldehyde was selected for toxicity and carcinogenicity testing by the oral and inhalation routes of exposure in mice and rats.

No acute testing was required.

In the 14-day repeated dose oral study, (Litton Bionetics Study No. 12322-05) five mice (B6C3F1) of each sex were randomized into five dose groups (18.75, 37.5, 75, 150, 300 mg/kg body weight of Crotonaldehyde) and one control group (corn oil, vehicle) and gavaged on weekdays only for a total of 12 times. All animals at the 300 mg/kg dose level and 8/10 animals at the 150 mg/kg dose level died within the first week of compound administration. One female at the 150 mg/kg level died during the second week. No compound related deaths occurred in the other dose groups.

Dose related stomach lesions were noted at necropsy in the mice that survived until termination of the study. The stomach was determined by gross and microscopic pathologic examination to be the primary target organ. The final mean body weight was statistically significantly decreased in the low dose (18.75 mg/kg) females only. The mean brain weights of the 75 mg/kg males and the right kidney weights of the 37.5 and 75 mg/kg males were significantly increased while the right kidney weights of the 75 mg/kg females were significantly decreased. The right kidney/brain weight ratio of the 37.5 mg/kg males was significantly increased.

This regimen of Crotonaldehyde administration was associated with a spectrum of gastric lesions which consisted of necrosis, inflammation, ulceration and hyperplasia of the forestomach epithelium. Perforation of gastric ulcers resulted in secondary involvement of adjacent organs. There were no discernable compound related lesions in the lowest dose groups (18.75 mg/kg) and this was considered to be the no-effect dose level.

In the 13-week subchronic study ten mice (B6C3F1) of each sex were randomized into five dose groups (2.5, 5, 10, 20 and 40 mg per Crotonaldehyde per kilogram body weight) and one control group (corn oil, vehicle), and gavaged on weekdays only for 13 weeks. There were no deaths during this study.

L:49/1

ABSTRACT (Continued)

There were no dose related clinical observations. A slight decrease in body weight gain for the treated male groups when compared to controls was noted from Week 4 to the end of the study. Mean body weights were decreased for the 40 mg/kg females from Week 7 until termination. There were no treatment related gross lesions at necropsy. Several statistically significant differences were noted in organ weights and organ/body weight ratios. These differences were not considered to be of toxicological significance. No changes were noted for the sperm morphology and vaginal cytology studies.

This regimen of crotonaldehyde was associated with a high incidence of epithelial hyperplasia in the forestomachs of males and females from the 40 mg/kg group. In addition, there was a low incidence of chronic active inflammation in the forestomach of the 40 mg/kg males. The results of this subchronic study suggest that the 20 mg/kg dose level can be considered to be a "no effect" dose level.

This report has been prepared by a laboratory that conducted these studies under the direction and support of the National Toxicology Program. The contents and conclusions have not been reviewed by the NTP staff at this time and therefore do not necessarily represent the position of the NTP

HAZLETON LABORATORIES AMERICA, INC.
PROJECT NO. 12322-09

TABLE A - THIRTEEN WEEK SUBCHRONIC STUDY OF CROTONALDEHYDE

SUMMARY OF TOXICOLOGICAL DATA IN MICE

MALE

DOSE (mg/kg)	MORTALITY DEAD/ TREATED	MEAN WEIGHT GAIN IN GRAMS (% DIFFERENCE FROM CONTROLS)[a]	CLINICAL OBSERVATIONS[b]	GROSS NECROPSY OBSERVATIONS[c,d]	SPECIAL TOXICO-LOGIC STUDIES	PATHOLOGIC OBSERVATIONS (INCIDENCE)
0	0/10	9.8	NSF	NSF	N/A	NSF
2.5	0/10	8.3 (-6.3)	NSF	brain wt dec heart wt dec rt kidney wt dec liver wt dec rt testicle wt dec heart/BW ratio dec rt kidney/BW ratio dec liver/BW ratio dec	N/A	NSF
5	0/10	8.4 (-5.3)	NSF	liver wt dec liver/BW ratio dec	N/A	NSF
10	0/10	8.6 (-3.7)	NSF	liver wt dec liver/BW ratio dec	N/A	NSF
20	0/10	7.1 (-9.0)	NSF	liver wt dec brain/BW ratio inc	N/A	NSF
40	0/10	8.3 (-5.3)	NSF	rt testicle/BW ratio inc	N/A	Stomach 10/10

HAZLETON LABORATORIES AMERICA, INC.
PROJECT NO. 12322-09

TABLE A - THIRTEEN WEEK SUBCHRONIC STUDY OF CROTONALDEHYDE (Continued)

SUMMARY OF TOXICOLOGICAL DATA IN MICE

FEMALE

DOSE (mg/kg)	MORTALITY DEAD/ TREATED	MEAN WEIGHT GAIN IN GRAMS (Δ DIFFERENCE FROM CONTROLS)[a]	CLINICAL OBSERVATIONS[b]	GROSS NECROPSY OBSERVATIONS[c,d]	SPECIAL TOXICOLOGIC STUDIES	PATHOLOGIC OBSERVATIONS (INCIDENCE)
0	0/10	7.3	NSF	NSF	N/A	NSF
2.5	0/10	8.7 (7.5)	NSF	NSF	N/A	NSF
5	0/10	9.6 (8.8)	NSF	rt kidney wt inc	N/A	NSF
10	0/10	7.3 (0.4)	NSF	brain wt inc rt kidney wt inc	N/A	NSF
20	0/10	5.6 (-2.1)	NSF	brain wt inc brain/BW ratio inc	N/A	NSF
40	0/10	6.1 (-3.3)	NSF	brain wt inc rt kidney wt inc brain/BW ratio inc rt kidney/BW ratio inc liver/BW ratio inc	N/A	Stomach 8/10

901878

HAZLETON
LABORATORIES, AMERICA, INC.
1330 - 3 PICCARD DRIVE, ROCKVILLE, MARYLAND 20850

ABSTRACT

Crotonaldehyde is a representative alkyl-substituted vinyl aldehyde. It is one of seven aldehydes nominated by NTP for a variety of in-depth toxicity tests. This compound is considered to have great potential for exposure to humans. Oral ingestion and inhalation are considered the major routes of potential human exposure.

There is virtually no information in the literature on the toxicity of Crotonaldehyde by oral administration in rats and mice. There is suspicion of carcinogenic potential based on increased cancer incidence of workers exposed at an aldehyde manufacturing plant. Based on this background information, Crotonaldehyde was selected for toxicity and carcinogenicity testing by the oral and inhalation routes of exposure in mice and rats. No acute testing was required.

In the 14-day repeated dose oral study (Litton Bionetics Study No. 12322-04), five rats (F344) of each sex were randomized into five dose groups (18.75, 37.5, 75, 150, 300 mg/kg body weight of Crotonaldehyde) and one control group (corn oil, vehicle) and gavaged on weekdays only for a total of 12 times. All animals at the 300 mg/kg dose level and 9/10 animals at the 150 mg/kg dose level died within the first week of compound administration. No compound related deaths occurred in the other dose groups.

Dose related stomach lesions were noted at necropsy in the rats that survived until termination of the study. The stomach was determined by gross and microscopic pathologic examination to be the primary target organ. The final mean body weight of the 75 mg/kg dose groups were slightly decreased. The heart and lung weights of the 75 mg/kg females were significantly decreased. There was a significant increase of the liver/body weight ratio of the 75 mg/kg dose groups and a decrease in the lung/body weight ratios of the 37.5 and 75 mg/kg females. The heart/brain weight ratio for the 75 mg/kg females and the lung/brain weight ratios for the 37.5 and 75 mg/kg females were decreased.

This regimen of Crotonaldehyde administration was associated with a spectrum of gastric lesions which consisted of necrosis, inflammation, ulceration and hyperplasia of the forestomach epithelium. Perforation of gastric ulcers resulted in secondary involvement of adjacent organs. ~~There were mild gastric lesions present in the lowest dose groups~~ (18.75 mg/kg) and, therefore, a no-effect level was not evident in this study.

In the 13-week subchronic study, ten rats (F344) of each sex were randomized into five dose groups (2.5, 5, 10, 20 and 40 mg Crotonaldehyde per kilogram body weight), and one control group (corn oil, vehicle), and gavaged on weekdays only for 13 consecutive weeks. There were 26 deaths during the study, 10 of which were attributed to dosing accidents.

HAZLETON
LABORATORIES AMERICA, INC.
1030 B RICCARD DRIVE, ROCKVILLE, MARYLAND 20850

ABSTRACT (Continued)

Deaths were noted in the 10, 20 and 40 mg/kg male groups and all female groups except the 2.5 mg/kg group. Excluding the ten accidental deaths, three males and four females at 40 mg/kg, one male and four females at 20 mg/kg, two males and one female at 10 mg/kg, and one female at 5 mg/kg, did not survive to termination.

The 40 mg/kg males showed a decrease in body weight from Week 11 to Week 13, which was statistically significant at termination. Body weights were comparable in the females throughout the study. There were no notable clinical observations.

Several statistically significant changes were noted in hematology and clinical chemistry parameters but none of the changes appeared to be of toxicological significance since the changes were not dose-related or consistent over the duration of the study.

Dose related stomach lesions were noted at necropsy in rats that survived until termination. The body weights of the 40 mg/kg males were significantly decreased at necropsy, as were the thymus weights of the 40 mg/kg females. The brain/body weight ratio of the 5 mg/kg males and the rt testes/body weight ratio of the 10 mg/kg males were significantly decreased. A significant increase was seen in the liver/body weight ratio of the 20 mg/kg females. The thymus/body weight ratio of the 40 mg/kg females was significantly decreased.

This regimen of crotonadehyde was associated with a high incidence of epithelial hyperplasia in the forestomach of the 40 mg/kg males and females, with a lower incidence of similar lesions in the 20 and 10 mg/kg males and females. Compound administration also was associated with a high incidence of inflammation of the nasal cavity in the 40 mg/kg males and females, with a lower incidence of similar lesions in the 20 mg/kg males and females, and in females from the 10 and 5 mg/kg dose groups. Results of this subchronic study suggest that the 5 mg/kg is a "no effect" dose level for both male and female laboratory rats.

This report has been prepared by a laboratory that conducted these studies under the direction and support of the National Toxicology Program. The contents and conclusions have not been reviewed by the NTP staff at this time and therefore do not necessarily represent the position of the NTP

HAZLETON LABORATORIES AMERICA, INC.
PROJECT NO. 12322-08

TABLE A - THIRTEEN WEEK SUBCHRONIC STUDY OF CROTONALDEHYDE

SUMMARY OF TOXICOLOGICAL DATA IN RATS

MALE

DOSE (mg/kg)	MORTALITY DEAD/ TREATED	MEAN WEIGHT GAIN IN GRAMS (% DIFFERENCE FROM CONTROLS)[a]	CLINICAL[b] OBSERVATIONS	GROSS NECROPSY[c,d] OBSERVATIONS	SPECIAL[d] TOXICO-LOGIC STUDIES	PATHOLOGIC OBSERVATIONS (INCIDENCE)
0	0/10	178.6	NSF	NSF	--	NSF
2.5	0/10	182.6 (0.8)	NSF	NSF	NSF	NSF
5	0/10	191.8 (4.9)	NSF	brain/BW ratio dec	NSF	NSF
10	3*/10	186.8 (4.0)	NSF	rt testes/BW ratio dec	RBC[e] dec HGB[e] dec	Nose 3/10 Stomach 3/10
20	3**/10	172.2 (-0.7)	NSF	forestomach thickened 2/10	NSF	Nose 3/10 Stomach 3/10
40	5***/10	151.1 (-9.5)	NSF	Body wt dec forestomach thickened 1/10 forestomach nodule 3/10	MCH[e] inc MCHC[e] inc BUN[e] dec BUN/CRE[e] dec SEG[f] inc ALKP[f] dec	Nose 7/10 Stomach 8/10

*1/3 gavage trauma, **2/3 gavage trauma, ***2/5 gavage trauma

L:49/13

HAZLETON LABORATORIES AMERICA, INC.
PROJECT NO. 12322-08

TABLE A - THIRTEEN WEEK SUBCHRONIC STUDY OF CROTONALDEHYDE (Continued)

SUMMARY OF TOXICOLOGICAL DATA IN RATS

FEMALE

DOSE (mg/kg)	MORTALITY DEAD/ TREATED	MEAN WEIGHT GAIN IN GRAMS (% DIFFERENCE FROM CONTROLS)[a]	CLINICAL[b] OBSERVATIONS	GROSS NECROPSY[c,d] OBSERVATIONS	SPECIAL[d] TOXICO-LOGIC STUDIES	PATHOLOGIC OBSERVATIONS (INCIDENCE)
0	1*/10	89.6	NSF	NSF	--	Nose 1/10
2.5	0/10	89.9 (0.6)	NSF	NSF	NSF	NSF
5	1/10	89.4 (2.0)	NSF	NSF	NSF	Nose 1/10
10	1/10	88.1 (1.0)	NSF	NSF	GGT[f] dec EOSIN[g] dec	Nose 1/10 Stomach 1/10
20	7**/10	91.6 (1.5)	NSF	liver/BW ratio inc	GGT[f] dec	Nose 4/10 Stomach 4/10
40	5***/10	80.3 (-2.3)	NSF	thymus wt dec thymus/BW ratio dec forestomach thickened 5/10 forestomach nodule 2/10	ALKP[e] dec RBC[f] dec HGB[f] dec HCT[f] dec MCHC[f] inc MCV[g] inc MCH[g] inc	Nose 6/10 Stomach 8/10

*1/1 gavage truma, **3/7 gavage trauma, ***1/5 gavage trauma

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MAY 27 1987

NTP DATA UNIT

PATHOLOGY WORKING GROUP REVIEW

13-WEEK SUBCHRONIC TOXICITY TEST WITH CROTONALDEHYDE (C56279B) IN FISCHER 344 RATS AND B6C3F1 MICE

PWG Participants: Drs. L. Brennecke (Chairperson), M. Elweil, R. Hruby, S. Stefanski, A. Maklin, and M. Jokinen

Date: May 6, 1987
Site: NTP, Research Triangle Park, NC
Type of Study: Gavage, Corn Oil Vehicle
Contracting Laboratory: Hazleton Laboratories America, Inc.
Principal Investigator: G. Wolfe
Chemical Manager: J. French

The PWG convened to review selected slides from Fischer 344 rats and B6C3F1 mice of both sexes administered Crotonaldehyde by gavage for 13 weeks. The study pathologist (SP) was Dr. G. Parker, and the quality assessment pathologist (QAP) was Dr. M. Jokinen of Experimental Pathology Laboratories, Inc.

PWG CONCLUSIONS

The administration of crotonaldehyde by gavage five days per week for 13 weeks resulted in hyperplasia of the forestomach mucosa in male rats given 40, 20, or 10 mg/kg, in female rats given 40 or 20 mg/kg and in male and female mice given 40 mg/kg. In addition, the crotonaldehyde resulted in serous exudation and/or inflammation in the nose of male rats given 40, 20, or 10 mg/kg and female rats given 40, 20, 10, or 5 mg/kg. It was further concluded that nearly all of the early deaths in male and female rats were associated with gavage error.

REVIEW OF RAT STUDY DATA

Experimental Design

The study groups for the 13-week rat study were as follows:

GROUP NO.	DOSE (MG/KG)	NUMBER OF ANIMALS MALE	FEMALE
1	0	10	10
2	2.5	10	10
3	5	10	10
4	10	10	10
5	20	10	10
6	40	10	10

Mortality

Twenty-six rats (11 males, 15 females) died prior to termination of the study. Ten of these deaths in each sex were attributed to gavage trauma in the toxicology report. Mortality data are presented in tabular form by sex, dose group, and study week.

Mortality Table

Study Week

Group (Total M/F)	1	2	3	4	5	6	7	8	9	10	11	12	13
1 (0/1)					F*								
2 (0/0)													
3 (0/1)													F
4 (3/1)					M		M*				F	M	
5 (3/7)	M,F	F	F*			M*			F*F	M*	F*F		
6 (5/5)	M,F				M*,F	M*,F	F*				M	M	F

M = One male rat early death
F = One female rat early death

* = Death attributed to gavage trauma in toxicology report

Clinical Signs

None

Body Weight Gain

A decrease in body weights occurred in group 6 from Week 11 to Week 13. This was significant at the end of the study. There were no significant differences in the female groups over the course of the study.

Organ Weights

At necropsy there was a significant decrease (relative and absolute) in the thymus weights of group 6 females, an increased liver/body ratio of group 5 females, a decreased R. testis/body ratio of group 4 males, and a decreased brain/body ratio of group 3 males compared to controls. None are considered toxicologically significant.

Hematology and Clinical Chemistry - See attached Summaries from Toxicology Report (Attachment 1)

Statistically significant changes were observed in hematology and clinical chemistry parameters, but because the changes were not consistent or dose-related over the course of the study, they did not appear to be of toxicological significance.

Gross Pathology

At necropsy, treatment-related lesions were noted only in the forestomachs of group 6 males and females and group 5 males. These are presented in tabular format below.

Forestomach Lesions in Rats

	40 mg/kg		20 mg/kg	
Observation	Male	Female	Male	Female
Thickened	1/10	5/10	2/10	0/10
Nodule	3/10	2/10	0/10	0/10

REVIEW OF MOUSE STUDY DATA

Experimental Design

The study groups for the 13-week mouse study were as follows:

		NUMBERS OF ANIMALS	
GROUP NO.	DOSE (MG/KG)	MALE	FEMALE
1	0. (Vehicle Control)	10	10
2	2.5	10	10
3	5	10	10
4	10	10	10
5	20	10	10
6	40	10	10

Mortality

All mice survived to terminal sacrifice.

Clinical Signs

None

Body Weight Gain

There were no statistically significant differences between treated and control mice.

Organ Weights - See attached Summaries from Toxicology Report (Attachment 2)

There were numerous statistically significant changes in absolute organ weights and organ/body weight ratios of treated vs. controls. None of these differences were consistent or dose-related over the course of the study and thus were not determined to be toxicologically significant.

Hematology and Clinical Chemistry

No evaluation was performed on mice from this study.

Gross Pathology

No treatment-related lesions were noted at necropsy.

CONDUCT OF THE PWG

Prior to the PWG, the chairperson reviewed the laboratory report for Individual Animal and Summary Tables, other toxicologic data, and the SP's pathology narrative, the Quality Assessment report, and selected microscopic slides from the study. Slides reviewed included all noses, lungs, and forestomachs in rats as well as one potential neoplasm and all forestomachs in mice. From these, the chairperson selected a subset of 31 slides for PWG review. These included representative sections of rat lung within which foreign material (oil) was noted, representative sections of rat nose containing possible chemical-related changes, the diagnoses of which the SP and QAP routinely disagreed, and representative examples of possible chemical-related lesions in the forestomach of rats and mice.

HISTOPATHOLOGY

Lung

The PWG reviewed lung sections from five male and four female treated and control rats. Foreign material (oil) was noted by all PWG participants in eight sections and by two participants in the remaining lung section. The chairperson presented his findings from his review of all available lungs in the PWG slide set. These findings are presented in tabular format.

Foreign Material (Oil) in Rat Lungs

Dose Group (mg/kg)	No. of Lungs Examined		No. of Lungs with Oil Present	
	Early Deaths	Terminal Sacrifice	Early Deaths	Terminal Sacrifice
40 male	5	0	3 [a]	--
40 female	5	0	4 [a]	--
20 male	3	6	3	0
20 female	7	0	4 [b,c]	--
10 male	3	0	3	--
10 female	1	6	1	1 [d]
5 male	0	0	--	--
5 female	1	0	1	--
2.5 male	0	0	--	--
2.5 female	0	0	--	--
0 male	0	6	--	0
0 female	1	4	1	0

a) The remaining early death animals (9454, 9457, 9469) were categorized as gavage deaths in laboratory report and contained lesions in the mediastinum and/or esophagus indicative of gavage trauma.

b) Oil in one additional lung (9442) noted by PWG participants. Animal was categorized as a gavage death in laboratory report.

c) The two additional early death animals with no oil in lung or cause of death indicated were 9445 and 9448.

d) Animal No. 9426

The PWG concluded that while the oil in the lungs may not have killed the rats it was indicative that a gavage error had occurred, and because the majority of the rats which died early were in the higher dose groups it is likely that the crotonaldehyde in the lungs contributed to the death of the rats. It was further concluded that the substantial number of early deaths (26/120) in the rats in this study should not be considered as a criterion for establishing doses in the chronic study.

Nose

Sections of nose from seven male and female rats were reviewed by the PWG. The PWG concluded that the general change present was serous exudation and not acute inflammation as reported in the laboratory report. In addition, because the lesion was usually present in the early death rats, the apparent change in some rats may have been exacerbated by post mortem change. The exudates in the noses were thought to be reactions to the highly volatile crotonaldehyde, some of which may have been deposited in the posterior pharynx. As such, the nose should be considered a target tissue but not primarily due to a circulatory-delivered effect. A no-effect level was achieved at the 5.0 mg/kg dose in males and 2.5 mg/kg dose in females. However, the minimal to mild change should not be a major criterion in the dose determination. Oil droplets were present in several sections of nose.

Forestomach

The PWG reviewed sections of forestomach from male and female rats and female mice. A treatment-related hyperplasia of the forestomach mucosa was confirmed by the PWG. A "no-effect" level was confirmed at the 5 mg/kg dose in male rats. However, the change in the 1/10 female rats in the 10 mg/kg dose group was thought to be equivocal and that the female "no-effect" level should be 10 mg/kg.

The "no-effect" level was confirmed at the 20 mg/kg dose for male and female mice. However, 3/8 female mice in the 40 mg/kg dose group diagnosed by the SP as having mild epithelial hyperplasia were determined by the PWG to have no change, while 2/8 mice had equivocal or minimal change.

HISTOTECHNIQUE

The overall slide quality was acceptable as determined by the PWG. However, the PWG noted that many of the forestomach sections showed extensive folding as though the stomachs had not been perfused at necropsy or as though the stomachs were opened at necropsy. This made interpretation of possible proliferative changes in the forestomach particularly troublesome. In addition, numerous sections of noses were fractured and/or folded. It should also be noted that many but not all lung sections were present in duplicate on the same slide. Duplication can introduce a sampling bias into the study results although such was apparently not the case in this study.

SUMMARY

The PWG concurred with the SP and QAP that the forestomach was a target tissue for both species, both sexes. A "no-effect" level was confirmed in male rats at 5 mg/kg and in both sexes of mice at 20 mg/kg. Based upon forestomach lesions, the

"no-effect" level for female rats should be 10 mg/kg. The changes in the noses of rats was thought to be directly related to the highly volatile crotonaldehyde, but the minimal or mild change should not affect survival in the two-year study.

ACTION ITEMS

None

DOSAGE RECOMMENDATIONS FOR TWO -YEAR STUDY

The PWG recommended a maximum dose of 10 mg/kg for both male and female rats. The 10 mg/kg male group contained only two rats each with mild forestomach epithelial hyperplasia while the 10 mg/kg female group contained only one rat with an equivocal change. The 20 mg/kg dose group, however, contained males and females with moderate forestomach hyperplasia. The nasal lesion (serous exudate) was not considered to be a change that would have significant effect on the survival of rats for a two-year study.

The PWG recommended maximum dose levels of 20 mg/kg for male mice and 40 mg/kg for female mice. Only 2/10 female mice in the 40 mg/kg dose group were thought to have mild forestomach hyperplasia. The corresponding changes in the 40 mg/kg male dose group were more severe than those in the 20 mg/kg male dose group.

Lucas H. Brennecke

Lucas H. Brennecke, D.V.M.
Diplomate, A.C.V.P.
PWG Chairperson
26 May 1987

Study No. EN-407-901878-1
ES-90-030
263599H

SCANNED

Study Title

ACUTE AQUATIC EFFECTS OF CROTONALDEHYDE ON THREE FRESHWATER SPECIES: the Fathead Minnow, *Pimephales promelas*, the Gammarid (Sideswimmer), *Gammarus fasciatus*, and the Daphnid, *Daphnia magna*

Data Requirement

In Accordance with the US EPA, 1985. Toxic Substances, Testing Requirements; Final Rule and Proposed Rule. Federal Register: 40 CFR Part 797.1400 Fish Acute Toxicity Test (Amended 1987); 40 CFR Part 797.1300 Daphnid Acute Toxicity Test (Amended 1987); 40 CFR Part 797.1310 Gammarid Acute Toxicity Test; (as Amended in Testing Consent Order on Crotonaldehyde, 40 CFR Part 799).

Authors

Janice M. Beglinger

Robert J. O'Boyle

Study Completed on

August 27, 1990

Study Report Amended on

September 6, 1990

Performing Laboratory

Eastman Kodak Company

Health and Environment Laboratories

Rochester, New York 14652-3617

U.S.A.

Laboratory Project ID

Accession No. 901878 HAEL No. 90-0016

HAEL No. 90-0016 Study No. EN-407-901878-1

STUDY REPORT AMENDMENT

STUDY TITLE

ACUTE AQUATIC EFFECTS OF CROTONALDEHYDE ON THREE FRESHWATER SPECIES:
the Fathead Minnow, *Pimephales promelas*,
the Gammarid (Sideswimmer), *Gammarus fasciatus*,
and the Daphnid, *Daphnia magna*

Authors
Janice M. Beglinger
Robert J. O'Boyle

The study report has been amended to correct the "Actual Concentration (mg/L)" and "Recovery (%)" values reported for the analytical method validation performed on 3-22-90. The original values are incorrect due to an error in calculating the calibration curve. The correct values are approximately 10% higher than those reported originally; and, the corrected values more closely correspond to the values obtained on the second day of method validation (3-27-90). This correction results in a better day-to-day precision of the analytical method. It has no effect on the test article concentration data reported for this study.

Study Director: R. J. O'Boyle 9/6/90
Robert J. O'Boyle Date

HAEL No. 90-0016 Study No. EN-407-901878-1

EPA/TSCA Acute Aquatic Toxicity Test(s) of
Crotonaldehyde

In Accordance with the US EPA, 1985. Toxic Substances, Testing Requirements; Final Rule and Proposed Rule. Federal Register: 40 CFR Part 797.1400 Fish Acute Toxicity Test (Amended 1987); 40 CFR Part 797.1300 Daphnid Acute Toxicity Test (Amended 1987); 40 CFR Part 797.1310 Gammarid Acute Toxicity Test; (as Amended in Testing Consent Order on Crotonaldehyde, 40 CFR Part 799).

STATEMENT OF GLP COMPLIANCE

To the best of my knowledge and belief, these procedures were conducted in compliance with all Good Laboratory Practice standards of 40 CFR Part 792, November 29, 1983 (revised August 17, 1989). No unforeseen circumstances were observed that might have affected the quality or integrity of these procedures.

Study Director: R. J. O'Boyle 8/29/90
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STUDY TITLE

ACUTE AQUATIC EFFECTS OF CROTONALDEHYDE ON THREE FRESHWATER SPECIES:
the Fathead Minnow, Pimephales promelas,
the Gammarid (Sideswimmer), Gammarus fasciatus,
and the Daphnid, Daphnia magna

ABSTRACT

The acute toxicity of the test article Crotonaldehyde (HAEL No. 90-0016; Accession No. 901878) was determined in a flow-through, aquatic effects study that involved three freshwater aquatic species. Gammarus fasciatus (gammarids) and Pimephales promelas (fathead minnows) were exposed to the test article for 96 hours. Daphnia magna (daphnids) were exposed for the first 48 hours of the 96-hour study.

Static, preliminary bioassays were performed as part of methods development to establish the test article concentrations that would be appropriate for the three freshwater species used in the subsequent definitive test. This report describes the results of the flow-through, definitive study. Crotonaldehyde has appreciable aqueous solubility in water. The test article appeared to be completely dissolved at all of the exposure concentrations used in the definitive study. A total of 20 animals (of each species) were exposed to each of five dose concentrations (nominally 0.6, 1.2, 2.5, 5, and 10 mg/L). Two replicates per dose were used in the test (10 animals/replicate). Replicate results were pooled.

The pooled, 96-hour LC_{50} values (rounded to two significant figures) calculated from the study dose-response data were 2.7 mg/L for the gammarid and 0.84 mg/L for the fathead minnow. The daphnid 48-hour EC_{50} value (rounded to two significant figures) was 2.0 mg/L for pooled Replicates A and B.

The pooled, acute No Observed Effect Concentration (NOEC) value for fathead minnows was 0.51 mg/L. The gammarid NOEC value for pooled replicates A and B was 1.1 mg/L. The daphnid NOEC value was 0.61 mg/L for pooled replicates A and B. The NOEC values are based upon both lethal and sublethal effects.

The results of this study indicate that a continuous, acute exposure to Crotonaldehyde at concentrations as low as 1 - 2 mg/L may be expected to have an adverse effect on most freshwater aquatic organisms.

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HAEL No. 90-0016 Study No. EN-407-901878-1

TESTING FACILITY

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652-3617
USA

SPONSOR

Eastman Chemicals Division
Kingsport, Tennessee 37662
USA

DATE OF STUDY INITIATION February 7, 1990 (Date Protocol Signed by Study Director)

DATES OF CHEMICAL EXPOSURE *Pimephales promelas*: April 23 - 27, 1990
Gammarus fasciatus: April 23 - 27, 1990
Daphnia magna: April 23 - 25, 1990

DATE OF STUDY COMPLETION August 27, 1990

DATE OF STUDY REPORT AMENDMENT September 6, 1990

STUDY PARTICIPANTS

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PURPOSE/OBJECTIVE

To permit a more accurate and complete assessment of environmental impact, juveniles of three freshwater, aquatic species were exposed to five concentrations of Crotonaldehyde (Accession No. 901878), in a flow-through, aquatic effects test. Gammarus fasciatus (gammarids) and Pimephales promelas (fathead minnows) were exposed to the test article for 96 hours. Daphnia magna (daphnids) were exposed for the first 48 hours of the 96-hour study. The test article solutions were prepared through the direct addition of appropriate aliquots of a test article stock solution to the test vessels. Species were chosen for this test based on their ability to be reared within the laboratory as well as their ecological importance. The objectives of the test were: (1) to determine if acute exposure to concentrations of the test article would affect species adversely; and, (2) if appropriate, provide either a 48-hr EC_{50} (effect concentration for 50% of the daphnid test population) or a 96-hr LC_{50} (lethal concentration for 50% of the test population) value and the highest acute No Observed Effect Concentration (NOEC) for each species.

The exposure of each of the three freshwater species to Crotonaldehyde was initiated simultaneously. The animals were observed for signs of stress, as well as mortality (or immobility), when exposed to the test article. Mortality was used as the requisite biological endpoint for two species to calculate the 96-hour LC_{50} values. In the case of daphnids, immobilization was the requisite endpoint used to calculate the 48-hour EC_{50} value. For the purpose of calculating or estimating the highest acute NOEC, either mortality (or immobility) or signs of stress would serve as the requisite endpoint. The results of this study may be useful in determining the likelihood of acute aquatic effects if Crotonaldehyde enters a freshwater environment.

TEST ARTICLE

Identity: Crotonaldehyde CAS Registry No.: 4170-30-3
Sample Reference Identification (SRID) No.: Lot No. 90-7
Sample Laboratory No. (HAEL No.): 90-0016

Physical/Chemical Properties:

Composition & Solubility: The test article is a clear liquid with appreciable aqueous solubility.

Purity: 98.1% initial (7.1% water) and 99.5% final (6.3% water); using GC/FID analysis (Attachments 4a, 4b, and 4c).

MATERIALS AND METHODS

Test System

Genus and Species	Common Name
Daphnia magna	Daphnid
Gammarus fasciatus	Gammarid (Sideswimmer)
Pimephales promelas	Fathead Minnow

Source of Fathead Minnows

Juvenile fathead minnows used in this study were obtained from the Eco-Chem Testing Group Minnow Breeding Facility, Health and Environment Laboratories, Building 306, Kodak Park, Rochester, New York, 14652-3617, and were designated HAEL Lot 12/89. An organism origination statement is prepared for each lot of fish and is kept on file in the Central Archive of Non-Test Data currently located in Room 145, Building 306, Kodak Park.

Source of Invertebrate Test Species

Invertebrates are routinely reared in colonies maintained within the Eco-Chem Testing Group Facility. An organism origination statement and taxonomic verification are kept in the Central Archive of Non-Test Data for the acute aquatic effects testing area currently in Room 145, Building 306, Kodak Park.

Source of Dilution Water

The water used in this test was pumped from Lake Ontario by the Kodak Park Lake Station Water Treatment Facility into a large underground storage reservoir located near the Environmental Sciences Section testing facility (Building 306, Kodak Park). This water is subsequently pumped into the laboratory where it passes through a series of 3-micron (pore size) polypropylene filter tubes, then through a series of activated-powdered carbon filter tubes, and finally through another set of 3-micron polypropylene filter tubes. The filtered water stream next received 150 ppb of $Na_2S_2O_3$ via a chemical injection system. This treatment further reduces trace levels of residual chlorine. The filtered-treated water is subsequently tempered to 20 ± 2°C by passage through a heat-exchange unit. This filtered-treated-

tempered water supply is distributed throughout the testing facility through stainless steel piping. Upon reaching the laboratory, the filtered-treated-tempered water cascades through a column degassing unit into an open, aeration basin for seasoning prior to use. The test diluent water is drawn from this source. Representative values for the hardness and the total alkalinity (both as $CaCO_3$) of the laboratory diluent water for the study period were 120 mg/L and 99 mg/L, respectively. Refer to Attachment 2, Summary of Chemical Characterization of Dilution Water.

Acclimation Procedure

All aquatic organisms were acclimated to the diluent water since the same filtered-treated-tempered water and the filtered, compressed air used for all laboratory water aeration processes was supplied continuously to the stainless steel rearing tanks used for testing. The test minnows and invertebrate colonies are maintained in this water for at least two weeks before they or their young are used for testing.

Size and Number of Organisms Used at Each Concentration

For each test vessel ten juveniles of each species, as uniform in size as possible, were collected from the rearing colonies and holding tanks. Sequential randomization was accomplished by allocating no more than 50% of any one set of test organisms (20% for fish) at any one time into each collection vessel. Biological loading was kept below 0.8 g wet weight per liter of exposure solution. The average wet weights for two randomly-chosen sets of minnows were determined at test start (Replicate A = 0.28 g and Replicate B = 0.31 g). These minnows were subsequently used as the diluent water control sets of test organisms. Mean lengths of these same minnow controls at test end were determined to be 25.3 mm and 26.1 mm for Replicates A and B, respectively. The average wet weights for two randomly-chosen sets of gammarids (sacrificed for measurements and not exposed to the test article) were determined at test start, with Set #1 = 8.5 mg and Set #2 = 9.4 mg. The representative average lengths of these same gammarids at test start was 4.2 mm for both Sets #1 and #2.

Preparation of Test Solutions

Preliminary bioassays were performed (as part of methods development) with all three freshwater species using Crotonaldehyde as the test article.

The dose levels employed in the subsequent definitive study were determined by these results. All exposure solutions (test article doses and diluent water controls) were prepared in replicates of two for the definitive study.

The test article was introduced into replicate test vessels at nominal concentrations of 0.6, 1.2, 2.5, 5.0, and 10.0 mg/L using the following procedure: The laboratory diluent water was sparged with nitrogen for approximately 24 hours prior to adding the test article (in an attempt to reduce oxidative hydrolysis of the test article). Vigorous mixing with a power stirrer was employed to achieve complete dissolution of 20.0 g of the test article in 200 L of diluent water. This stock solution (@ 100 mg/L) container was sealed with a plastic bag and tape. Argon was continually introduced into the bag onto the solution surface in an attempt to form a non-oxidizing atmosphere over the aqueous stock solution. (The 100 mg/L stock solution continuously supplied the diluter throughout the study, and was renewed at time 48 hours as described above.) Each exposure dose was prepared by spiking with an appropriate amount of the stock solution at test start, in replicates of two. The aqueous stock solution of the test article was proportionally delivered to the appropriate test vessels throughout the study by the gas-driven, siphon type diluter. Samples were removed for eventual analysis by CQSD at times 0 (prior to introducing the test organisms into the exposure solutions), 48 hours (aged exposure solutions & fresh stock), and 96 hours (or at test end per dose; i.e., after complete mortality/immobility was observed).

Apparatus and Test Conditions (Refer to Figure 1.)

This flow-through (6.7 vessel volumes/day), multispecies study was performed in Pyrex® 30.5 cm cuboidal tanks equipped with overflow standpipes. Two tanks, each containing 15 L of exposure solution, were prepared for each dose level tested. The test article doses were positioned by pairs ("A" and "B") in a restrictively-randomized pattern within the temperature-controlled diluter basin (i.e., a water bath, tempered to 20 ± 1°C). The minnows were placed in the main volume of the glass test vessel. The other two test organisms were isolated by species in stainless steel screen baskets (of appropriate mesh to prevent escape) suspended from 1 rpm motors. Approximately half of the volume of the partially-submerged basket was continually exchanged with the main vessel volume through the action of the motors. The lighting regime was 16 hours of light and 8 hours of darkness.

Test Procedure

The procedures used in this test were based on accepted methodologies.[1-8] Observations for mortality/immobility, signs of stress, temperature, dissolved oxygen, and pH were made during the test at times 0, 24, 48, 72, and 96 hours.

Ten test organisms of each species were placed into each of the test vessels and control vessels at the initiation of the 96-hour (total) study. Organisms were considered dead if gentle prodding produced no reaction and if no breathing movements were visible. The daphnid endpoint was immobility and that test end was after 48 hours of exposure. Daphnid immobilization was defined as lack of movement except for minor activity of the appendages.

Analytical Procedure

Samples were removed for analysis at times 0, 48 (aged exposure solutions & fresh stock), and 96 hours (or test end per dose; i.e., complete mortality or daphnid immobility). A sample (≃ 50 mL) of each replicate test article dose solution and diluent water control was drawn from mid-depth of each test vessel (or from inside the wire mesh baskets). These samples were submitted immediately to the Chemicals Quality Services Division (CQSD) for test article concentration analysis by GC/ECD (refer to Attachment 7).

LC_{50}/EC_{50} Calculation

A computer program developed by C.E. Stephan[9] and ASTM[10] is used to compute point and interval (i.e., 95% confidence interval) estimates of the LC_{50} or EC_{50} values. The program requires the following data: the concentration of the test substance, either nominal or measured, the number of organisms exposed, and the number of organisms that die or are immobilized. Data for the control vessels are not entered because the program does not have the capability of adjusting for control mortality/immobility. The program computes point and interval estimates of the LC_{50}/EC_{50} values using three approaches. The concentration is $\log_{10}$ transformed for all methods (refer to Attachment 8 for a description of each method).

QUALITY ASSURANCE MONITORING

See the attached report of Quality Assurance Monitoring (Attachment 10).

Good Laboratory Practice Statement

This study was conducted according to: Good Laboratory Practice Regulations for Nonclinical Laboratory Studies as promulgated by the Food and Drug Administration; 21 CFR Part 58, December 22, 1978 (revised September 4, 1987); Environmental Protection Agency Good Laboratory Practice Standards 40 CFR Part 792, November 29, 1983 (revised August 17, 1989); and Organization of Economic Cooperation and Development Principles of Good Laboratory Practice specified in Annex 2 of the OECD Guidelines for Testing of Chemicals [C(81) 30(Final)] as required by Council Directive 89/569/EEC (October 28, 1989).

DATA STORAGE AND RECORDS RETENTION

All raw data and summaries of data will be retained for at least ten years in the Health and Environment Laboratories Archive currently located in Bldg. 320 of the Eastman Kodak Company, Kodak Park, Rochester, NY, 14652-3615.

WATER QUALITY DURING TEST

The temperature in all test vessels remained at 20°C during the test. The pH value extremes were 8.2 and 8.4. Dissolved oxygen reading extremes were 8.7 and 9.1 mg/L. The dissolved organic carbon concentrations measured in the diluent water supply during the study were 2.7, 2.6, and 2.6 mg/L at times 0, 48, and 96 hours, respectively. These values are considered to be acceptable for the organisms used in this test,[1-7] and are not likely to have caused any adverse effects.

TEST RESULTS

Raw data for biological effects are provided in Attachments 6 and 9. A summary is provided in Tables 1A-1C (requisite endpoint mortality or immobility). The 96-hour LC_{50}/48-hour EC_{50} values per species calculated by a computer program are provided in Attachment 8 and summarized in Table 3. Table 3 also includes the No Observed Effect Concentration (NOEC) values.

TEST VALIDITY

The following criteria for a valid test were met during the study:

A. The diluent water control immobilization/mortality did not exceed 10%.

B. The dissolved oxygen concentration did not fall below 6.6 mg/L.

C. No abnormal occurrences (i.e., laboratory accidents) that might have compromised the integrity of the data were noted during the study.

PROTOCOL/STANDARD OPERATING PROCEDURE DEVIATIONS

A protocol deviation was not necessary, but the test did deviate from the multispecies SOP No. EN-4035 by using only three of the seven species listed.

DISCUSSION AND INTERPRETATION

Static, preliminary bioassays with Crotonaldehyde using the fathead minnow (Pimephales promelas), the gammarid (Gammarus fasciatus), and the daphnid (Daphnia magna) were performed as part of methods development (refer to Attachment 9). The initial methods development was performed using some Crotonaldehyde obtained commercially (not the test article lot), but the minnow portion of the preliminary bioassay was repeated and confirmed at a later date using the test article lot. The dose levels employed in the subsequent definitive study were determined from the results of these bioassays. These static bioassay data indicated a nominal, acute No Observed Effect Concentration (NOEC) of 1 mg/L for all three of the test species.

In the subsequent flow-through, definitive, multispecies study, the test article Crotonaldehyde caused complete mortality (of all three species) in the two highest exposure concentrations (nominally 5 and 10 mg/L) in both replicate dose series by the end of the respective exposure periods. All of the minnows were dead by time 24 hours at the nominal dose of 2.5 mg/L. More than half of the gammarids survived until test end at 2.5 mg/L (Replicate A: 8 activity depressed, 2 dead; Replicate B: 6 activity depressed, 4 dead), but few daphnids exhibited normal mobility in that concentration by the end of the 48-hour exposure period (Replicate A: 3 normal, 1 activity depressed, 6 immobile; Replicate B: 1 normal, 9 immobile). At the second lowest dose (nominally 1.2 mg/L), three minnows survived at test end (Replicate A: 1 normal, 9 dead; Replicate B: 2 normal, 8 dead) while all of the gammarids appeared normal. There were nine daphnids mobile in both replicates of 1.2 mg/L (Replicate A: 8 normal, 1 activity depressed, 1 missing, and presumed dead; Replicate B: 9 normal, 1 immobile). The lowest nominal dose (0.6 mg/L) had all minnows, gammarids, and daphnids normal at test end except for one missing daphnid (presumed dead) in Replicate B. No mortality or adverse effects were observed in the diluent water controls of the minnows and gammarids. The daphnid diluent water controls had nine mobile daphnids remaining in each replicate (1 immobile in Replicate A; 1 missing, and presumed dead, in Replicate B). Table 1 summarizes the survival/mobility data.

Crotonaldehyde is described as being appreciably soluble in water (refer to Attachment 1). The test article appeared to be completely dissolved at the stock solution concentration (nominally 100 mg/L), and in all the resulting exposure doses, under the conditions used in this study (i.e., 20°C laboratory diluent water). All exposure solutions were sampled for analysis at test start, time 48 hours (just prior to stock solution renewal), and time 96 hours (or test end per exposure dose). Refer to Attachment 7 (CQSD report) and to Table 2 for a summary of the analytical results for each exposure solution.

Some of the containment baskets used to confine the daphnids and gammarids were sampled when the surrounding dose solutions were sampled for analysis (refer to Protocol Amendment 3 included on p. 68 and to the Records of Collection and Analysis of Test Solutions in Attachment 7). This procedure was intended to confirm that the external solution concentrations were representative of those occurring within the baskets. The test article concentrations reported in Attachment 7 indicate that the values obtained from inside the respective baskets were representative of the external solutions (given the apparent, inherent variability of the analytical method employed in this study). The reported values for inside the baskets were not used in calculating mean exposure concentrations since so few data points exist. The external solution values were used in all endpoint calculations. These endpoint values still constitute a conservative assessment of environmental risk since they are based on exposure concentrations that appear to be only slightly higher.

The analyzed concentrations for each replicate test article solution and diluent water control obtained throughout the study were averaged to produce a mean exposure value per dose. These mean exposure concentrations were used in all endpoint calculations for this study. The dose-response curves per species (pooled Replicates A and B) at times 48 and 96 hours are plotted in Figures 1-3.

The EC_{50}/LC_{50} Calculator Program defaults to the most appropriate statistical method depending on the distribution of the dose response within the data set (refer to Attachment 8 for method descriptions and the test calculations). Non-linear Interpolation was used to calculate the pooled EC_{50}/LC_{50} values for all three species of test organisms, and the associated 95% confidence intervals were generated by the Binomial Method.

The pooled, 48-hour LC_{50} value (rounded to two significant figures) for gammarids and fathead minnows were 6.1 mg/L and 1.2 mg/L, respectively. The acute No Observed Effect Concentration (NOEC) value at 48 hours was 2.5 mg/L for gammarids and 0.61 mg/L for fathead minnows. (Note: The customary study endpoints for these test organisms are 96-hour values, as listed below.)

The pooled, 96-hour LC_{50} values (rounded to two significant figures) calculated from the dose-response data were 2.7 mg/L for the gammarid and 0.84 mg/L for the fathead minnow. The daphnid 48-hour EC_{50} value (rounded to two significant figures) was 2.0 mg/L for pooled Replicates A and B.

The pooled, acute NOEC value for fathead minnows was 0.51 mg/L. The gammarid NOEC value for pooled replicates A and B was 1.1 mg/L. The daphnid NOEC value was 0.61 mg/L for pooled replicates A and B. The NOEC values are based upon both lethal and sublethal effects.

CONCLUSIONS

The results of this study indicate that a continuous, acute exposure to Crotonaldehyde at concentrations as low as 1 - 2 mg/L may be expected to have an adverse effect on most freshwater aquatic organisms.

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TABLE 1A. Survival of Aquatic Organisms

Species: Fathead Minnow (*Pimephales promelas*)

Test Vessel	Aim Conc. (mg/L)	Test Conc. Pooled, mg/L (48-)/96-hour *	Survival at 24 hr	48 hr	72 hr	96 hr
DWC A	0	ND	10	10	10	10
DWC B	0		10	10	10	10
Test A	0.6	(0.61)/0.51	10	10	10	10
Test B	0.6		10	10	10	10
Test A	1.2	(1.3)/1.1	10	3	1	1
Test B	1.2		9	6	2	2
Test A	2.5	(2.5)/2.5	0	0	0	0
Test B	2.5		0	0	0	0
Test A	5.0	(4.7)/4.7	0	0	0	0
Test B	5.0		0	0	0	0
Test A	10.0	(10)/10	0	0	0	0
Test B	10.0		0	0	0	0

* Test article concentrations determined by a GC/ECD analytical procedure (refer to Attachment 7 for a description and the raw data from the analyses). The measured values shown are the averaged analyzed concentrations in replicate exposure solution samples taken at test times 0, 48, and 96 hours. The 48- and 96-hour mean exposure values are rounded to a maximum of two significant figures.

DWC = Diluent Water Control

ND = None Detected (at the method lower limit of detection of 0.005 mg/L)

TABLE 1B. Survival of Aquatic Organisms

Species: Gammarid (*Gammarus fasciatus*)

Test Vessel	Aim Conc. (mg/L)	Test Conc. Pooled, mg/L (48-)/96-hour *	Survival at 24 hr	48 hr	72 hr	96 hr
DWC A	0	ND	10	10	10	10
DWC B	0		10	10	10	10
Test A	0.6	(0.61)/0.51	10	10	10	10
Test B	0.6		10	10	10	10
Test A	1.2	(1.3)/1.1	10	10	10	10
Test B	1.2		10	10	10	10
Test A	2.5	(2.5)/2.3	10	10	10	8
Test B	2.5		10	10	9	6
Test A	5.0	(4.7)/4.5	10	9	1	0
Test B	5.0		10	9	1	0
Test A	10.0	(9.3)/9.3	10	0	0	0
Test B	10.0		9	0	0	0

* Test article concentrations determined by a GC/ECD analytical procedure (refer to Attachment 7 for a description and the raw data from the analyses). The measured values shown are the averaged analyzed concentrations in replicate exposure solution samples taken at test times 0, 48, and 96 hours. The 48- and 96-hour mean exposure values are rounded to a maximum of two significant figures.

DWC = Diluent Water Control

ND = None Detected (at the method lower limit of detection of 0.005 mg/L)

TABLE 1C. Survival of Aquatic Organisms

Species: Daphnid (Daphnia magna)

Test Vessel	Aim Conc. (mg/L)	Test Conc. Pooled (mg/L)*	Number of Daphnids Mobile at 24 hr	48 hr
DWC A	0	ND	10	9
DWC B	0		10	9
Test A	0.6	0.61	10	10
Test B	0.6		10	9
Test A	1.2	1.3	10	9
Test B	1.2		10	9
Test A	2.5	2.5	10	4
Test B	2.5		10	1
Test A	5	4.7	0	0
Test B	5		0	0
Test A	10	10	0	0
Test B	10		0	0

* Test article concentrations determined by a GC/ECD analytical procedure (refer to Attachment 7 for a description and the raw data from the analyses). The measured values shown are the averaged analyzed concentrations in replicate exposure solution samples taken at test times 0 and 48 hours. The 48-hour mean exposure values are rounded to a maximum of two significant figures.

DWC = Diluent Water Control

ND = None Detected (at the method lower limit of detection of 0.005 mg/L)

TABLE 2. Summary of Analytical Results

Nominal, as mg/L	Measured Concentrations, mg/L 0 hours		48 hours	96 hours	(48-)/96-hour Mean, mg/L Dm	Gf	Pp
DWC (A)	ND		ND	ND	ND	ND	ND
DWC (B)	ND		ND	ND	ND	ND	ND
0.6 (A)	0.543		0.669	0.311	(0.606)	(0.606)/0.508	(0.606)/0.508
0.6 (B)	0.543		0.686	0.311	(0.614)	(0.614)/0.513	(0.614)/0.513
1.2 (A)	1.27		1.38	0.740	(1.32)	(1.32)/1.13	(1.32)/1.13
1.2 (B)	1.31		1.13	0.590	(1.22)	(1.22)/1.01	(1.22)/1.01
		24 hrs.					
2.5 (A)	2.58	2.59 §	2.33	1.55	(2.50)	(2.50)/2.26	2.58 *
2.5 (B)	2.69	2.25 §	2.66	1.58	(2.53)	(2.53)/2.30	2.47 *
5 (A)	5.11	3.71 §	4.84	3.82 §§	(4.41) *	(4.55)/4.37 §§	4.41 *
5 (B)	5.54	4.30 §	4.82	4.06 §§	(4.92) *	(4.89)/4.68 §§	4.92 *
10 (A)	11.4	7.92 §	7.84	-----	(9.66) *	(9.05)/9.05 **	9.66 *
10 (B)	12.4	8.65 §	7.75	-----	(10.5) *	(9.60)/9.60 **	10.5 *

Note: Test article concentrations determined by using a GC/ECD analytical procedure (refer to Attachment 7 for a method description and the raw data from the analyses). The measured values shown are the averages of the analyzed concentrations in the replicate exposure solutions sampled at times 0, 48, and 96 hours. These mean exposure values per replicate were pooled, rounded to a maximum of two significant figures, and were used to calculate all test endpoints (see Table 3).

Dm = *Daphnia magna* (48-hour value)

Gf = *Gammarus fasciatus*

Pp = *Pimephales promelas*

DWC = Diluent Water Control

ND = None Detected (at the method lower limit of detection of 0.005 mg/L).

§ = Dose analyzed due to complete mortality of at least one species at 24 hours.

* = Mean value for 24 hours. Substituted for 96-hour (or 48-hour) mean since complete minnow mortality (or daphnid immobility) was noted at that time.

§§ = Dose analyzed due to complete mortality of gammarids at 74 hours. The dose was then discontinued due to complete mortality of all three test species.

** = Mean value for 48 hours.

TABLE 3. Calculated LC_{50}/EC_{50} and NOEC Values for Aquatic Organisms

Note: All endpoint values were calculated after pooling the dose-response data from Replicates A and B, and are rounded to a maximum of two significant figures.

Species	$LC_{50}/(EC_{50})$ (mg/L)	95% Confidence Interval as mg/L (Lower, Upper)	Method of Calculation	Highest Acute NOEC (mg/L)
Fathead Minnow				
48-hr Values:	1.2	(0.61, 2.5)	N-L I	0.61
96-hr Values:	0.84	(0.51, 1.1)	N-L I	0.51
Gammarid				
48-hr Values:	6.1	(4.7, 9.3)	N-L I	2.5
96-hr Values:	2.7	(1.1, 4.5)	N-L I	1.1
Daphnid				
48-hr Values:	(2.0) †	(1.3, 2.5)	N-L I	0.61

NOEC = No Observed Effect Concentration

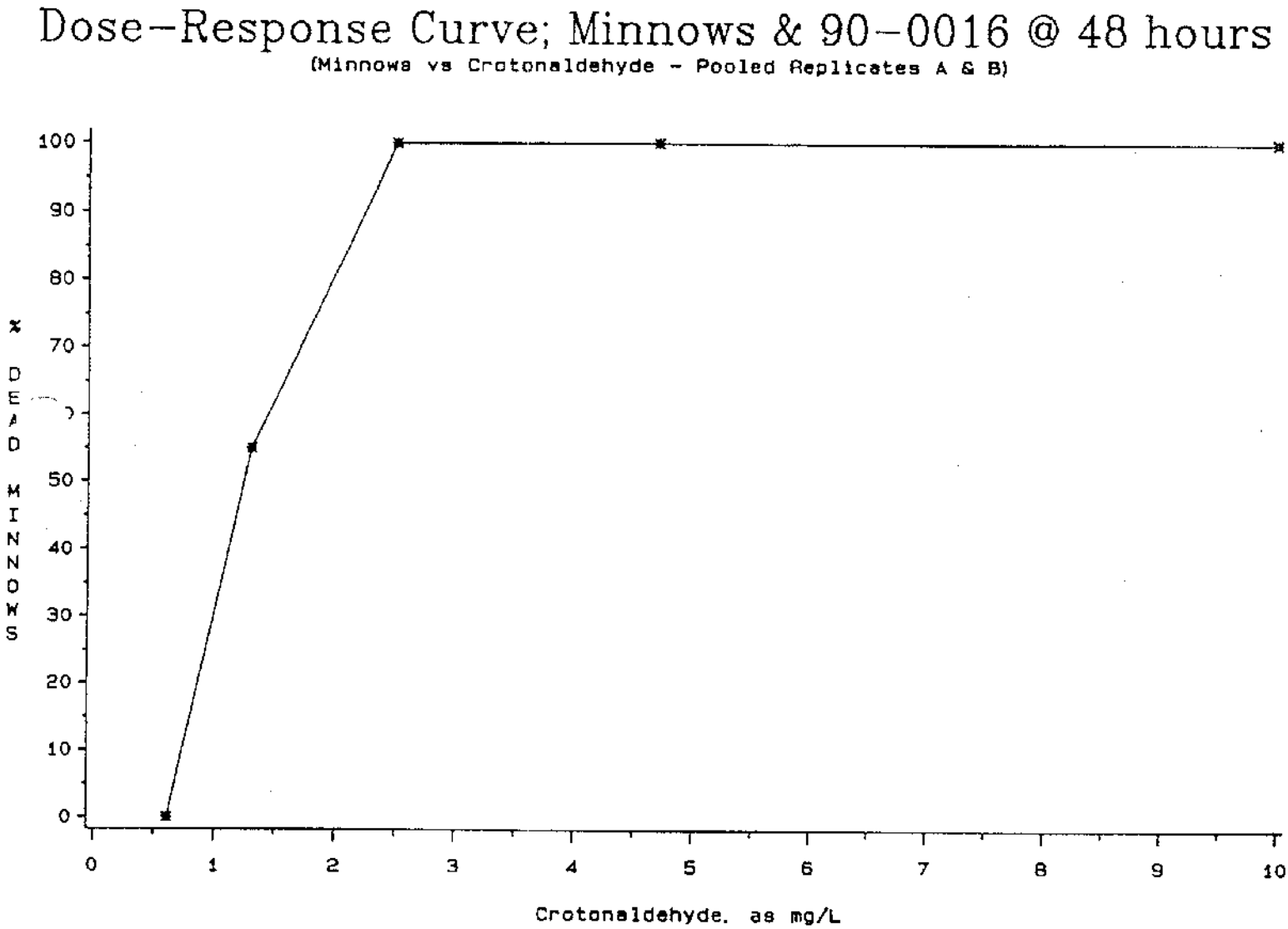
N-L I = Non-linear Interpolation was used to calculate the LC_{50}/EC_{50} values; the associated 95% Confidence Intervals were determined by the Binomial Method. See Attachment 8 for a description of these methods.

† = Only a 48-hour EC_{50}, based on immobilization rather than mortality, was computable from the study data due to limited analyses of doses.

HAEL No. 90-0016 Study No. EN-407-901878-1

FIGURE 1a. Dose-Response Curve for Fathead Minnows at Time 48 Hours

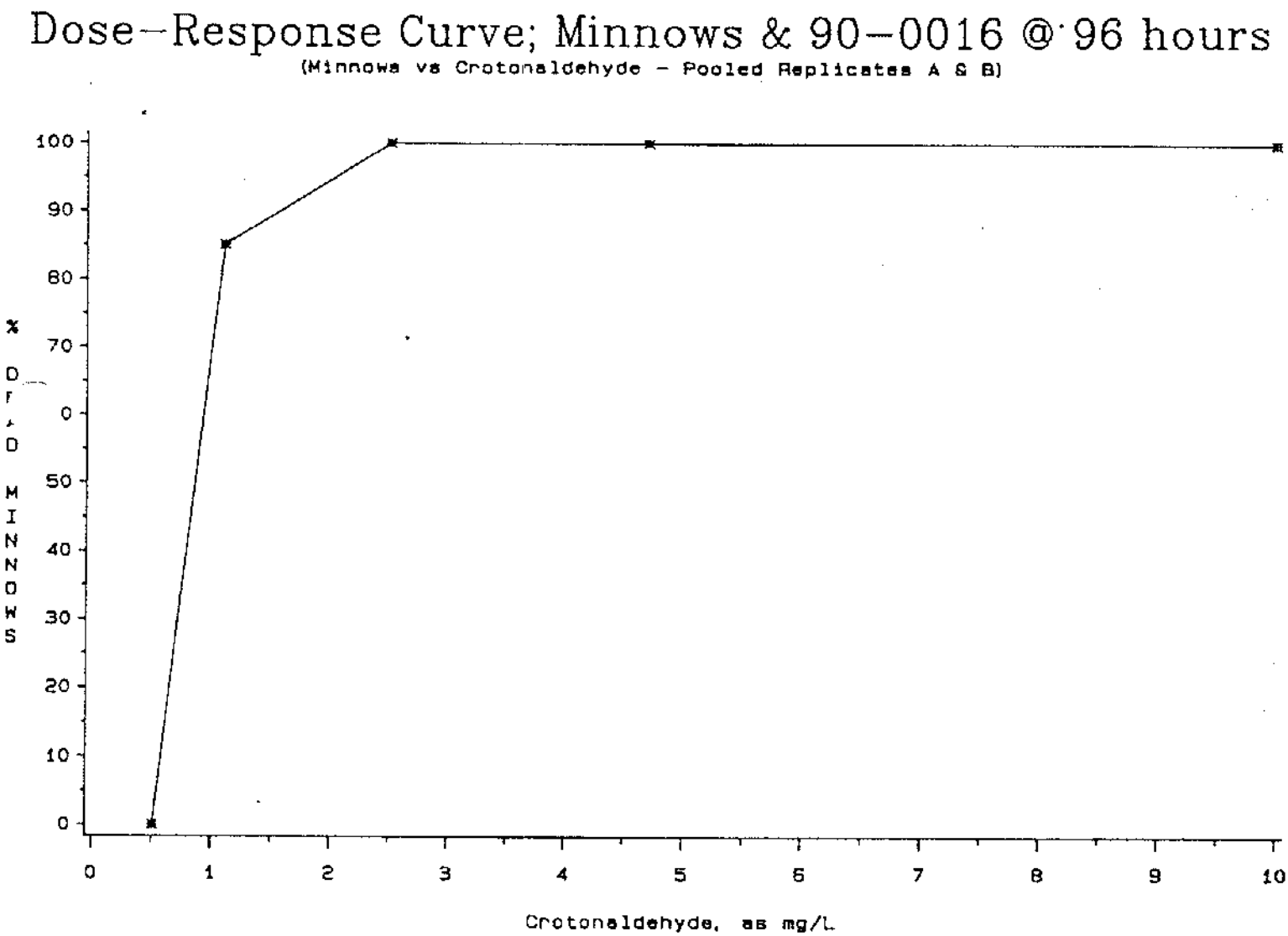
Pooled Replicates A & B



HAEL No. 90-0016 Study No. EN-407-901878-1

FIGURE 1b. Dose-Response Curve for Fathead Minnows at Time 96 Hours

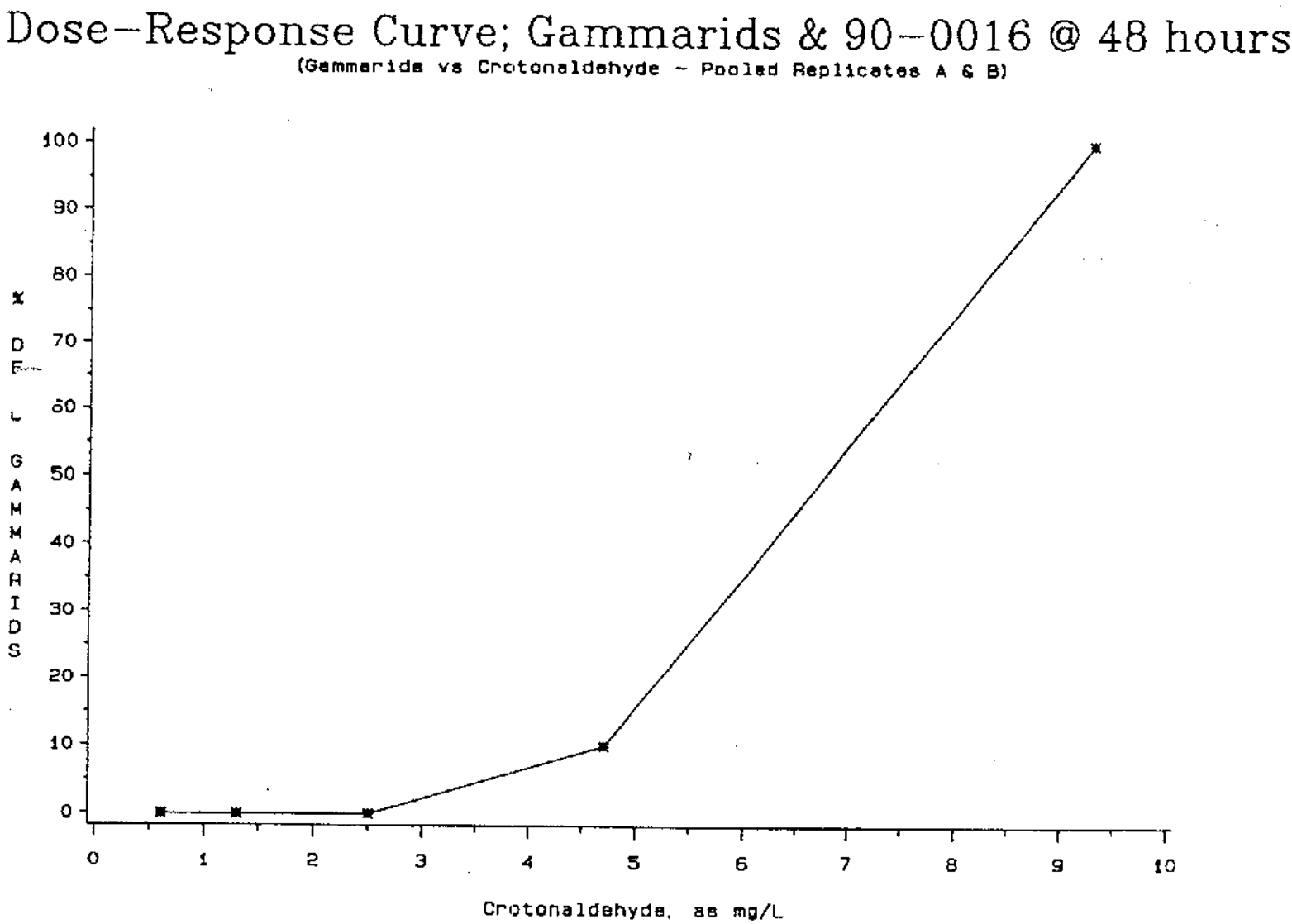
Pooled Replicates A & B



HAEL No. 90-0016 Study No. EN-407-901878-1

FIGURE 2a. Dose-Response Curve for Gammarids at Time 48 Hours

Pooled Replicates A & B



HAEL No. 90-0016 Study No. EN-407-901878-1

FIGURE 2b. Dose-Response Curve for Gammarids at Time 96 Hours

Pooled Replicates A & B

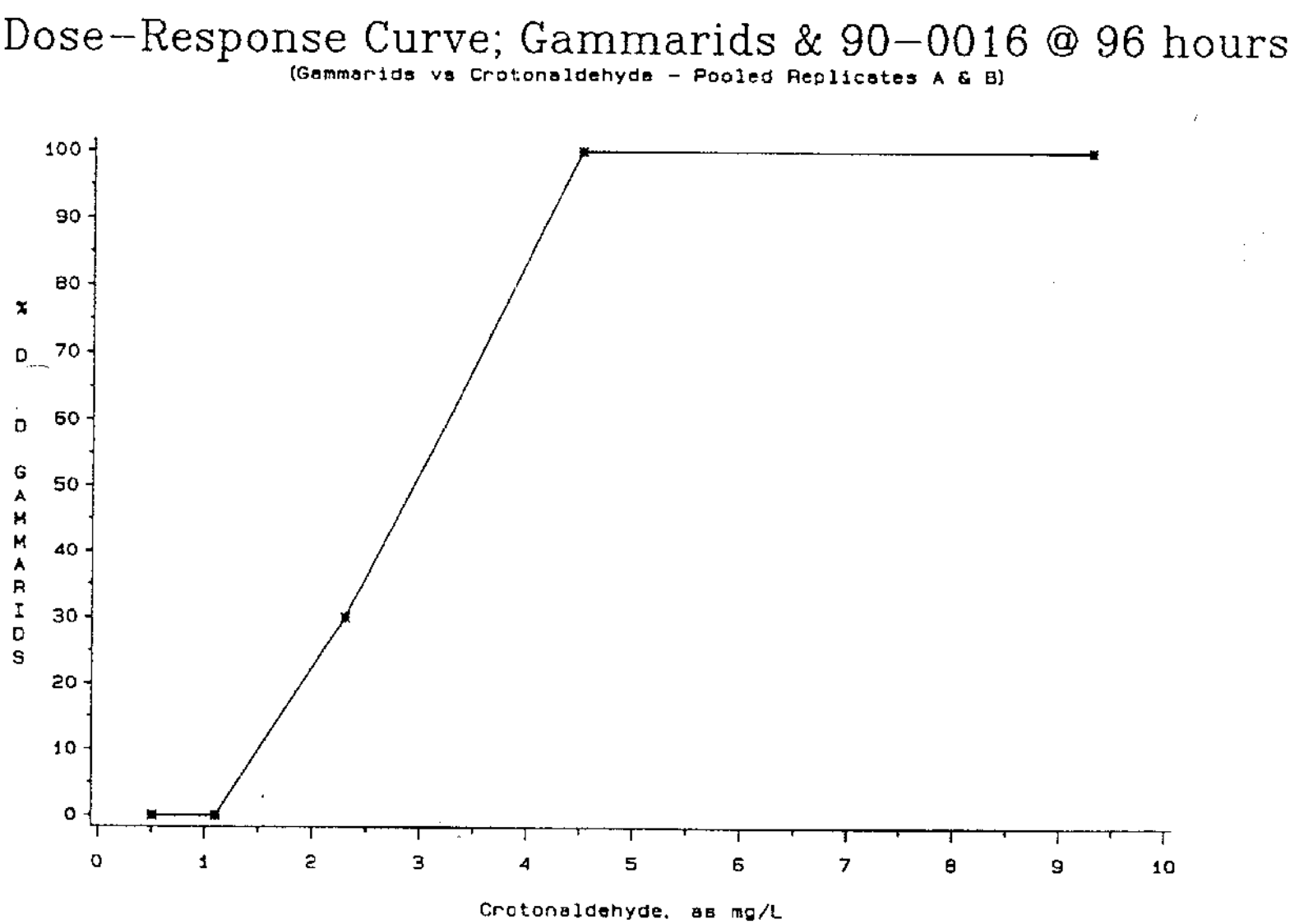


FIGURE 3. Dose-Response Curve for Daphnids at Test End (48 Hours)

Pooled Replicates A & B

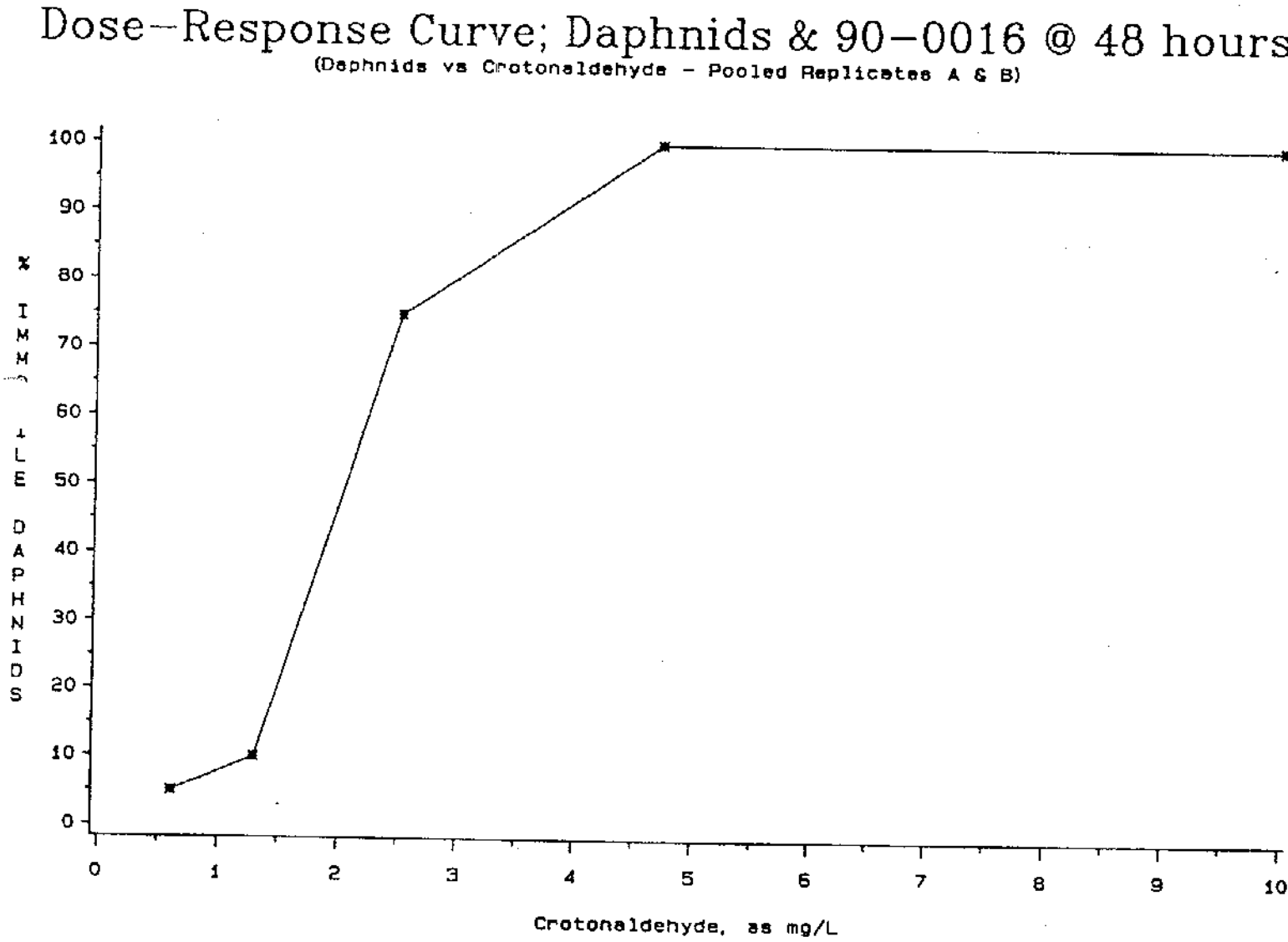
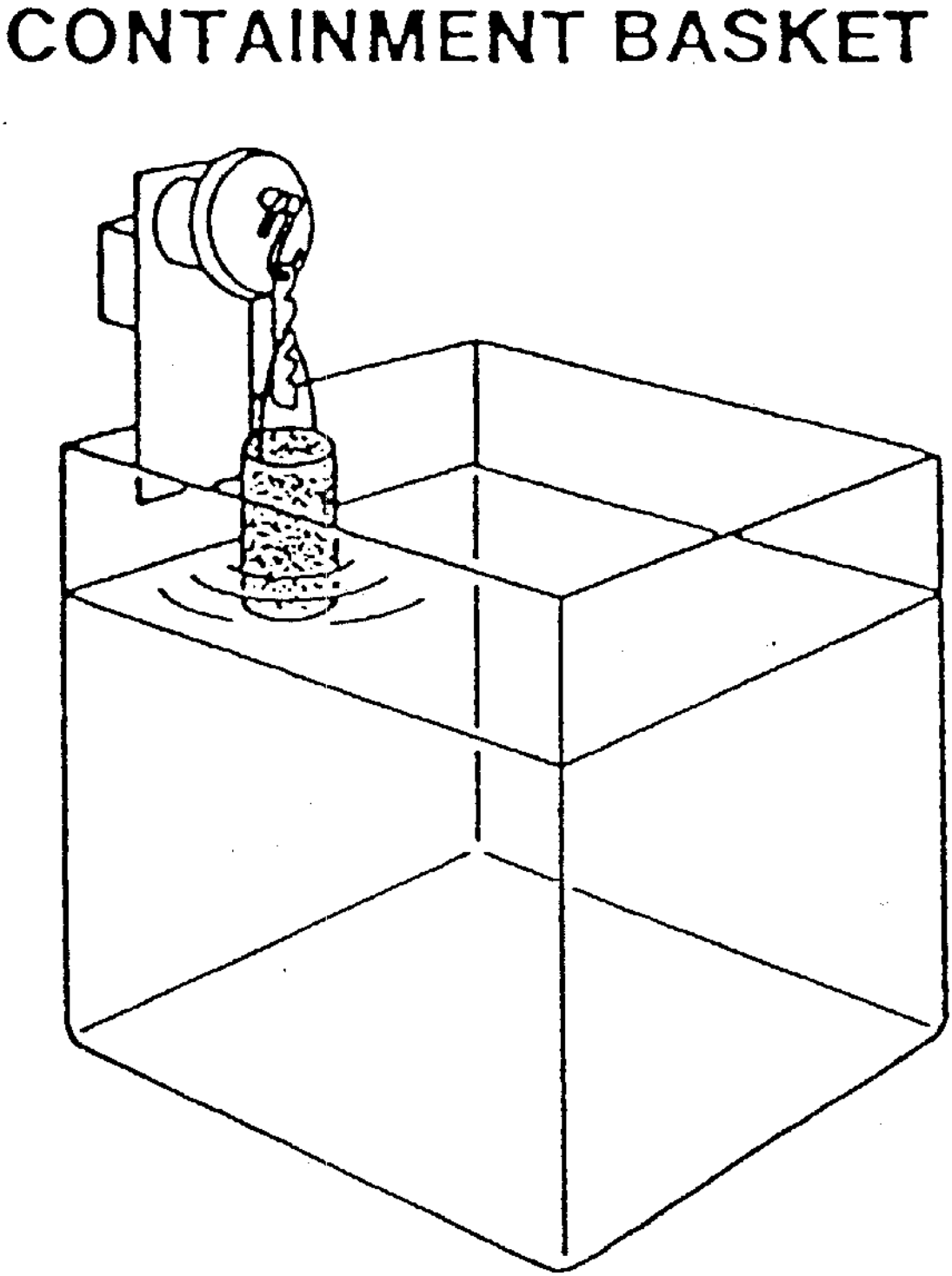


FIGURE 4. Containment Basket In 20 L Test Vessel



ATTACHMENT 1. Product Specification Sheet (Supplied by Sponsor)

901878

MATERIAL SAFETY DATA SHEET

EASTMAN CHEMICAL PRODUCTS, INC.
EASTMAN KODAK COMPANY
Kingsport, Tennessee 37662

For Health Hazard Information, Call: (615) 229-6094

For Other Information, Call Your Eastman Representative

Eastman Operator: (615) 229-2000 Date of Preparation 08-24-87

SECTION I. IDENTIFICATION

-- Name:

Crotonaldehyde

-- Synonyms: PM 161; 2-Butenal,
-- Formula: C_4H_6O
-- Molecular Weight: 70.09

SECTION II. PRODUCT AND COMPONENT HAZARD DATA

A. COMPONENTS:	Approx Weight %	CAS Reg No	Eastman Kodak No
Crotonaldehyde*	92	4170-30-3	901878
Water	8		

See Section VI-A for information on exposure limits.

B. PRECAUTIONARY LABEL STATEMENTS:

DANGER! FLAMMABLE
MAY BE FATAL IF INHALED OR ABSORBED THROUGH THE SKIN
CAUSES SKIN AND EYE BURNS
HARMFUL IF SWALLOWED
VAPOR EXTREMELY IRRITATING
MAY FORM EXPLOSIVE PEROXIDES
MAY POLYMERIZE

SECTION III. PHYSICAL DATA (1)

-- Appearance and Odor: Clear, colorless liquid; pungent, suffocating odor; lachrymator.
-- Boiling Point: 84°C (183°F).
-- Specific Gravity (H_2O = 1): 0.871.
-- Vapor Pressure: 32 mm Hg at 20°C.
-- Percent Volatile by Volume: Approx 1.0.
-- Vapor Density (Air = 1): 2.41.
-- Evaporation Rate (ethyl ether = 1): 0.2.
-- Solubility in Water: Appreciable.

ATTACHMENT 2. Summary of Chemical Characterization of Dilution Water

CHEMICAL CHARACTERISTICS OF BUILDING 306 DILUTION WATER — STATISTICAL ANALYSIS

AGGREGATE HISTORICAL DATA FROM 1981
(All values are reported as mg/L unless otherwise stated)

PARAMETERS *	MOST RECENT VALUES **	MEAN	S.D.	LOW	HIGH	N
1 pH (determined @ 20 C)	8.4	7.96	0.24	7.50	8.40	30
2 Conductivity, as umhos/cm	310	313.59	14.75	268.00	357.00	39
3 Total Hardness (as CaCO3)	120	129.21	5.79	110.00	140.00	39
4 Total Organic Carbon	2.3	2.70	2.12	0.90	10.00	19
5 Total Residual Cl2, as ug/L	<(3)	3.46	2.92	1.50	10.60	16
6 Acidity (as CaCO3)	0	0.00	0.00	0.00	0.00	6
7 Alkalinity (as CaCO3)	99	93.08	5.47	84.00	110.00	39
8 Non-carbonate Hardness (as CaCO3)	21	36.15	7.74	14.00	52.00	39
9 Total Dissolved Solids	110	178.51	23.26	110.00	240.00	39
10 Turbidity, as NTU	0.4	0.13	0.09	0.10	0.40	10
11 Total Suspended Solids	ND(5)	2.50	0.00	2.50	2.50	6
12 Total NH3 (as N)	ND(0.014)	0.05	0.03	0.00	0.14	38
13 Organic Nitrogen	0.2	0.18	0.09	0.07	0.38	18
14 NO3- (as N)	0.37	0.27	0.11	0.00	0.53	39
15 NO2- (as N)	ND(0.01)	0.01	0.004	0.001	0.02	39
16 Chloride by IC (Cl-)	24	25.21	1.70	21.00	29.00	39
17 Fluoride by ISE (F-)	<(0.5)	0.33	0.14	0.10	0.50	19
18 Sulfate by IC (SO4=)	35	29.77	4.65	23.00	47.00	39
19 Total Cyanide (CN-)	ND(0.003)	0.002	0.001	0.001	0.007	19
20 Total Phosphorus (P)	ND(0.02)	0.014	0.007	0.000	0.030	19
21 Molybdenum-Reactive Dissolved SiO2	0.66	0.40	0.17	0.10	0.70	20
Total Metals by ICP/AES:						
22 Aluminum	0.16	0.23	0.09	0.05	0.50	39
23 Barium	0.02	0.02	0.003	0.01	0.03	39
24 Boron	<(0.004)	0.01	0.004	.00	0.01	3
25 Cadmium	<(0.003)	0.004	0.003	0.001	0.01	39
26 Chromium	0.007	0.01	0.01	0.002	0.04	39
27 Cobalt	<(0.003)	0.01	0.01	0.002	0.02	39
28 Copper	<(0.003)	0.01	0.02	0.000	0.14	39
29 Iron	0.02	0.01	0.01	0.001	0.05	39
30 Lead	<(0.01)	0.10	0.12	.00	0.35	39
31 Manganese	0.008	0.01	0.003	0.001	0.02	39
32 Nickel	<(0.007)	0.02	0.02	0.004	0.04	39
33 Silver	<(0.003)	0.01	0.005	.00	0.02	36
34 Zinc	0.006	0.01	0.02	0.002	0.09	39
35 Arsenic by Hydride Generation AAS, as ug/L	ND(6)	2.44	1.67	0.60	5.00	29
36 Mercury by Hydride Generation AAS, as ug/L	ND(0.2)	0.1	.00	0.1	0.1	7

ND( ) = Not Detected at (Method Detection Limit)
<( ) = Less than (Limit of Quantification)
NT = Not Tested for this Parameter

* Note: Parameters reported as ND( ) or <( ) are entered for statistical analysis by halving the parenthetical value (in accordance with the Central Limit Theory for Averages).

** Sampling Date: January 30, 1990

HAEL No. 90-0016 Study No. EN-407-901878-1

ATTACHMENT 3. Chemical Distribution Record

HAEL Chemical Distribution Record

HAEL Lab. No.	90-0016	EN Accession No.	~~901876~~	SRID No.	Lot No. 90-7
Chemical Name	Crotonaldehyde (901878 LRH 2/8/90) (cont)				
Source of Test Substance	~~Texas Eastman Co.~~ ① Tennessee Eastman Co.			Sponsor	TEC/Coord. Wade Francisco (x-3152)
Container(s) Description	1 large glass amber bottle				
Date Test Substance Received	2-5-90	Date Test Substance Processed	2-8-90	By: HRH	Expiration Date: 2-8-95
Date Test Substance Received from C&RI (for testing areas only)	2/8/90 JWG				
Physical State (circle one)	(Liquid) Solid Other	Appearance	clear		
Impurities with %	N/A				
Storage Conditions	~~Room Temperature~~ Refrigerate, and cover with nitrogen or Argon after each use ①				
Weight or Volume (circle one) of Bottle, Cap, and Contents	4348.5g			Weight or Volume (circle one) of Test Substance Sent	3121.2g
Balance / Make	Mettler	Model	PE6000	KP ID No.	1A20916

① Entry error, JWG, 2/9/90

Initials	Date	Area	Purpose	Scale Make, Model, KP ID No.	Initial Bottle Cap & Contents (Wt.) or Vol.	Amount Removed Wt. or Vol.	Amount Remaining Bottle/Cap/Contents (Wt.) or Vol.
-	02/09/90	B-306 ECT-HAEL	Confirmation Long-term Weighing	Mettler PE 4400 (#2) ID # 255850 #589461	4348.2g	0.0g	4348.2g
mm	2-12-90	B-306	Algal Methods	"	4347.9g	6.8g	4341.1g
O'B.	3/13/90	B-306	Sample to CQSD	" O'B.	4341.2g	29.2g	4312.0g
mm	3/19/90	B-306	Algal Range Finding Test	"	4312.5g	1.2g	4311.3g
O'B.	3/22/90	B-306	AAE Meth. Des.	" O'B.	4310.3g	27.6g	4282.7g
mm	4-2-90	B-306	Algal Refinding Test	"	4283.0g	0.8g	4282.2g
O'B.	4/18/90	B-306	AAE Dose Rep (4/23/90)	" O'B.	4280.8g ✓✓	20.7g (20.00g used)	4259.9g
O'B.	4/24/90	B-306	AAE Renewal (4/25/90)	" O'B.	4260.1g ✓✓	20.4g (20.00g used)	4239.7g
O'B.	4/27/90	B-306	Om Dose Prep (4/30/90)	" O'B.	4240.0g ✓✓	4.2g (4.00g used)	4235.8g
O'B.	5/1/90	B-306	Om Dose Renewal (5/2/90)	" O'B.	4235.5g ✓✓	4.6g (4.00g used)	4230.9g
O'B.	5/14/90	B-306	Purity/Stability (H2O)	" O'B.	4230.9g ✓✓	30.9g (to CQSD)	4200.0g
mm	6-21-90	B-306	Methods Closed Bottle	"	4200.0g	removed 1.1g (used 1.1g)	4198.9g
O'B.	6/22/90	B-306	Om Dose Rep (6/25/90)	" O'B.	4198.0g ✓✓	4.7g (used 4.00g)	4193.3g
O'B.	6/26/90	B-306	Om Dose Renewal (6/27/90)	" O'B.	4193.1g ✓✓	4.2g (used 4.00g)	4188.9g
O'B.	6/26/90	B-306	Purity Assay/CQSD	" O'B.	4188.9g	31.8g	4157.1g
mm	7-26-90	B-306	Closed Bottle Test	" mm	4156.8g	1.6g	4165.2g

Chem Dist Form 2/89 plm

ATTACHMENT 4a. Initial Purity Report

CHEMICALS QUALITY SERVICES REPORT FOR: ETS

STUDY DIRECTOR: Heather Watson DATE RECEIVED: 3/1/90

REQUESTED BY: Heather Watson DATE ANALYZED: 3/20/90

REQUEST NO.: 149292 DATE REPORTED: 6/6/90

CQS JOB NO.: 3HGY501

STUDY TYPE: Algal Acute Toxicity

PHASE: Purity Analysis

HAEL NO.: 90-0016

ACCESSION NO.: 901878

AUTHOR: Nancy Porter

SAMPLES (LABELS AND DESCRIPTION): One sample of 90-0016, Accession # 901878, for purity analysis. Lot # 90-7

FOR ADDITIONAL INFORMATION OR FURTHER WORK: Barry Remington @85010

REPORT: A purity analysis was performed on the test chemical using GC/FID on a HP 5890, and water analysis by Karl Fisher.

The results are as follows:

Sample I.D.	Area % by GC/FID	% Water by Karl Fisher
90-0016 Lot # 90-7	98.1%	7.1%

n = 3 (GC/FID)
standard deviation = 0.12

Analyst N. Porter Date 6/6/90

Reviewed by Robert T. Ambrose Date 6/6/90

HAEL No. 90-0016 Study No. EN-407-901878-1

ATTACHMENT 4b. Final Purity Report

CHEMICALS QUALITY SERVICES REPORT FOR: 8306

STUDY DIRECTOR: Robert T. Ambrose

REQUESTED BY: Rob O'Boyle

CQS JOB # : 3HGVS01

PHASE: Purity Determination

REQUEST # : 142470
DATE RECEIVED: 05/14/90
DATE ANALYZED: 05/18/90
DATE REPORTED: 05/18/90
AUTHOR: Beth Isaacs

HAEL#: 90-0016
ACC.#: 901878
SAMPLE REF.#: Lot 90-7

FOR ADDITIONAL INFORMATION OR FURTHER WORK CONTACT: Robert Ambrose X2592S
Beth Isaacs X8503S

RESULTS: The purity of the test chemical was determined by a combination of a water analysis by Karl Fisher titration and a gas chromatographic analysis with flame ionization detection. The results are as follows:

Water (%)	Area % (GC/FID)	
6.3	Run 1	99.7
	Run 2	99.1
	Run 3	99.7
	Mean	99.5
	Std Dev	0.3464

Scientist Beth Isaacs (signature) Date 6/7/90

Reviewed by Robert T Ambrose (signature) Date 6/7/90

ATTACHMENT 4c. Instrumental Conditions for Purity Analysis

Instrumental Conditions for GC Analysis

Instrument:	Hewlett Packard 5890
Column:	DB-WAX; 30m; wide bore (0.25 um film thickness)
Column Pressure:	7 psi
Carrier Gas:	Helium
Initial Temp:	50 °C
Initial Time:	4 min.
Rate:	10 °C/min.
Final Temp:	250 °C
Final Time:	7 min.
Injection Port:	250 °C
Injection Type:	Split
Injection Vol:	1 uL
Detector:	FID
Detector Temp:	250 °C
Solvent:	n-Propanol
Split Flow:	120 cc/min.

ATTACHMENT 5. Protocol for Definitive Test

ENVIRONMENTAL SCIENCES SECTION
ENVIRONMENTAL TECHNICAL SERVICES
HEALTH AND ENVIRONMENT LABORATORIES

Protocol No. EN-407-901878-1
Revision No.:
Date: 3-12-87

Protocol: **Determination of the Acute Aquatic Effects of Test Article on the Fathead Minnow, Pimephales promelas, the Sideswimmer (Gammarid), Gammarus fasciatus, and the Daphnid, Daphnia magna (Performed as a Multispecies Simultaneous Exposure)**

In Accordance with the US EPA, 1985. Toxic Substances, Testing Requirements; Final Rule and Proposed Rule. Federal Register: 40 CFR Part 797.1400 Fish Acute Toxicity Test; 40 CFR Part 797.1300 Daphnid Acute Toxicity Test; 40 CFR Part 797.1310 Gammarid Acute Toxicity Test; (All Tests Amended 1987)

Test Article: Crotonaldehyde

Accession No. 901878 HAEL No. 90-0016

Approved by: R.J. O'Boyle — Study Director — 2/7/'90 — Date

Approved by: Roderick D. Stewe — Sponsor (ECD) — 2/8/90 — Date

Note: The attached protocol will be performed in compliance with the Standard Operating Procedure (SOP) No. EN-4002. The testing procedure will deviate from SOP No. EN-4002 by using only three (listed in title) of the seven aquatic species described in the SOP.

ATTACHMENT 5. Protocol for Definitive Test

Health and Environment Laboratories Management and
Quality Assurance Unit Approvals

Approved: Joseph W. Gorsuch — 2/2/90
Joseph W. Gorsuch — Date
Project Manager
Environmental Sciences Section

Approved: M. Susan James — 2/2/90
Quality Assurance Unit — Date

Approved: John R Owen — 2/2/90
John R. Owen — Date
Unit Director
Environmental Sciences Section

ATTACHMENT 5. Protocol for Definitive Test

Protocol: Determination of the Acute Aquatic Effects of Test Article on the Fathead Minnow, *Pimephales promelas*, the Sideswimmer (Gammarid), *Gammarus fasciatus*, and the Daphnid, *Daphnia magna* (Performed as a Multispecies Simultaneous Exposure)

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I. Test Administration

A. Testing Facility

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652-3617
U.S.A.

B. Sponsor

Eastman Chemical Division
Kingsport, Tennessee 37662
U.S.A.

C. Dates of Experiment

Start Date: 4/23/90
Test Article Exposure Completion Date: 4/27/90 (4/25/90 daphnids only)
Estimated Study Completion Date: 5/27/90

ATTACHMENT 5. Protocol for Definitive Test

D. Test Type
1. ____ Range-Finding/ X Definitive
2. 2 Replicates (10 animals per replicate)
3. ____ Static/ X Dynamic/; 70 mL/Min (At least 6 test vessel volumes per day; a proportionating diluter system will be used.)
4. 15 L/Test Vessel Vol. (Daphnids & gammarids to be confined in stainless-steel mesh, 150 mL baskets suspended from 1 rpm motors.)
5. X Fluorescent/____ Xenon-Arc Illumination (Sixteen-Hour Photoperiod with a 20-Minute Transition Period)
6. (X) Aeration (May Be Used at ≈ 0.05 L/Minute, if Necessary)
7. ____ Nominal/ X Measured Test Article Concentrations @ 0.6, 1.2, 2.5, 5, and 10 mg/L; 100 mg/L stock solution feeding diluter will be renewed at time 48 hours. Samples will be removed for analysis @ times "0", 48 (aged solutions & fresh stock), and 96 hours (or test end per dose; i.e., complete mortality). The daphnid endpoint is immobility and test end is at time 48 hours.

II. Test and Control Substance(s)

A. Test Article
1. Identity: Crotonaldehyde
2. Accession No.: 901878 HAEL No.: 90-0016
Sample Reference Identification No. (Lot No.): Lot No. 90-7
CAS Registry No.: 4170-30-3
3. Other Information (Composition, Stability, Purity, etc.):
The test article is a clear liquid, appreciably soluble in water.

B. Control Substance(s)
1. Negative Control: Laboratory diluent water (seasoned @ 20°C).
2. Positive Control: None required for this test.
3. Carrier Solvent Control: None needed for this test.

III. Test System

A. Test Organisms
1. The fathead minnows, *Pimephales promelas*, were obtained from:
Eco-Chem Testing Group Minnow Breeding Facility
Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York, U.S.A. 14652-3617
Lot Designation: HAEL Lot 12/89

2. The neonate (< 24-hours old) daphnids, *Daphnia magna*, and the juvenile sideswimmers (gammarids), *Gammarus fasciatus*, were obtained from:
Eco-Chem Testing Group Aquatic Animal Colonies
Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York, U.S.A. 14652-3617

B. Test Vessel Identification
The test system will be identified by a placard placed above the exposure vessels. The placard will include the designation HAEL No. 90-0016 (identifying the test article), the test species codes (i.e., Pp, Dm, and Gf), and the test start date. In addition, the location of each of the invertebrate species will be identified within the test system by appropriate labelling of the 1 rpm motors that

ATTACHMENT 5. Protocol for Definitive Test

continually move the containment baskets up and down within each exposure solution (test article doses and diluent water controls).

C. Endpoint Calculations

A computer program developed by C.E. Stephan and the American Society for Testing and Materials (ASTM) is used to compute point and interval (i.e., 95% confidence limits) estimates of the 96-hour LC_{50} value and 48-hour daphnid EC_{50} value. The 24-, 48-, and 72-hour values should be calculated whenever there is sufficient mortality (or daphnid immobility at test time 24 hours) data to determine such values. The program requires the concentration of the test substance (either nominal or measured), the number of organisms exposed, and the number of organisms that die (or are immobilized). Control data are not used because the program does not have the capability of adjusting for control mortality/immobility. The program computes point and interval estimates of the LC_{50}/EC_{50} value using three approaches. (The concentration is $\log_{10}$-transformed for all methods.)

IV. Reporting and Records

A. Reporting

The report must contain:

1. Name and address of the testing facility.
2. Statement of GLP Compliance signed by Study Director and Sponsor.
3. Dates of the study.
4. Procedures stated in the protocol, including any changes or amendments to the original protocol.
5. Methods used in analyzing the data.
6. Identification of test and reference substances.
7. Description of the test methods.
8. Description of the test system.
9. Description of the test article concentration.
10. Description of any circumstances that may have affected the quality or integrity of the study.
11. A copy of all raw data and summaries of data.
12. A copy of the protocol and any amendments.
13. Name of the Study Director and of all other persons involved in the study, including supervision.
14. Summary and analysis of results, including any statistical analyses performed to obtain an LC_{50} (or daphnid EC_{50}) value.
15. Graph of the concentration effect (dose-response) relationship.
16. Proof of validity of the test.
17. Stability of the test substance.
18. Signed and dated reports of the scientists involved in the study.
19. Location of all specimens, raw data, and final reports.
20. Quality Assurance Statement.

B. Records

All records will be retained for at least ten years by the Health and Environment Laboratories:

1. All raw data and summaries of data.
2. The protocol and any protocol amendments.
3. Summaries of training and experience for all persons involved in the study.
4. Standard operating procedures.
5. Final reports.

ATTACHMENT 5. Protocol for Definitive Test

V. Appendix 1. EPA Consent Order Protocol for Fish (Fathead Minnow) Acute Toxicity Test (Section 797.1400)

Section 797.1400 Fish [FATHEAD MINNOW] acute toxicity test.

(a) Purpose. This guideline may be used to develop data on the acute toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 et seq.). This guideline prescribes tests to be used to develop data on the acute toxicity of chemicals to fish. The United States Environmental Protection Agency will use data from these tests in assessing the hazard of a chemical to the environment.

(b) Definitions. The definitions in section 3 of the Toxic Substances Control Act (TSCA), and the definitions in Part 792 Good Laboratory Practice Standards of this chapter apply to this test guideline. The following definitions also apply to this guideline:

(1) "Acclimation" means the physiological compensation by test organisms to new environmental conditions (e.g., temperature, hardness, pH).

(2) "Acute toxicity test" means a method used to determine the concentration of a substance that produces a toxic effect on a specified percentage of test organisms in a short period of time (e.g., 96 hours). In this guideline, death is used as the measure of toxicity.

(3) "Carrier" means a solvent used to dissolve a test substance prior to delivery to the test chamber.

(4) "Conditioning" means the exposure of construction materials, test chambers, and testing apparatus to dilution water or to test solutions prior to the start of a test in order to minimize the sorption of the test substance onto the test facilities or the leaching of substances from the test facilities into the dilution water or test solution.

(5) "Death" means the lack of opercular movement by a test fish.

(6) "Flow-through" means a continuous or an intermittent passage of test solution or dilution water through a test chamber, or a holding or acclimation tank with no recycling.

(7) "Incipient LC_{50}" means the test substance concentration, calculated from experimentally-derived mortality data, that is lethal to 50 percent of a test population when exposure to the test substance is continued until the mean increase in mortality does not exceed 10 percent in any concentration over a 24-hour period.

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(8) "LC_{50}" means that test substance concentration, calculated from experimentally-derived mortality data, that is lethal to 50 percent of a test population during continuous exposure over a specified period of time.

(9) "Loading" means the ratio of fish biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber or passing through it in a 24 hour period.

(10) "Static" means the test solution is not renewed during the period of the test.

(11) "Test solution" means the test substance and the dilution water in which the test substance is dissolved or suspended.

(c) Test procedures—(1) Summary of the test. (i) Test chambers are filled with appropriate volumes of dilution water. If a flow-through test is performed, the flow of dilution water through each chamber is adjusted to the rate desired.

(ii) The test substance is introduced into each test chamber. In a flow-through test, the amount of test substance which is added to the dilution water is adjusted to establish and maintain the desired concentration of test substance in each test chamber.

(iii) Test fish which have been acclimated in accordance with the test design are introduced into the test and control chambers by stratified random assignment.

(iv) Fish in the test and control chambers are observed periodically during the test; dead fish are removed at least twice each day and the findings are recorded.

(v) The dissolved oxygen concentration, pH, temperature and the concentration of test substance are measured at intervals in selected test chambers.

(vi) Concentration-response curves and LC_{50} values for the test substance are developed from the mortality data collected during the test.

(2) (Reserved)

(3) Range-finding test. If the toxicity of the test substance is not already known, a range finding test should be performed to determine the range of concentrations to be used in the definitive test.

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V. Appendix 1. EPA Consent Order Protocol for Fish (Fathead Minnow) Acute Toxicity Test (Section 797.1400)

The highest concentration of test substance for use in the range finding test should not exceed its solubility in water or the permissible amount of the carrier used. [NOMINAL STATIC CONCENTRATIONS MAY BE USED FOR THE RANGE-FINDING TEST.]

(4) Definitive test. (i) A minimum of 20 fish ~~should~~ [SHALL] be exposed to each of five or more test substance concentrations. The range of concentrations to which the fish are exposed should be such that in 96 hours there are at least two partial mortality exposures bracketing 50 percent survival. [RESTRICTED RANDOMIZATION WILL BE USED. THE PARTIAL MORTALITY WILL NOT BE REQUIRED FOR A STEEP SLOPE DOSE RESPONSE CURVE.]

(ii) For exposure to each concentration of a test substance, an equal number of test fish ~~should~~ [SHALL] be placed in two or more replicate test chambers. The distribution of individual fish among the test chambers should be ~~randomized~~ [IMPARTIAL].

(iii) Every test ~~should~~ [SHALL] include a control consisting of the same dilution water, conditions, procedures and fish from the same group used in the test, except that none of the test substance is added.

(iv) Mortality data collected during the test are used to calculate a 96-hour LC_{50}. The 24-, 48-, and 72-hour values should be calculated whenever there is sufficient mortality data to determine such values. If the 96-hour LC_{50} is less than 50 percent of the estimated 48-hour LC_{50} in a flow-through test, the test ~~should~~ [SHALL] be continued until the mean increase in mortality at any test concentration does not exceed 10 percent over a 24-hour period or until 14 days.

(v) Test fish ~~should~~ [SHALL] not be fed while they are being exposed to the test substance under static conditions or during the first 96 hours of flow-through testing. If the test continues past 96 hours, the fish should be fed a suitable food at a maintenance level every other day beginning on test day 5. Any excess food and the fecal material should be removed when observed.

(5) Test results. (i) Death is the primary criterion used in this test guideline to evaluate the toxicity of the test substance.

(ii) In addition to death, any abnormal behavior such as, but not limited to, erratic swimming, loss of reflex, increased excitability, lethargy, or any changes in appearance or physiology such as discoloration, excessive mucous production, hyperventilation, opaque eyes, curved spine, or hemorrhaging ~~should~~ [SHALL] be recorded.

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(iii) Observations on compound solubility ~~should~~ [SHALL] be recorded. The investigator ~~should~~ [SHALL] report the appearance of surface slicks, precipitates, or material adhering to the sides of the test chamber.

(iv) Each test and control chamber ~~should~~ [SHALL] be checked for dead fish and observations recorded at 24, 48, 72, and 96 hours after the beginning of the test or within one hour of the designated times. If the test is continued past 96 hours, additional observations ~~should~~ [SHALL] be made every 24 hours until termination.

(v) The mortality data is used to calculate LC_{50} and their 95 percent confidence limits, and to plot concentration-response curves for each time interval whenever sufficient data exists. The methods recommended for use in calculating LC_{50}'s include probit, logit, binomial, and moving average angle.

(vi) A test is unacceptable if more than 10 percent of the control fish die or exhibit abnormal behavior during a 96-hour test. If a flow-through test is continued past 96 hours, the maximum allowable additional mortality is 10 percent.

(6) Analytical measurements—(i) Water quality analysis. (A) The hardness, acidity, alkalinity, pH, conductivity, TOC or COD, and particulate matter of the dilution water should be measured ~~at the beginning of each static test and at the beginning and end of each flow-through test~~ [MONTHLY]. The month to month variation of the above values should be less than 10 percent and the pH should vary less than 0.4 units. [FOR THE ANALYTICAL REQUIREMENTS OF THE DILUENT WATER, THE ATTACHED AGGREGATE DATA SUMMARY WILL BE SUBSTITUTED. THE MEASURED RESIDUAL CHLORINE SHOULD BE LESS THAN 0.01 mg/L.]

(B) During static tests, the dissolved oxygen concentration, temperature and pH should be measured in each test chamber at the beginning of the test and as often as needed thereafter to document changes from the initial levels. The test solution volume should not be reduced by more than 10 percent as a result of these measurements.

(C) During flow-through tests, dissolved oxygen, temperature and pH measurements ~~should~~ [SHALL] be made in each chamber at the beginning of the test and every ~~48~~ [24] hours thereafter until the end of the test.

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(ii) Collection of samples for measurement of test substance. Test solution samples to be analyzed for the test substance should be taken midway between the top, bottom, and sides of the test chamber. These samples should not include any surface scum or material dislodged from the bottom or sides. Samples should be analyzed immediately or handled and stored in a manner which minimizes loss of test substance through microbial degradation, photodegradation, chemical reaction, volitalization, or sorption.

(iii) Measurement of test substance. ~~(A) For static tests the concentration of dissolved test substance (that which passes through a 0.45 micron filter) should be measured at a minimum in each test chamber at the beginning (0 hour, before fish are added) and at the end of the test.~~ [(A) DEIONIZED OR DISTILLED WATER SHOULD BE USED IN MAKING STOCK SOLUTIONS OF THE TEST SUBSTANCE. STANDARD ANALYTICAL METHODS SHOULD BE USED WHENEVER AVAILABLE IN PERFORMING THE ANALYSES. THE ANALYTICAL METHOD USED TO MEASURE THE AMOUNT OF TEST SUBSTANCE IN A SAMPLE SHALL BE VALIDATED BEFORE BEGINNING THE TEST BY APPROPRIATED LABORATORY PRACTICES. AN ANALYTICAL METHOD IS NOT ACCEPTABLE IF LIKELY DEGRADATION PRODUCTS OF THE TEST SUBSTANCE, SUCH AS HYDROLYSIS AND OXIDATION PRODUCTS, GIVE POSITIVE OR NEGATIVE INTERFERENCES WHICH CANNOT BE SYSTEMATICALLY IDENTIFIED AND CORRECTED MATHEMATICALLY.] During flow-through tests, the concentration of dissolved test substance ~~should~~ [SHALL] be measured as follows:

(1) In each chamber at 0-hour. [REAL-TIME ANALYSIS OF THE TEST SOLUTION FOR THE TEST ARTICLE WILL BE CONDUCTED AT 0, 48, AND 96 HOURS.]

(2) In each chamber at 96-hours and every 4 days thereafter, as long as the test is continued.

(3) In at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system.

~~(B) Filters and their holders used for determining the dissolved test substance concentrations should be rinsed with several volumes of distilled water and undergo a final rinse with test solution. Glass or stainless steel filter holders are best for organic test substances, while plastic holders are best for metals. The sample should be filtered within 30 minutes after it is taken from the test chamber.~~

~~(C) The analytical methods used to measure the amount of test substance in a sample should [SHALL] be validated before beginning the test. The accuracy of a method should be verified by a method such as using known additions.~~

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~~This involves adding a known amount(s) of the test substance to three water samples taken from a chamber containing (of) dilution water and the same number and species of fish as are used in the test. The nominal concentration(s) of the test substance in these samples should span the concentration range to be used in the test. Validation of the analytical method should be performed on at least two separate days prior to starting the test.~~

~~(D) An analytical method is not acceptable if likely degradation products of the test substance give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test.~~

~~(E) In addition to analyzing samples of test solution, at least one reagent blank containing all reagents used should also be analyzed.~~

~~(F) If the measured concentrations of dissolved test substances are considerably lower (e.g., (50 percent) than the nominal concentrations, the total test substance concentration should be measured in the highest test concentration.~~

[(B) THE ANALYTICAL METHOD FOR THE TEST SUBSTANCE SHALL BE VALIDATED PRIOR TO BEGINNING THE TEST. A PROCEDURE SUCH AS USING KNOWN ADDITIONS MAY BE USED. THIS INVOLVES ADDING KNOWN AMOUNTS OF THE TEST SUBSTANCE TO THREE OR MORE SAMPLES OF DILUTION WATER. THE NOMINAL CONCENTRATIONS OF THE TEST SUBSTANCE IN THESE SAMPLES SHOULD SPAN THE CONCENTRATION RANGE TO BE USED IN THE TEST. BOTH DISSOLVED TEST SUBSTANCE (THAT WHICH PASSES THROUGH A 0.45 MICRON FILTER) AND TOTAL TEST SUBSTANCE SHALL BE MEASURED IN EACH SAMPLE. IF THE MEASURED CONCENTRATIONS OF DISSOLVED TEST SUBSTANCE ARE GREATER THAN 80% OF THE MEASURED CONCENTRATIONS OF TOTAL TEST SUBSTANCE, THEN ONLY TOTAL TEST SUBSTANCE SHALL BE MEASURED DURING THE TEST. HOWEVER, IF THE MEASURED CONCENTRATIONS OF DISSOLVED TEST SUBSTANCE ARE LESS THAN 80% OF THE MEASURED CONCENTRATIONS OF TOTAL TEST SUBSTANCE, THEN ONLY DISSOLVED TEST SUBSTANCE SHALL BE MEASURED DURING THE TEST. VALIDATION OF THE ANALYTICAL METHOD SHOULD BE PERFORMED ON AT LEAST TWO SEPARATE DAYS PRIOR TO STARTING THE TEST.]

[(C) IN ADDITION TO ANALYZING SAMPLES OF TEST SOLUTION, AT LEAST ONE REAGENT BLANK, CONTAINING ALL REAGENTS USED, SHOULD ALSO BE ANALYZED.]

[(D) FILTERS AND THEIR HOLDERS USED FOR DETERMINING THE DISSOLVED TEST SUBSTANCE CONCENTRATIONS SHOULD BE PREWASHED WITH SEVERAL VOLUMES OF DISTILLED WATER OR DILUTION WATER AND UNDERGO A FINAL RINSE WITH TEST SOLUTION. GLASS OR STAINLESS STEEL FILTER HOLDERS ARE BEST FOR ORGANIC SUBSTANCES, WHILE PLASTIC HOLDERS ARE BEST FOR METALS. THE SAMPLE SHOULD BE FILTERED WITHIN 30 MINUTES AFTER IT IS TAKEN FROM THE TEST CHAMBER.]

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V. Appendix 1. EPA Consent Order Protocol for Fish (Fathead Minnow) Acute Toxicity Test (Section 797.1400)

~~(C)~~ [E] Among replicate test chambers, the measured concentrations should not vary more than 20 percent. The measured concentration of the test substance in any chamber during the test should not vary more than 30 percent from the measured concentration prior to initiation of the test.

~~(D)~~ [F] The mean measured concentration of ~~dissolved~~ [THE] test substance ~~should~~ [SHALL] be used to calculate all LC_{50}'s and to plot all concentration-response curves.

(d) Test conditions—(i) Test species. (i) Selection. The test species for this test are the rainbow trout (Salmo gairdneri), ~~bluegill (Lepomis macrochirus)~~ and fathead minnow (Pimephales promelas). The particular species of fish to be used will be prescribed in the test rule. [FOR THIS PROTOCOL THE TEST SPECIES WILL BE FATHEAD MINNOW.]

(ii) Age and condition of fish. (A) Juvenile fish ~~should~~ [SHALL] be used. Fish used in a particular test ~~should~~ [SHALL] be the same age and be of normal size and appearance for their age. The longest fish ~~should~~ [SHALL] not be more than twice the length of the shortest.

(B) All newly acquired fish should be quarantined and observed for at least 14 days prior to use in a test.

(C) Fish ~~should~~ [SHALL] not be used for a test if they appear stressed or if more than five percent die during the 48 hours immediately prior to the test.

(iii) Acclimation of test fish. (A) If the holding water is not from the same source as the test dilution water, acclimation to the dilution water should be done gradually over a 48-hour period. The fish should then be held an additional 14 days in the dilution water prior to testing. Any changes in water temperature should not exceed 3°C per day. Fish should be held for a minimum of 7 days at the test temperature prior to testing.

(B) During the final 48-hours of acclimation, fish should be maintained in facilities with background colors and light intensities similar to those of the testing area and should not be fed.

(2) Facilities—(i) General. Facilities needed to perform this test include:

(A) Flow-through tanks for holding and acclimating fish.

(B) A mechanism for controlling and maintaining the water temperature during the holding, acclimation and test periods.

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(C) Apparatus for straining particulate matter, removing gas bubbles, or insufficient dissolved oxygen, respectively.

(D) Apparatus for providing a 16-hour light and 8-hour dark photoperiod with a 15- to 30-minute transition period.

(E) Chambers for exposing test fish to the test substance.

(F) A test substance delivery system for flow-through tests.

(ii) Construction materials. Construction materials and commercially purchased equipment that may contact the stock solution, test solution, or dilution water should not contain substances that can be leached or dissolved into aqueous solutions in quantities that can alter the test results. Materials and equipment that contact stock or test solutions should be chosen to minimize sorption of test chemicals. Glass, stainless steel, and perfluorocarbon plastic should be used whenever possible. Concrete, fiberglass, or plastic (e.g., PVC) may be used for holding tanks, acclimation tanks, and water supply systems, but they should be used to remove rust particles. Rubber, copper, brass, galvanized metal, epoxy glues, and lead should not come in contact with the dilution water, stock solution, or test solution.

(iii) Test substance delivery system. In flow-through tests, diluters, metering pump systems or other suitable devices should be used to deliver the test substance to the test chambers. The system used should be calibrated before each test. Calibration includes determining the flow rate through each chamber and the concentration of the test substance delivered to each chamber. The general operation of the test substance delivery system should be checked twice daily during a test. The 24-hour flow rate through a test chamber [SHOULD BE A MINIMUM OF SIX TANK VOLUMES]. During a test, the flow rates should not vary more than 10 percent from one test chamber to another or from one time to any other.

(iv) Test chambers. Test chambers made of stainless steel should be welded, not soldered. Test chambers made of glass should be fused or bonded using clear silicone adhesive. As little adhesive as possible should be left exposed in the interior of the chamber.

(v) Cleaning of test system. Test substance delivery systems and test chambers should be cleaned before each test. They should be washed with detergent and then rinsed in sequence with clean water, pesticide-free acetone, clean water, and 5 percent nitric acid, followed by two or more changes of dilution water.

(vi) Dilution water. (A) Clean surface or ground water reconstituted water, or dechlorinated tap water is acceptable as dilution water if the test fish will survive in it for the duration

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of the holding, acclimating, and testing periods without showing signs of stress, such as discoloration, hemorrhaging, disorientation or other unusual behavior. The quality of the dilution water should be constant and should meet the following specifications measured at least twice a year [FOR THE ANALYTICAL REQUIREMENTS OF THE DILUENT WATER, THE ATTACHED DILUTION WATER AGGREGATE HISTORICAL DATA SUMMARY WILL BE SUBSTITUTED. THE MEASURED RESIDUAL CHLORINE SHOULD BE LESS THAN 0.01 mg/L.]:

Substance	Maximum Concentration	
Particulate matter	20	mg/liter.
Total organic carbon or	~~2~~ [3]	mg/liter.
Chemical oxygen demand	5	mg/liter.
Un-ionized ammonia	~~1~~ [20]	µg/liter.
Residual chlorine	~~1~~ [10]	µg/liter.
Total organophosphorus pesticides	50	µg/liter.
Total organochlorine pesticides plus polychlorinated biphenyls (PCBs).	50	µg/liter.
or organic chlorine	25	µg/liter.

(B) The concentration of dissolved oxygen in the dilution water should be between 90 and 100 percent saturation; 9.8 to 10.8 mg/l for tests with trout, and 8.0 to 8.9 mg/l for tests with bluegill or fathead minnow at sea level. If necessary, the dilution water can be aerated before the addition of the test substance. All reconstituted water should be aerated before use. Buffered soft water should be aerated before but not after the addition of buffers.

(C) If disease organisms are present in the dilution water in sufficient numbers to cause infection, they should be killed or removed by suitable equipment.

(D) Glass distilled or carbon filtered deionized water with a conductivity less than 1 micromho/cm is acceptable for use in making reconstituted water. If the reconstituted water is prepared from a ground or surface water source, conductivity, and total organic carbon (TOC) or chemical oxygen demand (COD) should be measured on each batch.

(vii) Carriers. (A) Distilled water [STOCK SOLUTIONS WILL BE MADE WITH DILUTION WATER.] should be used in making stock solutions of the test substance. If the stock volume however is more than 10 percent of the test solution volume, dilution water should be used. If a carrier is absolutely necessary to dissolve the test substance, the volume used should not exceed the minimum volume necessary to dissolve or suspend the test substance in the test solution. If the test substance is a mixture, formulation, or commercial product, none of the ingredients is considered a carrier unless an extra amount is used to prepare the stock solution.

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V. Appendix 1. EPA Consent Order Protocol for Fish (Fathead Minnow) Acute Toxicity Test (Section 797.1400)

(B) Triethylene glycol and dimethyl formamide are the preferred carriers, but acetone can also be used. The concentration of triethylene glycol in the test solution should not exceed 80 mg/l [0.1 ML/L]. The concentration of dimethyl formamide or acetone in the test solution should not exceed ~~5/0 mg/l~~ [0.1 ML/L].

(3) Test parameters—(i) Loading. The number of fish placed in a test chamber should not be so great as to affect the results of the test. The loading ~~should~~ [SHALL] not be so great that the test substance concentrations are decreased by more than 20 percent due to uptake by the fish. In static tests, loading should not exceed 0.5 grams of fish per liter of solution in the test chamber at any one time. In flow-through tests loading should not exceed 0.5 grams of fish per liter of test solution passing through the chamber in 24 hours. These loading rates should be sufficient to maintain the dissolved oxygen concentration above the recommended levels and the ammonia concentration below 20 µg/l.

(ii) Dissolved oxygen concentration. (A) During static tests with rainbow trout the dissolved oxygen in each test chamber should be greater than 5.5 mg/l. In tests with bluegill and fathead minnows, the DO should be maintained above 4.5 mg/l.

(B) During flow-through tests the dissolved oxygen concentration ~~should~~ [SHALL] be maintained above 8.2 mg/l in tests with trout and above 6.6 mg/l in tests with bluegills or fathead minnows.

(iii) Temperature. The test temperature ~~should~~ [SHALL] be ~~22~~ [20] ± ~~1~~[2] °C for ~~bluegill and~~ fathead minnows, and 12 ± ~~1~~[2]°C for rainbow trout. The temperature ~~should~~ [SHALL] be measured at least hourly in one test chamber.

(iv) Light. A 16-hour light and 8-hour dark photoperiod with a 15- to 30-minute transition period should be maintained.

(e) Reporting. The sponsor ~~should~~ [SHALL] submit to the EPA all data developed by the test that are suggestive or predictive of toxicity. In addition to the reporting requirements prescribed in Part 792—Good Laboratory Practice Standards of this chapter, the reported test data ~~should~~ [SHALL] include the following:

(1) The source of the dilution water, a description of any pretreatment, and the measured hardness, acidity, alkalinity, pH, conductivity, TOC or COD and particulate matter.

(2) A description of the test chambers, the depth and volume of solution in the chamber, the specific way the test was begun (e.g., conditioning, test substance additions), and for flow-through tests, a description of the test substance delivery system.

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V. Appendix 1. EPA Consent Order Protocol for Fish (Fathead Minnow) Acute Toxicity Test (Section 797.1400)

(3) Detailed information about the test fish, including the scientific name and method of verification, average weight (grams, wet weight), standard length, age, source, history, observed diseases, treatments, and mortalities, acclimation procedures, and food used.

(4) The number of replicates used, the number of organisms per replicate, the loading rate, and the flow rate for flow-through tests.

(5) The measured DO, pH, and temperature and the lighting regime.

(6) The solvent used, the test substance concentration in the stock solution, the highest solvent concentration in the test solution and a description of the solubility determinations in water and solvents if used.

(7) The concentration of the test substance in each test chamber just before the start of the test and at all subsequent sampling periods.

(8) The number of dead and live tests organisms, the percentage of organisms that died, and the number that showed any abnormal effects in the control and in each test chamber at each observation period.

(9) The 96-hour LC_{50}, and when sufficient data have been generated, the 24-, 48-, 72-, and incipient LC_{50} values, their 95 percent confidence limits, and the methods used to calculate the LC_{50} values and their confidence limits.

(10) When observed, the observed no effect concentration (the highest concentration tested at which there were no mortalities or abnormal behavioral or physiological effects).

(11) The concentration-response curve at each observation period for which a LC_{50} was calculated.

(12) Methods and data records of ~~all chemical~~ analyses of ~~water quality parameters and~~ test substance concentrations, including method validations and reagent blanks.

[FOR THE ANALYTICAL REQUIREMENTS OF THE DILUENT WATER, THE ATTACHED DILUTION WATER AGGREGATE HISTORICAL DATA SUMMARY WILL BE SUBSTITUTED. LIMITS OF DETECTION SHALL BE INCLUDED.]

ATTACHMENT 5. Protocol for Definitive Test

V. Appendix 2. EPA Consent Order Protocol for Daphnid Acute Toxicity Test (Section 797.1300)

Section 797.1300 Daphnid acute toxicity test.

(a) Purpose. This guideline is intended for use in developing data on the acute toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003. 15 U.S.C. 2601 et seq.). This guideline prescribes an acute toxicity test in which daphnids (Daphnia magna or D. pulex) are exposed to a chemical in static ~~and~~ [OR] flow-through systems. The United States Environmental Protection Agency will use data from this test in assessing the hazard a chemical may present to the aquatic environment.

(b) Definitions. The definitions in section 3 of the Toxic Substances Control Act (TSCA), and the definitions in Part 792—Good Laboratory Practice Standards of this chapter apply to this test guideline. In addition, the following definitions apply to this guideline:

(1) "Brood stock" means the animals which are cultured to produce test organisms through reproduction.

(2) "EC_{50}" means that experimentally derived concentration of test substance in dilution water that is calculated to affect 50 percent of a test population during continuous exposure over a specified period of time. In this guideline, the effect measured is immobilization.

(3) "Ephippium" means a resting egg which develops under the carapace in response to stress conditions in daphnids.

(4) "Flow-through" means a continuous or intermittent passage of test solution [, TEST SUBSTANCE] or dilution water through a test chamber or culture tank with no recyling.

(5) "Immobilization" means the lack of movement by the test organisms except for minor activity of the appendages.

(6) "Loading" means the ratio of daphnid biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber at a point in time, or passing through the test chamber during a specific interval.

(7) "Static system" means a test system in which the test solution and test organisms are placed in the test chamber and kept there for the duration of the test without renewal of the test solution.

(c) Test procedures--(1) Summary of the test. (i) Test chambers are filled with appropriate volumes of dilution water. In the flow-through test, the flow of dilution water through each chamber is adjusted to the rate desired. The test chemical is introduced

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into each treatment chamber. The addition of test chemical in the flow-through system is conducted at a rate which is sufficient to establish and maintain the desired concentration in the test chamber. The test is started within 30 minutes after the test chemical has been added and uniformly distributed in static test chambers or after the concentration of test chemical in each flow-through test chamber reaches the prescribed level and remains stable. At the initiation of the test, daphnids which have been cultured and acclimated in accordance with the test design, are randomly placed into the test chambers. Daphnids in the test chambers are observed periodically during the test, the immobile daphnids removed, and the findings recorded.

(ii) Dissolved oxygen concentration, pH, temperature, the concentration of test chemical and other water quality parameters are measured at specified intervals in selected test chambers. Data are collected during the test to develop concentration-response curves and determine EC_{50} values for the test chemical.

(2) (Reserved)

(3) Range-finding test. (i) A range-finding test should be conducted to establish test solution concentrations for the definitive test. [NOMINAL STATIC CONCENTRATIONS MAY BE USED FOR THE RANGE-FINDING TEST.]

(ii) The daphnids should be exposed to a series of widely spaced concentrations of the test chemical (e.g., 1, 10, 100 mg/l, etc.), usually under static conditions.

(iii) A minimum of five daphnids should be exposed to each concentration of test chemical for a period of 48 hours. The exposure period may be shortened if data suitable for the purpose of the range-finding test can be obtained in less time. No replicates are required and nominal concentrations of the chemical are acceptable.

(4) Definitive test. (i) The purpose of the definitive test is to determine concentration-response curves and the 24- and 48-hour EC_{50} values with the minimum amount of testing beyond the range-finding test.

(ii) A minimum of 20 daphnids per concentration shall be exposed to five or more concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32, and 64 mg/l). An equal number of daphnids shall be placed in two or more replicates. If solvents, solubilizing agents or emulsifiers have to be used, they shall be commonly used carriers and ~~shall~~ [SHOULD] not possess a synergistic or antagonistic

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effect on the toxicity of the test chemical. The concentration of solvent should not exceed 0.1 ~~ml/l~~ [ml/l]. The concentration ranges ~~shall~~ [SHOULD] be selected to determine the concentration-response curves, EC_{50} values at 24 and 48 hours. Concentration of test chemical in test solutions should be analyzed prior to use.

(iii) Every test shall include controls consisting of the same dilution water, conditions, procedures and daphnids from the same population (culture container), except that none of the chemical is added.

(iv) The dissolved oxygen concentration, temperature and pH shall be measured at the beginning and end of the test in each chamber.

(v) The test duration is 48 hours. The test is unacceptable if more than 10 percent of the control organisms are immobilized during the 48-hour test period. Each test chamber shall be checked for immobilized daphnids at 24 and 48 hours after the beginning of the test. [WHENEVER POSSIBLE,] concentration-response curves and 24-hour and 48-hour EC_{50} values for immobilization shall be determined along with their 95 percent confidence limits.

(vi) In addition to immobility, any abnormal behavior or appearance shall also be reported.

(vii) Test organisms shall be impartially distributed among test chambers in such a manner that test results show no significant bias from the distributions. In addition, test chambers within the testing area shall be positioned in a random manner or in a way in which appropriate statistical analyses can be used to determine the variation due to placement. [RESTRICTED RANDOMIZATION WILL BE USED.]

(viii) The concentration of the test chemical in the chambers should be measured as often as is feasible during the test. In the static test the concentration of test chemical shall be measured, at a minimum, at the beginning of the test and at the end of the test in each test chamber. In the flow-through test the concentration of test chemical shall be measured at a minimum:

(A) In each chamber at the beginning of the test and at 48 hours after the start of the test;

(B) In at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system. Among replicate test chambers of a treatment concentration, the measured concentration of the test chemical shall not vary more than ± 20 percent.

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(5) (Reserved) [MORE THAN ONE SPECIES OF TEST ORGANISM MAY BE INCLUDED IN A TEST VESSEL AT THE SAME TIME. APPROPRIATE ISOLATION TECHNIQUES WILL BE USED.]

(6) Analytical measurements—(i) Test chemical. [(A)] Deionized [OR DISTILLED] water should be used in making stock solutions of the test chemical. Standard analytical methods should be used whenever available in performing the analyses. The analytical method used to measure the amount of test chemical in a sample shall be validated before beginning the test by appropriate laboratory practices. Any analytical method is not acceptable if likely degradation products of the test chemical, such as hydrolysis and oxidation products, give positive or negative interferences which cannot be systematically identified and corrected mathematically.

[(B) THE ANALYTICAL METHOD FOR THE TEST SUBSTANCE SHALL BE VALIDATED PRIOR TO BEGINNING THE TEST. A PROCEDURE SUCH AS USING KNOWN ADDITIONS MAY BE USED. THIS INVOLVES ADDING KNOWN AMOUNTS OF THE TEST SUBSTANCE TO THREE OR MORE SAMPLES OF DILUTION WATER. THE NOMINAL CONCENTRATIONS OF THE TEST SUBSTANCE IN THESE SAMPLES SHOULD SPAN THE CONCENTRATION RANGE TO BE USED IN THE TEST. BOTH DISSOLVED TEST SUBSTANCE (THAT WHICH PASSES THROUGH A 0.45 MICRON FILTER) AND TOTAL TEST SUBSTANCE SHALL BE MEASURED IN EACH SAMPLE. IF THE MEASURED CONCENTRATIONS OF DISSOLVED TEST SUBSTANCE ARE GREATER THAN 80% OF THE MEASURED CONCENTRATIONS OF TOTAL TEST SUBSTANCE, THEN ONLY TOTAL TEST SUBSTANCE SHALL BE MEASURED DURING THE TEST. HOWEVER, IF THE MEASURED CONCENTRATIONS OF DISSOLVED TEST SUBSTANCE ARE LESS THAN 80% OF THE MEASURED CONCENTRATIONS OF TOTAL TEST SUBSTANCE, THEN ONLY DISSOLVED TEST SUBSTANCE SHALL BE MEASURED DURING THE TEST. VALIDATION OF THE ANALYTICAL METHOD SHOULD BE PERFORMED ON AT LEAST TWO SEPARATE DAYS PRIOR TO STARTING THE TEST.]

[(C) IN ADDITION TO ANALYZING SAMPLES OF TEST SOLUTION, AT LEAST ONE REAGENT BLANK, CONTAINING ALL REAGENTS USED, SHOULD ALSO BE ANALYZED.]

[(D) FILTERS AND THEIR HOLDERS USED FOR DETERMINING THE DISSOLVED TEST SUBSTANCE CONCENTRATIONS SHOULD BE PREWASHED WITH SEVERAL VOLUMES OF DISTILLED WATER OR DILUTION WATER AND UNDERGO A FINAL RINSE WITH TEST SOLUTION. GLASS OR STAINLESS STEEL FILTER HOLDERS ARE BEST FOR ORGANIC SUBSTANCES, WHILE PLASTIC HOLDERS ARE BEST FOR METALS. THE SAMPLE SHOULD BE FILTERED WITHIN 30 MINUTES AFTER IT IS TAKEN FROM THE TEST CHAMBER.]

(ii) Numerical. The number of immobilized daphnids should be counted during each definitive test. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration-response curves. A 24- and 48-hour EC_{50} and corresponding 95 percent interval should be calculated [WHENEVER POSSIBLE].

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(d) Test conditions--(1) Test species—(i) Selection (A) The cladocerane, Daphnia magna ~~or D. pulex, are~~ [IS] the species to be used in this test. ~~Either species may be utilized for testing of a particular chemical.~~ The species identity of the test organisms should be verified using appropriate systematic keys. First instar daphnids, ~~≤~~ 24 hours old, are to be used to start the test. ≤ Typographical error during transcription - R. O'Boyle 2/14/90

(B) Daphnids to be used in acute toxicity tests should be cultured at the test facility. Records should be kept regarding the source of the initial stock and culturing techniques. All organisms used for a particular test should have originated from the same culture population.

(C) Daphnids shall not be used for a test (1) if cultures contain ephippia; (2) if adults in the cultures do not produce young before day 12; (3) if more than 20 percent of the culture stock die during the 2 days preceding the test; (4) if adults in the culture do not produce an average of at least 3 young per adult per day over the 7-day period prior to the test and (5) if daphnids have been used in any portion of a previous test, either in a treatment or in a control. [THE CULTURE DATA THAT IS CURRENTLY COLLECTED DAILY WILL SUFFICE THAT THE COLONY IS ROBUST AND DOES NOT CONTAIN EPHIPPIA.]

(ii) Acclimation. (A) Brood daphnids shall be maintained in 100 percent dilution water at the test temperature for at least 48 hours prior to the start of the test. This is easily accomplished by culturing them in dilution water at the test temperature. During production of neonates, daphnids should not be fed.

(B) During culturing and acclimation to the dilution water, daphnids should be maintained in facilities with background colors and light intensities similar to those of the testing area.

(iii) Care and handling. (A) Daphnids should be cultured in dilution water under similar environmental conditions to those used in the test. Organisms should be handled as little as possible. When handling is necessary it should be done as gently, carefully and quickly as possible. During culturing and acclimation, daphnids should be observed carefully for ephippia and other signs of stress, physical damage and mortality. Dead and abnormal individuals shall be discarded. Organisms that touch dry surfaces or are dropped or injured in handling shall be discarded.

(B) Smooth glass tubes (I.D. greater than 5 mm) equipped with rubber bulb should be used for transferring daphnids with minimal culture media carry-over. Care should be exercised to introduce the daphnids below the surface of any solution to avoid trapping air under the carapace.

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(iv) Feeding. A variety of foods (e.g., unicellular green algae) have been demonstrated to be adequate for daphnid culture. Daphnids shall not be fed during testing.

(2) Facilities—(i) Apparatus. (A) Facilities needed to perform this test include: (1) Containers for culturing and acclimating daphnids; (2) a mechanism for controlling and maintaining the water temperature during the culturing, acclimation, and test periods; (3) apparatus for straining particulate matter, removing gas bubbles, or aerating the water as necessary; and (4) an apparatus for providing a 16-hour light and 8-hour dark photoperiod with a 15 to 30 minute transition period. In addition, the flow-through system shall contain appropriate test chambers in which to expose daphnids to the test chemical and an appropriate test substance delivery system.

(B) Facilities should be well ventilated and free of fumes and disturbances that may affect the test organisms.

(C) Test chambers ~~shall~~ [SHOULD] be loosely covered to reduce the loss of test solution or dilution water due to evaporation and to minimize the entry of dust or other particulates into the solutions. [FLOW-THROUGH TEST VESSEL COVERING IS OPTIONAL.]

(ii) Construction materials. (A) Materials and equipment that contact test solutions should be chosen to minimize sorption of test chemicals from the dilution water and should not contain substances that can be leached into aqueous solution in quantities that can affect the test results.

(B) For static tests, daphnids can be conveniently exposed to the test chemical in 250 ml beakers or other suitable containers.

(C) For flow-through tests, daphnids can be exposed in glass or stainless steel containers with stainless steel or nylon screen bottoms. The containers should be suspended in the test chamber in such a manner to insure that the test solution flows regularly into and out of the container and that the daphnids are always submerged in at least 5 centimeters of test solution. Test chambers can be constructed using 250 ml beakers or other suitable containers equipped with screened overflow holes, standpipes or V-shaped notches.

(iii) Dilution water. (A) Surface or ground water, reconstituted water or dechlorinated tap water are acceptable as dilution water if daphnids will survive in it for the duration of the culturing, acclimation and testing periods without showing signs of stress. The quality of the dilution water should be constant and should meet the following specifications:

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Substance	Maximum Concentration	
Particulate matter	20	mg/liter.
Total organic carbon or	~~1~~ [3]	mg/liter.
Chemical oxygen demand	5	mg/liter.
Un-ionized ammonia	~~1~~ [20]	µg/liter.
Residual chlorine	~~12~~ [10]	µg/liter.
Total organophosphorus pesticides	50	ng/liter.
Total organochlorine pesticides plus polychlorinated biphenyls (PCBs)	50	ng/liter.
or organic chlorine	25	ng/liter.

(B) The above water quality parameters under paragraph (d)(2)(iii)(A) of this section ~~shall~~ [SHOULD] be measured at least twice a year or whenever it is suspected that these characteristics may have changed significantly. If dechlorinated tap water is used, daily chlorine analysis shall be performed. [FOR THE ANALYTICAL REQUIREMENTS OF THE DILUENT WATER, THE ATTACHED DILUTION WATER AGGREGATE HISTORICAL DATA SUMMARY WILL BE SUBSTITUTED. THE MEASURED RESIDUAL CHLORINE SHOULD BE LESS THAN 0.01 mg/l.]

(C) If the diluent water is from a ground or surface water source, conductivity and total organic carbon (TOC) or chemical oxygen demand (COD) shall be measured. Reconstituted water can be made by adding specific amounts of reagent-grade chemicals to deionized or distilled water. Glass distilled or carbon-filtered deionized water with a conductivity of less than 1 µohm/cm is acceptable as the diluent for making reconstituted water.

(iv) Cleaning. All test equipment and test chambers shall be cleaned before each use using standard laboratory procedures.

(v) Test substance delivery system. In flow-through tests, proportional diluters, metering pump systems or other suitable devices should be used to deliver test chemical to the test chambers. The system shall be calibrated before each test. Calibration includes determining the flow rate through each chamber and the concentration of the test chemical in each chamber. The general operation of the test substance delivery system should be checked twice daily during a test. The 24-hour flow through a test chamber ~~shall~~ [SHOULD] be equal to at least 5 times the volume of the test chamber. During a test, the flow rates should not vary more than 10 percent from any one test chamber to another.

(3) Test parameters. Environmental parameters of the water contained in test chambers shall be maintained as specified below:

(i) The test temperature shall be 20°C. Excursions from the test temperature shall be no greater than ± 2°C.

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(ii) Dissolved oxygen concentration between 60 and 105 percent saturation. Aeration, if needed to achieve this level, shall be done before the addition of the test chemical. All treatment and control chambers shall be given the same aeration treatment.

(iii) The number of daphnids placed in a test chamber shall not affect test results. Loading shall not exceed 40 daphnids per liter test solution in the static system. In the flow-through test, loading limits will vary depending on the flow rate of dilution water. Loading shall not cause the dissolved oxygen concentration to fall below the recommended levels.

(iv) Photoperiod of 16-hours light and 8-hours darkness.

(e) Reporting. The sponsor shall submit to the U.S. EPA all data developed by the test that are suggestive or predictive of acute toxicity and all concomitant gross toxicological manifestations. In addition to the reporting requirements prescribed in Part 792—Good Laboratory Practice Standards of this chapter, the reporting of test data shall include the following:

(1) The name of the test, sponsor, testing laboratory, study director, principal investigator, and dates of testing.

(2) A detailed description of the test chemical including its source, lot number, composition (identity and concentration of major ingredients and major impurities), known physical and chemical properties, and any carriers or other additives used and their concentrations.

(3) The source of the dilution water, its chemical characteristics (e.g., conductivity, hardness, pH, etc.), and a description of any pretreatment.

(4) Detailed information about the daphnids used as brood stock, including the scientific name and method of verification, age, source, treatments, feeding history, acclimation procedures, and culture methods. The age of the daphnids used in the test shall be reported.

(5) A description of the test chambers, the volume of solution in the chambers, the way the test was begun (e.g., conditioning, test chemical additions), the number of test organisms per test chamber, the number of replicates per treatment, the lighting, the method of test chemical introduction or the test substance delivery system and flow rate (in flow-through test) expressed as volume additions per 24 hours.

(6) The concentration of the test chemical in each test chamber at times designated for static and flow-through tests.

(7) The number and percentage of organisms that were immobilized or showed any adverse effects in each test chamber at each observation period.

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(8) Utilizing the average measured test chemical concentration, concentration-response curves should be fitted to immobilization data at 24 and 48 hours. A statistical test of goodness-of-fit should be performed and the results reported. [REAL-TIME ANALYSIS OF THE TEST SOLUTION FOR THE TEST ARTICLE WILL BE CONDUCTED AT 0, 24, AND 48 HOURS.]

(9) The 24- and 48-hour EC_{50} values and their respective 95 percent confidence limits using the mean measured test chemical concentration and the methods used to calculate both the EC_{50} values and their confidence limits.

(10) All chemical analyses of ~~water quality and~~ test chemical concentrations, including methods, method validations and reagent blanks. [FOR THE ANAYLICAL REQUIREMENTS OF THE DILUENT WATER, THE ATTACHED AGGREGATE HISTORICAL DATA SUMMARY WILL BE SUBSTITUTED. LIMITS OF DETECTION FOR EACH OF THE ANALYTES SHALL BE INCLUDED.]

(11) The data records of the culture, acclimation and test temperatures.

(12) Any deviation from this test guideline and anything unusual about the test, e.g., diluter failure, temperature fluctuations, etc.

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Section 797.1310 Gammarid acute toxicity test.

(a) Purpose. This guideline is intended for use in developing data on the acute toxicity of chemical substances and mixtures subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003 (15 U.S.C. 2601 et. seq.)). This guideline describes a test to develop data on the acute toxicity of chemicals to gammarids. The United States Environmental Protection Agency (EPA) will use data from this test in assessing the hazard of a chemical to aquatic organisms.

(b) Definitions. The definitions in Section 3 of the Toxic Substances Control Act (TSCA) and in Part 792—Good Laboratory Practice Standards of this chapter apply to this test guideline. The following definitions also apply to this guideline:

(1) "Death" means the lack of reaction of a test organism to gentle prodding.

(2) "Flow-through" means a continuous or intermittent passage of test solution or dilution water through a test chamber or a holding or acclimation tank with no recyling.

(3) "LC_{50}" means the experimentally derived concentration of test substance that is expected to kill 50 percent of a test population during continuous exposure over a specified period of time.

(4) "Loading" means the ratio of the biomass of gammarids (grams, wet weight) to the volume (liters) of test solution in either a test chamber or passing through it in a 24-hour period.

(5) "Solvent" means a substance (e.g., acetone) which is combined with the test substance to facilitate introduction of the test substance into the dilution water.

(6) "Static system" means a test chamber in which the test solution is not renewed during the period of the test.

(c) Test procedures—(1) Summary of the test. In preparation for the test, test chambers are filled with appropriate volumes of dilution water. If a flow-through test is performed, the flow of dilution water through each chamber is adjusted to the rate desired. In a static test, the test substance is introduced into each test chamber. In a flow-through test, the rate at which the test substance is added is adjusted to establish and maintain the desired concentration of test substance in each test chamber. The test is started by randomly introducing gammarids which have been acclimated to the test conditions into the test chambers. Gammarids in the test chambers are observed periodically during the test:

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the dead gammarids are removed and the findings recorded. Dissolved oxygen concentration, pH, temperature and the concentration of test substance in test chambers are measured at specified intervals. Data collected during the test are used to develop concentration-response curves and LC_{50} values for the test substance.

(2) (Reserved)

(3) Range-finding test. (i) A range-finding test should be conducted to establish test substance concentrations for the definitive test. [NOMINAL STATIC CONCENTRATIONS MAY BE USED FOR THE RANGE-FINDING TEST.]

(ii) The gammarids shall be exposed to a series of widely spaced concentrations of the test chemical (e.g., 1, 10, 100 mg/l, etc.), usually under static conditions.

(iii) A minimum of five gammarids should be exposed to each concentration of test chemical for a period of 96 hours. The exposure period may be shortened if data suitable for determining concentrations in the definitive test can be obtained in less time. Nominal concentrations of the chemical may be acceptable.

(4) Definitive test. (i) The purpose of the definitive test is to determine the 24, 48, 72 and 96-hour LC_{50} values and the concentration-response curves. [THE CONCENTRATION RESPONSE CURVES WILL BE BASED ON ANALYSIS AT 0, 48, AND 96 HOURS.]

(ii) A minimum of 20 gammarids per concentration shall be exposed to five or more concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32, 64 mg/L). The range and number of concentrations to which the organisms are exposed ~~should~~ [SHOULD] be such that in 96 hours, there is at least one concentration resulting in mortality greater than 50 and less than 100 percent, and one concentration causing greater than zero and less than 50 percent mortality. An equal number of gammarids ~~may~~ [SHALL] be placed in two or more replicate test chambers. Solvents should be avoided, if possible. If solvents have to be used, a solvent control, as well as a dilution control, shall be tested at the highest solvent concentration employed in the treatments. The solvent should not be toxic or have an effect on the toxicity of the test chemical. The concentration of solvent should not exceed 0.1 ml/L.

(iii) Every test shall include a concurrent control using gammarids from the same population or culture container. The control group shall be exposed to the same dilution water, conditions and procedures, except that none of the chemical is added to the chamber.

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(iv) The dissolved oxygen concentration, temperature and pH shall be measured at the beginning of the test and at 24, 48, 72 and 96 hours in at least one replicate each of the control and the highest, lowest and middle test concentrations.

(v) The test duration is 96 hours. The test is unacceptable if more than 10 percent of the control organisms die during the test.

(vi) In addition to death, any abnormal behavior or appearance shall also be reported.

(vii) Gammarids shall be ~~randomly~~ [IMPARTIALLY] assigned to the test chambers. Test chambers shall be positioned within the testing area in a random manner or in a way in which appropriate statistical analyses can be used to determine whether there is any variation due to placement. [RESTRICTED RANDOMIZATION WILL BE USED.]

(viii) Gammarids shall be introduced into the test chambers after the test substance has been added.

(ix) Observations on compound solubility shall be recorded. The investigator should report the appearance of surface slicks, precipitates, or material adhering to the sides of the test chambers.

(5) (Reserved) [MORE THAN ONE SPECIES OF TEST ORGANISM MAY BE INCLUDED IN A TEST VESSEL AT THE SAME TIME. APPROPRIATE ISOLATION TECHNIQUES WILL BE USED.]

(6) Analytical measurements—(i) Water quality analysis. The hardness, acidity, alkalinity, pH, conductivity, TOC or COD, and particulate matter of the dilution water ~~shall~~ [SHOULD] be measured at the beginning of each test. [FOR THE ANALYTICAL REQUIREMENTS OF THE DILUENT WATER, THE ATTACHED DILUTION WATER AGGREGATE HISTORICAL DATA SUMMARY WILL BE SUBSTITUTED.]

(ii) Collection of samples for measurement of test substance. Samples to be analyzed for the test substance concentrations shall be taken midway between the top, bottom, and sides of the test chamber. These samples should not include any surface scum or material dislodged from the bottom or sides. Samples shall be analyzed immediately or handled and stored in a manner which minimizes loss of test substance through microbial degradation, photodegradation, chemical reaction, volatilization, or sorption.

(iii) Measurement of test substance. ~~(A) For static tests, the concentration of dissolved test substance (that which passes through a 0.45 micron filter) shall be measured, at a minimum, in each test chamber at the beginning (zero hour, before gammarids are added)~~

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~~and at the end of the test.~~ [(A) DEIONIZED OR DISTILLED WATER SHOULD BE USED IN MAKING STOCK SOLUTIONS OF THE TEST SUBSTANCE. STANDARD ANALYTICAL METHODS SHOULD BE USED WHENEVER AVAILABLE IN PERFORMING THE ANALYSES. THE ANALYTICAL METHOD USED TO MEASURE THE AMOUNT OF TEST SUBSTANCE IN A SAMPLE SHALL BE VALIDATED BEFORE BEGINNING THE TEST BY APPROPRIATED LABORATORY PRACTICES. AN ANALYTICAL METHOD IS NOT ACCEPTABLE IF LIKELY DEGRADATION PRODUCTS OF THE TEST SUBSTANCE, SUCH AS HYDROLYSIS AND OXIDATION PRODUCTS, GIVE POSITIVE OR NEGATIVE INTERFERENCES WHICH CANNOT BE SYSTEMATICALLY IDENTIFIED AND CORRECTED MATHEMATICALLY.] During flow-through tests, the concentration of ~~dissolved~~ [THE] test substance shall be measured in each test chamber at least as often as a 0 [, 48,] and 96-hours and in at least one chamber whenever a malfunction of the test substance delivery system is observed.

~~(B) The analytical methods used to measure the amount of test substance in a sample shall be validated before beginning the test. This involves adding a known amount(s) of the test substance to each of three water samples taken from a chamber containing [OE] dilution water and the same number of gammarids as are used in the test. The nominal concentrations of the test substance in these samples should span the concentration range to be used in the test. Validation of the analytical method should be performed on at least two separate days prior to starting the test.~~

~~(C) An analytical method is not acceptable if likely degradation products of the test substance give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test.~~

[(B) THE ANALYTICAL METHOD FOR THE TEST SUBSTANCE SHALL BE VALIDATED PRIOR TO BEGINNING THE TEST. A PROCEDURE SUCH AS USING KNOWN ADDITIONS MAY BE USED. THIS INVOLVES ADDING KNOWN AMOUNTS OF THE TEST SUBSTANCE TO THREE OR MORE SAMPLES OF DILUTION WATER. THE NOMINAL CONCENTRATIONS OF THE TEST SUBSTANCE IN THESE SAMPLES SHOULD SPAN THE CONCENTRATION RANGE TO BE USED IN THE TEST. BOTH DISSOLVED TEST SUBSTANCE (THAT WHICH PASSES THROUGH A 0.45 MICRON FILTER) AND TOTAL TEST SUBSTANCE SHALL BE MEASURED IN EACH SAMPLE. IF THE MEASURED CONCENTRATIONS OF DISSOLVED TEST SUBSTANCE ARE GREATER THAN 80% OF THE MEASURED CONCENTRATIONS OF TOTAL TEST SUBSTANCE, THEN ONLY TOTAL TEST SUBSTANCE SHALL BE MEASURED DURING THE TEST. HOWEVER, IF THE MEASURED CONCENTRATIONS OF DISSOLVED TEST SUBSTANCE ARE LESS THAN 80% OF THE MEASURED CONCENTRATIONS OF TOTAL TEST SUBSTANCE, THEN ONLY DISSOLVED TEST SUBSTANCE SHALL BE MEASURED DURING THE TEST. VALIDATION OF THE ANALYTICAL METHOD SHOULD BE PERFORMED ON AT LEAST TWO SEPARATE DAYS PRIOR TO STARTING THE TEST.]

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[(C) IN ADDITION TO ANALYZING SAMPLES OF TEST SOLUTION, AT LEAST ONE REAGENT BLANK, CONTAINING ALL REAGENTS USED, SHOULD ALSO BE ANALYZED.]

[(D) FILTERS AND THEIR HOLDERS USED FOR DETERMINING THE DISSOLVED TEST SUBSTANCE CONCENTRATIONS SHOULD BE PREWASHED WITH SEVERAL VOLUMES OF DISTILLED WATER OR DILUTION WATER AND UNDERGO A FINAL RINSE WITH TEST SOLUTION. GLASS OR STAINLESS STEEL FILTER HOLDERS ARE BEST FOR ORGANIC SUBSTANCES, WHILE PLASTIC HOLDERS ARE BEST FOR METALS. THE SAMPLE SHOULD BE FILTERED WITHIN 30 MINUTES AFTER IT IS TAKEN FROM THE TEST CHAMBER.]

~~(D)~~[(E)] Among replicate test chambers the measured concentrations shall [SHOULD] not vary more than 20 percent. The measured concentration of the test substance in any chamber during the test shall [SHOULD] not vary more than plus or minus 30 percent from the measured concentration in that chamber at zero time.

~~(E)~~[(F)] The mean measured concentration of ~~dissolved~~ [THE] test substance shall be used to calculate all LC_{50}'s and to plot all concentration-response curves.

(d) Test conditions—(1) Test species--(i) Selection (A) The amphipod~~s~~ Gammarus fasciatus~~, G. pseudolimnaeus and G. lacustris are~~ [IS] specified for this test.

(B) Gammarids can be cultured in the laboratory or collected from natural sources. If collected, they must be held in the laboratory for at least 14 days prior to testing.

(C) Gammarids used in a particular test shall [illegible]

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(iv) Feeding. The organisms shall not be fed during testing. During culturing, holding, and acclimation, a sufficient quantity of deciduous leaves, such as maple, aspen or birch, should be placed in the culture and holding containers to cover the bottom with several layers. These leaves should be aged for at least 30 days in a flow-through system before putting them in the aquaria. [A 48-HOUR FLOW-THROUGH WATER WASH AT APPROXIMATELY 18U°C WILL BE USED FOR LEAF PREPARATION.] As these leaves are eaten, more aged leaves should be added. Pelleted fish food may also be added.

(2) Facilities—(i) Apparatus. (A) Facilities needed to perform this test include: (1) Containers for culturing and acclimating and testing gammarids; (2) containers for aging leaves under flow-through conditions; (3) a mechanism for controlling and maintaining the water temperature during the culturing, acclimation and test periods; (4) apparatus for straining particulate matter, removing gas bubbles, or aerating the dilution water, as necessary; and (5) an apparatus for providing a 16-hour light and 8-hour dark photoperiod with a 15- to 30-minute transition period.

(B) Facilities ~~shall~~ [SHOULD] be well ventilated and free of fumes and disturbances that may affect the test organisms.

(C) Test chambers shall be covered loosely to reduce the loss of test solution or dilution water due to evaporation and to minimize the entry of dust or other particulates into the solutions. [FLOW-THROUGH TEST VESSEL COVERING IS OPTIONAL.]

(ii) Construction materials. Construction materials and equipment that may contact the stock solution, test solution, or dilution water ~~shall~~ [SHOULD] not contain substances that can be leached or dissolved into aqueous solutions in quantities that can alter the test results. Materials and equipment that contact stock or test solutions should be chosen to minimize sorption of test chemicals. Glass, stainless steel, and perfluorocarbon plastic should be used whenever possible. Concrete, fiberglass, or plastic (e.g., PVC) may be used for holding tanks, acclimation tanks, and water supply systems, but they should be aged prior to use. Rubber, copper, brass, galvanized metal, and lead should not come in contact with the dilution water, stock solution, or test solution.

(iii) Test substance delivery system. In flow-through tests, diluters, metering pump systems or other suitable devices shall be

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ATTACHMENT 5. Protocol for Definitive Test

V. Appendix 3. EPA Consent Order Protocol for Gammarid Acute Toxicity Test (Section 797.1310)

(iv) Test chambers. Test chambers shall contain at least one liter of test solution. Test chambers made of stainless steel should be welded, not soldered. Test chambers made of glass should be glued using clear silicone adhesive. As little adhesive as possible should be left exposed in the interior of the chamber. A substrate, such as a bent piece of stainless steel screen, should be placed on the bottom of each test chamber to provide cover for the gammarids.

(v) Cleaning of test system. Test substance delivery systems and test chambers should be cleaned before each test. They should be washed with detergent and then rinsed sequentially with clean water, pesticide-free acetone, clean water, and 5 percent nitric acid, followed by two or more changes of dilution water.

(vi) Dilution water. (A) Clean surface or ground water, reconstituted water, or dechlorinated tap water is acceptable as dilution water if gammarids will survive in it for the duration of the culturing, acclimating, and testing periods without showing signs of stress. The quality of the dilution water should be constant enough that the month-to-month variation in hardness, acidity, alkalinity, conductivity, TOC or COD, and particulate matter is not more than 10 percent. The pH should be constant within 0.4 unit. In addition, the dilution water should meet the following specifications measured at least twice a year (FOR THE ANALYTICAL REQUIREMENTS OF THE DILUENT WATER, THE ATTACHED DILUTION WATER AGGREGATE HISTORICAL DATA SUMMARY WILL BE SUBSTITUTED. THE MEASURED RESIDUAL CHLORINE SHOULD BE LESS THAN 0.01 mg/L.):

Substance	Maximum Concentration	
Particulate matter	20	mg/L.
Total organic carbon (TOC) or	≠ [3]	mg/L.
Chemical oxygen demand (COD)	5	mg/L.
Boron fluoride	100	µg/L.
Un-ionized ammonia	≠ [20]	µg/L.
Aluminum, arsenic chromium, cobalt, copper iron, lead, nickel, zinc	1	µg/L.
Residual chlorine	≠ [10]	µg/L.
Cadmium, mercury, silver	100	ng/L.
Total organophosphorus pesticides	50	ng/L.
Total organochlorine pesticides plus polychlorinated biphenyls (PCBs).	50	ng/L.
or organic chlorine	25	ng/L.

(B) If the diluent water is from a ground or surface water source, conductivity and total organic carbon (TOC) or chemical oxygen demand (COD) shall be measured. Reconstituted water can be made by adding specific amounts or reagent-grade chemicals to deionized or distilled water.

ATTACHMENT 5. Protocol for Definitive Test

V. Appendix 3. EPA Consent Order Protocol for Gammarid Acute Toxicity Test (Section 797.1310)

Glass distilled or carbon-filtered deionized water with a conductivity less than 1 micromho/cm is acceptable as the diluent for making reconstituted water.

(C) The concentration of dissolved oxygen in the dilution water shall be between 90 and 100 percent saturation. If necessary, the dilution water can be aerated before the addition of the test substance. All reconstituted water should be aerated before use.

(3) Test parameters. Environmental parameters during the test shall be maintained as specified below:

(i) Water temperature of ~~18~~ [20] ± 1°C. [A TEST TEMPERATURE OF 20 ± 2°C WILL ALSO BE ACCEPTABLE.]

(ii) Dissolved oxygen concentration between 60 and 105 percent saturation.

(iii) The number of gammarids placed in a test chamber shall not be so great as to affect the results of the test. Ten gammarids per liter is the recommended level of loading for a static test. Loading requirements for the flow-through test will vary depending on the flow rate of dilution water. The loading should not cause the dissolved oxygen concentration to fall below the recommended levels.

(iv) Photoperiod of 16 hours light and 8 hours darkness.

(e) Reporting. (1) The sponsor shall submit to the EPA all data developed by the test that are suggestive or predictive of toxicity. In addition, the test report shall include, but not necessarily be limited to, the following information:

(i) Name and address of the facility performing the study and the dates on which the study was initiated and completed.

(ii) Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

(iii) Statistical methods employed for analyzing the data.

(iv) The test substance identified by name, Chemical Abstracts (CAS) number or code number, source, lot or batch number, strength, purity, and composition or other appropriate characteristics.

(v) Stability of the test substance under the conditions of the test.

(vi) A description of the methods used, including:

ATTACHMENT 5. Protocol for Definitive Test

V. Appendix 3. EPA Consent Order Protocol for Gammarid Acute Toxicity Test (Section 797.1310)

(A) The source of the dilution water, its chemical characteristics (e.g., hardness, pH, etc.) and a description of any pretreatment.

(B) A description of the test substance delivery system, test chambers, the depth and volume of solution in the chamber, the way the test was begun (e.g., test substance addition), the loading, the lighting, and the flow rate.

(C) Frequency and methods of measurements and observations.

(vii) The scientific name, weight, length, source, and history of the organisms used, and the acclimation procedures and food used.

(viii) The concentrations tested, the number of gammarids and replicates per test concentration. The reported results ~~should~~ [SHALL] include:

(A) The results of dissolved oxygen, pH and temperature measurements.

(B) If solvents are used, the name and source of the solvent, the nominal concentration of the test substance in the stock solution, the highest solvent concentration in the test solution and a description of the solubility determinations in water and solvents.

(C) The measured concentration of the test substance in each test chamber just before the start of the test and at all subsequent sampling periods.

(D) The number of dead and live test organisms, the percentage of organisms that died, and the number that showed any abnormal effects in each test chamber at each observation period.

(E) The 96-hour LC_{50} and the 95 percent confidence limits. When sufficient data have been generated, the 24, 48 and 72-hour LC_{50} values also. These calculations should be made using the mean measured test substance concentrations. [REAL-TIME ANALYSIS OF THE TEST SOLUTION FOR THE TEST ARTICLE WILL BE CONDUCTED AT 0, 48, AND 96 HOURS.]

(F) The observed no-effect concentration (the highest concentration tested at which there were no mortalities or abnormal behavioral or physiological effects), if any.

(G) Methods and data for ~~all chemical~~ analyses of ~~water quality and~~ test substance concentrations, including method validations and reagent blanks. [FOR THE ANALYTICAL REQUIREMENTS OF THE DILUENT WATER, THE ATTACHED DILUTION WATER AGGREGATE HISTORICAL DATA SUMMARY WILL BE SUBSTITUTED. UNITS OF DETECTION SHALL BE INCLUDED]

ATTACHMENT 5. Protocol for Definitive Test

V. Appendix 3. EPA Consent Order Protocol for Gammarid Acute Toxicity Test (Section 797.1310)

(ix) A description of all circumstances that may have affected the quality or integrity of the data.

(x) The name of the sponsor, study director, principal investigator, names of other scientists or professionals, and the names of all supervisory personnel involved in the study.

(xi) A description of the transformations, calculations, or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis. Results of the analysis of data should include the calculated LC_{50} value, 95 percent confidence limits, slope of the transformed concentration response line, and the results of a goodness-of-fit test (e.g., chi-square test).

(xii) The signed and dated reports of each of the individual scientists or other professionals involved in the study, including each person who, at the request or direction of the testing facility or sponsor, conducted an analysis or evaluation of data or specimens from the study after data generation was completed.

(xiii) The locations where all specimens, raw data, and the final report are stored.

(xiv) The statement prepared and signed by the quality assurance unit.

ATTACHMENT 5. Protocol for Definitive Test

VI. Attachment 1. Protocol Amendments (to be prepared only if necessary)

Protocol: Determination of the Acute Aquatic Effects of Test Article on the Fathead Minnow, Pimephales promelas, the Sideswimmer (Gammarid), Gammarus fasciatus, and the Daphnid, Daphnia magna (Performed as a Multispecies Simultaneous Exposure)

Test Article: Crotonaldehyde

Accession No. 901878 HAEL No. 90-0016

1.) The gammarid 5 mg/L exposure solutions were sampled at time 74 hours since complete mortality had occurred by that time. Only a single nearly-dead gammarid remained in each replicate at time 72 hours so the individuals were observed periodically until they were determined to be dead. It was deemed more appropriate to sample at 74 hours rather than wait overnight until the sampling scheduled for test end at 96 hours.

2.) The replicate doses nominally set at 2.5, 5, and 10 mg/L were sampled for analysis at time 24 hours since all the fathead minnows in those exposure solutions had died by that time. The analyzed values obtained will be used as the test end exposure concentrations in all endpoint calculations for the minnows because later samplings would be irrelevant.

3.) Some of the containment baskets used to confine the daphnids and gammarids were sampled when the surrounding dose solutions were sampled for analysis. This procedure should confirm that the external solution concentrations are representative of those occurring within the baskets.

4.) It was not feasible due to analytical limitations to strictly comply with each Consent Order in regard to the references to "Real-time analysis of the test solution for the test article will be conducted at 0, 48 (24-daphnid), and 96 (48-daphnid) hours." The procedure used during the three studies is that described on page 3 of this protocol: "Samples will be removed for analysis @ times 0, 48 (aged solutions & fresh stock), and 96 hours (or test end per dose; i.e., complete mortality). The daphnid endpoint is immobility and test end is at time 48 hours." It is felt that this interpretation will yield endpoint values ultimately more representative of the dose-response relationship.

5.) Any references to "concentration response curves" contained within the Consent Orders shall be interpreted to require the use of analyzed dose values obtained from samples taken at test start, prior to stock solution renewal at time 48 hours, and at test end per species per dose (i.e., complete fish/gammarid mortality or daphnid immobilization).

Approved by: R. J. O'Boyle 4/25/'90
Study Director Date

Approved by: Roderick D. Gerwe 05/11/90
Sponsor Date

3791e

ATTACHMENT 6. Acute Aquatic Effects Data Forms (Replicates A & B)

ACCESSION NO	HAEL NO	TEST ARTICLE	SRID NO
901878	90-0016	Crotonaldehyde	Lot No. 90-7

TIME, DATE & INITIALS	TIME PROTO-COL	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 0.0 mg/L OBSERVATIONS Pp	Gf	Dm	DWC	STARTING CONDITIONS FL, DY REMARKS (Diluent Water Controls) "A"
09:15, 4/23/'90, O'B.	0	20	9.0	8.3	10N	10N	10N	AW=0.28 g	(Pp only - wet weights)
9:30, 4/24/90, JB	24	20	8.8	8.3	10N	10N	10N		
8:30, 4/25/90, JB	48	20	8.8	8.3	10N	10N	9N	(1510)	
08:35, 4/26/'90, O'B.	72	20	8.8	8.3	10N	10N	—		
8:40, 4/27/90, JB	96	20	8.8	8.3	10N	10N	—		

TIME, DATE & INITIALS	TIME PROTO-COL	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 0.0 mg/L OBSERVATIONS Pp	Gf	Dm	DWC	STARTING CONDITIONS FL, DY REMARKS (Diluent Water Controls) "B"
09:15, 4/23/'90, O'B.	0	20	9.0	8.3	10N	10N	10N	AW=0.31 g	(Pp only - wet weights)
9:30, 4/24/90, JB	24	20	8.9	8.3	10N	10N	10N		
8:30, 4/25/90, JB	48	20	8.8	8.3	10N	10N	9N, 1M		exoskeletons present - remains of Dm?
08:35, 4/26/90, O'B.	72	20	8.8	8.3	10N	10N	—		
8:40, 4/27/90, JB	96	20	8.8	8.3	10N	10N	—		

TIME, DATE & INITIALS	TIME PROTO-COL	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 0.6 mg/L OBSERVATIONS Pp	Gf	Dm		STARTING CONDITIONS FL, DY REMARKS "A"
09:20, 4/23/'90, O'B.	0	20	9.0	8.3	10N	10N	10N		Clear Solution
9:35, 4/24/90, JB	24	20	8.9	8.3	10N	10N	10	(8N, 2PS)	
8:30, 4/25/90, JB	48	20	8.8	8.3	10N	10N	10N		
08:40, 4/26/'90, O'B.	72	20	8.8	8.3	10N	10N	—		
8:45, 4/27/90, JB	96	20	8.7	8.3	10N	10N	—		

TIME, DATE & INITIALS	TIME PROTO-COL	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 0.6 mg/L OBSERVATIONS Pp	Gf	Dm		STARTING CONDITIONS FL, DY REMARKS "B"
09:20, 4/23/'90, O'B.	0	20	9.0	8.3	10N	10N	10N		Clear Solution
9:35, 4/24/90, JB	24	20	8.9	8.3	10N	10N	10	(8N, 2PS)	
8:30, 4/25/90, JB	48	20	8.9	8.3	10N	10N	9N	1M	
08:40, 4/26/90, O'B.	72	20	8.8	8.3	10N	10N	—		
8:45, 4/27/90, JB	96	20	8.7	8.3	10N	10N	—		

Abbr. used to describe test organisms' condition
AD - Activity Depressed; AA - Activity Accelerated; PS - Position Bottom; PS - Position Surface; FR - Fragmentation; GD - Gill Rate Depressed; GA - Gill Rate Accelerated; I - Immobilized; N - Normal; ND - Near Death; AO - Abnormal Orientations; SH - Shriveling; T - Tremors; W - Withdrawn; M - Missing; D - Dead

Abbr. used to describe test conditions
AR - Aeration; DY - Dynamic; FL - Fluorescent Lab Lighting; AW - Avg. Wgt. Fish; PRECIP. - Precipitate; RECIRC. - Recirculating; SOLN - Solution; ST - Static; X - Xenon Arc Lighting

ACUTE AQUATIC EFFECTS

TEST ORGANISM: Pimephales promelas (Pp) see pp. 3,5

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SIGNATURE	DATE	REVIEWED BY	DATE
Janice M. Beglinger	4/27/90	R.J. O'Boyle	4/27/90

HAEL No. 90-0016 Study No. EN-407-901878-1

ATTACHMENT 6. Acute Aquatic Effects Data Forms (Replicates A & B)

ACCESSION NO	HAEL NO	TEST ARTICLE	SRID NO
901878	90-0016	Crotonaldehyde	Lot No. 90-7

Refer to pp. 4, 6.

Test Type

1. _____ Range-Finding/ X Definitive
2. 2 Replicates (10 animals per replicate)
3. _____ Static/ X Dynamic/; 70 mL/Min (At least 6 test vessel volumes per day; a proportionating diluter system will be used.)
4. 15 L/Test Vessel Vol. (Daphnids & gammarids to be confined in stainless-steel mesh, 150 mL baskets suspended from 1 rpm motors.)
5. X Fluorescent/_____ Xenon-Arc Illumination (Sixteen-Hour Photoperiod with a 20-Minute Transition Period)
6. (X) Aeration (May Be Used at = 0.05 L/Minute, if Necessary)
7. _____ Nominal/ X Measured Test Article Concentrations @ 0.6, 1.2, 2.5, 5, and 10 mg/L; 100 mg/L stock solution feeding diluter will be renewed at time 48 hours. Samples will be removed for analysis @ times "0", 48 (aged solutions & fresh stock), and 96 hours (or test end per dose; i.e., complete mortality). The daphnid endpoint is immobility and test end is at time 48 hours.

Randomly-placed test vessels within diluter basin: correction, R.O'B, 4/23/90

Replicate B:	2.5 mg/L	5 mg/L	0.6 mg/L	1.2 mg/L	10 mg/L	Diluent Water	X
Replicate A:						Controls	X

Empty positions due to ventilator located here.

FRONT (North) — R.J. O'Boyle, 4/23/90

Acute Aquatic Effects Summary:

	Daphnid, EC_{50} (NOEC)	Gammarid LC_{50} (NOEC)	Minnow
Replicate A:	2.1 (0.6)	3.1 (1.2)	0.92 (0.6)
Replicate B:	1.6 (1.2)	2.7 (1.2)	0.97 (0.6)
Pooled Replicates:	1.8 (0.6)	2.9 (1.2)	0.95 (0.6)

— R.J. O'Boyle, 4/27/90

Note: all endpoint values are for nominal dose concentrations + are expressed as mg/L.
Daphnia only — R.J. O'Boyle 4/25/90
Remainder — R.J. O'Boyle, 4/27/90

ACUTE AQUATIC EFFECTS

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SIGNATURE	DATE	REVIEWED BY	DATE
Janice H. Boylan	4/27/90	R.J. O'Boyle	4/27/90

ATTACHMENT 6. Acute Aquatic Effects Data Forms (Replicates A & B)

ACCESSION NO	HAEL NO	TEST ARTICLE	SRID NO.
901878	90-0016	Crotonaldehyde	Lot No. 90-7

TIME, DATE & INITIALS	TIME PROTO COU	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 1.2 mg/L OBSERVATIONS Pp	Gf	Dm		STARTING CONDITIONS FL, DY REMARKS "A"
09:20, 4/23/'90, O'B.	0	20	9.0	8.3	10N	10N	10N		Clear Solution
9:40 4/24/90, JB	24	20	9.0	8.2	10 9N 1AD	10N	10N		
8:40, 4/25/90, JB	48	20	8.8	8.3	3 [illegible]	10N	9	8N, 1AD, 1N	
08:45, 4/26/90, O'B	72	20	8.8	8.3	1AD	10N	—		
8:45, 4/27/90, JB	96	20	8.8	8.3	1N	10N	—		

TIME, DATE & INITIALS	TIME PROTO COU	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 1.2 mg/L OBSERVATIONS Pp	Gf	Dm		STARTING CONDITIONS FL, DY REMARKS "B"
09:20, 4/23/'90, O'B.	0	20	9.0	8.3	10N	10N	10N		Clear Solution
9:40 4/24/90, JB	24	20	9.0	8.2	9N 1D	10N	10N		
8:40 4/25/90, JB	48	20	8.8	8.3	6 [illegible]	10N	9N	1T	
08:45, 4/26/90, O'B.	72	20	8.8	8.3	2AD	10N	—		
8:45, 4/27/90, JB	96	20	8.7	8.3	2N	10N	—		correction 4/27/90, JMB

TIME, DATE & INITIALS	TIME PROTO COU	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 2.5 mg/L OBSERVATIONS Pp	Gf	Dm		STARTING CONDITIONS FL, DY REMARKS "A"
09:25, 4/23/'90, O'B.	0	20	9.1	8.3	10N	10N	10N		Clear Solution
9:45 4/24/90, JB	24	20	9.0	8.3	0	10N	10N		
8:40 4/25/90, JB	48	20	9.0	8.3	—	10N	4	3N, 1AD, 6T, D	
08:55 4/26/90, O'B.	72	20	9.0	8.3	—	10AD	—		
8:50, 4/27/90, JB	96	20	8.9	8.3	—	8AD	—		

TIME, DATE & INITIALS	TIME PROTO COU	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 2.5 mg/L OBSERVATIONS Pp	Gf	Dm		STARTING CONDITIONS FL, DY REMARKS "B"
09:25, 4/23/'90, O'B.	0	20	9.1	8.4	10N	10N	10N		Clear solution
9:45, 4/24/90, JB	24	20	9.0	8.3	0	10N	10	6N, 4P	
8:40, 4/25/90, JB	48	20	9.0	8.3	—	10N	1N	9D/T	
08:55 4/26/90, O'B.	72	20	8.9	8.3	—	9AD	—		
8:55, 4/27/90, JB	96	20	8.9	8.3	—	6AD	—		

Abbr. used to describe test organisms' condition: AD - Activity Depressed; AA - Activity Accelerated; PB - Position Bottom; PS - Position Surface; FR - Fragmentation; GO - Gill Rate Depressed; GA - Gill Rate Accelerated; I - Immobilized; N - Normal; NO - Near Death; AO - Abnormal Orientations; SH - Shrinking; T - Tremors; W - Windrown; M - Missing; D - Dead

Abbr. used to describe test conditions: AR - Aeration; DY - Dynamic; FL - Fluorescent Lab Lighting; AW - Avg. Wgt. Fish; PRECIP. - Precipitate; RECIRC. - Recirculating; SOLN - Solution; ST - Static; X - Xenon Arc Lighting

ACUTE AQUATIC EFFECTS

TEST ORGANISM: Gammarus fasciatus 'Gf'

Health and Environment Laboratories see pp. 1, 5

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United States of America

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SIGNATURE	DATE	REVIEWED BY	DATE
Janice H Boylinger	4/27/90	R.J. O'Boyle	4/27/'90

ATTACHMENT 6. Acute Aquatic Effects Data Forms (Replicates A & B)

ACCESSION NO	HAEL NO	TEST ARTICLE	SRID NO.
901878	90-0016	Crotonaldehyde	Lot No. 90-7

Refer to pp. 2, 6.

Test Procedure

A. Preparation of Exposure Solutions (from Aqueous Stock Solution)

1. Stock Solution Preparation (in Laboratory Diluent Water):
   a. Amount Test Article: 20.0 g; Final Volume Water: 200 L
   b. Test Article Concentration in the Stock Solution: 100 mg/L

2. Exposure Solution Preparation (by initially "spiking" tanks with appropriate volumes of stock solution; doses continually renewed from stock by using gas-driven, proportionating diluter system): mL/cycle*
   a. Conc. 10 mg/L; Stock Solution 1500 mL; Total Vol 15 L (28 mL)
   b. Conc. 5 mg/L; Stock Solution 750 mL; Total Vol 15 L (14 mL)
   c. Conc. 2.5 mg/L; Stock Solution 375 mL; Total Vol 15 L (7 mL)
   d. Conc. 1.2 mg/L; Stock Solution 188 mL; Total Vol. 15 L (3.5 mL)
   e. Conc. 0.6 mg/L; Stock Solution 94 mL; Total Vol 15 L (1.8 mL)

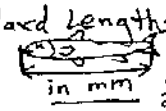
* The diluter cycles every two minutes and delivers a total of 280 mL per cycle to the test system (i.e., flow/replicate = 70 mL/minute).

3. Test Article Solution Preparation Date: 4/23/90 — R.J. O'Boyle 4/23/90

Remarks: All exposure solutions (test article doses and diluent water controls) will prepared in replicates of two, and the 100 mg/L stock solution will be renewed (i.e., remade fresh) at test time 48 hours.

Note: Stock solution water was sparged with N_2 for ≈24 hours prior to adding test article (initially + @ renewal). The stock drum was plastic bagged + taped, and Argon was continuously introduced, throughout test. — R.J. O'Boyle 6/22/90

Standard Lengths of Fathead Minnow Controls @ Test End: (after euthanized with Finquel®)



	A		B
1 -	23	1 -	24
2 -	21	2 -	22
3 -	22	3 -	25
4 -	26	4 -	28
5 -	26	5 -	25
6 -	27	6 -	26
7 -	28	7 -	27
8 -	28	8 -	30
9 -	26	9 -	27
10 -	26	10 -	27
$\bar{X}_{10}$ =	25.3 mm (Avg. Wet Wt = 0.28g at Test Start)	$\bar{X}_{10}$ =	26.1 mm (Avg. Wet Wt. = 0.31g at Test Start)

∴ Replicate A: 25.3 mm
Replicate B: 26.1 mm
mean Lengths @ Test End
— R.J. O'Boyle, 4/27/90

ACUTE AQUATIC EFFECTS

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SIGNATURE	DATE	REVIEWED BY	DATE
Janice M. Beglingir	4/27/90	R.J. O'Boyle	4/27/90

ATTACHMENT 6. Acute Aquatic Effects Data Forms (Replicates A & B)

ACCESSION NO	HAEL NO	TEST ARTICLE	SRID NO.
901878	90-0016	Crotonaldehyde	Lot No. 90-7

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 5 mg/L OBSERVATIONS Pp	Gf	Dm		STARTING CONDITIONS FL, DY REMARKS "A"
09:25, 4/23/'90, O'B.	0	20	9.0	8.4	10N	10N	10N		Clear Solution
9:50, 4/24/90, JB	24	20	8.9	8.3	0	10AD	10I		
8:50, 4/25/90, JB	48	20	9.0	8.3	—	9AD	0		
08:55, 4/26/90, O'B.	72	20	8.9	8.3	—	1AD	—		
10:55, 4/26/'90, O'B.	74				—	0	—	Sampled for analysis	Dose discontinued 14:00 R.O'B., 4/26/'90

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 5 mg/L OBSERVATIONS Pp	Gf	Dm		STARTING CONDITIONS FL, DY REMARKS "B"
09:25, 4/23/'90, O'B.	0	20	9.1	8.4	10N	10N	10N		Clear Solution
9:50, 4/24/90, JB	24	20	9.0	8.3	0	10AD	10I		
8:50, 4/25/90, JB	48	20	9.0	8.3	—	9AD	0		
08:55, 4/26/90, O'B.	72	20	9.0	8.3	—	1AD	—		
10:55, 4/26/'90, O'B.	74				—	0	—	Sampled for analysis	Dose discontinued 14:00 R.O'B., 4/26/'90

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 10 mg/L OBSERVATIONS Pp	Gf	Dm		STARTING CONDITIONS FL, DY REMARKS "A"
09:30, 4/23/'90, O'B.	0	20	8.9	8.4	10N	10N	10N		Clear Solution
9:55, 4/24/90, JB	24	20	9.0	8.3	0	10AD	10I		
8:50, 4/25/90, JB	48	20	8.9	8.3	—	0	0	10 mg/L dose discontinued ~~13:00~~ 14:00 on 4/25/'90 - R.O'Boyle	Error R.O'B. 4/25/90

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 10 mg/L OBSERVATIONS Pp	Gf	Dm		STARTING CONDITIONS FL, DY REMARKS "B"
09:30, 4/23/'90, O'B.	0	20	8.9	8.4	10N	10N	10N		Clear Solution
9:55, 4/24/90, JB	24	20	9.0	8.3	0	9AD 10	10I		
8:50, 4/25/90, JB	48	20	9.0	8.3	—	0	0	10 mg/L dose discontinued ~~13:00~~ 14:00 on 4/25/'90 - R.O'Boyle	Error R.O'B. 4/25/90

Abbr. used to describe test organisms' condition

AD - Activity Depressed
AA - Activity Accelerated
PB - Position Bottom
PS - Position Surface
FR - Fragmentation
GD - Gill Rate Depressed
GA - Gill Rate Accelerated
I - Immobilized
N - Normal
NO - New Death
AO - Abnormal Orientations
SH - Shrivelling
T - Tremors
W - Windmill
M - Missing
D - Dead

Abbr. used to describe test conditions

AR - Aeration
DY - Dynamic
FL - Fluorescent Lab Lighting
AW - Avg. Wgt. Flan
PRECIP. - Precipitate
RECIRC. - Recirculating
SOLN. - Solution
ST - Static
X - Xenon Arc Lighting

ACUTE AQUATIC EFFECTS

TEST ORGANISM: Daphnia magna (Dm)

Health and Environment Laboratories see pp. 1, 3
Eastman Kodak Company
Rochester, New York 14650
United States of America

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SIGNATURE	DATE	REVIEWED BY	DATE
Janice H Beglinger	4/27/90	R.J. O'Boyle	4/27/'90

ATTACHMENT 6. Acute Aquatic Effects Data Forms (Replicates A & B)

ACCESSION NO	HAEL NO	TEST ARTICLE	SAMO NO
901878	90-0016	Crotonaldehyde	Lot No. 90-7

Refer to pp. 2, 4.

Gammarus fasciatus (Gf) wet weights + lengths @ Test start: 14 groups of 10 Gf each were collected and 12 groups were used in 96-hr exposure. The remaining two sets were sacrificed by immersion in 70% CH_3OH, allowed to air dry for approximately 1 hour (positioned on absorbent tissue), and were "wet weighed" after taring the weigh dish to 0.00000g (Mettler AE 240).

Set #1: 0.08501g/10 Gf = 8.5 mg/Gf

set #2: 0.09367g/10 Gf = 9.4 mg/Gf (correction, R.O'B., 4/23/'90)

These organisms were selected impartially/randomly from among the initial total of 12 sets, and are consequently deemed to be representative of the test organisms. The representative lengths were also determined from these two sets of Gf:

set #1, mm	set #2, mm
4	4
5	5
4	4
5	5
5	4
5	5
4	3
3	3
4	4
3	5
4.2 mm $\bar{X}_{10}$	4.2 mm

(The Gf were loosely curled, and the measurements* were made from head to tail:
* correction, R.O'B., 4/23/'90

The representative "length" of the gammarids will be considered to be 4.2 mm @ test start.
— R.J. O'Boyle, 4/23/'90.

ACUTE AQUATIC EFFECTS

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14650
United States of America

PAGE 6 OF 6

SIGNATURE	DATE	REVIEWED BY	DATE
Janice M. Beglinger	4/27/90	R.J. O'Boyle	4/27/90

Attachment 7. Excerpts from Analytical Summary Report (page 1 of 13)

3HGV580/3HGV581
90-0016

Crotonaldehyde
901878

TESTING FACILITY

Environmental Analytical Services Unit
Chemicals Quality Services Division
Bldg-320
Eastman Kodak Company
Rochester, New York 14652-3615

SPONSOR

Environmental Technical Services
Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652

DATE OF STUDY INITIATION: 03/20/90

DATES OF EXPERIMENT

Experiment Start Date: 04/23/90

Experiment Termination Date: 04/27/90

STUDY PARTICIPANTS

Chemicals Quality Services Division

Barry W. Remington, Laboratory Technician
Elizabeth M. Isaacs, Laboratory Technician

Robert Ambrose, Chemist

STUDY DIRECTOR

R.J. O'Boyle

INTRODUCTION

The Environmental Protection Agency (EPA) is concerned about the potential environmental impact of releases of quantities of Crotonaldehyde into the Holston River at the Kingsport, Tennessee Kodak plant where the test chemical is manufactured. Kodak has volunteered to perform numerous aquatic tests on the test chemical through Testing Consent Order Docket No. OPTS-42108. The second test, a diluent water study, was performed where concentration determinations were carried out using GC/ECD.

Attachment 7. Excerpts from Analytical Summary Report (page 2 of 13)

3HGV580/3HGV581
90-0016

Crotonaldehyde
901878

REQUEST FOR ANALYSIS

Method Validation		03-22-90 03-27-90	148783
Purity		03-20-90	149292
Multi Species Test	Time 0	04-23-90	105123
	Time 24	04-24-90	105123
	Time 48	04-25-90	105123
	Time 72	04-26-90	105123
	Time 96	04-27-90	105123

TEST ARTICLE

Name: Crotonaldehyde
HAEL No.: 90-0016
Accession No.: 901878
Sample Reference No.: 90-7

3HGV580/3HGV581
90-0016

Crotonaldehyde
901878

MATERIALS AND METHODS

Title: Analysis of Crotonaldehyde in Diluent Water

Abstract: Samples containing crotonaldehyde were diluted (as necessary), derivitized, extracted and analyzed by gas chromatography with electron capture detection (GC/ECD). The method detection limit was 2 ug/L.

Procedure:

Materials:

1. O-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride (PFBOA), Aldrich c/n 19,448-4
2. Sodium thiosulfate, Aldrich c/n 21,726-3
3. Sulfuric acid, Eastman Chemical c/n 13063
4. Diluent water (B-306)
5. EDTA (Eastman Kodak Company)
6. Distilled water
7. Hexane, Baker Resi-analyzed c/n 9262-03
8. Culture tubes, 16 x 125 mm, Kimble c/n 45066-A
9. Disposable Pasteur pipets, VWR Scientific c/n 14672-200
10. Assorted volumetric flasks (10, 25, 50, 100 and 1000 mL)
11. Volumetric pipet (1 mL)
12. Assorted SMI micropettors (50 to 1000 uL)
12. Capillary gas chromatograph
13. Capillary gas chromatographic column, DB-5, 30 meter, 0.25 um film thickness, J&W Scientific c/n 009-5032
14. Chromatographic data handling system

Attachment 7. Excerpts from Analytical Summary Report (page 4 of 13)

3HGV580/3HGV581
90-0016

Crotonaldehyde
901878

Reagent Preparation:

1. 0.10 g of PFBOA was weighed to a 100.0 mL volumetric flask and diluted it to the mark with distilled water. This solution was prepared fresh daily.

2. 1.58 g of sodium thiosulfate was weighed to a 100.0 mL volumetric flask and diluted to the mark with distilled water. (0.1 M solution). This solution was good for one (1) week.

Standard Preparation:

1. Stock Approximately 0.100 g of test chemical was weighed to a 100.0 mL volumetric flask and diluted to the mark with diluent water , containing EDTA. This solution was prepared fresh daily.

2. Intermediate An 0.5 mL aliquot of the stock solution was taken and diluted to 100.0 mL with diluent water, containing EDTA.

3. Working Up to seven (7) aliquots ranging from 25 to 175 uL of the intermediate solution were taken and the aliquots transferred to separate 25.0 mL volumetric flasks. The flasks were diluted to the mark with diluent water, containing EDTA.

Sample Preparation:

1. All samples were diluted down to 20 ug/L with diluent water, containing EDTA, using at least 25 mL volumetric flasks.

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90-0016

Crotonaldehyde
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Derivatization:

1. One (1.0) mL of the 1 mg/mL PFBOA solution was added to a culture tube.
2. 200 uL of the 0.1 M sodium thiosulfate solution was added to the culture tube and mixed.
3. Ten (10.0) mL of diluent water containing EDTA was added and mixed. Allow to stand for two (2) hours.
4. Steps 1-3 were repeated for all of the working standards.
5. Steps 1-3 were repeated for all samples.

Extraction:

1. One (1) drop of concentrated sulfuric acid was added to each culture tube and mixed.
2. Two (2.0) mL of hexane were added to each culture tube and then shaken for 30 seconds.
3. The culture tubes were allowed to stand for fifteen (15) minutes.
4. Approximately half of the extract was transferred to two (2) separate GC vials and capped.
5. Step 4 was repeated for all samples.

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Crotonaldehyde
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Analyses:

1. The samples were then analyzed by GC/ECD using the following conditions:

Instrument: Perkin-Elmer 8500 Gas Chromatograph

Column: J&W DB-5 30 meter 0.25 um film thickness

Pressure: 10 psig

Oven Program: 80 C (held for 2 min.) to 250 C @ 10 C/min. held @ 250 C for 1 min.

Injector Temp: 250 C

Type: Splitless (2 min. purge delay)

Split Flow: 60 cc/min.

Inj. Volume: 1 uL

Detector: Electron Capture

Temp.: 300 C

Make-up: Nitrogen

Flow: 55 cc/min.

Calculations:

1. Data reduction was performed using a chromatography data handling system such as Multichrom (a VG, Inc. product). The cis-and trans- isomers were added together and treated as one component whenever possible.

Attachment 7. Excerpts from Analytical Summary Report (page 7 of 13)

3HGV580/3HGV581
90-0016

Crotonaldehyde
901878

METHOD VALIDATION

3-27-90

Theoretical Conc. (mg/L)	Actual Conc. (mg/L)	Recovery (%)
0.620 filtered	0.62	99.5
	0.62	101
	0.62	101
0.620 unfiltered	0.63	102
	0.64	103
	0.64	104
2.55 filtered	2.50	98.0
	2.22	87.1
	2.41	94.5
2.55 unfiltered	2.46	96.5
	2.49	97.7
	2.46	96.5
9.97 filtered	9.96	100
	10.1	101
	9.52	95.5
9.97 unfiltered	9.93	99.6
	9.76	97.9
	9.55	95.8

Attachment 7. Excerpts from Analytical Summary Report (page 8 of 13)

3HGV580/3HGV581
90-0016

Crotonaldehyde
901878

METHOD VALIDATION

3-22-90

Theoretical Conc. (mg/L)	Actual Conc. (mg/L)	Recovery (%)
0.70866 filtered	0.61	85.7
	0.67	94.2
	0.66	92.7
0.70866 unfiltered	0.72	101
	0.70	98.3
	0.74	104
2.8575 filtered	4.09	143
	2.85	99.9
	2.81	98.5
2.8575 unfiltered	3.03	106
	3.11	109
	3.10	108
11.43 filtered	19.7	172
	11.4	99.6
	11.5	101
11.43 unfiltered	14.4	126
	12.3	108
	12.8	112

Attachment 7. Excerpts from Analytical Summary Report (page 9 of 13)

3HGV580/3HGV581
90-0016

Crotonaldehyde
901878

MULTI SPECIES TEST

Sample	Theor Conc (mg/L)	Time 0 Actual Conc. (mg/L)	Time 24 Actual Conc. (mg/L)	Time 48 Actual Conc. (mg/L)	Time 72 Actual Conc. (mg/L)	Time 96 Actual Conc. (mg/L)
DWC-A	0	<0.005	NA	<0.005	NA	<0.005
DWC-B	0	<0.005	NA	<0.005	NA	<0.005
0.6-A	0.625	0.543	NA	0.669	NA	0.311
0.6-B	0.625	0.543	NA	0.686	NA	0.311
1.2-A	1.25	1.27	NA	1.38	NA	0.740
1.2-B	1.25	1.31	NA	1.13	NA	0.590
1.2-A inside	NA	NA	NA	0.866	NA	NA
1.2-B inside	NA	NA	NA	NA	NA	0.366
2.5-A	2.5	2.58	2.59	2.33	NA	1.55
2.5-B	2.5	2.69	2.25	2.66	NA	1.58
5.0-A	5.0	5.11	3.71	4.84	3.82	NA
5.0-B	5.0	5.54	4.30	4.82	4.06	NA
5.0-A inside	NA	NA	NA	NA	3.22	NA
5.0-B inside	NA	NA	NA	3.71	NA	NA
10-A	10	11.4	7.92	7.84	NA	NA
10-B	10	12.4	8.65	7.75	NA	NA
STOCK	NA	NA	NA	123	NA	NA
EXT BLK	ND	ND	<0.005	<0.005	<0.005	<0.005

Method Quantification Limit = 0.005 mg/L

NA= Not Analyzed (Sample not received)
ND= None Detected

Attachment 7. Excerpts from Analytical Summary Report (page 10 of 13)

3HGV580/3HGV581
90-0016

Crotonaldehyde
901878

PERTINENT MATHEMATICAL EQUATIONS

Calculations of the concentration of the test chemical were done using the linear regression equation for the standards run with each day:

$y = mx + b$ where: y = area
m = slope
x = concentration (ug/L)
b = y-intercept

All samples were corrected using the appropriate dilution factor to obtain the concentration in mg/L.

$$\text{Dilution Factor} = \frac{\text{Total Volume(mL)}}{\text{Aliquoted Volume(mL)}}$$

Scientist Beth Isaacs (signature) Date 7-3-90

HAEL No. 90-0016 Study No. EN-407-901878-1

Attachment 7. Analytical Summary Report (page 11 of 13)

Record of Collection and Analysis of Exposure Solutions at Test Start

RECORD OF COLLECTION AND ANALYSIS OF TEST SOLUTIONS

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14650
United States of America

Protocol No.: EN-407-901878-1 HAEL No.: 90-0016 Accession No.: 901878 Multispecies

Concentration/Replicate	Sample No.	Sampled by			Sample Prepared for Analysis			Sample Analyzed		
		Date	Time	Person	Date	Time	Person	Date	Time	Person
DWC - "A"	1	4/23/90	09:30	R.O'Boyle	4-23-90	10:30 am	BLR	4-23-90	2:00 pm	BLR
DWC - "B"	2									
0.6 mg/L - "A"	3									
0.6 - "B"	4									
1.2 - "A"	5									
1.2 - "B"	6									
2.5 - "A"	7									
2.5 - "B"	8									
- "A"	9									
5 - "B"	10									
10 - "A"	11									
10 ✓O'B. - "B"	12	✓O'B.	✓O'B.	✓O'B.	BR	BLR	BLR	BLR	BLR	BLR
2.5 mg/L - "A"	U	4/24/90	08:30	R.O'Boyle	4-24-90	9:30 am	BD	4-24-90	12:30 pm	BD
2.5 - "B"	V									
5 - "A"	W									
5 - "B"	X									
10 - "A"	Y									
10 ✓O'B. - "B"	Z	✓O'B.	✓O'B.	✓O'B.	BD	BD	BD	BD	BD	BD

KEY:
DWC - Dilution Water Control
CSC - Carrier Solvent Control

REMARKS: ≈ 50 mL of each exposure solution submitted to CQSD @ test start (Time "0")
Samples U-Z taken @ time 24 hrs.
Due to complete minnow mortality in these doses - R.O'Boyle - 4/24/90
(and possible daphnid immobilization in 10 mg/L)

- R.J. O'Boyle 4/23/90

HAEL No. 90-0016 Study No. EN-407-901878-1

Attachment 7. Analytical Summary Report (page 12 of 13)
(Record of Collection and Analysis of Exposure Solutions at Time 48 Hours)

RECORD OF COLLECTION AND ANALYSIS OF TEST SOLUTIONS

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14650
United States of America

Protocol No.: EN-407-901878-1 HAEL No.: 90-0016 Accession No.: 901878 Multispecies

Concentration/Replicate	Sample No.	Sampled by			Sample Prepared for Analysis			Sample Analyzed		
		Date	Time	Person	Date	Time	Person	Date	Time	Person
DWC - "A"	13	4/25/90	08:40	R.O'Boyle	4-25-90	9:30am	B.D	4-25-90	4:30pm	B.D
DWC - "B"	14									
0.6 mg/L - "A"	15									
0.6 - "B"	16									
1.2 - "A"	17									
1.2 - "B"	18									
2.5 - "A"	19									
2.5 - "B"	20									
5 - "A"	21									
5 - "B"	22									
10 - "A"	23									
10 - "B"	24	↓O'B.	↓O'B.	↓O'B.						
100 mg/L Fresh Stock Solution (Supplying Diluter)	25	4/25/90	10:15	R.O'Boyle						
1.2 mg/L - "A"	"a"	4/25/90	08:30	R.O'Boyle						
5 mg/L - "B" (Both sampled inside Daphnia baskets)	"b"	4/25/90	08:30	R.O'Boyle	B.D	B.D	B.D		B.D	B.D

KEY:
DWC - Dilution Water Control
CSC - Carrier Solvent Control

REMARKS: ≈50 mL of each exposure solution (sampled prior to midtest renewal) submitted to CQSD @ time 48 hours (Test end for Daphnia magna)
— R.J. O'Boyle, 4/25/90

HAEL No. 90-0016 Study No. EN-407-901878-1

Attachment 7. Analytical Summary Report (page 13 of 13)
(Record of Collection and Analysis of Exposure Solutions at Test End)

RECORD OF COLLECTION AND ANALYSIS OF TEST SOLUTIONS

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14650
United States of America

Protocol No.: EN-407-901878-1 HAEL No.: 90-0016 Accession No.: 901878 Multispecies

Concentration/Replicate	Sample No.	Sampled by			Sample Prepared for Analysis			Sample Analyzed		
		Date	Time	Person	Date	Time	Person	Date	Time	Person
DWC – "A"	26	4/27/90	08:50	R.O'Boyle	4-27-90	10:00am	BD	4-27-90	4:00pm	BD
DWC – "B"	27									
0.6 mg/L – "A"	28									
0.6 – "B"	29									
1.2 – "A"	30									
1.2 – "B"	31									
2.5 – "A"	32									
All * 2.5 – "B"	33	✓O'B.	✓O'B.	✓O'B.	✓BD	✓BD	✓BD	✓BD	✓BD	✓BD
5 – "A"	34	4/26/90	11:00	R.O'Boyle	4-26-90	11:00am	BD	4-26-90	4:00pm	BD
5 ✓O'B. – "B"	35	4/26/90	11:00	R.O'Boyle	↓BD	↓BD	↓BD	↓BD	↓BD	↓BD
~~10 – "A"~~										
~~10 ✓O'B – "B"~~										
(Concentration dose discontinued on 4/25/90)	R.O'Boyle 4/26/90									
1.2 mg/L – "B"	"C"	4/27/90	08:55	R.O'Boyle	4-27-90	10:00am	BD	4-27-90	4:00pm	BD
* 5 mg/L – "A"	"d"	4/26/90	11:00	R.O'Boyle	4-26-90	11:00am	BD	4-26-90	4:00pm	BD
(Both sampled inside containment baskets)										

KEY:
DWC - Dilution Water Control
CSC - Carrier Solvent Control

REMARKS: ≈ 50 mL of each exposure solution submitted @ test end per dose (usually @ Time 96 hrs) From R.O'B. 4/27/90
* 5 mg/L doses sampled @ ≈ 74 hrs due to complete gammarid mortality; Dose then discontinued. R.O'Boyle, 4/26/90
– R.J. O'Boyle, 4/27/90

ATTACHMENT 8. LC_{50}/EC_{50} Calculator Program Methods and Calculations
(page 1 of 4)

a) Non-linear Interpolation between the concentrations that bracket 50% test population mortality gives an approximate point estimate, and the Binomial Method gives a conservative 95% confidence interval. In the case of a test utilizing a single effective concentration, this program, by use of the Binomial Method, is capable of indicating at a conservative 95% confidence interval whether or not the LC_{50} value lies above or below the test concentration.

b) The Moving Average Method gives both a point and interval estimate. This output is included only when two consecutive dose level sets cause death in the test population.

c) The Probit Method gives both a point and interval estimate. This output is included only when there are at least two consecutive sets of partial test population mortality. Additionally, when the number of effective concentrations is at least three, a chi-square statistic is included to test the goodness-of-fit for the Probit Model.

Note: Calculating an EC_{50} for *Daphnia magna* requires the input of immobilization data rather than mortality as with an LC_{50}.

ATTACHMENT 8. LC_{50}/EC_{50} Calculator Program Methods and Calculations
(page 2 of 4)

Fathead Minnow

Pooled Replicates A & B:

(48-Hour Values)

CONC.,MG/L	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROB.(PERCENT)
10	20	20	100	LESS THAN 0.001
4.7	20	20	100	LESS THAN 0.001
2.5	20	20	100	LESS THAN 0.001
1.3	20	11	55	33.1812
.61	20	0	0	LESS THAN 0.001

THE BINOMIAL TEST SHOWS THAT .61 AND 2.5 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 1.24 MG/L IS OBTAINED BY NONLINEAR INTERPOLATION BETWEEN .61 AND 1.3

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

(96-Hour Values)

CONC.,MG/L	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROB.(PERCENT)
10	20	20	100	LESS THAN 0.001
4.7	20	20	100	LESS THAN 0.001
2.5	20	20	100	LESS THAN 0.001
1.1	20	17	85	.074482
.51	20	0	0	LESS THAN 0.001

THE BINOMIAL TEST SHOWS THAT .51 AND 1.1 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 0.84 MG/L IS OBTAINED BY NONLINEAR INTERPOLATION BETWEEN .51 AND 1.1

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

ATTACHMENT 8. LC_{50}/EC_{50} Calculator Program Methods and Calculations

(page 3 of 4)

Gammarid

Pooled Replicates A & B:

(48-Hour Values)

CONC.,MG/L	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROB.(PERCENT)
9.3	20	20	100	LESS THAN 0.001
4.7	20	2	10	1.10626E-02
2.5	20	0	0	LESS THAN 0.001
1.3	20	0	0	LESS THAN 0.001
.61	20	0	0	LESS THAN 0.001

THE BINOMIAL TEST SHOWS THAT 4.7 AND 9.3 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 6.14 MG/L IS OBTAINED BY NONLINEAR INTERPOLATION BETWEEN 4.7 AND 9.3

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

(96-Hour Values)

CONC.,MG/L	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROB.(PERCENT)
9.3	20	20	100	LESS THAN 0.001
4.5	20	20	100	LESS THAN 0.001
2.3	20	6	30	3.91769
1.1	20	0	0	LESS THAN 0.001
.51	20	0	0	LESS THAN 0.001

THE BINOMIAL TEST SHOWS THAT 1.1 AND 4.5 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 2.68 MG/L IS OBTAINED BY NONLINEAR INTERPOLATION BETWEEN 2.3 AND 4.5

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

ATTACHMENT 8. LC_{50}/EC_{50} Calculator Program Methods and Calculations
(page 4 of 4)

Daphnid (48-Hour Values Only)

Pooled Replicates A & B:

****

CONC.,MG/L	NUMBER EXPOSED	NUMBER IMMOBILE	PERCENT IMMOBILE	BINOMIAL PROB.(PERCENT)
10	20	20	100	LESS THAN 0.001
4.7	20	20	100	LESS THAN 0.001
2.5	20	15	75	1.33018
1.3	20	2	10	1.10626E-02
.61	20	1	5	1.04904E-03

THE BINOMIAL TEST SHOWS THAT 1.3 AND 2.5 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 EC50 OF 1.98 MG/L IS OBTAINED BY NONLINEAR INTERPOLATION BETWEEN 1.3 AND 2.5

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

SPAN	G	EC50	95 PERCENT CONFIDENCE LIMITS	
3	6.61953E-02	1.87604	1.53802	2.30075
2	7.60283E-02	2.00791	1.66137	2.34295
1	.200686	1.97075	1.6981	2.39182

AN EC50 CALCULATED 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
4	.1348	1	6.83689	7.72821E-02

SLOPE = 4.98921
95 PERCENT CONFIDENCE LIMITS = 3.15742 AND 6.821

EC50 = 1.87089 MG/L
95 PERCENT CONFIDENCE LIMITS = 1.53275 AND 2.2635

EC1 = .639415 MG/L
95 PERCENT CONFIDENCE LIMITS = .32452 AND .893692

****

ATTACHMENT 9. Preliminary Bioassay Data Forms

Fathead Minnow

ACCESSION NO.	HAEL NO.	TEST ARTICLE	SRID NO.
901878	Meth. Dev.	Crotonaldehyde (H-C-C=C-C-H)	B15B

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 1 L °C	D.O.	pH	AIM CONCENTRATION 0 mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS (DWC = Diluent Water Control)
09:00, 10/24/'89, O'B	0	20	—	—	3	N	
15:00, 10/24/'89, O'B	6	20	—	—	3	N	
09:00, 10/25/'89, O'B	24	20	—	—	3	N	
09:00, 10/26/'89, O'B	48	20	—	—	3	N	
09:00, 10/27/'89, O'B	72	20	—	—	3	N; Exploratory R-F	Test Discontinued @ 72 hours.

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 1 L °C	D.O.	pH	AIM CONCENTRATION 1 mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS
09:00, 10/24/'89, O'B	0	20	—	—	3	N	
15:00, 10/24/'89, O'B	6	20	—	—	3	N	
09:00, 10/25/'89, O'B	24	20	—	—	3	N	
09:00, 10/26/'89, O'B	48	20	—	—	3	N	
09:00, 10/27/'89, O'B	72	20	—	—	3	N; Exploratory R-F	Test Discontinued @ 72 hours.

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 1 L °C	D.O.	pH	AIM CONCENTRATION 10 mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS
09:00, 10/24/'89, O'B	0	20	—	—	3	N	
15:00, 10/24/'89, O'B	6	20	—	—	0	(3 Dead)	

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 1 L °C	D.O.	pH	AIM CONCENTRATION 100 mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS
09:00, 10/24/'89, O'B	0	20	—	—	3	N	
15:00, 10/24/'89, O'B	6	20	—	—	0	(3 Dead)	

Note: 3 Pp in 1000 mg/L solution all Dead in 5 minutes.

Abbr. used to describe test organisms' condition
AD - Activity Depressed
AA - Activity Accelerated
PB - Position Bottom
PS - Position Surface
FR - Fragmentation
GD - Gill Rate Depressed
GA - Gill Rate Accelerated
I - Immobilized
N - Normal
ND - Near Death
AO - Abnormal Orientations
SH - Shriveling
T - Tremors
W - Withdrawn
M - Missing
D - Dead

Abbr. used to describe test conditions
AR - Aeration
DY - Dynamic
FL - Fluorescent Lab Lighting
AW - Avg. Wgt. Fish
PRECIP. - Precipitate
RECIRC. - Recirculating
SOLN. - Solution
ST - Static
X - Xenon Arc Lighting

ACUTE AQUATIC EFFECTS

TEST ORGANISM: Pimephales promelas (Pp)

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PAGE 1 OF 2

SIGNATURE	DATE	REVIEWED BY	DATE
R.J. O'Boyle	10/24/'89	[signature]	12/17/89

10/27/'89 correction, R.O'Boyle, 8/23/90

HAEL No. 90-0016 Study No. EN-407-901878-1

ATTACHMENT 9. Preliminary Bioassay Data Forms

Fathead Minnow

ACCESSION NO.	HAEL NO.	TEST ARTICLE	SRID NO.
901878	Meth. Dev.	Crotonaldehyde	B15B

AAE Summary:

72-hr LC_{50} (static) = 3.2 mg/L (nominal)

NOEC = 1 mg/L (nominal)

– R. J. O'Boyle
10/26/'89
27 Error R.O'B., 10/27/'89.

ACUTE AQUATIC EFFECTS

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United States of America

PAGE 2 OF 2

SIGNATURE	DATE	REVIEWED BY	DATE
R. J. O'Boyle	10/24/89	[illegible]	10/27/89

10/27/89 Correction, R.O'Boyle, 6/23/90

ATTACHMENT 9. Preliminary Bioassay Data Forms

Gammarid

ACCESSION NO.	HAEL NO.	TEST ARTICLE	SRID NO.
901878	Meth. Dev.	Crotonaldehyde ($H_2C-C=C-C-H$)	B15B

TIME, DATE & INITIALS	TIME (PROTOCOL)	TEST VOLUME 200 mL °C	D.O.	pH	AIM CONCENTRATION 0.0 mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS (DWC = Diluent Water Control)
10:00, 10/26/'89, O'B.	0	20	—	—	10	N	
14:00, 10/26/'89, O'B.	4	20	—	—	10	N	
09:30, 10/27/'89, O'B.	24	20	—	—	10	N (Solution Renewed)	
15:00, 10/30/'89, O'B.	96	20	—	—	10	N	

Error R.O'B. 10/26/'89

TIME, DATE & INITIALS	TIME (PROTOCOL)	TEST VOLUME 200 mL °C	D.O.	pH	AIM CONCENTRATION 1 mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS
10:00, 10/26/'89, O'B.	0	20	—	—	10	N	
14:00, 10/26/'89, O'B.	4	20	—	—	10	N	
09:30, 10/27/'89, O'B.	24	20	—	—	10	N (Solution Renewed)	
15:00, 10/30/'89, O'B.	96	20	—	—	10	N	

Error R.O'B. 10/26/'89

TIME, DATE & INITIALS	TIME (PROTOCOL)	TEST VOLUME 200 mL °C	D.O.	pH	AIM CONCENTRATION 10 mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS
10:00, 10/26/'89, O'B.	0	20	—	—	10	N	
14:00, 10/26/'89, O'B.	4	20	—	—	10	N	
09:30, 10/27/'89, O'B.	24	20	—	—	10	AD (Solution Renewed)	
15:00, 10/30/'89, O'B.	96	20	—	—	0	10D	

Error R.O'B. 10/26/'89

TIME, DATE & INITIALS	TIME (PROTOCOL)	TEST VOLUME 200 mL °C	D.O.	pH	AIM CONCENTRATION 100 mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS
10:00, 10/26/'89, O'B.	0	20	—	—	10	N	
14:00, 10/26/'89, O'B.	4	20	—	—	10	ND/Dead	
09:30, 10/27/'89, O'B.	24	20	—	—	0	(All Definitely Dead)	

Abbr. used to describe test organisms' condition
AD - Activity Depressed
AA - Activity Accelerated
PB - Position Bottom
PS - Position Surface
FR - Fragmentation
GD - Gill Rate Depressed
GA - Gill Rate Accelerated
I - Immobilized
N - Normal
ND - Near Death
AO - Abnormal Orientations
SH - Shriveling
T - Tremors
W - Withdrawn
M - Missing
D - Dead

Abbr. used to describe test conditions
AR - Aeration
DY - Dynamic
FL - Fluorescent Lab Lighting
AW - Avg. Wgt. Fish
PRECIP. - Precipitate
RECIRC. - Recirculating
SOLN. - Solution
ST - Static
X - Xenon Arc Lighting

ACUTE AQUATIC EFFECTS

TEST ORGANISM: Gammarus fasciatus Gp

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United States of America

PAGE 1 OF 2

SIGNATURE	DATE	REVIEWED BY	DATE
R.J. O'Boyle	10/27/'89	[signature]	12/13/89

30 error R.O'B. 10/30/'89

HAEL No. 90-0016 Study No. EN-407-901878-1

ATTACHMENT 9. Preliminary Bioassay Data Forms

Gammarid

ACCESSION NO.	HAEL NO.	TEST ARTICLE	SRID NO.
901878	Meth. Des.	Crotonaldehyde ($H_3C-C(=O)-C=C-H$)	B15B

AAE Summary:

LC_{50} (static) = 3.2 mg/L (nominal)
NOEC = 1 mg/L (nominal)

– R.J. O'Boyle
10/30/'89

ACUTE AQUATIC EFFECTS

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SIGNATURE	DATE	REVIEWED BY	DATE
R.J. O'Boyle	10/30/'89	[illegible]	11/13/89

ATTACHMENT 9. Preliminary Bioassay Data Forms

Daphnid

ACCESSION NO	HAEL NO.	TEST ARTICLE	SRID NO
901878	Meth. Dev.	Crotonaldehyde ($H_2C=C(H)-C(H)=C(H)-C(=O)-H$)	B15B

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 100 mL °C	D.O.	pH	AIM CONCENTRATION 0 mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS (DWC = Diluent Water Control)
09:00, 10/24/'89, O'B.	0	20	—	—	10	N	
15:00, 10/24/'89, O'B.	6	20	—	—	10	N	
09:00, 10/25/'89, O'B.	24	20	—	—	10	N	
09:00, 10/26/'89, O'B.	48	20	—	—	10	N	

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 100 mL °C	D.O.	pH	AIM CONCENTRATION 1 mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS
09:00, 10/24/'89, O'B.	0	20	—	—	10	N	
15:00, 10/24/'89, O'B.	6	20	—	—	10	N	
09:00, 10/25/'89, O'B.	24	20	—	—	10	N	
09:00, 10/26/'89, O'B.	48	20	—	—	10	N	

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 100 mL °C	D.O.	pH	AIM CONCENTRATION 10 mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS
09:00, 10/24/'89, O'B.	0	20	—	—	10	N	
15:00, 10/24/'89, O'B.	6	20	—	—	7	5N, 2AD (3I)	
09:00, 10/25/'89, O'B.	24	20	—	—	0	(All Dead)	

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 100 mL °C	D.O.	pH	AIM CONCENTRATION 100 mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS
09:00, 10/24/'89, O'B.	0	20	—	—	10	N	
15:00, 10/24/'89, O'B.	6	20	—	—	0	(10 D)	

Note: 10 Dm in 1000 mg/L Solution all Dead in 5 minutes.

Abbr. used to describe test organisms' condition
AD - Activity Depressed
AA - Activity Accelerated
PB - Position Bottom
PS - Position Surface
FR - Fragmentation
GD - Gill Rate Depressed
GA - Gill Rate Accelerated
I - Immobilized
N - Normal
ND - Near Death
AO - Abnormal Orientations
SH - Shriveling
T - Tremors
W - Withdrawn
M - Missing
D - Dead

Abbr. used to describe test conditions
AR - Aeration
DY - Dynamic
FL - Fluorescent Lab Lighting
AW - Avg. Wgt. Fish
PRECIP - Precipitate
RECIRC - Recirculating
SOLN. - Solution
ST - Static
X - Xenon Arc Lighting

ACUTE AQUATIC EFFECTS

TEST ORGANISM: Daphnia magna 1Dm

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PAGE 1 OF 2

SIGNATURE	DATE	REVIEWED BY	DATE
R.J. O'Boyle	10/26/'89	[signature]	12/13/89

HAEL No. 90-0016 Study No. EN-407-901878-1

ATTACHMENT 9. Preliminary Bioassay Data Forms

Daphnid

ACCESSION NO.	HAEL NO.	TEST ARTICLE	SRID NO.
901878	Meth. Dev.	Crotonaldehyde	B15B

AAE Summary:

EC_{50} (static) = 3.2 mg/L (nominal)
NOEC = 1 mg/L (nominal)

— R.J. O'Boyle
10/26/89

ACUTE AQUATIC EFFECTS

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PAGE 2 OF 2

SIGNATURE	DATE	REVIEWED BY	DATE
R.J. O'Boyle	10/26/89	[illegible]	12/11/89

HAEL No. 90-0016

Study No. EN-407-901878-1

ATTACHMENT 9. Preliminary Bioassay Data Forms

Fathead Minnow (3/29/90)

Methods Development

ACCESSION NO.	HAEL NO.	TEST ARTICLE	SRID NO. (Lot No.)
901878	90-0016	Crotonaldehyde	90-7

AIM CONCENTRATION 0.1mg/L — TEST VOLUME 20L — STARTING CONDITIONS ST, FL

TIME, DATE & INITIALS	TIME (PROTOCOL)	°C	D.O.	pH	OBSERVATIONS		REMARKS
8:45, 3/29/90, RB	0	20	8.8	8.0	10	N	Pp
15:00, 3/29/90, RB	6	—	—	—	10	N	Vessels on this page
8:55, 3/30/90, RB	24	—	—	—	10	N	were all loosely covered
10:00, 3/31/90, RB	48	—	—	—	10	N	throughout exposure.
11:00, 4/1/90, RB	72	—	—	—	10	N	- R.D. Boyle, 4/2/90
16:00, 4/2/90, RB	96	20	—	—	10	N	

AIM CONCENTRATION 1mg/L — TEST VOLUME 20L — STARTING CONDITIONS ST, FL

TIME, DATE & INITIALS	TIME (PROTOCOL)	°C	D.O.	pH	OBSERVATIONS		REMARKS
8:45, 3/29/90, RB	0	20	8.9	8.3	10	N	Pp
15:00, 3/29/90, RB	6	—	—	—	10	N	
8:55, 3/30/90, RB	24	—	—	—	10	N	
10:00, 3/31/90, RB	48	—	—	—	10	N	
11:00, 4/1/90, RB	72	—	—	—	10	N	
16:00, 4/2/90, RB	96	20	—	—	10	N	

AIM CONCENTRATION 10mg/L — TEST VOLUME 20L — STARTING CONDITIONS ST, FL

TIME, DATE & INITIALS	TIME (PROTOCOL)	°C	D.O.	pH	OBSERVATIONS		REMARKS
8:45, 3/29/90, RB	0	20	8.9	8.3	10	N	Pp
15:00, 3/29/90	6	20	8.8	8.4	0	(10 D)	Reaction RB 4/29/90

AIM CONCENTRATION 0.1mg/L — TEST VOLUME 20L — STARTING CONDITIONS ST, FL

TIME, DATE & INITIALS	TIME (PROTOCOL)	°C	D.O.	pH	OBSERVATIONS		REMARKS
8:45, 3/29/90, RB	0	12	10.6	8.3	10	N	Sg
15:00, 3/29/90	6	—	—	—	10	N	
8:55, 3/30/90	24	—	—	—	10	N	
10:00, 3/31/90, RB	48	—	—	—	10	N	
11:00, 4/1/90, RB	72	—	—	—	10	N	
16:00, 4/2/90, RB	96	12	—	—	10	N	

Error R.D.B. 4/1/90

Abbr. used to describe test organisms' condition: AD - Activity Depressed; AA - Activity Accelerated; PB - Position Bottom; PS - Position Surface; FR - Fragmentation; GD - Gill Rate Depressed; GA - Gill Rate Accelerated; I - Immobilized; N - Normal; MD - Near Death; AO - Abnormal Orientations; SH - Shriveling; T - Tremors; W - Withdrawn; M - Missing; D - Dead

Abbr. used to describe test conditions: AR - Aeration; DY - Dynamic; FL - Fluorescent Lab Lighting; AW - Avg. Wgt. Run; PRECIP. - Precipitate; RECIRC. - Recirculating; SOLN. - Solution; ST - Static; X - Xenon Arc Lighting

ACUTE AQUATIC EFFECTS

TEST ORGANISM: Pimephales promelas (SS) (Pp) Salmo gairdneri

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PAGE 1 OF 2

SIGNATURE	DATE	REVIEWED BY	DATE
Janice M. Beglinger	4/3/90	R.D. Boyle	4/2/90

HAEL No. 90-0016 Study No. EN-407-901878-1

ATTACHMENT 9. Preliminary Bioassay Data Forms

Fathead Minnow (3/29/90)

ACCESSION NO.	HAEL NO.	TEST ARTICLE	SPRD NO. (Lot/No.)
901878	90-0016	Crotonaldehyde	90-7

R-F Test Results: (0.26 mL/22.2 L = 10 mg/L, nominal)
Most recent methods development with test article and fathead minnows suggest that 0.6, 1.2, 2.5, 5.0, and 10 mg/L is still an appropriate dose series for definitive test (even though flow through).

In addition, a nominal dose of 0.1 mg/L does not appear to cause adverse effects in rainbow trout. Consequently, the dosing scheme for the definitive test (flow-through) is tentative set at 0.12, 0.25, 0.5, 1.0, & 2.0 mg/L

— R. O'Boyle
4/3/90.

ACUTE AQUATIC EFFECTS

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Rochester, New York 14650
United States of America

PAGE 2 OF 2

SIGNATURE	DATE	REVIEWED BY	DATE
Janice M. Beglinger	4/3/90	R. O'Boyle	4/2/90

ATTACHMENT 10. Quality Assurance Unit Statements (page 1 of 2)

ANALYTICAL QUALITY ASSURANCE INSPECTION STATEMENT
(CFR 58.35(B)(7) 792.35(B)(7) 160.35(B)(7))

STUDY: 90-0016-7 STUDY DIRECTOR: OBOYLE, R.
ANALYTICAL DIRECTOR: AMBROSE, R.
ACCESSION NUMBER: 901878
CQS JOB NUMBER: 3HGV581

STUDY TYPE: ACUTE AQUATIC EFFECTS: MULTI-SPECIES TEST

M J Boccaci
(AUDITOR, QUALITY ASSURANCE UNIT)

8-20-90
DATE

THIS STUDY WAS INSPECTED BY 1 OR MORE PERSONS OF THE QUALITY ASSURANCE UNIT OF MQAO, EASTMAN KODAK COMPANY, ROCHESTER, N.Y. AND WRITTEN STATUS REPORTS WERE SUBMITTED ON THE FOLLOWING DATES:

INSPECT DATES	REQUEST NUMBER	PHASE(S) INSPECTED	STATUS REPORT DATES
04/25/90	105123	DOSE VERIFICATION PHASE INSPECTION	04/25/90
06/06/90	149292	PURITY TEST REPORT INSPECTION	06/01/90
06/07/90	142470	PURITY TEST REPORT INSPECTION	06/30/90
07/02/90	105123	DOSE VERIFICATION TEST REPORT INSPECTION	07/02/90
07/02/90	148783	METHOD VALIDATION	07/02/90

ATTACHMENT 10. Quality Assurance Unit Statements (page 2 of 2)

Q.A. INSPECTION STATEMENT
(CFR 58.35(B)(7) 792.35(B)(7) 160.35(B)(7))

STUDY: 90-0016-1 STUDY DIRECTOR: O'BOYLE,R.J. PAGE 1
ACCESSION NUMBER: 901878 09/06/90

STUDY TYPE: ACUTE AQUATIC EFFECTS TEST (MULTI-SPECIES-FL.)

M. Susan Jones 9/6/90
(AUDITOR, QUALITY ASSURANCE UNIT) DATE

TO THE BEST OF MY KNOWLEDGE, THIS FINAL REPORT ACCURATELY DESCRIBES THE METHODS AND STANDARD OPERATING PROCEDURES, AND THE REPORTED RESULTS ACCURATELY REFLECT THE RAW DATA. THIS STUDY WAS INSPECTED BY 1 OR MORE PERSONS OF THE QUALITY ASSURANCE UNIT OF THE HAEL, EASTMAN KODAK COMPANY, ROCHESTER, N.Y. AND WRITTEN STATUS REPORTS WERE SUBMITTED ON THE FOLLOWING DATES:

INSPECTION DATES	PHASE(S) INSPECTED	STATUS REPORT DATES
04/03/90	PROTOCOL SUBMISSION	
04/23/90	TEST ARTICLE-CARRIER-MIXTURE PREPARATION PH, TEMPERATURE, AND DISSOLVED OXYGEN READINGS SAMPLE COLLECTION TEST SYSTEM ADDITION TO TEST ARTICLE-CARRIER-MIXTURE TEST SYSTEM ADDITION TO CONTROL ARTICLE CLINICAL SIGNS AT 0 HR. 0 HR SAMPLES SENT TO CQSD FOR ANALYSIS	08/22/90
05/17/90	PROTOCOL AMENDMENT AMENDMENT OF 4/25/90 RECEIVED	
08/22/90	FINAL REPORT REVIEW	08/22/90
09/06/90	FINAL REPORT REVIEW AMENDED REPORT	09/06/90

HAEL No. 90-0016 Study No. EN-407-901878-1

DIS No.: 263599H

Prepared by Janice M. Beglinger Date 8/22/90
Janice M. Beglinger
Principal Investigator-Biology
Eco-Chem Testing Group

Approved by Joseph W. Gorsuch Date 8/23/90
Joseph W. Gorsuch
Group Leader
Eco-Chem Testing Group

Approved by John R. Owen Date 8/24/90
John R. Owen
Unit Director
Environmental Sciences Section

Reviewed by M. Susan James Date 8/23/90
Quality Assurance Unit
Health and Environment Laboratories

Prepared by R. J. O'Boyle Date 8/29/'90
Robert J. O'Boyle
Study Director
Eco-Chem Testing Group

Approved by Roderick D. Gerwe Date August 27 1990
Roderick D. Gerwe
Sponsor
Eastman Chemicals Division

4097e

Study No. EN-421-901878-2
ES-90-029
263598G

SCANNED

Study Title

ACUTE AQUATIC EFFECTS OF
CROTONALDEHYDE
ON THE RAINBOW TROUT, *Oncorhynchus mykiss*

Data Requirement

In Accordance with the US EPA, 1985. Toxic Substances, Testing Requirements; Final Rule and Proposed Rule. Federal Register: 40 CFR Part 797.1400 Fish Acute Toxicity Test (Amended 1987); (as Amended in Testing Consent Order on Crotonaldehyde, 40 CFR Part 799)

Authors

Janice M. Beglinger
Robert J. O'Boyle

Study Completed on

October 4, 1990

Performing Laboratory

Eastman Kodak Company
Health and Environment Laboratories
Rochester, New York 14652-3617
U.S.A.

Laboratory Project ID

Accession No. 901878 HAEL No. 90-0016

HAEL No. 90-0016 Study No. EN-421-901878-2

EPA/TSCA Acute Fish (Rainbow Trout) Toxicity Test of Crotonaldehyde

In Accordance with the US EPA, 1985. Toxic Substances, Testing Requirements; Final Rule and Proposed Rule. Federal Register: 40 CFR Part 797.1400 Fish Acute Toxicity Test (Amended 1987); (as Amended in Testing Consent Order on Crotonaldehyde, 40 CFR Part 799)

STATEMENT OF GLP COMPLIANCE

To the best of my knowledge and belief, these procedures were conducted in compliance with all Good Laboratory Practice standards of 40 CFR Part 792, November 29, 1983 (revised August 17, 1989). No unforeseen circumstances were observed that might have affected the quality or integrity of these procedures.

Study Director: R. J. O'Boyle 10/4/90

Robert J. O'Boyle
Eco-Chem Testing Group
Environmental Sciences Section
Health and Environment Laboratories
Eastman Kodak Company
Rochester, NY 14652-3617

Date

Sponsor: Roderick D. Gerwe 9/28/90

Roderick D. Gerwe
Eastman Chemical Company
Kingsport, TN 37662

Date

STUDY TITLE

ACUTE AQUATIC EFFECTS OF CROTONALDEHYDE
ON THE RAINBOW TROUT, Oncorhynchus mykiss

ABSTRACT

The acute toxicity of the test article Crotonaldehyde (Accession No. 901878) to the rainbow trout (Oncorhynchus mykiss) was determined in two 96-hour, flow-through, aquatic effects studies. The initial test was not successful due to the malfunction of the analytical instrumentation. Thus, the dose-response data obtained in the initial study ("I") are strictly nominal and are presented in this report merely to confirm the results of the second study.

A static, preliminary bioassay was performed as part of methods development to establish the test article concentrations that would be appropriate for the two, subsequent, definitive studies. This report chiefly describes the results of the second, definitive study ("II"). Crotonaldehyde has appreciable aqueous solubility in water. The test article appeared to be completely dissolved at all of the exposure concentrations used in the definitive study. A total of 20 trout were exposed to each of five dose concentrations (nominally 0.12, 0.25, 0.5, 1, and 2 mg/L). Two replicates per dose were used in the test (10 animals/replicate). Replicate results were pooled.

The endpoint values for Study I are strictly nominal and rounded to a maximum of two significant figures. The 96-hour LC_{50} value for Study I for rainbow trout was calculated to be 0.71 mg/L for pooled Replicates A and B. The pooled, acute No Observed Effect Concentration (NOEC) value for Study I was determined to be 0.25 mg/L. The NOEC values for both Study I and II are based on both lethal and sublethal effects.

The 96-hour LC_{50} value for Study II (rounded to two significant figures) for rainbow trout was calculated from the analyzed doses to be 0.65 mg/L for pooled Replicates A and B. The pooled, acute NOEC value for Study II (rounded to two significant figures) for rainbow trout was determined to be 0.27 mg/L.

The results of this study indicate that an isolated or intermittent exposure to a concentration of Crotonaldehyde less than or equal to 0.27 mg/L is unlikely to have an adverse effect on rainbow trout (Oncorhynchus mykiss).

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TESTING FACILITY

Health and Environment Laboratories
Eastman Kodak Company
Rochester, NY 14652-3617
U.S.A.

SPONSOR

Eastman Chemical Company
Kingsport, Tennessee 37662
U.S.A.

DATE OF STUDY INITIATION March 23, 1990 (Definitive Protocol Signed)

DATES OF CHEMICAL EXPOSURE April 30 - May 4, 1990 (Definitive Study I)
June 25 - June 29, 1990 (Definitive Study II)

DATE OF STUDY COMPLETION October 4, 1990

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PURPOSE/OBJECTIVE

To allow for a more accurate and complete assessment of environmental impact, juvenile rainbow trout were exposed to five concentrations of the test article Crotonaldehyde (Accession No. 901878) in two 96-hour, flow-through, aquatic effects tests. The initial test was not successful due to the malfunction of the analytical instrumentation. Thus, the dose-response data obtained in the initial study ("I") are strictly nominal and are presented in this report merely to confirm the results of the second study. This report chiefly describes the results of the second, definitive study ("II").

The test article solutions were prepared through the direct addition of appropriate aliquots of a test article stock solution to the test vessels. The rainbow trout, Oncorhynchus mykiss, was chosen for this test based on its ecological importance as a representative freshwater species of fish which can be maintained within the laboratory. The objectives of the test were: (1) to determine if acute exposure to concentrations of the test article would affect fish adversely; and, (2) if appropriate, provide an estimate of the 96-hour LC_{50} value and the highest acute No Observed Effect Concentration (NOEC). In this test, the rainbow trout were observed for signs of stress, as well as mortality, when exposed to the test article. For the purpose of calculating or estimating a 96-hour LC_{50} value, death would serve as the requisite endpoint. For the purpose of calculating or estimating the highest acute NOEC, either mortality or signs of stress would serve as the requisite endpoint. The information from this test can be used to determine the likelihood of acute aquatic effects if Crotonaldehyde enters a freshwater environment.

TEST ARTICLE

Identity: Crotonaldehyde CAS Registry No.: 4170-30-3
Sample Reference Identification (SRID) No.: Lot No. 90-7
Sample Laboratory No. (HAEL No.): 90-0016

Physical/Chemical Properties:

Composition & Solubility: The test article is a clear liquid with appreciable aqueous solubility.

Purity: 99.5% initial (6.3% water) and 99.7% final (6.7% water); using GC/FID analysis (Attachments 4a, 4b, and 4c).

MATERIALS AND METHODS

Test System

Phylum - Chordata
Class - Osteichthyes
Order - Salmoniformes
Family - Salmonidae
Genus - *Oncorhynchus*
Species - *mykiss*

Source of Rainbow Trout

Juvenile rainbow trout were obtained from Mt. Lassen Trout Farm, Red Bluff, CA, and held in the Eco-Chem Testing Group animal colony room for at least two weeks prior to use. The fish used in Study I were designated MLTF Lot 4/90 and those in Study II were MLTF Lot 6/90.

Source of Dilution Water

The water used in this test was pumped from Lake Ontario by the Kodak Park Lake Station Water Treatment Facility into a large underground storage reservoir located near the Environmental Sciences Section testing facility (Building 306, Kodak Park). This water is subsequently pumped into the laboratory where it passes through a series of 3-micron (pore size) polypropylene filter tubes, then through a series of activated-powdered carbon filter tubes, and finally through another set of 3-micron polypropylene filter tubes. The filtered water stream next received 150 ppb of $Na_2S_2O_3$ via a chemical injection system. This treatment further reduces trace levels of residual chlorine. The filtered-treated water is subsequently tempered to 20 ± 2°C by passage through a heat-exchange unit. This filtered-treated-tempered water supply is distributed throughout the testing facility through stainless steel piping. Upon reaching the laboratory, the filtered-treated-tempered water cascades through a column degassing unit into an open, aeration basin for seasoning prior to use. The water is then further tempered to 12 ± 1°C by a submersible-coil refrigeration unit. The test diluent water is drawn from this source. Representative values for the hardness and the total alkalinity (both as $CaCO_3$) of the laboratory diluent water for the study period were 120 and 99 mg/L, respectively. Refer to Attachment 2, Summary of Chemical Characterization of Dilution Water.

Acclimation Procedure

All test organisms were acclimated to the diluent water prior to the test. The same filtered-treated-tempered water and compressed air used for all laboratory water/aeration processes during the test were supplied continuously to the stainless steel holding tanks. All aquatic organism populations used in the laboratory are maintained in this water for at least two weeks prior to testing.

Size and Number of Rainbow Trout Used at Each Concentration

Juvenile rainbow trout, as uniform in size as possible, were collected from the stainless steel holding tanks. Sequential randomization was accomplished by allocating to each vessel no more than 20% of any one set of test organisms at a time. Biological loading within test vessels was kept below 1.0 g wet weight per liter of test solution. The average wet weights for two randomly-chosen sets of trout were determined at test start (Study II: Replicate A = 0.61 g and Replicate B = 0.55 g). These rainbow trout were subsequently used as the control sets of test organisms. Mean lengths of these same trout at test end were determined to be 33.9 mm and 32.8 mm for Replicates A and B, respectively.

Preparation of the Test Solutions

A preliminary bioassay was performed (as part of methods development) with rainbow trout using Crotonaldehyde as the test article. The dose levels employed in the subsequent definitive studies were determined by these results. Test article solutions and dilution water controls were prepared in replicates of two for the definitive study.

The test article was introduced into replicate test vessels at nominal concentrations of 0.12, 0.25, 0.5, 1.0, and 2.0 mg/L using the following procedure: The laboratory diluent water was sparged with nitrogen for 24 hours prior to adding the test article. Vigorous mixing with a power stirrer was employed to achieve complete dissolution of 4.00 g of the test article in 200 L of diluent water. The test article container was sealed with a plastic bag, and argon was continuously injected to form a "blanket" over the aqueous stock solution. This stock solution continuously supplied the diluter throughout the study. The aqueous stock solution of the test article (@ 20 mg/L) was proportionally delivered to the appropriate test vessels throughout the study by the gas-driven, siphon type diluter. The 20 mg/L stock solution feeding

the diluter was renewed at time 48 hours. All test article dose concentrations and the diluent water controls were prepared in replicates of two. Each test vessel was spiked with an appropriate amount of the stock solution. Samples were removed for analysis at times "0" (prior to introducing the fish to the exposure solutions), 48 hours (aged solutions & fresh stock), and 96 hours (or test end per dose; i.e., complete mortality).

Apparatus and Test Conditions

This acute aquatic effects bioassay was performed in seamless glass 30.5 cm cuboidal Pyrex® chromatography jars containing 15 L of the exposure solution. The light/dark cycle of the lighting regime was 16 hours on and 8 hours off with a 20-minute transition period. The test temperature was maintained at 12 ± 1°C through the use of a refrigerated water bath.

Test Procedure

The procedures used in this test were based on accepted methodologies.[1-6] Ten test organisms were placed into each of the replicate test article solution and diluent water control vessels. Observations for signs of mortality and stress, in comparison to the controls, were made during the test at times 0, 24, 48, 72, and 96 hours. Temperature, dissolved oxygen, and pH were measured at times 0, 24, 48, 72, and 96 hours.

Analytical Procedures

A 50 mL sample of each replicate test article solution and diluent water control was drawn from approximately mid-depth of each test vessel at test times 0 and 96 hours (or at the end of exposure per dose). These samples were submitted at those times to the Chemicals Quality Services Division (CQSD) for test article concentration analysis by GC/ECD (refer to Attachment 7).

LC_{50} Calculation

A computer program developed by C.E. Stephan[7] and ASTM[8] was used to compute point and interval (i.e., confidence interval) estimates of the LC_{50}. The program requires entering the concentration of the test substance (either nominal or measured), the number of organisms exposed, and the number of organisms that died. Data for the control vessels are not entered as the program does not have the capability of adjusting for control mortality. The program computes point and interval estimates of the LC_{50} value using three approaches (refer to Attachment 8 for descriptions).

QUALITY ASSURANCE MONITORING

See the attached reports of Quality Assurance Monitoring (Attachment 10).

Good Laboratory Practice Statement

This study was conducted according to: Good Laboratory Practice Regulations for Nonclinical Laboratory Studies as promulgated by the Food and Drug Administration; 21 CFR Part 58, December 22, 1978 (revised September 4, 1987); Environmental Protection Agency Good Laboratory Practice Standards 40 CFR Part 792, November 29, 1983 (revised August 17, 1989); and Organization of Economic Cooperation and Development Principles of Good Laboratory Practice specified in Annex 2 of the OECD Guidelines for Testing of Chemicals [C(81) 30(Final)] as required by Council Directive 89/569/EEC (October 28, 1989).

DATA STORAGE AND RECORDS RETENTION

All raw data and summaries of data will be retained for at least ten years in the Health and Environment Laboratories Archive currently located in Bldg. 320 of the Eastman Kodak Company, Kodak Park, Rochester, NY, 14652-3615.

WATER QUALITY DURING TEST

During Study I the temperature in all test vessels remained at 12°C and pH value extremes were 8.1 and 8.4. Dissolved oxygen reading extremes were 9.1 and 10.5 mg/L. Averages for the dissolved organic carbon readings taken during Study I were 2.6, 2.2, and 2.4 mg/L. (Due to an oversight by the analytical area, DOC readings are not available for Study II. A DOC mean value for 1990 using 11 data points is 2.3 mg/L.) The temperature in all test vessels remained at 12°C during Study II. The pH value extremes were 8.2 and 8.5. Dissolved oxygen reading extremes were 10.2 and 10.8 mg/L. These values are considered to be acceptable for the organisms used in this test,[1-6] and are not likely to have caused any adverse effects.

TEST RESULTS

A summary of the analyses of the test article exposure solutions and diluent water controls for definitive Study II is presented in Attachment 7. The average of each of the analyses in duplicate at test times 0, 48, and 96 hours (or at the end of the exposure per dose) was derived, as were the consequent mean 96-hour exposure concentrations for each replicate dose series. These values are summarized in Table 2. (The initial test was not successful due to the malfunction of the analytical instrumentation. Thus,

the dose-response data obtained in Study I are strictly nominal.)

Raw data for biological effects and physical/chemical parameters are provided in Attachment 6 and summarized in Table 1 (requisite endpoint mortality). The output from the LC_{50} calculator program is provided in Attachment 8 and summarized in Table 3.

TEST VALIDITY

The following criteria for a valid test were met during the study.

A. The control mortality was not greater than 10% when adverse effects were noted.

B. The concentration of the test article did not fall below 80% of the initial concentration.

C. The dissolved oxygen level did not fall below 60% of the initial oxygen level when adverse effects were observed in the highest dose.

D. No abnormal occurrences (i.e., laboratory accidents) that might have influenced the outcome of the test were noted.

PROTOCOL DEVIATIONS

A protocol deviation was not necessary.

DISCUSSION AND INTERPRETATION

Static, preliminary bioassays with Crotonaldehyde using rainbow trout (Oncorhynchus mykiss) were performed as part of methods development (refer to Attachment 9). The dose levels used in the subsequent definitive studies were determined from the results of these bioassays. The methods development data provided a nominal, acute No Observed Effect Concentration (NOEC) of 0.1 mg/L.

The acute toxicity of the test article Crotonaldehyde (90-0016) to the rainbow trout was determined in two 96-hour, flow-through, aquatic effects studies. The initial test was not successful due to the malfunction of the analytical instrumentation. Thus, the dose-response data obtained in the initial study ("I") are strictly nominal and are presented in this report merely to confirm the results of the second study ("II").

Crotonaldehyde in Study I caused complete mortality in the two highest exposure concentrations (nominally 1.0 and 2.0 mg/L) in both replicate dose series. All trout in the remaining doses (nominally 0.12, 0.25, and 0.5 mg/L)

were normal at test end. The trout at the 0.5 mg/L (nominal) concentration were somewhat darker in color than the controls, but were exhibiting normal behavior. The 48-hour, No Observed Effect Concentration (NOEC) was consequently lowered to 0.25 mg/L (nominal) since color change may be indicative of stress in rainbow trout. No mortality or adverse effects were observed in the dilution water controls. Refer to Table 1a for a summary of the biological effects noted during Study I.

Crotonaldehyde was lethal at the top doses of 1 and 2 mg/L (nominal) used in the subsequent, flow-through, definitive, 96-hour Study II. At the nominal concentration of 0.5 mg/L five trout died (Replicate A: 2 normal, 5 activity depressed, 3 dead; Replicate B: 6 normal, 2 activity depressed, 2 dead.) It should be noted that the trout at the 0.5 mg/L (nominal) dose were also somewhat darker in color than the controls (as in Study I). All of the trout exposed in both replicates of the 0.25 and 0.12 mg/L (nominal) concentrations appeared to be normal at test end, as were the dilution water controls. Refer to Table 1b for a summary of the biological effects noted for Study II.

Crotonaldehyde is described as being appreciably soluble in water (Attachment 1). The test article appeared to be completely dissolved at the stock solution concentration (nominally 20 mg/L), and in all the resulting doses, under the conditions used in this study (i.e., 12°C laboratory diluent water). All exposure solutions were sampled for analysis at test start, time 48, and time 96 hours (or test end per exposure). Refer to Attachment 7 (CQSD report) and to Tables 1 and 2 for the analyzed test article concentrations for each exposure solution.

The analyzed concentrations for each replicate test article solution and control obtained throughout Study II were averaged to produce a mean exposure value per dose. The mean exposure concentrations were pooled (i.e., combined replicates A and B by averaging) and used in all endpoint calculations for this study. The dose-response curves (at 48 and 96 hours) for Studies I and II are plotted in Figures 1a & b and 2a & b, respectively.

The analytical data contained in Attachment 7 and summarized in Table 1 indicate that the measured concentrations in the middle dose replicates of Study II (nominally 0.5 mg/L) were anomalously high (> 0.9 mg/L) at test end.

These elevated values at time 96 hours are inexplicable since the diluter calibration was checked and documented daily, and it is not credible that the exposure apparatus could be responsible for raising the concentration so much over the nominal dose level. CQSD personnel who performed the analyses were also not able to suggest a plausible explanation. Consequently, these anomalous values were discounted and dropped as outliers from the mean exposure concentration calculations. The 48-hour mean for the middle dose was substituted in the accepted study endpoint calculations for the mean that would have been derived if the (>) 0.9 mg/L had been included. Attachment 8 includes the LC_{50} calculations from the dose-response data using both possible mean exposure values for the middle dose, and the difference is not significant. The LC_{50} is 0.1 mg/L higher if the discounted analytical results from time 96 hours are employed in the endpoint calculations. The lower LC_{50} value that was calculated by using the 48-hour mean for the middle dose would be the more conservative approach to assessing environmental risk, so that lower LC_{50} alone will be considered the acceptable value obtained from the study data.

The LC_{50} Calculator Program defaults to the most appropriate statistical method depending on the distribution of the dose response within the data set. Refer to Attachment 8 for method descriptions and the test calculations. (The Study II dose-response data were entered both with and without the anomalous 0.5 mg/L concentrations at time 96 hours, but the values determined <u>without</u> using those suspect dose levels were the ones accepted as being valid.) Non-linear Interpolation was used to calculate the 48- and 96-hour LC_{50} values for Study I (nominal doses) and the 96-hour values for Study II (measured doses). The associated 95% confidence intervals were generated by the Binomial Method. The 48-hour LC_{50} value (and 95% confidence interval) for Study II were calculated by the Probit Method.

The pooled, 48-hour LC_{50} values (rounded to two significant figures) for the rainbow trout used in Studies I and II were 0.89 mg/L and 1.1 mg/L, repsectively. The acute No Observed Effect Concentration (NOEC) values at 48 hours were 0.25 mg/L for Study I and 0.28 mg/L for Study II. (<u>Note</u>: The customary study endpoints for these test organisms are 96-hour values, as listed below.) The NOEC values for both Study I and II are based on both lethal and sublethal effects.

The endpoint values for Study I are strictly nominal and rounded to a maximum of two significant figures. The 96-hour LC_{50} value for Study I for rainbow trout was calculated to be 0.71 mg/L for pooled Replicates A and B. The pooled, acute (96-hour) NOEC value for Study I was determined to be 0.25 mg/L.

The 96-hour LC_{50} value for Study II (rounded to two significant figures) for rainbow trout was calculated from the analyzed doses to be 0.65 mg/L for pooled Replicates A and B. The pooled, acute (96-hour) NOEC value for Study II (rounded to two significant figures) for rainbow trout was determined to be 0.27 mg/L.

CONCLUSIONS

The results of this study indicate that an isolated or intermittent exposure to a concentration of Crotonaldehyde less than or equal to 0.27 mg/L is unlikely to have an adverse effect on rainbow trout (Oncorhynchus mykiss).

REFERENCES

1. American Society for Testing and Materials (ASTM). 1988. Annual Book of ASTM Standards. Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians, ASTM Standard E 729-88; American Society for Testing and Materials, Philadelphia, PA. Volume 11.04, pp. 304-323.

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TABLE 1a. *Oncorhynchus mykiss* Survival during Study I

Test Vessel	Aim Conc. (mg/L)	Test Conc. (mg/L)*	Survival at 24 hr	48 hr	72 hr	96 hr
DWC A	0	*	10	10	10	10
DWC B	0	*	10	10	10	10
Test A	0.12	*	10	10	10	10
Test B	0.12	*	10	10	10	10
Test A	0.25	*	10	10	10	10
Test B	0.25	*	10	10	10	10
Test A	0.5	*	10	10	10	10
Test B	0.5	*	10	10	10	10
Test A	1.0	*	10	4	0	0
Test B	1.0	*	10	3	0	0
Test A	2.0	*	0	0	0	0
Test B	2.0	*	0	0	0	0

* The initial test was not successful due to the malfunction of the analytical instrumentation. Thus, the dose-response data obtained in Study I are strictly nominal and are presented in this report merely to confirm the results of the second study. (Refer to Attachment 7 for an analytical methods description and the raw data from the analyses of Study II.)

DWC = Diluent Water Control

TABLE 1b. *Oncorhynchus mykiss* Survival during Study II

Test Vessel	Aim Conc. (mg/L)	Test Conc. Pooled, mg/L (48-)/96-hour *	Survival at 24 hr	48 hr	72 hr	96 hr
DWC A	0	ND	10	10	10	10
DWC B	0		10	10	10	10
Test A	0.12	(0.14)/0.13	10	10	10	10
Test B	0.12		10	10	10	10
Test A	0.25	(0.28)/0.27	10	10	10	10
Test B	0.25		10	10	10	10
Test A	0.5	(0.55)/0.55 †	10	10	9	7
Test B	0.5		10	9	9	8
Test A	1.0	(1.1)/1.0 [72-hour mean]	10	6	0	0
Test B	1.0		9	5	0	0
Test A	2.0	(2.1)/2.1 [24-hour mean]	0	0	0	0
Test B	2.0		0	0	0	0

* Test article concentrations determined by a GC/ECD analytical procedure (refer to Attachment 7 for an analytical methods description and the raw data from the analyses). The measured values shown are the averaged analyzed concentrations in replicate exposure solution samples taken at test times 0, 48, and 96 hours. The 48- and 96-hour mean exposure values are rounded to a maximum of two significant figures.

DWC = Diluent Water Control

ND = None Detected (at the method lower limit of detection of 0.005 mg/L)

† = The 48-hour value was substituted because the 96-hour analyzed values were discounted due to anomalously high concentrations. (Refer to the section of this report entitled Discussion and Interpretation, beginning on p. 11.)

TABLE 2. Summary of Analytical Results for Study II

Nominal, mg/L		Measured Concentrations, as mg/L 0 hours	48 hours	96 hours	(48-)/96-hour Mean, mg/L
DWC	(A)	ND	ND	ND	ND
DWC	(B)	ND	ND	ND	ND
0.12	(A)	0.142	0.137	0.113	(0.140)/0.131
0.12	(B)	0.136	0.141	0.127	(0.138)/0.135
0.25	(A)	0.296	0.266	0.246	(0.281)/0.269
0.25	(B)	0.298	0.263	0.248	(0.280)/0.270
0.5	(A)	0.578	0.550	> 0.9 †	(0.564)/0.676
0.5	(B)	0.544	0.531	> 0.9 †	(0.538)/0.658
1.0	(A)	1.10	0.975	0.954 [72 hrs]	(1.038)/1.010 *
1.0	(B)	1.08	1.047	0.921 [72 hrs]	(1.064)/1.016 *
2.0	(A)	2.20	2.16 [24 hrs]	-----	(2.180)/2.180 **
2.0	(B)	2.06	2.05 [24 hrs]	-----	(2.055)/2.055 **

Note: Test article concentrations determined by using a GC/ECD analytical procedure (refer to Attachment 7 for a method description and the raw data from the analyses). The measured values shown are the averages of the analyzed concentrations in the pooled replicate exposure solution samples taken at test times 0, 48, and 96 hours. These mean exposure values per replicate were pooled, rounded to a maximum of two significant figures, and were used to calculate all test endpoints (see Table 3).

DWC = Diluent Water Control

ND = None Detected (at the method lower limit of detection of 0.005 mg/L).

† = The peak heights for the 0.5 mg/L samples were greater than the heights for the highest standard (and only a greater than value could be reported).

* = Mean value for 72 hours. Substituted for 96-hour mean since complete trout mortality was noted at 72 hours.

** = Mean value for 24 hours. Substituted for 48- and 96-hour mean since complete trout mortality was noted at 24 hours.

TABLE 3. Calculated LC_{50} and NOEC Values for *Oncorhynchus mykiss*

Study I

Endpoint values were calculated after pooling the nominal dose-response data from Replicates A & B, and are rounded to a maximum of two significant figures.

Pooled Replicate Dose Series	LC_{50} Value (mg/L)* (95% CI)	Acute NOEC Value (mg/L)
A and B (48-hour)	0.89 (0.5, 2)	0.5
A and B (96-hour)	0.71 (0.5, 1)	0.25

Study II

Endpoint values were calculated after pooling the analyzed dose-response data from Replicates A & B, and are rounded to a maximum of two significant figures.

Pooled Replicate Dose Series	LC_{50} Value (mg/L) (95% CI)	Acute NOEC Value (mg/L)
A and B (48-hour)	1.1 † (0.90, 1.3)	0.28
A and B (96-hour)	0.65 * (0.55, 1.0)	0.27

* Results calculated using Non-linear Interpolation. The associated 95% Confidence Intervals were determined by the Binomial Method. See Attachment 8 for a description of these methods.

† Results calculated using the Probit Method. See Attachment 8 for a description of these methods.

FIGURE 1a. Dose-Response Curve for Study I Trout at Time 48 Hours

Pooled Replicates A & B

Nominal Values

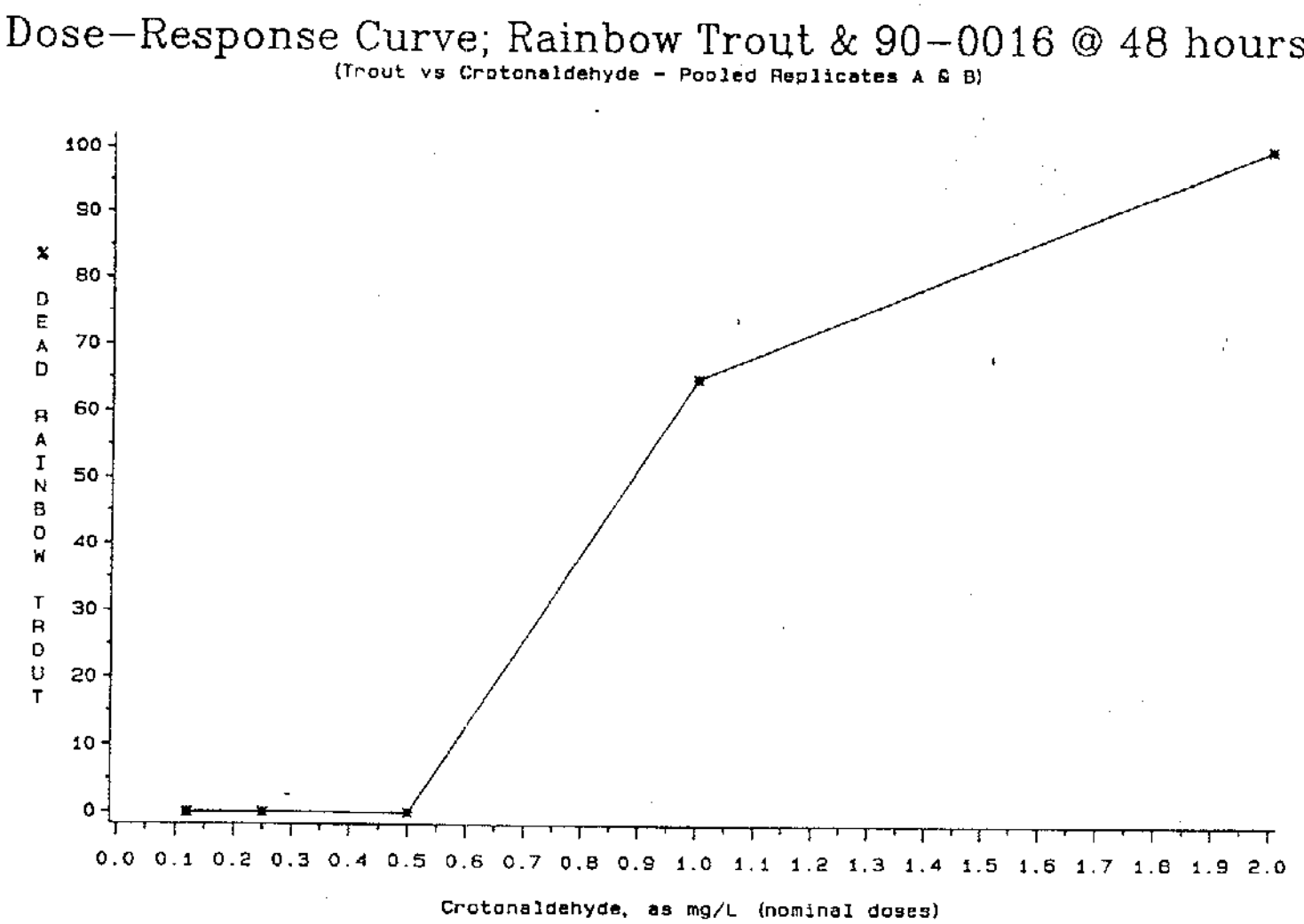


FIGURE 1b. Dose-Response Curve for Study I Trout at Time 96 Hours

Pooled Replicates A & B

Nominal Values

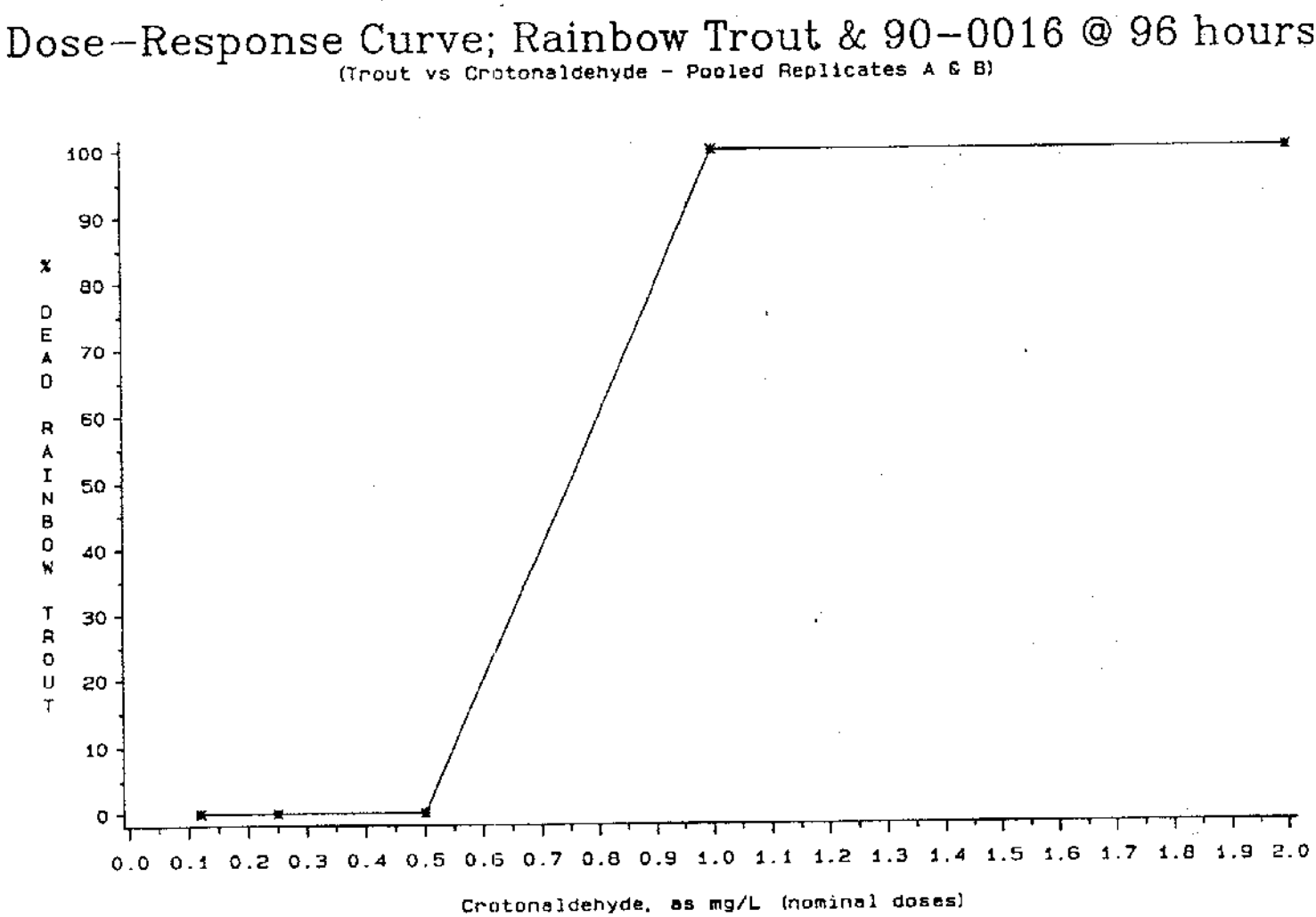


FIGURE 2a. Dose-Response Curve for Study II Trout at Time 48 Hours

Pooled Replicates A & B

Analyzed Values

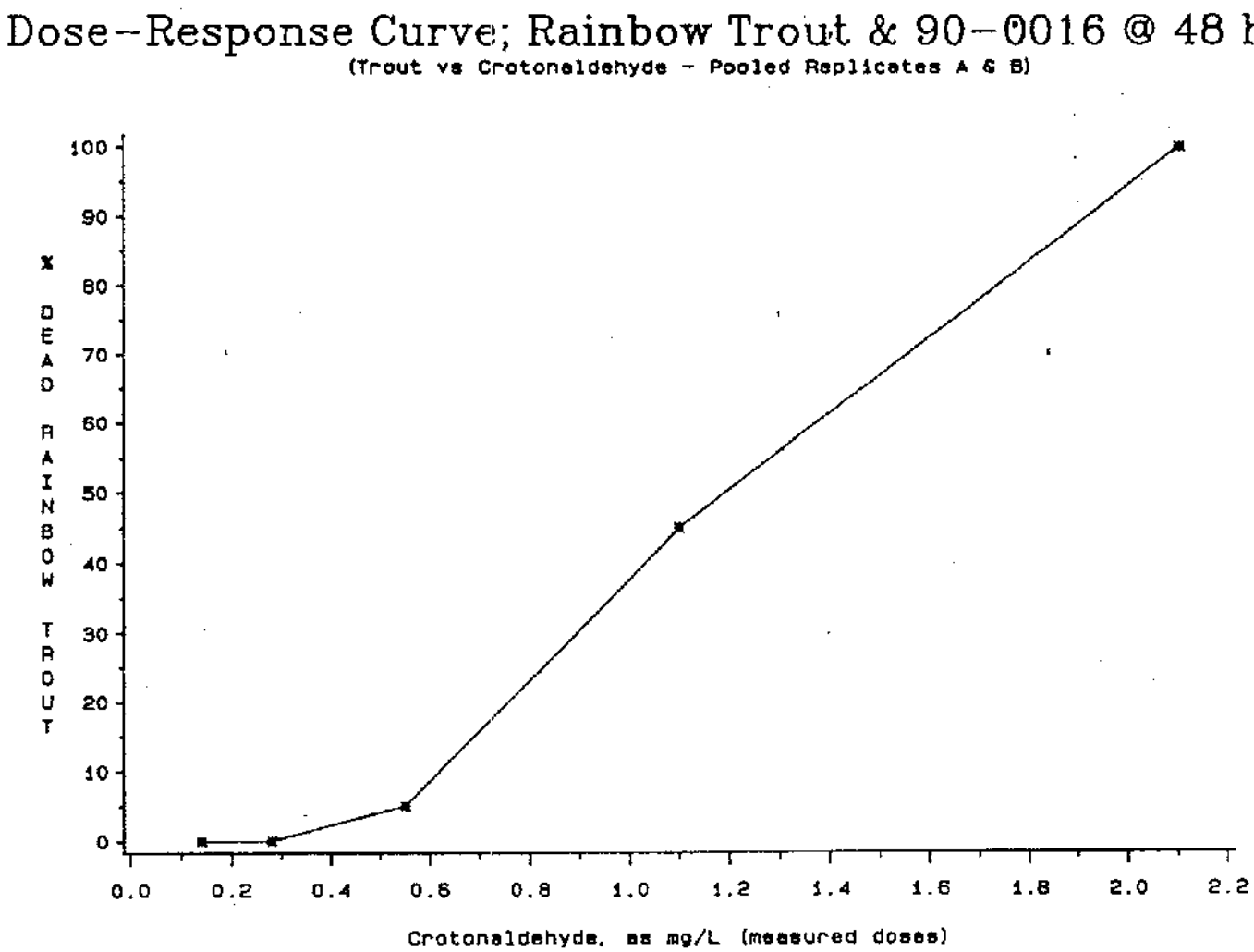
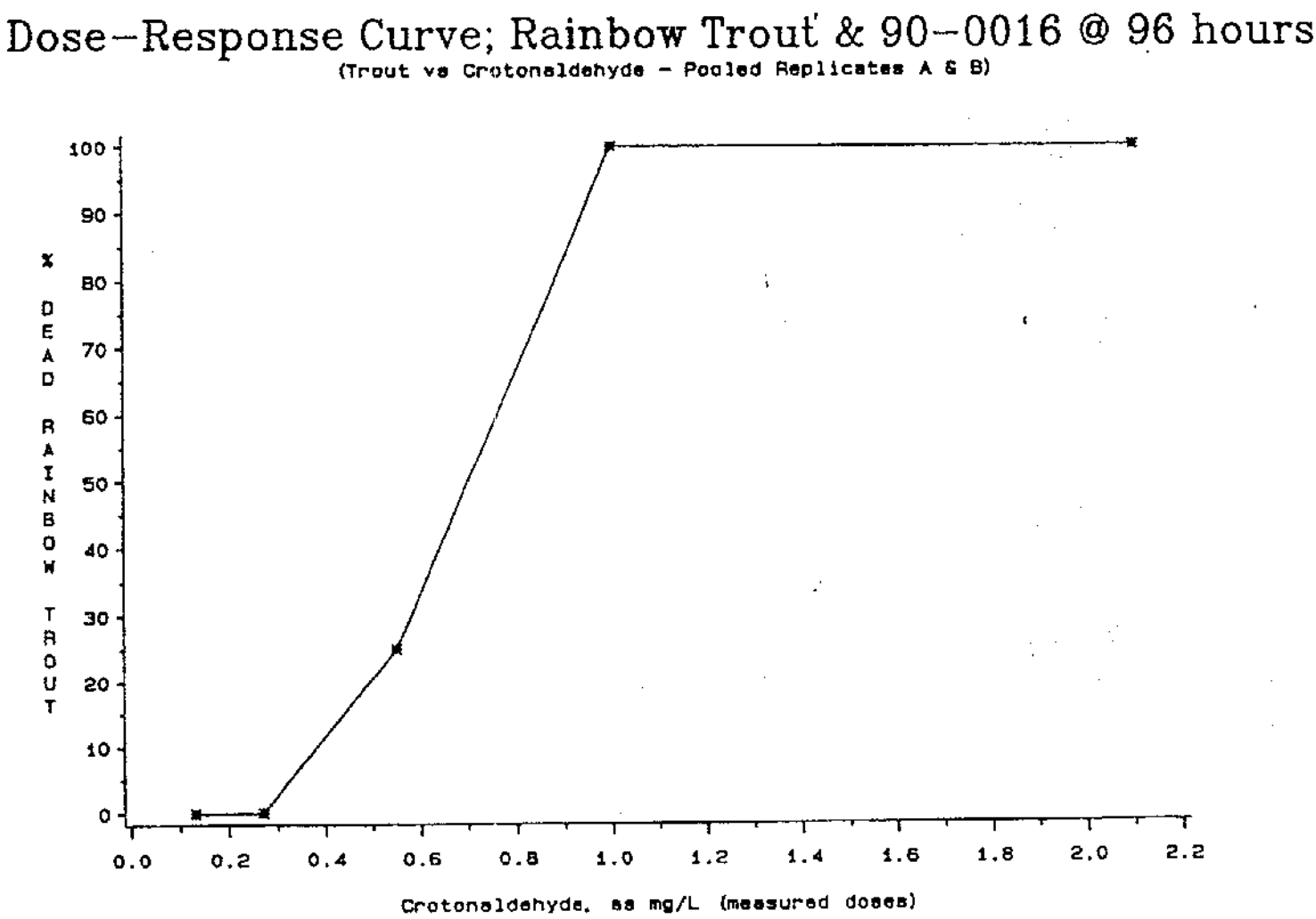


FIGURE 2b. Dose-Response Curve for Study II Trout at Time 96 Hours

Pooled Replicates A & B

Analyzed Values



ATTACHMENT 1. Product Specification Sheet (Supplied by Sponsor)

MATERIAL SAFETY DATA SHEET

EASTMAN CHEMICAL PRODUCTS, INC.
EASTMAN KODAK COMPANY
Kingsport, Tennessee 37662

For Health Hazard Information, Call: (615) 229-6094

For Other Information, Call Your Eastman Representative

Eastman Operator: (615) 229-2000 Date of Preparation 08-24-87

SECTION I. IDENTIFICATION

-- Name:

Crotonaldehyde

-- Synonyms: PM 161; 2-Butenal.
-- Formula: C_4H_6O
-- Molecular Weight: 70.09

SECTION II. PRODUCT AND COMPONENT HAZARD DATA

A. COMPONENTS:	Approx Weight %	CAS Reg No	Eastman Kodak No
Crotonaldehyde*	92	4170-30-3	901878
Water	8		

See Section VI-A for information on exposure limits.

B. PRECAUTIONARY LABEL STATEMENTS:

DANGER! FLAMMABLE
MAY BE FATAL IF INHALED OR ABSORBED THROUGH THE SKIN
CAUSES SKIN AND EYE BURNS
HARMFUL IF SWALLOWED
VAPOR EXTREMELY IRRITATING
MAY FORM EXPLOSIVE PEROXIDES
MAY POLYMERIZE

SECTION III. PHYSICAL DATA (1)

-- Appearance and Odor: Clear, colorless liquid; pungent, suffocating odor; lachrymator.
-- Boiling Point: 84°C (183°F).
-- Specific Gravity (H_2O = 1): 0.871.
-- Vapor Pressure: 32 mm Hg at 20°C.
-- Percent Volatile by Volume: Approx 1.0.
-- Vapor Density (Air = 1): 2.41.
-- Evaporation Rate (ethyl ether = 1): 0.2.
-- Solubility in Water: Appreciable.

ATTACHMENT 2. Summary of Chemical Characterization of Dilution Water

CHEMICAL CHARACTERISTICS OF BUILDING 306 DILUTION WATER — STATISTICAL ANALYSIS

AGGREGATE HISTORICAL DATA FROM 1981
(All values are reported as mg/L unless otherwise stated)

PARAMETERS *	MOST RECENT VALUES **	MEAN	S.D.	LOW	HIGH	N
1 pH (determined @ 20 C)	8.4	7.96	0.24	7.50	8.40	30
2 Conductivity, as umhos/cm	310	313.59	14.75	268.00	357.00	39
3 Total Hardness (as CaCO3)	120	129.21	5.79	110.00	140.00	39
4 Total Organic Carbon	2.3	2.70	2.12	0.90	10.00	19
5 Total Residual Cl2, as ug/L	<(3)	3.46	2.92	1.50	10.60	16
6 Acidity (as CaCO3)	0	0.00	0.00	0.00	0.00	6
7 Alkalinity (as CaCO3)	99	93.08	5.47	84.00	110.00	39
8 Non-carbonate Hardness (as CaCO3)	21	36.15	7.74	14.00	52.00	39
9 Total Dissolved Solids	110	178.51	23.26	110.00	240.00	39
10 Turbidity, as NTU	0.4	0.13	0.09	0.10	0.40	10
11 Total Suspended Solids	ND(5)	2.50	0.00	2.50	2.50	6
12 Total NH3 (as N)	ND(0.014)	0.05	0.03	0.00	0.14	38
13 Organic Nitrogen	0.2	0.18	0.09	0.07	0.38	18
14 NO3- (as N)	0.37	0.27	0.11	0.00	0.53	39
15 NO2- (as N)	ND(0.01)	0.01	0.004	0.001	0.02	39
16 Chloride by IC (Cl-)	24	25.21	1.70	21.00	29.00	39
17 Fluoride by ISE (F-)	<(0.5)	0.33	0.14	0.10	0.50	19
18 Sulfate by IC (SO4=)	35	29.77	4.65	23.00	47.00	39
19 Total Cyanide (CN-)	ND(0.003)	0.002	0.001	0.001	0.007	19
20 Total Phosphorus (P)	ND(0.02)	0.014	0.007	0.000	0.030	19
21 Molybdenum-Reactive Dissolved SiO2	0.66	0.40	0.17	0.10	0.70	20
Total Metals by ICP/AES:						
22 Aluminum	0.16	0.23	0.09	0.05	0.50	39
23 Barium	0.02	0.02	0.003	0.01	0.03	39
24 Boron	<(0.004)	0.01	0.004	.00	0.01	3
25 Cadmium	<(0.003)	0.004	0.003	0.001	0.01	39
26 Chromium	0.007	0.01	0.01	0.002	0.04	39
27 Cobalt	<(0.003)	0.01	0.01	0.002	0.02	39
28 Copper	<(0.003)	0.01	0.02	0.000	0.14	39
29 Iron	0.02	0.01	0.01	0.001	0.05	39
30 Lead	<(0.01)	0.10	0.12	.00	0.35	39
31 Manganese	0.008	0.01	0.003	0.001	0.02	39
32 Nickel	<(0.007)	0.02	0.02	0.004	0.04	39
33 Silver	<(0.003)	0.01	0.005	.00	0.02	36
34 Zinc	0.006	0.01	0.02	0.002	0.09	39
35 Arsenic by Hydride Generation AAS, as ug/L	ND(6)	2.44	1.67	0.60	5.00	29
36 Mercury by Hydride Generation AAS, as ug/L	ND(0.2)	0.1	.00	0.1	0.1	7

ND( ) = Not Detected at (Method Detection Limit)
<( ) = Less than (Limit of Quantification)
NT = Not Tested for this Parameter

* Note: Parameters reported as ND( ) or <( ) are entered for statistical analysis by halving the parenthetical value (in accordance with the Central Limit Theory for Averages).

** Sampling Date: January 30, 1990

ATTACHMENT 3. Chemical Distribution Record

HAEL Chemical Distribution Record

HAEL Lab. No.	90-0016	IDX Accession No.	~~901876~~	SRID No.	Lot No. 90-7
Chemical Name	Crotonaldehyde (901878 RH 2/8/90) (error)				
Source of Test Substance	~~Texas Eastman Co.~~ ① Tennessee Eastman Co.			Sponsor	TEC/Coord. Wade Francisco (K-3152) JWG 2/7/90
Containerial Description	1 large glass amber bottle				
Date Test Substance Received	2-5-90	Date Test Substance Processed	2-8-90	By: HRH	Expiration Date 2-8-95
Date Test Substance Received from C&RI (for testing areas only)	2/8/90 JWG				
Physical State (circle one)	Liquid Solid Other	Appearance	clear		
Impurities with %	N/A				
Storage Conditions	~~Room Temperature~~ Refrigerate, and cover with nitrogen or Argon after each use ①				
Weight or Volume (circle one) of Bottle, Cap, and Contents	4348.5g	Weight or Volume (circle one) of Test Substance Sent	3121.2g		
Balance / Make	Mettler	Model	PE6000	KP ID No.	1920916

① Entry error, JWG, 2/9/90

Initials	Date	Area	Purpose	Scale Make, Model, KP ID No.	Initial Bottle Cap & Contents Wt. or Vol.	Amount Removed Wt. or Vol.	Amount Remaining Bottle/Cap/Contents Wt. or Vol.
[illegible]	02/09/90	B-306 ECT-HAEL	Confirmation Log-in Weighing	Mettler PE 4400 (#2) ID = 255950 #589461	4348.2g	0.0g	4348.2g
	2-12-90	B-306	Algal Methods	"	4347.9g	6.8g	4341.1g
O'B.	3/13/90	B-306	Sample to CQSD	" O'B.	4341.2g	29.2g	4312.0g
mm	3/19/90	B-306	Algal Range Finding Test	"	4312.5g	1.2g	4311.3g
O'B.	3/22/90	B-306	AAE Meth. Des.	" O'B.	4310.3g	27.6g	4282.7g
mm	4-2-90	B-306	Algal Definitive Test	"	4283.0g	0.8g used 0.1g	4282.2g
O'B.	4/18/90	B-306	AAE Dose Prep (4/23/90)	" O'B.	4280.8g ✓✓	20.7g (20.00g used)	4259.9g
O'B.	4/24/90	B-306	AAE Renewal (4/25/90)	" O'B.	4260.1g ✓✓	20.4g (20.00g used)	4239.7g
O'B.	4/27/90	B-306	Om Dose Prep (4/30/90)	" O'B.	4240.0g ✓✓	4.2g (4.00g used)	4235.8g
O'B.	5/1/90	B-306	Om Dose Renewal (5/2/90)	" O'B.	4235.5g ✓✓	4.6g (4.00g used)	4230.9g
O'B.	5/14/90	B-306	Purity/Stability (H2O)	" O'B.	4230.9g ✓✓	30.9g (to CQSD)	4200.0g
mm	6-21-90	B-306	Methods Closed Bottle	"	4200.0g	removed 1.1g (used 0.1g)	4198.9g
O'B.	6/22/90	B-306	Om Dose Rep (6/25/90)	" O'B.	4198.0g ✓✓	4.7g (used 4.00g)	4193.3g
O'B.	6/26/90	B-306	Om Dose Renewal (6/27/90)	" O'B.	4193.1g ✓✓	4.2g (used 4.00g)	4188.9g
O'B.	6/26/90	B-306	Purity Assay/CQSD	" O'B.	4188.9g	31.8g	4157.1g
mm	7-26-90	B-306	Closed Bottle Test	" mm	4156.8g	1.6g	4165.2g

Chem Dist Form 2/89 plm

ATTACHMENT 4a. Initial Purity Report

CHEMICALS QUALITY SERVICES REPORT FOR: 8306

STUDY DIRECTOR: Robert T. Ambrose

REQUESTED BY: Rob O'Boyle

CQS JOB # : 3H6V50!

PHASE: Purity Determination

REQUEST # : 142470
DATE RECEIVED: 05/14/90
DATE ANALYZED: 05/18/90
DATE REPORTED: 05/18/90
AUTHOR: Beth Isaacs

HAEL#: 90-0016
ACC.#: 901878
SAMPLE REF.#: Lot 90-7

FOR ADDITIONAL INFORMATION OR FURTHER WORK CONTACT: Robert Ambrose X25925
Beth Isaacs X85035

RESULTS: The purity of the test chemical was determined by a combination of a water analysis by Karl Fisher titration and a gas chromatographic analysis with flame ionization detection. The results are as follows:

Water (%)	Area % (GC/FID)	
6.3	Run 1	99.7
	Run 2	99.1
	Run 3	99.7
	Mean	99.5
	Std Dev	0.3464

Scientist Beth Isaacs (signature) Date 6/7/90

Reviewed by Robert T Ambrose (signature) Date 6/7/90

ATTACHMENT 4b. Final Purity Report

CHEMICALS QUALITY SERVICES REPORT FOR: 8306

STUDY DIRECTOR: Rob O'Boyle

REQUESTED BY: Rob O'Boyle

CQS JOB # : 3PC1101

PHASE: Purity Determination

REQUEST # : 142464
DATE RECEIVED: 06/19/90
DATE ANALYZED: 07/24/90
DATE REPORTED: 07/24/90
AUTHOR: Beth Isaacs

HAEL#: 90-0016
ACC.#: 901878
SAMPLE REF.#: Lot 90-7

FOR ADDITIONAL INFORMATION OR FURTHER WORK CONTACT: Robert Ambrose X25925
Beth Isaacs X85035

TESTING FACILITY
Chemicals Quality Services Division, Bldg-320
Eastman Kodak Company
Rochester, New York 14652
U.S.A.

DATA STORAGE AND RECORD RETENTION
All raw data and summaries of data will be retained for ten years by the Environmental Analytical Services, Chemicals Quality Services Division in Building 320 of Eastman Kodak Company, Kodak Park, Rochester, New York 14650.

RESULTS: The purity of the test chemical was determined by a combination of a water analysis by Karl Fisher titration and a gas chromatographic analysis with flame ionization detection. The results are as follows:

Water (%)	Area % (GC/FID)	
6.7	Run 1	99.7
	Run 2	99.7
	Run 3	99.7
	Mean	99.7
	Std Dev	0.0000

Scientist Beth Isaacs (signature) Date 7-24-90

Reviewed by Robert T Ambrose (signature) Date 9/13/90

ATTACHMENT 4c. Instrumental Conditions for Purity Analysis

Instrumental Conditions for GC Analysis

Instrument:	Hewlett Packard 5890
Column:	DB-WAX; 30m; wide bore (0.25 um film thickness)
Column Pressure:	7 psi
Carrier Gas:	Helium
Initial Temp:	50 °C
Initial Time:	4 min.
Rate:	10 °C/min.
Final Temp:	250 °C
Final Time:	7 min.
Injection Port:	250 °C
Injection Type:	Split
Injection Vol:	1 uL
Detector:	FID
Detector Temp:	250 °C
Solvent:	n-Propanol
Split Flow:	120 cc/min.

ATTACHMENT 5a. Protocol for Study I (Excerpts)

ENVIRONMENTAL SCIENCES SECTION
ENVIRONMENTAL TECHNICAL SERVICES
HEALTH AND ENVIRONMENT LABORATORIES

Protocol No. EN-421-901878-1
Revision No.:
Date: 7-25-88

Protocol: Determination of the Acute Aquatic Effects of Test Article on the Rainbow Trout, *Oncorhynchus mykiss*

In Accordance with the US EPA, 1985. Toxic Substances, Testing Requirements; Final Rule and Proposed Rule. Federal Register: 40 CFR Part 797.1400 Fish Acute Toxicity Test; (Amended 1987)

Test Article: Crotonaldehyde

Accession No. 901878 HAEL No. 90-0016

Approved by: R. J. O'Boyle 3/23/'90
Study Director Date

Approved by: R. D. Dowe 4/10/90
Sponsor (ECD) Date

Note: The attached protocol will be performed in compliance with the Standard Operating Procedure (SOP) No. EN-4035.

ATTACHMENT 5a. Protocol for Study I (Excerpts)

Health and Environment Laboratories Management and
Quality Assurance Unit Approvals

Approved: Joseph W. Gorsuch — 3/25/90

Joseph W. Gorsuch
Project Manager
Environmental Sciences Section

Date

Approved: M. Susan James — 3/27/90

Quality Assurance Unit

Date

Approved: John R. Owen — 3/26/90

John R. Owen
Unit Director
Environmental Sciences Section

Date

ATTACHMENT 5a. Protocol for Study I (Excerpts)

Protocol: Determination of the Acute Aquatic Effects of Test Article on the Rainbow Trout, *Oncorhynchus mykiss*

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I. Test Administration

A. Testing Facility
Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652-3617
U.S.A.

B. Sponsor
Eastman Chemical Division
Kingsport, Tennessee 37662
U.S.A.

C. Dates of Experiment
Start Date: 4/30/90
Test Article Exposure Completion Date: 5/4/90
Estimated Study Completion Date: 6/1/90

ATTACHMENT 5a. Protocol for Study I (Excerpts)

D. Test Type
1. _____ Range-Finding/ X Definitive
2. 2 Replicates (10 animals per replicate)
3. _____ Static/ X Dynamic/; 70 mL/Min (At least 6 test vessel volumes per day; a proportionating diluter system will be used.)
4. 15 L/Test Vessel Vol.
5. X Fluorescent/_____ Xenon-Arc Illumination (Sixteen-Hour Photoperiod with a 20-Minute Transition Period)
6. (X) Aeration (May Be Used at ≈ 0.05 L/Minute, if Necessary)
7. _____ Nominal/ X Measured Test Article Concentrations @ 0.12, 0.25, 0.5, 1, and 2 mg/L; 20 mg/L stock solution feeding diluter will be renewed at time 48 hours. Samples will be removed for analysis @ times "0", 48 (aged solutions & fresh stock), and 96 hours (or test end per dose; i.e., complete mortality).

II. Test and Control Substance(s)

A. Test Article
1. Identity: Crotonaldehyde
2. Accession No.: 901878 HAEL No.: 90-0016
Sample Reference Identification No. (Lot No.): Lot No. 90-7
CAS Registry No.: 4170-30-3
3. Other Information (Composition, Stability, Purity, etc.):
The test article is a clear liquid, appreciably soluble in water.

B. Control Substance(s)
1. Negative Control: Laboratory diluent water (seasoned @ 12°C).
2. Positive Control: None required for this test.
3. Carrier Solvent Control: None needed for this test.

III. Test System

A. Test Organisms
1. The rainbow trout, Oncorhynchus mykiss (formerly Salmo gairdneri), were obtained from:
Mt. Lassen Trout Farm
Red Bluff, CA 96080
USA

Lot Designation: MLTF Lot 4/90

B. Test Vessel Identification
The test system will be identified by a placard placed above the exposure vessels. The placard will include the designation HAEL No. 90-0016 (identifying the test article), the test species code (Om), and the test start date.

C. Endpoint Calculations
A computer program developed by C.E. Stephan and the American Society for Testing and Materials (ASTM) is used to compute point and interval (i.e., 95% confidence limits) estimates of the 96-hour LC_{50} value. The 24-, 48-, and 72-hour values should be calculated whenever there is sufficient mortality data to determine such values. The program requires the concentration of the test substance (either nominal or measured), the number of organisms exposed, and the number of

ATTACHMENT 5a. Protocol for Study I (Excerpts)

organisms that die. Control data are not used because the program does not have the capability of adjusting for control mortality. The program computes point and interval estimates of the LC_{50} value using three approaches. (The concentration is $\log_{10}$-transformed for all methods.)

IV. Reporting and Records

A. Reporting

The report must contain:

1. Name and address of the testing facility.
2. Statement of GLP Compliance signed by Study Director and Sponsor.
3. Dates of the study.
4. Procedures stated in the protocol, including any changes or amendments to the original protocol.
5. Methods used in analyzing the data.
6. Identification of test and reference substances.
7. Description of the test methods.
8. Description of the test system.
9. Description of the test article concentration.
10. Description of any circumstances that may have affected the quality or integrity of the study.
11. A copy of all raw data and summaries of data.
12. A copy of the protocol and any amendments.
13. Name of the Study Director and of all other persons involved in the study, including supervision.
14. Summary and analysis of results, including any statistical analyses performed to obtain an LC_{50} value.
15. Graph of the concentration effect (dose-response) relationship.
16. Proof of validity of the test.
17. Stability of the test substance.
18. Signed and dated reports of the scientists involved in the study.
19. Location of all specimens, raw data, and final reports.
20. Quality Assurance Statement.

B. Records

All records will be retained for at least ten years by the Health and Environment Laboratories:

1. All raw data and summaries of data.
2. The protocol and any protocol amendments.
3. Summaries of training and experience for all persons involved in the study.
4. Standard operating procedures.
5. Final reports.

ATTACHMENT 5a. Protocol for Study I (Excerpts)

VI. Attachment 1. Protocol Amendments (to be prepared only if necessary)

Protocol: Determination of the Acute Aquatic Effects of Test Article on the Rainbow Trout, Oncorhynchus mykiss

Test Article: Crotonaldehyde

Accession No. 901878 HAEL No. 90-0016

1.) The 2 mg/L exposure solutions were sampled at time 24 hours since complete mortality had occurred by that time. It was deemed more appropriate to sample at 24 hours rather than wait overnight until the sampling scheduled for test midpoint (and stock solution renewal) at 48 hours. Similarly, the 1 mg/L exposure solutions were sampled at time 72 hours (also due to complete mortality) rather than at test end at 96 hours. The analyzed values obtained will be used as the test end exposure concentrations in all endpoint calculations for the rainbow trout because later samplings would be irrelevant to the dose response.

2.) It was not feasible due to an instrumentation malfunction to strictly comply with the Consent Order in regard to the reference to "Real-time analysis of the test solution for the test article will be conducted at 0, 48, and 96 hours." The samples taken at time 0 and 24 hours had to be prepared again and re-analyzed at time 72 hours. These repeat measurements were performed along with the samples collected at times 48 hours (not yet analyzed due to instrumental timing constraints but stabilized after preparation) and 72 hours. All exposure solution samples submitted for analysis were stored refrigerated in amber, glass containers (nearly full to brim and tightly capped). Crotonaldehyde concentration stability under the preservation conditions used will be verified and documented by CQSD subsequent to the end of this study.

3.) In reference to Amendments 1 and 2, the sampling procedure used during this trout study is that described on page 3 of this protocol: "Samples will be removed for analysis @ times 0, 48 (aged solutions & fresh stock), and 96 hours (or test end per dose; i.e., complete mortality)." It is felt that this interpretation will yield endpoint values ultimately more representative of the dose-response relationship.

Approved by: R. J. O'Boyle (Study Director) 5/3/90 Date

Approved by: Roderick D. Konwe (Sponsor) 05/11/90 Date

3990e

ATTACHMENT 5b. Protocol for Study II

ENVIRONMENTAL SCIENCES SECTION
ENVIRONMENTAL TECHNICAL SERVICES
HEALTH AND ENVIRONMENT LABORATORIES

Protocol No. EN-421-901878-2
Revision No.:
Date: 7-25-88

Protocol: Determination of the Acute Aquatic Effects of Test Article on the Rainbow Trout, *Oncorhynchus mykiss*

In Accordance with the US EPA, 1985. Toxic Substances, Testing Requirements; Final Rule and Proposed Rule. Federal Register: 40 CFR Part 797.1400 Fish Acute Toxicity Test; (Amended 1987)

Test Article: Crotonaldehyde

Accession No. 901878 HAEL No. 90-0016

Approved by: R. J. O'Boyle 6/6/90
Study Director Date

Approved by: Roderick D. Stowe 6/19/90
Sponsor (ECD) Date

Note: The attached protocol will be performed in compliance with the Standard Operating Procedure (SOP) No. EN-4035.

ATTACHMENT 5b. Protocol for Study II

Health and Environment Laboratories Management and
Quality Assurance Unit Approvals

Approved: Joseph W. Gorsuch — 6/6/90
Joseph W. Gorsuch
Project Manager
Environmental Sciences Section
Date

Approved: M. Susan Jones — 6/6/90
Quality Assurance Unit
Date

Approved: John R. Owen — 6/11/90
John R. Owen
Unit Director
Environmental Sciences Section
Date

ATTACHMENT 5b. Protocol for Study II

Protocol: Determination of the Acute Aquatic Effects of Test Article on the Rainbow Trout, Oncorhynchus mykiss

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I. Test Administration

A. Testing Facility
Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652-3617
U.S.A.

B. Sponsor
Eastman Chemical Division
Kingsport, Tennessee 37662
U.S.A.

C. Dates of Experiment
Start Date: 6/25/90
Test Article Exposure Completion Date: 6/29/90
Estimated Study Completion Date: 9/1/90

ATTACHMENT 5b. Protocol for Study II

D. Test Type
1. ____ Range-Finding/ X Definitive
2. 2 Replicates (10 animals per replicate)
3. ____ Static/ X Dynamic/; 70 mL/Min (At least 6 test vessel volumes per day; a proportionating diluter system will be used.)
4. 15 L/Test Vessel Vol.
5. X Fluorescent/____ Xenon-Arc Illumination (Sixteen-Hour Photoperiod with a 20-Minute Transition Period)
6. (X) Aeration (May Be Used at ≈ 0.05 L/Minute, if Necessary)
7. ____ Nominal/ X Measured Test Article Concentrations @ 0.12, 0.25, 0.5, 1, and 2 mg/L; 20 mg/L stock solution feeding diluter will be renewed at time 48 hours. Samples will be removed for analysis @ times "0", 48 (aged solutions & fresh stock), and 96 hours (or test end per dose; i.e., complete mortality).

Note: The sampling schedule/procedure described above supersedes that described in Attachment 1 of this protocol. If complete mortality is observed in a dose at 24 hours, it is deemed more appropriate to sample at 24 hours rather than wait overnight until the sampling scheduled for test midpoint (and stock solution renewal) at 48 hours. Similarly, complete mortality at 72 hours will trigger a sampling of the dose(s) involved at that time rather than wait until time 96 hours. The analyzed values obtained will be used as the test end exposure concentrations in all endpoint calculations for the rainbow trout because any later samplings of those exposure solutions would be irrelevant to the dose response. It is felt that this interpretation will yield endpoint values ultimately more representative of the dose-response relationship.

II. Test and Control Substance(s)

A. Test Article
1. Identity: Crotonaldehyde
2. Accession No.: 901878 HAEL No.: 90-0016
Sample Reference Identification No. (Lot No.): Lot No. 90-7
CAS Registry No.: 4170-30-3
3. Other Information (Composition, Stability, Purity, etc.):
The test article is a clear liquid, appreciably soluble in water.

B. Control Substance(s)
1. Negative Control: Laboratory diluent water (seasoned @ 12°C).
2. Positive Control: None required for this test.
3. Carrier Solvent Control: None needed for this test.

III. Test System

A. Test Organisms
1. The rainbow trout, Oncorhynchus mykiss (formerly Salmo gairdneri), were obtained from:
Mt. Lassen Trout Farm
Red Bluff, CA 96080
USA

Lot Designation: MLTF Lot 6/90

B. Test Vessel Identification
The test system will be identified by a placard placed above the exposure vessels. The placard will include the designation HAEL

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No. 90-0016 (identifying the test article), the test species code (Om), and the test start date.

C. Endpoint Calculations

A computer program developed by C.E. Stephan and the American Society for Testing and Materials (ASTM) is used to compute point and interval (i.e., 95% confidence limits) estimates of the 96-hour LC_{50} value. The 24-, 48-, and 72-hour values should be calculated whenever there is sufficient mortality data to determine such values. The program requires the concentration of the test substance (either nominal or measured), the number of organisms exposed, and the number of organisms that die. Control data are not used because the program does not have the capability of adjusting for control mortality. The program computes point and interval estimates of the LC_{50} value using three approaches. (The concentration is $\log_{10}$-transformed for all methods.)

IV. Reporting and Records

A. Reporting

The report must contain:

1. Name and address of the testing facility.
2. Statement of GLP Compliance signed by Study Director and Sponsor.
3. Dates of the study.
4. Procedures stated in the protocol, including any changes or amendments to the original protocol.
5. Methods used in analyzing the data.
6. Identification of test and reference substances.
7. Description of the test methods.
8. Description of the test system.
9. Description of the test article concentration.
10. Description of any circumstances that may have affected the quality or integrity of the study.
11. A copy of all raw data and summaries of data.
12. A copy of the protocol and any amendments.
13. Name of the Study Director and of all other persons involved in the study, including supervision.
14. Summary and analysis of results, including any statistical analyses performed to obtain an LC_{50} value.
15. Graph of the concentration effect (dose-response) relationship.
16. Proof of validity of the test.
17. Stability of the test substance.
18. Signed and dated reports of the scientists involved in the study.
19. Location of all specimens, raw data, and final reports.
20. Quality Assurance Statement.

B. Records

All records will be retained for at least ten years by the Health and Environment Laboratories:

1. All raw data and summaries of data.
2. The protocol and any protocol amendments.
3. Summaries of training and experience for all persons involved in the study.
4. Standard operating procedures.
5. Final reports.

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V. Appendix 1. EPA Consent Order Protocol for Fish (Rainbow Trout) Acute Toxicity Test (Section 797.1400)

Section 797.1400 Fish (RAINBOW TROUT) acute toxicity test.

(a) Purpose. This guideline may be used to develop data on the acute toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 et seq.). This guideline prescribes tests to be used to develop data on the acute toxicity of chemicals to fish. The United States Environmental Protection Agency will use data from these tests in assessing the hazard of a chemical to the environment.

(b) Definitions. The definitions in section 3 of the Toxic Substances Control Act (TSCA), and the definitions in Part 792 Good Laboratory Practice Standards of this chapter apply to this test guideline. The following definitions also apply to this guideline:

(1) "Acclimation" means the physiological compensation by test organisms to new environmental conditions (e.g., temperature, hardness, pH).

(2) "Acute toxicity test" means a method used to determine the concentration of a substance that produces a toxic effect on a specified percentage of test organisms in a short period of time (e.g., 96 hours). In this guideline, death is used as the measure of toxicity.

(3) "Carrier" means a solvent used to dissolve a test substance prior to delivery to the test chamber.

(4) "Conditioning" means the exposure of construction materials, test chambers, and testing apparatus to dilution water or to test solutions prior to the start of a test in order to minimize the sorption of the test substance onto the test facilities or the leaching of substances from the test facilities into the dilution water or test solution.

(5) "Death" means the lack of opercular movement by a test fish.

(6) "Flow-through" means a continuous or an intermittent passage of test solution or dilution water through a test chamber, or a holding or acclimation tank with no recycling.

(7) "Incipient LC_{50}" means the test substance concentration, calculated from experimentally-derived mortality data, that is lethal to 50 percent of a test population when exposure to the test substance is continued until the mean increase in mortality does not exceed 10 percent in any concentration over a 24-hour period.

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(8) "LC_{50}" means that test substance concentration, calculated from experimentally-derived mortality data, that is lethal to 50 percent of a test population during continuous exposure over a specified period of time.

(9) "Loading" means the ratio of fish biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber or passing through it in a 24 hour period.

(10) "Static" means the test solution is not renewed during the period of the test.

(11) "Test solution" means the test substance and the dilution water in which the test substance is dissolved or suspended.

(c) Test procedures—(1) Summary of the test. (i) Test chambers are filled with appropriate volumes of dilution water. If a flow-through test is performed, the flow of dilution water through each chamber is adjusted to the rate desired.

(ii) The test substance is introduced into each test chamber. In a flow-through test, the amount of test substance which is added to the dilution water is adjusted to establish and maintain the desired concentration of test substance in each test chamber.

(iii) Test fish which have been acclimated in accordance with the test design are introduced into the test and control chambers by stratified random assignment.

(iv) Fish in the test and control chambers are observed periodically during the test; dead fish are removed at least twice each day and the findings are recorded.

(v) The dissolved oxygen concentration, pH, temperature and the concentration of test substance are measured at intervals in selected test chambers.

(vi) Concentration-response curves and LC_{50} values for the test substance are developed from the mortality data collected during the test.

(2) (Reserved)

(3) Range-finding test. If the toxicity of the test substance is not already known, a range finding test should be performed to determine the range of concentrations to be used in the definitive test.

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V. Appendix 1. EPA Consent Order Protocol for Fish (Rainbow Trout) Acute Toxicity Test (Section 797.1400)

The highest concentration of test substance for use in the range finding test should not exceed its solubility in water or the permissible amount of the carrier used. [NOMINAL STATIC CONCENTRATIONS MAY BE USED FOR THE RANGE-FINDING TEST.]

(4) Definitive test. (i) A minimum of 20 fish ~~should~~ [SHALL] be exposed to each of five or more test substance concentrations. The range of concentrations to which the fish are exposed should be such that in 96 hours there are at least two partial mortality exposures bracketing 50 percent survival. [RESTRICTED RANDOMIZATION WILL BE USED. THE PARTIAL MORTALITY WILL NOT BE REQUIRED FOR A STEEP SLOPE DOSE RESPONSE CURVE.]

(ii) For exposure to each concentration of a test substance, an equal number of test fish ~~should~~ [SHALL] be placed in two or more replicate test chambers. The distribution of individual fish among the test chambers should be ~~randomized~~ [IMPARTIAL].

(iii) Every test ~~should~~ [SHALL] include a control consisting of the same dilution water, conditions, procedures and fish from the same group used in the test, except that none of the test substance is added.

(iv) Mortality data collected during the test are used to calculate a 96-hour LC_{50}. The 24-, 48-, and 72-hour values should be calculated whenever there is sufficient mortality data to determine such values. If the 96-hour LC_{50} is less than 50 percent of the estimated 48-hour LC_{50} in a flow-through test, the test ~~should~~ [SHALL] be continued until the mean increase in mortality at any test concentration does not exceed 10 percent over a 24-hour period or until 14 days.

(v) Test fish ~~should~~ [SHALL] not be fed while they are being exposed to the test substance under static conditions or during the first 96 hours of flow-through testing. If the test continues past 96 hours, the fish should be fed a suitable food at a maintenance level every other day beginning on test day 5. Any excess food and the fecal material should be removed when observed.

(5) Test results. (i) Death is the primary criterion used in this test guideline to evaluate the toxicity of the test substance.

(ii) In addition to death, any abnormal behavior such as, but not limited to, erratic swimming, loss of reflex, increased excitability, lethargy, or any changes in appearance or physiology such as discoloration, excessive mucous production, hyperventilation, opaque eyes, curved spine, or hemorrhaging ~~should~~ [SHALL] be recorded.

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(iii) Observations on compound solubility ~~should~~ [SHALL] be recorded. The investigator ~~should~~ [SHALL] report the appearance of surface slicks, precipitates, or material adhering to the sides of the test chamber.

(iv) Each test and control chamber ~~should~~ [SHALL] be checked for dead fish and observations recorded at 24, 48, 72, and 96 hours after the beginning of the test or within one hour of the designated times. If the test is continued past 96 hours, additional observations ~~should~~ [SHALL] be made every 24 hours until termination.

(v) The mortality data is used to calculate LC_{50} and their 95 percent confidence limits, and to plot concentration-response curves for each time interval whenever sufficient data exists. The methods recommended for use in calculating LC_{50}'s include probit, logit, binomial, and moving average angle.

(vi) A test is unacceptable if more than 10 percent of the control fish die or exhibit abnormal behavior during a 96-hour test. If a flow-through test is continued past 96 hours, the maximum allowable additional mortality is 10 percent.

(6) Analytical measurements—(i) Water quality analysis. (A) The hardness, acidity, alkalinity, pH, conductivity, TOC or COD, and particulate matter of the dilution water should be measured ~~at the beginning of each static test and at the beginning and end of each flow-through test~~ [MONTHLY]. The month to month variation of the above values should be less than 10 percent and the pH should vary less than 0.4 units. [FOR THE ANALYTICAL REQUIREMENTS OF THE DILUENT WATER, THE ATTACHED AGGREGATE DATA SUMMARY WILL BE SUBSTITUTED. THE MEASURED RESIDUAL CHLORINE SHOULD BE LESS THAN 0.01 mg/L.]

(B) During static tests, the dissolved oxygen concentration, temperature and pH should be measured in each test chamber at the beginning of the test and as often as needed thereafter to document changes from the initial levels. The test solution volume should not be reduced by more than 10 percent as a result of these measurements.

(C) During flow-through tests, dissolved oxygen, temperature and pH measurements ~~should~~ [SHALL] be made in each chamber at the beginning of the test and every ~~48~~ [24] hours thereafter until the end of the test.

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(ii) Collection of samples for measurement of test substance. Test solution samples to be analyzed for the test substance should be taken midway between the top, bottom, and sides of the test chamber. These samples should not include any surface scum or material dislodged from the bottom or sides. Samples should be analyzed immediately or handled and stored in a manner which minimizes loss of test substance through microbial degradation, photodegradation, chemical reaction, volitalization, or sorption.

(iii) Measurement of test substance. ~~(A) For static tests, the concentration of dissolved test substance (that which passes through a 0.45 micron filter) should be measured at a minimum in each test chamber at the beginning (0-hour, before fish are added) and at the end of the test.~~ [(A) DEIONIZED OR DISTILLED WATER SHOULD BE USED IN MAKING STOCK SOLUTIONS OF THE TEST SUBSTANCE. STANDARD ANALYTICAL METHODS SHOULD BE USED WHENEVER AVAILABLE IN PERFORMING THE ANALYSES. THE ANALYTICAL METHOD USED TO MEASURE THE AMOUNT OF TEST SUBSTANCE IN A SAMPLE SHALL BE VALIDATED BEFORE BEGINNING THE TEST BY APPROPRIATED LABORATORY PRACTICES. AN ANALYTICAL METHOD IS NOT ACCEPTABLE IF LIKELY DEGRADATION PRODUCTS OF THE TEST SUBSTANCE, SUCH AS HYDROLYSIS AND OXIDATION PRODUCTS, GIVE POSITIVE OR NEGATIVE INTERFERENCES WHICH CANNOT BE SYSTEMATICALLY IDENTIFIED AND CORRECTED MATHEMATICALLY.] During flow-through tests, the concentration of dissolved test substance ~~should~~ [SHALL] be measured as follows:

(1) In each chamber at 0-hour. [REAL-TIME ANALYSIS OF THE TEST SOLUTION FOR THE TEST ARTICLE WILL BE CONDUCTED AT 0, 48, AND 96 HOURS.]

(2) In each chamber at 96-hours and every 4 days thereafter, as long as the test is continued.

(3) In at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system.

~~(B) Filters and their holders used for determining the dissolved test substance concentrations should be prerinsed with several volumes of distilled water and undergo a final rinse with test solution. Glass or stainless steel filter holders are best for organic test substances, while plastic holders are best for metals. The sample should be filtered within 30 minutes after it is taken from the test chamber.~~

~~(C) The analytical methods used to measure the amount of test substance in a sample should [SHALL] be validated before beginning the test. The accuracy of a method should be verified by a method such as using known additions.~~

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~~This involves adding a known amount(s) of the test substance to three water samples taken from a chamber containing (of) dilution water and the same number and species of fish as are used in the test. The nominal concentration(s) of the test substance in those samples should span the concentration range to be used in the test. Validation of the analytical method should be performed on at least two separate days prior to starting the test.~~

~~(B) An analytical method is not acceptable if likely degradation products of the test substance give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test.~~

~~(C) In addition to analyzing samples of test solution, at least one reagent blank, containing all reagents used, should also be analyzed.~~

~~(F) If the measured concentrations of dissolved test substances are considerably lower (e.g., <50 percent) than the nominal concentrations, the total test substance concentration should be measured in the highest test concentration.~~

[(B) THE ANALYTICAL METHOD FOR THE TEST SUBSTANCE SHALL BE VALIDATED PRIOR TO BEGINNING THE TEST. A PROCEDURE SUCH AS USING KNOWN ADDITIONS MAY BE USED. THIS INVOLVES ADDING KNOWN AMOUNTS OF THE TEST SUBSTANCE TO THREE OR MORE SAMPLES OF DILUTION WATER. THE NOMINAL CONCENTRATIONS OF THE TEST SUBSTANCE IN THESE SAMPLES SHOULD SPAN THE CONCENTRATION RANGE TO BE USED IN THE TEST. BOTH DISSOLVED TEST SUBSTANCE (THAT WHICH PASSES THROUGH A 0.45 MICRON FILTER) AND TOTAL TEST SUBSTANCE SHALL BE MEASURED IN EACH SAMPLE. IF THE MEASURED CONCENTRATIONS OF DISSOLVED TEST SUBSTANCE ARE GREATER THAN 80% OF THE MEASURED CONCENTRATIONS OF TOTAL TEST SUBSTANCE, THEN ONLY TOTAL TEST SUBSTANCE SHALL BE MEASURED DURING THE TEST. HOWEVER, IF THE MEASURED CONCENTRATIONS OF DISSOLVED TEST SUBSTANCE ARE LESS THAN 80% OF THE MEASURED CONCENTRATIONS OF TOTAL TEST SUBSTANCE, THEN ONLY DISSOLVED TEST SUBSTANCE SHALL BE MEASURED DURING THE TEST. VALIDATION OF THE ANALYTICAL METHOD SHOULD BE PERFORMED ON AT LEAST TWO SEPARATE DAYS PRIOR TO STARTING THE TEST.]

[(C) IN ADDITION TO ANALYZING SAMPLES OF TEST SOLUTION, AT LEAST ONE REAGENT BLANK, CONTAINING ALL REAGENTS USED, SHOULD ALSO BE ANALYZED.]

[(D) FILTERS AND THEIR HOLDERS USED FOR DETERMINING THE DISSOLVED TEST SUBSTANCE CONCENTRATIONS SHOULD BE PREWASHED WITH SEVERAL VOLUMES OF DISTILLED WATER OR DILUTION WATER AND UNDERGO A FINAL RINSE WITH TEST SOLUTION. GLASS OR STAINLESS STEEL FILTER HOLDERS ARE BEST FOR ORGANIC SUBSTANCES, WHILE PLASTIC HOLDERS ARE BEST FOR METALS. THE SAMPLE SHOULD BE FILTERED WITHIN 30 MINUTES AFTER IT IS TAKEN FROM THE TEST CHAMBER.]

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~~(C)~~ [E] Among replicate test chambers, the measured concentrations should not vary more than 20 percent. The measured concentration of the test substance in any chamber during the test should not vary more than 30 percent from the measured concentration prior to initiation of the test.

~~(D)~~ [F] The mean measured concentration of ~~dissolved~~ [THE] test substance ~~should~~ [SHALL] be used to calculate all LC_{50}'s and to plot all concentration-response curves.

(d) Test conditions—(1) Test species. (i) Selection. The test species for this test are the rainbow trout [(ONCORHYNCHUS MYKISS) FORMERLY] {Salmo gairdneri}, ~~bluegill (Lepomis macrochirus)~~ and fathead minnow (Pimephales promelas). The particular species of fish to be used will be prescribed in the test rule. [FOR THIS PROTOCOL THE TEST SPECIES WILL BE RAINBOW TROUT.]

(ii) Age and condition of fish. (A) Juvenile fish ~~should~~ [SHALL] be used. Fish used in a particular test ~~should~~ [SHALL] be the same age and be of normal size and appearance for their age. The longest fish ~~should~~ [SHALL] not be more than twice the length of the shortest.

(B) All newly acquired fish should be quarantined and observed for at least 14 days prior to use in a test.

(C) Fish ~~should~~ [SHALL] not be used for a test if they appear stressed or if more than five percent die during the 48 hours immediately prior to the test.

(iii) Acclimation of test fish. (A) If the holding water is not from the same source as the test dilution water, acclimation to the dilution water should be done gradually over a 48-hour period. The fish should then be held an additional 14 days in the dilution water prior to testing. Any changes in water temperature should not exceed 3°C per day. Fish should be held for a minimum of 7 days at the test temperature prior to testing.

(B) During the final 48-hours of acclimation, fish should be maintained in facilities with background colors and light intensities similar to those of the testing area and should not be fed.

(2) Facilities—(i) General. Facilities needed to perform this test include:

(A) Flow-through tanks for holding and acclimating fish.

(B) A mechanism for controlling and maintaining the water temperature during the holding, acclimation and test periods.

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V. Appendix 1. EPA Consent Order Protocol for Fish (Rainbow Trout) Acute Toxicity Test (Section 797.1400)

(C) Apparatus for straining particulate matter, removing gas bubbles, or insufficient dissolved oxygen, respectively.

(D) Apparatus for providing a 16-hour light and 8-hour dark photoperiod with a 15- to 30-minute transition period.

(E) Chambers for exposing test fish to the test substance.

(F) A test substance delivery system for flow-through tests.

(ii) Construction materials. Construction materials and commercially purchased equipment that may contact the stock solution, test solution, or dilution water should not contain substances that can be leached or dissolved into aqueous solutions in quantities that can alter the test results. Materials and equipment that contact stock or test solutions should be chosen to minimize sorption of test chemicals. Glass, stainless steel, and perfluorocarbon plastic should be used whenever possible. Concrete, fiberglass, or plastic (e.g., PVC) may be used for holding tanks, acclimation tanks, and water supply systems, but they should be used to remove rust particles. Rubber, copper, brass, galvanized metal, epoxy glues, and lead should not come in contact with the dilution water, stock solution, or test solution.

(iii) Test substance delivery system. In flow-through tests, diluters, metering pump systems or other suitable devices should be used to deliver the test substance to the test chambers. The system used should be calibrated before each test. Calibration includes determining the flow rate through each chamber and the concentration of the test substance delivered to each chamber. The general operation of the test substance delivery system should be checked twice daily during a test. The 24-hour flow rate through a test chamber [SHOULD BE A MINIMUM OF SIX TANK VOLUMES]. During a test, the flow rates should not vary more than 10 percent from one test chamber to another or from one time to any other.

(iv) Test chambers. Test chambers made of stainless steel should be welded, not soldered. Test chambers made of glass should be fused or bonded using clear silicone adhesive. As little adhesive as possible should be left exposed in the interior of the chamber.

(v) Cleaning of test system. Test substance delivery systems and test chambers should be cleaned before each test. They should be washed with detergent and then rinsed in sequence with clean water, pesticide-free acetone, clean water, and 5 percent nitric acid, followed by two or more changes of dilution water.

(vi) Dilution water. (A) Clean surface or ground water reconstituted water, or dechlorinated tap water is acceptable as dilution water if the test fish will survive in it for the duration

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of the holding, acclimating, and testing periods without showing signs of stress, such as discoloration, hemorrhaging, disorientation or other unusual behavior. The quality of the dilution water should be constant and should meet the following specifications measured at least twice a year [FOR THE ANALYTICAL REQUIREMENTS OF THE DILUENT WATER, THE ATTACHED DILUTION WATER AGGREGATE HISTORICAL DATA SUMMARY WILL BE SUBSTITUTED. THE MEASURED RESIDUAL CHLORINE SHOULD BE LESS THAN 0.01 mg/L.]:

Substance	Maximum Concentration	
Particulate matter	20	mg/liter.
Total organic carbon or	~~2~~ [3]	mg/liter.
Chemical oxygen demand	5	mg/liter.
Un-ionized ammonia	~~1~~ [20]	µg/liter.
Residual chlorine	~~1~~ [10]	µg/liter.
Total organophosphorus pesticides	50	µg/liter.
Total organochlorine pesticides plus polychlorinated biphenyls (PCBs)	50	µg/liter.
or organic chlorine	25	µg/liter.

(B) The concentration of dissolved oxygen in the dilution water should be between 90 and 100 percent saturation; 9.8 to 10.8 mg/l for tests with trout, and 8.0 to 8.9 mg/l for tests with bluegill or fathead minnow at sea level. If necessary, the dilution water can be aerated before the addition of the test substance. All reconstituted water should be aerated before use. Buffered soft water should be aerated before but not after the addition of buffers.

(C) If disease organisms are present in the dilution water in sufficient numbers to cause infection, they should be killed or removed by suitable equipment.

(D) Glass distilled or carbon filtered deionized water with a conductivity less than 1 micromho/cm is acceptable for use in making reconstituted water. If the reconstituted water is prepared from a ground or surface water source, conductivity, and total organic carbon (TOC) or chemical oxygen demand (COD) should be measured on each batch.

(vii) Carriers. (A) Distilled water [STOCK SOLUTIONS WILL BE MADE WITH DILUTION WATER.] should be used in making stock solutions of the test substance. If the stock volume however is more than 10 percent of the test solution volume, dilution water should be used. If a carrier is absolutely necessary to dissolve the test substance, the volume used should not exceed the minimum volume necessary to dissolve or suspend the test substance in the test solution. If the test substance is a mixture, formulation, or commercial product, none of the ingredients is considered a carrier unless an extra amount is used to prepare the stock solution.

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(B) Triethylene glycol and dimethyl formamide are the preferred carriers, but acetone can also be used. The concentration of triethylene glycol in the test solution should not exceed ~~80 mg/l~~ [0.1 ML/L]. The concentration of dimethyl formamide or acetone in the test solution should not exceed ~~500 mg/l~~ [0.1 ML/L].

(3) Test parameters—(i) Loading. The number of fish placed in a test chamber should not be so great as to affect the results of the test. The loading ~~should~~ [SHALL] not be so great that the test substance concentrations are decreased by more than 20 percent due to uptake by the fish. In static tests, loading should not exceed 0.5 grams of fish per liter of solution in the test chamber at any one time. In flow-through tests loading should not exceed 0.5 grams of fish per liter of test solution passing through the chamber in 24 hours. These loading rates should be sufficient to maintain the dissolved oxygen concentration above the recommended levels and the ammonia concentration below 20 µg/l.

(ii) Dissolved oxygen concentration. (A) During static tests with rainbow trout the dissolved oxygen in each test chamber should be greater than 5.5 mg/l. In tests with bluegill and fathead minnows, the DO should be maintained above 4.5 mg/l.

(B) During flow-through tests the dissolved oxygen concentration ~~should~~ [SHALL] be maintained above 8.2 mg/l in tests with trout and above 6.6 mg/l in tests with bluegills or fathead minnows.

(iii) Temperature. The test temperature ~~should~~ [SHALL] be ~~17~~ [20] ± ~~1~~[2] °C for ~~bluegill and~~ fathead minnows, and 12 ± ~~1~~[2]°C for rainbow trout. The temperature ~~should~~ [SHALL] be measured at least hourly in one test chamber.

(iv) Light. A 16-hour light and 8-hour dark photoperiod with a 15- to 30-minute transition period should be maintained.

(e) Reporting. The sponsor ~~should~~ [SHALL] submit to the EPA all data developed by the test that are suggestive or predictive of toxicity. In addition to the reporting requirements prescribed in Part 792—Good Laboratory Practice Standards of this chapter, the reported test data ~~should~~ [SHALL] include the following:

(1) The source of the dilution water, a description of any pretreatment, and the measured hardness, acidity, alkalinity, pH, conductivity, TOC or COD and particulate matter.

(2) A description of the test chambers, the depth and volume of solution in the chamber, the specific way the test was begun (e.g., conditioning, test substance additions), and for flow-through tests, a description of the test substance delivery system.

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(3) Detailed information about the test fish, including the scientific name and method of verification, average weight (grams, wet weight), standard length, age, source, history, observed diseases, treatments, and mortalities, acclimation procedures, and food used.

(4) The number of replicates used, the number of organisms per replicate, the loading rate, and the flow rate for flow-through tests.

(5) The measured DO, pH, and temperature and the lighting regime.

(6) The solvent used, the test substance concentration in the stock solution, the highest solvent concentration in the test solution and a description of the solubility determinations in water and solvents if used.

(7) The concentration of the test substance in each test chamber just before the start of the test and at all subsequent sampling periods.

(8) The number of dead and live tests organisms, the percentage of organisms that died, and the number that showed any abnormal effects in the control and in each test chamber at each observation period.

(9) The 96-hour LC_{50}, and when sufficient data have been generated, the 24-, 48-, 72-, and incipient LC_{50} values, their 95 percent confidence limits, and the methods used to calculate the LC_{50} values and their confidence limits.

(10) When observed, the observed no effect concentration (the highest concentration tested at which there were no mortalities or abnormal behavioral or physiological effects).

(11) The concentration-response curve at each observation period for which a LC_{50} was calculated.

(12) Methods and data records of ~~all chemical~~ analyses of ~~water quality parameters~~ and test substance concentrations, including method validations and reagent blanks.

[FOR THE ANALYTICAL REQUIREMENTS OF THE DILUENT WATER, THE ATTACHED DILUTION WATER AGGREGATE HISTORICAL DATA SUMMARY WILL BE SUBSTITUTED. LIMITS OF DETECTION SHALL BE INCLUDED.]

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VI. Attachment 1. Protocol Amendments (to be prepared only if necessary)

Protocol: Determination of the Acute Aquatic Effects of Test Article on the Rainbow Trout, *Oncorhynchus mykiss*

Test Article: Crotonaldehyde

Accession No. 901878 HAEL No. 90-0016

Approved by: _____ Study Director _____ Date

Approved by: _____ Sponsor _____ Date

4134e

HAEL No. 90-0016 Study No. EN-421-901878-2

ATTACHMENT 6a. Acute Aquatic Effects Data Forms (Study I)

ACCESSION NO.	HAEL NO.	TEST ARTICLE	SRID NO.
901878	90-0016	Crotonaldehyde	Lot No. 90-7

TIME, DATE & INITIALS	TIME	TEST VOLUME 15L			AIM CONCENTRATION 0.0 mg/l		STARTING CONDITIONS DY, FL
	PHOTO COU	°C	D.O.	pH	OBSERVATIONS DWC		REMARKS (Diluent Water Control) "A"
8:45 4/30/90, OTB	0	12	10.4	8.3	10	N; AW = 0.75 g	(Wet Weight X10)
9:30 5/1/90 RB	24	12	9.2	8.2	10	N	
9:45 5/2/90 RB	48	12	9.4	8.2	10	N	
8:40 5/3/90 RB	72	12	9.6	8.3	10	N	
9:15 5/4/90 RB	96	12	9.6	8.3	10	N	correction 5/3/90 RB

TIME, DATE & INITIALS	TIME	TEST VOLUME 15L			AIM CONCENTRATION 0.0 mg/l		STARTING CONDITIONS DY, FL
	PHOTO COU	°C	D.O.	pH	OBSERVATIONS DWC		REMARKS (Diluent Water Control) "B"
8:45 4/30/90 OTB	0	12	10.5	8.3	10	W; AW = 0.87 g	(Wet Weight X10)
9:30 5/1/90 RB	24	12	9.3	8.2	10	N	
9:45 5/2/90 RB	48	12	9.4	8.1	10	N	
8:40 5/3/90 RB	72	12	9.4	8.3	10	N	
9:15 5/4/90 RB	96	12	9.6	8.4	10	N	

TIME, DATE & INITIALS	TIME	TEST VOLUME 15L			AIM CONCENTRATION 0.12 mg/l		STARTING CONDITIONS DY, FL
	PHOTO COU	°C	D.O.	pH	OBSERVATIONS		REMARKS "A"
8:45 4/30/90 OTB	0	12	10.4	8.3	10	N	Clear Solution
9:40 5/1/90 RB	24	12	9.1	8.2	10	N	
9:50 5/2/90 RB	48	12	9.3	8.2	10	N	
8:40 5/3/90 RB	72	12	9.4	8.3	10	N	
9:15 5/4/90 RB	96	12	9.5	8.3	10	N	

TIME, DATE & INITIALS	TIME	TEST VOLUME 15L			AIM CONCENTRATION 0.12 mg/l		STARTING CONDITIONS DY, FL
	PHOTO COU	°C	D.O.	pH	OBSERVATIONS		REMARKS "B"
8:45 4/30/90 OTB	0	12	10.4	8.3	10	N	Clear Solution
9:40 5/1/90 RB	24	12	9.3	8.2	10	N	
9:50 5/2/90 RB	48	12	9.3	8.2	10	N	
8:40 5/3/90 RB	72	12	9.4	8.3	10	N	
9:15 5/4/90 RB	96	12	9.6	8.3	10	N	

Abbr. used to describe test organisms' condition

AD - Activity Depressed
AA - Activity Accelerated
PB - Position Bottom
PS - Position Surface
FR - Fragmentation
GD - Gill Rate Depressed
GA - Gill Rate Accelerated
I - Immobilized
N - Normal
ND - Near Death
AO - Abnormal Orientations
SH - Shrivelling
T - Tremors
W - Withdrawn
M - Missing
D - Dead

Abbr. used to describe test conditions

AR - Aeration
DY - Dynamic
FL - Fluorescent Lab Lighting
AW - Avg. Wgt. Fish
PRECIP. - Precipitate
RECIRC - Recirculating
SOLN. - Solution
ST - Static
X - Xenon Arc Lighting

ACUTE AQUATIC EFFECTS

TEST ORGANISM: Oncorhynchus mykiss (m)

Health and Environment Laboratories
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United States of America

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SIGNATURE	DATE	REVIEWED BY	DATE
Janice M. Boylinger	5/4/90	R.J. O'Boyle	5/4/90

ATTACHMENT 6a. Acute Aquatic Effects Data Forms (Study I)

ACCESSION NO.	HAEL NO.	TEST ARTICLE	SRID NO.
901878	90-0016	Crotonaldehyde	Lot No. 90-7

Refer to pp. 4, 6.

Test Type

1. _____ Range-Finding/ X Definitive
2. 2 Replicates (10 animals per replicate)
3. _____ Static/ X Dynamic/; 70 mL/Min (At least 6 test vessel volumes per day; a proportionating diluter system will be used.)
4. 15 L/Test Vessel Vol.
5. X Fluorescent/_____ Xenon-Arc Illumination (Sixteen-Hour Photoperiod with a 20-Minute Transition Period)
6. (X) Aeration (May Be Used at ≈ 0.05 L/Minute, if Necessary)
7. _____ Nominal/ X Measured Test Article Concentrations @ 0.12, 0.25, 0.5, 1, and 2 mg/L; 20 mg/L stock solution feeding diluter will be renewed at time 48 hours. Samples will be removed for analysis @ times "0", 48 (aged solutions & fresh stock), and 96 hours (or test end per dose; i.e., complete mortality).

Random Placement of Test Vessels within Diluter Basin:

Replicate							
B:						Diluent	Empty Positions due to Ventilator located here, above test vessels.
	0.5 mg/L	1 mg/L	0.12 mg/L	0.25 mg/L	2 mg/L	Water	
A:						Controls	

FRONT (North) – R.J. O'Boyle, 4/30/90

Note: The stock solution water was sparged with N_2 for ≈ 24 hours prior to adding test article. The stock drum was plastic bagged + taped, and Argon was injected, throughout the study. These procedures were performed initially + @ the renewal. – R.J. O'Boyle 8/22/90.

Acute Aquatic Effects Test Summary: (Nominal concentrations only)

Replicate	LC_{50} (mg/L)	NOEC
A:	0.71	0.25
B:	0.71	0.25
Pooled:	0.71	0.25

– R.J. O'Boyle, 5/4/90

ACUTE AQUATIC EFFECTS

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SIGNATURE	DATE	REVIEWED BY	DATE
Janice M. Beglinger	5/4/90	R.J. O'Boyle	5/4/90

HAEL No. 90-0016 Study No. EN-421-901878-2

ATTACHMENT 6a. Acute Aquatic Effects Data Forms (Study I)

ACCESSION NO	HAEL NO.	TEST ARTICLE	SAID NO.
901878	90-0016	Crotonaldehyde	Lot No. 90-7

TIME, DATE & INITIALS	TIME (PHOTO COU)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 0.25 mg/L OBSERVATIONS		STARTING CONDITIONS DY, FL REMARKS "A"
08:50 4/30/90 DB	0	12	10.2	8.3	10	N	Clear Solution
9:40 5/1/90 JB	24	12	9.1	8.2	10	N	
9:50 5/2/90 JB	48	12	9.2	8.2	10	N	
8:40 5/3/90 JB	72	12	9.4	8.3	10	N	
9:15 5/4/90 JB	96	12	9.5	8.3	10	N	

TIME, DATE & INITIALS	TIME (PHOTO COU)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 0.25 mg/L OBSERVATIONS		STARTING CONDITIONS DY, FL REMARKS "B"
08:50 4/30/90 DB	0	12	10.3	8.3	10	N	Clear Solution
9:40 5/1/90 JB	24	12	9.2	8.2	10	N	
9:50 5/2/90 JB	48	12	9.4	8.2	10	N	
8:40 5/3/90 JB	72	12	9.4	8.3	10	N	
9:15 5/4/90 JB	96	12	9.5	8.3	10	N	

TIME, DATE & INITIALS	TIME (PHOTO COU)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 0.5 mg/L OBSERVATIONS		STARTING CONDITIONS DY, FL REMARKS "A"
08:50 4/30/90 DB	0	12	10.4	8.3	10	N	Clear Solution
9:45 5/1/90 JB	24	12	9.1	8.2	10	N	
9:55 5/2/90 JB	48	12	9.3	8.2	10	N	
8:40 5/3/90 JB	72	12	9.4	8.3	10	N	
9:15 5/4/90 JB	96	12	9.4	8.3	10	N	behavior, but color is somewhat darker than controls

TIME, DATE & INITIALS	TIME (PHOTO COU)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 0.5 mg/L OBSERVATIONS		STARTING CONDITIONS DY, FL REMARKS "B"
08:50 4/30/90 DB	0	12	10.3	8.3	10	N	Clear Solution
9:45 5/1/90 JB	24	12	9.1	8.2	10	N	
9:50 5/2/90 JB	48	12	9.2	8.2	10	N	
8:40 5/3/90 JB	72	12	9.3	8.3	10	N	
9:15 5/4/90 JB	96	12	9.4	8.3	10	N	behavior, but color is somewhat darker than controls

Abbr. used to describe test organisms' condition

AD - Activity Decreased
AA - Activity Accelerated
PB - Position Bottom
PS - Position Surface
FR - Fragmentation
GD - Gill Rate Decreased
GA - Gill Rate Accelerated
I - Immobilized
N - Normal
ND - Near Death
AO - Abnormal Orientation
SH - Shivering
T - Tremors
W - Withdrawn
M - Molting
D - Dead

Abbr. used to describe test conditions

AR - Aeration
DY - Dynamic
FL - Fluorescent Lab Lighting
AW - Avg. Wgt. Fish
PRECIP. - Precipitate
RECIRC. - Recirculating
SOLN. - Solution
ST - Static
X - Xenon Arc Lighting

ACUTE AQUATIC EFFECTS

TEST ORGANISM: Oncorhynchus mykiss (Om)

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SIGNATURE	DATE	REVIEWED BY	DATE
Janice M. Beglinger	5/4/90	R.J. O'Boyle	5/4/90

ATTACHMENT 6a. Acute Aquatic Effects Data Forms (Study I)

ACCESSION NO.	HAEL NO.	TEST ARTICLE	SAID NO.
901878	90-0016	Crotonaldehyde	Lot No. 90-7

Refer to pp. 2, 6.

Test Procedure

A. Preparation of Exposure Solutions (from Aqueous Stock Solution)

1. Stock Solution Preparation (in Laboratory Diluent Water):
   a. Amount Test Article: 4.00 g; Final Volume Water: 200 L
   b. Test Article Concentration in the Stock Solution: 20 mg/L

2. Exposure Solution Preparation (by initially "spiking" tanks with appropriate volumes of stock solution; doses continually renewed from stock by using gas-driven, proportionating diluter system): mL/cycle*
   a. Conc. 2 mg/L; Stock Solution 1500 mL; Total Vol. 15 L (28 mL)
   b. Conc. 1 mg/L; Stock Solution 750 mL; Total Vol. 15 L (14 mL)
   c. Conc. 0.5 mg/L; Stock Solution 375 mL; Total Vol. 15 L (7 mL)
   d. Conc. 0.25 mg/L; Stock Solution 188 mL; Total Vol. 15 L (3.5 mL)
   e. Conc. 0.12 mg/L; Stock Solution 94 mL; Total Vol. 15 L (1.8 mL)

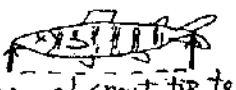
* The diluter cycles every two minutes and delivers a total of 280 mL per cycle to the test system (i.e., flow/replicate = 70 mL/minute).

3. Test Article Solution Preparation Date: 4/30/90 – R.J. O'Boyle 4/30/'90

Remarks: All exposure solutions (test article doses and diluent water controls) will prepared in replicates of two, and the 20 mg/L stock solution will be renewed (i.e., remade fresh) at test time 48 hours.

Length of Control Trout @ Test End:

Replicate A (in mm)	Replicate B
1 – 36	1 – 39
2 – 40	2 – 42
3 – 35	3 – 37
4 – 37	4 – 42
5 – 30	5 – 39
6 – 39	6 – 34
7 – 36	7 – 36
8 – 39	8 – 37
9 – 36	9 – 36
10 – 40	10 – 39
$\bar{X}_{10}$ = 36.8 mm	$\bar{X}_{10}$ = 38.1 mm
(wet wt. @ test start = 0.78 g)	(wet wt. @ test start = 0.87 g)



measured snout tip to caudal peduncle following over-dosing with Finquel & patted dry.
– R.J. O'Boyle 5/4/'90

ACUTE AQUATIC EFFECTS

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SIGNATURE	DATE	REVIEWED BY	DATE
Janice H Beglinger	5/4/90	R.J. O'Boyle	5/4/'90

ATTACHMENT 6a. Acute Aquatic Effects Data Forms (Study I)

ACCESSION NO.	HAEL NO.	TEST ARTICLE	SRID NO.
901878	90-0016	Crotonaldehyde	Lot No. 90-7

TIME, DATE & INITIALS	TIME (PROTOCOL)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 1 mg/L OBSERVATIONS		STARTING CONDITIONS DY, FL REMARKS "A"
08:55 4/30/90, O'B	0	12	10.3	8.3	10	N	Clear Solution
9:50 5/1/90, BS	24	12	9.2	8.2	10	AD (5PS)	
10:00 5/2/90 BS	48	12	9.4	8.2	4	PS/AD (6D)	(dark color)
8:40, 5/3/90, BS	72	12	9.5	8.4	0	(4D)	Dose discontinued 13:00, R.O'B 5/3/90

TIME, DATE & INITIALS	TIME (PROTOCOL)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 1 mg/L OBSERVATIONS		STARTING CONDITIONS DY, FL REMARKS "B"
08:55 4/30/90 O'B	0	12	10.4	8.3	10	N	Clear Solution
9:50, 5/1/90, BS	24	12	9.3	8.2	10	AD (5PS)	
10:00 5/2/90 BS	48	12	9.4	8.3	3	AD (7D)	(dark color)
8:40, 5/3/90, BS	72	12	9.5	8.4	0	(3D)	Dose discontinued 13:00, R.O'B, 5/3/90

TIME, DATE & INITIALS	TIME (PROTOCOL)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 2 mg/L OBSERVATIONS		STARTING CONDITIONS DY, FL REMARKS "A"
08:55 4/30/90 O'B	0	12	10.2	8.3	10	N	Clear Solution
9:50, 5/1/90, BS	24	12	9.3	8.3	0	(10D)	Dose discontinued 13:00 - R.O'B 5/1/90

TIME, DATE & INITIALS	TIME (PROTOCOL)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 2 mg/L OBSERVATIONS		STARTING CONDITIONS DY, FL REMARKS "B"
08:55 4/30/90 O'B	0	12	10.3	8.3	10	N	Clear Solution
9:50, 5/1/90, BS	24	12	9.3	8.3	0	(10D)	Dose discontinued 13:00 - R.O'B 5/1/90.

Abbr. used to describe test organisms' condition

AD - Activity Depressed
AA - Activity Accelerated
PB - Position Bottom
PS - Position Surface
FR - Fragmentation
DD - Gill Rate Depressed
GA - Gill Rate Accelerated
I - Immobilized
N - Normal
HD - Heat Death
AO - Abnormal Orientations
SH - Shriveling
T - Tremors
W - Withdrawn
M - Missing
D - Dead

Abbr. used to describe test conditions

AR - Aeration
DY - Dynamic
FL - Fluorescent Lab Lighting
AW - Aerg. Mgt. Fish
PRECIP. - Precipitate
RECIRC. - Recirculating
SOLN. - Solution
ST - Static
X - Xenon Arc Lighting

ACUTE AQUATIC EFFECTS

TEST ORGANISM: Oncorhynchus mykiss (Om)

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SIGNATURE	DATE	REVIEWED BY	DATE
Janice M. Beglinger	5/4/90	R.J. O'Boyle	5/4/90

ATTACHMENT 6a. Acute Aquatic Effects Data Forms (Study I)

ACCESSION NO.	HAEL NO.	TEST ARTICLE	SRID NO.
901878	90-0016	Crotonaldehyde	Lot No. 90-7

Refer to pp. 2, 4.

ACUTE AQUATIC EFFECTS

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SIGNATURE	DATE	REVIEWED BY	DATE
Francis M. Beglinger	5/4/90	R. J. O'Boyle	5/4/90

ATTACHMENT 6b. Acute Aquatic Effects Data Forms (Study II)

ACCESSION NO.	HAEL NO.	TEST ARTICLE	SRID NO.
901878	90-0016	Crotonaldehyde	Lot No. 90-7

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 0.0 mg/L OBSERVATIONS DWC		STARTING CONDITIONS FL, DY REMARKS (Diluent Water Control) "A"
8:40, 6/25/'90, JB	0	12	10.8	8.3	10	N; AW = 0.61 g	(Wet Wt. X10)
9:00, 6/26/90, JB	24	12	10.3	8.2	10	N	
9:10, 6/27/90, JB	48	12	10.4	8.2	10	N	
8:40, 6/28/90, JB	72	12	10.4	8.3	10	N	
8:55, 6/29/90, JB	96	12	10.2	8.3	10	N	

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 0.0 mg/L OBSERVATIONS DWC		STARTING CONDITIONS FL, DY REMARKS (Diluent Water Control) "B"
8:40, 6/25/'90, JB	0	12	10.6	8.3	10	N; AW = 0.55 g.	(Wet Wt. X10) correction [illegible]
9:00, 6/26/90, JB	24	12	10.4	8.2	10	N	
9:10, 6/27/90, JB	48	12	10.4	8.2	10	N	
8:40, 6/28/90, JB	72	12	10.4	8.2	10	N	
8:55, 6/29/90, JB	96	12	10.4	8.3	10	N	

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 0.12 mg/L OBSERVATIONS		STARTING CONDITIONS FL, DY REMARKS "A"
8:45, 6/25/'90, JB	0	12	10.4	8.2	10	N	
9:00, 6/26/90, JB	24	12	10.4	8.2	10	N	
9:10, 6/27/90, JB	48	12	10.4	8.2	10	N	
8:45, 6/28/90, JB	72	12	10.4	8.2	10	N	
9:00, 6/29/90, JB	96	12	10.2	8.3	10	N	

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 0.12 mg/L OBSERVATIONS		STARTING CONDITIONS FL, DY REMARKS "B"
8:45, 6/25/'90, JB	0	12	10.4	8.2	10	N	
9:05, 6/26/90, JB	24	12	10.5	8.2	10	N	
9:10, 6/27/90, JB	48	12	10.5	8.2	10	N	
8:45, 6/28/90, JB	72	12	10.4	8.2	10	N	
9:00, 6/29/90, JB	96	12	10.2	8.2	10	N	

Abbr. used to describe test organisms' condition
AD - Activity Depressed
AA - Activity Accelerated
PB - Position Bottom
PS - Position Surface
FR - Fragmentation
GD - Gill Rate Depressed
GA - Gill Rate Accelerated
I - Immobilized
N - Normal
HD - Near Death
AO - Abnormal Orientations
SH - Shrivaling
T - Tremors
W - Withdrawn
M - Missing
D - Dead

Abbr. used to describe test conditions
AR - Aeration
DY - Dynamic
FL - Fluorescent Lab Lighting
AW - Avg. Wgt. Fish
PRECIP. - Precipitate
RECIRC. - Recirculating
SOLN. - Solution
ST - Static
X - Xenon Arc Lighting

ACUTE AQUATIC EFFECTS

TEST ORGANISM: Oncorhynchus mykiss 10 m

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SIGNATURE	DATE	REVIEWED BY	DATE
Janice M. Beglinger	6/29/90	R.J. O'Boyle	6/29/90

ATTACHMENT 6b. Acute Aquatic Effects Data Forms (Study II)

ACCESSION NO	HAEL NO	TEST ARTICLE	SRID NO.
901878	90-0016	Crotonaldehyde	Lot No. 90-7

Refer to pp. 4, 6.

Test Type

1. ____ Range-Finding/ X Definitive
2. 2 Replicates (10 animals per replicate)
3. ____ Static/ X Dynamic/; 70 mL/Min (At least 6 test vessel volumes per day; a proportionating diluter system will be used.)
4. 15 L/Test Vessel Vol.
5. X Fluorescent/____ Xenon-Arc Illumination (Sixteen-Hour Photoperiod with a 20-Minute Transition Period)
6. (X) Aeration (May Be Used at ≈ 0.05 L/Minute, if Necessary)
7. ____ Nominal/ X Measured Test Article Concentrations @ 0.12, 0.25, 0.5, 1, and 2 mg/L; 20 mg/L stock solution feeding diluter will be renewed at time 48 hours. Samples will be removed for analysis @ times "0", 48 (aged solutions & fresh stock), and 96 hours (or test end per dose; i.e., complete mortality).

Distribution of Test Vessels within Refrigerated Diluter Basin (Replicates A + B:

B:	0.5 mg/L	1 mg/L	0.12 mg/L	0.25 mg/L	2 mg/L	Diluent Water	Two Spaces
A:						Controls	Not Used!

Ventilation Exhaust

FRONT (North) — R. J. O'Boyle 6/25/90

Acute Aquatic Effects Summary: (Nominal Concentrations only)

Replicate	LC_{50} (mg/L)	NOEC
A:	0.59	0.25
B:	0.63	0.25
Pooled: (Combined A+B)	0.61	0.25

— R. J. O'Boyle 6/29/'90

ACUTE AQUATIC EFFECTS

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PAGE 2 OF 6

SIGNATURE	DATE	REVIEWED BY	DATE
Janice H. Beplington	6/29/90	R. J. O'Boyle	6/29/'90

ATTACHMENT 6b. Acute Aquatic Effects Data Forms (Study II)

ACCESSION NO.	HAEL NO	TEST ARTICLE	SAID NO
901878	90-0016	Crotonaldehyde	Lot No. 90-7

TIME, DATE & INITIALS	TIME (PHOTO COU)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 0.25 mg/L OBSERVATIONS		STARTING CONDITIONS FL, DY REMARKS "A"
8:50, 6/25/90	0	12	10.5	8.3	10	N	
9:10, 6/26/90	24	12	10.5	8.2	10	N	
9:15, 6/27/90	48	12	10.5	8.2	10	N	
8:45, 6/28/90	72	12	10.4	8.2	10	N	
9:05, 6/29/90	96	12	10.3	8.2	10	N	

TIME, DATE & INITIALS	TIME (PHOTO COU)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 0.25 mg/L OBSERVATIONS		STARTING CONDITIONS FL, DY REMARKS "B"
8:50, 6/25/90	0	12	10.5	8.3	10	N	
9:10, 6/26/90	24	12	10.4	8.2	10	N	
9:15, 6/27/90	48	12	10.5	8.2	10	N	
8:45, 6/28/90	72	12	10.5	8.2	10	N	
9:05, 6/29/90	96	12	10.3	8.2	10	N	

TIME, DATE & INITIALS	TIME (PHOTO COU)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 0.5 mg/L OBSERVATIONS		STARTING CONDITIONS FL, DY REMARKS "A"
8:55, 6/25/90	0	12	10.4	8.3	10	N	
9:10, 6/26/90	24	12	10.5	8.4	10	N	
9:15, 6/27/90	48	12	10.4	8.2	10	N	
8:50, 6/28/90	72	12	10.5	8.2	9	N (darker color than DWC) (1D)	
9:05, 6/29/90	96	12	10.4	8.2	7	2N, 5AD (2D)	darker color than controls correction 6/29/90

TIME, DATE & INITIALS	TIME (PHOTO COU)	TEST VOLUME 15L °C	D.O.	pH	AIM CONCENTRATION 0.5 mg/L OBSERVATIONS		STARTING CONDITIONS FL, DY REMARKS "B"
8:55, 6/25/90	0	12	10.5	8.4	10	N	
9:10, 6/26/90	24	12	10.6	8.2	10	N	
9:15, 6/27/90	48	12	10.5	8.2	9	N (1D)	
8:50, 6/28/90	72	12	10.6	8.3	9	N (darker color than DWC)	
9:05, 6/29/90	96	12	10.3	8.2	8	6N, 2AD (1D)	darker color than controls

Abbr. used to describe test organisms' condition

AD - Activity Depressed
AA - Activity Accelerated
PB - Position Bottom
PS - Position Surface
FR - Fragmentation
GD - Gill Rate Depressed
GA - Gill Rate Accelerated
I - Immobilized
N - Normal
ND - Near Death
AO - Abnormal Orientations
SH - Shriveling
T - Tremors
W - Withdrawn
M - Missing
D - Dead

Abbr. used to describe test conditions

AR - Aeration
DY - Dynamic
FL - Fluorescent Lab Lighting
AW - Avg. Wgt. Fish
PRECIP. - Precipitate
RECIRC. - Recirculating
SOLN. - Solution
ST - Static
X - Xenon Arc Lighting

ACUTE AQUATIC EFFECTS

TEST ORGANISM: Oncorhynchus mykiss 10d

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SIGNATURE	DATE	REVIEWED BY	DATE
Janice H. Boylan	6/29/90	R.J. O'Boyle	6/29/90

ATTACHMENT 6b. Acute Aquatic Effects Data Forms (Study II)

ACCESSION NO	HAEL NO	TEST ARTICLE	SRID NO.
901878	90-0016	Crotonaldehyde	Lot No. 90-7

Refer to pp. 2, 6.

Test Procedure

A. Preparation of Exposure Solutions (from Aqueous Stock Solution)

Note: The stock solution water was sparged with N_2 for ≈ 24 hours prior to adding test article. The stock drum was plastic bagged + taped, and Argon injected, throughout study. (initially + @ renewal) — R.O'Boyle 6/29/'90

1. Stock Solution Preparation (in Laboratory Diluent Water):
   a. Amount Test Article: 4.00 g; Final Volume Water: 200 L
   b. Test Article Concentration in the Stock Solution: 20 mg/L

2. Exposure Solution Preparation (by initially "spiking" tanks with appropriate volumes of stock solution; doses continually renewed from stock by using gas-driven, proportionating diluter system): mL/cycle*
   a. Conc. 2 mg/L; Stock Solution 1500 mL; Total Vol. 15 L (28 mL)
   b. Conc. 1 mg/L; Stock Solution 750 mL; Total Vol. 15 L (14 mL)
   c. Conc. 0.5 mg/L; Stock Solution 375 mL; Total Vol. 15 L (7 mL)
   d. Conc. 0.25 mg/L; Stock Solution 188 mL; Total Vol. 15 L (3.5 mL)
   e. Conc. 0.12 mg/L; Stock Solution 94 mL; Total Vol. 15 L (1.8 mL)

* The diluter cycles every two minutes and delivers a total of 280 mL per cycle to the test system (i.e., flow/replicate = 70 mL/minute).

3. Test Article Solution Preparation Date: 6/25/90 — R.O'Boyle 6/25/'90

Remarks: All exposure solutions (test article doses and diluent water controls) will prepared in replicates of two, and the 20 mg/L stock solution will be renewed (i.e., remade fresh) at test time 48 hours.

Lengths of Control Trout @ Test End: (in mm)

Replicate A	Replicate B
1- 35	1- 31
2- 35	2- 33
3- 33	3- 36
4- 35	4- 37
5- 33	5- 34
6- 36	6- 31
7- 31	7- 35
8- 33	8- 30
9- 33	9- 29
10- 35	10- 32
$\bar{X}_{10}$ = 33.9 mm (wet wt @ test start = 0.61 g)	$\bar{X}_{10}$ = 32.8 (wet wt. @ test start = 0.55 g)

Measured snout tip to caudal peduncle following over-dosing with Finquel® + patted dry. — R.J. O'Boyle, 6/29/'90

ACUTE AQUATIC EFFECTS

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14650
United States of America

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SIGNATURE	DATE	REVIEWED BY	DATE
Janice H. Reglinger	6/29/90	R.J. O'Boyle	6/29/'90

ATTACHMENT 6b. Acute Aquatic Effects Data Forms (Study II)

ACCESSION NO	HAEL NO	TEST ARTICLE	SAID NO
901878	90-0016	Crotonaldehyde	Lot No. 90-7

TIME, DATE & INITIALS	TIME	TEST VOLUME 15L			AIM CONCENTRATION 1 mg/l	STARTING CONDITIONS FL, DY
	PROTO -COLI	°C	D.O.	pH	OBSERVATIONS	REMARKS "A"
8:55, 6/25/90	0	12	10.4	8.3	10 N	
9:15, 6/26/90	24	12	10.6	8.3	10 PS (dark color)	
9:20, 6/27/90	48	12	10.5	8.2	6 SAD, WD (dark)	(4D)
8:50, 6/28/90	72	12	10.6	8.3	0 (6D)	Dose discontinued @ 13:00 R.O'Boyle, 6/29/90

TIME, DATE & INITIALS	TIME	TEST VOLUME 15L			AIM CONCENTRATION 1 mg/l	STARTING CONDITIONS FL, DY
	PROTO -COLI	°C	D.O.	pH	OBSERVATIONS	REMARKS "B"
8:55, 6/25/90	0	12	10.4	8.3	10 N	
9:15, 6/26/90	24	12	10.7	8.4	9 PS (1D) (dark color)	
9:20, 6/27/90	48	12	10.6	8.2	5 AD (dark) (5D)	
8:50, 6/28/90	72	12	10.6	8.3	0 (5D)	correction 6/28/90 Dose discontinued @ 13:00 on 6/28/90 - R.O'B., 6/29/90

TIME, DATE & INITIALS	TIME	TEST VOLUME 15L			AIM CONCENTRATION 2 mg/l	STARTING CONDITIONS FL, DY
	PROTO -COLI	°C	D.O.	pH	OBSERVATIONS	REMARKS "A"
9:00, 6/25/90	0	12	10.3	8.3	10 N	clear solution
9:20, 6/26/90	24	12	10.6	8.5	0 (10D)	Dose discontinued @ 13:00 R.O'Boyle, 6/26/90

TIME, DATE & INITIALS	TIME	TEST VOLUME 15L			AIM CONCENTRATION 2 mg/l	STARTING CONDITIONS FL, DY
	PROTO -COLI	°C	D.O.	pH	OBSERVATIONS	REMARKS "B"
9:00, 6/25/90	0	12	10.3	8.3	10 N	clear solution
9:20, 6/26/90	24	12	10.8	8.3	0 (10D)	Dose discontinued @ 13:00 R.O'Boyle, 6/26/90

Abbr. used to describe test organisms' condition

AD - Activity Depressed
AA - Activity Accelerated
PB - Position Bottom
PS - Position Surface
FR - Fragmentation
GD - Gill Rate Depressed
GA - Gill Rate Accelerated
I - Immobilized
N - Normal
NO - Near Death
AO - Abnormal Observations
SH - Shivering
T - Tremors
W - Windrows
M - Mixing
D - Dead

Abbr. used to describe test conditions

AR - Aeration
DY - Dynamic
FL - Fluorescent Lab Lighting
AW - Avg. Wgt. Fish
PRECIP. - Precipitate
RECIPC - Reciprocating
SOLN - Solution
ST - Static
X - Xenon Arc Lighting

ACUTE AQUATIC EFFECTS

TEST ORGANISM: Oncorhynchus mykiss (m)

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14650
United States of America

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SIGNATURE	DATE	REVIEWED BY	DATE
Janice M. Beglinger	6/25/90	R. J. O'Boyle	6/29/90

ATTACHMENT 6b. Acute Aquatic Effects Data Forms (Study II)

ACCESSION NO.	HAEL NO.	TEST ARTICLE	SRIO NO.
901878	90-0016	Crotonaldehyde	Lot No. 90-7

Refer to pp. 2, 4.

ACUTE AQUATIC EFFECTS

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14650
United States of America

PAGE 6 OF 6

SIGNATURE	DATE	REVIEWED BY	DATE
Janice M Belanger	6/29/90	R.J. O'Boyle	6/29/90

Attachment 7. Excerpts from Analytical Summary Report (Study II)

(page 1 of 13)

3HGV580/3HGV581
90-0016

Crotonaldehyde
901878

TESTING FACILITY

Environmental Analytical Services Unit
Chemicals Quality Services Division
Bldg-320
Eastman Kodak Company
Rochester, New York 14652-3615

SPONSOR

Environmental Technical Services
Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652

DATE OF STUDY INITIATION: 03/22/90

DATES OF EXPERIMENT

Experiment Start Date: 06/25/90

Experiment Termination Date: 07/06/90

STUDY PARTICIPANTS

Chemicals Quality Services Division

Barry W. Remington, Laboratory Technician
Elizabeth H. Isaacs, Laboratory Technician

Robert Ambrose, Chemist

STUDY DIRECTOR

R.J. O'Boyle

INTRODUCTION

The Environmental Protection Agency (EPA) is concerned about the potential environmental impact of releases of quantities of Crotonaldehyde into the Holston River at the Kingsport, Tennessee Kodak plant where the test chemical is manufactured. Kodak has volunteered to perform numerous aquatic tests on the test chemical through Testing Consent Order Docket No. OPTS-42108. The second test, a diluent water study, was performed where concentration determinations were carried out using GC/ECD.

Attachment 7. Excerpts from Analytical Summary Report (Study II)

(page 2 of 13)

3HGV580/3HGV581
90-0016

Crotonaldehyde
901878

REQUEST FOR ANALYSIS

Method Validation		03-22-90 03-27-90	148783
Purity		03-20-90	149292
Trout Study Test	Time 0	06-25-90	142463
	Time 24	06-26-90	142463
	Time 48	06-27-90	142463
	Time 72	06-28-90	142463
	Time 96	06-29-90	142463

TEST ARTICLE

Name: Crotonaldehyde
HAEL No.: 90-0016
Accession No.: 901878
Sample Reference No.: 90-7

Attachment 7. Excerpts from Analytical Summary Report (Study II)
(page 3 of 13)

3HGV580/3HGV581
90-0016

Crotonaldehyde
901878

MATERIALS AND METHODS

Title: Analysis of Crotonaldehyde in Diluent Water

Abstract: Samples containing crotonaldehyde were diluted (as necessary), derivatized, extracted and analyzed by gas chromatography with electron capture detection (GC/ECD). The method detection limit was 2 ug/L.

Procedure:

Materials:

1. O-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride (PFBOA), Aldrich c/n 19,448-4
2. Sodium thiosulfate, Aldrich c/n 21,726-3
3. Sulfuric acid, Eastman Chemical c/n 13063
4. Diluent water (B-306)
5. EDTA (Eastman Kodak Company)
6. Distilled water
7. Hexane, Baker Resi-analyzed c/n 9262-03
8. Culture tubes, 16 x 125 mm, Kimble c/n 45066-A
9. Disposable Pasteur pipets, VWR Scientific c/n 14672-200
10. Assorted volumetric flasks (10, 25, 50, 100 and 1000 mL)
11. Volumetric pipet (1 mL)
12. Assorted SMI micropettors (50 to 1000 uL)
12. Capillary gas chromatograph
13. Capillary gas chromatographic column, DB-5, 30 meter, 0.25 um film thickness, J&W Scientific c/n 009-5032
14. Chromatographic data handling system

Attachment 7. Excerpts from Analytical Summary Report (Study II)
(page 4 of 13)

3HGV580/3HGV581
90-0016

Crotonaldehyde
901878

Reagent Preparation:

1. 0.10 g of PFBOA was weighed to a 100.0 mL volumetric flask and diluted it to the mark with distilled water. This solution was prepared fresh daily.

2. 1.58 g of sodium thiosulfate was weighed to a 100.0 mL volumetric flask and diluted to the mark with distilled water. (0.1 M solution). This solution was good for one (1) week.

Standard Preparation:

1. Stock Approximately 0.100 g of test chemical was weighed to a 100.0 mL volumetric flask and diluted to the mark with diluent water , containing EDTA. This solution was prepared fresh daily.

2. Intermediate An 0.5 mL aliquot of the stock solution was taken and diluted to 100.0 mL with diluent water, containing EDTA.

3. Working Up to seven (7) aliquots ranging from 25 to 175 uL of the intermediate solution were taken and the aliquots transferred to separate 25.0 mL volumetric flasks. The flasks were diluted to the mark with diluent water, containing EDTA.

Sample Preparation:

1. All samples were diluted down to 20 ug/L with diluent water, containing EDTA, using at least 25 mL volumetric flasks.

Attachment 7. Excerpts from Analytical Summary Report (Study II)
(page 5 of 13)

3HGV580/3HGV581
90-0016

Crotonaldehyde
901878

Derivatization:

1. One (1.0) mL of the 1 mg/mL PFBOA solution was added to a culture tube.
2. 200 uL of the 0.1 M sodium thiosulfate solution was added to the culture tube and mixed.
3. Ten (10.0) mL of diluent water containing EDTA was added and mixed. Allow to stand for two (2) hours.
4. Steps 1-3 were repeated for all of the working standards.
5. Steps 1-3 were repeated for all samples.

Extraction:

1. One (1) drop of concentrated sulfuric acid was added to each culture tube and mixed.
2. Two (2.0) mL of hexane were added to each culture tube and then shaken for 30 seconds.
3. The culture tubes were allowed to stand for fifteen (15) minutes.
4. Approximately half of the extract was transferred to two (2) separate GC vials and capped.
5. Step 4 was repeated for all samples.

Attachment 7. Excerpts from Analytical Summary Report (Study II)
(page 6 of 13)

3HGV580/3HGV581
90-0016

Crotonaldehyde
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Analyses:

1. The samples were then analyzed by GC/ECD using the following conditions:

Instrument: Perkin-Elmer 8500 Gas Chromatograph

Column: J&W DB-5 30 meter 0.25 um film thickness

Pressure: 10 psig

Oven Program: 80 C (held for 2 min.) to 250 C @ 10 C/min. held @ 250 C for 1 min.

Injector Temp: 250 C

Type: Splitless (2 min. purge delay)

Split Flow: 60 cc/min.

Inj. Volume: 1 uL

Detector: Electron Capture

Temp.: 300 C

Make-up: Nitrogen

Flow: 55 cc/min.

Calculations:

1. Data reduction was performed using a chromatography data handling system such as Multichrom (a VG, Inc. product). The cis-and trans- isomers were added together and treated as one component whenever possible.

Attachment 7. Excerpts from Analytical Summary Report (Study II)
(page 7 of 13)

3HGV580/3HGV581
90-0016

Crotonaldehyde
901878

METHOD VALIDATION

3-27-90

Theoretical Conc. (mg/L)	Actual Conc. (mg/L)	Recovery (%)
0.620 filtered	0.62	99.5
	0.62	101
	0.62	101
0.620 unfiltered	0.63	102
	0.64	103
	0.64	104
2.55 filtered	2.50	98.0
	2.22	87.1
	2.41	94.5
2.55 unfiltered	2.46	96.5
	2.49	97.7
	2.46	96.5
9.97 filtered	9.96	100
	10.1	101
	9.52	95.5
9.97 unfiltered	9.93	99.6
	9.76	97.9
	9.55	95.8

Attachment 7. Excerpts from Analytical Summary Report (Study II)
(page 8 of 13)

3HGV580/3HGV581
90-0016

Crotonaldehyde
901878

METHOD VALIDATION

3-22-90

Theoretical Conc. (mg/L)	Actual Conc. (mg/L)	Recovery (%)
0.70866 filtered	0.61	85.7
	0.67	94.2
	0.66	92.7
0.70866 unfiltered	0.72	101
	0.70	98.3
	0.74	104
2.8575 filtered	4.09	143
	2.85	99.9
	2.81	98.5
2.8575 unfiltered	3.03	106
	3.11	109
	3.10	108
11.43 filtered	19.7	172
	11.4	99.6
	11.5	101
11.43 unfiltered	14.4	126
	12.3	108
	12.8	112

Attachment 7. Excerpts from Analytical Summary Report (Study II)

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3HGV580/3HGV581
90-0016

Crotonaldehyde
901878

TROUT STUDY TEST

Sample	Theor Conc (mg/L)	Time 0 Actual Conc. (mg/L)	Time 24 Actual Conc. (mg/L)	Time 48 Actual Conc. (mg/L)	Time 72 Actual Conc. (mg/L)	Time 96 Actual Conc. (mg/L)
DWC-A	0	<0.005	NA	<0.005	NA	<0.005
DWC-B	0	<0.005	NA	<0.005	NA	<0.005
0.12-A	0.12	0.142	NA	0.137	NA	0.113
0.12-B	0.12	0.136	NA	0.141	NA	0.127
0.25-A	0.25	0.296	NA	0.266	NA	0.246
0.25-B	0.25	0.298	NA	0.263	NA	0.248
0.5-A	0.5	0.578	NA	0.550	NA	>0.9*
0.5-B	0.5	0.544	NA	0.531	NA	>0.9*
1.0-A	1.0	1.10	NA	0.975	0.954	NA
1.0-B	1.0	1.08	NA	1.047	0.921	NA
2.0-A	2.0	2.20	2.16	NA	NA	NA
2.0-B	2.0	2.06	2.05	NA	NA	NA
STOCK-A	20	NA	NA	27.44	NA	NA
STOCK-B	20	NA	NA	27.90	NA	NA
EXT BLK	0	<0.005	<0.005	<0.005	<0.005	<0.005

Method Quantification Limit = 0.005 mg/L

NA = Not Analyzed (Sample not received)
ND = None Detected

* = the heights for the 0.5 mg/L samples are greater than the heights for the highest standard. Therefore, only a greater than number can be reported.

Attachment 7. Excerpts from Analytical Summary Report (Study II)

(page 10 of 13)

3HGV580/3HGV581
90-0015

Crotonaldehyde
901878

PERTINENT MATHEMATICAL EQUATIONS

Calculations of the concentration of the test chemical were done using the linear regression equation for the standards run with each day:

$y = mx + b$ where: y = area
m = slope
x = concentration (ug/L)
b = y-intercept

All samples were corrected using the appropriate dilution factor to obtain the concentration in mg/L.

$$\text{Dilution Factor} = \frac{\text{Total Volume(mL)}}{\text{Aliquoted Volume(mL)}}$$

Scientist Beth Ascacs (signature) Date 8-1-90

Attachment 7. Analytical Summary Report (Study II) (page 11 of 13)

Record of Collection and Analysis of Exposure Solutions at Test Start

RECORD OF COLLECTION AND ANALYSIS OF TEST SOLUTIONS

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14650
United States of America

Protocol No.: EN-421-901878-2 HAEL No.: 90-0016 Accession No.: 901878 Rainbow Trout - 2

Concentration/Replicate	Sample No.	Sampled by			Sample Prepared for Analysis			Sample Analyzed		
		Date	Time	Person	Date	Time	Person	Date	Time	Person
DWC - "A"	1	6/25/90	09:00	R.O'Boyle	6-25-90	10:00am	BD	6-26-90	3:30pm	BD
DWC - "B"	2	↓	↓	↓	↓	↓	↓	↓	↓	↓
0.12 mg/L - "A"	3	↓	↓	↓	↓	↓	↓	↓	↓	↓
0.12 - "B"	4	↓	↓	↓	↓	↓	↓	↓	↓	↓
0.25 - "A"	5	↓	↓	↓	↓	↓	↓	↓	↓	↓
0.25 - "B"	6	↓	↓	↓	↓	↓	↓	↓	↓	↓
0.5 - "A"	7	↓	↓	↓	↓	↓	↓	↓	↓	↓
0.5 - "B"	8	↓	↓	↓	↓	↓	↓	↓	↓	↓
1 - "A"	9	↓	↓	↓	↓	↓	↓	↓	↓	↓
1 - "B"	10	↓	↓	↓	↓	↓	↓	↓	↓	↓
2 - "A"	11	↓	↓	↓	↓	↓	↓	↓	↓	↓
2 ↓O'B. - "B"	12	↓O'B.	↓O'B.	↓O'B.	↓BD	↓BD	↓BD	↓BD	↓BD	↓BD
2 mg/L - "A"	13	6/26/90	08:30	R.O'Boyle	6-26-90	9:00am	BD	6-30-90	1:30pm	BD
2 mg/L - "B"	14	6/26/90	08:30	R.O'Boyle	↓BD	↓BD	BD	↓BD	↓BD	↓BD
(complete mortality @ 24 hours - R.O'Boyle 6/26/90)										

KEY:
DWC - Dilution Water Control
CSC - Carrier Solvent Control

REMARKS: ≈ 50 mL of each exposure solution sampled for analysis @ time "0" hr
- R.O'Boyle 6/25/90

HAEL No. 90-0016 Study No. EN-421-901878-2

Attachment 7. Analytical Summary Report (Study II) (page 12 of 13)
(Record of Collection and Analysis of Exposure Solutions at Time 48 Hours)

RECORD OF COLLECTION AND ANALYSIS OF TEST SOLUTIONS

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14650
United States of America

Protocol No.: EN-421-901878-2 HAEL No.: 90-0016 Accession No.: 901878 Rainbow Trout - 2

Concentration/Replicate	Sample No.	Sampled by			Sample Prepared for Analysis			Sample Analyzed		
		Date	Time	Person	Date	Time	Person	Date	Time	Person
DWC - "A"	15	6/27/90	09:00	R.O'Boyle	6-27-90	10:30am	BD	~~6-30-90~~ 7-1-90 error BD 7-1-90	12:30p	BD
DWC - "B"	16									
0.12 mg/L - "A"	17									
0.12 - "B"	18									
0.25 - "A"	19									
0.25 - "B"	20									
0.5 - "A"	21									
0.5 - "B"	22									
1 - "A"	23									
1 - "B"	24	O'B.	O'B.	O'B.	BD	BD	BD	BD	BD	BD
20mg/L "Fresh" Stock (Sampled @ 48 hr Renewal of Doses)	25	6/27/90	10:00	R.O'Boyle	6-27-90	11:30am	BD	BD	BD	BD
1mg/L - "A"	26	6/28/90	08:45	R.O'Boyle	6-28-90	10:00am	BD	7-5-90	error BD 7-5-90 5:30pm ~~10:00am~~	BD
1mg/L - "B"	27	6/28/90	08:45	R.O'Boyle	BD	BD	BD	BD	BD	BD
(Test end per doses, complete trout mortality)										

"Aged" Solutions

KEY:
DWC - Dilution Water Control
CSC - Carrier Solvent Control

REMARKS: ≈ 50 mL of each exposure solution sampled for analysis @ time 48 hrs. - R.O'Boyle 6/27/90

HAEL No. 90-0016 Study No. EN-421-901878-2

Attachment 7. Analytical Summary Report (Study II) (page 13 of 13)
(Record of Collection and Analysis of Exposure Solutions at Test End)

RECORD OF COLLECTION AND ANALYSIS OF TEST SOLUTIONS

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14650
United States of America

Protocol No.: EN-421-901878-2 HAEL No.: 90-0016 Accession No.: 901878 Rainbow Trout-2

Concentration/Replicate	Sample No.	Sampled by			Sample Prepared for Analysis			Sample Analyzed		
		Date	Time	Person	Date	Time	Person	Date	Time	Person
DWC - "A"	28	6/29/90	09:00	R. O'Boyle	6/29/90	9:30am	BD	7-6-90	2:00pm	BD
DWC - "B"	29	↓	↓	↓	↓	↓	↓	↓	↓	↓
0.12 mg/L - "A"	30	↓	↓	↓	↓	↓	↓	↓	↓	↓
0.12 - "B"	31	↓	↓	↓	↓	↓	↓	↓	↓	↓
0.25 - "A"	32	↓	↓	↓	↓	↓	↓	↓	↓	↓
0.25 - "B"	33	↓	↓	↓	↓	↓	↓	↓	↓	↓
0.5 - "A"	34	↓	↓	↓	↓	↓	↓	↓	↓	↓
0.5 - "B"	35	↓ O'B.	↓ O'B.	↓ O'B.	↓ BD	↓ BD	↓ BD	↓ BD	↓ BD	↓ BD

KEY:
DWC - Dilution Water Control
CSC - Carrier Solvent Control

REMARKS: ≈ 50 mL of each exposure solution sampled for analysis @ time 96 hrs
- R. O'Boyle
6/29/90

ATTACHMENT 8. LC_{50} Calculator Program Methods and Calculations

(page 1 of 3)

a) Non-linear Interpolation between the concentrations that bracket 50% test population mortality gives an approximate point estimate, and the Binomial Method gives a conservative 95% confidence interval. In the case of a test utilizing a single effective concentration, this program, by use of the Binomial Method, is capable of indicating at a conservative 95% confidence interval whether or not the LC_{50} value lies above or below the test concentration.

b) The Moving Average Method gives both a point and interval estimate. This output is included only when two consecutive dose level sets cause death in the test population.

c) The Probit Method gives both a point and interval estimate. This output is included only when there are at least two consecutive sets of partial test population mortality. Additionally, when the number of effective concentrations is at least three, a chi-square statistic is included to test the goodness-of-fit for the Probit Model.

ATTACHMENT 8. LC_{50} Calculator Program Methods and Calculations

(page 2 of 3)

Study I:

Pooled Replicates A & B: 48-Hour Nominal Values

****

CONC.,MG/L	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROB.(PERCENT)
2	20	20	100	LESS THAN 0.001
1	20	13	64.99998	9.462305
.5	20	0	0	LESS THAN 0.001
.25	20	0	0	LESS THAN 0.001
.12	20	0	0	LESS THAN 0.001

THE BINOMIAL TEST SHOWS THAT .5 AND 2 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 0.89 MG/L IS OBTAINED BY NONLINEAR INTERPOLATION BETWEEN .5 AND 1

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.

****

Pooled Replicates A & B: 96-Hour Nominal Values

****

CONC.,MG/L	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROB.(PERCENT)
2	20	20	100	LESS THAN 0.001
1	20	20	100	LESS THAN 0.001
.5	20	0	0	LESS THAN 0.001
.25	20	0	0	LESS THAN 0.001
.12	20	0	0	LESS THAN 0.001

THE BINOMIAL TEST SHOWS THAT .5 AND 1 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 0.71 MG/L IS OBTAINED BY NONLINEAR INTERPOLATION BETWEEN .5 AND 1

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.

****

ATTACHMENT 8. LC_{50} Calculator Program Methods and Calculations

(page 3 of 3)

Study II:

Pooled Replicates A & B; 48-Hour Analyzed Values

CONC.,MG/L	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROB.(PERCENT)
2.1	20	20	100	LESS THAN 0.001
1.1	20	9	45	33.1812
.55	20	1	5	1.04904E-03
.28	20	0	0	LESS THAN 0.001
.14	20	0	0	LESS THAN 0.001

——RESULTS CALCULATED,USING THE PROBIT METHOD——

ITERATIONS	G	H	CHI-SQUARE	PROBABILITY
6	.174787	1	1.46941	.689347

SLOPE = 6.68898
95 PERCENT CONFIDENCE LIMITS = 3.89249 AND 9.48548

LC50 = 1.07619 MG/L
95 PERCENT CONFIDENCE LIMITS = .902373 AND 1.27543

LC1 = .483178 MG/L
95 PERCENT CONFIDENCE LIMITS = .259746 AND .636221

Pooled Replicates A & B: 96-Hour Analyzed Values

(Not accepted as valid due to discounted analyses of middle dose at 96 hours.)

****

CONC.,MG/L	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROB.(PERCENT)
2.1	20	20	100	LESS THAN 0.001
1	20	20	100	LESS THAN 0.001
.67	20	5	25	1.33018
.27	20	0	0	LESS THAN 0.001
.13	20	0	0	LESS THAN 0.001

THE BINOMIAL TEST SHOWS THAT .67 AND 1 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 0.75 MG/L IS OBTAINED BY
NONLINEAR INTERPOLATION BETWEEN .67 AND 1

Pooled Replicates A & B; 96-Hour Analyzed Values (Valid)

(These results are accepted as valid; middle dose is a 48-hour mean value.)

****

CONC.,MG/L	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROB.(PERCENT)
2.1	20	20	100	LESS THAN 0.001
1	20	20	100	LESS THAN 0.001
.55	20	5	25	1.33018
.27	20	0	0	LESS THAN 0.001
.13	20	0	0	LESS THAN 0.001

THE BINOMIAL TEST SHOWS THAT .55 AND 1 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 0.65 MG/L IS OBTAINED BY
NONLINEAR INTERPOLATION BETWEEN .55 AND 1

ATTACHMENT 9. Preliminary Bioassay Data Forms

3/26 - 3/28/90

ACCESSION NO	HAEL NO.	TEST ARTICLE	SRID NO. (Lot No.)
901878	90-0016	Crotonaldehyde	90-7

correction 3/26/90 JB — Methods Development

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 20L °C	D.O.	pH	AIM CONCENTRATION DWC OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS
10:15, 3/26/90, JB	0	12	10.6	8.3	10	N	Avg. wet wt. = 0.59g
15:15, 3/26/90, JB	6	—	—	—	10	N	
9:35, 3/27/90, JB	24	12	10.0	8.1	10	N	
8:45, 3/28/90, JB	48	12	—	—	10	N	

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 20L °C	D.O.	pH	AIM CONCENTRATION 1mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS
10:15, 3/26/90, JB	0	12	10.6	8.2	10	N	
15:15, 3/26/90, JB	6	—	—	—	10	GA/N	
9:45, 3/27/90, JB	24	12	10.2	8.2	10	PS (gills flared)	
8:45, 3/28/90, JB	48	12	10.1	7.9	0	(10D)	

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 20L °C	D.O.	pH	AIM CONCENTRATION 10mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS
10:20, 3/26/90, JB	0	12	10.7	8.4	10	N	
15:15, 3/26/90, JB	6	—	10.6	8.5	0	(10D)	

errors JB 3/26/90

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 20L °C	D.O.	pH	AIM CONCENTRATION 100mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS
10:20, 3/26/90, JB	0	12	10.8	8.4	10	N	
11:50, 3/26/90, JB	1.5	12	—	—	0	(All Dead)	

Abbr. used to describe test organisms' condition: AD - Activity Depressed; AA - Activity Accelerated; PB - Position Bottom; PS - Position Surface; FR - Fragmentation; GD - Gill Rate Depressed; GA - Gill Rate Accelerated; I - Immobilized; N - Normal; ND - Near Death; AO - Abnormal Orientations; SH - Shrivaling; T - Tremors; W - Withdrawn; M - Missing; D - Dead

Abbr. used to describe test conditions: AR - Aeration; DY - Dynamic; FL - Fluorescent Lab Lighting; AW - Avg. Wgt. Fish; PRECIP - Precipitate; RECIRC - Recirculating; SOLN - Solution; ST - Static; X - Xenon Arc Lighting

ACUTE AQUATIC EFFECTS

TEST ORGANISM: Salmo gairdneri Sg

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14650
United States of America

PAGE 1 OF 2

SIGNATURE	DATE	REVIEWED BY	DATE
Janice M. Beylinger	3/29/90	R. J. O'Boyle	3/29/90

Note: Salmo gairdneri was the previous nomenclature for Oncorhynchus mykiss.

ATTACHMENT 9. Preliminary Bioassay Data Forms

3/26 - 3/28/90

ACCESSION NO.	HAEL NO.	TEST ARTICLE	SPID NO. (Lot No.)
901878	90-0016	Crotonaldehyde	90-7

Conclusions: sp. gravity of 90-0016 = 0.85 ∴ 1/0.85 = x1.18*

Doses prepared by diluting 2.6 mL 90-0016 (2.22 g x 1.18* = 2.6 mL) in 22.2 L H_2O = 100 mg/L. Serial dilution of 100 mg/L yielded other doses. Since all were lethal to trout, a nominal dose of 0.1 mg/L will be tested (starting 3/29/90).

– R.J. O'Boyle
3/28/90

ACUTE AQUATIC EFFECTS

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14650
United States of America

PAGE 2 OF 2

SIGNATURE	DATE	REVIEWED BY	DATE
Janice H. Bylinger	3/29/90	R.J. O'Boyle	3/29/90

ATTACHMENT 9. Preliminary Bioassay Data Forms

3/29 - 4/2/90

Methods Development

ACCESSION NO	HAEL NO.	TEST ARTICLE	SRID NO. (Lot No.)
901878	90-0016	Crotonaldehyde	90-7

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 20L °C	D.O.	pH	AIM CONCENTRATION 0.1mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS Pp
8:45, 3/29/90, JB	0	20	8.8	8.0	10	N	Vessels on this page
15:00, 3/29/90, JB	6	—	—	—	10	N	were all loosely covered
8:55, 3/30/90, JB	24	—	—	—	10	N	throughout exposure.
10:00, 3/31/90, O'B	48	—	—	—	10	N	- R.O'Boyle, 4/2/90
11:00, 4/1/90, O'B	72	—	—	—	10	N	
16:00, 4/2/90, JB	96	20	—	—	10	N	

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 20L °C	D.O.	pH	AIM CONCENTRATION 1mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS Pp
8:45, 3/29/90, JB	0	20	8.9	8.3	10	N	
15:00, 3/29/90, JB	6	—	—	—	10	N	
8:55, 3/30/90, JB	24	—	—	—	10	N	
10:00, 3/31/90, O'B	48	—	—	—	10	N	
11:00, 4/1/90, O'B	72	—	—	—	10	N	
16:00, 4/2/90, JB	96	20	—	—	10	N	

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 20L °C	D.O.	pH	AIM CONCENTRATION 10mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS Pp
8:45, 3/29/90, JB	0	20	8.9	8.3	10	N	
15:00, 3/29/90,	6	20	8.8	8.4	0	(10D)	Junction JB 3/29/90

TIME, DATE & INITIALS	TIME (PROTO-COL)	TEST VOLUME 20L °C	D.O.	pH	AIM CONCENTRATION 0.1mg/L OBSERVATIONS		STARTING CONDITIONS ST, FL REMARKS Sg
8:45, 3/29/90, JB	0	12	10.6	8.3	10	N	
15:00, 3/29/90, JB	6	—	—	—	10	N	
8:55, 3/30/90, JB	24	—	—	—	10	N	
10:00, 3/31/90, O'B	48	—	—	—	10	N	
11:00, 4/1/90, O'B	72	—	—	—	10	N	
16:00, 4/2/90, JB	96	12	—	—	10	N	

Error R.O'B. 4/1/90

Abbr. used to describe test organisms' condition
AD - Activity Depressed
AA - Activity Accelerated
PB - Position Bottom
PS - Position Surface
FR - Fragmentation
GD - Gill Rate Depressed
GA - Gill Rate Accelerated
I - Immobilized
N - Normal
NO - Near Death
AO - Abnormal Orientations
SH - Shrivelling
T - Tremors
W - Windmill
M - Missing
D - Dead

Abbr. used to describe test conditions
AR - Aeration
DY - Dynamic
FL - Fluorescent Lab Lighting
AW - Avg. Wgt. Fish
PRECIP. - Precipitate
RECIRC. - Recirculating
SOLK. - Solution
ST - Static
X - Xenon Arc Lighting

ACUTE AQUATIC EFFECTS

TEST ORGANISM: Salmo gairdneri (Sg) Pp Pimephales promelas

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14650
United States of America

PAGE 1 OF 2

SIGNATURE	DATE	REVIEWED BY	DATE
Janice M. Beglinger	4/3/90	R.O'Boyle	4/2/90

Note: Salmo gairdneri was the previous nomenclature for Oncorhynchus mykiss.

ATTACHMENT 9. Preliminary Bioassay Data Forms

3/29 - 4/2/90

ACCESSION NO.	HAEL NO.	TEST ARTICLE	SPID NO. (lot/lb)
901878	90-0016	Crotonaldehyde	90-7

R-F Test Results: (0.26 mL/22.2 L = 10 mg/L, nominal)

Most recent methods development with test article and fathead minnows suggest that 0.6, 1.2, 2.5, 5.0, and 10 mg/L is still an appropriate dose series for definitive test (even though flow through).

In addition, a nominal dose of 0.1 mg/L does not appear to cause adverse effects in rainbow trout. Consequently, the dosing scheme for the definitive test (flow-through) is tentative set at 0.12, 0.25, 0.5, 1.0, + 2.0 mg/L

— R. O'Boyle
4/3/90.

ACUTE AQUATIC EFFECTS

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14650
United States of America

PAGE 2 OF 2

SIGNATURE	DATE	REVIEWED BY	DATE
Janice M. Beglinger	4/3/90	R. O'Boyle	4/2/'90

ATTACHMENT 10. Quality Assurance Unit Statements (page 1 of 2)

ANALYTICAL QUALITY ASSURANCE INSPECTION STATEMENT
(CFR 58.35(B)(7) 792.35(B)(7) 160.35(B)(7))

STUDY: 90-0016-7 STUDY DIRECTOR: OBOYLE, R.
ANALYTICAL DIRECTOR: AMBROSE, R.
ACCESSION NUMBER: 901878
CQS JOB NUMBER: 3HGV501

STUDY TYPE: ACUTE AQUATIC EFFECTS - RAINBOW TROUT

M J Bossarine 9-26-90
(AUDITOR, QUALITY ASSURANCE UNIT) DATE

THIS STUDY WAS INSPECTED BY 1 OR MORE PERSONS OF THE QUALITY ASSURANCE UNIT OF MQAO, EASTMAN KODAK COMPANY, ROCHESTER, N.Y. AND WRITTEN STATUS REPORTS WERE SUBMITTED ON THE FOLLOWING DATES:

INSPECT DATES	REQUEST NUMBER	PHASE(S) INSPECTED	STATUS REPORT DATES
06/07/90	142470	PURITY TEST REPORT INSPECTION	05/30/90
08/29/90	142463	DOSE VERIFICATION TEST REPORT INSPECTION TIMES 0, 24, 48, 72, AND 96 METHOD VALIDATION (REQUEST NUMBER 148783)	08/29/90
09/21/90	142464	PURITY TEST REPORT INSPECTION	09/21/90

ATTACHMENT 10. Quality Assurance Unit Statements (page 2 of 2)

Q.A. INSPECTION STATEMENT
(CFR 58.35(B)(7) 792.35(B)(7) 160.35(B)(7))

STUDY: 90-0016-1 STUDY DIRECTOR: O'BOYLE,R.J.
ACCESSION NUMBER: 901878

PAGE 1
09/26/90

STUDY TYPE: ACUTE AQUATIC EFFECTS (SPECIAL)

M. Susan James 9/26/90
(AUDITOR, QUALITY ASSURANCE UNIT) DATE

TO THE BEST OF MY KNOWLEDGE, THIS FINAL REPORT ACCURATELY DESCRIBES THE METHODS AND STANDARD OPERATING PROCEDURES, AND THE REPORTED RESULTS ACCURATELY REFLECT THE RAW DATA. THIS STUDY WAS INSPECTED BY 1 OR MORE PERSONS OF THE QUALITY ASSURANCE UNIT OF THE HAEL, EASTMAN KODAK COMPANY, ROCHESTER, N.Y. AND WRITTEN STATUS REPORTS WERE SUBMITTED ON THE FOLLOWING DATES:

INSPECTION DATES	PHASE(S) INSPECTED	STATUS REPORT DATES
04/18/90	PROTOCOL SUBMISSION	
04/30/90	SAMPLE COLLECTION	09/26/90
	SAMPLE ANALYSIS	
	TEST SYSTEM RANDOMIZATION	
	TEST SYSTEM ADDITION TO CONTROL ARTICLE	
	TEST SYSTEM ADDITION TO TEST ARTICLE-CARRIER-MIXTURE	
	SAMPLES SENT TO CQSD FOR ANALYSIS	
05/17/90	PROTOCOL AMENDMENT	
	AMENDMENT OF 5/3/90 RECEIVED	
06/22/90	PROTOCOL SUBMISSION	
06/25/90	INSTRUMENT CALIBRATION INSPECTION	09/26/90
	TEST ARTICLE-CARRIER-MIXTURE PREPARATION	
	PH, TEMPERATURE, AND DISSOLVED OXYGEN READINGS	
	TEST SYSTEM ADDITION TO CONTROL ARTICLE	
	TEST SYSTEM ADDITION TO TEST ARTICLE-CARRIER-MIXTURE	
	SAMPLE COLLECTION	
	DATA RECORDING	
	DO, PH AND THERMOMETER CALIBRATED	
	FLOW RATE CALIBRATION	
	0 HR SAMPLE SENT TO CQSD FOR ANALYSIS	
06/28/90	CLINICAL SIGNS AT 72 HRS.	09/26/90
	PH, TEMPERATURE, AND DISSOLVED OXYGEN READINGS	
	SAMPLE COLLECTION	
	72 HR TEST ARTICLE SAMPLES COLLECTED FROM 1 MG/L DOSE	
09/26/90	FINAL REPORT REVIEW	09/26/90

HAEL No. 90-0016

Study No. EN-421-901878-2

DIS No.: 263598G

Prepared by Janice M. Beglinger
Janice M. Beglinger
Principal Investigator-Biology
Eco-Chem Testing Group

Date 9/25/90

Approved by Joseph W. Gorsuch
Joseph W. Gorsuch
Group Leader
Eco-Chem Testing Group

Date 9/25/90

Approved by John R. Owen
John R. Owen
Unit Director
Environmental Sciences Section

Date 9/25/90

Reviewed by M. Susan James
Quality Assurance Unit
Health and Environment Laboratories

Date 9/26/90

Prepared by R.J. O'Boyle
Robert J. O'Boyle
Study Director
Eco-Chem Testing Group

Date 10/4/'90

Approved by Roderick D. Gerwe
Roderick D. Gerwe
Sponsor
Eastman Chemical Company

Date 9/28/90

4087e

Study No. EN-1050-901878-1

ES-90-032

263601B

EPA/TSCA ALGAL ACUTE TOXICITY TEST

(using the Federal Register Guideline 40 CFR 797.1050)

OF

Crotonaldehyde

HAEL No. 90-0016 Accession No. 901878

CAS Reg. No. 4170-30-3

By

Heather M. Watson

Environmental Sciences Section

Health and Environment Laboratories

Eastman Kodak Company

Rochester, New York 14652-3617

Completion Date: August 15, 1990

HAEL No. 90-0016

Study No. EN-1050-901878-1

EPA/TSCA Algal Acute Toxicity Test of Crotonaldehyde

Conducted in Accordance with the Federal Register Guideline 40 CFR 797.1050 with the May 20, 1987 Amendments.

STATEMENT OF GLP COMPLIANCE

To the best of my knowledge and belief, these procedures were conducted in compliance with all Good Laboratory Practice standards of 40 CFR Part 792, November 29, 1983 (revised August 17, 1989). No unforeseen circumstances were observed that might have affected the quality or integrity of these procedures.

Study Director: Heather M. Watson — 7-19-90 Date

Heather M. Watson
Eco-Chem Testing Group
Environmental Sciences Section
Health and Environment Laboratories
Eastman Kodak Company
Rochester, NY 14652-3617

Sponsor: Roderick D. Gerwe — 8-19-90 Date

Roderick D. Gerwe
Tennessee Eastman Company
Kingsport, TN 37662

HAEL No. 90-0016 Study No. EN-1050-901878-1

EPA/TSCA Algal Acute Toxicity Test of Crotonaldehyde

Conducted in Accordance with the Federal Register Guideline
40 CFR 797.1050 with the May 20, 1987 Amendments.

Abstract

A 96-hour algal acute toxicity test (Federal Register Guideline 40 CFR 797.1050 with Amendments of 5-20-87 and the November 7, 1989 Consent Agreement with the EPA) was performed on cultures of Selenastrum capricornutum maintained at 24°C with mean illumination of 404 foot-candles. Cultures were exposed to six concentrations of the test chemical over several algal generations with three replicate test flasks per concentration. Owing to the volatility of the test substance, a closed system was used instead of the usual foam-stoppered flasks. Four sets of flasks were used so that each set could be discarded after flasks had been opened to make analyses. Cell counts were made at 24-hour intervals using a Coulter Counter, calibrated by performing a microscopic count with a hemacytometer, and growth curves were plotted from these data. Computer graphs were generated from the cell count data using the "SAS User's Guide: Statistics" to plot the percent inhibition versus the log concentration and to derive EC_x values and their 95% confidence limits.

The 96-hour EC_x values were as follows: EC_{10}, <0.385 mg/L; EC_{50}, <0.881 mg/L; and EC_{90}, <1.982 mg/L. The EC_{50} value was 8.492 mg/L at 24 hours, 0.646 mg/L at 48 hours, and 0.597 mg/L at 72 hours. These values are based on the mean measured concentrations for each time period except that some final values were below the detection limit of the analytical method; in these cases the value of the lower limit of detection was used. Microscopic observations showed that the test chemical did not cause changes in cell size, shape, or color and did not cause flocculation or aggregation of algal cells. The test substance was algicidal, however, after exposure for 96 hours to the two high doses (starting concentrations of 10.6 and 5.2 mg/L): cells removed from these flasks and placed into fresh medium did not resume growth even after 17 days in culture.

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HAEL No. 90-0016

Study No. EN-1050-901878-1

Testing Facility

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652-3617
USA

Sponsor

Tennessee Eastman Company
Kingsport, TN 37662
USA

Date of Study Initiation

February 1, 1990

Date of Study Completion

August 15, 1990

Dates of Experiment

Volatility Pre-test Start Date: 2-12-90	Termination Date: 2-16-90
Range-Finding Test Start Date: 3-19-90	Termination Date: 3-23-90
Definitive Test Start Date: 4-2-90	Termination Date: 4-6-90
Sub-culturing Start Date: 4-6-90	Termination Date: 4-23-90

Study Participants

Environmental Sciences Section:

J.W. Gorsuch, Group Leader, Eco-Chem Testing Group
M.M. McWilliams, Technician, Eco-Chem Testing Group
J.R. Owen, Unit Director, Environmental Sciences Section
H.M. Watson, Principal Investigator and Study Director, Eco-Chem Testing Group

Chemicals Quality Services Division (CQSD) Participants:

R.A. Ambrose
E.M. Isaacs
B.W. Remington
K.A. Robillard
L.J. Wieszczyk

Quality Technology and Applied Statistics (QTS) Participant:

M.L. Ritter

Test Guideline

Federal Register Guideline 40 CFR 797.1050[1] with the May 20, 1987 Amendments[2] and the November 7, 1989 Consent Agreement with the EPA.

Purpose/Objective

The purpose of this test is to determine the effects of a chemical substance on the growth of a unicellular green algal species. The effect on algal growth in relation to an untreated control culture is determined over a 96-hour period. Exponentially growing cultures of algae are exposed to various concentrations of the test substance over several algal generations under defined conditions of temperature, light, and culture medium. The concentration range in which effects are likely to occur is determined on the basis of results from an earlier range-finding test, unless these data are already available. Algal cells are counted using a hemacytometer and a Coulter Counter. Results are expressed in terms of the 96-hour EC_x values, where x = 10, 50, or 90. The EC_x value is defined as the experimentally derived chemical concentration that is calculated to effect X% of the test criterion. Values are also determined at 24, 48, and 72 hours when sufficient data have been generated.

Test Substance

Identity: Crotonaldehyde

Lot No.: 90-7

Sample Laboratory No.: 90-0016

Composition: Determined by CQSD (Attachment 4)

Purity: Initial, 98.1%; final, 99.5% (area) by HPLC

Stability (determined by CQSD (Attachment 4):

Shelf-life stability was provided by storing and removing test substance under nitrogen or argon; stability was confirmed by repeating the purity assay after the start of testing.

Reference Substance

No reference substance was used.

Materials and Methods

The details described here are documented in the data sheets (Attachment 1).

A. Test System

The test alga, Selenastrum capricornutum ATCC 22662, was obtained from the American Type Culture Collection, Rockville, Maryland on 11-11-87 and was

subsequently cultured in our laboratory. A 5-day culture, passage no. 5 in liquid algal medium (Table 1), was used as the source of test inoculum. Several previous passages were performed in order to confirm exponential growth under the conditions of the test.

A Range-Finding Test was conducted using two replicates per dose and the results were used to derive the concentrations to be tested in the Definitive Test (Figure 6).

B. Preparation of Test Solutions

Sterile algal Medium (Table 1) was prepared using high quality distilled water. Six concentrations of the test chemical were prepared, starting from a stock solution. Test chemical (105 mg) was dissolved in approximately 900 mL of sterile algal medium and the solution was brought to 1.0 L with sterile algal medium resulting in a 105 mg/L stock solution. The pH was adjusted to 7.5 ± 0.1 using 0.1 N NaOH. Serial dilutions were performed from the stock solution using sterile algal medium to achieve six nominal dose concentrations of 10, 5, 2.5, 1.25, 0.625, and 0.1 mg/L (Attachment 1, p 39). Dilutions were performed aseptically.

C. Test Procedure

All steps in the procedure were carried out aseptically. Owing to the volatility of the test substance, four times as many flasks as usual were needed so that at each analysis time, the flasks just opened for analysis could be discarded. A closed system was used instead of foam-stoppered flasks. Sterile 250-mL Erlenmeyer flasks were conditioned by adding 1-2 mL of the appropriate test solution, swirling to coat the flask, and then removing the solution by pipet. A total of 84 flasks were prepared. Twelve flasks were conditioned for each of the 6 test concentrations and twelve flasks were conditioned for the negative controls (using algal medium). Chemical controls were not needed because the results of the work done in conjunction with the Range-Finding test had shown no significant adsorption of the test substance to the algae (concentrations in flasks with algae were similar to concentrations in chemical controls without algae by the end of the 96-hour test period - Attachment 2, p 74).

A hemacytometer count was made to determine the number of algal cells in the Erlenmeyer flask serving as inoculum source for the test. Enough cells were inoculated into each test chemical solution and into the negative control solution to give a cell concentration of 10^4 cells/mL. Sterile 100-mL volumetric flasks were used to dispense inoculated solutions into the appropriate sets of conditioned flasks. The flasks were sealed and transferred to the incubators in random order[8] where they were maintained according to Protocol at a temperature of 24 ± 2°C and shaken at approximately 100 rpm. A mean illumination of approximately 400 ft-candles was maintained. Flasks were rotated randomly at 24-hour intervals.

D. Test Measurements

1. Culture conditions: temperature, light intensity and rpm values were recorded at test start and on each analysis day (24, 48, 72, and 96 hours from the start of the test).
2. The pH of solutions was measured at the start and end of the test.
3. Concentration determinations were made by CQSD (Attachment 2, p 75) on each analysis day after pooling the triplicate samples from each dose (the pooling procedure was evaluated earlier and proved acceptable, see Attachment 2, p 72).
4. Cell concentrations were measured on each analysis day. Flasks were swirled to achieve a uniform cell suspension, 2 mL were removed from each of the three negative controls to prepare a composite sample for calibration of the Coulter Counter, and a further amount was removed for counting cell numbers in each flask. A hemacytometer count was performed initially to calibrate the Coulter Counter which was then used to make all subsequent cell counts.
5. Algistatic versus algicidal effects were evaluated by taking 0.5 mL aliquots containing algae from the two high doses (in which growth was maximally inhibited) and placing them in flasks containing 100 mL of fresh medium. This subculture was observed at 3, 9, and 17 days for evidence of growth.

E. Data Evaluation and Interpretation

The measured cell concentrations in the test cultures and controls were

tabulated, together with the concentrations of the test substance and the time of measurement. Lotus 1-2-3® was used for data calculations and tabulations including those for calibrating the Coulter Counter (i.e., deriving a linear regression from the calibration data and using this to correct test count values). The mean value of the cell concentration for each test substance concentration and for the controls was plotted against time to produce growth curves. Graphs were also plotted to show concentration values and their percent loss over time.

Computer graphs were generated from the cell count data using the SAS User's Guide: Statistics[7] to plot the percent inhibition versus the log concentration and to derive EC_x values and their 95% confidence limits (see Calculations). The statistical procedure used to analyze the data was performed by QTS (Attachment 3, p 83).

F. Calculations

EC_{50} values for the algal toxicity test can be estimated by regression analysis using the PROC REG procedure in the SAS software package[7]. The independent variable is the concentration of the test compound, and the dependent variable is defined as:

$$P = 100(C - \bar{Y})/\bar{C}$$

where Y = the measured dry weight or cell density for a particular treatment replicate,

$\bar{Y}$ = mean Y,

$\bar{C}$ = the mean measurement of the control replicates, and

P = the percent difference from the control mean for the particular treatment replicate.

The data analysis begins with a plot of P against the concentration of the test compound. Outliers can often be identified from this plot, and they should be investigated and reported. Also, concentrations at the extreme left or right with flat response can be identified for exclusion from the model fitting procedure. Using the remaining data, an appropriate regression model

(usually a linear or quadratic model will suffice) is fit to the data. Sometimes a transformer of the concentration values may be necessary, especially when the concentrations are spaced an order of magnitude apart. The regression analysis produces a fitted model or equation:

$$P = a + b(CONC) \qquad \text{(linear model)}$$

or

$$P = a + b(CONC) + c(CONC)^2 \qquad \text{(quadratic model)}$$

The EC_x is estimated by substituting X for P and solving for CONC, and 95% confidence limits for the EC_x are obtained graphically from a plot of the 95% confidence bounds for the regression line. The confidence bounds produced by the regression analysis must be adjusted to account for the uncertainty in the control mean. This can be accomplished by applying an adjustment factor F to the variance of the regression residuals. F is derived as follows:

$$\begin{aligned}
Var(P\ CONC) &= 100^2\ Var(Y/\bar{C}) \\
&= 100^2\ [Var(Y)/\bar{C}^2 + Y^2\ Var(\bar{C})/\bar{C}^4] \quad \text{(by error propagation)} \\
&= 100^2\ [Var(Y)/\bar{C}^2 + (1-P/100)^2\ Var(\bar{C})/\bar{C}^2] \\
&= 100^2\ [Var(Y)/\bar{C}^2 + (1-P/100)^2\ Var(Y)/(n\bar{C}^2)] \quad \text{(assuming that } Var(C) = Var(Y)\text{)} \\
&= [100^2\ Var(Y)/\bar{C}^2]\ [1 + (1-P/100)^2/n]
\end{aligned}$$

where n is the number of replicates for the control mean.

The first bracketed term is estimated by the variance of the regression residuals when the model fits. The second term is the adjustment factor F, which varies depending upon the predicted value P at each value of the concentration of the test compound.

Quality Assurance Monitoring

See the report of Quality Assurance Monitoring (Attachment 5, p 106-109).

Good Laboratory Practice Statement

This study was conducted according to: Good Laboratory Practice Regulations for Nonclinical Laboratory Studies as promulgated by the Food and Drug Administration; 21 CFR Part 58, December 22, 1978 (revised September 4, 1987); Environmental Protection Agency Good Laboratory Practice Standard 40 CFR Part 792, November 29, 1983 (revised August 17, 1989); and Organization of Economic Cooperation and Development Principles of Good Laboratory Practice specified in Annex 2 of the OECD Guidelines for Testing of Chemicals [C(81) 30(Final)] as required by Council Directive 89/569/EEC (October 28, 1989).

Data Storage and Records Retention

All raw data and summaries of data will be retained for at least ten years by the Health and Environment Laboratories, Eastman Kodak Company, Rochester, New York 14652-3617.

Results

Definitive Test growth curves are shown in Figure 1. The graphs of Percent Inhibition vs % Log Concentration (with 95% confidence limits) are shown in Figures 2-5. The Range-Finding Test graph of Nominal Concentration vs % Inhibition is shown in Figure 6 and the graphs showing definitive test concentrations and percent losses over time in Figure 7. The Range-Finding and Definitive Test Data Worksheets are found in Attachment 1, the chemical concentration report in Attachment 2, and the statistical analysis in Attachment 3.

The pH value for the algal medium was 7.5 ± 0.1. Starting pH values for the test and control solutions were within the range 7.1 to 7.3. By the end of the test, pH values were in the range 6.8 - 10.1 (Attachment 1, p 40). A mean temperature of 24°C was maintained (range 24-25°C); mean illumination was 404 foot-candles (range 396-411), and rpm were 100 (range 100-101), see Attachment 1, p 32-34.

The cell count results show cells in the control flasks exhibiting log

growth, increasing from 10^4 cells/mL at time 0 to a mean of 5.05×10^5 cells/mL at 96 hours (Attachment 1, p 55). The 96-hour EC_x values with their upper and lower confidence limits were estimated to be: EC_{10}, <0.385 mg/L (0.447 - 0.316); EC_{50}, <0.881 mg/L (1.00 - 0.794); EC_{90}, <1.982 mg/L (2.754 - 1.905). EC_{50} values at 24, 48, and 72 hours were 8.492 mg/L, 0.646 mg/L, and 0.597 mg/L, respectively.

Microscopic observations showed that the test chemical did not cause changes in cell size, shape, or color and did not cause flocculation or aggregation of algal cells. Growth was not observed, however, in the subcultures from the two top doses which had been diluted from final test concentrations of 5.7 and 0.56 mg/L to no effect levels.

There was a considerable decrease in dose concentrations over the 96-hour test period (Figure 7 and Attachment 1, p 56). Measured starting concentrations were 10.6, 5.2, 2.7, 1.2, 0.6, and 0.1 mg/L. At 96 hours these values had dropped to 5.7, 0.6, <0.25, <0.090, <0.062 and <0.01 mg/L, respectively (the four low values were below the limit of detection of the analytical method - see Attachment 2, p 75).

Test Validity

Logarithmic growth was observed in the controls. This meets the criterion for a valid test.

Protocol Deviations

None.

Discussion

Crotonaldehyde was highly volatile under the conditions of this test. This was first determined in a pre-test (Attachment 1, p 26 - 28 and Attachment 2, p 73) and is the reason that the algal test was performed in a closed system. Even in a closed system, however, a considerable loss of starting material took place. Greater percentage losses occurred at low doses than at high ones (Figure 7). For example, at 72 hours a loss of 92% of crotonaldehyde was observed from a starting concentration of 1.2 mg/L compared to a loss of 45%

at the highest dose tested, 10.6 mg/L, over the same time period. By the end of the test four of the six doses were below the detection limit of the analytical method (Attachment 1, p 56 and Attachment 2, p 75). In order to calculate the EC_x values the lower limit of detection was used as the final concentration. The value of the lower limit of detection was averaged with the measured, time 0 concentration to give an estimated mean concentration over the period of the test. For this reason and because the statistical method needed a minimum of three measured doses, 96-hour EC_X values were reported as <x mg/L.

Data interpretation was complicated due to a combination of three factors: in addition to the high volatility/low concentration difficulty mentioned above, there was considerable test substance toxicity, and third, the use of a closed system limits algal growth due to an insufficiency of carbon dioxide needed to support high numbers of algal cells. As cell numbers increase and the available CO_2 is depleted, the pH in the test vessels rises. For example, the controls (with the highest cell concentration), had a final pH of 10.1 (Attachment 1, p 40). After 96 hours, algal cells in a closed system may number only about 4-5 x 10^5 cells/mL compared to 3-4 x 10^6 cells/mL in a normal foam-stoppered system. This makes it difficult to distinguish between carbon-dioxide-limited growth and toxicity-limited growth. The problem of interpretation can be seen by careful analysis of the growth curves (Figure 1) which seem to fall into three distinct patterns. At the three low doses (labelled Conc. #4, Conc. #5, and Conc. #6 on the growth curves), quite similar numbers of cells are present at 96 hours compared to the controls and therefore both toxicity and CO_2 depletion may be causing effects. This is complicated, however, by the fact that Conc. #6 was below the detection limit of the analytical method at 48 hours, Conc. #5 at 72 hours, and Conc. #4 at 96 hours. It seems likely that for these low doses, the effect of toxicity diminished over time due to volatilization while the importance of CO_2 limitation increased over time. Another pattern is evident in the growth curve for Conc. #3. It is unusual in that growth plateaus between 24 and 72 hours but then resumes, possibly because enough crotonaldehde had

volatilized by 72 hours, with a resulting lessening of toxicity. This indicates an algistatic effect at a starting concentration of 2.7 mg/L. There appeared to be relatively few cells present in these test vessels so that CO_2 depletion should not be a factor. The third type of pattern can be seen at the two high doses (labelled Conc. #1 and Conc. #2 on the growth curves). Some growth takes place but plateaus at 24 hours with a low number of cells. Again, toxicity rather than lack of CO_2 appears to be the mechanism. In addition, lack of renewed algal growth in the two high-dose subcultures indicates that crotonaldehyde was algicidal after a 96-hour exposure to starting concentrations of 10.6 and 5.2 mg/L. In the environment, however, it seems highly unlikely that crotonaldehyde would remain in solution at algicidal concentrations due to its high volatility.

Conclusion

The 96-hour EC_x values for crotonaldehyde were as follows: EC_{10}, <0.385 mg/L; EC_{50}, <0.881 mg/L; and EC_{90}, <1.982 mg/L. The EC_{50} value was 8.492 mg/L at 24 hours, 0.646 mg/L at 48 hours, and 0.597 mg/L at 72 hours. Crotonaldehyde was algicidal at 10.6 and 5.2 mg/L.

References

1. Algal Acute Toxicity Test, Guideline 40 CFR 797.1050, Federal Register, 50, 188, (September 27, 1985).
2. Amendments to Algal Acute Toxicity Test, Guideline 40 CFR 797.1050, Federal Register, 52, 97, (May 20, 1987).
3. Freshwater Algae Acute Toxicity Test, Guideline 40 CFR 797.1060, Federal Register, 50, 188, (September 27, 1985).
4. U.S. EPA: Algal Assay Procedure: Bottle Test, National Environmental Research Center, Corvallis, Oregon (1971).
5. U.S. EPA: The Selenastrum capricornutum Printz Algal Assay Bottle Test, EPA-600/9-78-018 (July 1978).
6. American Society for Quality Control, in Glossary and Tables for Statistical Quality Control, pp. 84-85.

7. SAS Institute Inc. SAS User's Guide: Statistics, 1982 Edition. Cary, NC: SAS Institute Inc., (1982).

8. American Society for Quality Control, in Glossary and Tables for Statistical Quality Control, pp. 84-85.

Table 1. Composition of Algal Medium and Stock Solutions[1]

Stock Solutions	Test Conc. (per 1.0 L)	Stock Conc. (per 500 mL)
I. $NaNO_3$ (sodium nitrate)	25.500 mg	12.75 g
II. $MgCl_2 \cdot 6H_2O$ (magnesium chloride)	12.164 mg	6.082 g
III. $CaCl_2 \cdot 2H_2O$ (calcium chloride)	4.410 mg	2.205 g
IV. Micronutrients		
H_3BO_3 (boric acid)	185.520 µg	92.760 mg
$MnCl_2 \cdot 4H_2O$ (manganese chloride)	415.610 µg	207.690 mg
$ZnCl_2$ (zinc chloride)	3.271 µg	1.635 mg
$FeCl_3 \cdot 6H_2O$ (ferric chloride)	160.000 µg	79.880 mg
$CoCl_2 \cdot 6H_2O$ (cobalt chloride)	1.428 µg	0.714 mg
$NaMoO_4 \cdot 2H_2O$ (sodium molybdate)	7.260 µg	3.630 mg
$CuCl_2 \cdot 2H_2O$ (cupric chloride)	0.012 µg	0.006 mg
$Na_2EDTA \cdot 2H_2O$ (disodium EDTA)[2]	300.000 µg	150.000 mg
V. $MgSO_4 \cdot 7H_2O$ (magnesium sulphate)	14.700 mg	7.350 g
VI. K_2HPO_4 (potassium phosphate)	1.044 mg	0.522 g
VII. $NaHCO_3$ (sodium bicarbonate)	15.000 mg	7.500 g

Solution Preparation

Stock solutions I-VII may be made up in advance, filter-sterilized through 0.45 µm membranes (or autoclaved), and refrigerated until needed (for a period of 6 months). If desired, reagent salts I-IV can be combined into one 500 mL solution.

Algal medium is made up by adding 1.0 mL of each stock solution in the order given to approximately 900 mL of distilled water and diluting to 1.0 L. The pH is adjusted to 7.5 ± 0.1 using 0.1 N NaOH or HCl and the medium is then sterilized as before.

(1) U.S. EPA: The *Selenastrum capricornutum* Printz Algal Assay Bottle Test, EPA-600/9-78-018 (July 1978).

Table 2. Summary of EC_x Values[1]

Time (hours)	Effect EC_x	Concentration (mg/L)	Upper 95% Conf. Limit	Lower 95% Conf. Limit	Regression Model Used
24	EC_{10}	0.282	0.363	0.199	linear
	EC_{50}	8.492	>10.00	6.026	linear
	EC_{90}	258.7	ne[2]	ne[2]	linear
48	EC_{10}	0.045	0.076	0.025	linear
	EC_{50}	0.646	0.871	0.468	linear
	EC_{90}	9.247	>10.00	5.750	linear
72	EC_{10}	0.089	0.126	0.056	linear
	EC_{50}	0.597	0.794	0.457	linear
	EC_{90}	4.027	6.310	2.818	linear
96	EC_{10}	<0.385	0.447	0.316	cubic
	EC_{50}	<0.881	1.000	0.794	cubic
	EC_{90}	<1.982	2.754	1.905	cubic

Notes

(1) x = 10, 50, or 90

(2) ne = not estimable from graph

Figure 1. Definitive Test Growth Curves

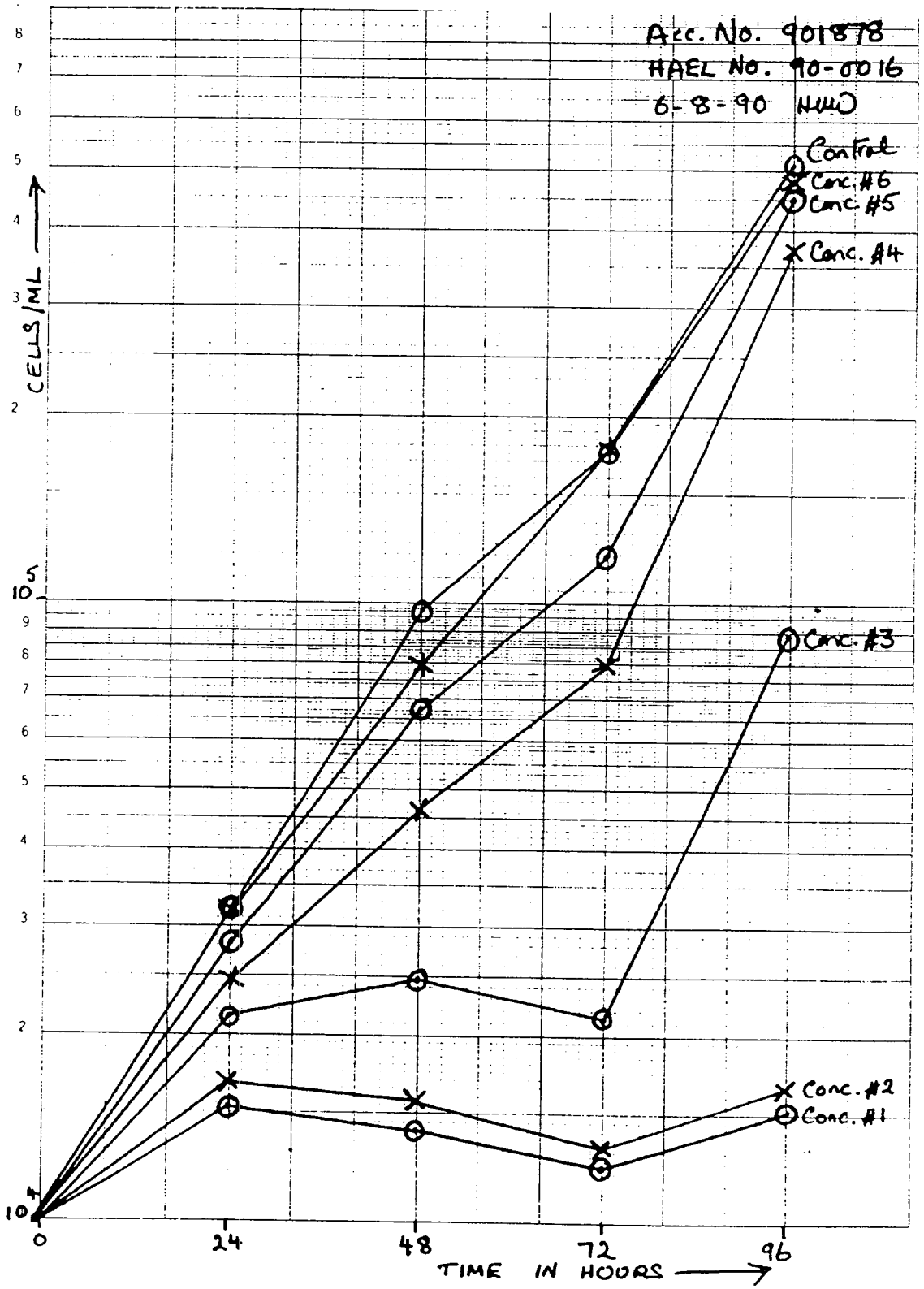


Figure 2. 24-Hour Definitive Test Graph (Fitted Linear Model)

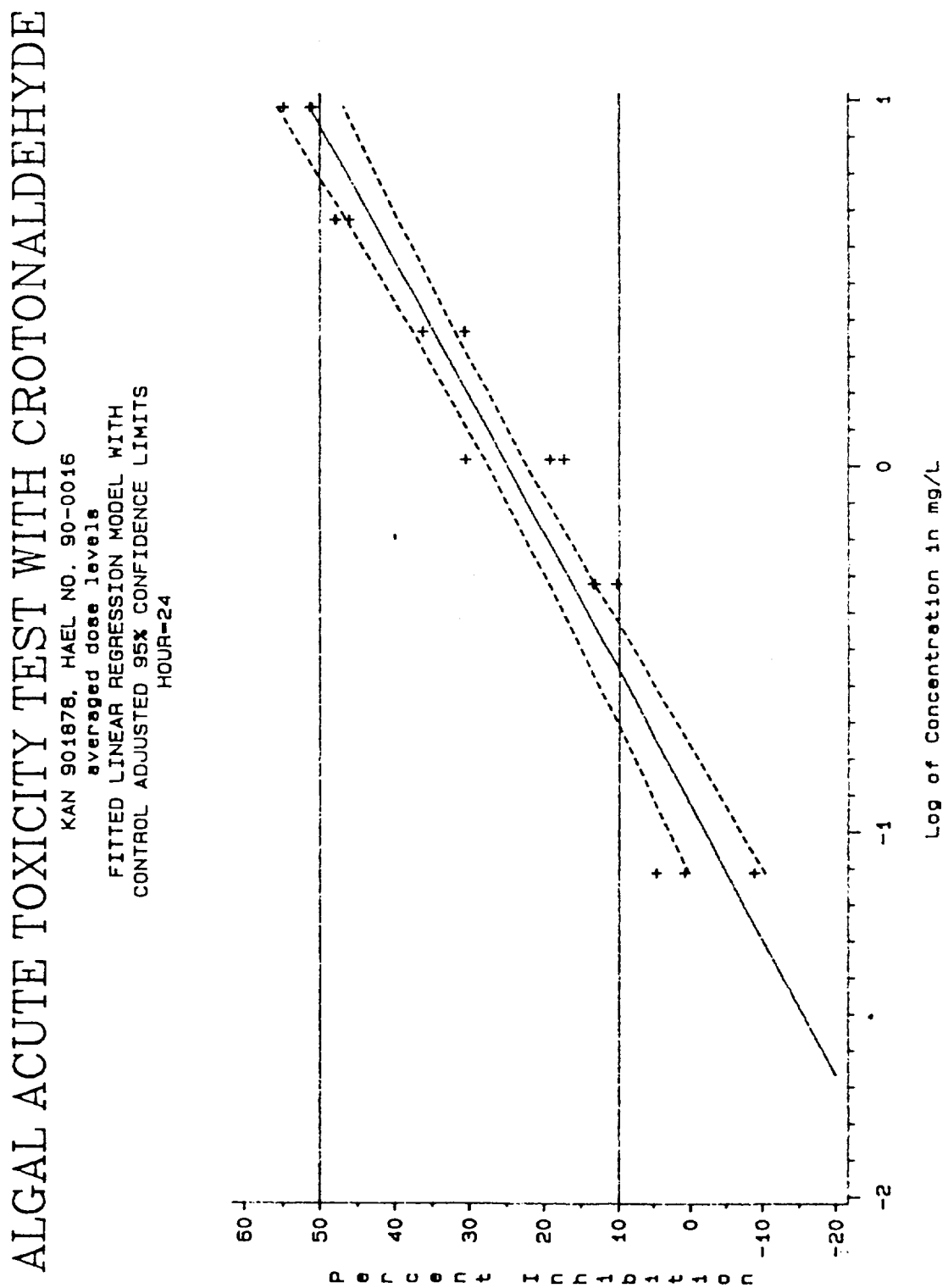


Figure 3. 48-Hour Definitive Test Graph (Fitted Linear Model)

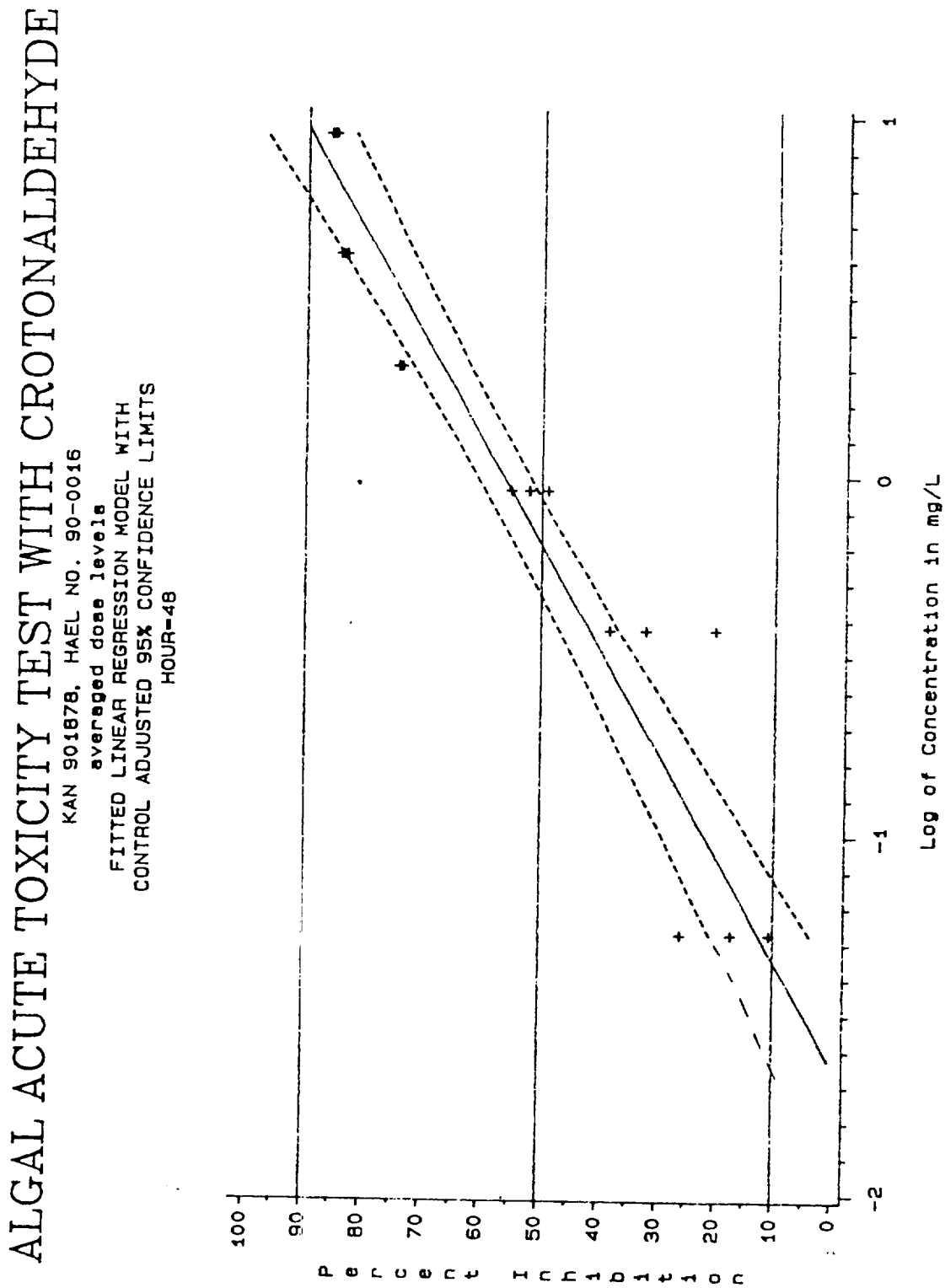


Figure 4. 72-Hour Definitive Test Graph (Fitted Linear Model)

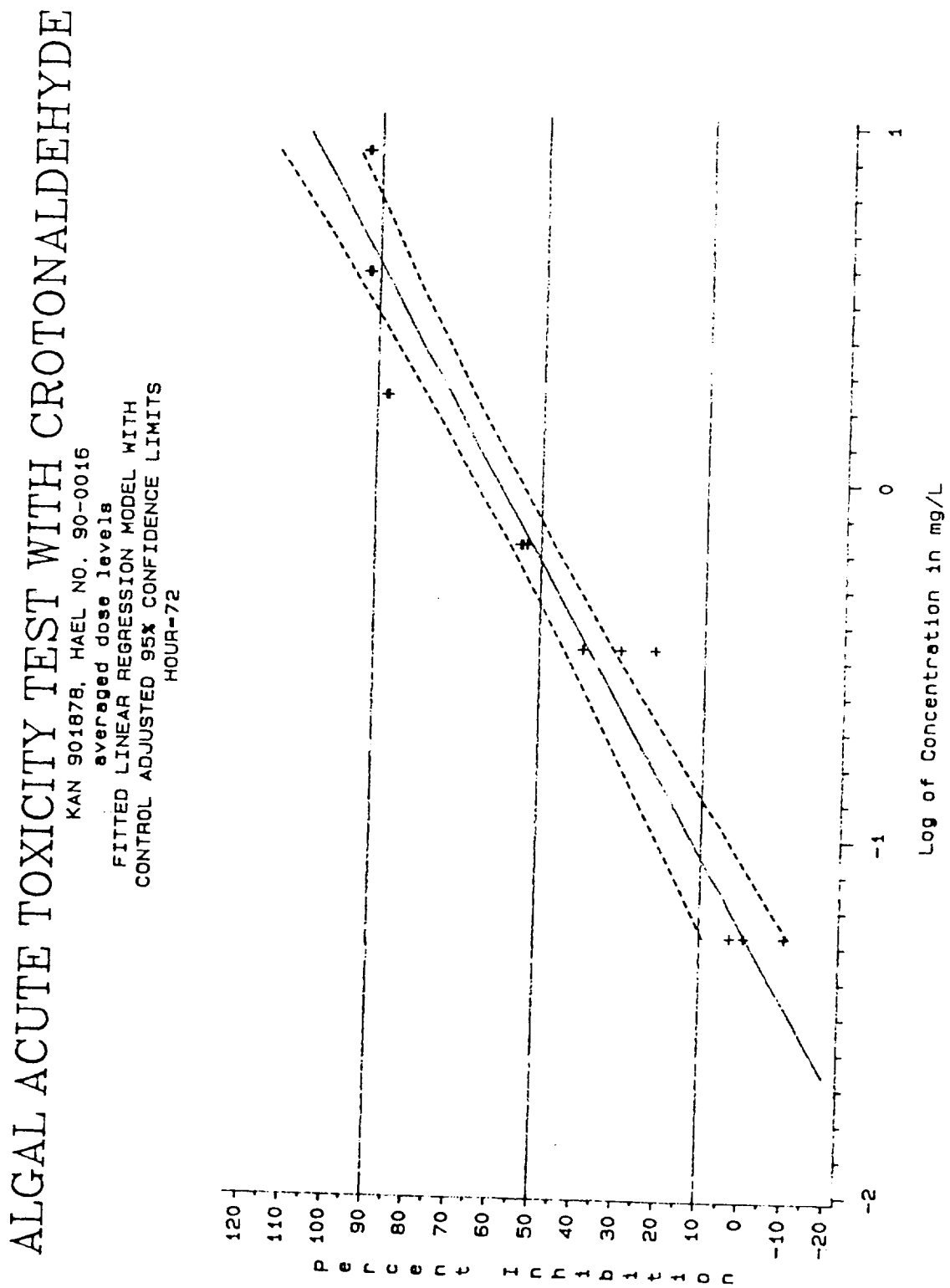


Figure 5. 96-Hour Definitive Test Graph (Fitted Cubic Model)

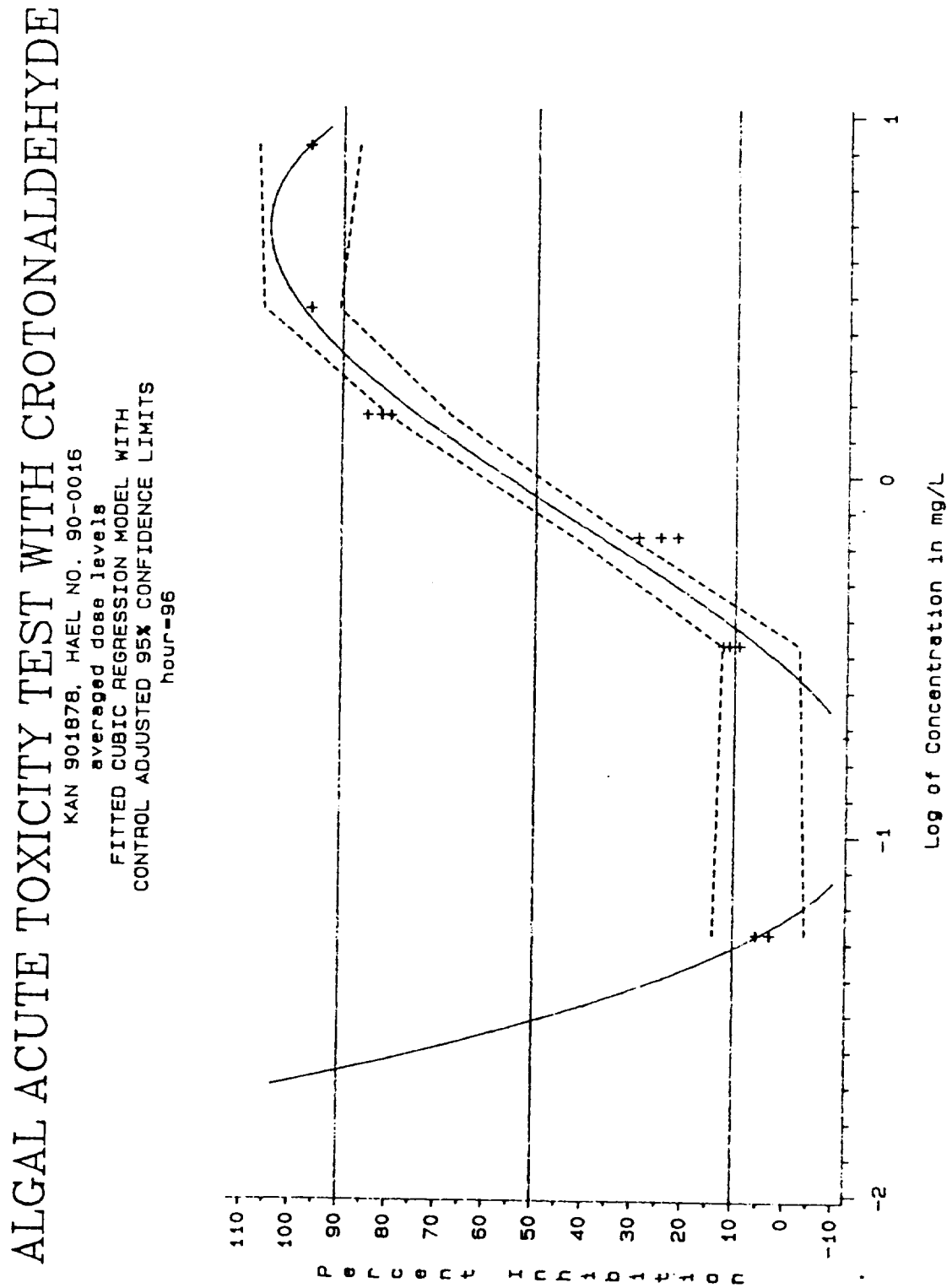


Figure 6. Range-Finding Test Graph of % Inhibition vs Nominal Concentration

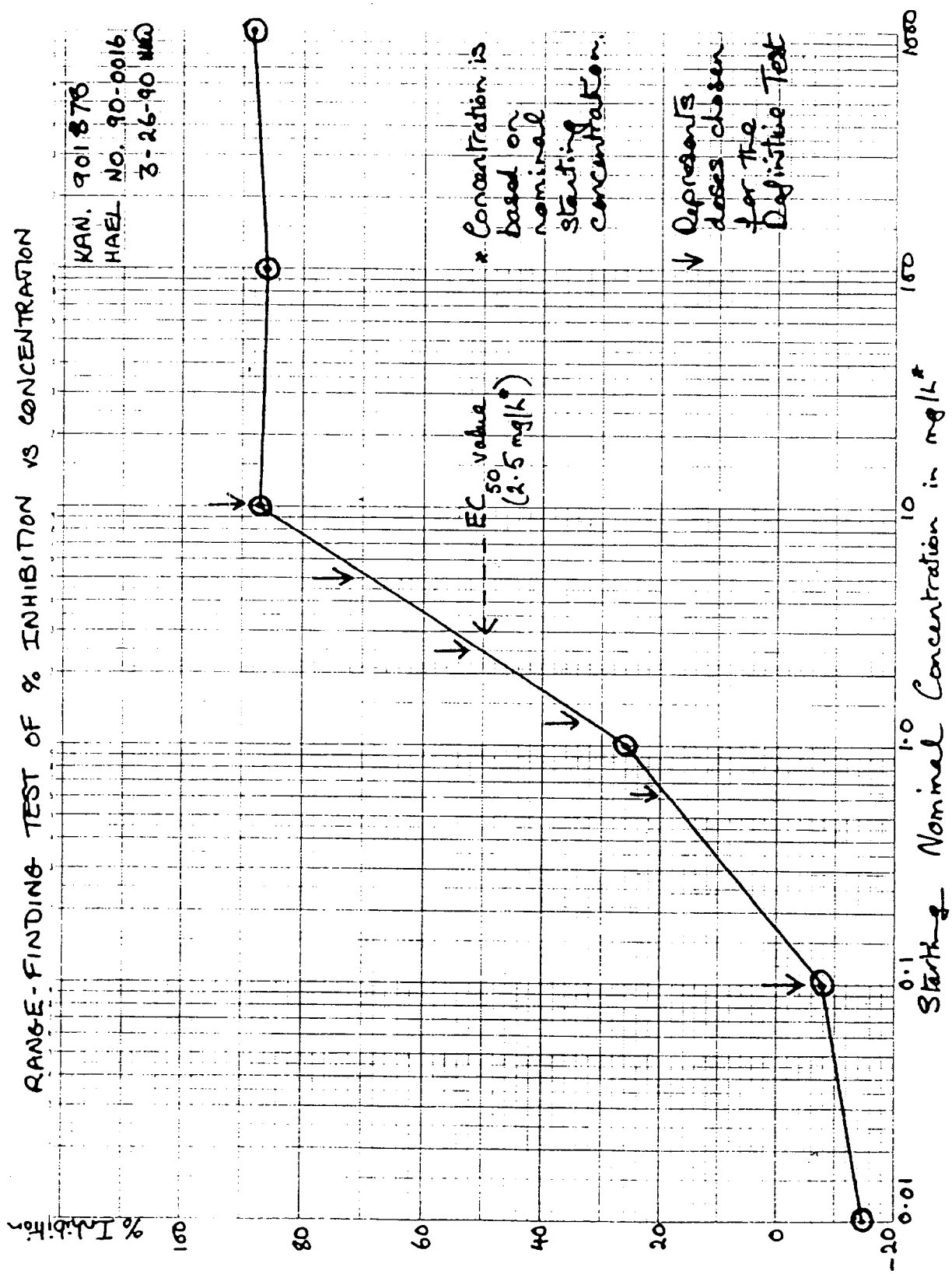
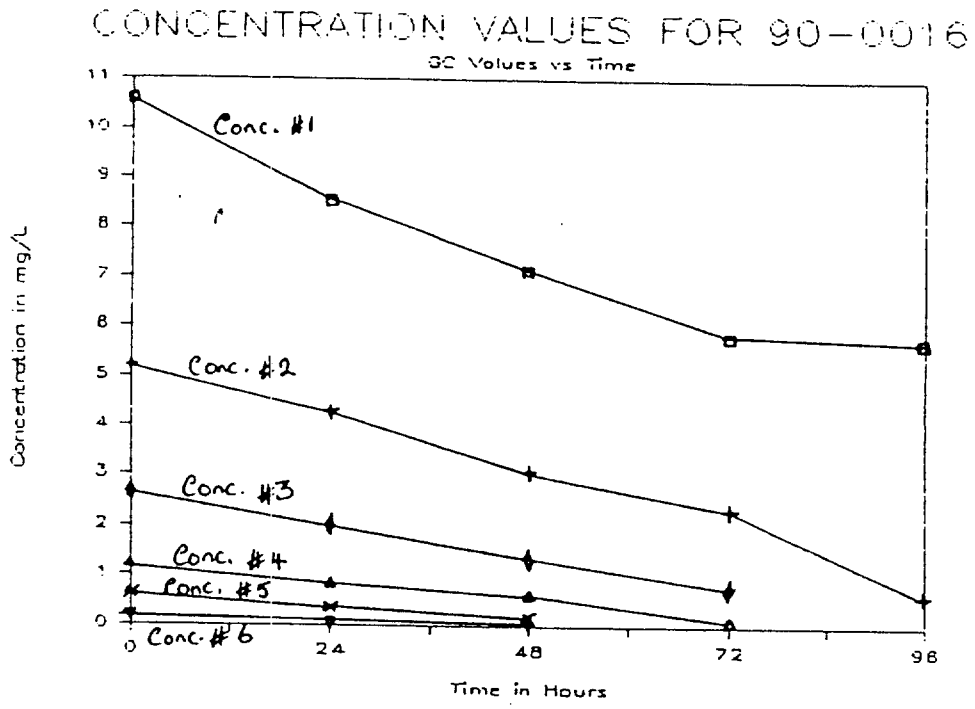
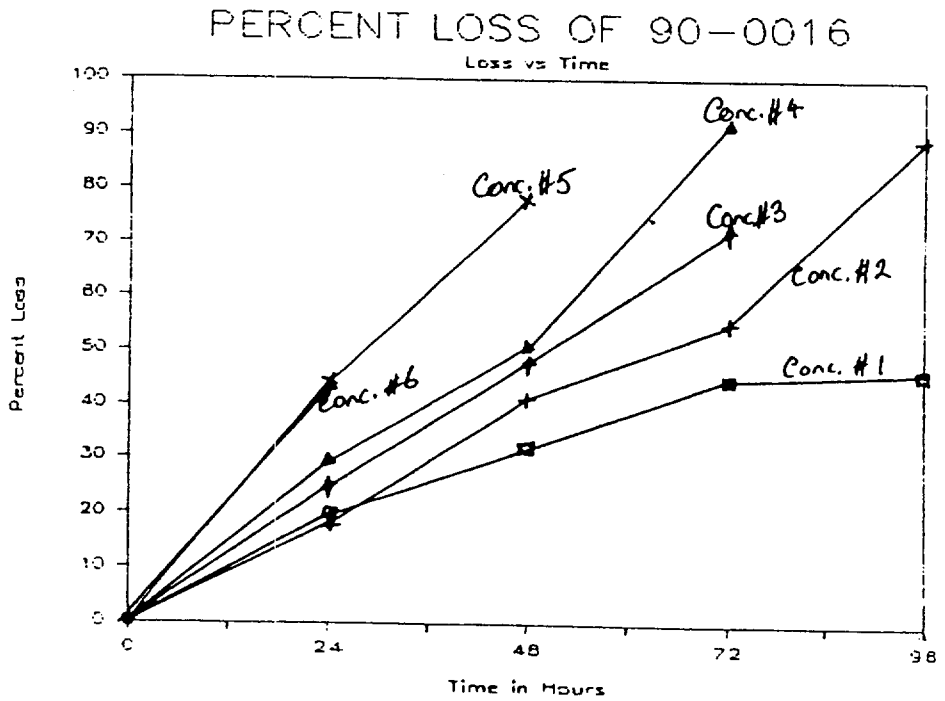


Figure 7. Graphs Showing Concentration Values and Percent Losses Over Time





NOTE: No data points are drawn once the lower limit of detection is reached

Attachment 1. Range-Finding and Definitive Test Data Worksheets

-1-

EPA/TSCA ALGAL ACUTE TOXICITY TEST
(Federal Register Guideline 40 CFR 797.1050 and Amendments of 5-20-87)
WORKSHEET SUMMARY

Chemical Name Crotonaldehyde

Accession No. 901878 HAEL No. 90-0016
Sample Ref. No. Lot No. 90-7 CAS Reg. No. 4170-30-3
Range-Finding Experimental Start Date 3-19-90 Termination Date 3-23-90
Definitive Test Experimental Start Date 4-2-90 Termination Date 4-6-90

SUMMARY: A 96-hour effects test using the alga Selenastrum capricornutum as the test organism showed EC_X values as follows: 96-hr EC_{50}, < 0.385 mg/L; EC_{50} < 0.881 mg/L; EC_{90}, < 1.982 mg/L. The EC_{50} at 24 hrs was 8.429 mg/L; at 48 hrs, 0.646 mg/L; & at 72 hrs, 0.597 mg/L. Based on mean, measured concentrations over each time period.*

SOURCE AND STRAIN OF ALGAE: Selenastrum capricornutum ATCC 22662
TEST CONCENTRATIONS: 10.6, 5.2, 2.7, 1.2, 0.6, and 0.1 mg/L**
METHOD FOR DATA HANDLING: PROC REG procedure in SAS Users Guide

TEST VALIDITY: Mean cell concentration in control cultures reached log growth within 96 hours Yes (Yes/No).

ANY SPECIAL CIRCUMSTANCES RELATING TO THIS SPECIFIC TEST OR TEST CHEMICAL: Owing to chemical volatility a closed system was used for the Range-Finding & Definitive Tests

REMARKS: *Some final values were below the detection limit of the analytical method, & in these cases the mean was calculated using the value of the lower limit of detection.
**Measured starting concentrations. Final concentrations were 5.7, 0.6, < 0.25, < 0.125, < 0.062, & < 0.01 mg/L, respectively (< the limit of detection). The chemical was algicidal at starting concentrations of 10.6 and 5.2 mg/L.

Prepared by Heather M. Watson Date 6-12-90

-2a-

Acc. No. 901878
HAEL No. 90-0016
Test Type: ALGAL/TSCA

TEST CHEMICAL WORKSHEET-SOLUBILITY/VOLATILITY METHODS

Chemical Name Crotonaldehyde

Sample Ref. No. Lot No. 90-7
Molecular Weight 70.09 Molecular Formula C_4H_6O
Appearance: Solid ___ Liquid ✓ Aqueous Solution ___ Color Colorless

Methods Development to Determine Concentrations for Range-Finding Test: Not needed
Aim concentration _____ mg/L
Actual amount in approx. ___ mL algal medium ___ mg Stir Time _____
Add algal medium and bring to volume (_____ mL)
Starting pH: _____ Final pH: _____ Adjustment: _____
TOC _____ mg/L, DOC _____ mg/L for concentration of _____ mg/L
Theoretical TOC for _____ mg/L of this chemical: _____ mg/L (if known)
Solubilizing agent (solvent) _____ (if needed)
Aim concentration (10^4 x highest test concentration) _____ g/L
Soluble at this concentration _____ (Yes/No)
Volatility and Photolysis Determinations (if needed) Yes* (Yes/No)
Test Chemical concentration _____ mg/L of algal medium
No. of flasks used: closed-system _____ foam-stoppered _____
Starting TOC of Chemical _____ mg/L, of Medium _____ mg/L
Loss of TOC after 96 hours: closed-system _____% foam-stoppered _____%
Balance Used: _____

Further Methods Development Required: _____

Conclusions for Range-Finding Test: Must be run in closed system

REMARKS: *Pre-test to determine volatility (see 2b & 2c) was run. Preliminary results indicated losses in order of 60% from foam-stoppered flasks vs 16% closed flasks (at high dose).

Prepared by Heather N. Watson Date 2-27-90

-2b-

Croton-aldehyde Pre-test Methods
2-12-90 thru' 2-16-90

Acc. No 901878
HAEL No. 90-0016
Lot No. 90-7

I. Objective

To determine the magnitude of losses over the 96-hour algal test period from closed flasks vs foam-stoppered flasks by analyzing samples of each dose at Time 0 & Time 96 hours, as volatility measure

II. Algal Medium Prep.

6.0L prepared on 2-9-90
Starting pH = 7.458 (no adjustment needed)

III Dose Prep.

① Aim conc^ns (wt/vol): 1000, 100, 10, 1, 0.1, and 0.01 mg/L
② Actual wt = 950 mg in approx. 900 mL algal medium
Brought to vol (1000 mL)
Balance used: Mettler AE163 EQID #269160
Prep. Date: 2-12-90
Start pH: 6.929 Final pH: 7.510
pH adjusted with 1 drop 1N NaOH, 5 dr. 0.1N HCl
Filter sterilized? No. Danger of volatilization
③ Diluted using sterile algal medium

950 mg/1000 mL volumetric → 950 mg/L ... ①
50 mL of ① / 500 mL " → 95 mg/L .... ②
50 mL of ② / 500 mL " → 9.5 mg/L .... ③
50 mL of ③ / 500 mL " → 0.95 mg/L ... ④
50 mL of ④ / 500 mL " → 0.095 mg/L ... ⑤
50 mL of ⑤ / 500 mL " → 0.0095 mg/L ... ⑥

-2c-

IV Method

① 100 mL of each dose (+ 100 mL of algal medium, for negative control) placed into 2 series of flasks:

a) foam-stoppered
b) closed

② Flasks incubated (randomly) + rotated every 24 hours at test conditions

V Analyses

By CQSD at Time 0 + Time 96 hours (Feb 12 + Feb 16, 1990)

VI. Preliminary Results (by phone, for guestimate only)

Time 0 (theoretical) (nominal)		Time 96 hours in mg/L Foam	Loss	Closed	Loss
950	Conc #1	380	60%	800	16%
95	" 2	33	65%	75	21%
9.5	" 3	2.5	74%	7.5	21%
0.95	" 4	0.4	58%	0.8	16%
0.095	" 5	0.25	?	0.24	?
0.0095	" 6	N.D.		ND	

ND = None detected

VII Conclusion

Use closed system

Prepared by: Heather Watson 2-27-90

-3-

Acc. No. 901878
HAEL No. 90-0016
Test Type: ALGAL/TSCA

TOC/DOC DATA FROM CARBON ANALYZER (IF NEEDED)

Not Needed

Prepared by Heather R Watson Date 2-27-90

-4-

Acc. No. 90-0016
HAEL No. 901878
Test Type: ALGAL/TSCA

SOLUBLE CHEMICAL WORKSHEET (RANGE-FINDING TEST)

Test Chemical Water Solubility is: Ready ✓ Limited ____

Test Concentrations: 1000, 100, 10, 1, 0.1, and 0.01 mg/L

Test Chemical is Volatile: Yes

Flasks Needed:

No. of flasks per test conc: 2 Closed (foam stoppers/closed system)

No. of flasks per control (foam stoppers): ____

No. of flasks per control (closed system): 2

Other flasks (purpose): Another set of flasks will be used as chemical controls (i.e., without algae)

Dilution Method for Test Chemical: dilution series is based on aim concⁿ

For concentration (1), Aim concentration: 1000 mg/L;

Actual amount in approx. 900 mL algal medium: 1,000 mg

Preparation Date: 3-19-90 Stir Time shaken

Add algal medium and bring to volume (1000 mL) using sterile algal medium*

Starting pH: 6.946 Final pH: 6.946 Adjustment: ____

Filter sterilized: No** (Yes/No) Date used: 3-19-90

Balance Used: Mettler PC4400 EQ10* 255800

Test Conc. (1) 1000 mg per 1000 mL = 1000 mg/L (based on aim concentration)

Test Conc. (2) 100 mL of (1) per 1000 mL = 100 mg/L*

Test Conc. (3) 100 mL of (2) per 1000 mL = 10 mg/L*

Test Conc. (4) 100 mL of (3) per 1000 mL = 1.0 mg/L*

Test Conc. (5) 100 mL of (4) per 1000 mL = 0.1 mg/L*

Test Conc. (6) 100 mL of (5) per 1000 mL = 0.01 mg/L*

Test Conc. (7) ____ mL of (—) per ____ mL = ____ mg/L*

*Sterile algal medium (pH 7.5 ± 0.1) is used for dilutions (2) onwards

Preparation of Flasks: Add 1-2 mL of the appropriate solution, swirl it around the flask, and remove it (to condition each flask)

Inoculation with Algal Cells (see Attachment 5 for cell count)

Entry error HMW 3-19-

1. Add 1.95 mL of algal suspension per ~~500~~ 250 mL of each Test Conc. to give 10^4 cells/mL
2. Add 100 mL of each inoculated Test Conc. to the appropriate flasks
3. Add 100 mL of inoculated medium to negative control flask

** Sterile algal medium was used (instead of filtering under vacuum which would have risked volatalization of the test chemical)

Prepared by [signature] Date 3-19-90
Michele McWilliams 3-19-90

-5-

Acc. No. 90-0016
HAEL No. 901878
Test Type: ALGAL/TSCA

RANGE-FINDING TEST PREPARATION AND pH RECORD

Flask serving as source of algal cell suspension (passage no/age): Pass #3/5 days
No. of 1 mm^2 Hemacytometer Squares Counted: 10 (each square 0.1 mm deep)
Cells/mL of source = Mean no. of cells per square x 10^4 x dilution factor

Upper Chamber	Lower Chamber	Total	Dilution Factor	Cells/mL of Source
69	69	138	10	1.38×10^6
59	66	125	10	1.25×10^6
61	63	124	10	1.24×10^6

Mean no. of cells in Source Flask: 1.29×10^6 cells/mL (X)
Amount of source algal suspension needed to supply 1.0 x 10^4 cells/mL in 100 mL is: $\frac{(1 \times 10^4)}{X} \times 100$ mL = 0.78 mL (i.e. 7.8 mL/L)

pH Record

Volume of medium prepared: 10 L; starting pH 5.913; final pH 7.425
pH adjustment: 11 drops 6 N NaOH + 2 drops 1 N NaOH

Conc.	Flasks	Start	Finish	Comments
Conc. #1	1-2	6.535	7.879	Final pH chem control Flasks 3-4 7.730
Conc. #2	3-4 5-6	7.511	7.684	" " " " " 7-8 7.695
Conc. #3	9-10	7.634	7.675	" " " " " 11-12 7.667
Conc. #4	13-14	7.662	7.808	" " " " " 15-16 7.713
Conc. #5	17-18	7.655	8.202	" " " " " 19-20 7.655
Conc. #6	21-22	7.685	8.109	Final pH Chem Control Flasks 23-24 7.695
Conc. #7	—	—	—	—
Solvent Control	—	—	—	—
Control	25-26	7.695	8.024	Final pH Chem Control Flasks 27-28 7.751

REMARKS:

Prepared by Michele M. Williams Date 3-23-90

-6a-

Acc. No. 90-0016
HAEL No. 901878
Test Type: ALGAL/TSCA

INCUBATION RECORD

aintain the following conditions (record every 24 hours):

Temperature of 24 ± 2°C

Shaker speed approx. 100 rpm

Illumination of approx. 400 foot-candles

Incubate flasks by assigning to positions randomly (using random number tables) and randomize flasks every 24 hours

Record of Incubator Conditions

Test	Date	Light (2) (foot candles)	Temperature (1)	rpm
Range-Finding	3-19-90	399.7*	24°C	100
	3-20-90	411.4*	24°C (min 24°C, max 25°C)	101
	3-21-90	402.1*	24°C (min 23°C, max 24°C)	100
	3-22-90	399.8*	24°C (min 24°C, max 25°C)	100
	3-23-90	399.7*	24°C (min 24°C, max 24°C)	100
Definitive Inc #3	4-2-90	402.4*	24°C	100
	4-3-90	405.8*	(min 24°C max 25°C) 24°C	100
	4-4-90	406.6*	24°C (min 24°C max 25°C)	100
	4-5-90	406.6*	24°C (min 24°C max 25°C)	100
	4-6-90	403.4*	24°C (min 24°C max 25°C)	100
Definitive Means (all incubators)		404.4 ± 5.2	24°C (range 24° to 25°C)	100.2 ± 0.39

(1) Temperature is taken at 24-hour intervals and max/min is also recorded.

(2) Light measurements are made at 24-hour intervals. The mean of these measurements is recorded.

REMARKS: *mean of all flasks

Prepared by Michael M. Williams Date 4-6-90
Heather Watson 4-9-90

-66-

Acc. No. 90-0016
HAEL No. 901878
Test Type: ALGAL/TSCA

INCUBATION RECORD

Maintain the following conditions (record every 24 hours):

Temperature of 24 ± 2°C

Shaker speed approx. 100 rpm

Illumination of approx. 400 foot-candles

Incubate flasks by assigning to positions randomly (using random number tables) and randomize flasks every 24 hours

Record of Incubator Conditions

Test	Date	Light(2) (foot candles)	Temperature(1)	rpm
Range-Finding	—	—	—	—
	—	—	—	—
	—	—	—	—
	—	—	—	—
	—	—	—	—
Definitive	4-2-90	411.3*	24°C	100
	4-3-90	~~318.6~~ 313.8*	24°C (min 24°C max 25°C)	100
Inc #2	4-4-90	411.3*	24°C (min 24°C max 24°C)	100
entry error 4-3-90 mr	—	—	—	—
	—	—	—	—

(1) Temperature is taken at 24-hour intervals and max/min is also recorded.

(2) Light measurements are made at 24-hour intervals. The mean of these measurements is recorded.

REMARKS: *mean of all flasks

Prepared by Michael McWilliams Date 4-4-90

-6C-

Acc. No. 90-0016
HAEL No. 901878
Test Type: ALGAL/TSCA

INCUBATION RECORD

Maintain the following conditions (record every 24 hours):

Temperature of 24 ± 2°C

Shaker speed approx. 100 rpm

Illumination of approx. 400 foot-candles

Incubate flasks by assigning to positions randomly (using random number tables) and randomize flasks every 24 hours

Record of Incubator Conditions

Test	Date	Light(2) (foot candles)	Temperature(1)	rpm
Range-Finding	—	—	—	—
	—	—	—	—
	—	—	—	—
	—	—	—	—
	—	—	—	—
Definitive Inc #1	4-2-90	400.7*	24°C	100
	4-3-90	396.5*	24°C (min 24°C max 24°C)	100
	4-4-90	400.4*	24°C (min 24°C max 24°C)	101
	4-5-90	397.1*	24°C (min 24°C max 25°C)	101
	—	—	—	—

(1) Temperature is taken at 24-hour intervals and max/min is also recorded.

(2) Light measurements are made at 24-hour intervals. The mean of these measurements is recorded.

REMARKS: *mean of all flasks

Prepared by Michael McWilliams Date 4-5-90

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Acc. No. 901878
HAEL No. 90-0016
Test Type: ALGAL/TSCA

RANGE-FINDING TEST RESULTS (Coulter Counter)

I. Hemacytometer Counts:

No. of Hemacytometer Squares Counted (below) 10 Date 3-23-90

Flask Cells/mL = Mean no. of Cells per Square x 10^4 x Dilution

Flask	Upper Chamber	Lower Chamber	Total	Dilution	Flask Cells/mL
~~1~~	56	57	113	0	1.13×10^5
~~2~~	47	56	103	0	1.03×10^5
~~3~~	42	41	83	0	8.30×10^4

II. Coulter Counter Calibration:

Flask # ~~1~~ Mean Hemacytometer Count: 9.97×10^4 Cells/mL in flask

Coulter Counts (for calibration):

Vial	Value 1	Value 2	Value 3	Dilution Factor
1	12430	12583	12857	4 from flask ~~1~~
2	6265	6440	6148	2 from Vial 1
3	3226	3215	3076	2 from Vial 2
4	1648	1618	1574	2 from Vial 3
5	1017	926	872	2 from Vial 4
6	503	461	430	2 from Vial 5
Isoton	48	43	46	4
Isoton	42	34	46	

Dilution from Flask # ~~1~~ to Vial # 1 was 5 mL from flask + 15 mL Isoton

III. Test Flask Counts:

Date of Counts made with Coulter Counter (below): 3-23-90 after ~~72~~ 96 hours

Flask	Value 1	Value 2	Value 3	Dilution:	Flask+Isoton
1	1306	1361	1314	5	2mL + 8mL
2	1191	1186	1241		
~~3~~ 5	1626	1625	1842		
~~4~~ 6	1125	1169	1190		
~~5~~ 9	1312	1338	1331		
~~6~~ 10	1245	1293	1187		
~~7~~ 13	7247	7146	7221		
~~8~~ 14	7573	7410	7457		
~~9~~ 17	10546	10633	10401		
~~10~~ 18	10818	10873	10916		
~~11~~ 21	11752	11778	11717		
~~12~~ 22	11111	11112	11103		
~~13~~ 25	7574	7529	7397		
~~14~~ 26	12346	12335	12404	5	2mL + 8mL
Isoton	45	29	44	5	4mL + 16mL
Isoton	51	35	27		

* = Control ** = Solvent Control ~~1~~ composite sample flask 25+26 (3mL each)

Note: Isoton is the diluent used; the count for "Isoton" is made on algal medium diluted with isoton (for a background value). The Coulter Counter counts 0.5 mL of sample (this dilution is taken into account in the worksheet).

Prepared by Michele McWilliams Date 3-23-90

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Acc. No. 901878
HAEL No. 90-0016
Test Type: ALGAL/TSCA

COULTER COUNTER CALIBRATION WORKSHEET (RANGE-FINDING)

A. Coulter Counts

MEDIUM(*)	DATE TIME	VALUE1	VALUE2	VALUE3	MEAN	CORRECTED MEAN (x)	CELL CONC (y)
ISOTON		48	43	46			
ISOTON	3-23-90	42	34	46	43.2		
CONC. 1		12430	12583	12857	12623.3	25160.3	24925.0
CONC. 2	96 hours	6265	6440	6148	6284.3	12482.3	12462.5
CONC. 3		3226	3215	3076	3172.3	6258.3	6231.3
CONC. 4		1648	1618	1574	1613.3	3140.3	3115.6
CONC. 5		1017	926	872	938.3	1790.3	1557.8
CONC. 6		503	461	430	464.7	843.0	778.9

(*) Six Isoton (diluent) background counts were made and averaged
(x) Corrected Mean = (Mean of Coulter Counter Values - Mean Isoton Value) x 2
(y) Conc. 1 is determined by a hemacytometer cell count plus dilution (see Attachment 15)
Conc. 2-5 are serial dilutions from Conc. 1 (see Attachment 15)

B. Coulter Counter Linear Regression Worksheet

X	Y	PRED Y	2.5	97.5		
25160.3	24925.0	24985.68162	-14777.37306	64748.73629	6	=N
12482.3	12462.5	12383.29536	2460.27325	22266.31747	49674.50000	=SUM OF X
6258.3	6231.3	6166.59777	1401.24267	10931.95287	49071.10000	=SUM OF Y
3140.3	3115.6	3062.27529	-9043.91454	15168.46513	841792135.45000	=SUM OF X^2
1790.3	1557.8	1718.19725	-13567.35742	17003.75192	828139020.35000	=SUM OF Y^2
843.0	778.9	775.05271	-16741.57263	18291.67805	834907946.76000	=SUM OF X*Y
					8279.08333	=X MEAN
					8178.51667	=Y MEAN
					430532810.40833	=SUM OF X^2 - ((SUM OF X)^2)/N
					426810211.14833	=SUM OF Y^2 - ((SUM OF Y)^2)/N
					428644220.60167	=SUM OF X*Y - (SUM OF X)*(SUM OF Y)/N
					0.99561	=SLOPE b
					-64.24936	=INTERCEPT a
					0.99995	= r (COEFFICIENT OF CORRELATION)
					107.58230	=SD OF POINTS ABOUT THE FITTED LINE

Prepared by Heather Watson Date 3-26-90

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Acc. No. 901878
HAEL No. 90-0016
Test Type: Range-Finding

RANGE-FINDING TEST WORKSHEET - 72 HOURS

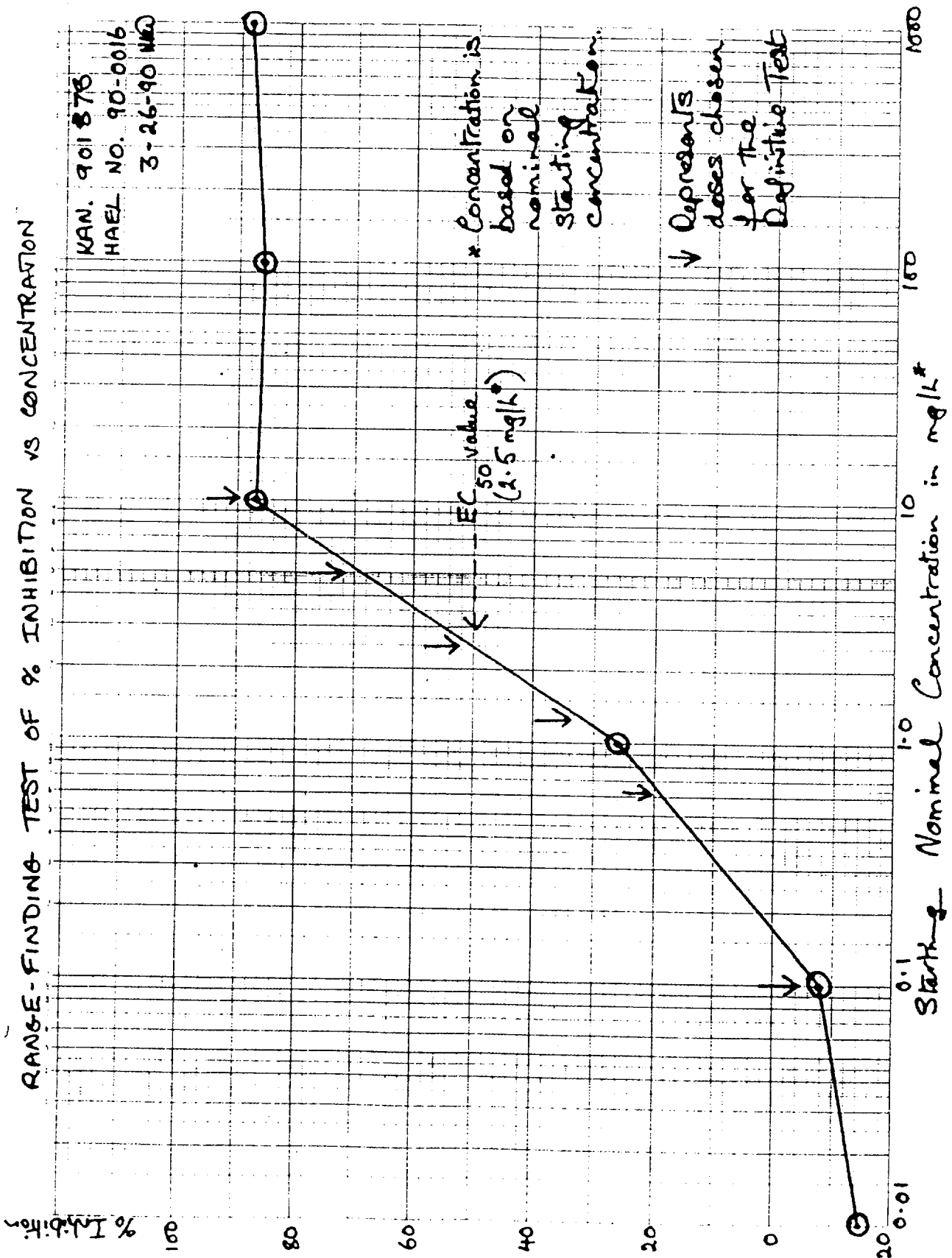
MEDIUM	FLASK CONC.	VALUE1	VALUE2	VALUE3	MEAN	CORRECTED MEAN (x)	FLASK COUNTS (y)	PERCENT INHIBITION
ISOTON		45	29	44				
ISOTON		51	35	27	38.5			
FLASK 1	1000.00	1306	1361	1314				
FLASK 2	1000.00	1191	1186	1241	1266.5	12280.0	12162	86%
FLASK 5	100.00	1626	1625	1842				
FLASK 6	100.00	1125	1169	1190	1429.5	13910.0	13785	86%
FLASK 9	10.00	1312	1338	1331				
FLASK 10	10.00	1245	1293	1187	1284.3	12458.3	12339	87%
FLASK 13	1.00	7249	7146	7221				
FLASK 14	1.00	7573	7410	7457	7342.7	73041.7	72657	26%
FLASK 17	0.10	10546	10633	10401				
FLASK 18	0.10	10816	10873	10916	10697.8	106593.3	106061	-8%
FLASK 21	0.01	11752	11778	11717				
FLASK 22	0.01	11111	11112	11103	11426.8	113903.3	113339	-15%
CONTROL	0	7574	7529	7397				
CONTROL	0	12346	12335	12404	9930.8	98923.3	98425	

Flask Conc. is in mg/L
Six Isoton (diluent) background counts were made and averaged
Corrected Mean (x) = (Mean of the 3 Coulter Counter Values - Mean Isoton Value) x 2 x Dilution (Attachment 7)
Flask Counts (y) = y value in the regression equation: y = bx + a (Attachment 8)
Where b = 0.99561
a =-64.2493

REMARKS: Concentration is nominal starting concentration

Prepared by Heather M Watson Date 3-26-90

-10-


RANGE-FINDING TEST OF % INHIBITION VS CONCENTRATION
KAN. 901878
HAEL NO. 90-0016
3-26-90
* Concentration is based on nominal starting concentration.
EC50 value (2.5 mg/L*)
Represents doses chosen for the Definitive Test
% Inhibition
Starting Nominal Concentration in mg/L*
0.01
0.1
1.0
10
100
1000
20
0
40
60
80
100

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Acc. No. 901878
HAEL No. 90-0016
Test Type: ALGAL/TSCA

SOLUBLE CHEMICAL PREPARATION WORKSHEET (DEFINITIVE TEST)

Test Chemical Water Solubility is: Ready ✓ Limited ____

Test Concentrations: 10, 5, 2.5, 1.25, 0.625, + 0.1 mg/L

Test Chemical is Volatile: Yes. Closed system will be used with 4X as many flasks as usual. At the end of each day's analysis, the flasks that are analyzed will be discarded

Flasks Needed:

No. of flasks per test conc: 12 Closed (foam stoppers/closed system)

No. of flasks per control (foam stoppers): —

No. of flasks per control (closed system): 12 (TOTAL FLASKS NEEDED = 84)

Dilution Method for Test Chemical:

For stock concentration (S), Aim concentration: 100 mg/L;

Actual amount in approx. 900 mL algal medium: ~~0.105~~ 105** mg entry error 4-2-90 mm

Preparation Date: 4-2-90 Stir Time Shaken

Add algal medium and bring to volume (1000 mL)

Starting pH: 7.274 Final pH: 7.558 Adjustment: 1 dr 0.1N NaOH

Filter sterilized: No** (Yes/No) Date used: 4-2-90

Balance Used: Mettler AE163 EQ10* 269160

Test Conc. (1) 200 mL of (S) per 2000 mL = 10 mg/L*
Test Conc. (2) 100 mL of (S) per 2000 mL = 5 mg/L*
Test Conc. (3) 50 mL of (S) per 2000 mL = 2.5 mg/L*
Test Conc. (4) 25 mL of (S) per 2000 mL = 1.25 mg/L*
Test Conc. (5) 12.5 mL of (S) per 2000 mL = 0.625 mg/L*
Test Conc. (6) 2 mL of (S) per 2000 mL = 0.1 mg/L*
Test Conc. (–) — mL of (–) per — mL = — mg/L*

Based on aim conc^n

*Sterile algal medium (pH 7.5 ± 0.1) is used for dilutions (2) onwards

Preparation of Flasks:

Add 1-2 mL of the appropriate solution, swirl it around the flask, and remove it to condition each flask

Inoculation with Algal Cells (see Attachment 12 for cell count)

1. Add 5.55 mL of algal suspension per 1500 mL of each Test Conc. to give 10^4 cells/mL
2. Add 100 mL of each inoculated Test Conc. to the appropriate flasks
3. Add 100 mL of inoculated medium to negative control flask

** Sterile algal medium was used instead of using filter sterilization which would have risked volatilization of the chemical

Prepared by Heather Nelson Date 4-2-90
Michele McWilliams 4-2-90

-12-

Acc. No. 901878
HAEL No. 90-0016
Test Type: ALGAL/TSCA

DEFINITIVE TEST PREPARATION AND pH RECORD

Flask serving as source of algal cell suspension (passage no/age): 5(5 days)
No. of 1 mm^2 Hemacytometer Squares Counted: 10 (each square 0.1 mm deep)
Cells/mL of source = Mean no. of cells per square x 10^4 x dilution factor

Upper Chamber	Lower Chamber	Total	Dilution Factor	Cells/mL of Source
118	155	273	10	2.73×10^6
116	146	262	10	2.62×10^6
147	130	277	10	2.77×10^6

Mean no. of cells in Source Flask: 2.71×10^6 cells/mL (X)
Amount of source algal suspension needed to supply 1.0 x 10^4 cells/mL in 100 mL is: $\frac{(1 \times 10^4)}{X} \times 100$ mL = 0.37 mL (i.e. 3.70 mL/L)

pH Record

Volume of medium prepared: 20 L; starting pH 7.440; final pH 7.440
pH adjustment: —

Conc.	Flasks*	Start	Finish	Comments
Conc. #1	1A-3D	7.149	8.923	
Conc. #2	4A-6D	7.232	7.022	
Conc. #3	7A-9D	7.299	6.810	
Conc. #4	10A-12D	7.281	9.725	
Conc. #5	13A-15D	7.196	9.830	
Conc. #6	16A-18D	7.304	9.911	
Conc. #7	—	—	—	—
Solvent Control	—	—	—	—
Control	19A-21D	7.270	10.107	

REMARKS: *Flasks "A" counted at 24 hrs, "B" at 48 hrs & so on

Prepared by Michele McWilliams Date 4-6-90
Heather Watson 4-6-90

-13-

Acc. No. 901878
HAEL No. 90-0016
Test Type: ALGAL/TSCA

DEFINITIVE TEST RESULTS - 24 hours (Flask Set A)

Date of Counts made with Coulter Counter (below): 4-3-90

Flask	Value 1	Value 2	Value 3	Dilution: Flask+Isoton	
1	1415	1355	1349	5	~~4mL + 16 mL~~ Corrected 4/3/90
2	1298	1194	1392		2mL + 8mL (for all)
3	1388	1430	1326		
4	1450	1437	1502		
5	1470	1497	1430		
6	1555	1472	1512		
7	1962	1957	1899		
8	2086	1844	1870		
9	1811	1796	1744		
10	2382	2243	2288		
11	1978	1957	1895		
12	2344	2222	2192		
13	2491	2416	2359		
14	2482	2559	2457		
15	2402	2395	2441		
16	2700	2802	2773		
17	2732	2648	2580		
18	3005	2977	3090		
19	2903	2890	2973		
20	2747	2730	2710		
21	2653	2761	2654		
22	—	—	—		
23	—	—	—		
24	—	—	—		2mL + 8 mL
Isoton	34	36	33	5	KW 4/3/90
Isoton	34	39	32		

Note: Isoton is the diluent used; the count for "Isoton" is made on algal medium diluted with isoton (for a background value). The Coulter Counter counts 0.5 mL of sample (dilution is taken into account in the worksheets).

No. of Hemacytometer Squares Counted (below) 10 Date 4-3-90

Flask	Upper Chamber	Lower Chamber	Total	Dilution	Flask Cells/mL
*	18	16	34	—	3.4×10^4
*	16	17	33	—	3.3×10^4
*	12	24	36	—	3.6×10^4

Cells in flasks 1-3 have a normal appearance

REMARKS: * 2 mL each from flasks 19-21 (composite sample)

Prepared by Michele Williams Date 4-3-90

Heather Walton 4-3-90

-14-

Acc. No. 901878
HAEL No. 90-0016
Test Type: ALGAL/TSCA

DEFINITIVE TEST RESULTS - 48 hours (Flask Set B)

Date of Counts made with Coulter Counter (below): 4-4-90

Flask	Value 1	Value 2	Value 3	Dilution:	Flask+Isoton
1	1309	1248	1251	5	2mL + 8mL
2	1286	1384	1258		
3	1349	1378	1322		
4	1562	1487	1506		
5	1488	1493	1438		
6	1477	1359	1448		
7	2282	2315	2281		
8	2218	2365	2249		
9	2294	2351	2365		
10	4578	4491	4480		
11	4237	4151	4296		
12	4052	3994	3840		
13	7048	7026	6990		
14	5966	5991	5945		
15	5506	5506	5322		
16	7347	7155	7345		
17	7842	7901	7870		
18 (6489)	6489	6552	6536		
19 MMW	8579	8599	8517		
20 6-7-90	8762	8858	8913		
21 (Original	8873	9064	8811		
22 hard to	–	–	–		
23 read)	–	–	–		
24	–	–	–		
Isoton	34	35	40	5	2 mL + 8 mL
Isoton	35	38	36		

Note: Isoton is the diluent used; the count for "Isoton" is made on algal medium diluted with isoton (for a background value). The Coulter Counter counts 0.5 mL of sample (dilution is taken into account in the worksheets).

No. of Hemacytometer Squares Counted (below) 10 Date 4-4-90

Flask	Upper Chamber	Lower Chamber	Total	Dilution	Flask Cells/mL
1	39	51	90	–	9.0×10^4
2	43	42	85	–	8.5×10^4
3	54	55	109	–	1.09×10^5

REMARKS: cells in flasks 1-3 have a normal appearance
~2 mL each from flasks 19-21 (composite sample)

Prepared by Michele McWilliams Date 4-4-90

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Acc. No. 901878
HAEL No. 90-0016
Test Type: ALGAL/TSCA

DEFINITIVE TEST RESULTS - 72 hours (Flask Set C)

Date of Counts made with Coulter Counter (below): 4-5-90

Flask	Value 1	Value 2	Value 3	Dilution	Flask+Isoton
1	1306	1257	1315	5	2 mL + 8 mL
2	1426	1412	1397		
3	1353	1403	1307		
4	1556	1499	1467		
5	1421	1360	1401		
6	1435	1455	1471		
7	2263	2280	2280		
8	2477	2385	2295		
9	2451	2303	2347	5	2 mL + 8 mL
10	4236	4222	4301	10	1 mL + 9 mL
11	4449	4253	4337		
12	4227	4189	4177		
13	5634	5594	5649		
14	6461	6504	6468		
15	7296	7223	7200		
16	10396	10038	10162		
17	9327	9318	9326		
18	9043	9049	8932		
19	8760	8866	8725		
20	9397	9506	9592		
21	9496	9524	9619	10	1 mL + 9 mL
22	—	—	—		
23	—	—	—		
24	—	—	—		
Isoton	36	37	37	5	2 mL + 8 mL
Isoton	24	47	35		

Note: Isoton is the diluent used; the count for "Isoton" is made on algal medium diluted with isoton (for a background value). The Coulter Counter counts 0.5 mL of sample (dilution is taken into account in the worksheets).

No. of Hemacytometer Squares Counted (below) 10 Date 4-5-90

Flask	Upper Chamber	Lower Chamber	Total	Dilution	Flask Cells/mL
1	98	98	196	–	1.96×10^5
2	73	100	173	–	1.73×10^5
3	85	114	199	–	1.99×10^5

REMARKS: cells in flasks 1-3 have a normal appearance
* 2 mL each from flasks 19-21 (composite sample)

Prepared by Michele McWilliams Date 4-5-90

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Acc. No. 901878
HAEL No. 90-0016
Test Type: ALGAL/TSCA

DEFINITIVE TEST RESULTS - 96 hours (Flask Set D)

Date of Counts made with Coulter Counter (below): 4-6-90

Flask	Value 1	Value 2	Value 3	Dilution:	Flask+Isoton
1	1237	1279	1210	5	2 mL + 8 mL
2	1340	1260	1251		
3	1267	1267	1291		
4	1324	1422	1443		
5	1385	1369	1405		
6	1474	1398	1403		
7	7375	7450	7237		
8	8215	8202	8074		
9	6147	6235	6265	5	2 mL + 8 mL
10	8033	7821	8257	20	0.5 mL + 9.5 mL
11	7138	7381	7240		
12	6968	8027	8067		
13	8958	8965	8998		
14	9173	9055	9079		
15	9260	9402	9218		
16	9851	9975	10038		
17	9629	9564	9856		
18	9644	9676	9741		
19	9830	9661	9682		
20	10459	10585	10415		
21	10372	10697	10381		
22	—	—	—		
23	—	—	—		
24	—	—	—	20	0.5 mL + 9.5 mL
Isoton	33	36	36	5	2 mL + 8 mL
Isoton	30	41	40		

Note: Isoton is the diluent used; the count for "Isoton" is made on algal medium diluted with isoton (for a background value). The Coulter Counter counts 0.5 mL of sample (dilution is taken into account in the worksheets).

No. of Hemacytometer Squares Counted (below) 10 Date 4-6-90

Flask	Upper Chamber	Lower Chamber	Total	Dilution	Flask Cells/mL
1	231	253	484	–	4.84 x 10^5
2	235	256	491	–	4.91 x 10^5
3	238	277	515	–	5.15 x 10^5

REMARKS: Cells in flasks 1-3 have a normal appearance
2 mL each from flasks 19-21 composite sample

Prepared by Heather R. Walton Date 4-6-90
Michele M. Williams 4-6-90

-17a-

Acc. No. 901878
HAEL No. 90-0016
Test Type: ALGAL/TSCA

COULTER COUNTER CALIBRATION RESULTS (24 and 48 hours)

TEST TIME 24-HOURS Date: 4-3-90 Sample: *2mL each from flasks 14-21 (composite sample)

Hemacytometer Count: 34, 33, 36, Mean 34 Dilution: —

No. of 1 mm^2 squares counted = 10 Cells/mL in Sample = 3.4×10^4

Coulter Counts:

Vial	Value 1	Value 2	Value 3	Dilution Factor
1	3706	3703	3627	4* from Sample
2	1955	1913	1944	2 from Vial 1
3	905	880	892	2 from Vial 2
4	569	524	507	2 from Vial 3
5	289	287	288	2 from Vial 4
—	—	—	—	— from Vial —
Isoton	27	22	22	4
Isoton	19	18	21	

Dilution from Sample to Vial #1 was 5 mL from Sample + 15 mL Isoton

TEST TIME 48-HOURS Date: 4-4-90 Sample: *2mL each from flasks 14-21 (composite sample)

Hemacytometer Count: 90, 85, 109, Mean 94.7 Dilution: —

No. of 1 mm^2 squares counted = 10 Cells/mL in Sample = 9.47×10^4

Coulter Counts:

Vial	Value 1	Value 2	Value 3	Dilution Factor
1	10807	10792	10888	4 from Sample *
2	5405	5470	5405	2 from Vial ____
3	2709	2753	2666	2 from Vial ____
4	1609	1432	1366	2 from Vial ____
5	778	783	735	2 from Vial ____
—				___ from Vial ____
Isoton	33	39	43	4
Isoton	33	29	40	

Dilution from Sample to Vial #1 was 5 mL from Sample + 15 mL Isoton

Note: Isoton is the diluent used; the count for "Isoton" is made on algal medium diluted with isoton (for a background value). The Coulter Counter counts 0.5 mL of sample (this dilution is taken into account in the worksheet).

Prepared by Heather Watson Date 4-4-90
Michele McWilliams 4-4-90

-17b-

Acc. No. 901878
HAEL No. 90-0016
Test Type: ALGAL/TSCA

COULTER COUNTER CALIBRATION RESULTS (72 and 96 hours)

TEST TIME 72-HOURS Date: 4-5-90 Sample: *2mL each from flasks 19-21 (composite sample)

Hemacytometer Count: 196, 173, 199, Mean 189 Dilution: —

No. of 1 mm^2 squares counted = 10 Cells/mL in Sample = 1.89×10^5

Coulter Counts:

Vial	Value 1	Value 2	Value 3	Dilution Factor
1	20004	20252	20311	5 from Sample *
2	10148	10128	10075	2 from Vial 1
3	5217	5108	5044	2 from Vial 2
4	2416	2406	2413	2 from Vial 3
5	1277	1219	1198	2 from Vial 4
6	—	—	—	2 from Vial —
Isoton	31	35	30	5
Isoton	29	21	34	

Dilution from Sample to Vial #1 was 4 mL from Sample + 16 mL Isoton

TEST TIME 96-HOURS Date: 4-6-90 Sample: *2mL each from flasks 19-21 (composite sample)

Hemacytometer Count: 484, 491, 515, Mean 497 Dilution: —

No. of 1 mm^2 squares counted = 10 Cells/mL in Sample = 4.97×10^5

Coulter Counts:

Vial	Value 1	Value 2	Value 3	Dilution Factor
1	21072	19974	19628	10 from Sample *
2	9909	9913	9716	2 from Vial 1
3	5216	5273	5021	2 from Vial 2
4	2680	2588	2537	2 from Vial 3
5	1320	1307	1365	2 from Vial 4
—				— from Vial —
Isoton	32	23	19	—
Isoton	21	36	32	

Dilution from Sample to Vial #1 was 2 mL from Sample + 18 mL Isoton

Note: Isoton is the diluent used; the count for "Isoton" is made on algal medium diluted with isoton (for a background value). The Coulter Counter counts 0.5 mL of sample (this dilution is taken into account in the worksheet).

Prepared by Heather Walton Date 4-6-90
Michele M. Williams 4-6-90

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Acc. No. 901878
HAEL No. 90-0016
Test Type: Definitive

COULTER COUNTER CALIBRATION WORKSHEET

MEDIUM(*)	DATE TIME	VALUE1	VALUE2	VALUE3	MEAN	CORRECTED MEAN (x)	CELL CONC (y)
ISOTON		27	22	22			
ISOTON	4-3-90	19	18	21	21.5		
CONC. 1		3706	3703	3627	3678.7	7314.3	8500.0
CONC. 2	24 hours	1955	1913	1944	1937.3	3831.7	4250.0
CONC. 3		905	880	892	892.3	1741.7	2125.0
CONC. 4		569	524	507	533.3	1023.7	1062.5
CONC. 5		289	287	288	288.0	533.0	531.3
ISOTON		33	39	43			
ISOTON	4-4-90	33	29	46	36.2		
CONC. 1		10807	10792	10888	10829.0	21585.7	23675.0
CONC. 2	48 hours	5405	5470	5405	5426.7	10781.0	11837.5
CONC. 3		2709	2753	2606	2689.3	5308.3	5918.8
CONC. 4		1609	1432	1366	1469.0	2865.7	2959.4
CONC. 5		776	783	735	765.3	1458.3	1479.7
ISOTON		31	35	30			
ISOTON	4-5-90	29	21	34	30.0		
CONC. 1		20004	20252	20311	20189.0	40318.0	37800.0
CONC. 2	72 hours	10148	10128	10075	10117.0	20174.0	18900.0
CONC. 3		5217	5108	5044	5123.0	10186.0	9450.0
CONC. 4		2416	2406	2413	2411.7	4763.3	4725.0
CONC. 5		1277	1219	1198	1231.3	2402.7	2362.5
ISOTON		32	23	19			
ISOTON	4-6-90	21	36	32	27.2		
CONC. 1		21072	19974	19628	20224.7	40395.0	49700.0
CONC. 2	96 hours	9909	9913	9716	9846.0	19637.7	24850.0
CONC. 3		5216	5273	5021	5170.0	10285.7	12425.0
CONC. 4		2680	2588	2537	2601.7	5149.0	6212.5
CONC. 5		1320	1307	1365	1330.7	2607.0	3106.3

(*) Six Isoton (diluent) background counts were made and averaged
(x) Corrected Mean = (Mean of Coulter Counter Values - Mean Isoton Value) x 2
(y) Conc. 1 is determined by a hemacytometer cell count plus dilution (see Attachment 15)
Conc. 2-5 are serial dilutions from Conc. 1 (see Attachment 15)

Remarks:

Prepared by Heather R Waton Date 4-9-90

-19a-

Acc. No. _____
HAEL No. _____
Test Type: ALGAL/TSCA

COULTER COUNTER CALIBRATION - LINEAR REGRESSION WORKSHEET (DEFINITIVE)

I	Y	PRED Y	2.5	97.5
7314.3	8500.0	8456.33844	-12131.22472	29043.90159
3831.7	4250.0	4393.62898	-1.77518	8789.03315
1741.7	2125.0	1955.49002	-3388.79233	7299.77237
1023.7	1062.5	1117.89013	-7563.19369	9798.97395
533.0	531.3	545.45243	-10417.16807	11508.07293

5 =N
14444.40000 =SUM OF I
16468.80000 =SUM OF Y
72546478.96000 =SUM OF I^2
96239310.94000 =SUM OF Y^2
83528251.65000 =SUM OF I*Y
2888.88000 =I MEAN
3293.76000 =Y MEAN
30818340.68800 =SUM OF I^2 - ((SUM OF I)^2)/N
41995036.25200 =SUM OF Y^2 - ((SUM OF Y)^2)/N
35951864.70600 =SUM OF I*Y - (SUM OF I)*(SUM OF Y)/N

TEST: EPA Algal Definitive - 24 hours
Accession No. 901878
HAEL No. 90-0016

1.16657 =SLOPE b
-76.33134 =INTERCEPT a
0.99935 = r (COEFFICIENT OF CORRELATION)

134.83027 =SD OF POINTS ABOUT THE FITTED LINE

I	Y	PRED Y	2.5	97.5
21585.7	23675.0	23714.09504	-8331.22898	55759.42105
10761.0	11837.5	11800.17695	6006.98581	17593.36809
5306.3	5918.8	5763.43990	-1757.49217	13284.37196
2865.7	2959.4	3072.28596	-10377.69422	16522.26614
1458.3	1479.7	1520.40116	-15349.24194	18390.04425

5 =N
41997.00000 =SUM OF I
45870.40000 =SUM OF Y
620668100.56000 =SUM OF I^2
746611785.14000 =SUM OF Y^2
680707062.53000 =SUM OF I*Y
8399.40000 =I MEAN
9174.08000 =Y MEAN
267918498.76000 =SUM OF I^2 - ((SUM OF I)^2)/N
325793065.90800 =SUM OF Y^2 - ((SUM OF Y)^2)/N
295423224.77000 =SUM OF I*Y - (SUM OF I)*(SUM OF Y)/N

TEST: EPA Algal Definitive - 48 hours
Accession No. 901878
HAEL No. 90-0016

1.10266 =SLOPE b
-87.60908 =INTERCEPT a
0.99994 = r (COEFFICIENT OF CORRELATION)

117.55580 =SD OF POINTS ABOUT THE FITTED LINE

Prepared by Heather M Watson Date 4-5-90

-19b-

Acc. No. _____
HAEL No. _____
Test Type: ALGAL/TSCA

COULTER COUNTER CALIBRATION - LINEAR REGRESSION WORKSHEET (DEFINITIVE)

I	Y	PRED Y	2.5	97.5		
40318.0	37800.0	37752.98853	-20193.71305	95699.69010	5	=N
20174.0	18900.0	18946.84688	8160.11917	29733.57459	77844.00000	=SUM OF I
10186.0	9450.0	9622.19722	-2984.49811	22228.89254	73237.50000	=SUM OF Y
4763.3	4725.0	4559.63438	-20741.42506	29860.71382	2164747990.18000	=SUM OF I^2
2402.7	2362.5	2355.82300	-28471.83203	33183.47802	1903259531.25000	=SUM OF Y^2
					2029749671.25000	=SUM OF I*Y
					15568.80000	=I MEAN
					14647.50000	=Y MEAN
					952810322.98000	=SUM OF I^2 - ((SUM OF I)^2)/N
					830513256.00000	=SUM OF Y^2 - ((SUM OF Y)^2)/N
					889529681.25000	=SUM OF I*Y - (SUM OF I)*(SUM OF Y)/N

TEST: EPA Algal Definitive - 72 hours
Accession No. 901878
HAEL No. 90-0016

0.93359 =SLOPE b
112.69767 =INTERCEPT a
0.99996 = r (COEFFICIENT OF CORRELATION)

143.11260 =SD OF POINTS ABOUT THE FITTED LINE

I	Y	PRED Y	2.5	97.5		
40395.0	49700.0	49921.35817	-93709.76915	193552.42550	5	=N
19637.7	24850.0	24236.54514	903.18468	47569.90561	78074.40000	=SUM OF I
10265.7	12425.0	12664.50198	-18236.59025	43565.59422	96293.80000	=SUM OF Y
5149.0	6212.5	6308.41622	-54359.40129	66976.23373	2156499560.78000	=SUM OF I^2
2607.0	3106.3	3162.97848	-72237.30672	78563.26368	3290237380.94000	=SUM OF Y^2
					2663514454.10000	=SUM OF I*Y
					15614.88000	=I MEAN
					19258.76000	=Y MEAN
					937377173.70800	=SUM OF I^2 - ((SUM OF I)^2)/N
					1435738197.25200	=SUM OF Y^2 - ((SUM OF Y)^2)/N
					1155898322.35600	=SUM OF I*Y - (SUM OF I)*(SUM OF Y)/N

TEST: EPA Algal Definitive - 96 hours
Accession No. 901878
HAEL No. 90-0016

1.23739 =SLOPE b
-62.89941 =INTERCEPT a
0.99983 = r (COEFFICIENT OF CORRELATION)

406.24290 =SD OF POINTS ABOUT THE FITTED LINE

Prepared by Heather Watson Date 4-9-90

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Acc. No. 901878
HAEL No. 90-0016
Test Type: Definitive

DEFINITVE TEST WORKSHEET - 48 HOURS

MEDIUM (x	FLASK CONC.	VALUE1	VALUE2	VALUE3	MEAN	CORRECTED MEAN (x)	FLASK COUNTS (y)	FLASK MEAN PER CONC.	S.D.	C.V.
ISOTON	0	34	35	40						
ISOTON	0	35	38	36	36.3					
FLASK 1	10	1309	1248	1251	1269.3	12330.0	13503			
FLASK 2	10	1286	1354	1258	1309.3	12730.0	13949			
FLASK 3	10	1349	1378	1322	1349.7	13133.3	14394	13950	381.6	3%
FLASK 4	5	1562	1487	1506	1518.3	14820.0	16254			
FLASK 5	5	1488	1493	1438	1473.0	14366.7	15754			
FLASK 6	5	1477	1359	1448	1428.0	13916.7	15258	15755	486.6	3%
FLASK 7	2.5	2282	2315	2281	2292.7	22363.3	24792			
FLASK 8	2.5	2218	2365	2249	2277.3	22410.0	24623			
FLASK 9	2.5	2294	2351	2365	2336.7	23003.3	25277	24897	277.3	1%
FLASK 10	1.25	4578	4491	4480	4516.3	44600.0	49312			
FLASK 11	1.25	4237	4151	4296	4228.0	41916.7	46132			
FLASK 12	1.25	4052	3994	3840	3962.0	39256.7	43199	46214	2496.1	5%
FLASK 13	0.625	7048	7026	6990	7021.3	69850.0	76933			
FLASK 14	0.625	5966	5991	5995	5984.0	59476.7	65495			
FLASK 15	0.625	5506	5506	5322	5444.7	54083.3	59546	67325	7214.6	11%
FLASK 16	0.1	7347	7155	7345	7282.3	72460.0	79811			
FLASK 17	0.1	7942	7901	7870	7871.0	78346.7	86302			
FLASK 18	0.1	6489	6552	6536	6525.7	64893.3	71468	79194	6071.9	8%
FLASK 19	0	8579	8599	8517	8565.0	85286.7	93955			
FLASK 20	0	8962	8958	8913	8944.3	89080.0	98137			
FLASK 21	0	8873	9064	8811	8916.0	88796.7	97825	96639	1992.4	2%

Flask Conc. is in ug/L
Six Isoton (diluent) background counts were made and averaged
Corrected Mean (x) = (Mean of the 3 Coulter Counter Values - Mean Isoton Value) x 2 x Dilution
Flask Counts (y) = y value in the regression equation: y = bx + a
Where b = 1.10266
a =-87.6090

REMARKS: Concentrations are nominal starting concentrations

Prepared by Heather Walton Date 4-4-90

-22-

Acc. No. 901878
HAEL No. 90-0016
Test Type: Definitive

DEFINITVE TEST WORKSHEET - 72 HOURS

MEDIUM (x	FLASK CONC.	VALUE1	VALUE2	VALUE3	MEAN	CORRECTED MEAN (x)	FLASK COUNTS (y)	FLASK MEAN PER CONC.	S.D.	C.V.
ISOTON	0	36	37	37						
ISOTON	0	24	47	35	36.0					
FLASK 1	10	1306	1257	1315	1292.7	12566.7	11845			
FLASK 2	10	1426	1412	1337	1391.7	13556.7	12769			
FLASK 3	10	1353	1403	1307	1354.3	13183.3	12421	12345	381.1	3%
FLASK 4	5	1556	1499	1467	1507.3	14713.3	13649			
FLASK 5	5	1421	1360	1401	1394.0	13580.0	12791			
FLASK 6	5	1435	1455	1471	1453.7	14176.7	13346	13329	432.2	3%
FLASK 7	2.5	2263	2280	2280	2274.3	22383.3	21016			
FLASK 8	2.5	2477	2385	2295	2385.7	23496.7	22049			
FLASK 9	2.5	2451	2303	2347	2367.0	23310.0	21875	21644	454.5	2%
FLASK 10	1.25	4236	4222	4301	4253.0	84340.0	78852			
FLASK 11	1.25	4449	4253	4337	4346.3	86206.7	80594			
FLASK 12	1.25	4222	4189	4177	4196.0	83200.0	77787	79078	1157.1	1%
FLASK 13	0.625	5634	5594	5649	5625.7	111793.3	104482			
FLASK 14	0.625	6461	6504	6468	6477.7	128833.3	120390			
FLASK 15	0.625	7296	7223	7200	7239.7	144073.3	134618	119830	12309.5	10%
FLASK 16	0.1	10396	10032	10162	10196.7	203213.3	189831			
FLASK 17	0.1	9327	9318	9336	9327.0	185820.0	173592			
FLASK 18	0.1	9043	9049	8932	9008.0	179440.0	167636	177020	9379.4	5%
FLASK 19	0	8760	8866	8775	8800.3	175286.7	163759			
FLASK 20	0	9397	9506	9592	9498.3	189246.7	176791			
FLASK 21	0	9496	9524	9619	9546.3	190206.7	177688	172746	6365.5	4%

Flask Conc. is in mg/L
Six Isoton (diluent) background counts were made and averaged
Corrected Mean (x) = (Mean of the 3 Coulter Counter Values - Mean Isoton Value) x 2 x Dilution
Flask Counts (y) = y value in the regression equation: y = bx + a
Where b = 0.93359
a =112.6976

REMARKS: Concentrations are nominal starting concentrations

Prepared by Heather Watson Date 4-5-90

-23-

Acc. No. 901878
HAEL No. 90-0016
Test Type: Definitive

DEFINITVE TEST WORKSHEET - 96 HOURS

MEDIUM (x	FLASK CONC.	VALUE1	VALUE2	VALUE3	MEAN	CORRECTED MEAN (x)	FLASK COUNTS (y)	FLASK MEAN PER CONC.	S.D.	C.V.
ISOTON	0	33	36	36						
ISOTON	0	30	41	40	36.0					
FLASK 1	10	1287	1279	1210	1258.7	12226.7	15066			
FLASK 2	10	1340	1260	1251	1283.7	12476.7	15376			
FLASK 3	10	1267	1267	1291	1275.0	12390.0	15268	15237	128.3	1%
FLASK 4	5	1324	1422	1443	1396.3	13603.3	16770			
FLASK 5	5	1385	1369	1405	1386.3	13503.3	16646			
FLASK 6	5	1444	1398	1403	1415.0	13790.0	17001	16805	147.0	1%
FLASK 7	2.5	7375	7450	7237	7354.0	73180.0	90489			
FLASK 8	2.5	8215	8202	8074	8163.7	81276.7	100508			
FLASK 9	2.5	6147	6335	6265	6215.7	61796.7	76404	89134	9887.1	11%
FLASK 10	1.25	8033	7821	8257	8037.0	320040.0	395951			
FLASK 11	1.25	7138	7381	7240	7253.0	288680.0	357147			
FLASK 12	1.25	6968	8027	8067	7687.3	306053.3	378644	377248	15872.7	4%
FLASK 13	0.625	8958	8965	8998	8973.7	357506.7	442312			
FLASK 14	0.625	9173	9055	9079	9102.3	362653.3	448681			
FLASK 15	0.625	9260	9402	9218	9293.3	370293.3	458134	449709	6500.1	1%
FLASK 16	0.1	9851	9975	10038	9954.7	396746.7	490867			
FLASK 17	0.1	9629	9564	9856	9683.0	385880.0	477421			
FLASK 18	0.1	9644	9676	9741	9687.0	386040.0	477619	481969	6292.5	1%
FLASK 19	0	9880	9661	9682	9741.0	388200.0	480292			
FLASK 20	0	10459	10585	10415	10486.3	418013.3	517183			
FLASK 21	0	10372	10697	10381	10483.3	417893.3	517034	504836	17355.6	3%

Flask Conc. is in mg/L
Six Isoton (diluent) background counts were made and averaged
Corrected Mean (x) = (Mean of the 3 Coulter Counter Values - Mean Isoton Value) x 2 x Dilution
Flask Counts (y) = y value in the regression equation: y = bx + a
Where b = 1.23739
a =-62.8894

REMARKS: Concentrations are nominal starting concentrations

Prepared by Heather Watson Date 4-9-90

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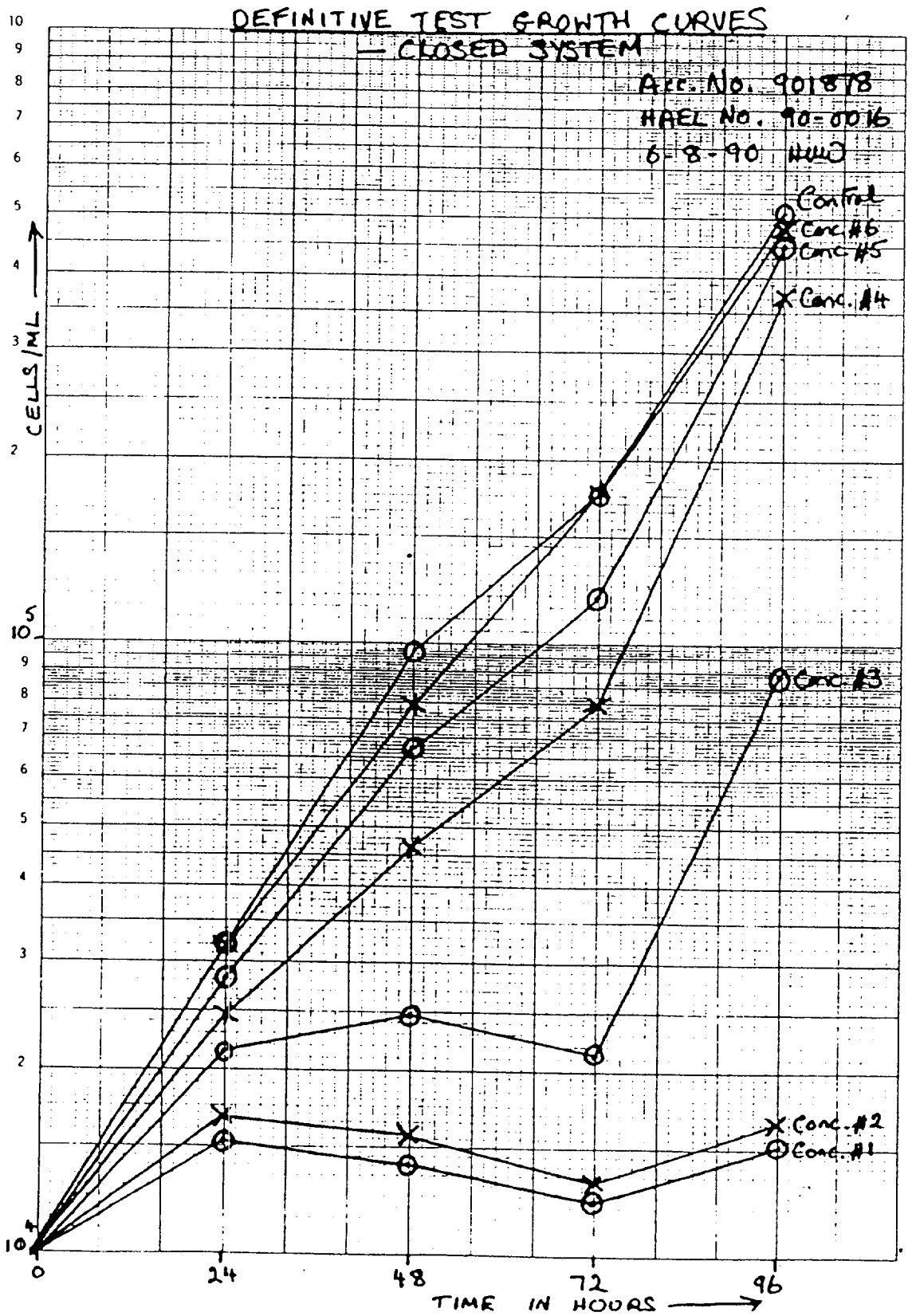
DEFINITIVE TEST GROWTH CURVES
— CLOSED SYSTEM
Acc. No. 901878
HAEL No. 90-0016
6-8-90
Control
Conc. #6
Conc. #5
Conc. #4
Conc. #3
Conc. #2
Conc. #1
CELLS/ML
TIME IN HOURS
0
24
48
72
96
10^4
10^5

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Acc. No. 901878
HAEL No. 90-0016
Test Type: Definitive

DEFINITIVE TEST RESULTS

FLASK DESCRIPTION	FLASK CONC.	0-HOURS	24-HOURS	48-HOURS	72-HOURS	96-HOURS
Test Conc. #1	10	10000	15186	13950	12345	15237
Test Conc. #2	5	10000	16791	15755	13329	16805
Test Conc. #3	2.5	10000	21540	24897	21644	89134
Test Conc. #4	1.25	10000	24796	46214	79078	377248
Test Conc. #5	0.625	10000	28030	67325	119830	449709
Test Conc. #6	0.1	10000	32322	79194	177020	481969
Control	0	10000	31954	96639	172746	504836

Flask Conc. is in mg/L

REMARKS: Concentrations are nominal starting concentrations

Prepared by Heather N Walton Date 4-9-90

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Acc. No. 901878
HAEL No. 90-0016
Test: Algal Definitive

CHEMICAL CONCENTRATION DATA WORKSHEET SHOWING GC VALUES

CONC NO.	NOMINAL CONC.	TIME 0 VALUES (MG/L)	TIME 0 MEAN (MG/L)	TIME 24 VALUES (MG/L)	TIME 24 MEAN (MG/L)	LOSS	TIME 48 VALUES (MG/L)	TIME 48 MEAN (MG/L)	LOSS	TIME 72 VALUES (MG/L)	TIME 72 MEAN (MG/L)	LOSS	TIME 96 VALUES (MG/L)	TIME 96 MEAN (MG/L)	LOSS	DETECT. LIMIT * (MG/L)
1	10.00	10.4000	10.6000	8.4000	8.5300	20%	6.9900	7.1750	32%	5.8000	5.8350	45%	5.690	5.730	46%	1.000
		10.8000		8.6600			7.3600			5.8700			5.770			
2	5.00	5.3600	5.2000	4.2700	4.2600	18%	3.0200	3.0600	41%	2.2000	2.3400	55%	0.560	0.563	89%	0.500
		5.0400		4.2500			3.1000			2.4800			0.566			
3	2.50	2.6600	2.6650	1.9900	2.0000	25%	1.3600	1.3750	48%	0.7450	0.7465	72%	N.D.	*	*	0.250
		2.6700		2.0100			1.3900			0.7480			N.D.			
4	1.25	1.2400	1.2250	0.8680	0.8605	30%	0.6350	0.6015	51%	0.0948	0.0970	92%	N.D.	*	*	~~0.125~~ 0.090
		1.2100		0.8530			0.5680			0.0992			N.D.			
5	0.625	0.6060	0.6130	0.3100	0.3410	44%	0.1240	0.1355	78%	N.D.	*	*	N.D.	*	*	0.062
		0.6200		0.3720			0.1470			N.D.			N.D.			
6	0.10	0.0961	0.0968	0.0566	0.0563	42%	N.D.	*	*	N.D.	*	*	N.D.	*	*	0.010
		0.0975		0.0559			N.D.			N.D.			N.D.			
Contr	0.00	N.D.	*	N.D.	*	*	N.D.	*	*	N.D.	*	*	N.D.	*	*	0.002
		N.D.		N.D.			N.D.			N.D.			N.D.			

Entry error 8/13/90 [illegible]

ND = None detected
* Calculated Detection Limit = 0.002 mg/L x Dilution Factor
GC Method Detection Limit = 0.002 mg/L

Prepared by Heather Watson Date 5-24-90

-27a-

STATISTICAL ANALYSIS FOR EPA/TSCA ALGAL ACUTE TOXICITY TEST

EC_{50} values for the algal toxicity test can be estimated by regression analysis using the PROC REG procedure in the SAS software package (1). The independent variable is the concentration of the test compound, and the dependent variable is defined as:

$$P = 100(\bar{C} - Y)/\bar{C}$$

where Y = the measured dry weight or cell density for a particular treatment replicate,

$\bar{C}$ = the mean measurement of the control replicates, and

P = the percent difference from the control mean for the particular treatment replicate.

The data analysis begins with a plot of P against the concentration of the test compound. Outliers can often be identified from this plot, and they should be investigated and reported. Also, concentrations at the extreme left or right with flat response can be identified for exclusion from the model fitting procedure. Using the remaining data, an appropriate regression model (usually a linear or quadratic model will suffice) is fit to the data. Sometimes a transformer of the concentration values may be necessary, especially when the concentrations are spaced an order of magnitude apart. The regression analysis produces a fitted model or equation:

$$P = a + b(CONC) \qquad \text{(linear model)}$$

or

$$P = a + b(CONC) + c(CONC)^2 \qquad \text{(quadratic model)}$$

The EC_x is estimated by substituting X for P and solving for CONC, and 95% confidence limits for the EC_x are obtained graphically from a plot of the 95% confidence bounds for the regression line. The confidence bounds produced by the regression analysis must be adjusted to account for the uncertainty in the control mean. This can be accomplished by applying an adjustment factor F to the variance of the regression residuals. F is derived as follows:

-25-

Acc. No. 901878
HAEL No. 90-0016
Test Type: Definitive

DEFINITIVE TEST RESULTS

FLASK DESCRIPTION	FLASK CONC.	0-HOURS	24-HOURS	48-HOURS	72-HOURS	96-HOURS
Test Conc. #1	10	10000	15186	13950	12345	15237
Test Conc. #2	5	10000	16791	15755	13329	16805
Test Conc. #3	2.5	10000	21540	24897	21644	89134
Test Conc. #4	1.25	10000	24796	46214	79078	377248
Test Conc. #5	0.625	10000	28030	67325	119830	449709
Test Conc. #6	0.1	10000	32322	79194	177020	481969
Control	0	10000	31954	96639	172746	504836

Flask Conc. is in mg/L

REMARKS: Concentrations are nominal starting concentrations

Prepared by Heather N Watson Date 4-9-90

-27b-

-2-

$$\begin{aligned}
Var(P\ CONC) &= 100^2\ Var(Y/\bar{C}) \\
&= 100^2\ [Var(Y)/\bar{C}^2 + Y^2\ Var(\bar{C})/\bar{C}^4] \text{ (by error propagation)} \\
&= 100^2\ [Var(Y)/\bar{C}^2 + (1-P/100)^2\ Var(\bar{C})/\bar{C}^2] \\
&= 100^2\ [Var(Y)/\bar{C}^2 + (1-P/100)^2\ Var(Y)/(n\bar{C}^2)] \text{ (assuming that } Var(C) = Var(Y)) \\
&= [100^2\ Var(Y)/\bar{C}^2]\ [1 + (1-P/100)^2\ /n]
\end{aligned}$$

where n is the number of replicates for the control mean.

The first bracketed term is estimated by the variance of the regression residuals when the model fits. The second term is the adjustment factor F, which varies depending upon the predicted value P at each value of the concentration of the test compound.

(1) SAS Institute Inc. SAS User's Guide: Statistics, 1982 Edition. Cary, NC: SAS Institute Inc., 1982.

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RECORD OF CULTURING TO DETERMINE IF TEST CHEMICAL IS ALGISTATIC OR ALGICIDAL

When growth is maximally inhibited, algicidal (killing) effects can be distinguished from algistatic (growth inhibiting) effects, if required, by removing aliquots (e.g., 0.5 mL) of test solution containing growth inhibited cells from the containers and culturing these with fresh media to dilute the chemical to a no-effect level. Cells are observed for growth for up to 9 days to see if the algistatic effect observed at 96 hours is reversible.

Test Substance: Crotonaldehyde

Accession No.: 901878 HAEL No.: 90-0016

Amount of Algal medium/flask: 100 mL Test Solution Aliquot: 0.5 mL

Flasks from which Test Solution used:

Flask #	Concentration	Start Date/Comments
1-3	10 mg/L*	4-6-90 (3 replicates)
4-5	5 mg/L*	4-6-90 (2 replicates - 3rd replicate inadvertently disposed of)

Results

Flask #	Date/Days in Culture	Growth/Comments
1-3	4-23-90/17	No growth visible in any flasks after 3, 9, or 17 days in culture.
4-5	4-23-90/17	

Conclusion

Crotonaldehyde was algicidal at starting nominal concentrations of 5 and 10 mg/L

REMARKS: *Nominal starting concentrations

Prepared by Heather M. Walton Date 4-23-90

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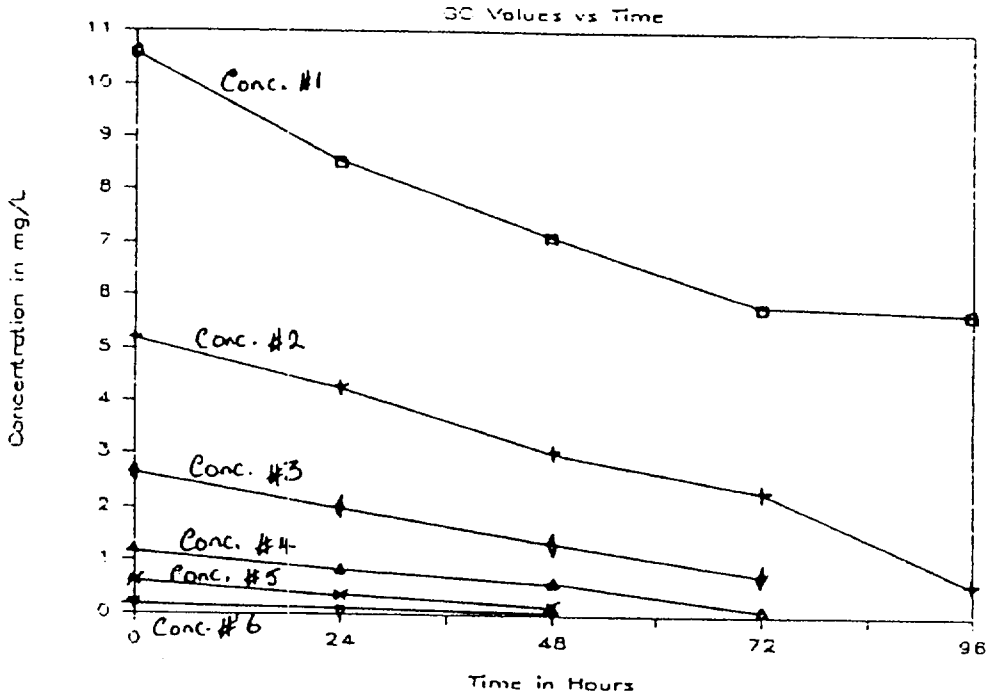
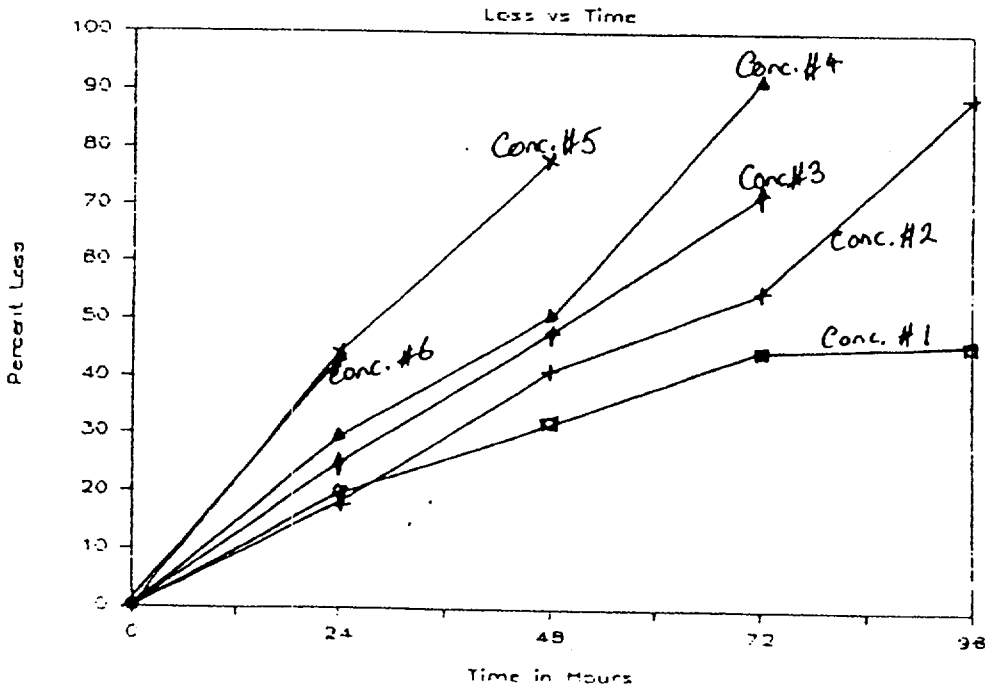
CONCENTRATION VALUES FOR 90-0016



PERCENT LOSS OF 90-0016



NOTE: No data points are drawn once the lower limit of detection is reached

Attachment 2. CQS Division Report on Chemical Concentration

ANALYTICAL SUPPORT FOR

EPA/TSCA ALGAL ACUTE TOXICITY TEST OF

CROTONALDEHYDE

Accession No. 901878 HAEL No. 90-0016

By

Environmental Analytical Services Unit

Chemicals Quality Services Division

B-320

Eastman Kodak Company

Rochester, New York 14652-3615

Completion Date: 04/20/90

ABSTRACT

Several steps were carried out to perform the concentration determination of test chemical in samples received. A method was developed and validated. In case filtering and pooling would be required, these steps were also validated. An algal pre-test was performed to confirm volatility in an open versus closed system. Then the actual range-finding and definitve tests were performed. Samples containing algal cells and test chemical were received for concentration determination. These samples were derivatized and extracted and then analyzed by gas chromatography with electron capture detection (GC/ECD).

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90-0016

Crotonaldehyde
901878

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TESTING FACILITY

Environmental Analytical Services Unit
Chemicals Quality Services Division
Bldg-320
Eastman Kodak Company
Rochester, New York 14652-3615

SPONSOR

Environmental Technical Services
Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652

DATE OF STUDY INITATION: 11/01/89

DATES OF EXPERIMENT

Experiment Start Date: 01/02/90

Experiment Termination Date: 04/17/90

STUDY PARTICIPANTS

Chemicals Quality Services Division

Barry W. Remington, Laboratory Technician
Elizabeth M. Isaacs, Laboratory Technician

Robert Ambrose, Chemist

STUDY DIRECTOR

Heather M. Watson

INTRODUCTION

The Environmental Protection Agency (EPA) is concerned about the potential environmental impact of releases of quantities of Crotonaldehyde into the Holston River at the Kingsport, Tennessee Kodak plant where the test chemical is manufactured. Kodak has volunteered to perform numerous aquatic tests on the test chemical through Testing Consent Order Docket No. OPTS-42108. The first test, an algal study, was performed where concentration determinations were carried out using GC/ECD.

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REQUEST FOR ANALYSIS

Method Validation	01-22-90	149293
Algal Pre-test Time 0	02-12-90	142424
Time 96	02-16-90	142423
Filtering Validation	03-13-90	149293
Algal Range Finding Time 0	03-19-90	142421
Time 96	03-23-90	142420
Purity	03-20-90	149292
Pooling Validation	03-30-90	149293
Algal Definitive Test Time 0	04-02-90	142473
Time 24	04-03-90	142474
Time 48	04-04-90	142475
Time 72	04-05-90	142476
Time 96	04-06-90	142477

TEST ARTICLE

Name: Crotonaldehyde
HAEL No.: 90-0016
Accession No.: 901878
Sample Reference No.: 90-7

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MATERIALS AND METHODS

Title: Analysis of Crotonaldehyde in Algal Medium

Abstract: Samples containing crotonaldehyde were diluted (as necessary), derivitized, extracted and analyzed by gas chromatography with electron capture detection (GC/ECD). The method detection limit was 2 ug/L.

Procedure:

Materials:

1. O-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride (PFBOA), Aldrich c/n 19,448-4
2. Sodium thiosulfate, Aldrich c/n 21,726-3
3. Sulfuric acid, Eastman Chemical c/n 13063
4. Algal medium (B-306)
5. Distilled water
6. Hexane, Baker Resi-analyzed c/n 9262-03
7. Culture tubes, 16 x 125 mm, Kimble c/n 45066-A
8. Disposable Pasteur pipets, VWR Scientific c/n 14672-200
9. Assorted volumetric flasks (25, 50 and 100 mL)
10. Assorted volumetric pipets (1, 2 and 10 mL)
11. Assorted SMI micropettors (50 to 1000 uL)
12. Capillary gas chromatograph
13. Capillary gas chromatographic column, DB-5, 30 meter, 0.25 um film thickness, J&W Scientific c/n 009-5032
14. Chromatographic data handling system

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Reagent Preparation:

1. 0.10 g of PFBOA was weighed to a 100.0 mL volumetric flask and diluted it to the mark with distilled water. This solution was prepared fresh daily.

2. 1.58 g of sodium thiosulfate was weighed to a 100.0 mL volumetric flask and diluted to the mark with distilled water. (0.1 M solution). This solution was good for one (1) week.

Standard Preparation:

1. Stock Approximately 0.100 g of test chemical was weighed to a 100.0 mL volumetric flask and diluted to the mark with algal medium. This solution was prepared fresh daily.

2. Intermediate An 0.5 mL aliquot of the stock solution was taken and diluted to 100.0 mL with algal medium.

3. Working Up to seven (7) aliquots ranging from 25 to 175 uL of the intermediate solution were taken and the aliquots tranferred to separate 25.0 mL volumetric flasks. The flasks were diluted to the mark with distilled water.

Sample Preparation:

1. Samples with expected concentrations greater than 10 mg/L were diluted down to a concentration range between 1-10 mg/L with algal medium.

2. All samples were then diluted down to 20 ug/L with distilled water using at least 25 mL volumetric flasks.

Note: Controls and samples with expected concentrations less than 30 ug/L were not diluted.

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Derivatization:

1. One (1.0) mL of the 1 mg/mL PFBOA solution was added to a culture tube.
2. 200 uL of the 0.1 M sodium thiosulfate solution was added to the culture tube and mixed.
3. Ten (10.0) mL of distilled water was added and mixed. Allow to stand for two (2) hours.
4. Steps 1-3 were repeated for all of the working standards.
5. Steps 1-3 were repeated for all samples.

Extraction:

1. One (1) drop of concentrated sulfuric acid was added to each culture tube and mixed.
2. Two (2.0) mL of hexane were added to each culture tube and then shaken for 30 seconds.
3. The culture tubes were allowed to stand for fifteen (15) minutes.
4. Approximately half of the extract was transferred to two (2) separate GC vials and capped.
5. Step 4 was repeated for all samples.

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Analyses:

1. The samples were then analyzed by GC/ECD using the following conditions:

Instrument: Perkin-Elmer 8500 Gas Chromatograph

Column: J&W DB-5 30 meter 0.25 um film thickness

Pressure: 10 psig

Oven Program: 80 C (held for 2 min.) to 250 C @ 10 C/min. held @ 250 C for 1 min.

Injector Temp: 250 C

Type: Splitless (2 min. purge delay)

Split Flow: 60 cc/min.

Inj. Volume: 1 uL

Detector: Electron Capture

Temp.: 300 C

Make-up: Nitrogen

Flow: 55 cc/min.

Calculations:

1. Data reduction was performed using a chromatography data handling system such as Multichrom (a VG, Inc. product). The cis- and trans- isomers were added together and treated as one component whenever possible.

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RESULTS

PURITY

The purity of the test chemical was determined by a combination of a water analysis by Karl Fisher titration and a gas chromatographic analysis with flame ionization detection. The results are as follows:

	Water (%)	Area % (GC/FID)
3-20-90	7.1	98.1

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METHOD VALIDATION

Theoretical Conc. (mg/L)	Actual Conc. (mg/L)	Recovery (%)
0.118	0.119	101
1.18	1.26	107
11.8	12.4	105
118	123	104
1180	1254	106

Filtering Validation

	Theoretical Conc. (mg/L)	Actual Conc. (mg/L)	Recovery (%)
Algal Medium	110	121	110
	1.10	1.15	104
Algal Cells	110	119	108
	1.10	0.902	82.0

Pooling Validation

	Theoretical Conc. (mg/L)	Actual Conc. (mg/L)	Recovery (%)
As Is	11.75	13.21	112
Pooled	11.75	13.81	118
As Is	1.175	1.123	95.6
Pooled	1.175	1.208	103
As Is	0.1175	0.1345	114
Pooled	0.1175	0.1418	121

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ALGAL PRE-TEST

Sample	Theoretical Conc. (mg/L)	TIME 0 Actual Conc. (mg/L)	TIME 96 'Open' Actual Conc. (mg/L)	TIME 96 'Closed' Actual Conc. (mg/L)
Control A	0.0	< 0.003	0.007	< 0.003
Control B	0.0	< 0.003	0.007	< 0.003
Conc. 6A	0.0095	0.0729	0.004	< 0.003
Conc. 6B	0.0095	0.0728	0.004	< 0.003
Conc. 5A	0.095	0.339	0.248	0.244
Conc. 5B	0.095	0.333	0.248	0.239
Conc. 4A	0.95	1.59	0.460	0.820
Conc. 4B	0.95	1.49	0.464	0.846
Conc. 3A	9.5	12.4	3.68	7.00
Conc. 3B	9.5	12.5	3.76	7.32
Conc. 2A	95	130	32.3	73.9
Conc. 2B	95	108	36.6	77.8
Conc. 1A	950	996	376	838
Conc. 1B	950	1048	384	826

Method Detection Limit = 0.002 mg/L

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ALGAL RANGE FINDING

Sample	Theoretical Conc. (mg/L)	TIME 0 Actual Conc. (mg/L)	TIME 96 Chemical Control Actual Conc. (mg/L)	TIME 96 Test Actual Conc. (mg/L)
Control A	0.0	N.D.	< 0.003	< 0.003
Control B	0.0	N.D.	< 0.003	< 0.003
Conc. 6A	0.010	0.0794	0.00585	< 0.003
Conc. 6B	0.010	0.0837	0.00553	< 0.003
Conc. 5A	0.10	0.107	0.0807	< 0.003
Conc. 5B	0.10	0.108	0.0820	< 0.003
Conc. 4A	1.0	1.10	0.847	0.0218
Conc. 4B	1.0	1.12	0.827	0.00312
Conc. 3A	10	11.1	8.62	7.50
Conc. 3B	10	11.2	8.24	6.80
Conc. 2A	100	108	102	98.4
Conc. 2B	100	110	101	95.5
Conc. 1A	1000	1210	1170	1070
Conc. 1B	1000	1180	1140	1109

N.D. = None Detected

Method Detection Limit = 0.002 mg/L

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ALGAL DEFINITIVE TEST

Sample	Theor Conc. (mg/L)	Time 0 Actual Conc. (mg/L)	Time 24 Actual Conc. (mg/L)	Time 48 Actual Conc. (mg/L)	Time 72 Actual Conc. (mg/L)	Time 96 Actual Conc. (mg/L)	Detect Limit (mg/L)
Control A	0.0	N.D.	N.D.	N.D.	N.D.	N.D.	0.002
Control B	0.0	N.D.	N.D.	N.D.	N.D.	N.D.	0.002
Conc. 6A	0.10	0.0975	0.0559	N.D.	N.D.	N.D.	0.010
Conc. 6B	0.10	0.0961	0.0566	N.D.	N.D.	N.D.	0.010
Conc. 5A	0.625	0.620	0.372	0.148	N.D.	N.D.	0.062
Conc. 5B	0.625	0.606	0.310	0.124	N.D.	N.D.	0.062
Conc. 4A	1.25	1.21	0.853	0.569	0.0948	N.D.	0.090
Conc. 4B	1.25	1.24	0.868	0.636	0.0903	N.D.	0.090
Conc. 3A	2.50	2.67	2.01	1.39	0.744	N.D.	0.25
Conc. 3B	2.50	2.66	1.99	1.36	0.740	N.D.	0.25
Conc. 2A	5.00	5.04	4.25	3.10	2.48	0.566	0.50
Conc. 2B	5.00	5.36	4.27	3.02	2.20	0.560	0.50
Conc. 1A	10.0	10.8	8.66	7.36	5.87	5.77	1.0
Conc. 1B	10.0	10.4	8.40	6.99	5.80	5.69	1.0

Method Detection Limit = 0.002 mg/L

Detection Limits = (0.002 mg/L) x (Dilution Factor)

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PERTINENT MATHEMATICAL EQUATIONS

Calculations of the concentration of the test chemical were done using the linear regression equation for the standards run with each day:

$y = mx + b$ where: y = area
m = slope
x = concentration (ug/L)
b = y-intercept

All samples were corrected using the appropriate dilution factor to obtain the concentration in mg/L.

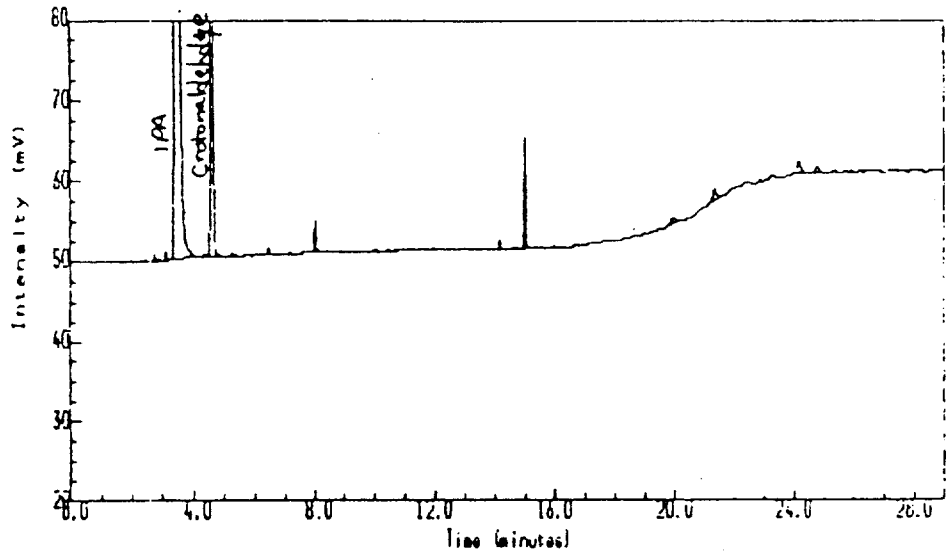
Scientist Barry M Remington Date 7-17-90
Scientist Robert T Ambrose Date 7/17/90
(signature)

FIGURE I

[DEFPROJECT] 10 CROTON,2,1
Reported on 20-MAR-1990 at 15:08

Injection Report

Acquired on 20-MAR-1990 at 12:31



EAS CHROMATOGRAPHY SYSTEM

Analyst Name : PORTER
Line Id :
Comment :
Method Title : CROTONALDEHYDE BY FID
Sample Name : 90-0016 DIL IN IPA FOR PURITY N2 PURGED
Sample Id :
Sample Type : Sample Amount=1.00000
Bottle No : 2

FIGURE 2

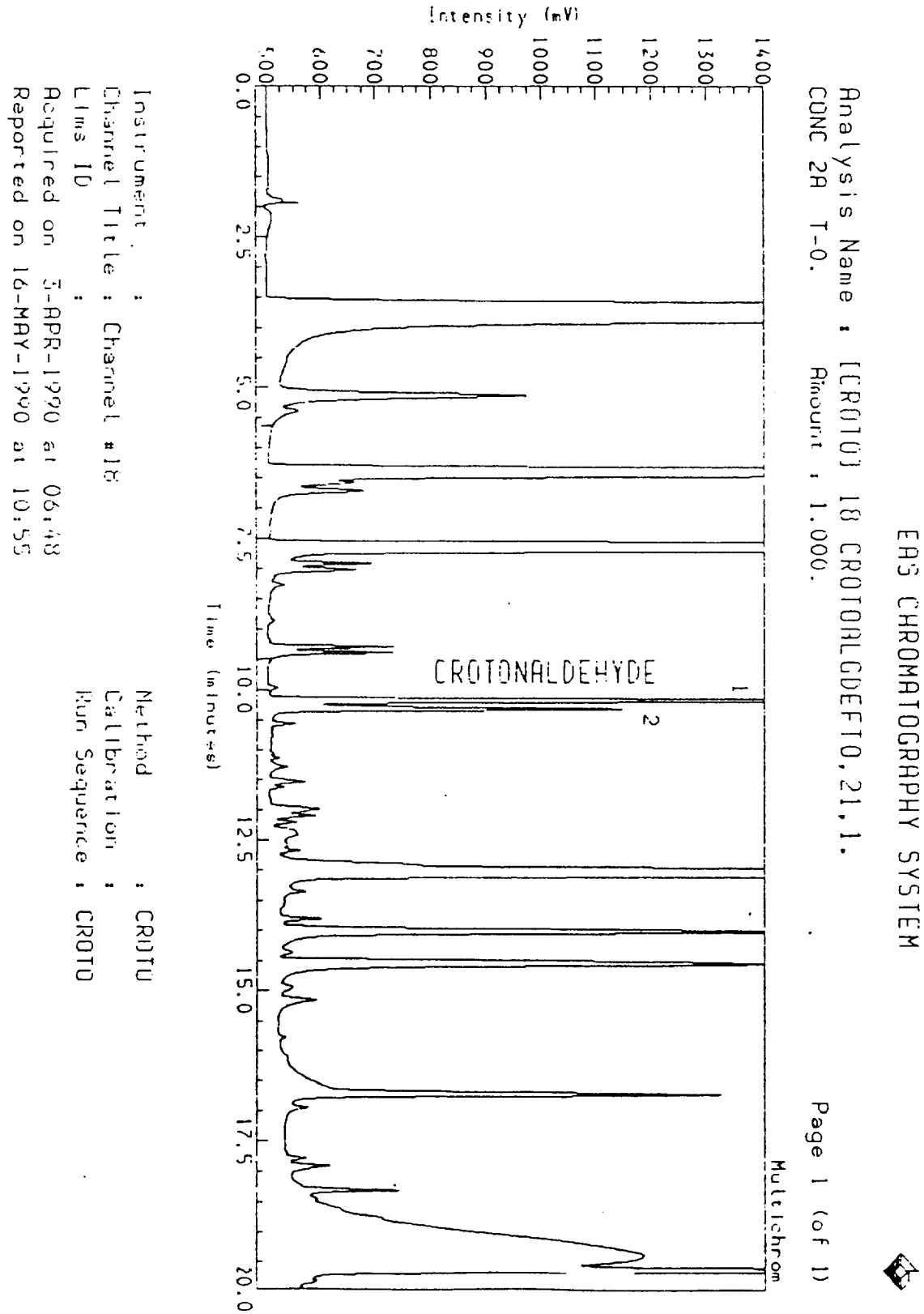

EAS CHROMATOGRAPHY SYSTEM
Analysis Name : [CROTO] 18 CROTONALDEHYDE,21,1.
CONC 2A T-0. Amount : 1.000.
Page 1 (of 1)
Intensity (mV)
1400
1300
1200
1100
1000
900
800
700
600
500
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
Time (minutes)
CROTONALDEHYDE
1
2
Multichrom
Instrument :
Channel Title : Channel # 18
Lims ID :
Acquired on 3-APR-1990 at 06:48
Reported on 16-MAY-1990 at 10:55
Method : CROTU
Calibration :
Run Sequence : CROTO

FIGURE 3

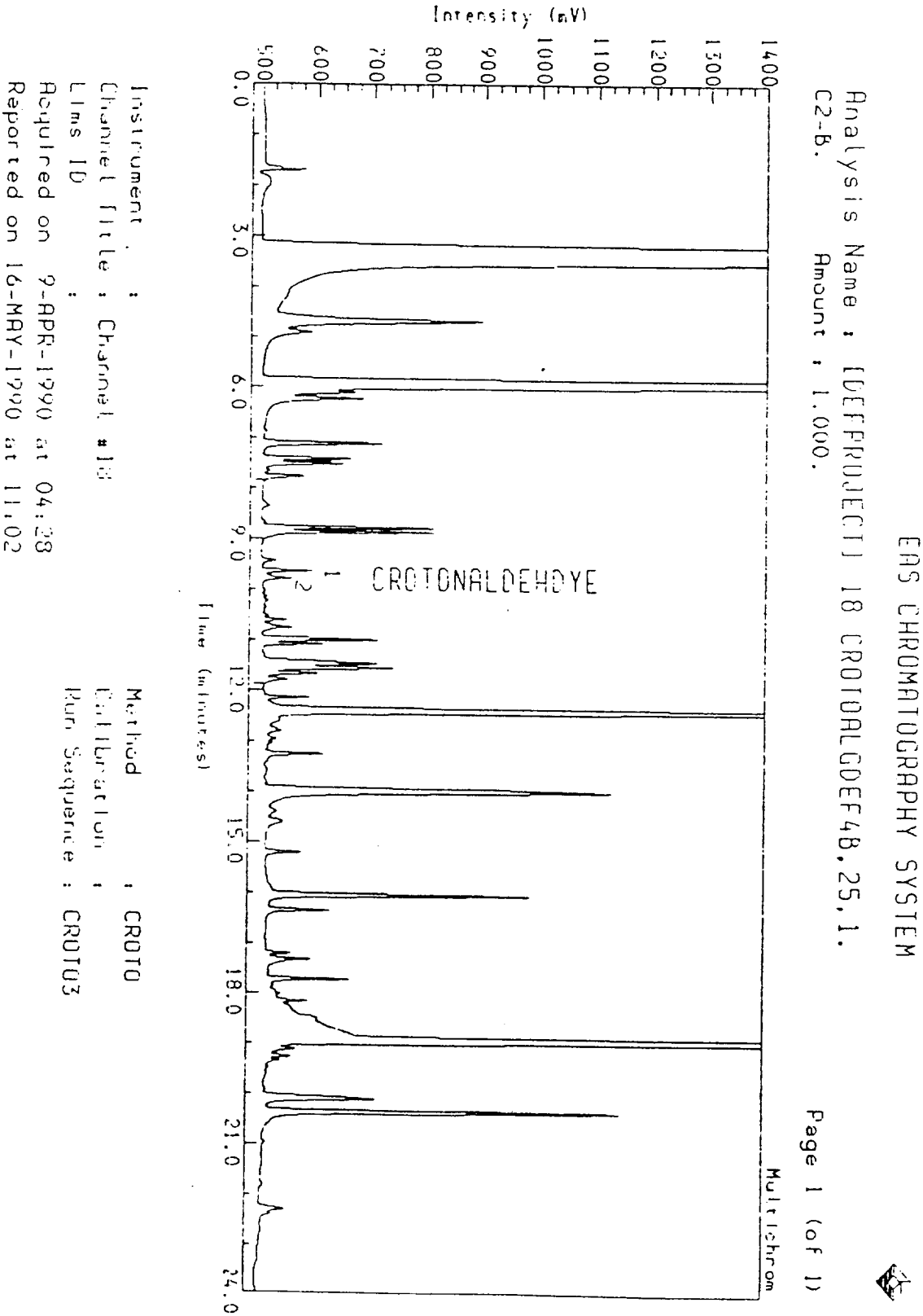
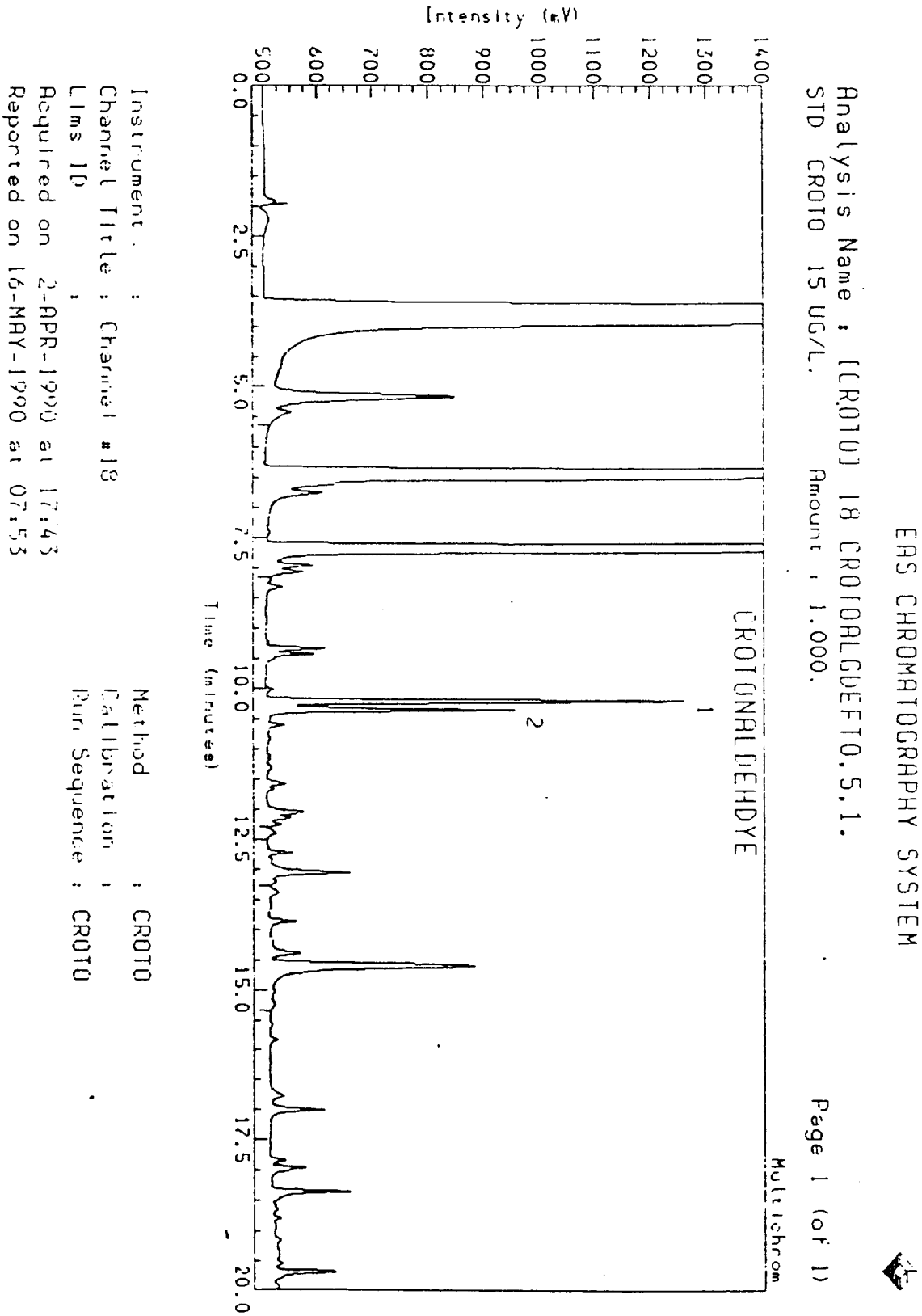

EAS CHROMATOGRAPHY SYSTEM
Analysis Name : EDEFPROJECT1 18 CROTOALDOEF4B.25.1.
C2-B. Amount : 1.000.
Page 1 (of 1)
Multichrom
Intensity (mV)
1400
1300
1200
1100
1000
900
800
700
600
500
0.0
3.0
6.0
9.0
12.0
15.0
18.0
21.0
24.0
Time (minutes)
1
2
CROTONALDEHDYE
Instrument :
Channel Title : Channel #18
Lims ID :
Acquired on 9-APR-1990 at 04:28
Reported on 16-MAY-1990 at 11:02
Method : CROTO
Calibration :
Run Sequence : CROTO3

FIGURE 4


EAS CHROMATOGRAPHY SYSTEM
Analysis Name : [CROTO] 18 CROTOALGUEFTO.5.1.
STD CROTO 15 UG/L.
Amount : 1.000.
Page 1 (of 1)
Multichrom
CROTONALDEHDYE
1
2
Intensity (µV)
1400
1300
1200
1100
1000
900
800
700
600
500
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
Time (minutes)
Instrument : Method : CROTO
Channel Title : Channel #18 Calibration :
Lims ID : Run Sequence : CROTO
Acquired on 2-APR-1990 at 17:43
Reported on 16-MAY-1990 at 07:53

Attachment 3. Report on Statistical Analysis

Results for the Algal Acute Toxicity Test with Crotonaldehyde

Averaged Dose Levels

Acc. No. 901878 Hael No. 90-0016

Time (hours)	Effective Concentration	Dose mg/L	Upper 95% Conf. Limit	Lower 95% Conf. Limit	Regression Model Used
24	EC10	0.282	0.363	0.199	linear
	EC50	8.492	>10.00	6.026	linear
	EC90	258.7	ne	ne	linear
48	EC10	0.045	0.076	<0.025	linear
	EC50	0.646	0.871	0.468	linear
	EC90	9.247	>10.00	5.750	linear
72	EC10	0.089	0.126	0.056	linear
	EC50	0.597	0.794	0.457	linear
	EC90	4.027	6.310	2.818	linear
96	EC10	0.385	0.447	0.316	cubic
	EC50	0.881	1.000	0.794	cubic
	EC90	1.982	2.754	1.905	cubic

ne - not estimable from the graph of the equation

Merrilee Ritter

Merrilee Ritter, Biostatistician
MQAO/QTAS, 2-320-KP
June 8, 1990

STATISTICAL ANALYSIS FOR EPA/TSCA ALGAL ACUTE TOXICITY TEST

EC_{50} values for the algal toxicity test can be estimated by regression analysis using the PROC REG procedure in the SAS software package (1). The independent variable is the concentration of the test compound, and the dependent variable is defined as:

$$P = 100(\bar{C} - Y)/\bar{C}$$

where Y = the measured dry weight or cell density for a particular treatment replicate,

$\bar{C}$ = the mean measurement of the control replicates, and

P = the percent difference from the control mean for the particular treatment replicate.

The data analysis begins with a plot of P against the concentration of the test compound. Outliers can often be identified from this plot, and they should be investigated and reported. Also, concentrations at the extreme left or right with flat response can be identified for exclusion from the model fitting procedure. Using the remaining data, an appropriate regression model (usually a linear or quadratic model will suffice) is fit to the data. Sometimes a transformer of the concentration values may be necessary, especially when the concentrations are spaced an order of magnitude apart. The regression analysis produces a fitted model or equation:

$$P = a + b(CONC) \qquad \text{(linear model)}$$

or

$$P = a + b(CONC) + c(CONC)^2 \qquad \text{(quadratic model)}$$

The EC_x is estimated by substituting X for P and solving for CONC, and 95% confidence limits for the EC_x are obtained graphically from a plot of the 95% confidence bounds for the regression line. The confidence bounds produced by the regression analysis must be adjusted to account for the uncertainty in the control mean. This can be accomplished by applying an adjustment factor F to the variance of the regression residuals. F is derived as follows:

-2-

$$\begin{aligned}
\mathrm{Var}(P\ \mathrm{CONC}) &= 100^2\ \mathrm{Var}(Y/\bar{C}) \\
&= 100^2\ [\mathrm{Var}(Y)/\bar{C}^2 + Y^2\ \mathrm{Var}(\bar{C})/\bar{C}^4] \text{ (by error propagation)} \\
&= 100^2\ [\mathrm{Var}(Y)/\bar{C}^2 + (1-P/100)^2\ \mathrm{Var}(\bar{C})/\bar{C}^2] \\
&= 100^2\ [\mathrm{Var}(Y)/\bar{C}^2 + (1-P/100)^2\ \mathrm{Var}(Y)/(n\bar{C}^2)] \text{ (assuming that } \mathrm{Var}(C) = \mathrm{Var}(Y)) \\
&= [100^2\ \mathrm{Var}(Y)/\bar{C}^2]\ [1 + (1-P/100)^2\ /n]
\end{aligned}$$

where n is the number of replicates for the control mean.

The first bracketed term is estimated by the variance of the regression residuals when the model fits. The second term is the adjustment factor F, which varies depending upon the predicted value P at each value of the concentration of the test compound.

(1) SAS Institute Inc. SAS User's Guide: Statistics, 1982 Edition. Cary, NC: SAS Institute Inc., 1982.

ALGAL ACUTE TOXICITY TEST WITH CROTONALDEHYDE
KAN 901878, HAEL NO 90-0016
AVERAGED DOSE LEVELS

9 39 THURSDAY, JUNE 7, 1990

HOUR=24

DEP VARIABLE COUNT PERCENT INHIBITION

ANALYSIS OF VARIANCE

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PROB>F
MODEL	1	6181.75514	6181.75514	227.631	0.0001
ERROR	16	434.50994	27.15687145		
C TOTAL	17	6616.26508			

ROOT MSE	5.211226	R-SQUARE	0.9343
DEP MEAN	27.6745	ADJ R-SQ	0.9302
C.V	18.83042		

PARAMETER ESTIMATES

VARIABLE	DF	PARAMETER ESTIMATE	STANDARD ERROR	T FOR H0: PARAMETER=0	PROB > |T|	VARIABLE LABEL
INTERCEP	1	24.94805225	1.24151969	20.095	0.0001	INTERCEPT
LCONC	1	26.96101754	1.78698229	15.087	0.0001	LOG OF CONCENTRATION IN MG/L

OBS	ACTUAL	PREDICT. VALUE	STD ERR PREDICT	LOWER95% MEAN	UPPER95% MEAN	RESIDUAL	STD ERR RESIDUAL	STUDENT RESIDUAL	-2 -1 0 1 2	COOK'S D
1	51.3801	51.3883	1.9948	47.1596	55.6170	-.008208	4.8143	-.001705		0.000
2	54.9696	51.3883	1.9948	47.1596	55.6170	3.5813	4.8143	0.7439	*	0.048
3	51.0765	51.3883	1.9948	47.1596	55.6170	-0.3118	4.8143	-0.0648		0.000
4	48.0941	43.1430	1.6000	39.7513	46.5347	4.9511	4.9595	0.9983	*	0.052
5	47.9971	43.1430	1.6000	39.7513	46.5347	4.8541	4.9595	0.9787	*	0.050
6	46.2696	43.1430	1.6000	39.7513	46.5347	3.1266	4.9595	0.6304	*	0.021
7	30.7035	34.8674	1.3176	32.0743	37.6605	-4.1639	5.0419	-0.8259	*	0.023
8	30.6785	34.8674	1.3176	32.0743	37.6605	-4.1889	5.0419	-0.8308	*	0.024
9	36.3867	34.8674	1.3176	32.0743	37.6605	1.5193	5.0419	0.3013		0.003
10	17.3781	25.4410	1.2372	22.8183	28.0637	-8.0629	5.0622	-1.5928	***	0.076
11	30.5564	25.4410	1.2372	22.8183	28.0637	5.1154	5.0622	1.0105	**	0.030
12	19.2652	25.4410	1.2372	22.8183	28.0637	-6.1758	5.0622	-1.2200	**	0.044
13	13.0813	16.2806	1.4419	13.2239	19.3372	-3.1993	5.0078	-0.6389	*	0.017
14	10.3336	16.2806	1.4419	13.2239	19.3372	-5.9470	5.0078	-1.1875	**	0.058
15	13.4224	16.2806	1.4419	13.2239	19.3372	-2.8582	5.0078	-0.5707	*	0.014
16	0.8043	-5.0733	2.4940	-10.3603	0.2137	5.8776	4.5757	1.2845	**	0.245
17	4.6379	-5.0733	2.4940	-10.3603	0.2137	9.7112	4.5757	2.1223	****	0.669
18	-8.8940	-5.0733	2.4940	-10.3603	0.2137	-3.8207	4.5757	-0.8350	*	0.104

SUM OF RESIDUALS	2.05835E-13
SUM OF SQUARED RESIDUALS	434.5099
PREDICTED RESID SS (PRESS)	580.3375

ALGAL ACUTE TOXICITY TEST WITH CROTONALDEHYDE
KAN 901878, HAEL NO. 90-0016
AVERAGED DOSE LEVELS

9:39 THURSDAY, JUNE 7, 1990

HOUR=48

DEP VARIABLE: COUNT PERCENT INHIBITION

ANALYSIS OF VARIANCE

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PROB>F
MODEL	1	11416.11906	11416.11906	163.121	0.0001
ERROR	16	1119.76927	69.98557939		
C TOTAL	17	12535.88833			

ROOT MSE	8.365738	R-SQUARE	0.9107
DEP MEAN	57.3436	ADJ R-SQ	0.9051
C.V	14.58879		

PARAMETER ESTIMATES

VARIABLE	DF	PARAMETER ESTIMATE	STANDARD ERROR	T FOR H0: PARAMETER=0	PROB > |T|	VARIABLE LABEL
INTERCEP	1	56.60739753	1.97266580	28.696	0.0001	INTERCEPT
LCONC	1	34.58395349	2.70781927	12.772	0.0001	LOG OF CONCENTRATION IN MG/L

OBS	ACTUAL	PREDICT VALUE	STD ERR PREDICT	LOWER95% MEAN	UPPER95% MEAN	RESIDUAL	STD ERR RESIDUAL	STUDENT RESIDUAL	-2 -1 0 1 2	COOK'S D
1	86.0222	89.4208	3.1931	82.6517	96.1899	-3.3986	7.7324	-0.4395		0.016
2	85.5659	89.4208	3.1931	82.6517	96.1899	-3.8549	7.7324	-0.4985		0.021
3	85.1054	89.4208	3.1931	82.6517	96.1899	-4.3154	7.7324	-0.5581	*	0.027
4	83.1807	77.9094	2.5458	72.5126	83.3062	5.2713	7.9690	0.6615	*	0.022
5	83.6981	77.9094	2.5458	72.5126	83.3062	5.7887	7.9690	0.7264	*	0.027
6	84.2113	77.9094	2.5458	72.5126	83.3062	6.3020	7.9690	0.7908	*	0.032
7	74.3458	67.1677	2.1165	62.6808	71.6545	7.1781	8.0936	0.8869	*	0.027
8	74.5206	67.1677	2.1165	62.6808	71.6545	7.3530	8.0936	0.9085	*	0.028
9	73.8439	67.1677	2.1165	62.6808	71.6545	6.6762	8.0936	0.8249	*	0.023
10	48.9730	55.2403	1.9787	51.0457	59.4349	-6.2673	8.1284	-0.7710	*	0.018
11	52.2636	55.2403	1.9787	51.0457	59.4349	-2.9767	8.1284	-0.3662		0.004
12	55.2986	55.2403	1.9787	51.0457	59.4349	0.0583	8.1284	0.071681		0.000
13	20.3914	41.8356	2.3157	36.9266	46.7446	-21.4443	8.0389	-2.6676	*****	0.295
14	32.2272	41.8356	2.3157	36.9266	46.7446	-9.6085	8.0389	-1.1953	**	0.059
15	38.3810	41.8356	2.3157	36.9266	46.7446	-3.4546	8.0389	-0.4297		0.008
16	17.4133	12.4878	4.0277	3.9494	21.0262	4.9254	7.3323	0.6717	*	0.068
17	10.6965	12.4878	4.0277	3.9494	21.0262	-1.7913	7.3323	-0.2443		0.009
18	26.0464	12.4878	4.0277	3.9494	21.0262	13.5586	7.3323	1.8492	***	0.516

SUM OF RESIDUALS	3.84137E-13
SUM OF SQUARED RESIDUALS	1119.769
PREDICTED RESID SS (PRESS)	1429.488

ALGAL ACUTE TOXICITY TEST WITH CROTONALDEHYDE
KAN 901878, HAEL NO. 90-0016
AVERAGED DOSE LEVELS

9:39 THURSDAY, JUNE 7, 1990

HOUR=72

P VARIABLE: COUNT PERCENT INHIBITION

ANALYSIS OF VARIANCE

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PROB>F
MODEL	1	21627.34230	21627.34230	212.563	0.0001
ERROR	16	1627.93238	101.74577		
C TOTAL	17	23255.27468			

ROOT MSE	10.08691	R SQUARE	0.9300
DEP MEAN	59.16487	ADJ R-SQ	0.9256
C.V.	17.04882		

PARAMETER ESTIMATES

VARIABLE	DF	PARAMETER ESTIMATE	STANDARD ERROR	T FOR H0: PARAMETER=0	PROB > |T|	VARIABLE LABEL
INTERCEP	1	60.80124535	2.38015553	25.545	0.0001	INTERCEPT
LCONC	1	48.24235884	3.30891147	14.580	0.0001	LOG OF CONCENTRATION IN MG/L

OBS	ACTUAL	PREDICT VALUE	STD ERR PREDICT	LOWER95% MEAN	UPPER95% MEAN	RESIDUAL	STD ERR RESIDUAL	STUDENT RESIDUAL	-2 -1 0 1 2	COOK'S D
1	93.1431	104.9	3.9378	96.5839	113.3	-11.7886	9.2865	-1.2694	| **| |	0.145
2	92.6082	104.9	3.9378	96.5839	113.3	-12.3235	9.2865	-1.3270	| **| |	0.158
3	92.8097	104.9	3.9378	96.5839	113.3	-12.1220	9.2865	-1.3053	| **| |	0.153
4	91.9830	88.6053	3.1193	81.9927	95.2179	3.3777	9.5925	0.3521	| | |	0.007
5	92.5955	88.6053	3.1193	81.9927	95.2179	3.9902	9.5925	0.4160	| | |	0.009
6	92.2730	88.6053	3.1193	81.9927	95.2179	3.6677	9.5925	0.3824	| | |	0.008
7	87.8376	71.9925	2.5351	66.6183	77.3666	15.8452	9.7632	1.6230	| |***|	0.089
8	87.2362	71.9925	2.5351	66.6183	77.3666	15.2437	9.7632	1.5614	| |***|	0.082
9	87.3369	71.9925	2.5351	66.6183	77.3666	15.3444	9.7632	1.5717	| |***|	0.083
10	54.3538	52.1273	2.4260	46.9844	57.2702	2.2265	9.7908	0.2274	| | |	0.002
11	53.3454	52.1273	2.4260	46.9844	57.2702	1.2180	9.7908	0.1244	| | |	0.000
12	54.9703	52.1273	2.4260	46.9844	57.2702	2.8430	9.7908	0.2904	| | |	0.003
13	39.5170	38.0751	2.7830	32.1754	43.9747	1.4419	9.6954	0.1487	| | |	0.001
14	30.3081	38.0751	2.7830	32.1754	43.9747	-7.7670	9.6954	-0.8011	| *| |	0.026
15	22.0717	38.0751	2.7830	32.1754	43.9747	-16.0034	9.6954	-1.6506	| ***| |	0.112
16	-9.8902	-0.7427	4.7473	-10.8064	9.3210	-9.1475	8.9000	-1.0278	| **| |	0.150
17	-0.4897	-0.7427	4.7473	-10.8064	9.3210	0.2530	8.9000	0.0284	| | |	0.000
18	2.9581	-0.7427	4.7473	-10.8064	9.3210	3.7008	8.9000	0.4158	| | |	0.025

SUM OF RESIDUALS 3.66596E-13
SUM OF SQUARED RESIDUALS 1627.932
PREDICTED RESID SS (PRESS) 2028.63

ALGAL ACUTE TOXICITY TEST WITH CROTONALDEHYDE
KAN 901878, HAEL NO. 90-0016
AVERAGED DOSE LEVELS

9 42 THURSDAY, JUNE 7, 1990

DEP VARIABLE: COUNT PERCENT INHIBITION

ANALYSIS OF VARIANCE

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PROB>F
MODEL	3	28057.60240	9352.53413	170.773	0.0001
ERROR	14	766.72400	54.76600015		
C TOTAL	17	28824.32640			

ROOT MSE	7.400405	R-SQUARE	0.9734
DEP MEAN	52.78643	ADJ R-SQ	0.9677
C.V.	14.01952		

PARAMETER ESTIMATES

VARIABLE	DF	PARAMETER ESTIMATE	STANDARD ERROR	T FOR H0: PARAMETER=0	PROB > |T|	VARIABLE LABEL
INTERCEP	1	56.52117860	2.46386603	22.940	0.0001	INTERCEPT
LCONC	1	113.42497	7.28392270	15.572	0.0001	LOG OF CONCENTRATION IN MG/L
LCONC2	1	-20.18074593	5.01681712	-4.023	0.0013	
LCONC3	1	-60.66781411	7.09463469	-8.551	0.0001	

OBS	ACTUAL	PREDICT VALUE	STD ERR PREDICT	LOWER95% MEAN	UPPER95% MEAN	RESIDUAL	STD ERR RESIDUAL	STUDENT RESIDUAL	-2 -1 0 1 2	COOK'S D
1	97.0153	97.1632	4.2214	88.1091	106.2	-0.1479	6.0783	-0.0243		0.000
2	96.9539	97.1632	4.2214	88.1091	106.2	-0.2093	6.0783	-0.0344		0.000
3	96.9751	97.1632	4.2214	88.1091	106.2	-0.1881	6.0783	-0.0309		0.000
4	96.6778	98.5040	3.1641	91.7177	105.3	-1.8262	6.6899	-0.2730		0.004
5	96.7023	98.5040	3.1641	91.7177	105.3	-1.8017	6.6899	-0.2693		0.004
6	96.6320	98.5040	3.1641	91.7177	105.3	-1.8720	6.6899	-0.2798		0.004
7	82.0752	74.2878	2.7706	68.3454	80.2302	7.7875	6.8622	1.1348	**	0.052
8	80.0907	74.2878	2.7706	68.3454	80.2302	5.8030	6.8622	0.8456	*	0.029
9	84.8654	74.2878	2.7706	68.3454	80.2302	10.5776	6.8622	1.5414	***	0.097
10	21.5683	36.8737	2.5360	31.4345	42.3129	-15.3054	6.9523	-2.2015	****!	0.161
11	29.2548	36.8737	2.5360	31.4345	42.3129	-7.6189	6.9523	-1.0959	**!	0.040
12	24.9965	36.8737	2.5360	31.4345	42.3129	-11.8772	6.9523	-1.7084	***!	0.097
13	12.3851	4.9524	3.5883	-2.7436	12.6485	7.4326	6.4723	1.1484	**	0.101
14	11.1236	4.9524	3.5883	-2.7436	12.6485	6.1712	6.4723	0.9535	*	0.070
15	9.2508	4.9524	3.5883	-2.7436	12.6485	4.2984	6.4723	0.6641	*	0.034
16	2.7670	4.9374	4.2668	-4.2139	14.0888	-2.1705	6.0465	-0.3590		0.016
17	5.4306	4.9374	4.2668	-4.2139	14.0888	0.4931	6.0465	0.0816		0.001
18	5.3913	4.9374	4.2668	-4.2139	14.0888	0.4539	6.0465	0.0751		0.001

SUM OF RESIDUALS	7.94254E-13
SUM OF SQUARED RESIDUALS	766.724
PREDICTED RESID SS (PRESS)	1053.282

Averaged Dose Levels HAEL 90-0016
Hour = 24, linear regression worksheet

EC_{50} 50 = 24.95 + 26.96 lconc
lconc = 0.929
Conc = 8.492 mg/l
u 95% lconc = >1 Conc = >10.0 mg/l
l 95% lconc = 0.78 Conc = 6.026 mg/l

EC_{10} 10 = 24.95 + 26.96 lconc
lconc = −0.55
Conc = 0.282 mg/l
u 95% lconc = −0.44 Conc = 0.363 mg/l
l 95% lconc = −0.70 Conc = 0.199 mg/l

EC_{90} Not estimable from graph
90 = 24.95 + 26.96 lconc
lconc = 2.413
Conc = 258.72 mg/l

Merilee Ritter
6/8/90

Averaged Dose Levels HAEL 90-0016
Hour = 48, linear regression worksheet

EC_{50}

50 = 56.61 + 34.58 lconc
lconc = -0.19
Conc = 0.646 mg/L
u 95% lconc = -0.06 Conc = 0.871
l 95% lconc = -0.33 Conc = 0.468 } mg/l

EC_{10}

10 = 56.61 + 34.58 lconc
lconc = -1.348
Conc = 0.045 mg/l
u 95% lconc = -1.12 Conc = 0.076
l 95% lconc = < -1.6 Conc = < 0.025 } mg/l

EC_{90}

90 = 56.61 + 34.58 lconc
lconc = 0.966
Conc = 9.247 mg/l
u 95% lconc = > 1 Conc = 10.0
l 95% lconc = 0.76 Conc = 5.75 } mg/l

Meridee Ritter
6/8/90

Averaged Dose Levels HAEL 90-0016
Hour = 72, linear regression worksheet

EC_{50} 50 = 60.80 + 48.24 lconc
lconc = -0.224
Conc = 0.597 mg/l
u 95% lconc = -0.10 Conc = 0.794 mg/l
l 95% lconc = -0.34 Conc = 0.457 mg/l

EC_{10} 10 = 60.80 + 48.24 lconc
lconc = -1.05
Conc = 0.089 mg/l
u 95% lconc = -0.90 Conc = 0.126 mg/l
l 95% lconc = -1.25 Conc = ~~1.563~~ 0.056 mg/l

EC_{90} 90 = 60.80 + 48.24 lconc
lconc = 0.605
Conc = 4.027 mg/l
u 95% lconc = 0.80 Conc = 6.310 mg/l
l 95% lconc = 0.45 Conc = 2.818 mg/l

Merilee Ritter
6/8/90

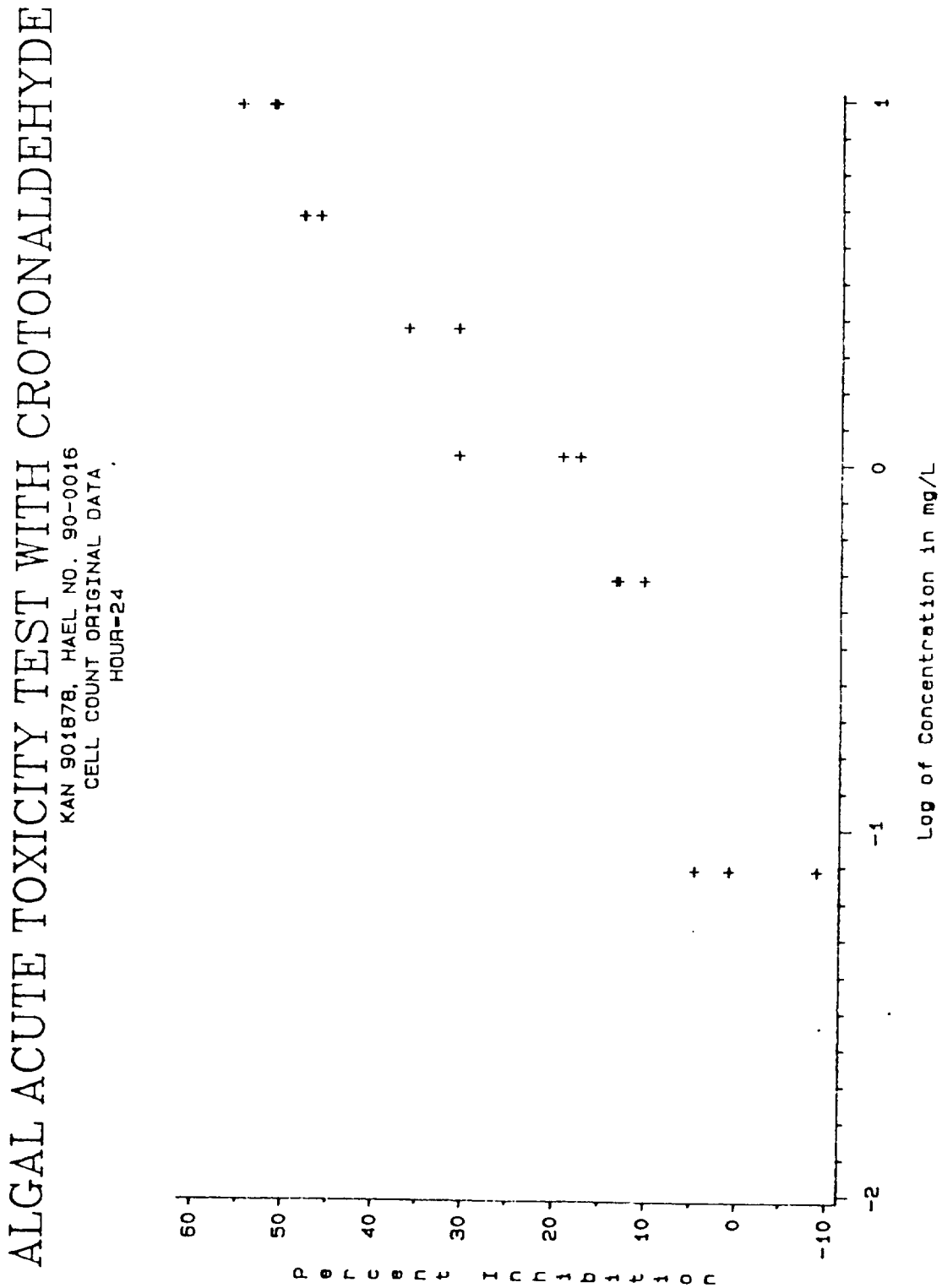
Averaged Dose Levels HAEL 90-0016
Hour = 96 Cubic Regression Worksheet

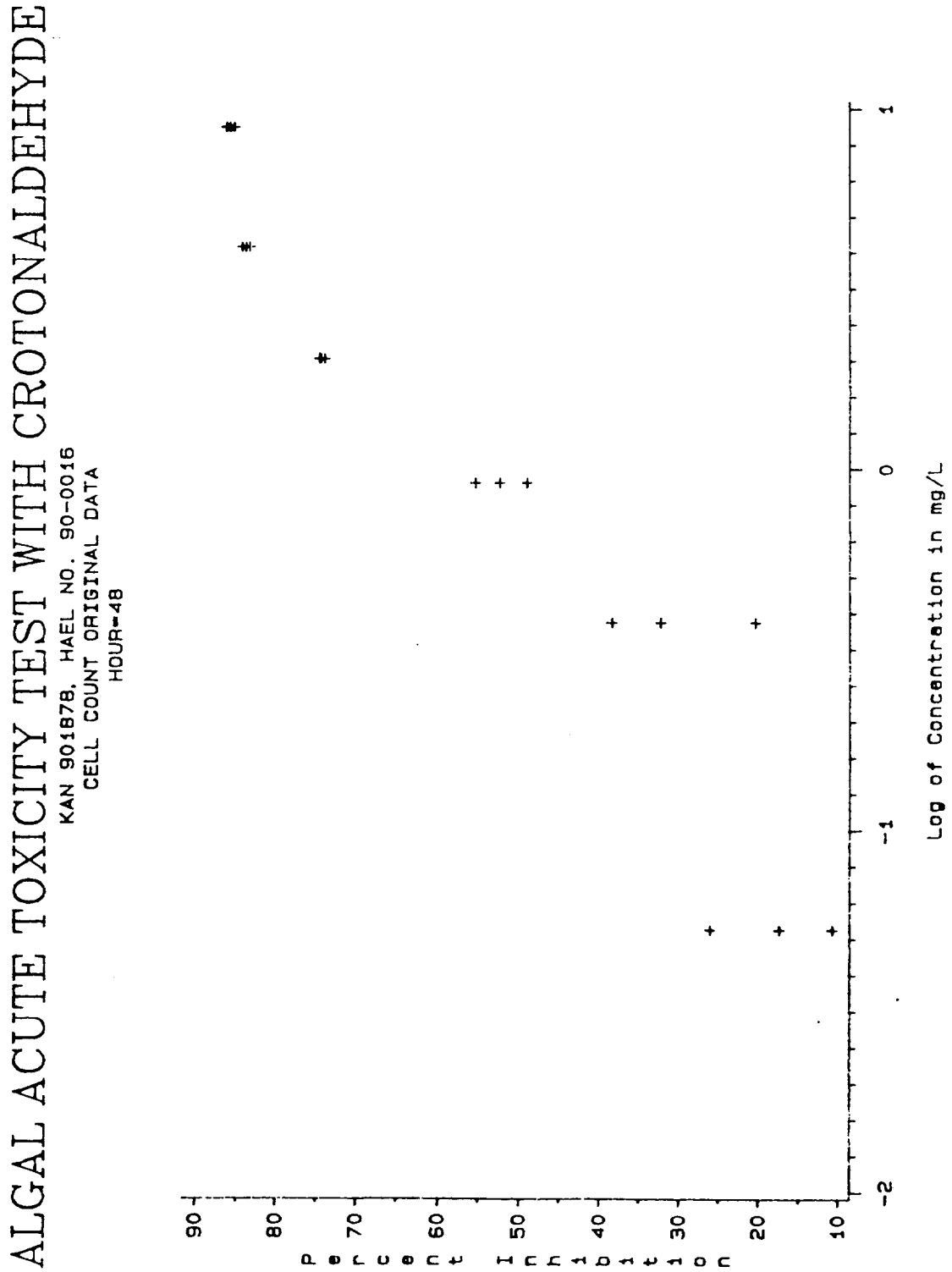
EC_{50} $50 = 56.52 + 113.42\,lconc - 20.18\,lconc^2 - 60.67\,lconc^3$
$lconc = -0.055$
$Conc = 0.881$ mg/l
u 95% $lconc = 0.0$ $Conc = 1.00$
l 95% $lconc = -0.1$ $Conc = 0.794$ mg/l

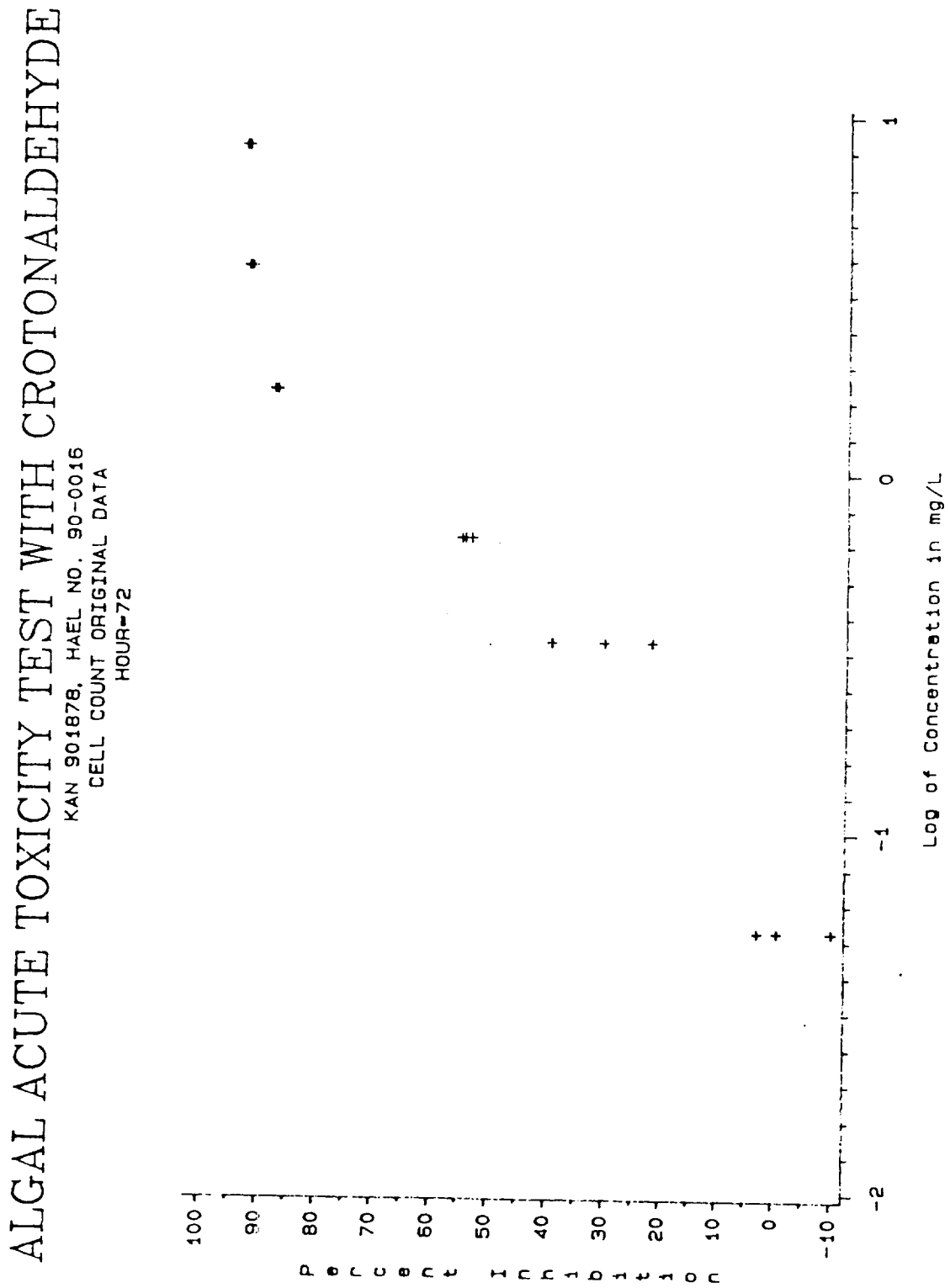
EC_{10} $10 = 56.52 + 113.42\,lconc - 20.18\,lconc^2 - 60.67\,lconc^3$
$lconc = -0.415$
$Conc = 0.385$ mg/l
u 95% $lconc = -0.35$ $Conc = 0.447$
l 95% $lconc =$ ~~-0.60~~ -0.50 $Conc = 0.316$ mg/l

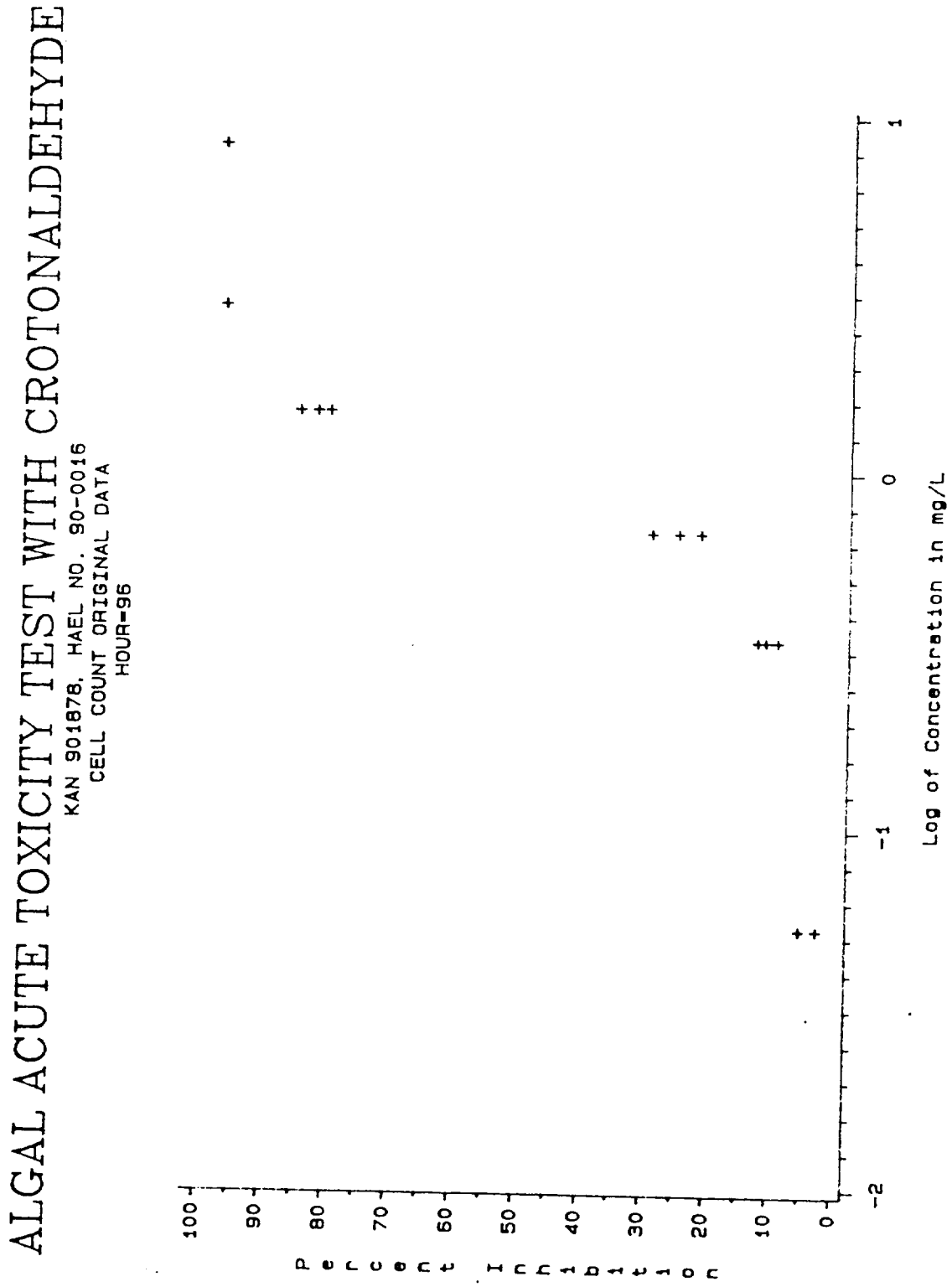
EC_{90} $90 = 56.52 + 113.42\,lconc - 20.18\,lconc^2 - 60.67\,lconc^3$
$lconc = 0.297$
$Conc = 1.982$ mg/l
u 95% $lconc = 0.44$ $Conc = 2.754$
l 95% $lconc = 0.28$ $Conc = 1.905$ mg/l

Merilee Ritter
6/8/90


ALGAL ACUTE TOXICITY TEST WITH CROTONALDEHYDE
KAN 901878, HAEL NO. 90-0016
CELL COUNT ORIGINAL DATA
HOUR=24
Percent Inhibition
60
50
40
30
20
10
0
-10
-2
-1
0
1
Log of Concentration in mg/L

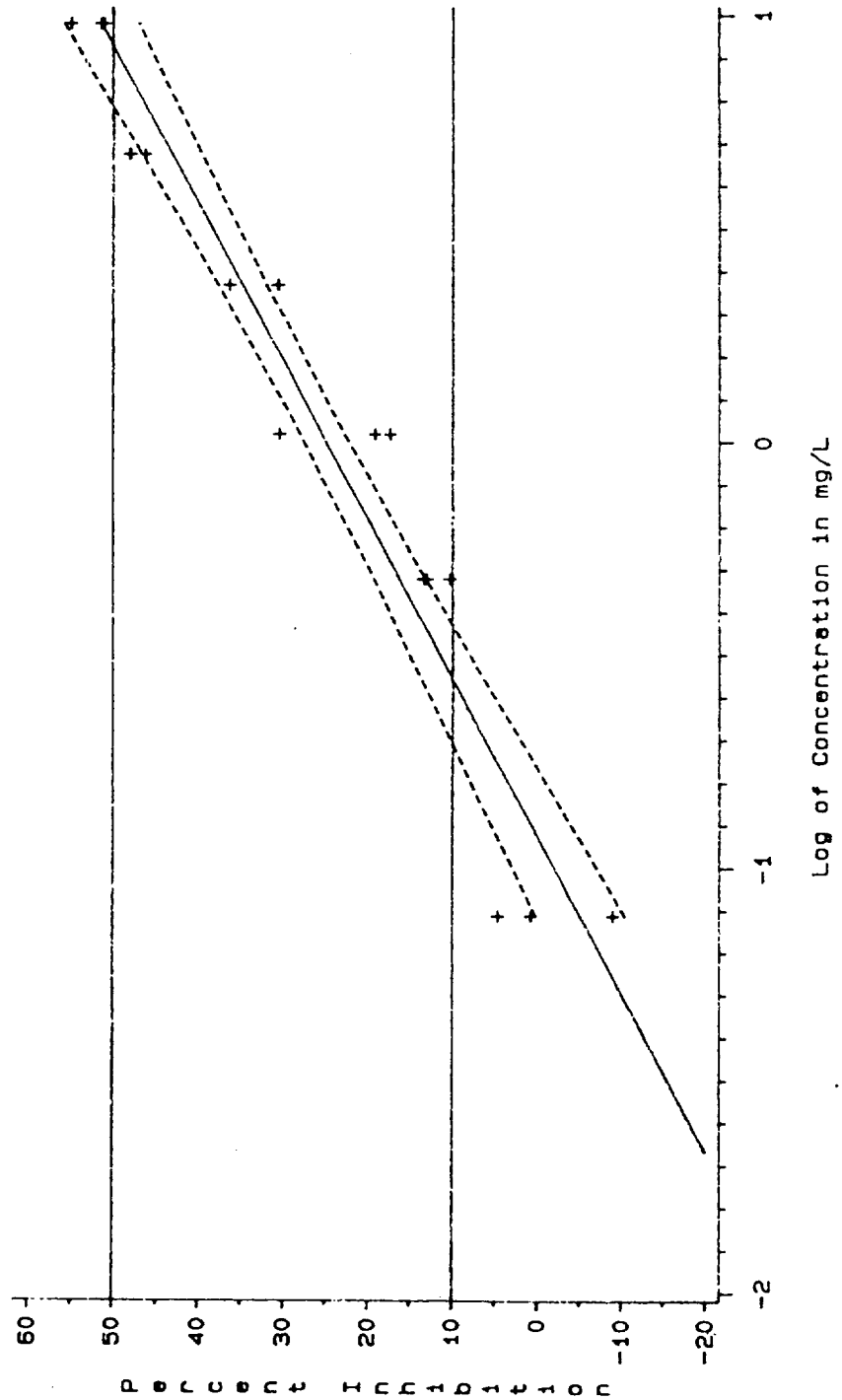

ALGAL ACUTE TOXICITY TEST WITH CROTONALDEHYDE
KAN 901878, HAEL NO. 90-0016
CELL COUNT ORIGINAL DATA
HOUR=48
Percent Inhibition
90
80
70
60
50
40
30
20
10
-2
-1
0
1
Log of Concentration in mg/L


ALGAL ACUTE TOXICITY TEST WITH CROTONALDEHYDE
KAN 901878, HAEL NO. 90-0016
CELL COUNT ORIGINAL DATA
HOUR=72
Percent Inhibition
100
90
80
70
60
50
40
30
20
10
0
-10
-2
-1
0
1
Log of Concentration in mg/L


ALGAL ACUTE TOXICITY TEST WITH CROTONALDEHYDE
KAN 901878, HAEL NO. 90-0016
CELL COUNT ORIGINAL DATA
HOUR=96
Percent Inhibition
100
90
80
70
60
50
40
30
20
10
0
-2
-1
0
1
Log of Concentration in mg/L

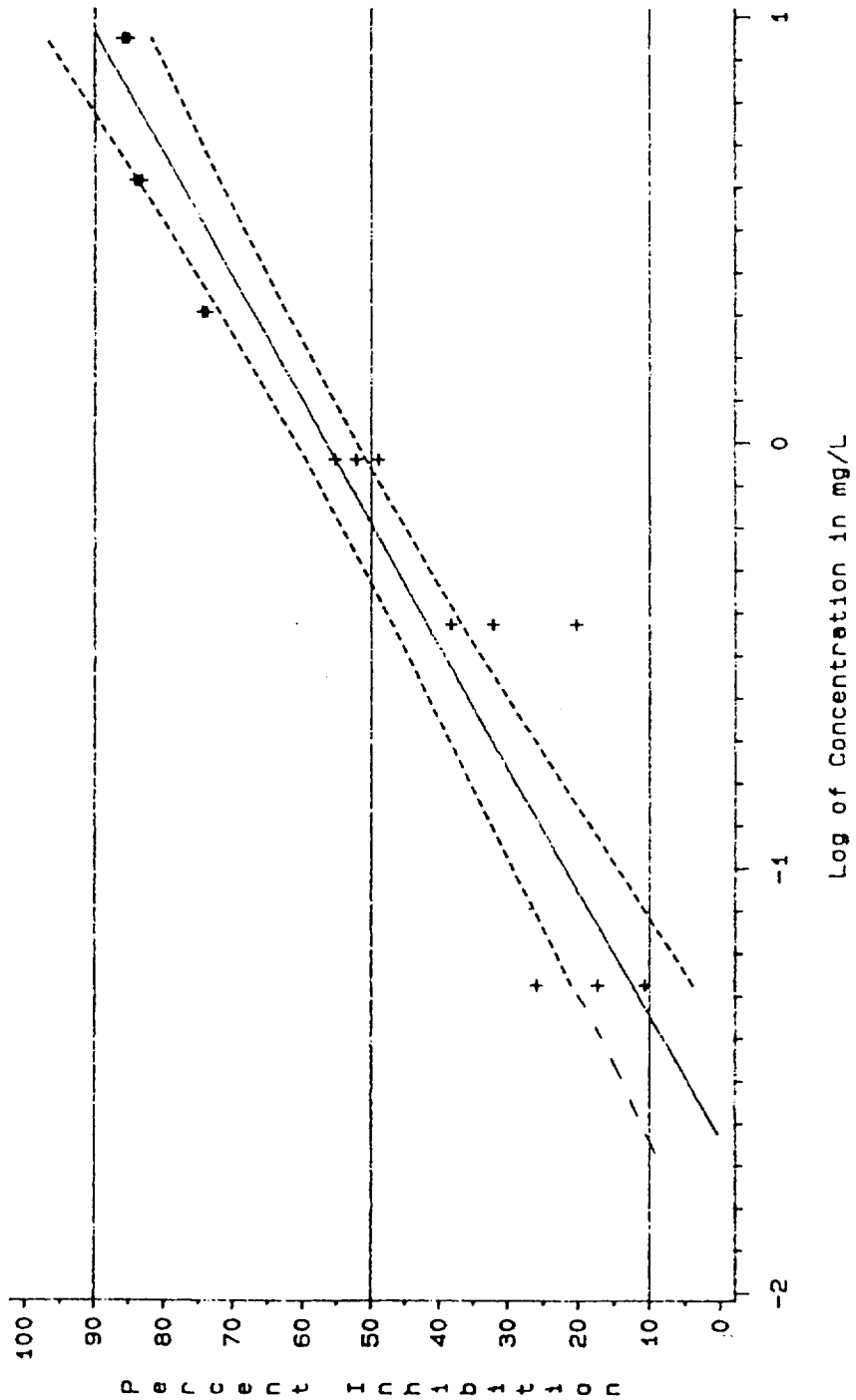
ALGAL ACUTE TOXICITY TEST WITH CROTONALDEHYDE

KAN 901878, HAEL NO. 90-0016
averaged dose levels
FITTED LINEAR REGRESSION MODEL WITH
CONTROL ADJUSTED 95% CONFIDENCE LIMITS
HOUR=24



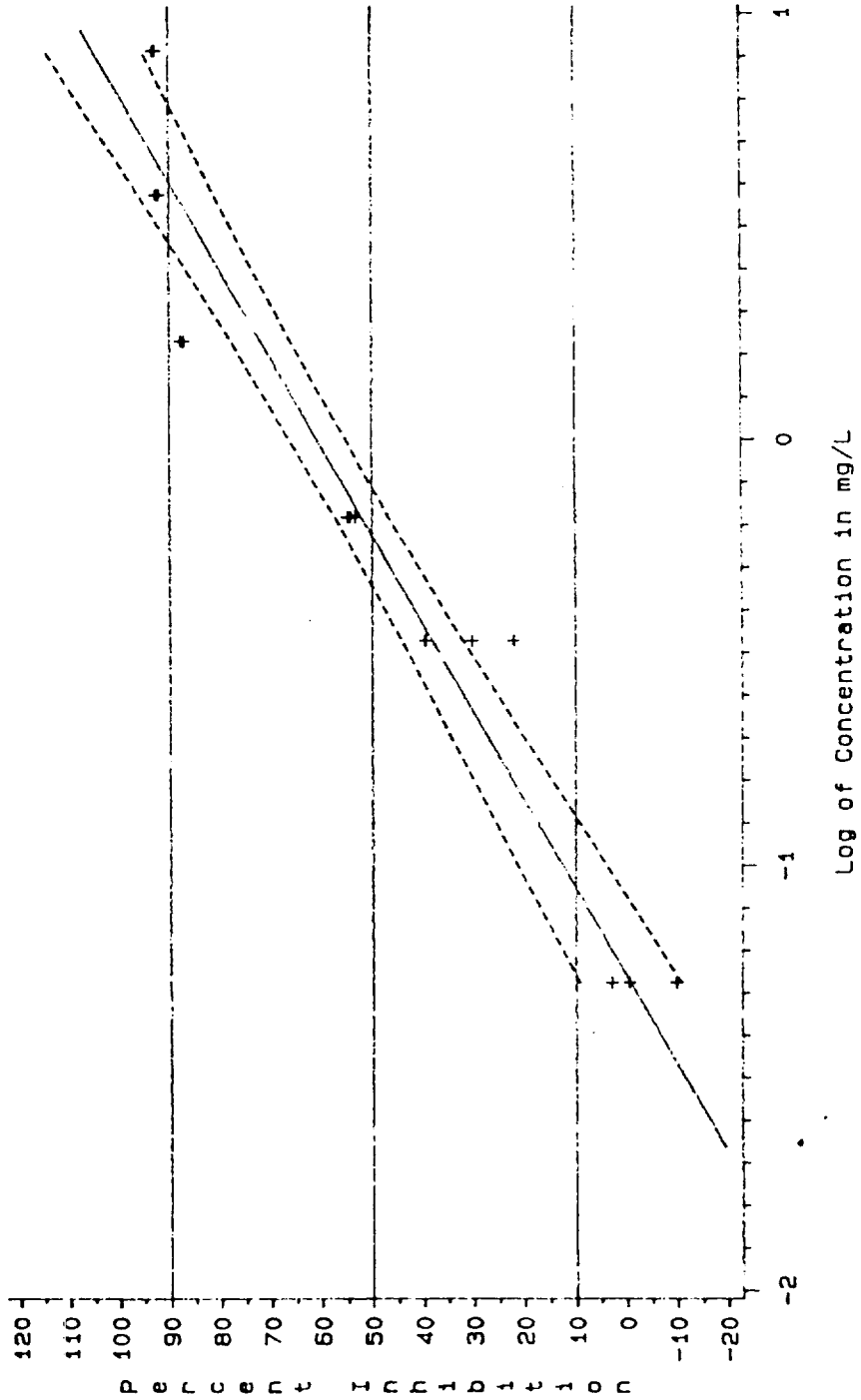
ALGAL ACUTE TOXICITY TEST WITH CROTONALDEHYDE

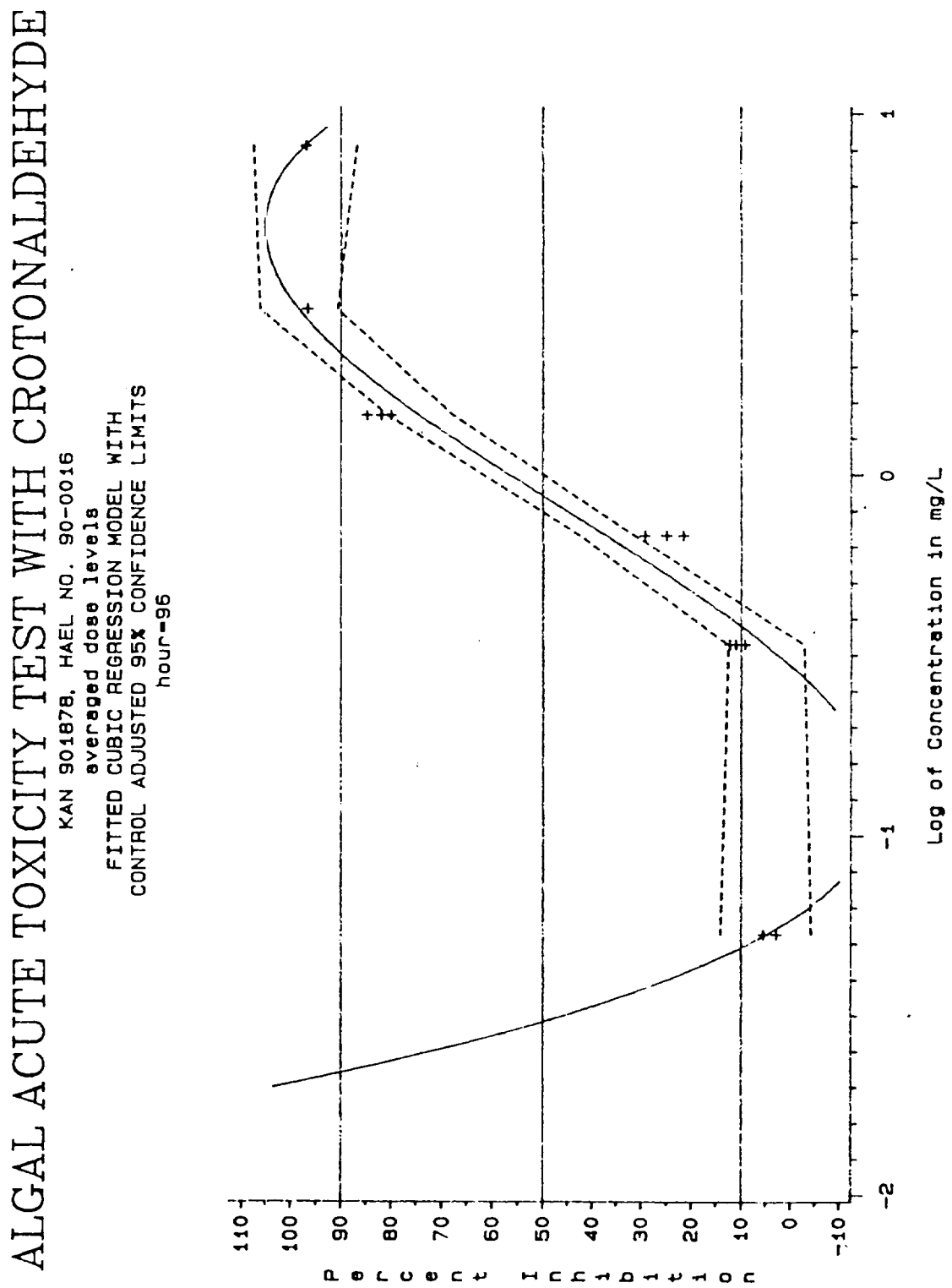
KAN 901878, HAEL NO. 90-0016
averaged dose levels
FITTED LINEAR REGRESSION MODEL WITH
CONTROL ADJUSTED 95% CONFIDENCE LIMITS
HOUR=48



ALGAL ACUTE TOXICITY TEST WITH CROTONALDEHYDE

KAN 901878, HAEL NO. 90-0016
averaged dose levels
FITTED LINEAR REGRESSION MODEL WITH
CONTROL ADJUSTED 95% CONFIDENCE LIMITS
HOUR=72




ALGAL ACUTE TOXICITY TEST WITH CROTONALDEHYDE
KAN 901878, HAEL NO. 90-0016
averaged dose levels
FITTED CUBIC REGRESSION MODEL WITH
CONTROL ADJUSTED 95% CONFIDENCE LIMITS
hour=96
Percent Inhibition
110
100
90
80
70
60
50
40
30
20
10
0
-10
-2
-1
0
1
Log of Concentration in mg/L

Attachment 4. CQS Division Report on Chemical Purity

CHEMICALS QUALITY SERVICES REPORT FOR: ETS

STUDY DIRECTOR: Heather Watson

DATE RECEIVED: 3/1/90

REQUESTED BY: Heather Watson

DATE ANALYZED: 3/20/90

REQUEST NO.: 149292

DATE REPORTED: 6/6/90

CQS JOB NO.: 3HGV501

STUDY TYPE: Algal Acute Toxicity

PHASE: Purity Analysis

HAEL NO.: 90-0016

ACCESSION NO.: 901878

AUTHOR: Nancy Porter

SAMPLES (LABELS AND DESCRIPTION): One sample of 90-0016, Accession # 901878, for purity analysis. Lot # 90-7

FOR ADDITIONAL INFORMATION OR FURTHER WORK: Barry Remington @85010

REPORT: A purity analysis was performed on the test chemical using GC/FID on a HP 5890, and water analysis by Karl Fisher.

The results are as follows:

Sample I.D.	Area % by GC/FID	% Water by Karl Fisher
90-0016 Lot # 90-7	98.1%	7.1%

n = 3 (GC/FID)
standard deviation = 0.12

Analyst N. Porter Date 6/6/90

Reviewed by Robert T Cambron Date 6/6/90

CHEMICALS QUALITY SERVICES REPORT FOR: B306

STUDY DIRECTOR: Robert T. Ambrose

REQUESTED BY: Rob O'Boyle

CQS JOB # : 3H6V501

PHASE: Purity Determination

REQUEST # : 142470
DATE RECEIVED: 05/14/90
DATE ANALYZED: 05/18/90
DATE REPORTED: 05/18/90
AUTHOR: Beth Isaacs

HAEL#: 90-0016
ACC.#: 901878
SAMPLE REF.#: Lot 90-7

FOR ADDITIONAL INFORMATION OR FURTHER WORK CONTACT: Robert Ambrose X25925
Beth Isaacs X85035

RESULTS: The purity of the test chemical was determined by a combination of a water analysis by Karl Fisher titration and a gas chromatographic analysis with flame ionization detection. The results are as follows:

Water (%)	Area % (GC/FID)	
6.3	Run 1	99.7
	Run 2	99.1
	Run 3	99.7
	Mean	99.5
	Std Dev	0.3464

Scientist Beth Isaacs (signature) Date 6/7/90

Reviewed by Robert T Ambrose (signature) Date 6/7/90

Instrumental Conditions for GC Analysis

Instrument:	Hewlett Packard 5890
Column:	DB-WAX; 30m; wide bore (0.25 um film thickness)
Column Pressure:	7 psi
Carrier Gas:	Helium
Initial Temp:	50 °C
Initial Time:	4 min.
Rate:	10 °C/min.
Final Temp:	250 °C
Final Time:	7 min.
Injection Port:	250 °C
Injection Type:	Split
Injection Vol:	1 uL
Detector:	FID
Detector Temp:	250 °C
Solvent:	n-Propanol
Split Flow:	120 cc/min.

Attachment 5. Quality Assurance Statements

Q.A. INSPECTION STATEMENT
(CFR 58.35(B)(7) 792.35(B)(7) 160.35(B)(7))

STUDY: 90-0016-1 STUDY DIRECTOR: WATSON,H.M. PAGE 1
ACCESSION NUMBER: 901878 08/06/90

STUDY TYPE: ALGAL ACUTE TOXICITY TEST

M. Susan James 8/14/90
(AUDITOR, QUALITY ASSURANCE UNIT) DATE

TO THE BEST OF MY KNOWLEDGE, THIS FINAL REPORT ACCURATELY DESCRIBES THE METHODS AND STANDARD OPERATING PROCEDURES, AND THE REPORTED RESULTS ACCURATELY REFLECT THE RAW DATA. THIS STUDY WAS INSPECTED BY 1 OR MORE PERSONS OF THE QUALITY ASSURANCE UNIT OF THE HAEL, EASTMAN KODAK COMPANY, ROCHESTER, N.Y. AND WRITTEN STATUS REPORTS WERE SUBMITTED ON THE FOLLOWING DATES:

INSPECTION DATES	PHASE(S) INSPECTED	STATUS REPORT DATES
03/16/90	PROTOCOL SUBMISSION	
03/19/90	TEST ARTICLE-CARRIER-MIXTURE PREPARATION RANGE-FINDING TEST	03/19/90
04/02/90	SOLUTION PREPARATION TEST ARTICLE WEIGHT TEST ARTICLE-CARRIER-MIXTURE PREPARATION PH ADJUSTMENT TEST ARTICLE-CARRIER-MIXTURE DILUTIONS CONTROL ARTICLE PREPARATION TEST SYSTEM ADDITION TO CONTROL ARTICLE TEST SYSTEM ADDITION TO TEST ARTICLE-CARRIER-MIXTURE DEFINITIVE TEST - DAY 0	08/06/90
08/06/90	FINAL REPORT REVIEW	08/06/90

ANALYTICAL QUALITY ASSURANCE INSPECTION STATEMENT
(CFR 58.35(B)(7) 792.35(B)(7) 160.35(B)(7))

STUDY: 90-0016-A STUDY DIRECTOR: WATSON, H.
ANALYTICAL DIRECTOR: AMBROSE, R.
ACCESSION NUMBER: 901878
CQS JOB NUMBER: 3HGV501

STUDY TYPE: ALGAL ACUTE TOXICITY

(AUDITOR, QUALITY ASSURANCE UNIT) 7-31-90 DATE

THIS STUDY WAS INSPECTED BY 1 OR MORE PERSONS OF THE QUALITY ASSURANCE UNIT OF MQAO, EASTMAN KODAK COMPANY, ROCHESTER, N.Y. AND WRITTEN STATUS REPORTS WERE SUBMITTED ON THE FOLLOWING DATES:

INSPECT DATES	REQUEST NUMBER	PHASE(S) INSPECTED	STATUS REPORT DATES
06/06/90	149292	PURITY TEST REPORT INSPECTION	06/01/90
06/07/90	142470	PURITY TEST REPORT INSPECTION	05/30/90

ANALYTICAL QUALITY ASSURANCE INSPECTION STATEMENT
(CFR 58.35(B)(7) 792.35(B)(7) 160.35(B)(7))

STUDY: 90-0016-A STUDY DIRECTOR: WATSON, H.
ANALYTICAL DIRECTOR:
ACCESSION NUMBER: 901878
CQS JOB NUMBER: 3HGV598

STUDY TYPE: EPA/TSCA ALGAL ACUTE TOXICITY TEST OF CROTONALDEHYDE

M J Boccaccio 6-1-90
(AUDITOR, QUALITY ASSURANCE UNIT) DATE

THIS STUDY WAS INSPECTED BY 1 OR MORE PERSONS OF THE QUALITY ASSURANCE UNIT OF QSO, EASTMAN KODAK COMPANY, ROCHESTER, N.Y. AND WRITTEN STATUS REPORTS WERE SUBMITTED ON THE FOLLOWING DATES:

INSPECT DATES	REQUEST NUMBER	PHASE(S) INSPECTED	STATUS REPORT DATES
01/22/90		METHOD VALIDATION PHASE INSPECTION	01/22/90
04/06/90	142477	CONCENTRATION DETERMINATION PHASE INSPECTION	04/06/90
06/01/90	142424	CONCENTRATION DETERMINATION PRE-TEST, T0 TEST REPORT INSPECTION	06/01/90
06/01/90	142476	CONCENTRATION DETERMINATION DEFINITIVE TEST - T72	06/01/90
06/01/90	142477	CONCENTRATION DETERMINATION DEFINITIVE TEST - T96	06/01/90
06/01/90	142423	CONCENTRATION DETERMINATION PRE-TEST, T96	06/01/90
06/01/90	149293	METHOD VALIDATION FILTERING VALIDATION TEST REPORT INSPECTION	06/01/90
06/01/90	149293	METHOD VALIDATION POOLING VALIDATION	06/01/90
06/01/90	142421 142420	CONCENTRATION DETERMINATION RANGE FINDING - T0 RANGE FINDING - T96	06/01/90
06/01/90	142473	CONCENTRATION DETERMINATION DEFINITIVE TEST - T0	06/01/90

ANALYTICAL QUALITY ASSURANCE INSPECTION STATEMENT
(CFR 58.35(B)(7) 792.35(B)(7) 160.35(B)(7))

STUDY: 90-0016-A STUDY DIRECTOR: WATSON, H.
ANALYTICAL DIRECTOR: AMBROSE, R.
ACCESSION NUMBER: 901878
CQS JOB NUMBER: 3HGV586

STUDY TYPE: EPA/TSCA ALGAL ACUTE TOXICITY TEST OF CROTONALDEHYDE

(AUDITOR, QUALITY ASSURANCE UNIT) 6-1-90 DATE

THIS STUDY WAS INSPECTED BY 1 OR MORE PERSONS OF THE QUALITY ASSURANCE UNIT OF QSO, EASTMAN KODAK COMPANY, ROCHESTER, N.Y. AND WRITTEN STATUS REPORTS WERE SUBMITTED ON THE FOLLOWING DATES:

INSPECT DATES	REQUEST NUMBER	PHASE(S) INSPECTED	STATUS REPORT DATES
06/01/90	142474	CONCENTRATION DETERMINATION DEFINITIVE TEST - T24 TEST REPORT INSPECTION	06/01/90
06/01/90	142475	CONCENTRATION DETERMINATION DEFINITIVE TEST - T48 TEST REPORT INSPECTION	06/01/90

Appendix 1. Chemical Distribution Record

HAEL Chemical Distribution Record

HAEL Lab. No.	90-0016	IDX Accession No.	~~901876~~	SHD No.	Lot No. 90-7
Chemical Name	Crotonaldehyde (901878 RH 2/8/90)				
Source of Test Substance	~~Texas Eastman Co.~~ ① Tennessee Eastman Co.	Sponsor	TEC/Coord. Wade Francisco (x-3152) JWG 2/9/90		
Container(s) Description	1 large glass amber bottle				
Date Test Substance Received	2-5-90	Date Test Substance Processed	2-8-90	By: HRH	Expiration Date: 2-8-95
Date Test Substance Received from C&R (for testing areas only)	2/8/90 JWG				
Physical State (circle one)	Liquid Solid Other	Appearance	clear		
Impurities with %	N/A				
Storage Conditions	~~Room Temperature~~ Refrigerate, and cover with nitrogen or Argon after each use ①				
Weight or Volume (circle one) of Bottle, Cap, and Contents	4348.5g	Weight or Volume (circle one) of Test Substance Sent	3121.2g		
Balance / Make	Mettler	Model	PE6000	KP ID No.	A20916

① Entry error, JWG, 2/9/90

Initials	Date	Area	Purpose	Scale Make, Model, KP ID No.	Initial Bottle Cap & Contents Wt. or Vol.	Amount Removed Wt. or Vol.	Amount Remaining Bottle/Cap/Contents Wt. or Vol.
[illegible]	02/09/90	B-306 EST-HAEL	Confirmation Log-in Weighing	Mettler PE 4400 (#2) ID # 255858 #589461	4348.2g	0.0g	4348.2g
HWW	2-12-90	B-306	Algal Methods	"	4347.9g	6.8g	4341.1g
O'B.	3/13/90	B-306	Sample to CQSD	" O'B.	4341.2g	29.2g	4312.0g
MM	3/19/90	B-306	Algal Range Finding Test	"	4312.5g	1.2g	4311.3g
O'B.	3/22/90	B-306	AAE Meth. Dev.	" O'B.	4310.3g	27.6g	4282.7g
MM	4-2-90	B-306	Algal Refractive Test	"	4283.0g	0.8g used	4282.2g
O'B.	4/18/90	B-306	AAE Dose Prep (for 4/23/90)	" O'B.	4280.8g ✓✓	20.7g (20.00g used)	4259.9g
O'B.	4/24/90	B-306	AAE Renewal (for 4/25/90)	" O'B.	4260.1g ✓✓	20.4g (20.00g used)	4239.7g
O'B.	4/27/90	B-306	Om Dose Prep (for 4/30/90)	" O'B.	4240.0g ✓✓	4.2g (4.00g used)	4235.8g
O'B.	5/1/90	B-306	Om Dose Renewal (for 5/2/90)	" O'B.	4235.5g ✓✓	4.6g (4.00g used)	4230.9g
O'B.	5/14/90	B-306	Purity/Stability (H2O)	" O'B.	4230.9g ✓✓	30.9g (to CQSD)	4200.0g

Chem Dist Form 2/89 plm

Appendix 2. Protocol

ENVIRONMENTAL SCIENCES SECTION
ENVIRONMENTAL TECHNICAL SERVICES
HEALTH AND ENVIRONMENT LABORATORIES

Protocol No. EN-1050-901878-1
Revision No.
Date: November 9, 1989

PROTOCOL: EPA/TSCA Algal Acute Toxicity Test
(using the Federal Register Guideline 40 CFR 797.1050
Amendments of 5-20-87 and EPA Consent Order of November 9, 1989)

Test Article: Crotonaldehyde

Accession No. 901878 HAEL No. 90-0016

Approved by: Heather N Watson — Study Director — 2-1-90 Date

Approved by: Roderick D. Stowe — Sponsor — 2/13/90 Date

3827e

Health and Environment Laboratories Management and Quality Assurance Unit Approvals

Approved: Joseph W. Gorsuch — 3/6/90

Joseph W. Gorsuch
Project Manager
Environmental Sciences Section
Health and Environment Laboratories

Date

Approved: M. Sue James — 3/7/90

Quality Assurance Unit
Health and Environment Laboratories

Date

Approved: John R Owen — 3/7/90

John R. Owen
Unit Director
Environmental Sciences Section
Health and Environment Laboratories

Date

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I. Test Administration

A. Testing Facility

Health and Environment Laboratories
Eastman Kodak Company
Rochester, NY 14652-3617
USA

B. Sponsor

Eastman Chemicals Division
Kingsport, TN 37662
USA

C. Dates of Experiment

1. Dates of Range-Finding Test (if applicable)

Start Date: 3-19-90 Chemical Exposure Completion Date: 3-23-90

2. Dates of Definitive Test

Start Date: 4-2-90 Chemical Exposure Completion Date: 4-6-90

3. Estimated Study Completion Date: 6-6-90

II. Test and Reference Substances

A. Test Substance

Name: Crotonaldehyde

HAEL No.: 90-0016 Accession No.: 901878

Sample Reference No.: Lot No. 90-7 CAS Reg. No.: 4170-30-3

Other Information (Composition, Stability, Purity, Solubility, etc.):

Test substance is volatile

Concentrations for Range-Finding Test: 1000, 100, 10, 1, 0.1, 0.01 mg/L

Concentrations for Definitive Test: _____ mg/L

or see Attachment 2 Yes (yes/no)

Analytical Method for Test Substance (Attachment 3): Yes (Yes/No)

B. Reference Substance (if used)

Name: None

CAS Reg. No.: ----

Concentration(s) used: ---- mg/L

III. Identification and Source of Alga

Identity: Selenastrum capricornutum ATCC 22662

Source: American Type Culture Collection, Maryland, USA

Date Obtained: 11-11-87

Other Information: Kept on agar, passaged in liquid medium prior to test

IV. Reporting and Records

A. Reporting

The report must contain:

1. Name and address of the testing facility.
2. Statement of GLP Compliance signed by study director and sponsor.
3. Dates of the study.
4. Procedures stated in the protocol, including any changes or amendments to the original protocol.
5. Methods used in analyzing the data.
6. Identification of test and reference substances.
7. Description of the test methods.
8. Description of the test system.
9. Description of the test substance concentration.
10. Description of any circumstances that may have affected the quality or integrity of the study.
11. A copy of all raw data and summaries of data.
12. A copy of the protocol and any amendments.
13. Name of the study director and of all other persons involved in the study, including supervision.
14. Summary and analysis of results, including any statistical analyses.
15. Growth curves.
16. Graph showing the concentration effect relationship.
17. Proof of validity of the test.
18. Stability of the test substance.
19. Signed and dated reports of the scientists involved in the study.
20. Location of all specimens, raw data, and final reports.
21. Quality Assurance Statement.

B. Records

All records will be retained for at least ten years by the Health and Environment Laboratories:

1. All raw data and summaries of data.
2. The protocol and any protocol amendments.
3. Summaries of training and experience for all persons involved in the study.
4. Standard operating procedures.
5. Final reports.

Attachment 1. Composition and Preparation of Algal Medium[1]

	Compound	Test Concentration (per 1.0 L)	Stock Concentration (per 500mL)
I.	$NaNO_3$ (sodium nitrate)	25.500 mg	12.75 g
II.	$MgCl_2 \cdot 6H_2O$ (magnesium chloride)	12.164 mg	6.082 g
III.	$CaCl_2 \cdot 2H_2O$ (calcium chloride)	4.410 mg	2.205 g
IV.	Micronutrients		
	H_3BO_3 (boric acid)	185.520 µg	92.760 mg
	$MnCl_2 \cdot 4H_2O$ (manganese chloride)	415.610 µg	207.690 mg
	$ZnCl_2$ (zinc chloride)	3.271 µg	1.635 mg
	$FeCl_3 \cdot 6H_2O$ (ferric chloride)	160.000 µg	79.880 mg
	$CoCl_2 \cdot 6H_2O$ (cobalt chloride)	1.428 µg	0.714 mg
	$NaMoO_4 \cdot 2H_2O$ (sodium molybdate)	7.260 µg	3.630 mg
	$CuCl_2 \cdot 2H_2O$ (cupric chloride)	0.012 µg	0.006 mg
	$Na_2EDTA \cdot 2H_2O$ (disodium EDTA)	300.000 µg	150.0 mg
V.	$MgSO_4 \cdot 7H_2O$ (magnesium sulphate)	14.700 mg	7.350 g
VI.	K_2HPO_4 (potassium phosphate)	1.044 mg	0.522 g
VII.	$NaHCO_3$ (sodium bicarbonate)	15.000 mg	7.500 g

Solution Preparation

Stock solutions I-VII may be made up in advance, filter-sterilized through 0.45 µm membranes (or autoclaved), and refrigerated until needed (for a period of 6 months). If desired, reagent salts I-IV can be combined into one 500 mL solution.

Algal medium is made up by adding 1.0 mL of each stock solution in the order given to approximately 900 mL of distilled water and diluting to 1.0 L. The pH is adjusted to 7.5 ± 0.1 using 0.1N NaOH or HCl and the medium is then sterilized as before.

(1) U.S. EPA: The *Selenastrum capricornutum* Printz Algal Assay Bottle Test, EPA-600/9-78-018 (July 1978).

Attachment 2. Documentation for Definitive Test Concentrations

The concentrations for the definitive test are documented after the completion of the range-finding test (where this is needed). A copy of this attachment will be filed with the Quality Assurance Unit (the original is filed with the rest of the protocol).

Concentrations to be Tested: 10, 5, 2.5, 1.25, 0.625, and 0.1 mg/L

Test Chemical Water Solubility is: Ready Yes Limited _____ Low _____

Solubilizing Vehicle (if needed): No Concentration _____

Test Chemical is Volatile: Yes. A closed system will be used with a total of 84 flasks (4X as many as usual). After each day's analyses, the flasks just analyzed will be discarded and a fresh, unopened set used for the next day's analyses.

Approved by Heather Watson
Study Director

3-26-90
Date

Attachment 3. Analytical Method for Test Substance

GC Yes HPLC _____ UV-VIS _____ OTHER _____

Instrument: Perkin Elmer 8500 Gas Chromatograph with electron capture detector

Column:

Conditions:

Detection:

Sample Size:

Calibration:

Sample Prep: Derivitization and extraction

Remarks: All further details will be provided in the final report

Appendix 1. EPA Consent Order Protocol for Algal Acute Toxicity Test (Section 797.1050)

Section 797.1050 Algal acute toxicity test.

(a) Purpose. This guideline is intended for use in developing data on the acute toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003. 15 U.S.C. 2601 et seq.). This guideline prescribes test procedures and conditions using freshwater and marine algae to develop data on the phytoxicity of chemicals. The United States Environmental Protection Agency (U.S. EPA) will use data from these tests in assessing the hazard of a chemical to the environment.

(b) Definitions. The definitions in section 3 of the Toxic Substances Control Act (TSCA), and the definitions in Part 792—Good Laboratory Practice Standards of this chapter apply to this test guideline. The following definitions also apply to this guideline:

(1) "Algicidal" means having the property of killing algae.

(2) "Algistatic" means having the property of inhibiting algal growth.

(3) "EC_X" means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

(4) "Growth" means a relative measure of the viability of an algal population based on the number and/or weight of algal cells per volume of nutrient medium or test solution in a specified period of time.

(5) "Static system" means a test container in which the test solution is not renewed during the period of the test.

(c) Test procedures—(1) Summary of the test. (i) In preparation for the test, fill test containers with appropriate volumes of nutrient medium and/or test solution. Start the test by introducing algae into the test and control containers in the growth chambers. Environmental conditions within the growth chambers are established at predetermined limits.

(ii) At the end of 96 hours enumerate the algal cells in all containers to determine inhibition or stimulation of growth in test containers compared to controls. Use data to define the concentration-response curve, and calculate the EC_{10}, EC_{50}, and EC_{90} values.

(2) (Reserved)

(3) Range-finding test. (i) A range-finding test should be conducted to determine:

(A) If definitive testing is necessary.

(B) Test chemical concentrations for the definitive test.

(ii) Algae are exposed to a widely spaced (e.g., log interval) chemical concentration series. The lowest value in the series, exclusive of controls, should be at the chemical's detection limit. The upper value, for water soluble compounds, should be the saturation concentration. ~~No replicates are required; and nominal concentrations of the chemical are acceptable unless definitive testing is not required.~~ [TWO REPLICATES PER CONCENTRATION SHALL BE USED.]

(iii) ~~The test is performed once for each of the recommended algal species or selected alternates.~~ Test chambers should contain equal volumes of test solution and approximately 1×10^4 Selenastrum cells/ml ~~or 7.7×10^4 Skeletonema cells/ml~~ of test solution. The algae should be exposed to each concentration of test chemical for up to 96 hours. The exposure period may be shortened if data suitable for the purposes of the range-finding test can be obtained in less time.

(iv) Definitive testing is not necessary if the highest chemical concentration tested (water saturation concentration or 1000 mg/l) results in less than a 50 percent reduction in growth or if the lowest concentration tested (analytical detection limit) results in greater than a 50 percent reduction in growth.

[(C) TEST CHEMICAL VOLATILITY AND ADSORPTION TO ALGAE.]

[(i) TEST CHEMICAL ANALYSES SHALL BE DETERMINED AT 0 AND 96 HOURS ON TEST CONTAINERS (WITH ALGAE) AND CHEMICAL CONTROL CONTAINERS (WITHOUT ALGAE).]

[(ii) ADSORPTION. IF, AT 96 HOURS, TEST CONTAINER CONCENTRATIONS HAVE DECREASED SIGNIFICANTLY OVER THE TEST PERIOD RELATIVE TO CHEMICAL CONTROL VALUES, ADSORPTION OF TEST CHEMICAL TO ALGAE IS INDICATED. IN THIS CASE, THE DEFINITIVE TEST SHALL ALSO BE RUN WITH CHEMICAL CONTROLS TO EVALUATE ADSORPTION.]

[(iii) VOLATILES. IF, AT 96 HOURS, TEST SAMPLE CONCENTRATIONS AND CHEMICAL CONTROL CONCENTRATIONS HAVE BOTH DECREASED > 15% OVER THE TEST PERIOD, VOLATILITY IS INDICATED AND A CLOSED SYSTEM SHALL BE USED FOR THE DEFINITIVE TEST.]

(4) Definitive test. (i) The purpose of the definitive test is to determine the concentration-response curves, the EC_{10}'s, EC_{50}'s, and EC_{90}'s for algal growth for each species tested, with a minimum amount of testing beyond the range-finding test.

(ii) Algae should be exposed to five or more concentrations of the test chemical in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 6, 8, 16, 32, and 64 mg/l). Algae shall be placed in a minimum of three replicate test containers for each concentration of test chemical and control. More than three replicates may be required to provide sufficient quantities of test solution for determination of test substance concentration at the end of the test. Each test chamber should contain equal volumes of test solution and approximately $1x10^4$ Selenastrum cells ml^{-1} ~~or $1.7x10^4$ Skeletonema cells/ml~~ of test solution. The chemical concentrations should result in greater than 90 percent of algal growth being inhibited or stimulated at the highest concentrations of test substance compared to controls.

(iii) Every test shall include a control consisting of the same nutrient medium, conditions, procedures, and algae from the same culture, except that none of the test substance is added. If a carrier is present in any of the test chambers, a separate carrier control is required.

(iv) The test begins when algae from 5- to 10-day-old stock cultures are placed in the test chambers containing test solutions having the appropriate concentrations of the test substance. Algal growth in controls should reach the logarithmic growth phase by 96 hours. [AT LEAST 4 SEPARATE GROUPS OF TEST CONTAINERS SHALL BE SET UP FOR VOLATILES. ONE GROUP SHALL BE SAMPLED AND ANALYZED FOR ALGAL GROWTH AT 24 HR. AND THE CONTAINERS DISCARDED; THE NEXT GROUP SHALL BE SAMPLED AT 48 HR. AND THE CONTAINERS DISCARDED; AND SIMILARLY AT 72 AND 96 HR.] ~~[DUE TO THE VOLATILITY OF ONCS, AT LEAST 4 SEPARATE GROUPS OF TEST CHAMBERS SHALL BE SET UP. ONE GROUP SHALL BE SAMPLED AT 24, 48, 72, AND 96 HR. AND THE TEST CHAMBERS TERMINATED AFTER SAMPLING.]~~ ~~If logarithmic growth cannot be demonstrated, the test shall be repeated.~~ At the end of 24, 48, 72, and 96 hours the algal growth response (number or weight of algal cells/ml) in all test containers and controls shall be determined by an indirect (spectrophotometry, electronic cell counters, dry weight, etc.) or a direct (actual microscopic cell count) method. Indirect methods should be calibrated by a direct microscopic count. The percentage inhibition or stimulation of growth for each concentration, EC_{10}, EC_{50}, EC_{90} and the concentration-response curves are determined from these counts.

(v) At the end of the definitive test, the following additional analyses of algal growth response ~~shall~~ [SHOULD] be performed:

(A) Determine whether the altered growth response between controls and test algae was due to a change in relative cell numbers, cell sizes or both. Also note any unusual cell shapes, color differences, flocculations, adherence of algae to test containers, or aggregation of algal cells.

(B) In test concentrations where growth is maximally inhibited, algistatic effects may be differentiated from algicidal effects by the following two methods for Skeletonema and by the second method for Selenastrum.

(1) Add 0.5 ml of a 0.1 percent solution (weight/volume) of Evans blue stain to a 1 milliliter aliquot of algae from a control container and to a 1 milliliter aliquot of algae from the test container having the lowest concentration of test chemical which completely inhibited algal growth (if algal growth was not completely inhibited, select an aliquot of algae for staining from the test container having the highest concentration of test chemical which inhibited algal growth). Wait 10 to 30 minutes, examine microscopically, and determine the percent of the cells which stain blue (indicating cell mortality). A staining control shall be performed concurrently using heat-killed or formaldehyde-preserved algal cells; 100 percent of these cells should stain blue.

(2) Remove 0.5 ml aliquots of test solution containing growth-inhibited algae from each replicate test container having the concentration of test substance evaluated in paragraph (c)(4)(v)(B)(1) of this section. Combine these aliquots into a new test container and add a sufficient volume of fresh nutrient medium to dilute the test chemical to a concentration which does not affect growth. Incubate this subculture under the environmental conditions used in the definitive test for a period of up to 9 days, and observe for algal growth to determine if the algistatic effect noted after the 96-hour test is reversible. This subculture test may be discontinued as soon as growth occurs.

(5) (Reserved)

(6) Analytical measurements—(i) Chemical. (A) Glass distilled or deionized water shall be used in the preparation of the nutrient medium. The pH of the test solution shall be measured in the control and test containers at the beginning and at the end of the definitive test. The concentration of test chemical in the test containers shall be determined at the beginning and end of the

definitive test by standard analytical methods which have been validated prior to the test. [VOLATILES SHALL HAVE TEST CHEMICAL CONCENTRATION DETERMINATIONS AT 0, 24, 48, 72, AND 96 HR.] An analytical method is unacceptable if likely degradation products of the chemical, such as hydrolysis and oxidation products, give positive or negative interference.

(B) At the end of the test and after aliquots have been removed for algal growth-response determinations, microscopic examination, mortal staining, or subculturing, the replicate test containers for each chemical concentration may be pooled into one sample. An aliquot of the pooled sample may then be taken and the concentration of test chemical determined. [PRIOR TO THE DEFINITIVE TEST, THE POOLING PROCEDURE WILL BE EVALUATED FOR CONTRIBUTING TO ANY LOSS OF TEST CHEMICAL. THE MEASURED CONCENTRATIONS OF TEST CHEMICAL IN THE POOLED SAMPLE SHALL BE CORRECTED FOR ANY LOSSES DURING THE POOLING PROCEDURE.] ~~In addition, the concentration of test chemical associated with the algae alone should be determined. Separate and concentrate the algal cells from the test solution by centrifuging or filtering the remaining pooled sample and measure the test substance concentration in the algal cell concentrate.~~

(ii) Numerical. Algal growth response (as percent of inhibition or stimulation in the test solutions compared to the controls) is calculated at the end of the test. Mean and standard deviation should be calculated and plotted for each treatment and control. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration response curves. The concentration response curves are plotted using the mean measured test solution concentrations obtained at the end of the test.

(d) Test conditions—(1) Test species. Species of algae recommended as test organisms for this test are the freshwater green alga, Selenastrum capricornutum, and the marine diatom, Skeletonema costatum. Algae to be used in acute toxicity tests may be initially obtained from commercial sources and subsequently cultured using sterile technique. Toxicity testing shall not be performed until algal cultures are shown to be actively growing (i.e., capable of logarithmic growth within the test period) in at least 2 subcultures lasting 7 days each prior to the start of the definitive test. All algae used for a particular test shall be from the same source and the same stock culture. Test algae shall not have been used in a previous test, either in a treatment or a control.

(2) Facilities--(i) General. (A) Facilities needed to perform this test include: a growth chamber or a controlled environment room that can hold the test containers and will maintain the air temperature, lighting intensity and photoperiod specified in this test guideline; apparatus for culturing and enumerating algae; a source of distilled and/or deionized water; and apparatus for carrying out analyses of the test chemical.

(B) Disposal facilities should be adequate to accommodate spent glassware, algae and test solutions at the end of the test and any bench covering, lab clothing, or other contaminated materials.

(ii) Test containers. Erlenmeyer flasks should be used for test containers. [FOR TESTING ~~OMOTE~~ [VOLATILES], THE TEST CONTAINERS SHALL BE CAPPED TO REDUCE CHEMICAL LOSS TO VOLATILITY.] The flasks may be of any volume between 125 and 500 ml as long as the same size is used throughout a test and the test solution volume does not exceed 50 percent of the flask volume.

(iii) Cleaning and sterilization. New test containers may contain substances which inhibit growth of algae. They shall therefore be cleaned thoroughly and used several times to culture algae before being used in toxicity testing. All glassware used in algal culturing or testing shall be cleaned and sterilized prior to use according to standard good laboratory practices.

(iv) Conditioning. Test containers should be conditioned by a rinse with the appropriate test solutions prior to the start of the test. Decant and add fresh test solutions after an appropriate conditioning period for the test chemical.

(v) Nutrient medium. (A) Formulation and sterilization of nutrient medium used for algal culture and preparation of test solutions should conform to those currently recommended by the U.S. EPA for freshwater and marine algal bioassays. ~~No chelating agents are to be included in the nutrient medium used for test solution preparation.~~ Nutrient medium should be freshly prepared for algal testing and may be dispensed in appropriate volumes in test containers and sterilized by autoclaving or filtration. The pH of the nutrient medium shall be 7.5 (± 0.1) for Selenastrum ~~and 8.1 (± 0.1) for Skeletonema~~ at the start of the test and may be adjusted prior to test chemical addition with 0.1N NaOH or HCl.

(1) Detailed information about the test organisms, including the scientific name, method of verification, and source.

(2) A description of the test chambers and containers, the volumes of solution in the containers, the way the test was begun (e.g., conditioning, test substance additions, etc.), the number of replicates, the temperature, the lighting, and method of incubation, oscillation rates, and type of apparatus.

(3) The concentration of the test chemical in the control and in each treatment at the end of the test and the pH of the solutions.

(4) The number of algal cells per milliliter in each treatment and control and the method used to derive these values at the beginning, 24, 48, and 72 hours, and end of the test; the percentage of inhibition or stimulation of growth relative to controls; and other adverse effect in the control and in each treatment.

(5) The 96-hour EC_{10}, EC_{50}, an EC_{90} values, and when sufficient data have been generated, the 24, 48, and 72 hour LC_{50}s and 96-percent confidence limits, the methods used to derive these values, the data used to define the shape of the concentration-response curve and the goodness-of-fit determination.

(6) Methods and data records of all chemical analyses of water quality and test substance concentrations, including method validations and reagent blanks.

(7) The results of any optional analyses such as: microscopic appearance of algae, size or color changes, percent mortality of cells and the fate of subcultured cells, the concentration of test substance associated with algae and the test solution supernate or filtrate.

(8) If the range-finding test showed that the highest concentration of the chemical tested (not less than 1000 mg/l or saturation concentration) had no effect on the algae, report the results and concentration and a statement that the chemical is of minimum phytotoxic concern.

(9) If the range-finding test showed greater than a 50 percent inhibition of algal growth at a test concentration below the analytical detection limit, report the results, concentration, and a statement that the chemical is phytotoxic below the analytical detection limit.

(B) Dilution water used for preparation of nutrient medium and test solutions should be filtered, deionized or glass distilled. Saltwater for marine algal nutrient medium and test solutions should be prepared by adding a commercial, synthetic, sea salt formulation or a modified synthetic seawater formulation to distilled/deionized water to a concentration of 30 parts per thousand.
(vi) Carriers. Nutrient medium shall be used in making stock solutions of the test chemical. If a carrier other than nutrient medium is absolutely necessary to dissolve the chemical, the volume used shall not exceed the minimum volume necessary to dissolve or suspend the chemical in the test solution.

(3) Test parameters. (i) The test temperature shall be 24°C for Selenastrum ~~and 20°C for Skeletonema~~. Excursions from test temperature shall be no greater than ± 2°C. Temperature should be recorded hourly during the test.

(ii) Test chambers containing Selenastrum shall be illuminated continuously ~~and those containing Skeletonema shall be provided a 14 hour light and 10 hour dark photoperiod with a 30 minute transition period~~ under fluorescent lamps providing ~~300±25~~ [60-65] uEin/m^2 sec (approximately 400 ft-c) measured adjacent to the test chambers at the level of test solution.

(iii) Stock algal cultures should be shaken twice daily by hand. Test containers shall be placed on a rotary shaking apparatus and oscillated at approximately 100 cycles/min for Selenastrum and at approximately 60 cycles/min for Skeletonema during the test. The rate of oscillation should be determined at least once daily during testing.

(iv) The pH of nutrient medium in which algae are subcultured shall be 7.5 (± 0.1) for Selenastrum ~~and 8.1 (± 0.1) for Skeletonema~~, and is not adjusted after the addition of the algae. The pH of all test solutions shall be measured at the beginning and end of the test.

(v) Light intensity shall be monitored at least daily during the test at the level of the test solution.

(e) Reporting. The sponsor shall submit to the EPA all data developed by the test that are suggestive or predictive of acute phytotoxicity. In addition to the general reporting requirements prescribed in Part 792—Good Laboratory Practice Standards of this chapter, the following shall be reported:

HAEL No. 90-0016 Study No. EN-1050-901878-1

DIS No. 263601B

Prepared by _____ Date 7-19-90
Heather M. Watson
Principal Investigator
Algal Acute Toxicity Test

Approved by _____ Date 7-19-90
Joseph W. Gorsuch
Group Leader
Eco-Chem Testing Group

Approved by _____ Date 7/23/90
John R. Owen
Unit Director
Environmental Sciences Section

Reviewed by _____ Date 8/14/90
Quality Assurance Unit
Health and Environment Laboratories

Approved by _____ Date 8/15/90
Bruce H. Klanderman
Director
Environmental Technical Services

Approved by _____ Date 8-15-90
Heather M. Watson
Study Director
Eco-Chem Testing Group

Approved by _____ Date 8/19/90
Roderick D. Gerwe
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Tennessee Eastman Company

4122e

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14650
United States of America
Telephone No. (716) 588-5447

ENVIRONMENTAL SAFETY DATA SHEET

CAS No. 4170-30-3 | Accession No. 901878
SRID No. Lot No. 90-7 | HAEL No. 90-0016

COMPOUND NAME(s) (Name from request sheet is first):

2-Butenal

Synonym(s): Crotonaldehyde

Molecular Formula: C_4H_6O **Molecular Wt.:** 70.09[1]

Structure: CH_3-CH=CH-CHO

ENVIRONMENTAL EVALUATION[2]

		Low(1)	Med(2)	High(3)
PERSISTENCE		--	2	--
BIOLOGICAL FATE	Biodegradation	--	2	--
ABIOTIC FATE	Chemical Degradation	--	2E	--
	Volatilization	--	2	--
BIOLOGICAL EFFECTS	Acute Aquatic	--	--	3
	Algal Acute Toxicity	--	--	3

ASSESSMENT: Single or intermittent aqueous exposures to chemical concentrations of $\leq$0.27 mg/L are not expected to have any adverse environmental effects.

E = Estimate

PHYSICAL-CHEMICAL PROPERTIES

Solubility:
Water: 181 g/L @ 20°C; 192 g/L @ 5°C[3]

Oxygen Demand: ThOD: 2.28 g O_2/g[4] COD: 97% of ThOD[5]

BOD_5: 97% of ThOD[5] BOD_{10}: 2.28 g O_2/g[5]

Other:
B.P.: 104-105°C[1]; this crotonaldehyde/water mixture 84°C (138°F)
M.P.: -74°C[1]
V.P.: 32 mm @ 20°C[6]

Assay: An initial purity analysis was carried out by Karl Fisher titration (for water) and using GC/FID on a HP 5890 gas chromatograph [for 2- butenal (crotonaldehyde) purity. The test chemical was found to contain 7.1% water, and the sample was found to be 98.1% pure (n = 3, std. dev. = 0. 12 with respect to 2-butenal. A final purity analysis was carried out by Karl Fisher titration (for water) and using GC/FID on a HP 5890 gas chromatograph [for 2-butenal (crotonaldehyde) purity]. The test chemical was found to contain 6.3% water, and the sample was found to be 100% pure (n = 3, std. dev. = 0. 00) with respect to 2-butenal.

Stability: Stable

BIODEGRADATION

A 28-day test for ready biodegradability using unacclimated microorganisms as the inoculum showed a 55% degradation of the test article as measured by oxygen uptake in the Closed Bottle Test.

Remarks: This chemical is moderately, but not readily, biodegradable. No toxicity was seen at the test concentration of 2.1 mg/L.

AQUATIC EFFECTS

Test Species (Acute)	Test Duration	EC50*/LC50** Point Estimate and (95% Confidence Limits)	No Observed Effect Concentration
Daphnid (EC50*) *Daphnia magna*	48 hours	2.0 mg/L[a] (1.3, 2.5 mg/L)	0.61 mg/L
Fathead Minnow (LC50**) *Pimephales promelas*	96 hours	0.84 mg/L[a] (0.51, 1.1 mg/L)	0.51 mg/L
Sideswimmer (LC50**) *Gammarus fasciatus*	96 hours	2.7 mg/L[a] (1.1, 4.5 mg/L)	1.1 mg/L
Rainbow Trout (LC50**) *Oncorhyncus mykiss*	96 hours	0.65 mg/L[a] (0.55, 1.0 mg/L)	0.27 mg/L
Bluegill Sunfish (LC50**) *Lepomis macrochirus*	96 hours	3.5 mg/L[d][5,7]	----
Tidewater Silversides(LC50**) *Menidia beryllina*	96 hours	1.3 mg/L[d][5,7]	----

*EC50 = Effect Concentration based on immobilization of 50% of population.
**LC50 = Lethal Concentration to 50% of the population.

Point Estimate Calculated By:

a = Non-Linear Interpolation
b = Binomial Method
c = Moving Average Method
d = Probit Method

Remarks: The endpoint values for the first four species presented above are all based on analyzed test article concentrations and the dose-response data from pooled replicates.

ALGAL ACUTE TOXICITY

96-hr EC50* = <0.881 mg/L

*EC50 = The concentration of test chemical that inhibits the growth of *Selenastrum capricornutum* by 50%.

Remarks: The chemical was algicidal at starting measured concentrations of 10.6 mg/L and 5.2 mg/L.

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7. Dawson, G.W., et al., J. Hazard. Mater., 1(4), 303-18 (1975/77).

Prepared by: M.S. Giancursio, P. W. Jenkins, R.J. O'Boyle, H.M. Watson

Co-ordinated by: Philip W. Jenkins Date: October 19, 1990

3904e/10-19-90

615-229-1

International Tanker Chartering, Inc.
Chartering Brokers
1160 Springfield Avenue
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TELEPHONE (201) 654-1220
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ANSWERBACK "ITC UR"

R:TRFES2

12 DECEMBER 89

PM 161
MSDS 10,597
901878

ATT: MRS. CAROLYN LINDSEY
TENNESSEE EASTMAN COMPANY

RE: CROTONALDEHYDE

ODFJELL HAVE COME BACK ASKING FOR ANSWERS TO THE FOLLOWING QUESTIONS ABOUT THE ABOVE PRODUCT:

1) IMO PRODUCT CLASSIFICATION NAME

2) MARPOL POLLUTION CATEGORY:

3) PRODUCT MELTING POINT DEG C: -76.5°C (Merck)

4) PRODUCT VISCOSITY 20 DEG C: .7CPS @ 25°C

5) COEFFICIENT OF CUBIC EXPANSION PER 1 DEG C: .0011

6) SOLUBILITY IN WATER PCT: 15.1% @ 20°C

7) CARRIAGE TEMPERATURE: Ambient Temperature

8) NITROGEN REQUIREMENTS: Positive pressure of nitrogen required

9) PH VALUE: Approx. 5

10) ACID VALUE: 15% as Acetic Acid

11) CORROSION PROPERTIES SUITABLE FOR STOWAGE IN:

A) STAINLESS 316 L: Yes

B) ZINC: No

C) PHINOLIC EPOXY: No

D) MILD STEEL: No

E) OTHERS: Aluminum

12) REACTIVITY DATA:

A) IS PRODUCT STABLE? No

B) IS PRODUCT INHIBITED? No

C) INHIBITOR TYPE/AMOUNT: None

Information was obtained from Dale Christian 1/17/90 LB

901878

PRELIMINARY TOXICITY STUDIES ON CROTONALDEHYDE AND TEC 2046

by

Harold C. Hodge

and

Elliott A. Maynard

From the

Division of Pharmacology & Toxicology
University of Rochester School of Medicine and Dentistry
Rochester 7, New York

Signed Harold C. Hodge

Date Nov. 21, 1947

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Resume. A series of preliminary acute toxicity tests have been carried out on crotonaldehyde and on TEC 2046. Only small groups of animals were employed, although tests were performed on several species. The substances were injected intraperitoneally in rats, mice, guinea pigs, and rabbits. Oral administration to rats was carried out. Skin applications were made on guinea pigs and rabbits. The approximate doses to kill the average animal of each species is indicated in the accompanying table:

Route	Species	Approximate Lethal Dose CA (mg/kg)	TEC 2046 (mg/kg)
Intraperitoneal	Rats	70	60
"	Mice	130	130 to 140
"	Guinea Pigs	20-35	10
"	Rabbit	5 to 50	>50
Oral	Rat	160	130
Skin	Guinea Pigs	500-1000	>1000
"	Rabbits	150- 200	>150

Species Variations. The species variations are large. Rabbits and guinea pigs are most susceptible, rats are intermediate, and mice are most

resistant. The approximate lethal doses of either crotonaldehyde or TEC 2046 are of the order of 10-35 mg/kg in guinea pigs, 5 to over 50 mg/kg in rabbits, 60-70 mg/kg in rats, and 130-140 mg/kg in mice. Oral administration reduces the toxicity by a factor of roughly 2 in the rat. When these materials are applied to the intact skin, large doses are required to kill; in rabbits, 150-200 mg/kg or more; and in guinea pigs 500-1000 or more mg/kg. When applied to the skin, it is apparent that guinea pigs are more resistant than rabbits by a factor of 5 or 6, at least.

Signs of Poisoning. When either material was administered intraperitoneally, the animals gave signs of intense irritation. Violent jumping following the administration; the animals would shut the eyes, rub the noses with paws, and move in violent gyrations. Clonic convulsions preceded death; red-stained tears were sometimes observed; the hearts continued to beat after respiration ceased; petechiae were sometimes observed on the surface of the gut; and occasionally bloody fluid was found in the peritoneal cavity.

Relative Toxicities of Crotonaldehyde and TEC 2046. From the approximate lethal doses, species by species, when these compounds were administered

intraperitoneally, orally or on the skin, crotonaldehyde and TEC 2046 exhibited the same order of toxicity in every instance. The impression was gained that TEC 2046 was somewhat less irritating when administered intraperitoneally than was crotonaldehyde, but this difference was not marked. Both compounds appeared to be highly toxic for guinea pigs and rabbits when given intraperitoneally, and moderately toxic for rats and mice.

Comparison with data of Smyth and Carpenter. Smyth and Carpenter in the Journal of Industrial Hygiene and Toxicology 26: 269 (1944) give typical results with their battery of range-finding tests on crotonaldehyde. In their table they report that the R.F. LD50 for a single dose administered orally to rats is 0.3 g/kg. Our value of 0.2 g/kg (160 mg/kg) is of the same order of magnitude and in general confirms the finding of Smyth and Carpenter. As far as industrial handling practices are concerned, Smyth and Carpenter indicate that from its oral toxicity in rats crotonaldehyde should be compared to ammonia (NH_3).

Their skin absorption test is conducted using a rubber cuff as recommended by the Food and Drug Administration. For materials which pass through

rubber, the collectable film of vinyl plastic or some other film may be used. On guinea pigs, the sample is placed on absorbent cotton which is placed on the clipped abdomen of the animal for 4 days. Using this procedure, they report the surprising value of 0.03 ml/kg for the R.F. LD50, and compare it as a hazard to ethylene chlorhydrin — a highly toxic material. As a matter of fact, in scanning the table included in the paper, it is seen that no other compound has the extraordinary degree of toxicity by this route. In fact, the next most toxic one requires about 6 times as much. Our experience with crotonaldehyde is entirely out of line with the findings of Smyth and Carpenter. In our guinea pigs, 0.5 to 1.0 ml/kg is lethal; that is, our guinea pigs require 15 to 30 times the dose described by Smyth and Carpenter.

Smyth and Carpenter also report that the longest time a group of 6 rats could breathe the saturated vapor with no deaths is one minute. This represents a high degree of toxicity as compared with the other compounds in their table. They indicate that, as far as its hazards in handling are concerned, crotonaldehyde should be compared with ethylene dichloride.

A frequent hazard in handling fluid chemicals is the exposure of the

eye. Smyth and Carpenter specify that the same precautions should be used in handling crotonaldehyde as are observed in their plant in handling acetic anhydride.

901878

PART I.

PRELIMINARY TOXICITY STUDIES ON CROTONALDEHYDE

Rats: Intraperitoneal Injection. Crotonaldehyde was administered intraperitoneally to male and female rats, in doses ranging from 5 to 100 mg per rat (Summary Table I). These rats included some young male rats and some old female rats (Tables II, III, IV, and V). The body weights of the young males were around 100 grams and of the old females, about 200 grams. The doses have all been recalculated on the basis of body weight, and range from 36 to 950 mg/kg.

Doses of 36 and of 47 mg/kg killed no rats; doses of 64 to 93 killed from none of 2 to 4 of 5, as shown in Table I; doses of 100 mg/kg and greater killed all the rest receiving such doses. Although so few rats were used in each group that the statistical analysis, the data were found to give a fairly good relationship when a probit kill was plotted against logarithm of the dose (Chart not shown). From this graph it appears that the dose to kill the average rat is of the order of 70 mg/kg.

When the solutions containing crotonaldehyde were administered the rats gave every evidence of profound irritation. They were hyperactive, squealed, shut their eyes, rubbed their noses, jumped about. Panting

progressed to gasping respirations. Respiration ceased, clonic convulsions were observed, and red tinged tears were seen about the eyes. The heart action continued after respiration ceased. Surviving animals on sacrifice several days later were generally completely normal in appearance.

SUMMARY TABLE[I]

INTRAPERITONEAL INJECTIONS OF CROTONALDEHYDE

RATS

Mg/kg/Dose	Dose/Rat	No. Rats	24-Hr. Mort.	% Mort.	Av. Bd. wt.
950	100	2	2	100	105
360	60	5	5	100	195
291	30	2	2	100	103
216	40	5	5	100	184
100	20	10	10	100	199
93	10	2	0	0	107
82	16	5	4	80	196
64	12	5	3	60	190
47	5	2	0	0	106
36	8	5	0	0	220

TABLE II

ACUTE TOXICITY OF CROTONALDEHYDE

RATS

INTRAPERITONEAL INJECTION

Injected by E. A. Maynard - 4 August 1947 - 11:00 A. M.

Group	Sex	Rat No.	Body Weight	Dose - C. A.	Mortality
A	♂	1	106 g	0.1 cc = 100 mg	Dead in 1 minute+
	♂	2	104 g	0.1 cc = 100 mg	Dead in 1 minute+
B	♂	1	90 g	0.03 cc = 30 mg	Dead in 3-4 minutes+
	♂	2	116 g	0.03 cc = 30 mg	Dead in 12 minutes+
C	♂	1	120 g	0.01 cc = 10 mg	None*
	♂	2	93 g	0.01 cc = 10 mg	None*

(1/10 solution of Crotonaldehyde in propylene glycol - 0.1 cc contains 10 mg.)

D	♂	1	110 g	0.05 cc = 5 mg	None*
	♂	2	102 g	0.05 cc = 5 mg	None*

* Rats were sacrificed on 8 August 1947 - all organs normal.

\+ Hyperactivity; gasping respiration; continued heart action following cessation of resp; watering of eyes; some slight nasal hemorrhage.

24 Hour Mortality

Dose	Mortality (24 hrs.)
100 mg	2/2
30 mg	2/2
10 mg	0/2
5 mg	0/2

TABLE III

ACUTE TOXICITY OF CROTONALDEHYDE

INTRAPERITONEAL INJECTION

(1/10 solution of Crotonaldehyde in water - 1 cc contains 100 mg.)

Injected by R. A. Maynard - 6 August 1947 - 10:30 A. M.

5 RATS - FEMALES - Dose 0.6 cc = 60 mg*

Rat No.	Body Weight	
1	190 g	Dead in 10 minutes
2	190 g	Dead in 10 minutes
3	210 g	Dead in 10 minutes
4	200 g	Dead in 10 minutes
5	186 g	Dead in 15 minutes

5 RATS - FEMALES - Dose 0.4 cc = 40 mg*

Rat No.	Body Weight	
1	180 g	Dead in 20 minutes
2	180 g	Dead in 20 minutes
3	180 g	Dead in 45 minutes
4	190 g	Dead in 45 minutes
5	190 g	Dead 8/6/47 - 5:00 P. M.

5 RATS - FEMALES - Dose 0.2 cc = 20 mg*

Rat No.	Body Weight	
1	190 g	Dead 8/7/47 - 9:00 A. M.
2	190 g	Dead 8/6/47 - 5:00 P. M.
3	220 g	Dead 8/7/47 - 9:00 A. M.
4	210 g	Dead 8/7/47 - 9:00 A. M.
5	200 g	Dead 8/6/47 - 5:00 P. M.

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* Squeal, shut eyes, rub noses, jump around, paving motions, clonic convulsions, tears - tinge of red, wild hyperactivity, gyrations, pop-eye, panting.

24 Hour Mortality

Dose	Mortality (24 hrs.)
60 mg	5/5
40 mg	5/5
20 mg	5/5

TABLE IV

ACUTE TOXICITY OF CROTONALDEHYDE

INTRAPERITONEAL INJECTION

(1/10 solution of Crotonaldehyde in water - 1 cc contains 100 mg.)

Injected by H. C. Hodge - 7 August 1947 - 11:00 A. M.

5 RATS - FEMALES - Dose 0.2 cc = 20 mg

Rat No.	Body Weight	
1	184 g	Dead 8/7/47 - 2:30 P. M.
2	192 g	Dead 8/7/47 - 2:30 P. M.
3	194 g	Dead 8/7/47 - 2:30 P. M.
4	190 g	Dead 8/7/47 - 2:30 P. M.
5	220 g	Dead 8/7/47 - 2:30 P. M.

5 RATS - FEMALES - Dose 0.16 cc = 16 mg

Rat No.	Body Weight	
1	194 g	Dead 8/7/47 - 2:30 P. M.
2	192 g	Dead 8/8/47 - 8:30 A. M.
3	204 g	Dead 8/9/47 - 8:30 A. M.
4	204 g	Dead 8/8/47 - 8:30 A. M.
5	184 g	Dead 8/8/47 - 8:30 A. M.

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24 Hour Mortality

Dose	Mortality (24 hrs.)
20 mg	5/5
16 mg	4/5 - 5/5 (48 hrs.)

TABLE V

ACUTE TOXICITY OF CROTONALDEHYDE

INTRAPERITONEAL INJECTION

(1/10 solution of Crotonaldehyde in water - 1 cc contains 100 mg.)

Injected by H. C. Hodge - 7 August 1947 - 11:10 A. M.

5 RATS - FEMALES - Dose 0.12 cc = 12 mg

Rat No.	Body Weight	
1	198 g	(Sacrificed 8/11/47) - Normal
2	194 g	Dead 8/8/47 - 8:30 A. M.
3	190 g	Dead 8/8/47 - 8:30 A. M.
4	160 g	Dead 8/9/47 - 8:30 A. M.
5	210 g	Dead 8/8/47 - 8:30 A. M.

5 RATS - FEMALES - Dose 0.08 cc = 8 mg

Rat No.	Body Weight	
1	198 g	(Sacrificed 8/11/47) - Normal
2	242 g	(Sacrificed 8/11/47)*
3	250 g	(Sacrificed 8/11/47)*
4	190 g	(Sacrificed 8/11/47) - Normal
5	220 g	Dead 8/11/47 - 8:30 A. M.

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* 2/4 sacrificed 8/11/47 had little hemorrhage on gut.

24 Hour Mortality

Dose	Mortality (24 hrs.)
12 mg	3/5 - 4/5 (48 hrs.)
8 mg	0/5

Mice: Intraperitoneal Injection. Crotonaldehyde was administered to adult female mice in doses ranging from 0.2 to 10 mg per mouse. (Summary Table VI). These mice were all of approximately the same size; however, they ranged from 18 to 30 grams in weight (Tables VII, VIII, IX, X XII, and XIII). On the basis of body weight the doses ranged from 8 to 400 mg/kg.

Except for one mouse of 10 receiving 40 mg/kg, all mice survived which received doses of 8, 40, 57, and 84 mg/kg. Only 2 of 15 mice given 105 mg/kg died. Five of 5 receiving 129 mg/kg died; but only 8 of 10 receiving 158 mg/kg died. Mice given 192 mg/kg or larger doses all died. The number of mice per group is too small for a strict statistical analysis; however, the data when plotted as probits versus logarithm of doses fell reasonably closely around the line from which the dose to kill the average mouse was estimated as of the order of 130 mg/kg.

SUMMARY TABLE VI

INTRAPERITONEAL INJECTIONS OF CROTONALDEHYDE

MICE

mg/kg/dose	Dose/Mouse	No. Mice	24-Hr. Mort.	% Mort.	Av. Bd. Wt.
400	10.0	5	5	100	25
210	5.0	10	10	100	24
192	4.0	5	5	100	21
158	3.5	10	8	80	22
129	3.0	5	5	100	23
105	2.5	15	2	13	24
84	2.0	5	0	0	24
57	1.5	5	0	0	26
40	1.0	10	1	10	25
8	0.2	5	0	0	24

TABLE VII

ACUTE TOXICITY OF CROTONALDEHYDE

INTRAPERITONEAL INJECTION

(1/10 solution of Crotonaldehyde in propylene glycol - 0.1 cc contains 10 mg.)

Injected by E. A. Maynard - 4 August 1947 - 11:10 A. M.

5 MICE - FEMALES - Dose 0.1 cc = 10 mg

Mouse No.	Body Weight	
1	27 g	Dead in 10-11 minutes
2	25 g	Dead in 10-11 minutes
3	24 g	Dead in 10-11 minutes
4	27 g	Dead in 15 minutes
5	22 g	Dead in 15 minutes

5 MICE - FEMALES - Dose 0.05 cc = 5 mg

Mouse No.	Body Weight	
1	25 g	Dead in 1 hour
2	22 g	Dead in 1 1/4 hour
3	26 g	Dead 8/4/47 - 3:30 P. M.
4	24 g	Dead 8/4/47 - 3:30 P. M.
5	22 g	Dead 8/4/47 - 3:30 P. M.

5 MICE - FEMALES - Dose 0.01 cc = 1 mg

Mouse No.	Body Weight	
1	25 g	Dead 8/5/47 - 8:00 A. M.
2	25 g	Dead 8/7/47 - 9:00 A. M.
3	25 g	Dead 8/7/47 - 9:00 A. M.
4	28 g	(Sacrificed 8/8/47) - Normal
5	23 g	(Sacrificed 8/8/47) - Normal

24 Hour Mortality

Dose	Mortality (24 hrs.)
10 mg	5/5
5 mg	5/5
1 mg	1/5

TABLE VII

ACUTE TOXICITY OF CROTONALDEHYDE

INTRAPERITONEAL INJECTION

(1/200 solution of Crotonaldehyde in water - 1 cc contains 5 mg.)

Injected by H. C. Hodge - 6 August 1947 - 11:00 A. M.

5 MICE - FEMALES - Dose .04 cc = 0.2 mg

Mouse No.	Body Weight	
1	26 g	(Sacrificed 8/8/47) - Normal
2	22 g	(Sacrificed 8/8/47) - Normal
3	26 g	(Sacrificed 8/8/47) - Normal
4	24 g	(Sacrificed 8/8/47) - Normal
5	22 g	(Sacrificed 8/8/47) - Normal

24 hour mortality = 0/5.

(1/200 solution of Crotonaldehyde in water - 1 cc contains 5 mg.)

Injected by E. A. Maynard - 8 August 1947 - 10:30 A. M.

5 MICE - FEMALES - Dose 1.0 cc = 5.0 mg

Mouse No.	Body Weight	
1	24 g	Dead 8/9/47 - 8:30 A. M.
2	22 g	Dead 8/8/47 - 2:30 P. M.
3	30 g	Dead 8/9/47 - 8:30 A. M.
4	20 g	Dead 8/9/47 - 8:30 A. M.
5	20 g	Dead 8/9/47 - 8:30 A. M.

24 hour mortality = 5/5.

TABLE IX

ACUTE TOXICITY OF CROTONALDEHYDE

INTRAPERITONEAL INJECTION

(1/200 solution of Crotonaldehyde in water - 1 cc contains 5 mg.)

Injected by H. G. Hodge - 7 August 1947 - 11:30 A. M.

5 MICE - FEMALES - Dose 0.3 cc = 1.5 mg

Mouse No.	Body Weight	
1	28 g	(Sacrificed 8/11/47) - Normal
2	24 g	(Sacrificed 8/11/47) - Normal
3	28 g	(Sacrificed 8/11/47) - Normal
4	26 g	(Sacrificed 8/11/47) - Normal
5	26 g	(Sacrificed 8/11/47) - Normal

5 MICE - FEMALES - Dose 0.2 cc = 1 mg

Mouse No.	Body Weight	
1	28 g	(Sacrificed 8/11/47) - Normal
2	26 g	(Sacrificed 8/11/47) - Normal
3	25 g	(Sacrificed 8/11/47) - Normal
4	24 g	(Sacrificed 8/11/47) - Normal
5	25 g	(Sacrificed 8/11/47) - Normal

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24 Hour Mortality

Dose	Mortality (24 hrs.)
1.5 mg	0/5
1 mg	0/5

TABLE X

ACUTE TOXICITY OF CROTONALDEHYDE

INTRAPERITONEAL INJECTION

(1/200 solution of Crotonaldehyde in water - 1 cc contains 5 mg.)

Injected by H. C. Hodge - 7 August 1947 - 11:20 A. M.

5 MICE - FEMALES - Dose 0.5 cc = 2.5 mg

Mouse No.	Body Weight	
1	25 g	Dead 8/9/47 - 8:30 A. M.
2	28 g	(Discarded)
3	26 g	Dead 8/8/47 - 8:30 A. M.
4	26 g	(Sacrificed 8/11/47) - Normal
5	26 g	(Sacrificed 8/11/47) - Normal

5 MICE - FEMALES - Dose 0.4 cc = 2.0 mg

Mouse No.	Body Weight	
1	25 g	(Sacrificed 8/11/47) - Normal
2	22 g	(Sacrificed 8/11/47) - Normal
3	23 g	(Sacrificed 8/11/47) - Normal
4	24 g	(Sacrificed 8/11/47) - Normal
5	26 g	(Sacrificed 8/11/47) - Normal

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24 Hour Mortality

Dose	Mortality (24 hrs.)
2.5 mg	1/5 - 2/5 (48 hrs.)
2.0 mg	0/5

ACUTE TOXICITY OF CROTONALDEHYDE

INTRAPERITONEAL INJECTION

(1/200 solution of Crotonaldehyde in water - 1 cc contains 5 mg.)

Injected by E. A. Maynard - 8 August 1947 - 10:40 A. M.

5 MICE - FEMALES - Dose 0.7 cc = 3.5 mg

Mouse No.	Body Weight	
1	18 g	Dead 8/9/47 - 8:30 A. M.
2	20 g	Dead 8/9/47 - 8:30 A. M.
3	23 g	Dead 8/9/47 - 8:30 A. M.
4	22 g	Dead 8/9/47 - 8:30 A. M.
5	22 g	Dead 8/9/47 - 8:30 A. M.

5 MICE - FEMALES - Dose 0.5 cc = 2.5 mg

Mouse No.	Body Weight	
1	23 g	(Sacrificed 8/11/47) - Normal
2	19 g	Dead 8/11/47 - 8:30 A. M.
3	22 g	(Sacrificed 8/11/47) - Normal
4	20 g	(Sacrificed 8/11/47) - Normal
5	23 g	(Sacrificed 8/11/47) - Normal

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24 Hour Mortality

Dose	Mortality (24 hrs.)
3.5 mg	5/5
2.5 mg	0/5

ACUTE TOXICITY OF CROTONALDEHYDE

INTRAPERITONEAL INJECTION

(1/200 solution of Crotonaldehyde in water - 1 cc contains 5 mg.)

Injected by E. A. Maynard - 29 August 1947 - 11:20 A. M.

5 MICE - FEMALES - Dose 0.5 cc = 2.5 mg

Mouse No.	Body weight	
1	20 g	Dead 8/30/47 - 7:00 A. M.
2	24 g	(Sacrificed 9/1/47) - Normal
3	24 g	(Sacrificed 9/1/47) - Normal
4	26 g	(Sacrificed 9/1/47) - Normal
5	26 g	(Sacrificed 9/1/47) - Normal

5 MICE - FEMALES - Dose 0.7 cc = 3.5 mg

Mouse No.	Body weight	
1	25 g	Dead 8/30/47 - 7:00 A. M.
2	20 g	Dead 8/30/47 - 7:00 A. M.
3	23 g	Dead 8/30/47 - 7:00 A. M.
4	20 g	(Sacrificed 9/1/47) - Normal
5	22 g	(Sacrificed 9/1/47) - Normal

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24 Hour Mortality

Dose	Mortality (24 hrs.)
3.5 mg	3/5
2.5 mg	1/5

TABLE XIII

ACUTE TOXICITY OF CROTONALDEHYDE

INTRAPERITONEAL INJECTION

(1/200 solution of Crotonaldehyde in water - 1 cc contains 5 mg.)

Injected by E. A. Maynard - 29 August 1947 - 11:30 A. M.

5 MICE - FEMALES - Dose 0.8 cc = 4 mg

Mouse No.	Body Weight	
1	24 g	Dead 8/30/47 - 7:00 A. M.
2	22 g	Dead 8/30/47 - 7:00 A. M.
3	22 g	Dead 8/30/47 - 7:00 A. M.
4	18 g	Dead 8/30/47 - 7:00 A. M.
5	20 g	Dead 8/30/47 - 7:00 A. M.

5 MICE - FEMALES - Dose 0.6 cc = 3 mg

Mouse No.	Body Weight	
1	30 g	(Sacrificed 9/1/47) - Normal
2	20 g	(Sacrificed 9/1/47) - Normal
3	20 g	(Sacrificed 9/1/47) - Normal
4	24 g	(Sacrificed 9/1/47) - Normal
5	22 g	(Sacrificed 9/1/47) - Normal

---

24 Hour Mortality

Dose	Mortality (24 hrs.)
4 mg	5/5
3 mg	0/5

Guinea Pigs: Intraperitoneal Injection. A few guinea pigs were given graded doses of crotonaldehyde, as indicated in Summary Table XIV. A program of decreasing dosage was instituted in which doses of 450 mg and less were administered to 1 guinea pig each. There was a considerable variation in body weight in the guinea pigs (See Tables XV, and XVI). One guinea pig weighed only 454 grams; the heaviest guinea pig weighed 1077 grams. When the doses were recalculated on the basis of body weight the range varies from 10 to 513 mg/kg.

The guinea pigs receiving 10 and 20 mg/kg survived. The guinea pigs which received 35 mg/kg and greater all died with the exception of 1 guinea pig which received 355 mg/kg. This guinea pig was the only black one; all the others were albino. The color difference and survival may be only coincidentally related. There is no explanation available as to why this animal survived for 4 days, whereas other guinea pigs given much smaller doses of the same solution died in a few hours.

The dose to kill the average guinea pig appears to lie somewhere in the range of 20 to 35 mg/kg.

SUMMARY TABLE XIV

INTRAPERITONEAL INJECTIONS OF CROTONALDEHYDE

GUINEA PIGS

Mg/kg/dose	Dose/Guinea Pig	No. Guinea Pigs	24-Hr. Mort.	% Mort.	Av. Bd. Wt.
1022	110	1	1	100	1077 g.
513	450	1	1	100	879 g.
353	250	1	0	0	709 g.
252	200	1	1	100	794 g.
107	70	2	2	100	652 g.
50	45	1	1	100	907 g.
35	20	1	1	100	566 g.
20	16	1	0	0	790 g.
10	4.5	1	0	0	454 g.

TABLE IV

ACUTE TOXICITY OF SECTONALENSIN

GUINEA PIGS

INTRAPERITONEAL INJECTION

Injected by E. A. Maynard - 4 August 1947 - 11:30 A. M.

Guinea Pig No.	Sex	Body Weight	Dose - C. A.	Mortality
1	♂	879 g	.45 cc = 450 mg	Dead in 10 minutes
2	♂	794 g	.2 cc = 200 mg	Dead 8/4/47 - 3:30 P. M.
3	♂	1077 g	.11 cc = 110 mg	Dead in 10 minutes
4	♂	709 g	0.25 cc = 250 mg	Died 8/8/47 *
5	♂	624 g	.07 cc = 70 mg	Dead 8/4/47 - 3:30 P. M.
6	♂	680 g	.07 cc = 70 mg	Dead 8/4/47 - 4:00 P. M.

* No. 4 Guinea Pig was black.

24 Hour Mortality

Dose	Mortality (24 hrs.)
450 mg	1/1
250 mg	0/1
200 mg	1/1
110 mg	1/1
70 mg	2/2

TABLE XVI

ACUTE TOXICITY OF CROTONALDEHYDE

INTRAPERITONEAL INJECTION

(1/20 solution of Crotonaldehyde in water - 1 cc contains 100 mg.)

Injected by E. A. Maynard - 8 August 1947 - 11:30 A. M.

1 Albino Guinea Pig - MALE - Dose 0.45 cc = 45 mg

Guinea Pig No.	Body Weight	
1	907 g	Dead 8/8/47 - 5:00 P. M.

1 Albino Guinea Pig - MALE - Dose 0.16 cc = 16 mg

Guinea Pig No.	Body Weight	
1	790 g	(Sacrificed 8/11/47) - Normal

Injected by E. A. Maynard - 12 August 1947 - 11:00 A. M.

1 Albino Guinea Pig - MALE - Dose 0.2 cc = 20 mg

Guinea Pig No.	Body Weight	
1	566 g	Dead 8/12/47 - 8:30 P. M.

Injected by E. A. Maynard - 13 August 1947 - 10:40 A. M.

1 Albino Guinea Pig - MALE - Dose 0.045 cc = 4.5 mg

Guinea Pig No.	Body Weight	
1	454 g	Dead 8/15/47 - 7:00 A. M.

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24 Hour Mortality

Dose	Mortality (24 hrs.)
45 mg	1/1
20 mg	1/1
16 mg	0/1
4.5 mg	0/1 - 1/1 (48 hrs.)

Rabbits: Intraperitoneal Injection. A few albino rabbits, mostly adult males, were given selected doses of croton aldehyde intraperitoneally (Summary Table XVII). Doses ranging from 19 to 1840 mg were given to one rabbit each. The rabbits ranged in body weight from 3.6 to 5.6 kg (Tables XVIII, XIX). When the doses were recalculated on the basis of body weight, the range was found to extend from 5 to 511 mg/kg.

The rabbits receiving 5 and 20 mg/kg, respectively, survived. For reasons unknown, the rabbit receiving 10 mg/kg died. All rabbits receiving 50 or more mg/kg died. The dose to kill the average rabbit would appear to lie someplace in the range of 5 to 50 mg/kg.

SUMMARY TABLE XVII

INTRAPERITONEAL INJECTIONS OF CROTONALDEHYDE

RABBITS

Mg/kg/Dose	Dose/Rabbit	No. Rabbits	24-Hr. Mort.	% Mort.	Av. Bd. Wt.
511	1840	1	1	100	3.6 kg.
245	1250	1	1	100	5.1 kg.
100	410	1	1	100	4.1 kg.
50	240	1	1	100	4.8 kg.
20	95	1	0	0	4.7 kg.
10	48	1	1	100	4.7 kg.
5	19	1	0	0	3.9 kg.

TABLE XVIII

ACUTE TOXICITY OF CROTONALDEHYDE

ALBINO RABBITS

INTRAPERITONEAL INJECTION

Injected by E. A. Maynard - 4 August 1947 - 11:50 A. M.

Rabbit No.	Sex	Body Weight	Dose - C. A.	Mortality
1	♂	3628 g	1.84 cc = 1840 mg	Dead in 12 minutes
2	♂	5102 g	1.25 cc = 1250 mg	Dead in 30 minutes
3	♂	4138 g	.41 cc = 410 mg	Dead 8/4/47 - 3:30 P. M.

24 Hour Mortality

Dose	Mortality (24 hrs.)
1840 mg	1/1
1250 mg	1/1
410 mg	1/1

TABLE XIX

ACUTE TOXICITY OF CROTONALDEHYDE

INTRAPERITONEAL INJECTION

(1/10 solution of Crotonaldehyde in water - 1 cc contains 100 mg.)

Injected by E. A. Maynard - 8 August 1947 - 11:40 A. M.

1 Albino Rabbit - MALE - Dose 2.4 cc = 240 mg

Rabbit No.	Body Weight	
1	4762 g	Dead 8/8/47 - 5:00 P. M.

1 Albino Rabbit - FEMALE - Dose 0.95 cc = 95 mg

Rabbit No.	Body Weight	
1	4735 g	Dead in 48 hours

Injected by E. A. Maynard - 12 August 1947 - 11:15 A. M.

1 Albino Rabbit - MALE - Dose 0.48 cc = 48 mg

Rabbit No.	Body Weight	
1	4705 g	Dead 8/12/47 - 3:30 P. M.

Injected by E. A. Maynard - 13 August 1947 - 11:00 A. M.

1 Albino Rabbit - MALE - Dose 0.19 cc = 19 mg

Rabbit No.	Body Weight	
1	3855 g	Lived 1 month

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24 Hour Mortality

Dose	Mortality (24 hrs.)
240 mg	1/1
95 mg	0/1 - 1/1 (48 hrs.)
48 mg	1/1
19 mg	0/1

Rats: Oral Administration. Small groups of young adult albino rats were given doses ranging from 10 to 70 mg of croton aldehyde by stomach tube. (Summary Table XX). Large doses produced death promptly. (Tables XXI and XXII). These young rats mostly weighed from 100 to 175 grams. When the doses were recalculated on the basis of body weight, the range of dose was found to extend from 70 to 483 mg/kg.

Doses of 838 mg/kg and greater killed all the rats. A dose of 192 mg/kg killed 4 of 6 rats; 126 mg/kg killed 1 of 6; and a dose of 70 mg/kg killed 2 of 9 rats. Although the groups are too small to afford opportunity for statistical evaluation, the data, when plotted as probits versus logarithm of dose, lay along the line which gave indication that the dose to kill the average rat was of the order of 160 mg/kg.

SUMMARY TABLE XX

ACUTE ORAL TOXICITY (Stomach Tube of Crotonaldehyde)

RATS

mg/kg/dose	Dose/wt	No. Rats	24-Hr. Mort.	% Mort.	Av. Bd. Wt.
483	70	2	2	100	144
481	65	1	1	100	135
470	50	1	1	100	106
238	25	2	2	100	105
192	30	6	4	67	157
126	20	6	1	17	160
70	10	9	2	22	142

SUMMARY TABLE XXI

ACUTE TOXICITY OF CROTONALDEHYDE

RATS

Stomach Tubed by Raymond Kesel - 4 August 1947 - 12:10 P. M.

Group	Sex	Rat No.	Body Weight	Dose - C. A.	Mortality
A	♂	1	106 g	.05 cc = 50 mg	Dead in 3 minutes
	♂	2	142 g	.07 cc = 70 mg	Dead in 10 minutes
	♂	3	145 g	.07 cc = 70 mg	Dead in 30 minutes
	♂	4	135 g	.065 cc = 65 mg	Dead in 30 minutes
B	♂	1	120 g	.03 cc = 30 mg	Dead in 10 minutes
	♂	2	104 g	.025 cc = 25 mg	Dead in 20 minutes
	♂	3	145 g	.02 cc = 20 mg	Dead 8/4/47 - 3:30 P. M.
	♂	4	105 g	.025 cc = 25 mg	Dead 8/4/47 - 3:30 P. M.
C	♂	1	142 g	.01 cc = 10 mg	None*
	♂	2	126 g	.01 cc = 10 mg	Dead 8/4/47 - 3:30 P. M.
	♂	3	90 g	.01 cc = 10 mg	Dead 8/4/47 - 3:30 P. M.
	♂	4	154 g	.01 cc = 10 mg	None*

* Rats were sacrificed on 8 August 1947 - all organs normal.

24 Hour Mortality

Dose	Mortality (24 hrs.)
70 mg	2/2
65 mg	1/1
50 mg	1/1
30 mg	1/1
25 mg	2/2
20 mg	1/1
10 mg	2/4

SUMMARY TABLE XXII

ACUTE TOXICITY OF CROTONALDEHYDE

(1/10 solution of Crotonaldehyde in water - 1 cc contains 100 mg.)

Stomach Tubed by Raymond Kesel - 12 August 1947 - 11:45 A. M.

5 RATS - MALES - Dose 0.3 cc = 30 mg

Rat No.	Body Weight	
1	186 g	(Sacrificed 8/14/47) - Normal
2	162 g	(Sacrificed 8/14/47) - Normal
3	178 g	Dead 8/12/47 - 8:30 P. M.
4	136 g	Dead 8/12/47 - 8:30 P. M.
5	160 g	Dead 8/12/47 - 8:30 P. M.

5 RATS - MALES - Dose 0.2 cc = 20 mg

Rat No.	Body Weight	
1	168 g	(Sacrificed 8/14/47) - Normal
2	156 g	(Sacrificed 8/14/47) - Normal
3	164 g	(Sacrificed 8/14/47) - Normal
4	174 g	(Sacrificed 8/14/47) - Normal
5	154 g	(Sacrificed 8/14/47) - Normal

5 RATS - MALES - Dose 0.1 cc = 10 mg

Rat No.	Body Weight	
1	172 g	(Sacrificed 8/14/47) - Normal
2	138 g	(Sacrificed 8/14/47) - Normal
3	147 g	(Sacrificed 8/14/47) - Normal
4	174 g	(Sacrificed 8/14/47) - Normal
5	134 g	(Sacrificed 8/14/47) - Normal

---

24 Hour Mortality

Dose	Mortality (24 hrs.)
30 mg	3/5
20 mg	0/5
10 mg	0/5

Guinea Pigs : Skin application. The only reference to the skin application of croton aldehyde we found in the literature led us to believe that extremely minute doses were highly corrosive and lethal. As a first test 30 mg of undiluted croton aldehyde were applied to the intact skin of a guinea pig without any attempt at covering or confining the material. The hair was clipped and the material spread from a syringe fitted with a needle. When the animal showed practically no evidence at all of the application, larger doses were applied under a watch glass held in place by adhesive tape which was backed up by a round-the-body wrapping of elastic bandage. As may be seen in Summary Table XXIII, doses of 24 to 450 mg were used. The guinea pigs varied considerably in body weight; the smallest one weighed 454 and the largest one weighed 964 g. When the doses were recalculated on the basis of body weight, the range was established from 31 to 990 mg/kg. The results of the individual tests are given in Tables XXIV, XXV, XXVI, and XXVII.

Surprisingly enough, no mortalities were observed up to a dose of 500 mg/kg. It was necessary to administer al ost double this amount to obtain

the lethal outcome. The dose to kill the average guinea pig by skin application of croton aldehyde apparently lies someplace between 500 and 1000 mg/kg.

SUMMARY TABLE XXIII

SKIN APPLICATIONS OF CROTONALDEHYDE

GUINEA PIGS

Mg/kg/dose	Dose/Guinea Pig	No. Guinea Pigs	24-Hr. Mort.	% Mort.	Av. Bd. Wt.
990	450	1	1	100	454 g.
504	400	1	0	0	794 g.
405	230	1	0	0	567 g.
299	170	1	0	0	567 g.
92	60	1	0	0	652 g.
31	30	1	0	0	964 g.
31	24	1	0	0	766 g.

Rabbits: Skin Application. Beginning with a dose of 30 mg/kg, a series of rabbits were given increasing amounts of croton aldehyde upon the skin in the same fashion as were the guinea pigs. Doses of 130 to 1130 mg were administered (Summary Table XXVIII). The rabbits were mostly adult male albinos, ranging in weight from 3.2 to 4.5 kg. Calculated on the basis of body weight, the dosages ranged from 30 to 305 mg/kg. Rabbits receiving doses of 153 and less mg/kg all survived. One rabbit given 197 and one given 305 mg/kg died. The dose to kill the average rabbit by skin application of croton aldehyde apparently lies someplace between 150 and 200 mg/kg.

In guinea pigs and in rabbits, the effect of croton aldehyde was to give a tanned appearance to the skin. Sometimes considerable edema was present; that is, the skin felt stiff and thick. Reddened areas were sometimes observed near the edge of the watch glass when it was removed 24 hours after the croton aldehyde was applied. The area under the watch glass was almost always yellow-brown. Corrosive ulceration was not observed.

SUMMARY TABLE XXVIII

SKIN APPLICATIONS OF CROTONALDEHYDE

RABBITS

Mg/kg/Dose	Dose/Rabbit	No. Rabbits	24-Hr. Mort.	% Mort.	Av. Bd. Wt.
305	1130	1	1	100	3.7 kg.
197	770	1	1	100	3.9 kg.
153	690	1	0	0	4.5 kg.
68	300	1	0	0	4.4 kg.
31	100	1	0	0	3.2 kg.
30	130	1	0	0	4.3 kg.

SKIN APPLICATION OF CROTONALDEHYDE
(material not confined)

Applied by E. A. Maynard - 8 August 1947 - 12:30 P. M.

1 Albino Rabbit - MALE - Dose 0.13 cc = 130 mg

Rabbit No.	Body Weight	
1	4252 g	Lived 1 month

1 Albino Guinea Pig - MALE - Dose 0.03 cc = 30 mg

Guinea Pig No.	Body Weight	
1	964 g	Lived 1 month

No 24 hour mortalities.

SUMMARY TABLE XIV

SKIN APPLICATION OF CROTONALDEHYDE
(material confined in watch glass)

Applied by E. A. Maynard - 11 August 1947 - 11:00 A. M.

1 Albino Rabbit - MALE - Dose 0.1 cc = 100 mg

Rabbit No.	Body Weight	
1	3175 g	Lived 1 month

1 Albino Guinea Pig - MALE - Dose 0.024 cc = 24 mg

Guinea Pig No.	Body Weight	
1	766 g	Lived 1 month

Applied by E. A. Maynard - 12 August 1947 - 11:30 A. M.

1 Albino Rabbit - MALE - Dose 0.3 cc = 300 mg *

Rabbit No.	Body Weight	
1	4422 g	Lived 1 month

1 Albino Guinea Pig - MALE - Dose 0.06 cc = 60 mg +

Guinea Pig No.	Body Weight	
1	652 g	Lived 1 month

---

* Some erythema under adhesive wrapping; skin thickened, reddened, particularly just beyond ventral rim of watch glass; Tanning or browning general over entire area of watch glass - no evidence of acute injury.

\+ Tanned (brown) area larger than watch glass - central area not highly edematous - pink areas throughout crypt area = reaction to adhesive - Guinea Pig was somewhat sensitive on browned area.

No 24 hour mortalities.

SUMMARY TABLE XXVI

SKIN APPLICATION OF CROTONALDEHYDE

(Material confined in watch glass)

Applied by E. A. Maynard - 14 August 1947 - 11:00 A. M.

1 Albino Rabbit - MALE - Dose 1.13 cc = 1130 mg

Rabbit No.	Body Weight	
1	3741 g	Dead 8/15/47 - 7:00 A. M.

24 hour mortality = 1/1.

1 Albino Guinea Pig - MALE - Dose 0.17 cc = 170 mg

Guinea Pig No.	Body Weight	
1	567 g	Lived 1 month

24 hour mortality = 0/1.

Applied by E. A. Maynard - 19 August 1947 - 11:00 A. M.

1 RABBIT - MALE - Dose 0.77 cc = 770 mg

Rabbit No.	Body Weight	
1	3855 g	Dead 8/20/47 - 7:00 A. M.

24 hour mortality = 1/1.

1 GUINEA PIG - MALE - Dose 0.23 cc = 230 mg

Guinea Pig No.	Body Weight	
1	567 g	Lived 1 month

24 hour mortality = 0/1.

SUMMARY TABLE XXVII

SKIN APPLICATION OF CROTONALDEHYDE
(Material confined in watch glass)

Applied by E. A. Maynard - 21 August 1947 - 11:00 A. M.

1 Albino Rabbit - MALE - Dose 0.69 cc = 690 mg

Rabbit No.	Body Weight	
1	4541 g	Lived 1 month

1 Albino Guinea Pig - MALE - Dose 0.4 cc = 400 mg

Guinea Pig No.	Body Weight	
1	794 g	Lived 1 month

No 24 hour mortalities.

SKIN APPLICATION OF CROTONALDEHYDE
(Material poulticed in gauze pad in watch glass)

Applied by E. A. Maynard - 26 August 1947 - 11:00 A. M.

1 Albino Guinea Pig - MALE - Dose 0.45 cc = 450 mg

Guinea Pig No.	Body Weight	
1	454 g	Dead 8/27/47 - 7:00 A. M.

24 hour mortality = 1/1.

Summary. A series of acute toxicity tests of croton aldehyde has been carried out. These are all preliminary tests using small groups of animals. Croton aldehyde was administered intraperitoneally to rats, mice, guinea pigs, and rabbits. The approximate dose to kill the average animal of each species is indicated in the table below. Rats were given croton aldehyde by stomach tube; Croton aldehyde was applied to the skin of guinea pigs and rabbits.

Route	Species	Approximate Lethal Dose of Croton Aldehyde, mg/kg
Intraperitoneal	Rats	70
"	Mice	130
"	Guinea Pigs	20 - 35
"	Rabbits	5 - 50
Oral	Rat	160
Skin	Guinea Pigs	500 - 1000
"	Rabbits	150 - 200

It is apparent that there are large species variations. Mice are most resistant to croton aldehyde given intraperitoneally; and in order of increasing susceptibility may be listed rats, guinea pigs, and rabbits. That guinea

pigs are more resistant than rabbits appears from the results of the skin tests in which doses 5 to 10 times the lethal dose for rabbits was required to kill the guinea pigs. The oral dose, as would be expected, was much larger than the intraperitoneal dose in rats. The skin dose is many times the intraperitoneal dose. In the guinea pigs, the factor appears to be of the order of 20 or more times as much croton aldehyde required on the skin as intraperitoneally. In th rabbit, the factor is not so large, but still 5 to 10 times as much croton aldehyde as may be required when placed on the skin.

When croton aldehyde was administered, especially by intraperitoneal injection, the animals gave evidence of intense irritation. This was true in each species. The animals reacted with violent activity. They would sometimes squeal, shut the eyes, rub noses with paws, girate violently. Clonic convulsions frequently preceeded death. Red-stained tears were sometimes observed. The heart continued to beat after respiratory motions ceased.

~~M Schenker~~

901878

PROBIT ANALYSIS ON LOG10(CONC)

ITERATION	INTERCEPT	SLOPE	MU	SIGMA
0	-3.76812676	3.39763479	2.58065605	0.29432239
1	-9.34732096	5.53167296	2.59366761	0.18077714
2	-9.89260336	5.74088032	2.59413235	0.17418931
3	-9.89848603	5.74313820	2.59413678	0.17412083
4	-9.89848672	5.74313846	2.59413678	0.17412082

COVARIANCE MATRIX

	INTERCEPT	SLOPE
INTERCEPT	34.93228007	-13.07601637
SLOPE	-13.07601637	4.89999298

COVARIANCE MATRIX

	MU	SIGMA
MU	0.00197239	-0.00192559
SIGMA	-0.00192559	0.00450400

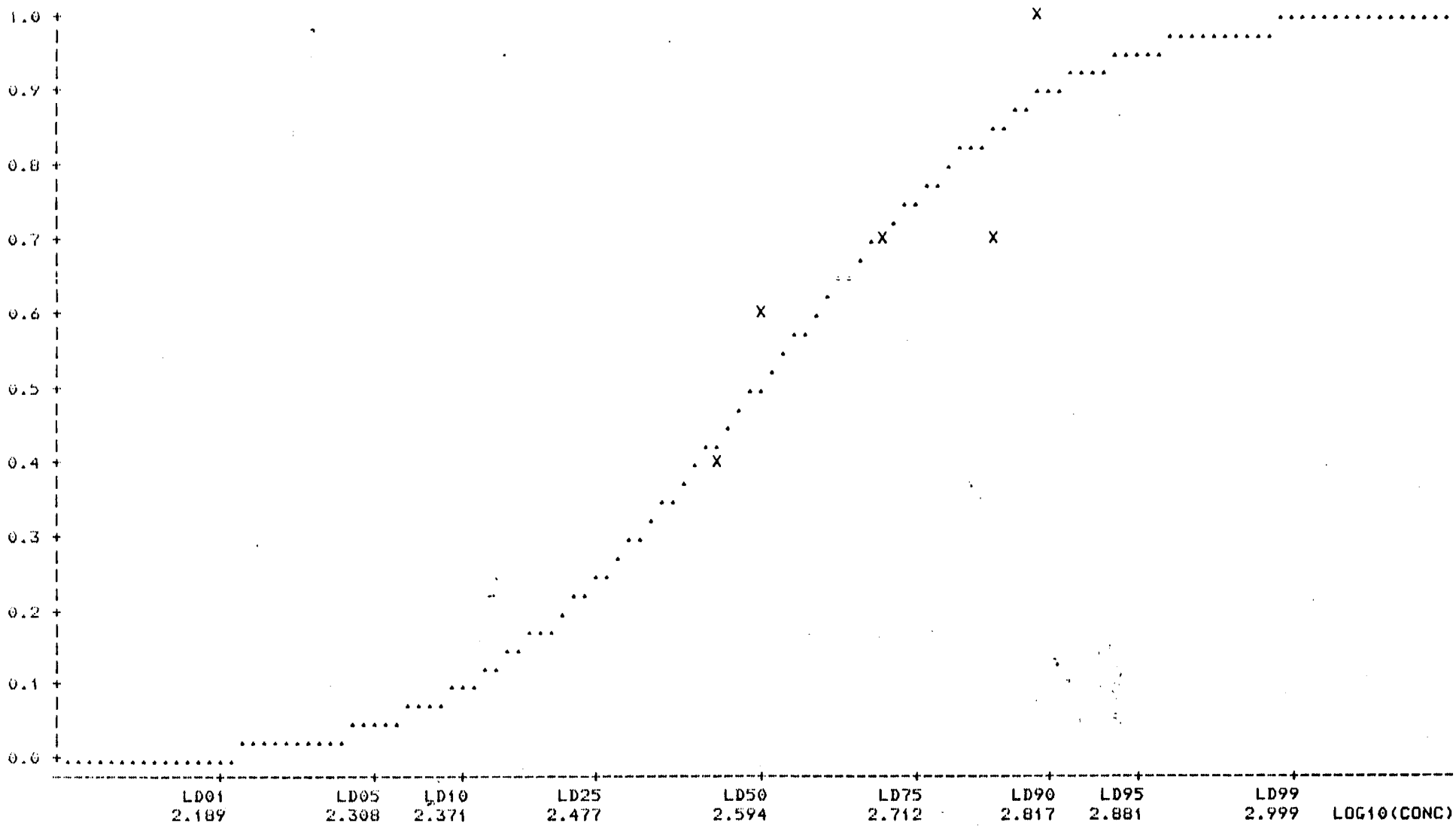
CHI-SQ = 3.1999 WITH 3 DF PROB > CHI-SQ = 0.3618

NOTE: SINCE THE CHI-SQUARE IS SMALL (P > 0.10), FIDUCIAL LIMITS WILL BE COMPUTED USING A T VALUE OF 1.96 .

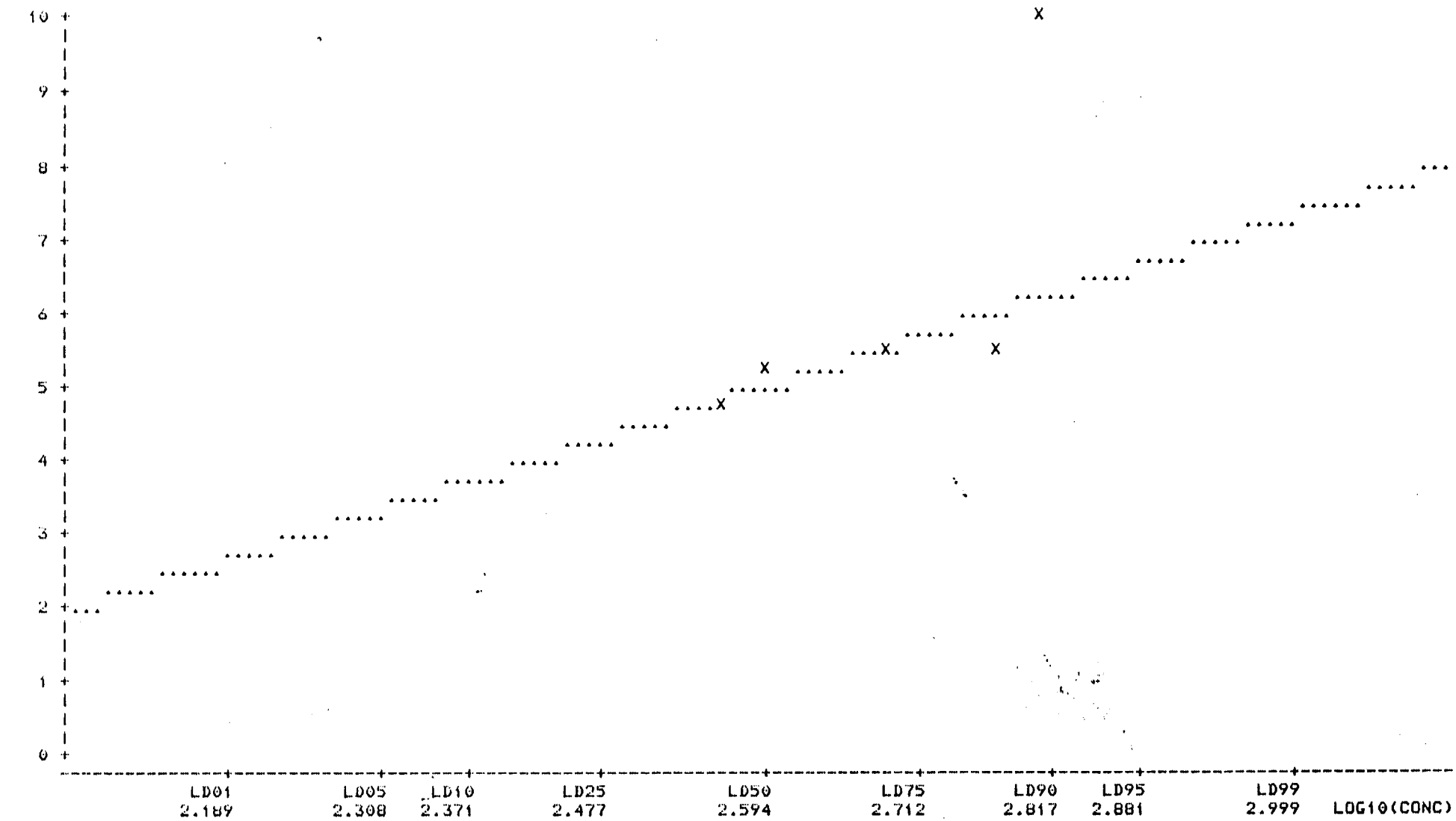
OBS	CONC	N	NDEAD
1	370	10	4
2	400	10	6
3	490	10	7
4	590	10	7
5	640	10	10

PROBABILITY

PROBIT ANALYSIS ON LOG10(CONC)



PROBIT ANALYSIS ON LOG10(CONC)



PROBIT ANALYSIS ON LOG10(CONC)

PROBABILITY	LOG10(CONC)	95 PERCENT FIDUCIAL LIMITS LOWER	UPPER	CONC	95 PERCENT FIDUCIAL LIMITS LOWER	UPPER
0.01	2.18907117	0.70174946	2.40149618	154.55076996	5.03210225	252.05550155
0.02	2.23653633	0.89517815	2.42919641	172.39962984	7.85557814	268.65591692
0.03	2.26665145	1.01782259	2.44685119	184.77850487	10.41891719	279.80224174
0.04	2.28930588	1.11003177	2.46018364	194.67307108	12.88343784	288.52512817
0.05	2.30773351	1.18499796	2.47106743	203.11103187	15.31080264	295.84717524
0.06	2.32341832	1.24877409	2.48036[illegible]	210.58058213	17.73266840	302.24777056
0.07	2.33717083	1.30466579	2.48854110	217.35559875	20.16813718	307.99317890
0.08	2.34948456	1.35468556	2.49588809	223.60657185	22.63005248	313.24784070
0.09	2.36068341	1.40015403	2.50259240	229.44754221	25.12777451	318.12104159
0.10	2.37099197	1.44198678	2.50878477	234.95893588	27.66857430	322.68944886
0.15	2.41367215	1.61491919	2.53468921	259.22217203	41.20208434	342.52258108
0.20	2.44759300	1.75194041	2.55569721	280.28057403	56.48594605	359.49860652
0.25	2.47669407	1.86904388	2.57416874	297.70505610	73.96800000	375.11872600
0.30	2.50282773	1.97368051	2.59128265	318.29347186	94.11969472	390.19585662
0.35	2.52704446	2.06997773	2.60780546	336.54602360	117.48372986	405.32693001
0.40	2.55002377	2.16045559	2.62438276	354.83281307	144.69568820	421.09759766
0.45	2.57225652	2.24668931	2.64172616	373.47068938	176.47748540	438.25427461
0.50	2.59413678	2.32951475	2.66083565	392.76861790	213.55746240	457.96854937
0.55	2.61601704	2.40889019	2.68339516	413.06370646	256.38356845	482.38651214
0.60	2.63824979	2.48333842	2.71252404	434.76020685	304.32555312	515.85071663
0.65	2.66122910	2.54923173	2.75368590	458.38362776	354.18627406	567.13428131
0.70	2.68544583	2.60220844	2.81352921	484.66965504	400.13674989	650.92238678
0.75	2.71157949	2.64221729	2.89527090	514.73001214	438.75015854	785.72560229
0.80	2.74068056	2.67410752	2.99895568	550.40270894	472.17992464	997.59824170
0.85	2.77460142	2.70319765	3.12789477	595.11571097	504.89102088	1342.43964693
0.90	2.81728159	2.73432651	3.29560276	656.57084559	542.40852695	1975.16215982
0.91	2.82759015	2.74135230	3.33660209	672.34185960	551.25469417	2170.71141044
0.92	2.83878900	2.74883963	3.38128754	689.90453158	560.84083771	2405.95519509
0.93	2.85110273	2.75692688	3.43056704	709.74563387	571.38243353	2695.05133771
0.94	2.86485524	2.76581017	3.48575344	732.58030557	583.19013971	3060.22557407
0.95	2.88054005	2.77578470	3.54885072	759.52145873	596.73938507	3538.75685799
0.96	2.89896768	2.78733132	3.62315408	792.44235659	612.81772699	4199.07936598
0.97	2.92162211	2.80132549	3.71470155	834.87626072	632.88600137	5184.43633815
0.98	2.95173723	2.81966749	3.83665876	894.82319277	660.18779644	6865.28793456
0.99	2.99920239	2.84814906	4.02930612	998.16511586	704.93497003	10698.08681408

Study No. EN-1040-901878-1

ES-90-031

263600A

READY BIODEGRADABILITY (CLOSED-BOTTLE) TEST

(using the Federal Register Guideline 40 CFR 796.3200)

OF

Crotonaldehyde

HAEL No. 90-0016 Accession No. 901878

CAS Reg. No. 4170-30-3

By

Heather M. Watson

Environmental Sciences Section

Health and Environment Laboratories

Eastman Kodak Company

Rochester, New York 14652-3617

Completion Date: September 27, 1990

HAEL No. 90-0016 Study No. EN-1040-901878-1

Ready Biodegradability (Closed Bottle) Test of
Crotonaldehyde

Conducted in Accordance with the Federal Register Guideline
40 CFR 796.3200

STATEMENT OF GLP COMPLIANCE

To the best of my knowledge and belief, these procedures were conducted in compliance with all Good Laboratory Practice standards of 40 CFR Part 792, November 29, 1983 (revised August 17, 1989). No unforeseen circumstances were observed that might have affected the quality or integrity of these procedures.

Study Director: Heather M. Watson 9-27-90
Date

Heather M. Watson
Eco-Chem Testing Group
Environmental Sciences Section
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Eastman Kodak Company
Rochester, NY 14652-3617

Sponsor: Roderick D. Gerwe 10-1-90
Date

Roderick D. Gerwe
Eastman Chemical Company
Kingsport, TN 37662

HAEL No. 90-0016

Study No. EN-1040-901878-1

Ready Biodegradability (Closed Bottle) Test of Crotonaldehyde

Conducted in Accordance with the Federal Register Guideline 40 CFR 796.3200

Abstract

A 28-day biodegradation test was performed to determine the biodegradability of crotonaldehyde using the Closed Bottle Test (Federal Register Guideline 40 CFR 796.3200 and the November 9, 1989 Consent Agreement with the EPA). The test inoculum was a sample of microorganisms from the secondary effluent of a domestic wastewater treatment plant. Biodegradability was determined by measuring the loss of dissolved oxygen from the test solutions contained in sealed BOD bottles. The degradation was calculated as the ratio of biochemical oxygen demand (BOD) to theoretical oxgen demand (ThOD) and expressed as a percentage. The concentration of the test chemical tested was 2.1 mg/L. An inhibition test to assess toxicity was performed at the same concentration of crotonaldehyde together with 2 mg/L of sodium benzoate, the positive control.

Crotonaldehyde showed 55% biodegradation during the 28-day Closed Bottle Test. This is not readily biodegradable under the strict definitions for this test which uses a very low concentration of unacclimated microorganisms (about 10/mL in this instance). No toxicity was seen at the concentration tested in the Inhibition Test. In fact, the biodegradation of crotonaldehyde was considerably enhanced in the presence of the positive control, sodium benzoate. This suggests that higher biodegradation values might be seen during wastewater treatment where other biodegradable substrates and a very high concentration of microorganisms are present.

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HAEL No. 90-0016

Study No. EN-1040-901878-1

Testing Facility

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652-3617
USA

Sponsor

Eastman Chemical Company
Kingsport, TN 37662
USA

Date of Study Initiation

July 16, 1990

Date of Study Completion

September 27, 1990

Dates of Experiment

Chemical Oxidation Pre-test Start Date: 6-21-90 Termination Date: 7-19-90
Test Inoculum Collection Date: 7-26-90
Experiment (Exposure) Start Date: 7-26-90 Termination Date: 8-23-90

Study Participants

Environmental Sciences Section:

O.K. Esmerian, Technician, Eco-Chem Testing Group
J.W. Gorsuch, Group Leader, Eco-Chem Testing Group
M.M. McWilliams, Technician, Eco-Chem Testing Group
J.R. Owen, Unit Director, Environmental Sciences Section
H.M. Watson, Principal Investigator and Study Director, Eco-Chem Testing Group

Chemicals Quality Services Division (CQSD) Participants:

R.A. Ambrose
E.M. Isaacs
B.W. Remington
K.A. Robillard

Test Guideline

Federal Register Guideline 40 CFR 796.3200 and the November 9, 1989 Consent Agreement with the EPA.

Purpose/Objective

The purpose of this test is to determine the ready biodegradability of the test substance by measuring the loss of oxygen from the test solution over the 28-day test period. The degradation is defined as the ratio of the biochemical oxygen demand (BOD) to either the theoretical oxygen demand (ThOD) or the chemical oxygen demand (COD) and is expressed as a percentage.

Test Substance

Identity: Crotonaldehyde

Lot No.: 90-7

Sample Laboratory No.: 90-0016

Composition (determined by CQSD, Attachment 3):

Purity: Initial, 99.5%; final, 100% by GC

Stability (determined by CQSD, Attachment 3):

Shelf-life stability was confirmed by repeating the purity assay after the completion of all testing.

Control Substance

Identity: Sodium benzoate; CAS Reg. No.: 532-32-1

Materials and Methods

A. Test System

Microoganisms were obtained from the secondary effluent of a domestic wastewater treatment plant (Van Lare Treatment Plant, Rochester, New York). Viability of the microorganisms was confirmed after inoculation of the test solutions and activity was checked by means of a positive control (reference) substance. In studies of this sort no attempt is made to identify the large variety of organisms present in the sample or the degree of absorption of the test and control substance.

B. Preparation of the Inoculum

A secondary effluent sample was collected, the sample was filtered through a coarse filter, and the first 200 mL were discarded. The remaining filtrate was aerated until used to inoculate the Closed Bottle Test.

C. Preparation of Test Solutions

High quality distilled water was used for the preparation of test solutions. Approximately 35 L were kept at room temperature (as close as possible to 20°C) and aerated strongly for four hours with carbon dioxide-free air obtained by passing air through a sodium hydroxide (8.0 g/L) trap. The water was used after standing for 20 hours to remove air bubbles. Dissolved oxygen (DO) was determined for control purposes. All transfer and filling operations using the air-saturated water were siphoned bubble-free and all test and control solutions were prepared from this water.

Two stock solutions were prepared in distilled water and added in the required amounts into the appropriate test bottles (see Test Procedure). Basal Salts Medium was prepared according to Table 1. A positive control stock solution of sodium benzoate (1.2 g/L) was used to check the activity of the inoculum. An aliquot of this stock solution was added to a large test bottle to provide the required final concentration of 2 mg/L in the Positive Control Bottle. The test chemical stock solution was prepared at a concentration of 1.27 g/L in distilled water. An aliquot of this stock solution was added to one of the large test bottles to provide a test concentration of 2.1 mg/L. The test chemical appeared to be a biodegradable substance (according to the results of the Chemical Oxidation Pre-Test, Appendix 3) and as it has a relatively high ThOD, 2.28 mg O_2/mg of test chemical, it was not tested at a second, higher concentration. An inhibition test was run, however, in order to evaluate potential toxicity towards the microorganisms. For this test, aliquots of both test and positive control stock solutions were added to a large bottle to achieve final concentrations of 2.1 mg/L of test chemical and 2 mg/L of positive control.

D. Test Procedure

All necessary manipulations before incubation were performed at about 20°C and care was taken to avoid introducing air bubbles during the preparation, inoculation, and siphoning of the solutions.

Five large bottles were used: one for uninoculated BSM (Oxygen Blank), one for inoculated BSM (Inoculation Blank), one for inoculated Positive Control Solution, one for inoculated Test Solution, and one for inoculated Inhibition Test Solution. First, the large bottles were filled by siphoning aerated, distilled water to about one-third of the final volume. Next, aliquots of the stock solutions were added in such amounts that the final required concentrations were attained. Then, the individual BSM stock solutions (Table 1) were pipetted into these bottles according to the final volume required. Finally, the solutions were made up to volume with distilled water using the siphon (which reached down to the bottom of the bottle). This ensured sufficient mixing. For details, refer to p 20-21.

The Inoculum Blank, Test, Positive Control, and Inhibition Test Solutions were inoculated with a small number of microorganisms (one drop from a pipet/L of final volume). These solutions and the BSM Oxygen Blank were siphoned immediately from the lower quarter (not from the bottom) of each large bottle to fill their respective group of BOD bottles. Enough BOD bottles were prepared for duplicate DO analyses on days 0, 5, 15, and 28. After the insertion of ground glass stoppers into the BOD bottles, a small amount of distilled water was poured around each stopper to form a seal. Finally, a plastic cap was placed over the top of the bottles which were then incubated at a temperature of approximately 20^{0}C in the dark.

E. Analytical Measurements

Dissolved Oxygen Analysis:

The course of degradation was followed by performing DO analyses on days 0, 5, 15, and 28 of the 28-day test. The BOD of test and control substances was determined in duplicate bottles for each analysis day (see Calculations).

F. Data Evaluation and Interpretation

Lotus 1-2-3® was used for data calculations and tabulations and also to generate the graph showing percentage biodegradation. No statistical evaluation of the data was performed.

Owing to the stringency of this test, a low result does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work would be necessary to establish this. Test articles showing a high oxygen uptake in this test should be regarded as easily biodegradable provided that this level is reached within the 10-day time window.

G. Calculations

The degradation is defined as the ratio of BOD to either the ThOD or the COD and expressed in percent. The two ways of calculation (percentage of the ThOD or percentage of the COD) may not give the same results.

The BOD is calculated as the difference of the oxygen depletion between the blank and a solution of test material under the conditions of the test. After division by the concentration (w/v) of the substance, the net oxygen depletion is obtained in milligrams of BOD/mg of substance.

$$\% \text{ biodegradation (of ThOD)} = \frac{\text{mg } O_2/\text{mg tested substance}}{\text{ThOD}} \times 100$$

$$= \frac{\text{BOD}}{\text{ThOD}} \times 100$$

where: ThOD = theoretical oxygen demand

Quality Assurance Monitoring

See the attached reports of Quality Assurance Monitoring (Attachment 4).

Good Laboratory Practice Statement

This study was conducted according to: Good Laboratory Practice Regulations for Nonclinical Laboratory Studies as promulgated by the Food and Drug Administration; 21 CFR Part 58, December 22, 1978 (revised September 4, 1987); Environmental Protection Agency Good Laboratory Practice

Standard 40 CFR Part 792, November 29, 1983 (revised August 17, 1989); and Organization of Economic Cooperation and Development Principles of Good Laboratory Practice specified in Annex 2 of the OECD Guidelines for Testing of Chemicals [C(81) 30(Final)] as required by Council Directive 89/569/EEC (October 28, 1989).

Data Storage and Records Retention

All raw data and summaries of data will be retained for at least ten years by the Health and Environment Laboratories, Eastman Kodak Company, Rochester, New York 14652-3617.

Results

The graph of percent biodegradation is shown in Figure 1. Values for the DO measurements, incubation temperature, and DO calibration are shown in Table 2. The Lotus 1-2-3® spreadsheet showing DO calculations is shown in Attachment 1 and all test data worksheets are shown in Attachment 2.

Percent biodegradation values were 32% by day 5, 45% by day 15, and 55% by day 28. Oxygen depletion in mg BOD/L was 6.2 for the inhibition test compared to individual values of 2.5 for the positive control and 2.6 for the test chemical (combined value, 5.1).

Microorganism numbers in the secondary effluent were estimated by Easy Cult™ dip slides and were about 10^5/mL. After dilution into the bottles this gave about 10 organisms/mL.

Test Validity

The positive control substance yielded $\geq$ 60% biodegradation within 28 days. The oxygen depletion in the Oxygen Blank did not exceed 0.3 mg O_2/L after five days and 0.4 mg O_2/L after 28 days; the Inoculum Blank did not exceed 0.5 mg O_2/L after five days, and 0.6 mg O_2/L after 15 and 28 days. These conditions fulfill the requirements for a valid test.

Protocol Deviations

None.

Discussion

The Closed Bottle Test is a useful test for volatiles and in addition, the test is useful where chemical toxicity prevents the choice of other biodegradation tests that require a higher concentration of the test chemical. It was therefore the test of choice for this chemical.

Crotonaldehyde showed 55% biodegradation by the end of the test. To pass the strict definition for ready biodegradability, 60% biodegradation would have to occur within a ten-day time window starting from the day on which a 10% value was first achieved (see Figure 1). The inhibition test showed that toxicity was not a factor. In fact, there appeared to be enhanced biodegradation in this test in which the test chemical and the positive control were both added to the same solution. This suggests that the presence of other biodegradable substrates may enhance the biodegradation of crotonaldehyde. If so, crotonaldehyde might well biodegrade during wastewater treatment.

Conclusion

Crotonaldehyde showed 55% biodegradation during the 28-day Closed Bottle Test. This is not readily biodegradable under the strict definitions for this test which uses a very low concentration of unacclimated microorganisms (about 10/mL in this instance). No toxicity was seen at the concentration tested in the Inhibition Test. In fact, the biodegradation of crotonaldehyde was considerably enhanced in the presence of the positive control, sodium benzoate. This suggests that higher biodegradation values might be seen during wastewater treatment where other biodegradable substrates and a very high concentration of microorganisms are present.

References

1. Degradation - Biotic Degradation: Closed-Bottle Test, 1984. Part C. Methods for the Determination of Ecotoxicity. Test Guideline C.6. Official Journal of the European Communities, 27, 188-197.
2. OECD, Paris, 1981. Test Guideline 301D, Decision of the Council C(81) 30 final.
3. Gerike, P., Fischer, W.K., 1979. A Correlation Study of Biodegradability Determinations with Various Chemicals in Various Tests. Ecotoxicology and Environmental Safety, 3, (2), 159-173.
4. Gerike, P., Fischer, W.K., 1981. A Correlation study of Biodegradability Determinations with Various Chemicals in Various Tests. II. Ecotoxicology and Environmental Safety, 5, (1), 45-55.
5. Ready Biodegradability: Closed Bottle Test, Guideline 40 CFR 796.3200,

Table 1. Composition of BSM and Stock Solutions

Stock Solutions:

I. Phosphate Solution

KH_2PO_4 (potassium dihydrogen phosphate):	8.50 g
K_2HPO_4 (dipotassium hydrogen phosphate):	21.75 g
$Na_2HPO_4 \cdot 2H_2O$ (disodium monohydrogen phosphate dihydrate):	33.30 g
NH_4Cl (ammonium chloride):	1.70 g

Dissolve in and make up to 1000 mL with distilled water.

The pH value should be 7.2.

II. Magnesium Sulfate Solution

$MgSO_4 \cdot 7H_2O$ (magnesium sulphate heptahydrate):	22.50 g

Dissolve in and make up to 1000 mL with distilled water.

III. Calcium Chloride Solution

$CaCl_2$ (calcium chloride):	27.50 g

Dissolve in and make up to 1000 mL with distilled water.

IV. Ferric Chloride Solution

$FeCl_3 \cdot 6H_2O$ (iron(III) chloride hexahydrate):	25.0 mg

Dissolve in and make up to 100 mL with distilled water.

Stock Solution Preparation

Stock solutions I-III were made up in advance, filter sterilized through 0.45 µm membranes, and refrigerated until needed (for a period of 6 months). Stock solution IV was made up immediately before use.

Test Medium

BSM was prepared immediately before use and contained 1.0 mL of each stock solution I-IV per liter of final solution.

Table 2. Dissolved Oxygen Measurements (mg/L)

Medium	Day 0		Day 5		Day 15		Day 28	
BSM	8.50	8.65	8.50	8.46	8.58	8.64	8.73	8.73
B+I	8.74	8.68	8.44	8.59	8.60	8.48	9.00	9.04
Pos.	8.67	8.67	6.88	6.85	6.55	6.47	6.79	6.32
Test	8.90	8.95	6.98	7.00	6.28	6.53	6.39	6.42
Inhib.	8.90	8.99	4.85	4.82	4.37	3.01	2.78	2.79

Where:

BSM = BSM (Oxygen Blank)

B+I = BSM + Inoculum (Inoculum Blank)

POS = Positive Control + BSM + Inoculum

Test = Test + BSM + Inoculum

Inhib. = Inhibition Test = Test + Positive Control + BSM + Inoculum

Incubation Temperature (^{o}C)

Day 0	Day 5	Day 15	Day 28
20	20	20	20.5

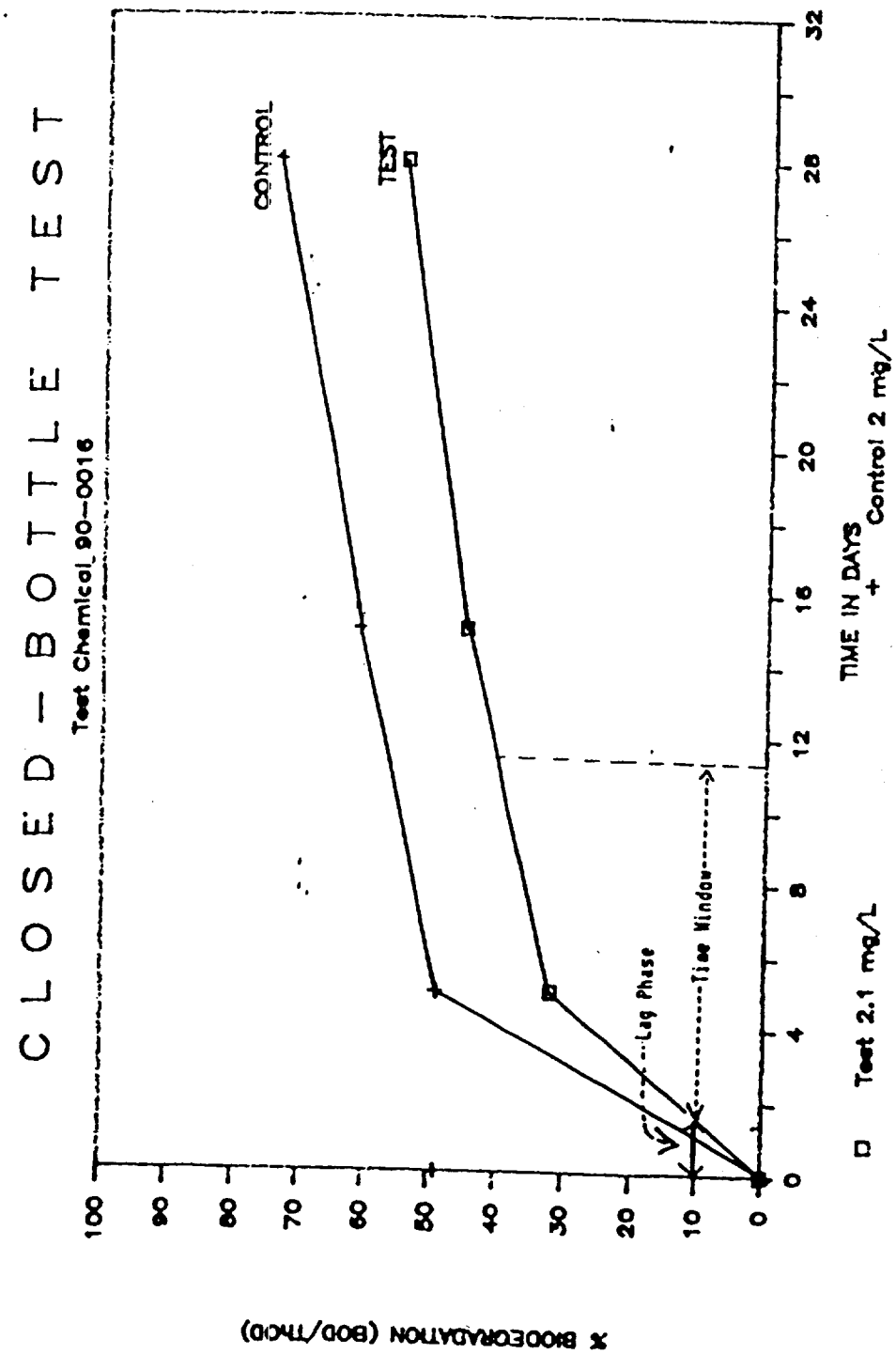
Calibration Method for DO Meter

Winkler Calibration

DO of distilled water used to set the test: 8.68 mg/L

HAEL No. 90-0016 Study No. EN-1040-901878-1

Figure 1. Graph Showing Percent Biodegradation



HAEL No. 90-0016 Study No. EN-1040-901878-1

Attachment 1. Lotus 1-2-3® Spreadsheet for Dissolved Oxygen Calculations

DISSOLVED OXYGEN WORKSHEET(A)

MEDIUM	DATE & DAY	VALUE 1	VALUE 2	MEAN(B)	LOSS(C) mg BOD/L(D)	mg BOD/mg(E)	% DEG.(F)
BSM	DAY 0	8.50	8.65	8.58			
B+I		8.74	8.68	8.71			
POS	7-26-90	8.67	8.67	8.67			
TEST #1		8.90	8.95	8.93			
INHIB. #1		8.90	8.99	8.95			
BSM	DAY 5	8.50	8.46	8.48	0.00		
B+I	7-31-90	8.44	8.59	8.52	0.06		
POS		6.88	6.85	6.87	1.71	1.7	0.83	49%
TEST #1		6.98	7.00	6.99	1.59	1.5	0.73	32%
INHIB. #1		4.85	4.82	4.84	3.74	3.7		
BSM	DAY 15	8.58	8.64	8.61	-0.04		
B+I	8-10-90	8.60	8.48	8.54	0.04		
POS		6.55	6.47	6.51	2.07	2.0	1.02	61%
TEST #1		6.28	6.53	6.41	2.17	2.1	1.02	45%
INHIB. #1		4.37	3.01	3.69	4.89	4.9		
BSM	DAY 28	8.73	8.73	8.73	-0.15		
B+I	8-23-90	9.00	9.04	9.02	-0.44		
POS		6.79	6.32	6.56	2.02	2.5	1.23	74%
TEST #1		6.39	6.42	6.41	2.17	2.6	1.25	55%
INHIB. #1		2.78	2.79	2.79	5.79	6.2		

(A) Values 1 and 2 are DO measurements in mg/L (see Data Worksheet 5)
(B) MEAN(B) = Mean of DO data from Dissolved Oxygen Meter
(C) LOSS(C) = DO depletion compared to BSM Time 0, for eg (BSM(B)-TEST(B) mg/L)
(D) mg BOD/L(D) = oxygen depletions as mg BOD/L after x days, for eg. (TEST(C) - B+I(C))
(E) mg BOD/mg(E) = mg BOD/L(D)/(mg chemical/L)
(F) % DEG. = BOD(E)/ThOD x 100

ThOD (Test Chemical) = 2.280 Conc. = 2.1 mg/L
ThOD (Positive Control) = 1.670 Conc. = 2 mg/L

Inhibition Test:
BOD/L of POS + TEST at 5, 15, and 28 days = 3.2, 4.1, and 5.1, respectively
BOD/L of INHIBITION at 5, 15, and 28 days = 3.7, 4.9, and 6.2, respectively

Conclusion: no inhibition is present because inhibition BOD values are greater than POS + TEST values

Prepared by Heather Watson Date 8-24-90

Attachment 2. Test Data Worksheets

-1-

CLOSED-BOTTLE TEST-ANNEX V

READY BIODEGRADABILITY TEST (BIOTIC DEGRADATION)

WORKSHEET SUMMARY

Chemical Name Crotonaldehyde

Accession No. 901878 HAEL No. 90-0016

Sample Ref. No. Lot No. 90-7 CAS Reg. No. 4170-30-3

Study Initiation Date 7-16-90 Date of Inoculum Collection 7-26-90

Experiment Start (Exposure) Date 7-26-90 Termination Date 8-23-90

Concentrations Tested 2.1 mg/L Inhibition Test Yes (Yes/No)*

SUMMARY: A 28 -day test for Ready Biodegradability using unacclimated microorganisms as the inoculum showed a 55 % degradation of the test chemical based on ThOD (ThOD or COD) as measured by loss of dissolved oxygen from solution, where % degradation = % BOD/ThOD (or % BOD/COD).

SOURCE OF MICROORGANISMS Secondary effluent from domestic wastewater treatment (Van Lare Treatment Plant, Rochester, NY)

TEST VALIDITY: The positive control, sodium benzoate, showed 74 % degradation. The requirements for a valid test were fulfilled Yes (Yes/No).

CONCLUSION: The test chemical is not readily biodegradable according to the strict definition of this test but considerable biodegradation was observed.

REMARKS:

*No inhibition was seen at 5, 15 or 28 days & so this suggests that crotonaldehyde is not toxic towards sludge microorganisms at 2.1 mg/L

Prepared by Heather M. Watson Date 9-12-90

-2-

Acc. No. 901878
HAEL No. 90-0016
Test Type Closed-Bottle

TEST CHEMICAL AND POSITIVE CONTROL WORKSHEET METHODS

I. Test Chemical Name Crotonaldehyde

Sample Ref. No. Lot No 90-7

Molecular Weight 70.09 Molecular Formula C_4H_6O

Appearance: Solid ___ Liquid ✓ Aqueous Solution ___ Color Colorless

1. The ThOD of the substance $C_c H_h Cl_{cl} N_n Na_{na} O_o P_p S_s$ of the molecular weight MW is calculated according to Protocol EN-104, Attachment 2, "Calculation of ThOD" where:

$$ThOD_{NH3} = \frac{16[2c + 1/2\,(h-cl-3n) + 3s + 5/2p + 1/2na - o]}{MW}$$

$= 2.28$ mg O_2/mg of test chemical

2. Amount(s) of Test Chemical:* 2.0 mg/L

Amount(s) of O_2 consumed during degradation 4.56 mg/L

II. Positive Control Chemical Name Sodium Benzoate

Molecular Weight 144.1 Molecular Formula $C_7H_5O_2Na$

ThOD 1.67 mg O_2/mg of Control Substance

Amount of Positive Control Chemical: 2 mg/L

Amount of O_2 consumed during degradation 3.34 mg/L

REMARKS:

*. Aim amount

Prepared by Heather Watson Date 7-16-90

-3-

Acc. No. 901878
HAEL No. 90-0016
Test Type Closed-Bottle

INOCULUM PREPARATION WORKSHEET

Inoculum Preparation:

Secondary Effluent*: Source Van dare Waste Treatment Plant

Date obtained 7-26-90

Filtered using coarse filter paper - discarded first 200 mL

Filtrate aerated until used

Laboratory Activated Sludge: Source ___/___

Date obtained _____

Filtered using coarse filter paper - discarded first 200 mL

Filtrate aerated until used

Soil: Source ___/___

Date obtained _____

Suspended 100 g/L in distilled water, settled _____ min.

Filtered supernatant using coarse filter paper- discarded first 200 mL

Filtrate aerated until used

Composite: Equal volumes of the three samples prepared above ___/___

Other ___—___

Date of inoculum use 7-26-90

NA = Not Applicable

REMARKS:

*10^5 orgs/ml were present (Easy cult™ dip slide)

Prepared by Heather M Weston Date 7-26-90

-4-

Acc. No. 901878
HAEL No. 90-0016
Test Type Closed-Bottle

PREPARATION OF TEST SOLUTIONS

I. Water:
Approx. Volume used 34-36 L Aeration time 4 hours
Standing time 20 hours Temperature 20.5 °C DO 8.68 mg/L

II. Test Chemical Solution(s):
Final concentration(s) required: 2.0 mg/L mg/L
1. Stock Solution Preparation:
Amount used (aim conc. 1200 mg/L) 127.1 mg/ 100 mL (Aim 120mg)
Balance used: AE 163 #269160 Prepared by: MM
2. Final Solution Preparation:
Amount used 10.0 mL Stock/ 6.0 L of final solution in bottle
to give final conc. #1 of 2.0 mg/L
Amount used NA mL Stock/ NA L of final solution in bottle
to give final conc. #2 of NA mg/L (if needed)

III. Positive Control Solution:
Final concentration required: 2.0 mg/L
1. Stock Solution Preparation: 120 Entry error HW 7-26-90
Amount used (aim conc. 1200 mg/L) ~~127.1~~ mg/ 100 mL (Aim 120mg)
Balance used: Mettler H45 #255798 Prepared by: MM
2. Final Solution Preparation: Entry error HW 7-26-90
Amount used 10.0 mL Stock/ 6.0 L of final solution in bottle
to give final conc. of 2.0 mg/L

IV. Procedure:
1. Siphon water (about one-third final volume) into large bottles
2. Add appropriate stock solutions by pipet (amounts above)
3. Add BSM stock solutions (1.0 mL/L of final solution)
4. Add 1 drop inoculum/L of final solution
5. Bring to volume using siphon to mix
6. Siphon into the appropriate bottles, stopper, seal with DW, and cap
No. of bottles per test series: 8 extras 4
No. of bottles per test conc. for specific analysis: 4 (Time 0 only)

REMARKS: NA - Not applicable

Prepared by Michele McWilliams Date 7-26-90
Heather Walton 7-26-90

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Acc. No. 901878
HAEL No. 90-0016
Test Type Closed-Bottle

INHIBITION TEST (if required)

Substances may easily and simply be tested for inhibitory effects using the Closed-Bottle Test results and one extra solution for each concentration to be tested:

- Series 1: Positive Control Solution (x mg/L) from Closed-Bottle Test
- Series 2: Test Chemical Solution(s) (y mg/L) from Closed-Bottle Test
- Series 3: Solution containing x mg/L of Positive Control and y mg/L of Test Chemical

The test chemical can be considered to be inhibitory to bacteria at the concentration tested if:

$$BOD_{series\ 3} < BOD_{series\ 1} + BOD_{series\ 2}$$

Procedure for Series 1 and 2: see Section IV, p4

Procedure for Series 3:

Add Test Chemical stock Solution:

I. Concentration 1:

Amount used 10.0 mL Stock/ 6.0 L of final solution in bottle to give final conc. of 2.0 mg/L

II. Concentration 2 (if required):

Amount used NA mL Stock/ NA L of final solution in bottle to give final conc. of NA mg/L

NA = Not applicable

Add Positive Control stock Solution:

Amount used 10.0 mL Stock/ 6.0 L of Final Solution in Bottle (s) to give final conc. of 2.0 mg/L

Prepared by [signature] Date 7-26-90

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Acc. No. 901878
HAEL No. 90-0016
Test Type Closed-Bottle

DO MEASUREMENTS (mg/L)

Medium	Day 0/7-26-90 (AWD) (mm)		Day 5/7-31-90 (AWD) (mm)		Day 15/8-10-90 (mm)		Day 28/8-23-90 (AWD) (mm)	
BSM	8.50	8.65	8.50	8.46	8.58	8.64	8.73	8.73
B+I	8.74	8.68	8.44	8.59	8.60	8.48	9.00	9.04
Pos.	8.67	8.67	6.88	6.85	6.55	6.47	6.79	6.32
Test #1	8.90	8.95	6.98	7.00	6.28	6.53	6.39	6.42
Test #2	NA	NA	NA	NA	NA	NA	NA	NA
Inhib. #1	8.90	8.99	4.85	4.82	4.37	3.01	2.78	2.79
Inhib. #2	NA	NA	NA	NA	NA	NA	NA	NA

Where:

Flask #

1-12 BSM = BSM (Oxygen Blank)

13-24 B+I = BSM + Inoculum (Inoculum Blank)

25-36 POS = Positive Control (Reference substance) + BSM + Inoculum

37-48 Test #1 = Test Conc. #1 + BSM + Inoculum

NA Test #2 = Test Conc. #2 + BSM + Inoculum

49-60 Inhib. #1 = Inhibition Test Conc. #1 + Positive Control + BSM + Inoculum

NA Inhib. #2 = Inhibition Test Conc. #2 + Positive Control + BSM + Inoculum

Labelled Other Extra bottles to measure DO in water & for Time 0 specific analysis to confirm dose concentration

Incubation Temperature (°C)	Day 0	Day 5	Day 15	Day 28
	20°C	20°C	20°C	20.5°C

NA = Not applicable

REMARKS: DO of water to set test = 8.68

Prepared by Heather Walton
Michele McWilliams

Date 8-23-90
8-23-90

-7-

Acc. No. 901878
HAEL No. 90-0016
Test Type: Closed-Bottle

OECD ANNEX V READY BIODEGRADABILITY TEST
DISSOLVED OXYGEN WORKSHEET(A)

MEDIUM	DATE & DAY	VALUE 1	VALUE 2	MEAN(B)	LOSS(C) mg BOD/L(D)	mg BOD/mg(E)	% DEG.(F)
BSM	DAY 0	8.50	8.65	8.58			
B+I		8.74	8.68	8.71			
POS	7-26-90	8.67	8.67	8.67			
TEST #1		8.90	8.95	8.93			
INHIB. #1		8.90	8.99	8.95			
BSM	DAY 5	8.50	8.46	8.48	0.00		
B+I	7-31-90	8.44	8.59	8.52	0.06		
POS		6.88	6.85	6.87	1.71	1.7	0.83	49%
TEST #1		6.98	7.00	6.99	1.59	1.5	0.73	32%
INHIB. #1		4.85	4.82	4.84	3.74	3.7		
BSM	DAY 15	8.58	8.64	8.61	-0.04		
B+I	8-10-90	8.60	8.48	8.54	0.04		
POS		6.55	6.47	6.51	2.07	2.0	1.02	61%
TEST #1		6.28	6.53	6.41	2.17	2.1	1.02	45%
INHIB. #1		4.37	3.01	3.69	4.89	4.9		
BSM	DAY 28	8.73	8.73	8.73	-0.15		
B+I	8-23-90	9.00	9.04	9.02	-0.44		
POS		6.79	6.32	6.56	2.02	2.5	1.23	74%
TEST #1		6.39	6.42	6.41	2.17	2.6	1.25	55%
INHIB. #1		2.78	2.79	2.79	5.79	6.2		

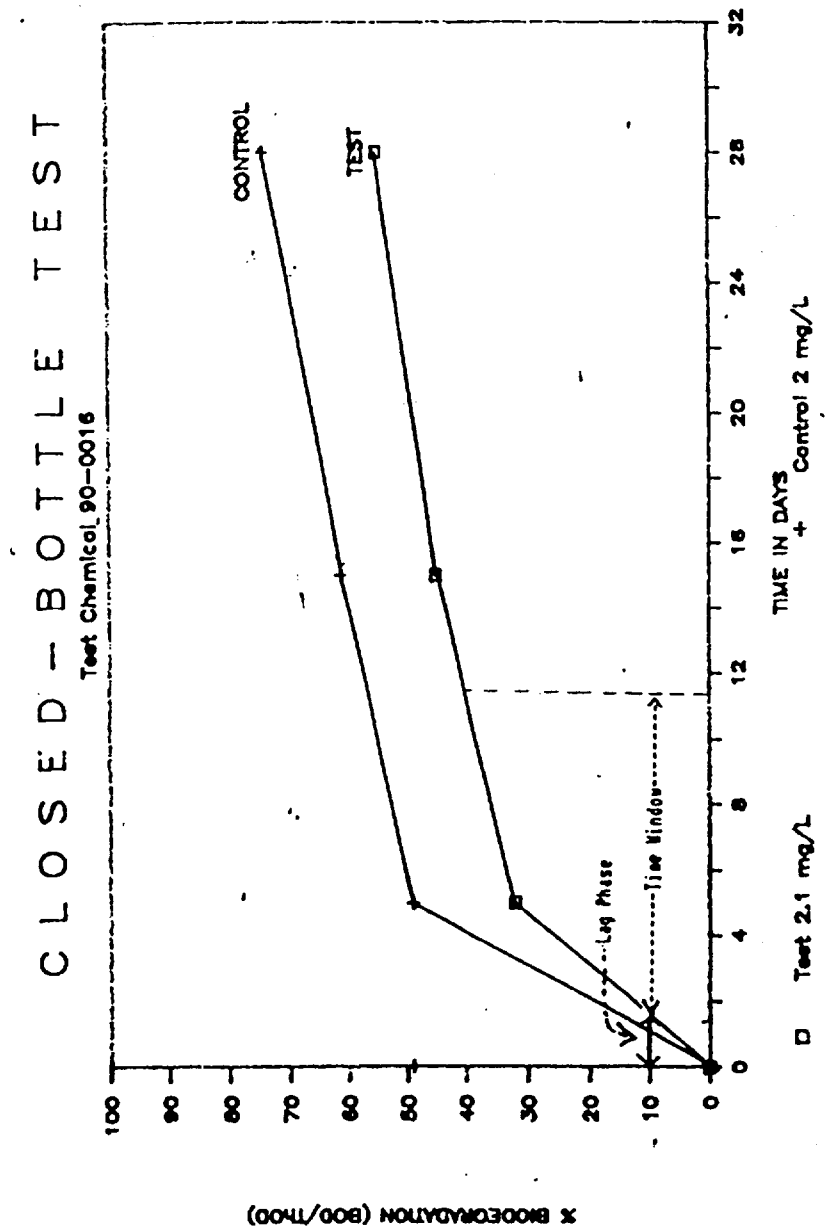
(A) Values 1 and 2 are DO measurements in mg/L (see Data Worksheet 5)
(B) MEAN(B) = Mean of DO data from Dissolved Oxygen Meter
(C) LOSS(C) = DO depletion compared to BSM Time 0, for eg (BSM(B)-TEST(B) mg/L)
(D) mg BOD/L(D) = oxygen depletions as mg BOD/L after x days, for eg. (TEST(C) - B+I(C))
(E) mg BOD/mg(E) = mg BOD/L(D)/(mg chemical/L)
(F) % DEG. = BOD(E)/ThOD x 100

ThOD (Test Chemical) = 2.280 Conc. = 2.1 mg/L
ThOD (Positive Control) = 1.670 Conc. = 2 mg/L

Inhibition Test:
BOD/L of POS + TEST at 5, 15, and 28 days = 3.2, 4.1, and 5.1, respectively
BOD/L of INHIBITION at 5, 15, and 28 days = 3.7, 4.9, and 6.2, respectively

Conclusion: no inhibition is present because inhibition BOD values are greater than POS + TEST values

Prepared by Heather Watson Date 8-24-90


CLOSED - BOTTLE TEST
Test Chemical 90-0016
CONTROL
TEST
Lag Phase
Time Window
TIME IN DAYS
% BIODEGRADATION (BOD/ThOD)
0
10
20
30
40
50
60
70
80
90
100
0
4
8
12
16
20
24
28
32
Test 2.1 mg/L
Control 2 mg/L

Attachment 3. CQS Division Reports on Chemical Purity

CHEMICALS QUALITY SERVICES REPORT FOR: B306

STUDY DIRECTOR: Robert T. Ambrose

REQUESTED BY: Rob O'Boyle

CQS JOB # : 3H6V501

PHASE: Purity Determination

REQUEST # : 142470
DATE RECEIVED: 05/14/90
DATE ANALYZED: 05/18/90
DATE REPORTED: 05/18/90
AUTHOR: Beth Isaacs

HAEL#: 90-0016
ACC.#: 901878
SAMPLE REF.#: Lot 90-7

FOR ADDITIONAL INFORMATION OR FURTHER WORK CONTACT: Robert Ambrose X25925
Beth Isaacs X85035

RESULTS: The purity of the test chemical was determined by a combination of a water analysis by Karl Fisher titration and a gas chromatographic analysis with flame ionization detection. The results are as follows:

Water (%)	Area % (GC/FID)	
6.3	Run 1	99.7
	Run 2	99.1
	Run 3	99.7
	Mean	99.5
	Std Dev	0.3464

Scientist Beth Isaacs (signature) Date 6/7/90

Reviewed by Robert T Ambrose (signature) Date 6/7/90

Instrumental Conditions for GC Analysis

Instrument:	Hewlett Packard 5890
Column:	DB-WAX; 30m; wide bore (0.25 um film thickness)
Column Pressure:	7 psi
Carrier Gas:	Helium
Initial Temp:	50 °C
Initial Time:	4 min.
Rate:	10 °C/min.
Final Temp:	250 °C
Final Time:	7 min.
Injection Port:	250 °C
Injection Type:	Split
Injection Vol:	1 uL
Detector:	FID
Detector Temp:	250 °C
Solvent:	n-Propanol
Split Flow:	120 cc/min.

CHEMICALS QUALITY SERVICES REPORT FOR: B306

STUDY DIRECTOR: H.M. Watson

REQUESTED BY: Heather Watson

CQS JOB # : 3PC1101

PHASE: Purity Determination

STUDY TYPE: Environmental Testing

REQUEST # : 142441
DATE RECEIVED: 08/21/90
DATE ANALYZED: 09/05/90
DATE REPORTED: 09/06/90
AUTHOR: Beth Isaacs

HAEL#: 90-0016
ACC.#: 901878
SAMPLE REF.#: Lot 90-7

FOR ADDITIONAL INFORMATION OR FURTHER WORK CONTACT: Robert Ambrose X25925
Beth Isaacs X85035

RESULTS: The purity of the test chemical was determined by a combination of a water analysis by Karl Fisher titration and a gas chromatographic analysis with flame ionization detection. The results are as follows:

Water (%)	Area % (GC/FID)	
6.3	Run 1	100
	Run 2	100
	Run 3	100
	Mean	100
	Std Dev	0.0000

Scientist Beth Isaacs (signature) Date 9-6-90

Reviewed by Robert Ambrose (signature) Date 9/10/90

Instrumental Conditions for GC Analysis

Instrument:	Hewlett Packard 5890
Column:	DB-WAX; 30m; wide bore (0.15 um film thickness)
Column Pressure:	7 psi
Carrier Gas:	Helium
Initial Temp:	50 °C
Initial Time:	4 min.
Rate:	10 °C/min.
Final Temp:	250 °C
Final Time:	7 min.
Injection Port:	250 °C
Injection Type:	Split
Injection Vol:	1 uL
Detector:	FID
Detector Temp:	250 °C
Solvent:	n-Propanol
Split Flow:	120 cc/min.

Attachment 4. Quality Assurance Statements

Q.A. INSPECTION STATEMENT
(CFR 58.35(B)(7) 792.35(B)(7) 160.35(B)(7))

STUDY: 90-0016-1 STUDY DIRECTOR: WATSON,H.M.
ACCESSION NUMBER: 901878

PAGE 1
09/24/90

STUDY TYPE: BIODEGRADATION (READY-CLOSED BOTTLE)

M. Sue Jones
(AUDITOR, QUALITY ASSURANCE UNIT)

9/25/90
DATE

TO THE BEST OF MY KNOWLEDGE, THIS FINAL REPORT ACCURATELY DESCRIBES THE METHODS AND STANDARD OPERATING PROCEDURES, AND THE REPORTED RESULTS ACCURATELY REFLECT THE RAW DATA. THIS STUDY WAS INSPECTED BY 1 OR MORE PERSONS OF THE QUALITY ASSURANCE UNIT OF THE HAEL, EASTMAN KODAK COMPANY, ROCHESTER, N.Y. AND WRITTEN STATUS REPORTS WERE SUBMITTED ON THE FOLLOWING DATES:

INSPECTION DATES	PHASE(S) INSPECTED	STATUS REPORT DATES
07/26/90	PROTOCOL SUBMISSION SOLUTION PREPARATION CONTROL ARTICLE WEIGHT TEST ARTICLE-CARRIER-MIXTURE PREPARATION BSM PREPARATION	09/24/90
08/23/90	SAMPLE ANALYSIS DAY 28 DO MEASUREMENTS	09/24/90
09/24/90	FINAL REPORT REVIEW	09/24/90

ANALYTICAL QUALITY ASSURANCE INSPECTION STATEMENT
(CFR 58.35(B)(7) 792.35(B)(7) 160.35(B)(7))

STUDY: 90-0016-Z STUDY DIRECTOR: WATSON, H.
ANALYTICAL DIRECTOR: AMBROSE, R.
ACCESSION NUMBER: 901878
CQS JOB NUMBER: 3HGV501

STUDY TYPE: ENVIRONMENTAL TESTING

(AUDITOR, QUALITY ASSURANCE UNIT) 9-18-90 DATE

THIS STUDY WAS INSPECTED BY 1 OR MORE PERSONS OF THE QUALITY ASSURANCE UNIT OF MQAO, EASTMAN KODAK COMPANY, ROCHESTER, N.Y. AND WRITTEN STATUS REPORTS WERE SUBMITTED ON THE FOLLOWING DATES:

INSPECT DATES	REQUEST NUMBER	PHASE(S) INSPECTED	STATUS REPORT DATES
06/07/90	142470	PURITY TEST REPORT INSPECTION	05/30/90
09/12/90	142441	PURITY TEST REPORT INSPECTION	09/12/90

Appendix 1. Chemical Distribution Record

HAEL Chemical Distribution Record

HAEL Lab No.	90-0016	~~901876~~	Lot No. 90-7
Chemical Name	Crotonaldehyde (901878 LRH 2/8/90) (over)		
Source of Test Substance	~~Texas Eastman Co.~~ ① Tennessee Eastman Co.	Sponsor	TEC/Gerd. Wade Francisco (x-315) JWG 4/1/90
Container/Description	Large glass amber bottle		
Date Test Substance Received	2-5-90	Date Test Substance Processed	2-8-90 By: HRH Expiration Date: 2-8-95
Date/Type Substance Received		Date Sent to MM	2/8/90 JWG
Physical State	Liquid Solid Other	Appearance	Clear
Impurities with %'s	N/A		
Storage Conditions	~~Room Temperature~~ Refrigerate, and cover with nitrogen or Argon after each use ①		
Gross Wt.	4348.5g	Net Wt. (Gross Wt. of Test Substance and Container minus Tare Wt. of Container)	3121.2g
Balance Type	Mettler	Model	PE6000	ID No.	A20916

① Entry error, JWG, 2/9/90

Initials	Date	Area	Purpose	Scale Make, Model, KP ID No.	Initial Bottle Cap & Contents (Wt. or Vol.)	Amount Removed Wt. or Vol.	Amount Remaining Bottle/Cap/Contents (Wt. or Vol.)
JW	02/09/90	B-306 ECT-HAEL	Confirmation Log-in Weighing	Mettler PE 4600 (02) ID # 35SCA02 #549461	4348.2g	0.0g	4348.2g
MMW	2-12-90	B-306	Algal Methods	"	4347.9g	6.8g	4341.1g
O'B.	3/13/90	B-306	Sample to (BSD)	" O'B.	4341.2g	29.2g	4312.0g
MM	3/07/90	B-306	Algal Range Finding	"	4312.5g	1.2g	4311.3g
O'B.	3/22/90	B-306	AAE Meth. Des.	" O'B.	4310.3g	27.6g	4282.7g
MM	4-2-90	B-306	Algal Reference Test	"	4283.0g	0.8g (est)	4282.2g
O'B.	4/18/90	B-306	AAE Dose Prep (4/23/90)	" O'B.	4280.8g ✓✓	20.7g (20.00g used)	4259.0g
O'B.	4/24/90	B-306	AAE Renewal (4/25/90)	" O'B	4260.1g ✓✓	20.4g (20.00g used)	4239.7g
O'B.	4/27/90	B-306	On Dose Prep (4/30/90)	" O'B.	4240.0g ✓✓	4.2g (4.00g used)	4235.8g
O'B.	5/1/90	B-306	On Dose Renewal (5/2/90)	" O'B.	4235.5g ✓✓	4.6g (4.00g used)	4230.9g
O'B.	5/14/90	B-306	Purity/Stability (H2O)	" O'B.	4230.9g ✓✓	30.9g (to CDSD)	4200.0g
MM	6-21-90	B-306	Methods Closed Bottle	"	4200.0g	amount 1.1g (est)	4198.9g
O'B.	6/22/90	B-306	On Dose Rep (6/25/90)	" O'B.	4198.0g ✓✓	4.7g (used 4.00g)	4193.3g
O'B.	6/26/90	B-306	On Dose Renewal (6/27/90)	" O'B.	4193.5g ✓✓	4.2g (used 4.00g)	4188.9g
O'B.	6/26/90	B-306	Purity Assay (CDSD)	" O'B.	4188.9g	31.8g	4157.1g
MM	7-26-90	B-306	Closed Bottle Test	" MM	4156.8g	1.6g	4165.2g

Chem Dist Form 2/89 plm

Appendix 2. Protocol

ENVIRONMENTAL SCIENCES SECTION

ENVIRONMENTAL TECHNICAL SERVICES

HEALTH AND ENVIRONMENT LABORATORIES

Protocol No. EN-1040-901878-1

Revision No.

Date: February 6, 1987

PROTOCOL: Determination of Ready Biodegradability (Biotic Degradation) using the Closed-Bottle Test

Test Article: Crotonaldehyde

Accession No. 901878

HAEL No. 90-0016

Approved by: Heather Watson
Study Director

7-16-90
Date

Approved by: Roderick D. Gerke
Sponsor

7/18/90
Date

4225e

Health and Environment Laboratories Management and Quality Assurance Unit Approvals

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Quality Assurance Unit
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Approved: John R Owen — 7/21/90

John R. Owen
Unit Director
Environmental Sciences Section
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Date

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I. Test Administration

A. Testing Facility

Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652-3617
U.S.A.

B. Sponsor

Eastman Chemicals Division
Kingsport, TN 37662
U.S.A.

C. Dates of Experiment

Study Initiation Date: 7-16-90
Start Date: 7-26-90
Chemical Exposure Completion Date: 8-23-90
Estimated Study Completion Date: 11-1-90
Inhibition Test: Yes (Yes/No)

II. Test and Control Substance

A. Test Substance

Name: Crotonaldehyde
HAEL No.: 90-0016 Accession No.: 901878
Sample Reference No.: Lot No. 90-7 CAS Reg. No.: 4170-30-3
Other Information (Composition, Stability, Purity, etc.): Volatile

Concentration(s) Tested: 2.0 mg/L
ThOD: 2.28 mg O_2/mg of Test Substance

B. Control Substance

Name: Sodium benzoate
CAS Reg. No.: 532-32-1
Concentration used: 2.0 mg/L ThOD: 1.67 mg O_2/mg

III. Source of Inoculum

Secondary Effluent: Van Lare Wastewater Treatment Plant, Rochester, NY

Soil: No

Laboratory Activated Sludge: No

Other: No

IV. Reporting and Records

A. Reporting

The report must contain:

1. Name and address of the testing facility.
2. Dates of the study.
3. Procedures stated in the protocol, including any changes or amendments to the original protocol.
4. Methods used in analyzing the data.
5. Identification of test and control substances.
6. Description of the test methods.
7. Description of the test system.
8. Description of the test substance concentration.
9. Description of any circumstances that may have affected the quality or integrity of the study.
10. Name of the study director and of all other persons involved in the study, including supervision.
11. Summary and analysis of results.
12. Graph of percentage degradation showing the lag phase, degradation phase, slope and time window ('time window' means a 10-day period, starting from the day that the observed level of biodegradation first exceeds 10%).
13. Method for COD Determinations (if made).
14. Proof of validity of the test.
15. Stability of the test substance.
16. Signed and dated reports of the scientists involved in the study.
17. Location of all specimens, raw data, and final reports.
18. Quality Assurance Statement.

B. Records

All records will be retained for at least ten years by the Health and Environment Laboratories:

1. All raw data and summaries of data.
2. The protocol and any protocol amendments.
3. Summaries of training and experience for all persons involved in the study.
4. Standard operating procedures.
5. Final reports.

Appendix 1. EPA Consent Order Protocol for Ready Biodegradability: Closed Bottle Test (Section 796.3200)

Section 796.3200 Ready biodegradability: Closed bottle test.

(a) Introductory information—(1) Prerequisites. The empirical formula of the test material is required so that the theoretical oxygen demand (TOD) may be calculated. If this is unknown, the chemical oxygen demand (COD) of the test material may serve as the reference point (6).

(2) Guidance information. (i) Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the result lies close to the "pass level."

(ii) Information on the toxicity of the chemical may be useful in the interpretation of low results and in the selection of appropriate test concentrations.

(3) Interpretation of results. Because of the stringency of this test, a measured BOD (biochemical oxygen demand) which is less than 60 percent of TOD (within 28 days) does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability. The possibility that nitrogen-containing compounds may affect the results should be considered.

(4) Qualifying statements. (i) The method is only applicable to those organic test materials which, at the concentration used in the test, are not inhibitory to bacteria.

(ii) This test has been found suitable by the OECD Expert Group Degradation/Accumulation for determining the ready biodegradability of organic chemicals under aerobic conditions. It has been tested in the OECD Laboratory Intercomparison Test Program (1978-1980).

(5) Recommendations. (i) Test chemicals giving a measured BOD which is greater than 60 percent of the TOD (within 28 days) should be regarded as readily biodegradable. This level must be reached within 10 days of biodegradation exceeding 10 percent.

(ii) If the test material is not soluble at the test concentration, special measures, such as the use of ultrasound dispersion, may have to be employed to achieve a good dispersion of test material.

(6) Standard documents. This test guideline is based on a paper found under paragraph (d)(8) of this section.

(b) Method—(1) Introduction purpose, scope, relevance, application and limits of test. (i) The purpose of the method is the measurement of the biodegradability of organic compounds in an aerobic, aqueous medium at a test concentration of 2 (standard concentration) to 10 mg/l of active material. Most data elaborated with this test pertain to water-soluble compounds; however, volatile compounds and those of low water solubility may also be tested, at least in principle.

(ii) Definitions and units. The degradation is stated as the biochemical oxygen demand (BOD) within 28 days as a percentage of either the theoretical oxygen demand (TOD) or the chemical oxygen demand (COD).

For example:

$$\text{Percent biodegradability} = \frac{\text{mg } O_2/\text{mg active substance}}{\text{TOD}} \times 100$$

or

$$\text{Percent biodegradability} = \frac{\text{mg } O_2/\text{mg active substance}}{\text{COD}} \times 100$$

where:

TOD = theoretical oxygen demand (calculation, see Data and reporting below)

COD = chemical oxygen demand determined experimentally.

(iii) Reference substances. In some cases when investigating a new substance, reference substances may be useful; however, specific reference substances cannot yet be recommended. In order to check the activity of the inoculum, the use of control substances is desirable. Aniline, sodium acetate, sodium benzoate, sodium n-dodecylsulfate and the sodium salt of linear alkylbenzenesulfonic acid (8,9) may be used for this purpose. They must exhibit a BOD which is]60 percent of their TOD within 28 days, otherwise the test is regarded as invalid and should be repeated using an inoculum from a different source.

(iv) Principle of the test method. A predetermined amount of the compound is dissolved in an inorganic medium (mineral nutrient solution), providing a usual concentration of 2 mg active substance per liter (AS/l). The solution is inoculated with a small number of micro-organisms from a mixed population and kept in closed bottles in the dark in a constant temperature bath at 20±1°C. The degradation is followed by oxygen analyses over a 28-day period. A control with inoculum, but without test material, is run parallel for the determination of oxygen blanks.

(v) Quality criteria—(A) Reproducibility. The reproducibility of the method is appropriate for a screening test for "ready biodegradability," which has solely an acceptance function, but is not sufficient for a final decision regarding biodegradability.

(B) Sensitivity. A starting concentration of 2 mg AS/l usually allows the determination of 95 percent degradation. Compounds with a low TOD may require higher starting concentrations.

(C) Specificity. The test is applicable for the biodegradability evaluation of organic compounds. Most experience has been gathered with water soluble compounds; however, the test leads itself, at least in principle, to the biodegradability evaluation of volatile and insoluble compounds.

(D) Possibility of standardization. The test is suited for standardization.

(E) Possibility of automation. Although the whole test cannot be automated, parts of it, e.g., the analysis may. The procedure is well suited, however, for being operated with whole series of test materials.

(2) Description of the test procedure—(i) Preparations-(A) Reagents—(1) Distilled or ion exchange water. Containing not more that 0.01 mg Cu/l, air saturated. Volume according to the need of the day, e.g., 50 l at room temperature, as close as possible to 20°C, aerated strongly for 20 minutes with compressed air. Generally, the water is ready for use after standing for 20 hours at 20°C. Oxygen is determined for control purposes. The concentration at 20°C should be about 9 mg O_2/l. All transfer and filling operations of the air saturated water must be conducted bubble-free by siphon.

(2) Standard dilution water for BOD determinations according to B5 of DEV (see paragraph (d)(1) of this section).

(i) Solution 1: 8.5g of KH_2PO, 21.75g of K_2HPO_4, 33.3g of $Na_2HPO_4 \cdot 2H_2O$, and 1.7g of NH_4Cl are dissolved in 500 ml of distilled water. The solution is made up to 1 l. The pH ought to be 7.2.

(ii) Solution 2: 22.5g of $MgSO_4 \cdot 7\ H_2O$/l.

(iii) Solution 3: 27.5g of $CaCl_2$/l.

(iv) Solution 4: 0.25g of $FeCl_3 \cdot 6\ H_2O$/l.

1 ml each of solutions ~~1 to 4~~ [(i) THROUGH (iv)] are added to 1 litre of aerated distilled water.

(B) Materials. (1) Calibrated 250-300 ml BOD bottles with glass stoppers (Uncalibrated narrow neck 250 ml bottles with glass stoppers whose volumes have been determined may be used instead without causing any appreciable error.) All glassware is cleaned according to the H23 procedure under paragraph (d)(2) of this section.

(2) Several 2, 3 and 5-l bottles with self-made litre marks for the preparation of the experiments and for filling of the BOD bottles.

(3) Pipettes of 1 to 10 ml volume.

(4) Funnels and coarse filter paper.

(5) Baby bottles for the preparation and shaking of the inoculum.

(6) Water bath [OR INCUBATOR] for keeping the bottles at constant temperature under the exclusion of light.

(C) Inoculation. The following inocula may be used:

(1) An aqueous suspension of unfertilized garden soil. 100g of unfertilized garden soil (soil from a greenhouse which is at constant temperature throughout the year is especially advantageous) are dispersed in chlorine-free tap water (1 liter). After 30 minutes the suspension is filtered through a coarse filter and the first 200 ml of the filtrate are discarded. The main part of the filtrate serves for the inoculation (1 drop from a pointed pipette per liter of final volume, see Procedure below). The inoculum is prepared immediately before the experiment. It must be aerated if it is to be kept for several hours. The number of bacteria may be determined according to the pour plate method or with nutrient pad sets. There should be no more than 10^3 to 10^5 bacteria per milliliter of final volume.

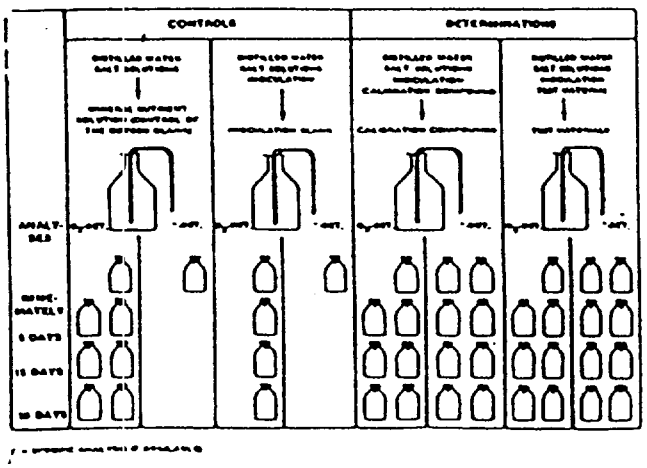
(2) Effluent from an activated sludge plant or trickling filter. Inoculation should preferably be made using a secoddary effluent of good quality collected from a treatment plant dealing with predominantly domestic sewage. The effluent must be kept under aerobic conditions in the period between sampling and application. To prepare the inoculum the sample is filtered through a coarse filter, the first 200 ml being discarded. The rest of the filtrate is kept aerobic until used. The inoculum must be used on the day of collection.

(3) Effluent from a strongly aerated laboratory activated sludge plant. The inoculum is prepared as described in previous section.

(4) Composite inoculum. Equal volumes of the three inoculum samples are combined, mixed well and the final inoculum drawn from this mixture. The suitability of the inoculum is checked by means of a control substance.

(ii) Procedure. (A)(1) Direct comparisons are always necessary in biological experiments. Therefore groups of parallel bottles are prepared for the determination of the BOD of the test and control substances in simultaneous experimental series, see the following Figure 1:

Figure 1—Scheme for the bottle arrangement for the closed bottle test



If chemical analyses are performed simultaneously a sufficient number of bottles--including the controls for the inoculum and the blank--have to be prepared (e.g., 7 or 15 parallel bottles are prepared for one test material for the 0, 5, 15 and 28-day tests after a sufficient volume of water has been prepared in large bottles (see Reagents above).

(2) These large bottles are first filled to about one third of their volume with distilled water by hose. Then the individual salt stock solutions are pipetted into these bottles according to the final volume, and the respective test or control materials are added in such amounts that final concentrations of 2 and sometimes 5 or 10 mg/l (higher concentrations only in case of poorly degradable compounds and those with a low TOD) are attained (see General considerations below). Subsequently the experimental solution is inoculated with 1 drop (from a pointed pipette) per liter of final volume, and the blank is inoculated similarly. Finally, the solutions are made up to volume with a hose which reaches down to the bottom of the flask, which achieves adequate mixing.

(3) Subsequently each prepared solution is filled immediately into the respective group of bottles by hose from the lower quarter (not from the bottom) of the bottle. Furthermore, the zero controls are analyzed or preserved for the later analyses (for the O_2 determination by precipitation with $MnCl_2$ and NaOH; for the surfactant determination by preservation with 50 mg/l of $HgCl_2$). The remaining parallels are placed in a water bath [OR INCUBATOR] at 20°C, kept in the dark, and removed after 5, 15 and 28 days, respectively, from the bath, and analyzed. The result is recorded as percentage biodegradability and should be visualized as a diagram.

(4) The test can be finished before the 28th day in the case where a plateau is observed before the 28th day. In the case where a degradation has obviously started on day 28 but has not reached a plateau on day 28, it is considered good practice to extend the experiment for one or two weeks longer.

(B) General considerations. (1) Each series is accompanied by complete parallel series for the determination of the blank, and the oxygen depletion without inoculation.

(2) The oxygen depletion without inoculation should not exceed 0.2 to 0.3 mg O_2/l after 5 days and 0.4 mg O_2/l after 28 days, respectively; the blank with inoculation should not exceed 0.4 to 0.5 mg O_2/l after 5 days and 0.5 to 0.6 mg O_2/l after 15 to 28 days. Typical values for the reference substances can be found in the literature references under paragraph (d)(8) and (9) of this section.

(3) Degradability is calculated by (i) subtracting the oxygen depletion result of the blank from that of the test substance and (ii) dividing this number by the concentration (W/V) of the test material. This calculation gives the oxygen depletion in mg O_2/mg of active material; this value is transformed to the "% biodegradability" according to Data and reporting under paragraph (c) of this section for the day under consideration.

(4) If the structure or elemental composition of the test material is unknown, or it is a mixture of unknown organic compounds, the chemical oxygen demand (COD) may serve as a reference instead of the TOD.

(5) The concentration of about 9 mg of dissolved oxygen per liter of dilution water at 20°C limits the possible starting concentration of the test material to about 2 mg/l in order to guarantee that an oxygen concentration of 4-5 mg/l remains after the oxidation of the test substrate. Substances which are only partially degraded or those which have a low TOD are advantageously tested in parallel experiments at 5 or even 10 mg/l starting concentrations.

(C) Inhibition test. (1) Organic and inorganic materials may easily and simply be tested for inhibitory effects in the closed bottle test:

(i) Series 1: 2 mg/l of a well-degraded compound (e.g., fatty alcohol + 10 moles ethylene oxide).

(ii) Series 2: x mg/l of test material (x is usually 2).

(iii) Series 3: 2 mg/l of the control compound plus x mg/l of test material.

(2) If the BOD values of series 3 correspond to the sum of those of series 1 and 2, the test material does not display inhibitory effects. This control experiment is always necessary if a negative or poor degradability result seems illogical in view of the structure of the test material, i.e., if there are indications that it may be caused by inhibition.

(iii) Analytical means. (A) Oxygen determination according to Winkler under paragraph (d)(3) of this section or electrometrically, if suitability is assured.

(B) In case of anionic surfactants: MBAS-determination according to B23 of DEV under paragraph (d)(2) of this section.

(C) In case of nonionic surfactants: BIAS (bismuth active substance) determination according to Wickbold under paragraph (d)(4) of this section.

(c) Data and reporting--(1) Treatment of results--(i) Calculation of the theoretical oxygen demand (TOD). (A) The TOD of the compound $C_cH_hCl_clN_nNa_nO_oP_pS_s$ of the molecular weight is calculated according to:

$$TOD_{NO_3} = \frac{16[2c+1/2(h-cl-3n)+3s+5/2p+1/2na-o}{mol.\ wt.}$$

(B) This calculation implied that C is mineralised to CO_2, H to H_2O, P to P_2O_3 and Na to Na_2O. Halogen is eliminated as hydrogen halide and nitrogen as ammonia. Example: Glucose $C_6H_{12}O_6$ mol. wt. = 180.

$$TOD = \frac{16(2\cdot6+1/2\cdot12-6)}{180} = 1.07 \text{ mg } O_2/\text{mg active substance}$$

(C) Molecular weights of salts other than those of the alkali metals are calculated on the assumption that the salts have been hydrolyzed.

(D) Sulfur is schematically oxidized to the state of +6. Example: Alkylbenzenesulfonate, LAS, $C_{18}H_{29}SO_3Na$, mol. wt. = 348.

$$TOD = \frac{16(36+29/2+3+1/2-3)}{348} = 2.34 \text{ mg } O_2/\text{mg active substance}$$

(E) In the case of nitrogen-containing compounds the nitrogen may be eliminated as ammonia, nitrite, or nitrate corresponding to different theoretical oxygen demands:

$$TOD_{NO_2} = \frac{16[2c+1/2(h-cl)+3s+3/2n+5/2p+1/2na-o]}{mol.\ wt.}$$

$$TOD_{NO_3} = \frac{16[2c+1/2(h-cl)+3s+5/2n+5/2p+1/2na-o]}{mol.\ wt.}$$

(F) Suppose full nitrate formation would have been observed by analysis in case of a secondary amine: $(C_{12}H_{25})_2NH$, mol. wt. = 353

$$TOD_{NO_3} = \frac{16(48+51/2+5/2)}{353} = 3.44 \text{ mg } O_2/\text{mg active substance}$$

(ii) Calculation of results. For the calculation of the percentage biodegradability after n days the determined oxygen depletions are divided by the concentration (W/V) of active substance.

Then—

$$\text{Percent biodegradability} = \frac{mgO_2/mgAS}{TOD} \times 100$$

or

$$\text{Percent biodegradability} = \frac{mgO_2/mgAS}{mgCOD/mgAS} \times 100$$

(2) Test report. The raw data may be listed in and evaluated according to the following form sheet:

(3) Interpretation of results. See paragraph (a) of this section.

Sample Form Sheet for the Closed Bottle Test

Exp. No..... .....
Date of start of test..... .....
Test/standard material..... .....
Theoretical test concentration..... mgAS/l
Inoculum..... .....

Analysis: (Winkler method or oxygen electrode)..... .....
TOD or COD of test material..... $mgO_2/mgAS$
Temp. of the dilution water after aeration..... $K(O_c)$
O_2-conc. of the distilled (deionized) water after aeration and standing before start of test..... mg O_2/l

-10- [796.3200 CB]

PART I: O_2 Determinations:

Culture medium	Flask No.	Analyses	mg O_2/l after x days		
			0	5	15
Mineral nutrient solution without test material and without inoculum	O_2	C_1			
	Control	C_2			
	Mean	$m_0 = \frac{C_1 + C_2}{2}$			
Mineral nutrient solution without test material but with inoculum	1	C_1			
	2	C_2			
	Mean blank	$m_1 = \frac{C_1 + C_2}{2}$			
Mineral nutrient solution with test material and with inoculum	1	C_1			
	2	C_2			
	Mean test material	$m_2 = \frac{C_1 + C_2}{2}$			

PART II: O_2 Depletions (mg BOD/l) after X days:

$$BOD_x = (m_0 - m_{xx}) - (m_0 - m_{xx})$$

(This difference is important as a check for the validity of the test.)

mg BOD/l after x days		
5	15	20

- 11 - [796.3200 CB]

PART III: Evaluation:

$$\text{Percent biodegradability (I)} = \frac{\text{mg BOD}_5/l}{\text{mg AS/l.TOD}}$$

or

$$\text{Percent biodegradability (II)} = \frac{\text{mg BOD}_5/l}{\text{mg AS/l.COD}}$$

	After 5 days		
	5	15	20
[illegible]			
[illegible]			

(d) Literature references. For additional background information on this test guideline the following references should be consulted.

(1) "Methode H5," Deutsche Einheitsverfahren zur Wasseruntersuchung, (Verlag Chemie: Weinheim, 1960).

(2) "Methode H23," Deutsche Einheitsverfahren zur Wasseruntersuchung, (Verlag Chemie: Weinheim, 1960).

(3) "Methode C2," Deutsche Einheitsverfahren zur Wasseruntersuchung, (Verlag Chemie: Weinheim, 1960).

(4) Wickbold, R. Wasser, 33:229. (1966).

(5) Wickbold, R. Tenside Detergents 8:61 (1971).

(6) "Oxygen Demand," Standard Methods for the Examination of Water and Wastewater, 12 Ed. American Public Health Association, American Water Works Association Water Pollution Control Federation, (1965) p. 510.

(7) Fischer, W. K., Fette, Seifen, Anstrichmittel, 65:37-32 (1963).

(8) Fischer, W. K. Gerike, P. and Schmid, R., Wasser und Abwasserforschung 7:99-118 (1974).

(9) Gerike, P. and Fischer, W. K., Ecotoxicology and Environmental Safety, Vol 3, No. 2, 159-173 (1979).

(6243Q)
0513M

Appendix 3. Data Worksheets and Chemical Analysis for Chemical Pre-test

-1-

Pre-Test Methods

CLOSED-BOTTLE TEST-ANNEX V

READY BIODEGRADABILITY TEST (BIOTIC DEGRADATION)

WORKSHEET SUMMARY

Chemical Name Crotonaldehyde Methods Development to assess chemical oxidation (i.e. Pre-test)

Accession No. 901878 HAEL No. 90-0016

Sample Ref. No. Lot No. 90-7 CAS Reg. No. 4170-30-3

Study Initiation Date NA Date of Inoculum Collection NA

Experiment Start (Exposure) Date 6-21-90 Termination Date 7-19-90

Concentrations Tested 2.1 mg/L Inhibition Test No (Yes/No)

SUMMARY: A NA -day test for Ready Biodegradability using unacclimated microorganisms as the inoculum showed a _____% degradation of the test chemical based on _____ (ThOD or COD) as measured by loss of dissolved oxygen from solution, where % degradation = % BOD/ThOD (or % BOD/COD).

NA = Not applicable

SOURCE OF MICROORGANISMS NA

TEST VALIDITY: The positive control, NA, showed _____% degradation. The requirements for a valid test were fulfilled _____(Yes/No).

CONCLUSION: 59% oxygen depletion was seen due to biodegradation (see Remarks)

REMARKS: Although no inoculum was used, tests using Easy Cult™ dip slides showed considerable microbial growth in the 15-day & 28-day BOD bottles, (non-sterile water was used to set this test). This clearly indicates the biodegradability of crotonaldehyde & the lack of need to separate/distinguish chemical abiotic oxidation. Analysis showed parent compound below the limit of detection by day 15

Prepared by Heather Walker Date 7-20-90

Approved by Heather Walker Date 7-20-90

-2-

Acc. No. 901878
HAEL No. 90-0016
Test Type Closed-Bottle

TEST CHEMICAL AND POSITIVE CONTROL WORKSHEET

I. Test Chemical Name Crotonaldehyde Methods (to assess potential chemical oxidation)
Sample Ref. No. Lot No - 90-7
Molecular Weight 70.09 Molecular Formula C_4H_6O
Appearance: Solid ___ Liquid ✓ Aqueous Solution ___ Color Colorless

1. The ThOD of the substance $C_cH_hCl_clN_nNa_naO_oP_pS_s$ of the molecular weight MW is calculated according to Protocol EN-104, Attachment 2, "Calculation of ThOD" where:

$$ThOD_{NH3} = \frac{16[2c + 1/2\,(h-cl-3n) + 3s + 5/2p + 1/2na - o]}{MW}$$

$= 2.28$ mg O_2/mg of test chemical

2. Amount(s) of Test Chemical: 2.0 mg/L mg/L
Amount(s) of O_2 consumed during degradation 4.56 mg/L

II. Positive Control Chemical Name Not applicable
Molecular Weight — Molecular Formula —
ThOD — mg O_2/mg of Control Substance
Amount of Positive Control Chemical: — mg/L
Amount of O_2 consumed during degradation — mg/L

REMARKS:

Prepared by Heather R Walton Date 6-19-90

-3-

Acc. No. 901878
HAEL No. 90-0016
Test Type Closed-Bottle METHODS

INOCULUM PREPARATION WORKSHEET

Inoculum Preparation:

Secondary Effluent: Source NA

Date obtained _____

Filtered using coarse filter paper - discarded first 200 mL

Filtrate aerated until used

Laboratory Activated Sludge: Source NA

Date obtained _____

Filtered using coarse filter paper - discarded first 200 mL

Filtrate aerated until used

Soil: Source NA

Date obtained _____

Suspended 100 g/L in distilled water, settled _____ min.

Filtered supernatant using coarse filter paper- discarded first 200 mL

Filtrate aerated until used

Composite: Equal volumes of the three samples prepared above _____

Other _____

Date of inoculum use _____

NA = Not Applicable

REMARKS:
No inoculum will be used for determination of chemical oxidation. In fact, all glassware will be autoclaved & solutions sterilized prior to preparation

Prepared by Heather N Walton Date 6-19-90

-4-

Acc. No. 901878
HAEL No. 90-0016
Test Type Closed-Bottle
METHODS

PREPARATION OF TEST SOLUTIONS

I. Water:
Approx. Volume used 15 L Aeration time 5 hours
Standing time 20 hours Temperature 20.5 °C DO 10.01 mg/L

II. Test Chemical Solution(s):
Final concentration(s) required: 2.0 mg/L mg/L
1. Stock Solution Preparation:
Amount used (aim conc. 1400 mg/L) 147.8 mg/ 100 mL (Aim 140mg)
Balance used: Mettler AE 163 #269160
2. Final Solution Preparation:
Amount used 10 mL Stock/ 7.0 L of final solution in bottle
to give final conc. #1 of 2.0 mg/L (Aim)
Amount used NA mL Stock/ NA L of final solution in bottle
to give final conc. #2 of NA mg/L (if needed)

III. Positive Control Solution:
Final concentration required: NA mg/L
1. Stock Solution Preparation:
Amount used (aim conc. — mg/L) — mg/ — mL
Balance used: —
2. Final Solution Preparation:
Amount used — mL Stock/ — L of final solution in bottle
to give final conc. of — mg/L

IV. Procedure:
1. Siphon water (about one-third final volume) into large bottles
2. Add appropriate stock solutions by pipet (amounts above)
3. Add BSM stock solutions (1.0 mL/L of final solution)
4. Add 1 drop inoculum/L of final solution
5. Bring to volume using siphon to mix
6. Siphon into the appropriate bottles, stopper, seal with DW, and cap
No. of bottles per test series: _____ extras _____
No. of bottles per test conc. for specific analysis: _____

REMARKS: NA = Not applicable

Prepared by Heather Watson Date 6-21-90

-5-

Acc. No. 901878
HAEL No. 90-0016
Test Type Closed-Bottle
METHODS

INHIBITION TEST (if required)

NOT REQUIRED

Substances may easily and simply be tested for inhibitory effects using the Closed-Bottle Test results and one extra solution for each concentration to be tested:

-Series 1: Positive Control Solution (x mg/L) from Closed-Bottle Test
-Series 2: Test Chemical Solution(s) (y mg/L) from Closed-Bottle Test
-Series 3: Solution containing x mg/L of Positive Control and y mg/L of Test Chemical

The test chemical can be considered to be inhibitory to bacteria at the concentration tested if:

$$BOD_{series\ 3} < BOD_{series\ 1} + BOD_{series\ 2}$$

Procedure for Series 1 and 2: see Section IV, p4

Procedure for Series 3:

Add Test Chemical stock Solution:

I. Concentration 1:

Amount used _____ mL Stock/_____ L of final solution in bottle to give final conc. of _____ mg/L

II. Concentration 2 (if required):

Amount used _____ mL Stock/_____ L of final solution in bottle to give final conc. of _____ mg/L

Add Positive Control stock Solution:

Amount used _____ mL Stock/_____ L of Final Solution in Bottle (s) to give final conc. of _____ mg/L

Prepared by Heather Walton Date 6-21-90

-6-

Acc. No. 901878
HAEL No. 90-0016
Test Type Closed-Bottle
METHODS

DO MEASUREMENTS (mg/L)

Medium	Day 0 6-21-90		Day 5 6-26-90		Day 15 7-6-90		Day 28 7-19-90	
BSM	10.17	10.00	10.21	10.35	10.25	10.19	9.58	10.06
B+I	NA	—	—	—	—	—	—	—
Pos.	NA	—	—	—	—	—	—	—
Test #1	10.22	10.20	10.04	9.79	7.55	7.60	7.45	7.30
Test #2	NA	—	—	—	—	—	—	—
Inhib. #1	NA	—	—	—	—	—	—	—
Inhib. #2	NA	—	—	—	—	—	—	—
Means: BSM	10.085		10.28		10.22		9.82	
Test #1	10.21		9.915		7.575		7.375	

Where:

BSM = BSM (Oxygen Blank)

B+I = BSM + Inoculum (Inoculum Blank)

POS = Positive Control (Reference substance) + BSM + Inoculum

Test #1 = Test Conc. #1 + BSM + Inoculum

Test #2 = Test Conc. #2 + BSM + Inoculum

Inhib. #1 = Inhibition Test Conc. #1 + Positive Control + BSM + Inoculum

Inhib. #2 = Inhibition Test Conc. #2 + Positive Control + BSM + Inoculum

Dip slides showed microbial growth in test BOD bottles at 15 days & at 28 days

Incubation Temperature (°C)

Day 0	Day 5	Day 15	Day 28
21	20.5	20°C	20.5

REMARKS: Degradation = Oxygen depletion / ThOD × 100% (for 2 mg/L in control)

At Day 5 = (10.21 − 9.915)/4.56 × 100% = 6.5% (6.2% @ 2.1 mg/L)

" Day 15 = (10.21 − 7.575)/4.56 × 100% = 57.8% (55.0% @ 2.1 mg/L)

" Day 28 = (10.21 − 7.375)/4.56 × 100% = 62.2% (59.2% @ 2.1 mg/L)

Prepared by Heather Watson Date 7-12-90

AUTHOR: Beth Isaacs
PHASE: Crotonaldehyde Methods-Concentration Determination
STUDY TYPE: Ready Biodegradation Test (Preliminary)
STUDY DIRECTOR: H.M. Watson

HAEL#: 90-0016
ACC.#: 901878
SAMPLE REF.#: Lot No. 90-7

Charge No.: 3PC1190
Date Received: 6/21/90-7/06/90
Date Analyzed: 6/21/90-7/06/90
Date Reported: 9-18-90

RESULTS: Samples were derivatized and extracted and then analyzed by gas chromatography with electron capture detection (GC/ECD).

Time	Request	Conc(mg/L) BSM A	Conc(mg/L) BSM B	Conc(mg/L) TEST A	Conc(mg/L) TEST B
0	142416	ND	ND	2.110	2.037
5	142414	ND	ND	1.772	1.296
15	142450	ND	ND	ND	ND

ND = None Detected

Duplicate samples were received and then prepared in duplicate. The average of the four injections was reported.

Scientist Beth Isaacs (signature) Date 9-18-90

Reviewed by Robert T Ambrose (signature) Date 9/19/90

GC/ECD Conditions:

Instrument:	Perkin-Elmer 8500 Gas Chromatograph
Column:	J&W DB-5 30 meter 0.25 um film thickness
Pressure:	10 psig
Oven Program:	80 °C (2 min. hold) to 250 °C @ 10 °C/min. (1 min. hold)
Injector Temp:	250 °C
Type:	Splitless (2 min. purge delay)
Split Flow:	60 cc/min
Inj. Volume:	1 uL
Detector:	Electron Capture
Temp.:	300 °C
Make-up:	Nitrogen
Flow:	55 cc/min.

HAEL No. 90-0016 Study No. EN-1040-901878-1

DIS No. 263600A

Prepared by _____ Date 9-24-90
Heather M. Watson
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Eco-Chem Testing Group

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Bruce H. Klanderman
Director
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Approved by _____ Date 9-27-90
Heather M. Watson
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Approved by _____ Date 10-1-90
Roderick D. Gerwe
Sponsor
Eastman Chemical Company

TOXICITY REPORT - E. K. CO. - LABORATORY OF INDUSTRIAL MEDICINE

901878

Chemical: Crotonaldehyde (60-508) Submitted by: D. W. Fassett, M.D.

INHALATION

Chemical Formula Type of Exposure	Animals* No. and Species	Conc.	Time	Mortality	Symptoms
1/4 L/min Air Flow thru gas washing bottle	3 R	37 mg/L 12,913 ppm	1/2 hr.	3/3	Gasping, convulsions, salivation, dark eyes, prostration. Death <1 hr.
1/4 L/min thru gas washing bottle with 3-1/2 L/min bypass.	3 R	6 mg/L 2094 ppm	1/2 hr.	3/3	Lacrimation, labored breathing, salivation, gasping. Death < 1 hr.
1/4 L/min thru gas washing bottle with 7 L/min. bypass.	3 R	3.6 mg/L 1256 ppm	2 hrs.	3/3	Gasping. Death in 1-2 hrs.
1/4 L/min air passing over compound in gas washing bottle, with 3-1/2 L/min bypass.	3 R	2.6 mg/L 907 ppm	2 hrs.	3/3	Gasping. Death in 2 hrs.
1/8 L/min air passing over compound in large gas washing bottle with 7 L/min bypass.	3 R	1.03 mg/L 359 ppm	6 hrs.	3/3	Gasping. Death in 3-1/2 hrs - 1 day.
1/3 L/min air passing over compound in small gas washing bottle with 7 L/min bypass.	4 R	.27-.31 mg/L 94-108 ppm	6 hrs/day 2 consecutive days and 2 days later.	1/4 died in 24 hrs. after 1st exposure. 2/4 died 24 hrs. after 3 rd exposure. other rat sacrificed day after last exposure.	Gasping, pink extremities, labored breathing.

4-26-61

* G.P. - Guinea Pig, M - Mouse,
R - Rat, RB - Rabbit

TOXICITY REPORT - E. K. CO. - LABORATORY OF INDUSTRIAL MEDICINE

Chemical: Crotonaldehyde (60-508) Submitted by: D. W. Fassett, M.D.

INHALATION

Chemical Formula Type of Exposure	Animals* No. and Species	Conc.	Time	Mortality	Symptoms
1/4 L/min air flow over beaker taped to chamber.	3 R	46.5 mg/L 16,229 ppm	43 min.	3/3	Gasping, pink extremities, tremors, prostration. Death in < 43 min.
1/4 L/min air flow over beaker taped to chamber with 7 L/min bypass.	3 R	0.38 mg/L 133 ppm	6 hrs.	2/3 in 24 hrs. 3/3 in 48 hrs.	Gasping, nasal irritation, pink extremities.
With beaker taped to chamber, air flow not directly over beaker with 7 L/min bypass.	3 R	0.48 mg/L 166 ppm	6 hrs.	2/3	Gasping, nasal irritation, pink extremities. Death in 1 day. In 2 wks, remaining rat's weight was - 2g.
Air flow in 15 mm diameter vial, 22 mm from top of cylinder with 7 L/min bypass	3 R	0.099 mg/L 35 ppm	6 hrs.	-	Pink extremities, slight nasal irritation, rough coat, labored breathing.
3-1/2 L/min bypass		.16-.28 mg/L 56-98 ppm	6 hrs.	-	Av. wt. gain in 2 wks. 3+ (14 gm av)

4-26-61

* G.P. - Guinea Pig, M - Mouse,
R - Rat, RB - Rabbit

901878

Crotonaldehyde (60-508)

Three rats exposed to atmospheres containing 0.27 mg/L for six hours were sacrificed one day after exposure. Three rats exposed to atmospheres containing 0.099 mg/L for six hours were sacrificed 27 days after exposure.

Pathology - The lungs are congested.

TOXICITY SUMMARY

for

CROTONALDEHYDE

Prepared by: J.A. Deyo

Product Safety & Regulatory Programs

Eastman Chemical Company

Kingsport, Tennessee 37662, USA

February 13, 1997

Toxicology of Crotonaldehyde : Overview

The following information on crotonaldehydye (CA) have been extracted from summaries and reviews identified through searches of various data bases such as RTECS, HSDB, CHEMID, CCRIS, TOXLINE, EMIC, EMICBACK, ETICBACK, and TSCATS. In addition, the toxicology of CA has been reviewed in text books such as Patty's Industrial Hygiene and Toxicology, and has had monographs prepared on it by both the International Agency for Research on Cancer (IARC) and by the Advisory Committee on Existing Chemicals of Environmental Relevance of the German Chemical Society: Gesellschaft Deutscher Chemiker. The raw data from studies identified through the above information sources were not available for direct review to confirm the accuracy of the reported findings. In addition, because most of the identified studies were not conducted under GLP assurances, the purity and the stability of test article in some of its vehicles (drinking water and rodent chow) are also unknown.

Crotonaldehyde is a member of a class of compounds known as unsaturated aliphatic aldehydes. The presence of the double bond considerably enhances the already potent reactivity of these aldehydes and their ability to bind to -SH and -NH groups as well as electrophiles. Crotonaldehyde exist as a liquid at room temperature but has a relatively high vapor pressure (32 mm Hg; 20° C). Any exposure to CA in occupational settings would occur primarily through inhalation or by accidental dermal or ocular contact with the liquid. Routine industrial hygiene analyses of air levels at Eastman facilities indicate its is well below the ACGIH-TLV of 2 ppm. The TLV was set at such a low level based on CA ability to induce severe burning sensations to the eyes and the respiratory tract. These effects lead to immediate avoidance reactions that self limit and minimize further exposure. As a result of its ability to induce such severe irritation and lacrimation at low levels, all manufacturing operations involving its synthesis, distribution, and use as a feedstock raw material are performed within closed systems at Eastman.

Several acute toxicity studies using different exposure routes have been conducted on CA. Results from these studies reveal that its potency is highly dependent on the route of exposure with lethality from intraperitoneal and inhalation exposures being roughly 3-4 times higher than that of oral or dermal administration. Actual lethal inhalation mg/kg dose levels are likely to be even much lower than what were calculated based on its ability to reduce respiration rates at very low levels in rodents. This difference in lethality between exposure routes is likely due to the high reactivity of CA and the consequences of damage to local tissues. Experiments in man confirm its ability to induce severe irritation, with levels as low as 5 ppm reported to induce significant irritation induced lacrimation. Although results from experimental exposures in laboratory animals indicate it is only a moderate irritant to the skin, significant burning sensations are reported by workers who have had accidental dermal contact. The current toxicity data base is believed to be sufficient in regard to understanding and managing the risk associated with acute exposure.

The weight of evidence from genetic toxicity studies on CA indicate it is a genotoxicant.

No information on the absorption or distribution of CA was identified in a literature search. Studies evaluating its metabolism are in general minimal, but indicate CA is a substrate for

aldehyde dehydrogenase and can form glutathione conjugates. Mercapturic acid metabolites have also been identified in rat urine. It is also reported that ip exposure to CA decreased cytochrome P450 enzyme and ethylmorphine N-demethylase levels.

Toxicity data are not available for evaluating its potential to induce reproductive and developmental effects in mammals. Malformations and mortality were observed when crotonaldehyde was directly applied to developing chick embryos. The significance of this test for risk assessment is questionable based on the reactive nature of CA. Significant mortality was induced even by the vehicle in this study. Acrolein (CA is methyl-acrolein), a closely related compound considered to be significantly more toxic than CA, did not induce reproductive toxicity in a two generation rat study or developmental toxicity in rabbits following oral exposure (1,2).

The effect of repeated oral exposures to CA have been investigated in several studies ranging in duration from 2-113 weeks. Results of these studies also substantiate the highly reactive nature of this compound, as toxicity, when induced, is primarily manifested at the point where test article is deposited, i.e., the stomach. Results of these studies also suggest that induction of oral toxicity is dependent upon test article concentration and/or the rate at which exposure occurs. For example, animals were able to well tolerate CA in their diet at a level which resulted in an estimated daily dose of 136-139 mg/kg over a 2 week period, while a single gavage bolus dose of 20 mg/kg killed 3/20 rats within 2 weeks. The primary target organ identified in studies in rats and mice following 13 weeks of gavage exposure of doses up to 40 mg/kg was the forestomach. In these studies, the NOEL in rats for stomach changes was 5 mg/kg in males and 10 mg/kg in females, and 20 mg/kg for both sexes of mice. Importantly, systemic toxicity did not appear to be manifested in any study except for that of a 113 week drinking water study. In this study, moderate to severe fatty change, focal necrosis, fibrosis, and cholestasis were reported in the livers of 10/23 animals receiving CA in their drinking water at a level of 6 mM. Although this study evaluated lifetime exposures of CA it was not robust enough to definitively confirm whether CA is or is not carcinogenic. Accordingly, when these data along with other studies were reviewed by IARC, CA was determined as not classifiable in regard to its carcinogenic potential.

Two subchronic inhalation studies of 8 and 13 weeks duration were reported, but only utilized single exposure levels of either 7 or 0.4 ppm, respectively. The only findings noted in the 8 week study were that of increased serum albumin, decreased "alpha-2-globulins" and an increase in liver and lung RNA. Effects reported in the 13 week study consisted alterations in motor activity and blood hemoglobin content. The toxicological significance of these findings, which were identified from Russian journals, are believed to be dubious.

Crotonaldehyde is considered to be readily biodegradable. Its long-term environmental fate is considered to be of minimal concern as it is broken down by a variety of mechanisms in air and water. Although it is very toxic to aquatic animal species on an acute basis, the no effect concentration is roughly half the acute lethality levels of most affected species indicating its effects are rapidly diminished upon dilution. Based on its physical chemical properties it is not expected to bioaccumulate.

Acute Toxicity

The acute toxicity of CA has been evaluated in several studies using many species and exposure routes. Its potency appears to be highly dependent on the route of exposure, with lethality from intraperitoneal and inhalation exposures being roughly 3-4 times higher than that of oral or dermal administration. This difference in lethality between exposure routes is likely due to the high reactivity of CA and ability to induce damage to local tissues. Results of acute CA exposures in laboratory animals and man indicate it induces severe irritation to ocular and respiratory tissues at very low levels, with levels as low as 3.5 and 23 ppm capable of reducing respiratory rates by 50% in mice and rats, respectively. Thus, the actual inhalation lethal dose levels are likely to be even much lower than what were calculated using normal physiological respiratory rates. The only organ noted in animals exposed to CA vapors to have gross pathological changes was the lung. Humans exposed to 5 and 45 ppm are reported to experience significant irritation and lacrimation. Although results from experimental exposures in laboratory animals indicate it is only a moderate irritant to the skin, significant burning sensations are reported by workers who have had accidental dermal exposures. There is one case report of a worker becoming sensitized to CA. Crotonaldehyde has been shown to induce various functional deficits in immunocytes and tracheal cilia under *in vitro* exposure conditions. Such findings are not unexpected based on the reactivity of CA.

Metabolism

No information on absorption or distribution was identified in a literature search. However, results from several studies indicate CA is a substrate for aldehyde dehydrogenase and can form glutathione conjugates (21,22). Mercapturic acid metabolites have also been identified in rat urine (23). An ip injection of 31.5 mg/kg decreased cytochrome P450 enzyme levels to 67% and ethylmorphine N-demethylase to 23% of control within 24 hours (24). Similarly, following *in vitro* exposure, aldehyde dehydrogenase and P450 enzyme activities were decreased (25, 26).

Reproductive Toxicity

There are no reproductive toxicity data available in mammals. However, malformations and mortality were observed when CA was directly applied to developing chick embryos. The significance of this test for risk assessment is questionable based on the reactive nature of CA. A mortality rate of 18% was induced even by the olive oil vehicle alone.

Genotoxicity

A multitude of studies have been conducted on CA investigating its potential to induce adverse effects on chromosomes, and in general CA should be considered a genotoxicant. Although quite a few mutational assays in bacteria have yielded negative results, the vast majority were positive. Similar positive mutagenic results were observed in studies using *Drosophila*. Such findings would not be unexpected as results from an equally numerous amount of studies have demonstrated CA to be capable of forming DNA adducts. Chromosomal aberrations have been observed in human lymphocytes (NAMALVA cell line) and sperm following both *in vitro* and *in vivo* exposure. Exposure to CA has also been shown to induce sister-chromatid exchange (SCE) and formation of micronuclei in various test systems. Studies looking at unscheduled DNA synthesis in rat hepatocyte cultures were negative.

Repeat-Dose Studies

The toxicological effects of CA following oral (gavage/dietary/drinking water) administration has been assessed in exposure periods up to 113 weeks in rats and 13-weeks in mice. Results of these studies suggest toxicity was quite dependent on the rate in which exposure occurred. Animals could tolerate doses of up to 136-139 mg/kg/day over 2 weeks without significant changes when CA was present in their diet, whereas doses as low as 20 mg/kg, when administered as a single bolus, induced mortality in 3/20 animals within two weeks. The major target organ identified in 13 week bolus dose studies up to 40 mg/kg was that of the stomach with major changes consisting of inflammation and hyperplasia. Test article administered by gavage also induced microscopic lesions in the nasal cavity of rats. However, this finding was not considered a result of systemic toxicity but was attributed to volatilization of test article. Lesions in the nasal cavity were not observed when exposure was spread out over time by placing test article in the feed or drinking water. Overall, systemic toxicity did not appear to be manifested in any study except for that of the 113 week drinking water study. In this study, moderate to severe fatty change, focal necrosis, fibrosis, and cholestasis were reported in the livers of 10/23 animals receiving CA in their drinking water at a level of 6 mM (approx. 16 mg/kg/day). Hepatic carcinomas occurred in 0/23, 2/27, and 0/23 animals, neoplastic nodules were present in livers of 0/23, 9/27, and 1/23 animals, while altered-foci were noted in 1/23, 23/27, and 13/23 animals. Although this study evaluated lifetime exposures of CA in male rats it was not considered robust enough to definitively confirm whether CA was or was not carcinogenic. Accordingly, when these data along with other studies were reviewed by IARC, CA was determined as not classifiable with regard to its carcinogenic potential.

Two single exposure level inhalation toxicity studies were identified from Russian journals. The only effects noted in an 8 week study in rats at a level of 7 ppm were that of increased serum albumin, decreased "alpha-2-globulins" and an increase in liver and lung RNA. Findings from a study in which rats and mice were exposed to 0.4 ppm for a period of 3 months consisted of alterations in motor activity and blood hemoglobin content. The toxicological significance of these findings is unclear.

Environmental Toxicity

Several studies have been conducted on CA to evaluate its potential effects on aquatic plants (algae) and organisms. Results of all studies clearly indicate CA is very toxic on an acute basis to all plants and animals tested. Although aquatic organisms appear to be very sensitive to CA, the no effect concentration for some was determined to be roughly half of the EC_{50} level. Experimental data suggest CA is not expected to bioaccumulate. This compound is considered to be readily biodegradable.

Acute Toxicity

Study Type	Exposure	Species	Results	Ref.
Acute Lethality	Oral	Rat	LD_{50} 206 mg/kg	3
		Rat	LD_{50} 300 mg/kg	4
		Rat	LD_{50} 220 mg/kg	5
		Rat	LD_{50} 160 mg/kg	6
		Mouse	LD_{50} 104 mg/kg	3
	Intravenous	Cat	LDLo 30 mg/kg (lowest lethal dose)	7
	Dermal	Rabbit	LD_{50} 380 mg/kg	8
		Rabbit	LD_{50} 150-200 mg/kg	8
		Guinea Pig	LD_{50} 500-1000 mg/kg	8
	Intraperitoneal	Rat	LD_{50} 70 mg/kg	6
		Mouse	LD_{50} 130 mg/kg	6
		Guinea Pig	LD_{50} 20-35 mg/kg	6
		Rabbit	LD_{50} 5-50 mg/kg	6
	Subcutaneous	Rat	LD_{50} 140 mg/kg	5
		Mouse	LD_{50} 160 mg/kg	5
	Inhalation	Rat	LC_{50} 600 ppm/0.5 hr (45 mg/kg)*	9
			LC_{50} 1500 ppm/0.5 hr (132 mg/kg)	5
			LC_{50} 380 ppm/1 hr (45 mg/kg)	9
			LC_{50} 85 ppm/4 hr (51 mg/kg)	9
		Mouse	LC_{50} 580 ppm/2 hr (142 mg/kg)	3
Irritation	Dermal	Rabbit	Moderate	10
		Guinea Pig	Moderate	10
	Ocular	Rabbit	Severe	8
	Respiratory	Human	4 ppm/15 minutes: highly irritating and lachrimatory	11
		Human	45 ppm/"few seconds": highly irritating and lachrimatory	12

*The mg/kg dose was based on a respiratory rate of 0.0156 M^3/hr and body weight of 0.3 kg for rats and 0.0015 M^3/hr and body weight of 0.035 kg for mice.

Acute Toxicity (cont)

Study Type	Exposure	Species	Results	Ref.
Sensitization	Dermal	??	An NTP report states that CA does not show any potential for skin sensitization in animal studies.	13
		Human	There is one case report of a textile worker who demonstrated sensitization.	14
Accidental Exposure	Ocular	Human	8 cases of corneal injury were reported as healed in 48 hours.	12

Other

- *In vitro* exposure to CA inhibited human polymorphonuclear leukocyte chemotaxis, adherence, and production of superoxide anion (15, 16, 17).
- CA has been shown to have adverse effects on trachea cilia function (18). Its effects were reported as the weakest relative to formaldehyde, acetaldehyde, and acrolein.
- Respiratory rates are reduced 50% following exposure to 3.5 ppm in mice and 23 ppm in rats (19, 20).

Reproductive Toxicity

Study Type	Exposure	Results		Ref.
Chick Embryo (single injection on day 3)	ug/embryo	Malformation Rate of Dead Embryos	Mortality	27
	0	7.9 %	17.8 %	
	1.75	10 %	21.4 %	
	8.76	31.8 %	39.7 %	
	17.5	55.2 %	72.3 %	
	87.6	Detection Impossible	100 %	

Genotoxicity

Study Type	Results	Ref.
Bacterial Assays with *E.coli* and *S. typhimurium*	Although some studies yielded negative results, findings from the majority of bacterial assays were considered to be positive.	28*
Drosophila melanogaster: sex-linked recessive lethal mutation	1 Positive (injection) and 1 negative (feed)	29
Drosophila melanogaster: heritable translocation	1 Positive (injection)	29
UDS in hepatocyte cultures	Negative	30
In vitro studies with NAMALVA lymphoma cell line -micronucleus -SCE	 Positive Positive	 31 31
Chromosomal aberrations in -CHO cells -sperm *in vivo* (ip inj.)/*in vitro* -NAMALVA cell line	 Positive Positive Positive (structural only, not numerical)	 32 33 31
DNA Adduct Formation	Several studies report DNA adducts are formed following *in vitro* and *in vivo* exposures.	28

*Although several references are identified in the IARC monograph many others were found in a literature search.

Repeat Dose Studies

Study Type	Species	Exposure	Dose	Results	Ref.
Subacute Toxicity	Mice	1 IP Injection over 1-5 days	4 mg/kg	A 5X rise in plasma LDH was noted following a single ip exposure. Repeated injections over 5 days induced some tolerance with progressively less pronounced effects. Histological examination revealed splenic and thymic atrophy.	34
Subacute Toxicity	Rat n=5/sex/ dose	2-Week Dietary	Actual doses (M/F) 19/17, 36/36, 73/68, and 139/136 mg/kg/day	There were no statistically significant changes between control and treated in mortality, clinical signs, body weight, food consumption, feed efficiency, and absolute or relative organ weights. No gross lesions were observed in any treatment group. The abstract did not note if histopathology was done.	35
Subacute Toxicity	Rat n=5/sex/ dose	2-Week Gavage	0, 18.75, 37.5, 75, 150, and 300 mg/kg/day in corn oil vehicle	Significant mortality occurred in the 150 and 300 mg/kg dose animals. The stomach was the primary target organ with evidence of inflammation, necrosis, ulceration, and hyperplasia. These lesions were present at the lowest dose and no NOEL was determined.	36
Subacute Toxicity	Mice n=5/sex/ dose	2-Week Gavage	0, 18.75, 37.5, 75, 150, and 300 mg/kg/day in corn oil vehicle	Significant mortality occurred in the 150 and 300 mg/kg dose animals. The stomach was the primary target organ with evidence of inflammation, necrosis, ulceration, and hyperplasia. The NOEL was reported as 18.75 mg/kg.	36
Subchronic Toxicity	Rat	8-Week Inhalation	20 mg/M^3 (7 ppm)	Only one dose was indicated and the only findings noted were that of increased serum albumin, decreased "alpha-2-globulins" and an increase in liver and lung RNA.	37
Subchronic Toxicity	Rat	3 Months	1.2 mg/M^3 (0.4 ppm)	The only effects reported in the reference were alterations in motor activity and blood hemoglobin content.	38
Subchronic Toxicity	Mouse	3 Months	1.2 mg/M^3 (0.4 ppm)	The only effects reported in the reference were alterations in motor activity and blood hemoglobin content.	38

Repeat Dose Studies (cont)

Study Type	Species	Exposure	Dose	Results	
Subchronic Toxicity	Rat n=10/sex/ dose	13-Week Gavage	0, 2.5, 5, 10, 20, and 40 mg/kg; 5 days/week in corn oil vehicle	Dose-related mortality was observed at doses ≥ 10 mg/kg. Body weights were sig. lower in males only at 40 mg/kg. There were no clinical signs, nor were changes in organ weights and clinical chemistries considered to be of toxicological significance. Doses of 20 and 40 mg/kg induced thickened stomachs, while microscopic changes of hyperplasia and hyperkeratosis were observed in the forestomach at dose levels ≥10 mg/kg. More severe inflammation, necrosis and ulceration were noted at the highest dose level. Acute inflammation was also noted in the nasal cavity of both sexes at 40 and 20 mg/kg and in females at 10 and 5 mg/kg. Nasal changes were not considered to have been a result of systemic toxicity. In females the NOEL was 2.5 mg/kg due to nasal effects but 10 mg/kg based on forestomach lesions. The NOEL in males was 5 mg/kg.	36
Subchronic Toxicity	Mice n=10/sex/ dose	13-Week Gavage	2.5, 5, 10, 20, and 40 mg/kg; 5 days/week in corn oil vehicle	No mortalities or gross lesions were noted. There were no clinical signs, nor were changes in organ weights and clinical chemistries considered to be of toxicological significance. No changes were noted for sperm morphology and vaginal cytology studies. Microscopic evidence of hyperplasia and inflammation were noted in the forestomach from high dose animals only. The NOEL for both sexes was 20 mg/kg.	36

Repeat Dose Studies (cont)

Study Type	Species	Exposure	Dose	Results	
Carcino-genicity	Rat (males only) n=23 and 27	113-Week Drinking water	0.6 and 6 mM (approx. 2 and 16 mg/kg/day)	Survival was similar for all groups. Body weights in the high dose group were lower throughout the study. No clinical chemistries was reported. Histological analysis of all other tissues revealed no difference in tumor rates between treated and control animals. Liver changes noted in 10/23 high dose animals consisted of moderate to severe fatty change, with focal necrosis, fibrosis, and cholestasis. In the respective treatment groups: hepatic carcinomas occurred in 0/23, 2/27, and 0/23; neoplastic nodules were present in 0/23, 9/27, and 1/23; while altered-foci were noted in 1/23, 23/27, and 13/23 animals.	39

Environmental Toxicity

Study Type	Results	Ref.
Acute Aquatic Toxicity (LC_{50})		
-Daphnia	2.0 ppm; 48-hr (NOEC 0.6 ppm)	40
-Fathead minnow	0.84 ppm; 96-hr (NOEC 0.51 ppm)	40
-Side-swimmer	2.7 ppm; 96-hr (NOEC 1.1 ppm)	40
-Rainbow trout	0.65 ppm; 96-hr (NOEC 0.27 ppm)	40
-Bluegill	3.5 ppm; 96-hr	40
-Tidewater Silversides	1.3 ppm; 96-hr	40
Acute Algae Effects (EC_{50}):		
24 hour	8.5 ppm	40
48 hour	0.65 ppm	40
72 hour	0.6 ppm	40
96 hour	<0.881 ppm	40
Plant Germination Effects (EC_{50}):		
-Lettuce seed	24 ppm	41
Plant Growth Effects (EC_{50}):		
-Barley	320 mg/M^3	42
-Wheat	340 mg/M^3	42
Oxygen Demand:		
ThOD	2.28 g O_2/g	10
COD	2.21 g O_2/g	10
BOD5	0.84-1.54 g O_2/g	10
Biodegradation	55% Degradation in a 28-day closed bottle test	40
Log P_{ow}	0.63	43

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