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TEXT SEARCHABLE DOCUMENT - 2009

Data Evaluation Report on the Chronic Toxicity of Ziram to Freshwater Invertebrates - Daphnia sp.

PMRA Submission Number {.....} EPA MRID No. 468233-01

Data Requirement:	PMRA Data Code	{.....}
	EPA DP Barcode	D323431
	OECD Data Point	{.....}
	EPA MRID	468233-01
	EPA Guideline	850.1300 (OPP §72-4b)

Test material: [^{14}C]Ziram **Radiochemical Purity:** 97%
Unlabeled Ziram Technical **Purity:** 98.2%
Common name Ziram
Chemical name: IUPAC: Zinc bis(dimethyldithiocarbamate)
CAS name: (*T*-4)-bis(Dimethylcarbamodithioato-κ*S*,κ*S*')zinc
CAS No.: 137-30-4
Synonyms: Ziram PHYTO

Primary Reviewer: Christie E. Padova **Signature:** Christie E. Padova
Staff Scientist, Dynamac Corporation **Date:** 10/16/06

Secondary Reviewer: Teri S. Myers **Signature:** Teri S Myers
Senior Scientist, Cambridge Environmental Inc. **Date:** 10/24/06

Primary Reviewer: ~~Allen Vaughan~~ Valerie Wolard, Biologist **Date:** 05/01/2009
EPA/OPP/EFED/ERB – V

Secondary Reviewer(s): {.....} **Date:** {.....}
{EPA/OECD/PMRA}

Reference/Submission No. {.....}

Company Code	{.....}	[For PMRA]
Active Code	{.....}	[For PMRA]
Use Site Category	{.....}	[For PMRA]
EPA PC Code	034805	

Date Evaluation Completed: {dd-mm-yyyy}

CITATION: Palmer, S.J., T.Z. Kendall, and H.O. Krueger. 2006. Ziram: A Flow-Through Life-Cycle Toxicity Test with the Cladoceran (*Daphnia magna*). Unpublished study performed by Wildlife International., Ltd., Easton, MD. Laboratory Project No. 602A-101. Study submitted by The Ziram Task Force c/o Cerexagri, Inc., King of Prussia, PA. Study initiated February 8, 2006 and submitted April 24, 2006.

DISCLAIMER: This document provides guidance for EPA and PMRA reviewers on how to complete a data evaluation record after reviewing a scientific study concerning the chronic toxicity of a pesticide to freshwater invertebrates. It is not intended to prescribe conditions to any external party for conducting this study nor to establish absolute criteria regarding the assessment of whether the study is scientifically sound and whether the study satisfies any applicable data requirements. Reviewers are expected to review and to determine for each study, on a case-by-case basis, whether it is scientifically sound and provides sufficient information to satisfy applicable data requirements. Studies that fail to meet any of the conditions may be accepted, if appropriate; similarly, studies that meet all of the conditions may be rejected, if appropriate. In sum, the reviewer is to take into account the totality of factors related to the test methodology and results in determining the acceptability of the study.

2084012

The 21-day-chronic toxicity of ziram to *Daphnia magna* was studied under flow-through conditions. Daphnids were exposed to a mixture of radiolabelled and unlabelled ziram at nominal concentrations of 0 (negative and solvent controls), 9.4, 19, 38, 75, and 150 µg ai/L. Mean measured concentrations were <LOQ for both negative and solvent controls, 9.3, 22, 39, 78 and 154 µg/L with TWA concentrations of <1.37 (<LOQ, controls), 9.2, 22, 39, 77, and 154 µg total residues/L, respectively. Length was the most sensitive endpoint, with significant reductions at the 78 and 154 µg total residues/L treatment levels. Based on this effect, the 21-day NOAEC and LOAEC were 39 and 78 µg total residues/L, respectively.

Mortality was significantly-reduced at the 154 µg total residues/L test level compared to the negative control (45 versus 100% survival, respectively). In addition, the number of offspring produced per reproductive day was also significantly-reduced at the 154 µg total residues/L level compared to the negative control (4.29 versus 11.24 young per day, respectively), although there was no treatment-related effect on the time of first brood release (visually-determined). The reviewer calculated 21-day moving average LC_{50} for survival was 144 (115-261) µg total residues/L. The reviewer calculated 21-day EC_{50} (with 95% C.I.) for reproduction was 130 (110-160) µg total residues/L.

Total length was the most sensitive endpoint, with statistically-significant reductions compared to the negative control at the 78 and 154 µg total residue/L levels (5.13 versus 4.80 and 4.77 mm, respectively). A similar effect on dry weight was not observed, with no statistically-significant differences at any treatment level.

This study is scientifically sound and does satisfy the guideline requirement for a chronic toxicity study with freshwater invertebrates.

Results Synopsis

Test Organism Age (eg. 1st instar): Neonates, <24 hours old
Test Type (Flow-through, Static, Static Renewal): Flow-through

LOAEC: 78 µg total residues/L
NOAEC: 39 µg total residues/L

Endpoint(s) Affected: First generation survival, reproduction, and terminal total lengths
Most Sensitive Endpoint(s): Total lengths

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I. MATERIALS AND METHODS:

GUIDELINE FOLLOWED: The study protocol was based on procedures outlined in the U.S. Environmental Protection Agency Series 850-Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1300: *Daphnid Chronic Toxicity Test*; OECD Guidelines for Testing of Chemicals, No. 211, *Daphnia magna Reproduction Test*; and ASTM Standard E 1193-97, *Standard guide for Conducting Daphnia magna Life-Cycle Toxicity Tests*. Deviations from OPPTS Guideline 850.1300 included:

1. Test samples were analyzed only for total radioactive residues using LSC. The radioactivity was not further characterized; therefore, the stability of ziram under actual use conditions was not verified.

2. From day 16 and thereafter, gentle aeration was added to each test chamber to ensure that dissolved oxygen levels remained >60% saturation. Aeration is generally not recommended for use; however, if used, it should not cause instability of test substance concentrations. In this study, as solutions were only analyzed for total radioactive residues, it was unknown if the aeration affected the stability of ziram in solution.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality claims statements were provided. This study was conducted in accordance with GLP Standards as published in 40 CFR Part 160 with the following exception: periodic analysis of well water for potential contaminants.

A. MATERIALS:

1. Test Material Ziram Technical and ^{14}C-Ziram

Description: Solids

Lot No./Batch No. : G4A0051877 (non-radiolabelled) and XV/36 (radiolabelled)

Purity: 98.2% (non-radiolabelled) and 97% (radiolabelled)

Stability of compound under test conditions: Unverified. Test samples collected on days 0, 7, 14, 21, 28, and 34 were analyzed for total radioactivity using LSC. All results were within 20% among replicate measurements; however, the radioactivity was not further characterized.

Storage conditions of test chemicals: Frozen

Physicochemical properties of Ziram.

Parameter	Values	Comments
Water solubility at 20EC	Not reported	
Vapor pressure	Not reported	
UV absorption	Not reported	
pKa	Not reported	
Kow	Not reported	

(OECD recommends water solubility, stability in water and light, pKa, Pow, and vapor pressure of test compound)

2. Test Organism:

Species: *Daphnia magna*, <24 hours old
EPA and OECD recommend Daphnia magna

Age of the parental stock: ≥15 days old
EPA recommends that young daphnids #24 hours old from a separate parental culture be used

Source: Laboratory cultures
EPA requires all test organisms must be produced from laboratory reared culture that has been maintained for at least 21 days at test conditions in dilution water with renewal of the culture medium at least three times per week.

B. STUDY DESIGN:

1. Experimental Conditions

a. Range-finding Study: The concentrations for the definitive study were selected in consultation with the Sponsor, and were based on exploratory range-finding toxicity data (not further specified).

b. Definitive Study

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Table 1: Experimental Parameters

Parameter	Details	Remarks *Criteria*
Parental acclimation: Period: Conditions: (same as test or not) Feeding: Health (any mortality observed):	≥15 days Same as test During culturing, the daphnids were fed two to four times daily with a mixture of yeast, cereal grass media, and trout chow (YCT), as well as a suspension of the freshwater green alga, *Selenastrum capricornutum.* No signs of disease or stress were observed in adults.	The adult daphnids used to supply the neonates had produced at least one prior brood. Adult daphnids in the general cultures had produced an average of at least three young per adult per day over the 7-day period prior to the test. *EPA recommends that prior to testing, daphnids that are at least 10-12 days old (those that have had at least one brood) should be separated from the culture, put in separate container and maintained for at least 21 days to insure that good health conditions are present*
Test condition: static renewal/flow-through: Type of dilution system- for flow through method: Flow rate: Renewal rate for static renewal:	Flow-through Continuous-flow serial diluter Approx. 5 volume additions per day N/A	The diluter was calibrated prior to test initiation and verified at approximately weekly intervals during the test. The general operation of the diluter was checked visually at least two times per day during testing. The flow splitting accuracy was checked weekly and varied by no more than ±10% of the mean for the two replicates. *(EPA requires consistent flow rate of 5-10 vol/24 hours, meter systems calibrated before study and checked twice daily during test period)*
Aeration, if any	Gentle aeration was added to each test chamber on day 16 to maintain DO levels >60% saturation. DO was monitored daily thereafter.	Aeration is generally considered OK for use if it does not cause instability of the test substance levels. As actual ziram concentrations were not determined, it was unknown if aeration affected the stability of ziram in solution. *EPA recommends test chambers should not be aerated*

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Parameter	Details	Remarks *Criteria*
Duration of the test	21 days	*Recommended duration is 21 days.*
Test vessel Material (glass/stainless steel): Size (for growth and reproduction/survival test): Fill volume:	Glass beakers with nylon mesh screens over two holes on opposite sides of each compartment. 300 mL 8.3-cm depth (not otherwise reported)	Two test compartments were suspended in each of two test chambers. Test chambers were 25-L Teflon-lined stainless steel aquaria filled with approx. 22 L (28.9-cm depth) of test solution. *1. Recommended Material: Glass, No. 316 stainless steel, or perfluorocarbon plastics* *2. Recommended Size: 250 ml with 200 ml fill volume; 100 ml with 80 ml fill volume* *OECD guideline recommends that parent animals be maintained individually; one per vessel, with 50 - 100 ml of medium in each vessel.*
Source of dilution water	Moderately-hard freshwater was obtained from a well approximately 40-m deep located on site. The well water was passed through a sand filter, aerated, filtered again (0.45 µm), and UV-sterilized prior to use.	During the 4-week period preceding the test, analysis of the dilution water yielded the following average values (4 measurements): specific conductance 299 µmhos/cm, hardness 136 mg/L as $CaCO_3$, alkalinity 181 mg as CaCO3, and pH 8.2. *Recommended source of dilution water includes unpolluted well or spring water that has been tested for contaminants, or appropriate reconstituted water (see ASTM for details).*

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Parameter	Details	Remarks *Criteria*
Water parameters: Hardness pH Dissolved oxygen Temperature Total Organic Carbon Particulate matter Metals Pesticides Chlorine	 132-144 mg/L as $CaCO_3$ 7.9-8.2 ≥6.0 mg/L (≥67% saturation) 19.5-20.5°C (constant throughout study) <1 mg C/L Not determined Calcium at 33.1 mg/L, chloride at 2.7 mg/L, fluoride at 0.56 mg/L, magnesium at 13.3 mg/L, potassium at 7.65 mg/L, and sodium at 19.1 mg/L (from periodic analysis) <LOD (from periodic analysis) Not determined	Dilution water sampling and results fulfilled all OPPTS criteria. Results of periodic analysis for pesticides, organics, and metals were provided from water collected on 12/15/05. ***Recommended hardness:*** *160 to 180 mg/L as $CaCO_3$; OECD recommends > 140 mg/L as $CaCO_3$* ***Recommended pH:*** *7.6 to8.0 pH should not deviate by more than 1.0 unit for more than 48 hours. OECD recommends that pH range be 6 - 9 and does not vary more than 1.5 units in any one test.* ***Recommended dissolved oxygen:*** *renewal should not drop below 50% for more than 48 hours.* *Recommended flow-through: ∃60% throughout test.* ***Recommended temperature:*** *20EC ∀ 2EC.; should not deviate from 20EC by more than 5EC for more than 48 hours. OECD recommends a range of 18 - 22°C; temperature should not vary more than ∀ 2°C* *OECD guideline recommends that total organic carbon < 2 mg/L*
Number of replicates	Two per level	Fulfills OPPTS guidance. *Number of replicates should include a control(s) and at least 5 test concentrations; dilution factor should not be greater than 50%..* *OECD recommends that at least 5 test concentrations be used in a geometric series with a separation factor not exceeding 3.2.*

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Parameter	Details	Remarks *Criteria*
Number of organisms: For growth and reproduction: For survival test:	20 per level (same)	The twenty daphnia were divided into four test compartments with five daphnia per compartment. There were two compartments per test chamber and two replicate chambers per level. Fulfills OPPTS guidance. *Recommended number of organisms include 22 daphnids/test concentration; 7 test chambers should contain 1 daphnid each, and 3 test chambers contain 5 daphnids each.* *OECD recommends holding a minimum of 10 daphnids individually for static tests.* *For flow-through tests, 40 animals should be divided into 4 groups of 10 animals at each test concentration.*
Treatment Concentrations: nominal: measured: TWA (reviewer-calculated):	0 (negative and solvent controls), 9.4, 19, 38, 75, and 150 µg ai/L <1.37 (<LOQ, controls), 9.3, 22, 39, 78, and 154 µg total residues/L <1.37 (<LOQ, controls), 9.2, 22, 39, 77, and 154 µg total residues/L	Total radioactive concentrations were determined using LSC at 0, 7, 14, 17, and 21 days. All measured concentrations were within 20% among replicates. The radioactivity was not characterized to determine what percentage was parent material.
Solvent (type, percentage, if used)	Dimethyl formamide, 0.1 ml/L	*Solvent concentration should not exceed 0.5 ml/L for static tests or 0.1 ml/L for flow-through tests.* *Recommended solvents include dimethylformamide, triethylene glycol, methanol, acetone and ethanol.* *OECD recommends # 0.1 ml/L of solvent.*

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Parameter	Details	Remarks *Criteria*
Lighting	16 hours light/8 hours dark, with 30-minute transition periods	Light intensity at test initiation was 172 lux over one representative test chamber. *Recommended photoperiod is 16 hours light and 8 hours of dark.*
Recovery of chemical: Frequency of measurement: LOD: LOQ:	94.9-125% of nominal Days 0, 7, 14, 17, and 21 Not reported 1.37 µg total residues/L	Based on LSC analysis of test samples.
Positive control {if used, indicate the chemical and concentrations}	N/A	
Other parameters, if any Feeding:	During testing, the daphnids were fed a mixture of yeast, cereal grass media, and trout chow (YCT), as well as a suspension of the freshwater green alga, *Selenastrum capricornutum*. Daphnids were fed three times per day through day 7 of the test and then two to four times per day for the remainder of the test. At each feeding, each chamber received 0.75 ml of YCT and 1.5 ml of algae.	It was reported that although the rate of feed given to the daphnids exceeded the OECD guideline recommended amount (of 0.1 to 0.2 mg C per daphnid per day), that an excess amount was fed in order to maintain sufficient feed in the flow-through system to support acceptable reproduction rates.

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2. Observations:

Table 2: Observations

Parameters	Details	Remarks
		Criteria
Data endpoints measured (list)	- Survival of first-generation daphnids - Immobilization of first-generation daphnids - First day of reproduction - Number of live young produced per reproductive day - Measurement of growth (total length, and dry weight) - Sub-lethal signs of toxicity	Mortality and immobility were assessed separately. --- *Recommended endpoints measured:* *- Survival of first-generation daphnids,* *- Number of young produced per female,* *- Dry weight (required) and length (optional) of each first generation daphnid alive at the end of the test,* *- Observations of other effects or clinical signs.*
Observation intervals	Each first-generation daphnid was observed daily. With the onset of reproduction, neonates were counted and discarded every Monday, Wednesday, and Friday. Growth was determined for each surviving first-generation daphnid at the end of the test.	
Were raw data included?	Yes	
Other observations, if any	N/A	

II. RESULTS AND DISCUSSION

A. MORTALITY:

After 21 days, survival averaged 100% for the negative control group, 95% for the solvent control group, and 100, 95, 95, 90, and 45% for the 9.3, 22, 39, 78, and 154 mg total residues/L treatment groups, respectively. Survival was statistically-reduced at the 154 mg total residues/L level ($p \leq 0.05$) compared to the pooled control. The 21-day LC_{50} was >78 µg total residues/L and estimated as approximately 144 µg total residues/L. The NOAEC was 78 µg total residues/L.

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Table 3: Effect of Ziram on Growth and Survival of Daphnia sp.

Treatment Mean Measured, µg total residues/L (and nominal, µg ai/L) concentrations	Mortality (dead or immobile)		First Day of Reproduction	Number of Live Young Produced Per Reproductive Day	Mean Length (mm)	Mean Dry Weight (mg)
	No. Dead	%				
Control (dilution water only)	0	0	8.2	11.24	5.13	0.69
Solvent control	1	5	8.0	14.35	5.25	0.73
9.3 (9.4)	0	0	8.5	11.97	5.15	0.81
22 (19)	1	5	8.8	11.71	5.03	0.84
39 (38)	1	5	8.5	14.82	5.10	0.84
78 (75)	2	10	8.2	10.90	4.80*	0.74
154 (150)	11	55*	8.3	4.29*	4.77*	0.69
NOAEC	78 µg total residues/L		154 µg total residues/L	78 µg total residues/L	39 µg total residues/L	154 µg total residues/L
LOAEC	154 µg total residues/L		>154 µg total residues/L	154 µg total residues/L	78 µg total residues/L	>154 µg total residues/L

* Statistically significant difference from the pooled control (mortality and growth) or from the solvent control (reproduction) at $p \leq 0.05$.

B. EFFECT ON REPRODUCTION:

No treatment-related effect on the time of first brood release was observed, with averages (reviewer-calculated) ranging from 8.0-8.8 days for all control and treatment groups. However, the number of offspring produced per reproductive day was statistically-reduced at the 154 µg total residues/L level compared to the solvent control (4.29 versus 14.35 young per day, respectively). No treatment-related differences were observed at the lower treatment levels. The 21-day EC_{50} (with 95% C.I.) for reproduction was 121 (99-152) µg total residues/L. The NOAEC for reproduction was 78 µg total residues/L.

Terminal length was the most sensitive endpoint, with statistically-reduced differences from the pooled control at the 78 and 154 µg total residues/L levels (5.19 mm compared to 4.80 and 4.77 mm, respectively). No other statistically-significant differences were observed at the lower treatment levels, or at any treatment level for dry weights of surviving daphnia. The NOAEC for length was 39 µg total residues/L.

C. REPORTED STATISTICS:

Data that were statistically analyzed included 1) first-generation survival, 2) the number of live young produced per reproductive day, 3) the mean total length of surviving first-generation daphnia at study termination, and 4) the mean dry weight of surviving first-generation daphnia at study termination. The time to fist brood release

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was recorded and visually assessed, but not statistically analyzed.

For all endpoints, responses from the negative and solvent control groups were compared using a t-test. No significant differences were observed for survival and growth, the controls were pooled for subsequent analyses. Significant differences were observed between the control responses regarding reproduction data, and therefore the data were compared to the solvent control responses. Survival data were analyzed using Chi-square and Fisher's Exact test to identify treatment groups that showed a statistically significant difference from controls ($p \leq 0.05$). Reproduction and growth data were checked for normality using the Chi-square and for homogeneity of variance using Levene's test ($p=0.01$), and were subsequently analyzed using analysis of variance (ANOVA) and Bonferroni's t-test to identify treatments that were significantly different from the control ($p \leq 0.05$).

The NOAEC and LOAEC were based on significance data. The binomial test indicated that the 21-day LC_{50} was >78 µg total residues/L, and an approximate LC_{50} was obtained by non-linear interpolation. For reproduction, the method for calculating the 21-day EC_{50} with 95% C.I. was not reported. All analyses were performed using TOXSTAT or SAS software programs and mean-measured concentrations.

D. VERIFICATION OF STATISTICAL RESULTS:

Statistical Method: Mortality data were analyzed using Fisher's Exact test. Reproduction, length, and dry weight data were analyzed using the Chi-square and Shapiro-Wilks tests for normality and the Hartley and Bartlett's tests for homogeneity of variances. Data did not require transformation to satisfy the assumptions of ANOVA. For all endpoints, the negative control was compared to the solvent control using a Student's t-Test; for reproduction, a significant difference was detected, with the solvent control reproduction greater than the negative control reproduction. Because the solvent did not appear to inhibit reproduction, this difference was not considered to have impacted the acceptability of the study. There were no differences between the negative and solvent control groups for any other endpoint and all treatment means were compared to the negative control groups. The NOAEC values were determined using ANOVA (dry weight), followed by Bonferroni's (reproduction) or William's (length) tests. These analyses were conducted using TOXSTAT statistical software. The LC_{50} was determined using the moving average method via Toxanal statistical software and the EC_{50} based on reproduction was determined using the Probit method via Nuthatch software. All estimates were calculated using the time-weighted average measured concentrations.

First generation survival:
NOAEC: 78 µg total residues/L
LOAEC: 154 µg total residues/L
LC_{50}: 144 µg total residues/L 95%C.I.: 115-261 µg total residues/L
Slope: N/A

Neonate production (reproduction):
NOAEC: 78 µg total residues/L
LOAEC: 154 µg total residues/L
EC_{50}: 130 µg total residues/L 95%C.I.: 110-160 µg total residues/L
Slope: 5.43±2.06

Total Lengths:
NOAEC: 39 µg total residues/L
LOAEC: 78 µg total residues/L

Dry Weight:
NOAEC: 154 µg total residues/L
LOAEC: >154 µg total residues/L

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Endpoint(s) Affected: Survival, Reproduction, Length
Most Sensitive Endpoint(s): Length

E. STUDY DEFICIENCIES:

This study is scientifically sound and provides useful data on the chronic toxicity of ziram to *Daphnia magna*. However, as test samples were only analyzed for total radioactive residues, the stability of ziram under test conditions was not determined. Furthermore, it was not determined if aeration had any affect on the stability of ziram in solution.

F. REVIEWER=S COMMENTS:

The reviewer's statistical results were identical to the study authors', despite the fact that the study author compared treatment responses to either the solvent or pooled control groups, while the reviewer compared treatment responses to the negative control groups. The study authors used the mean measured concentrations, which were not remarkably different from the reviewer-calculated TWA concentrations. The reviewer's EC_{50} value based on reproduction provided a slightly narrower 95% confidence interval than the study authors' estimate, so it was reported in the Executive Summary and Conclusions sections.

Dissolved oxygen concentrations were ≥8.3 mg/L (≥92% saturation) at test initiation and gradually declined to a minimum of 6.0 mg/L (67% of saturation) by day 16. Gentle aeration was initiated at that time, and concentrations were ≥7.0 mg/L (≥78% saturation) throughout the remainder of the test.

All test solutions appeared clear and colorless in the diluter mixing chambers and in the test chambers at test initiation and termination.

Experimental test dates were February 15 – March 8, 2006.

G. CONCLUSIONS:

This study is scientifically sound and is thus acceptable. Based upon treatment-related effects on terminal total lengths (the most sensitive endpoint), the NOAEC and LOAEC are 39 and 78 µg total residues/L, respectively. The 21-day LC_{50} for first-generation survival was approximately 144 µg total residues/L. The 21-day EC_{50} for reproduction was approximately 130 µg total residues/L.

LOAEC: 78 µg total residues/L
NOAEC: 39 µg total residues/L

Endpoint(s) Affected: First generation survival, reproduction, and terminal total lengths
Most Sensitive Endpoint(s): Total lengths

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III. REFERENCES:

U.S. Environmental Protection Agency. 1996. OPPTS Number 850.1300: *Daphnid Chronic Toxicity Test.* Series 850 - Ecological Effects Test Guidelines (*draft*).

Organization for Economic Cooperation and Development. 1997. Guideline 211: *Daphnia magna Reproduction Test.* OECD Guidelines for Testing of Chemicals.

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APPENDIX 1: OUTPUT OF REVIEWER'S STATISTICAL VERIFICATION:

SUMMARY OF FISHERS EXACT TESTS

GROUP	IDENTIFICATION	NUMBER EXPOSED	NUMBER DEAD	SIG (P=.05)
	CONTROL	20	0	
1	9.2	20	0	
2	22	20	1	
3	39	20	1	
4	**7.7**	**20**	**2**	
5	154	20	11	*

AN APPROXIMATE LC50 FOR THIS SET OF DATA IS 143.7908

RESULTS CALCULATED USING THE MOVING AVERAGE METHOD

SPAN	**G**	**LC50**	**95 PERCENT CONFIDENCE LIMITS**	
1	**.4025004**	**143.7907**	**115.0882**	**261.2652**

RESULTS CALCULATED USING THE PROBIT METHOD

ITERATIONS	G	H	GOODNESS OF FIT PROBABILITY
5	.2694578	1	.316334

SLOPE = 2.467984
95 PERCENT CONFIDENCE LIMITS = 1.18687 AND 3.749097

LC50 = 164.7476
95 PERCENT CONFIDENCE LIMITS = 113.6992 AND 394.6194

LC10 = 50.37661
95 PERCENT CONFIDENCE LIMITS = 24.63083 AND 71.06688

```
reproduction
File: 3301r          Transform: NO TRANSFORM

       t-test of Solvent and Blank Controls          Ho:GRP1 MEAN = GRP2 MEAN
-----------------------------------------------------------------------------
-
 GRP1 (SOLVENT CRTL) MEAN =    11.2400     CALCULATED t VALUE =    -2.4540
 GRP2 (BLANK CRTL) MEAN   =    14.3525     DEGREES OF FREEDOM =     6
 DIFFERENCE IN MEANS      =    -3.1125
-----------------------------------------------------------------------------
-
TABLE t VALUE (0.05 (2), 6) =  2.447**   SIGNIFICANT DIFFERENCE at alpha=0.05
TABLE t VALUE (0.01 (2), 6) =  3.707    NO significant difference at
alpha=0.01

Reproduction
File: 3301r        Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies
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INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	1.541	5.566	8.786	5.566	1.541
OBSERVED	0	8	6	9	0

Calculated Chi-Square goodness of fit test statistic = 7.1485
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.

Reproduction
File: 3301r Transform: NO TRANSFORMATION

Shapiro Wilks test for normality

D = 90.646

W = 0.931

Critical W (P = 0.05) (n = 23) = 0.914
Critical W (P = 0.01) (n = 23) = 0.881

Data PASS normality test at P=0.01 level. Continue analysis.

Reproduction
File: 3301r Transform: NO TRANSFORMATION

Hartley test for homogeneity of variance

Calculated H statistic (max Var/min Var) = 3.94
Closest, conservative, Table H statistic = 184.0 (alpha = 0.01)

Used for Table H ==> R (# groups) = 6, df (# reps-1) = 3
Actual values ==> R (# groups) = 6, df (# avg reps-1) = 2.83
(average df used)

Data PASS homogeneity test. Continue analysis.

NOTE: This test requires equal replicate sizes. If they are unequal but do not differ greatly, the Hartley test may still be used as an approximate test (average df are used).

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reproduction
File: 3301r        Transform: NO TRANSFORMATION

Bartletts test for homogeneity of variance
-----------------------------------------------------------------------------

Calculated B statistic =    1.28
Table Chi-square value =   15.09  (alpha = 0.01)
Table Chi-square value =   11.07  (alpha = 0.05)

Average df used in calculation  ==>   df (avg n - 1) =   2.83
Used for Chi-square table value ==>   df (#groups-1) =   5
-----------------------------------------------------------------------------

Data PASS homogeneity test at 0.01 level. Continue analysis.

NOTE: If groups have unequal replicate sizes the average replicate size is
      used to calculate the B statistic (see above).

reproduction
File: 3301r        Transform: NO TRANSFORMATION

                                ANOVA TABLE
-----------------------------------------------------------------------------

SOURCE             DF              SS                MS              F
-----------------------------------------------------------------------------
Between             5            199.059           39.812          7.467

Within (Error)     17             90.646            5.332
-----------------------------------------------------------------------------
Total              22            289.705
-----------------------------------------------------------------------------

  Critical F value =   2.81  (0.05,5,17)
  Since  F > Critical F  REJECT  Ho:All groups equal


reproduction
File: 3301r        Transform: NO TRANSFORMATION

     BONFERRONI T-TEST   -   TABLE 1 OF 2          Ho:Control<Treatment
-----------------------------------------------------------------------------
                                TRANSFORMED    MEAN CALCULATED IN
GROUP    IDENTIFICATION           MEAN          ORIGINAL UNITS    T STAT  SIG
----- --------------------     -----------    ------------------  ------  ---
  1            neg control      11.240             11.240
  2                    9.2      11.970             11.970         -0.447
  3                     22      11.705             11.705         -0.285
  4                     39      14.815             14.815         -2.190
  5                     77      10.903             10.903          0.207
  6                    154       4.290              4.290          3.941  *
-----------------------------------------------------------------------------
Bonferroni T table value =  2.57     (1 Tailed Value, P=0.05,  df=17,5)
```


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reproduction
File: 3301r Transform: NO TRANSFORMATION

BONFERRONI T-TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	neg control	4			
2	9.2	4	4.191	37.3	-0.730
3	22	4	4.191	37.3	-0.465
4	39	4	4.191	37.3	-3.575
5	77	4	4.191	37.3	0.337
6	154	3	4.527	40.3	6.950

reproduction
File: 3301r Transform: NO TRANSFORMATION

WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	neg control	4	11.240	11.240	12.433
2	9.2	4	11.970	11.970	12.433
3	22	4	11.705	11.705	12.433
4	39	4	14.815	14.815	12.433
5	77	4	10.903	10.903	10.903
6	154	3	4.290	4.290	4.290

reproduction
File: 3301r Transform: NO TRANSFORMATION

WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

IDENTIFICATION	ISOTONIZED MEAN	CALC. WILLIAMS	SIG P=.05	TABLE WILLIAMS	DEGREES OF FREEDOM
neg control	12.433				
9.2	12.433	0.730		1.74	k= 1, v=17
22	12.433	0.730		1.82	k= 2, v=17
39	12.433	0.730		1.85	k= 3, v=17
77	10.903	0.207		1.87	k= 4, v=17
154	4.290	3.941	*	1.87	k= 5, v=17

s = 2.309
Note: df used for table values are approximate when v > 20.

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Estimates of EC%

Parameter	Estimate	95% Bounds Lower	95% Bounds Upper	Std.Err.	Lower Bound /Estimate
EC5	65.	33.	1.3E+02	0.14	0.51
EC10	76.	43.	1.3E+02	0.12	0.58
EC25	98.	68.	1.4E+02	0.076	0.69
EC50	**1.3E+02**	**1.1E+02**	**1.6E+02**	**0.041**	**0.82**

Slope = 5.43 Std.Err. = 2.06

Goodness of fit: p = 0.21 based on DF= 3.0 17.

3301R : reproduction

Observed vs. Predicted Treatment Group Means

Dose	#Reps.	Obs. Mean	Pred. Mean	Obs. -Pred.	Pred. %Control	%Change
0.00	4.00	11.2	12.4	-1.17	100.	0.00
9.20	4.00	12.0	12.4	-0.436	100.	2.04e-08
22.0	4.00	11.7	12.4	-0.701	100.	0.00138
39.0	4.00	14.8	12.4	2.44	99.8	0.224
77.0	4.00	10.9	11.1	-0.161	89.2	10.8
154.	3.00	4.29	4.28	0.0149	34.5	65.5

length
File: 33011 Transform: NO TRANSFORM

t-test of Solvent and Blank Controls Ho:GRP1 MEAN = GRP2 MEAN

GRP1 (SOLVENT CRTL) MEAN = 5.1250 CALCULATED t VALUE = -1.3868
GRP2 (BLANK CRTL) MEAN = 5.2500 DEGREES OF FREEDOM = 6
DIFFERENCE IN MEANS = -0.1250

TABLE t VALUE (0.05 (2), 6) = 2.447 NO significant difference at alpha=0.05
TABLE t VALUE (0.01 (2), 6) = 3.707 NO significant difference at alpha=0.01

length
File: 33011 Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5

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```
EXPECTED     1.541          5.566              8.786           5.566       1.541
OBSERVED     0              8                  7               8           0

-------------------------------------------------------------------------------
Calculated Chi-Square goodness of fit test statistic =   5.5738
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.


length
File: 33011        Transform: NO TRANSFORMATION

Shapiro Wilks test for normality
-------------------------------------------------------------------------------

D =    0.712

W =    0.984

Critical W (P = 0.05) (n = 23) = 0.914
Critical W (P = 0.01) (n = 23) = 0.881
-------------------------------------------------------------------------------

Data PASS normality test at P=0.01 level. Continue analysis.


length
File: 33011         Transform: NO TRANSFORMATION

Hartley test for homogeneity of variance
-------------------------------------------------------------------------------

Calculated H statistic (max Var/min Var) =    5.26
Closest, conservative, Table H statistic =  184.0  (alpha = 0.01)

Used for Table H ==>     R (# groups) =   6,    df (# reps-1) =       3
Actual values    ==>     R (# groups) =   6,    df (# avg reps-1) =   2.83
                                                (average df used)

-------------------------------------------------------------------------------

Data PASS homogeneity test. Continue analysis.

NOTE: This test requires equal replicate sizes. If they are unequal
      but do not differ greatly, the Hartley test may still be used
      as an approximate test (average df are used).


length
File: 33011        Transform: NO TRANSFORMATION

Bartletts test for homogeneity of variance
```


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-----------------------------------------------------------------------------

Calculated B statistic =    3.63
Table Chi-square value =   15.09  (alpha = 0.01)
Table Chi-square value =   11.07  (alpha = 0.05)

Average df used in calculation  ==>   df (avg n - 1) =   2.83
Used for Chi-square table value ==>   df (#groups-1) =   5
-----------------------------------------------------------------------------

Data PASS homogeneity test at 0.01 level. Continue analysis.

NOTE: If groups have unequal replicate sizes the average replicate size is
      used to calculate the B statistic (see above).

length
File: 33011        Transform: NO TRANSFORMATION

                                ANOVA TABLE
-----------------------------------------------------------------------------

SOURCE              DF              SS                MS              F
-----------------------------------------------------------------------------
Between              5              0.518            0.104           2.476

Within (Error)      17              0.712            0.042
-----------------------------------------------------------------------------
Total               22              1.230
-----------------------------------------------------------------------------

  Critical F value =   2.81  (0.05,5,17)
  Since  F < Critical F  FAIL TO REJECT  Ho:All groups equal


length
File: 33011        Transform: NO TRANSFORMATION

      BONFERRONI T-TEST   -   TABLE 1 OF 2             Ho:Control<Treatment
-----------------------------------------------------------------------------
                                TRANSFORMED    MEAN CALCULATED IN
GROUP    IDENTIFICATION           MEAN          ORIGINAL UNITS    T STAT  SIG
----- --------------------     -----------    ------------------  ------  ---
  1            neg control       5.125              5.125
  2                    9.2       5.150              5.150         -0.173
  3                     22       5.025              5.025          0.690
  4                     39       5.100              5.100          0.173
  5                     77       4.800              4.800          2.243
  6                    154       4.767              4.767          2.289
-----------------------------------------------------------------------------
Bonferroni T table value =  2.57     (1 Tailed Value, P=0.05,  df=17,5)


length
File: 33011        Transform: NO TRANSFORMATION
```


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BONFERRONI T-TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	neg control	4			
2	9.2	4	0.372	7.3	-0.025
3	22	4	0.372	7.3	0.100
4	39	4	0.372	7.3	0.025
5	77	4	0.372	7.3	0.325
6	154	3	0.402	7.8	0.358

length
File: 33011 Transform: NO TRANSFORMATION

WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	neg control	4	5.125	5.125	5.138
2	9.2	4	5.150	5.150	5.138
3	22	4	5.025	5.025	5.063
4	39	4	5.100	5.100	5.063
5	77	4	4.800	4.800	4.800
6	154	3	4.767	4.767	4.767

length
File: 33011 Transform: NO TRANSFORMATION

WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

IDENTIFICATION	ISOTONIZED MEAN	CALC. WILLIAMS	SIG P=.05	TABLE WILLIAMS	DEGREES OF FREEDOM
neg control	5.138				
9.2	5.138	0.086		1.74	k= 1, v=17
22	5.063	0.432		1.82	k= 2, v=17
39	**5.063**	**0.432**		**1.85**	**k= 3, v=17**
77	4.800	2.246	*	1.87	k= 4, v=17
154	4.767	2.293	*	1.87	k= 5, v=17

s = 0.205
Note: df used for table values are approximate when v > 20.

dry weight
File: 3301w Transform: NO TRANSFORM

t-test of Solvent and Blank Controls Ho:GRP1 MEAN = GRP2 MEAN

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```
-----------------------------------------------------------------------------
-
 GRP1 (SOLVENT CRTL) MEAN =     0.6925     CALCULATED t VALUE =    -0.6450
 GRP2 (BLANK CRTL) MEAN   =     0.7325     DEGREES OF FREEDOM =     6
 DIFFERENCE IN MEANS      =    -0.0400
-----------------------------------------------------------------------------
-
TABLE t VALUE (0.05 (2), 6) =  2.447    NO significant difference at
alpha=0.05
TABLE t VALUE (0.01 (2), 6) =  3.707    NO significant difference at
alpha=0.01

dry weight
File: 3301w        Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies
-----------------------------------------------------------------------------

INTERVAL     <-1.5     -1.5 to <-0.5     -0.5 to 0.5     >0.5 to 1.5     >1.5
             ------    -------------     -----------     -----------     ----

EXPECTED     1.541         5.566             8.786           5.566       1.541
OBSERVED     0             7                 10              6           0

-----------------------------------------------------------------------------
Calculated Chi-Square goodness of fit test statistic =   3.6530
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.


dry weight
File: 3301w        Transform: NO TRANSFORMATION

Shapiro Wilks test for normality
-----------------------------------------------------------------------------

D =    0.341

W =    0.972

Critical W (P = 0.05) (n = 23) = 0.914
Critical W (P = 0.01) (n = 23) = 0.881
-----------------------------------------------------------------------------

Data PASS normality test at P=0.01 level. Continue analysis.


dry weight
File: 3301w        Transform: NO TRANSFORMATION

Hartley test for homogeneity of variance
-----------------------------------------------------------------------------
```


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```
Calculated H statistic (max Var/min Var) =    7.23
Closest, conservative, Table H statistic =  184.0   (alpha = 0.01)

Used for Table H ==>     R (# groups) =   6,    df (# reps-1) =        3
Actual values    ==>     R (# groups) =   6,    df (# avg reps-1) =    2.83
                                                (average df used)

-----------------------------------------------------------------------------

Data PASS homogeneity test. Continue analysis.

NOTE: This test requires equal replicate sizes. If they are unequal
      but do not differ greatly, the Hartley test may still be used
      as an approximate test (average df are used).


dry weight
File: 3301w        Transform: NO TRANSFORMATION

Bartletts test for homogeneity of variance
-----------------------------------------------------------------------------

Calculated B statistic =    3.87
Table Chi-square value =   15.09   (alpha = 0.01)
Table Chi-square value =   11.07   (alpha = 0.05)

Average df used in calculation  ==>   df (avg n - 1) =   2.83
Used for Chi-square table value ==>   df (#groups-1) =   5
-----------------------------------------------------------------------------

Data PASS homogeneity test at 0.01 level. Continue analysis.

NOTE: If groups have unequal replicate sizes the average replicate size is
      used to calculate the B statistic (see above).

dry weight
File: 3301w        Transform: NO TRANSFORMATION

                                ANOVA TABLE
-----------------------------------------------------------------------------

SOURCE              DF              SS                MS              F
-----------------------------------------------------------------------------
Between              5              0.091             0.018           0.900

Within (Error)      17              0.341             0.020
-----------------------------------------------------------------------------
Total               22              0.431
-----------------------------------------------------------------------------

  Critical F value =   2.81  (0.05,5,17)
  Since  F < Critical F  FAIL TO REJECT  Ho:All groups equal
```


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dry weight
File: 3301w Transform: NO TRANSFORMATION

BONFERRONI T-TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	neg control	0.693	0.693		
2	9.2	0.808	0.808	-1.150	
3	22	0.840	0.840	-1.475	
4	39	0.835	0.835	-1.425	
5	77	0.740	0.740	-0.475	
6	154	0.687	0.687	0.054	

Bonferroni T table value = 2.57 (1 Tailed Value, P=0.05, df=17,5)

dry weight
File: 3301w Transform: NO TRANSFORMATION

BONFERRONI T-TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	neg control	4			
2	9.2	4	0.257	37.1	-0.115
3	22	4	0.257	37.1	-0.148
4	39	4	0.257	37.1	-0.143
5	77	4	0.257	37.1	-0.048
6	154	3	0.277	40.0	0.006

dry weight
File: 3301w Transform: NO TRANSFORMATION

WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	neg control	4	0.693	0.693	0.794
2	9.2	4	0.808	0.808	0.794
3	22	4	0.840	0.840	0.794
4	39	4	0.835	0.835	0.794
5	77	4	0.740	0.740	0.740
6	154	3	0.687	0.687	0.687

dry weight

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File: 3301w Transform: NO TRANSFORMATION

WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

IDENTIFICATION	ISOTONIZED MEAN	CALC. WILLIAMS	SIG P=.05	TABLE WILLIAMS	DEGREES OF FREEDOM
neg control	0.794				
9.2	0.794	1.011		1.74	k= 1, v=17
22	0.794	1.011		1.82	k= 2, v=17
39	0.794	1.011		1.85	k= 3, v=17
77	0.740	0.474		1.87	k= 4, v=17
154	0.687	0.054		1.87	k= 5, v=17

s = 0.142
Note: df used for table values are approximate when v > 20.

APPENDIX 2: COPY OF REVIEWER'S TWA CALCULATIONS:

Nominal Concentration (ug ai/L)	Time (Day)	Measured Concentration (ug/L)	TWA (ug/L)
9.4	0	9.61	
	7	8.95	
	14	8.92	
	17	9.76	
	21	9.09	
			9.20119
19	0	21.2	
	7	21.5	
	14	21.2	
	17	23.7	
	21	20.5	
			21.65000
38	0	39.8	
	7	37.4	
	14	39.0	
	17	43.2	
	21	36.4	
			39.05238
75	0	78.9	
	7	71.5	
	14	77.5	
	17	87.9	
	21	75.3	
			77.25714

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150	0	147	
	7	149	
	14	153	
	17	179	
	21	144	
			154.14286

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