Data Evaluation Report on the Toxicity of Methyl Parathion Technical to Sheepshead Minnow (*Cyprinodon variegatus*), Early Life Cycle

PMRA Submission Number {.....} EPA MRID Number 467442-01

Data Requirement:	PMRA Data Code	{.....}
	EPA DP Barcode	D326572 and 326467
	OECD Data Point	{.....}
	EPA MRID	467442-01
	EPA Guideline	850.1400

Test material: Methyl Parathion Technical **Purity:** 97.4% (w:w)
Common name Methyl parathion
Chemical name: IUPAC: *O,O*-dimethyl *O*-4-nitrophenyl phosphorothioate
CAS name: Phosphorathioic acid, *O,O*-dimethyl *O*-(4-nitrophenyl) ester
CAS No.: 298-00-0
Synonyms: None Reported

Primary Reviewer: Christie E. Padova
Staff Scientist, Dynamac Corporation
Signature: Christie E. Padova
Date: 12/8/06

Secondary Reviewer: John Marton
Staff Scientist, Cambridge Environmental Inc.
Signature: John Marton
Date: 12/27/06

Primary Reviewer: Edward Odenkirchen, Biologist
EPA/OPP/EFED/ERB – I
Date: 2/14/09

Secondary Reviewer(s): Yan Donovan , Chemist
EPA/OPP/EFED/ERB – I
Date: 2/18/09 Yan Donovan

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

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CITATION: Hicks, S.L. 2006. Methyl Parathion Technical: Early Life-Stage Toxicity Test with the Sheepshead Minnow, *Cyprinodon Variegatus*, Under Flow-Through Conditions. Unpublished study performed by ABC Laboratories, Inc., Columbia, MO. Laboratory Project No. 49732. Study submitted by Cheminova, Inc., Washington, DC. Study initiated September 26, 2005 and submitted January 23, 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 toxicity of a pesticide to fish, early life cycle. 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.

EXECUTIVE SUMMARY:

The 38-day chronic toxicity of methyl parathion to the early life stage of sheepshead minnow (*Cyprinodon variegatus*) was studied under flow-through conditions. Newly-fertilized eggs/embryos (100/level, exact age not reported) of sheepshead minnow were exposed to methyl parathion at nominal concentrations of 0 (negative and solvent controls), 5.0, 10, 20, 40, 80, and 160 µg ai/L. TWA concentrations were <1.07 (<LOQ, controls), 4.57, 8.86, 17.7, 36.0, 74.5, and 148 µg ai/L, respectively. The test system was maintained at 24.5-25.5 °C, pH of 7.33-7.72, and salinity of 18.7-19.9‰. As survival did not fall below 50% for any treatment level, the 38-day LC_{50} for post-hatch survival was >148 µg ai/L. NOAEC and LOAEC values were 8.86 and 17.7 µg ai/L, respectively, based on treatment-related reductions in terminal growth (length and wet weight).

No treatment-related effects on time to 95% hatch or percent hatch were observed. In addition, no treatment-related signs of toxicity in post-hatch fry were observed. Fry survival, however, was statistically-reduced at the 148 µg ai/L level compared to the negative control (76 versus 90%, respectively). Terminal standard lengths and blotted wet weights were the most sensitive endpoints, with statistically-significant reductions compared to the negative control at the ≥17.7 µg ai/L levels. The mean standard length was 16 mm in the negative control, solvent control, 4.57, and 8.86 µg ai/L groups, and 15, 14, 13, and 9 mm in the 17.7, 36.0, 74.5, and 148 µg ai/L groups, respectively. Mean blotted wet weight was 0.134 and 0.136 mg in the negative and solvent control levels, respectively, and 0.134, 0.127, 0.112, 0.106, 0.093, and 0.041 mg in the 4.57, 8.86, 17.7, 36.0, 74.5, and 148 µg ai/L levels, respectively.

This study is scientifically sound and satisfies the guideline requirement for an early life toxicity study with sheepshead minnow.

Results Synopsis
Test Organism Size/Age (mean Weight or Length): Newly-fertilized embryos, exact age not reported
Test Type (Flow-through, Static, Static Renewal): Flow-through

Egg Hatchability:
EC_{50}: >148 µg ai/L 95% C.I.: N/A
Probit Slope: N/A
NOAEC: 148 µg ai/L
LOAEC: >148 µg ai/L

Fry Survival:
EC_{50}: >148 µg ai/L 95% C.I.: N/A
Probit Slope: N/A
NOAEC: 74.5 µg ai/L
LOAEC: 148 µg ai/L

Mean Standard Length:
EC_{50}: >148 µg ai/L 95% C.I.: N/A
Probit Slope: 1.51±0.168
NOAEC: 8.86 µg ai/L
LOAEC: 17.7 µg ai/L

Mean Blotted Wet Weight:
EC_{50}: 100 µg ai/L 95% C.I.: 88-120 µg ai/L
Probit Slope: 2.71±0.492
NOAEC: 8.86 µg ai/L
LOAEC: 17.7 µg ai/L
Endpoint(s) Affected: Fry Survival and Growth (Length and Wet Weight)

Most Sensitive Endpoint(s): Length and Wet Weight

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: The study protocol was based on procedures outlined in the U.S. EPA Series 850 – Ecological Effects Test Guidelines (draft), OPPTS No. 850.1400 *Fish Early Life-Stage Test.* Deviations from OPPTS No. 850.1400 included:

1. The exact age of the fertilized embryos used for testing was not reported.

2. The analytical variation among test sample results exceeded 20% at the nominal 5.0 µg ai/L level (lowest dose tested only). Variation at this level was 27%. At the remaining levels, analytical variation ranged from 6 to 15%.

3. Periodic analysis of the prepared dilution water was conducted in December 2004, approximately 10 months prior to the definitive study.

4. Raw data obtained during daily observations (including survival, time to hatch, and clinical signs of toxicity) were not provided.

These deviations do not affect the scientific soundness or acceptability of this study.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided. A No Data Confidentiality statement was included in the final report; however, it was not signed or dated.

A. MATERIALS:

1. Test Material Methyl Parathion Technical

Description: Amber solid

Lot No./Batch No. : 621-BSe-20A

Purity: 97.4% w:w

Stability of compound under test conditions: Verified. Test samples collected on days 0, 7, 14, 21, 28, and 38 were analyzed for methyl parathion using gas chromatography in conjunction with a nitrogen/phosphorous detector (GC/NPD). At all except the lowest treatment level, recoveries were within 20% among replicate measurements (range of 6-15%, reviewer-calculated). At the lowest treatment level (5.0 µg ai/L), the analytical variation was 27% among replicate measurements. These results indicate that methyl parathion was stable under the conditions of the test.

Storage conditions of test chemicals: Under refrigeration (approx. 4°C)

Physicochemical properties of Methyl Parathion.

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: Sheepshead minnow (*Cyprinodon variegates*) [*EPA recommends any of several freshwater fish species, including rainbow trout, brook trout, bluegill, fathead minnow, and channel catfish. See Standard Evaluation Procedure for listing of recommended species. OECD recommends rainbow trout, fathead minnows, zebra fish, and ricefish but does not exclude the use of other species.*]

Age /embryonic stage at test initiation: Newly-fertilized embryos, exact age not reported [*EPA recommends fish embryos 2 to 24 hours old.*]

Method of collection of the fertilized eggs: Testes from approximately ten male minnows were obtained from sacrificed fish and maintained in a glass dish containing fresh saltwater. Unfertilized eggs from approximately 46 female sheepshead minnows (previously injected twice with Human Chorionic Gonadoptropin solution; refer to Reviewer's Comments section) were collected by gently stroking the abdomen of anesthetized fish and subsequently collecting eggs into a glass dish containing fresh saltwater. The testes were macerated and rinsed into the glass dish containing the collected eggs. The milt and eggs were gently swirled and allowed to remain undisturbed for several minutes. The fertilized eggs were then rinsed with fresh saltwater and maintained in a glass dish at approximately 23°C prior to selection for testing.

Source: In-house cultures

B. STUDY DESIGN:

1. Experimental Conditions

a. Range-finding study: A 28-day flow-through range-finding study was conducted at nominal concentrations of 0 (negative and solvent controls), 6.5, 13, 25, 50, 100, and 200 µg ai/L. Two replicates, each containing 20 embryos, were exposed for a total of 40 embryos per concentration level. Egg hatchability was 78 and 90% in the negative and solvent controls, respectively, compared to 85,

85, 70, 83, 78, and 80% in the 6.5, 13, 25, 50, 100, and 200 µg ai/L levels, respectively. Fry mortality at the end of the 28-day exposure ranged from 6% in the 200 µg ai/L level to 16% in the 100 µg ai/L level, compared to 10 and 8% in the negative and solvent control groups, respectively. Standard length and blotted wet weight were measured in surviving fry from the negative control, solvent control, 6.5, 50, and 200 µg ai/L levels at study termination. The mean length was 13, 14, 13, 11, and 10 mm, respectively, and the mean wet weights were 0.076, 0.079, 0.079, 0.061, and 0.044 g, respectively.

The concentrations of methyl parathion were determined at the 6.5, 25, and 200 µg ai/L levels on days 15 and 28. Recoveries ranged from 80 to 90% of the nominal concentrations.

b. Definitive study

Table 1: Experimental Parameters

Parameter	Details	Remarks *Criteria*
Parental acclimation, if any Period: Conditions (same as test or not): Feeding (type, source, amount given, frequency): Health: (any mortality observed)	 Continuous Same as test Not reported No prophylactic or therapeutic disease treatments were administered in the 2 weeks prior to test initiation.	The mature sheepshead minnow were maintained in laboratory saltwater (20 ± 3‰) at a temperature of 23 ± 2°C.
Number of fertilized eggs/embryos in each treatment at test initiation	100 embryos/treatment level, divided into 25 embryos/cup, 1 cup/aquarium, and 4 replicate aquaria/treatment.	Fish were not thinned following hatching. *Each treatment should include a minimum of 20 embryos per replicate cup and a minimum of 30 fish per treatment for post-hatch exposure (OECD recommends at least 60 eggs, divided between at least 2 replicates)*

Parameter	Details	Remarks *Criteria*
Concentration of test material nominal: mean measured: TWA (reviewer-calculated):	0 (negative and solvent controls), 5.0, 10, 20, 40, 80, and 160 µg ai/L <1.07 (<LOQ, controls), 4.67, 8.94, 18.0, 36.2, 74.2, and 149 µg ai/L <1.07 (<LOQ, controls), 4.57, 8.86, 17.7, 36.0, 74.5, and 148 µg ai/L	Concentrations were corrected for test substance purity. Solutions were analyzed for ai at 0, 7, 14, 21, 28, and 38 days. Analytical variation was excessive at the 5.0 µg ai/L level only (27%, reviewer-calculated). This was due to the day-0 result, which was 121% of the mean-measured concentration. At the remaining treatment levels, variation ranged from 6 to 15%, which is within the 20% recommended limit. *A minimum of 5 concentrations and a control, all replicated, plus solvent control if appropriate should be used.* *- Toxicant concentration should be measured in one tank at each toxicant level every week.* *- One concentration should adversely affect a life stage and one concentration should not affect any life stage.* *OECD recommends that 5 concentrations be spaced by a constant factor not exceeding 3.2; concentrations of test substance in solution should be within ∀20% of the mean measured values.*
Solvent (type, percentage, if used)	Dimethyl formamide, 20 µl/L	*The solvent should not exceed 0.1 ml/L in a flow-through system.* *Recommended solvents include dimethylformamide, triethylene glycol, methanol, acetone, ethanol.* *OECD recommends that the solvent not have an effect on survival nor produce any other adverse effects;concentration should not be greater than 0.1 ml/L.*
Number of replicates control: solvent control: treated ones:	4 4 4/level	*Number of replicates should be 4 per concentration.* *A solvent control should be used in conjunction with a solubilizing agent.*

Parameter	Details	Remarks *Criteria*
Test condition static renewal/flow-through: type of dilution system for flow through method: flow rate: renewal rate for static renewal:	 Flow-through Intermittent-flow proportional diluter Approx. 7 volume additions per day N/A	The diluter was calibrated before the test and checked for normal operation twice daily during the test. *Intermittent flow proportional diluters or continuous flow serial diluters should be used. EPA recommends that flow rate to larval cups should provide 90% replacement in 8 to 12 hours (OECD recommends 5 test chamber volumes/24 hours). For static-renewal, OECD recommends 2 renewal procedures; either transfer eggs and larvae to new, clean vessels or reatain organisms in vessels and change at least 2/3 test water. A minimum of 5 toxicant concentrations with a dilution factor not greater than 0.5 and controls should be used.* *Toxicant Mixing:* *1) Mixing chamber is preferred;* *2) Aeration should not be used for mixing;* *3) The test solution should be completely mixed before introduction into the test system;* *4) Flow splitting accuracy should be within 10%.*
Aeration, if any	None used.	*Dilution water should be aerated to ensure DO concentration at or near 100% saturation. Test tanks and embryo cups should not be aerated.*
Duration of the test	38 days (28-days post-hatch)	Fulfills OPPTS requirement for this species. *Recommended test duration is 32 days for EPA. OECD recommendations for test duration are species specific and range from 28-60 days.*
Embryo cups, if used type/material (glass/stainless steel): size: fill volume:	 Glass jars with Nitex® screen replacing the bottom 9-cm diameter Not reported	The embryo cages were slowly oscillated vertically. *Recommended embryo cups are 120 ml glass jars with bottoms replaced with 40 mesh stainless steel or nylon screen.*

Parameter	Details	Remarks *Criteria*
Test vessel type/material: (glass/stainless steel) size: fill volume:	 Glass 15 x 31 x 22 cm 10 L	All chamber drains were covered with stainless steel screen to prevent fish escape. *Recommended test vessel is all glass or glass with stainless steel frame.*
Source of dilution water	Laboratory saltwater (20 ± 3‰) prepared using a commercial sea salt mix and laboratory freshwater consisting of well water that was de-mineralized by reverse-osmosis. The prepared dilution water was passed through a particulate filter and an UV sterilizer.	Results of periodic analysis of the dilution water for chlorinated hydrocarbons, metals, and organophosphates were provided (from water analyzed in December 2004). The following elements were detected: boron at 5.7 mg/L, calcium at 160 mg/L, magnesium at 580 mg/L, potassium at 200 mg/L, sodium at 5700 mg/L, nitrate at 0.39 mg/L, and total phosphorus at 0.12 mg/L. *Source of dilution water should be natural or reconstituted water; natural water should be sterilized with UV and tested for pesticides, heavy metals, and other possible contaminants.* *OECD accepts any water in which the test species show control survival at least as good as presented in SEP.*

Parameter	Details	Remarks *Criteria*
Water parameters hardness: pH: dissolved oxygen: temperature (s) (record all the temperatures used for different life stages): photoperiod: salinity (for marine or estuarine species): other measurements: interval of water quality measurements:	Not reported 7.33-7.72 5.13-6.64 mg/L (71-92% saturation) 24.5-25.5°C (all stages; maintained constant during the study) 16 hours light/8 hours dark, with 30-minute transition periods 18.7-19.9‰ N/A Temperature, pH, salinity, and DO were measured in all replicates of each level at test initiation and termination, and weekly throughout the study. Temperature was also continuously monitored in a centrally-located test chamber (20 µg ai/L replicate D).	Light intensity at the level of test solution, ranged from 450 to 547 on days 7, 14, and 35. *Recommended hardness: 40-48 mg/L as $CaCO_3$;* *Recommended pH:* 7.2 to 7.6 *Dissolved Oxygen (DO) should be measured at each concentration at least once a week;* *Freshwater parameters in a control and one concentration should be analyzed once a week.* *Temperature depends upon test species and should not deviate by more than 2EC from appropriate temperature.* *OECD recommends that DO concentration be between 60 - 90% saturation. As a minimum DO, salinity (if relevant) and temperature should be measured weekly, and pH and hardness at the beginning and end of the test. Temperature should be measured continuously.*
Post-hatch details when the post-hatch period began: number of hatched eggs (alevins)/ treatment released to the test chamber: on what day, the alevins were released from the incubation cups to the test chamber:	Day 10, when hatching was at least 95% complete in the solvent control chambers. All hatched larvae were released. Day 10	Percent hatch ranged from 72-88% in each negative control replicate (mean of 80%), and from 72-92% in each vehicle control replicate (mean of 84%). This fulfills the OPPTS minimum requirement of 75% for this species. *Percentage of embryos that produce live fry should be ≥ 50% in each control; percentage of hatch in any control embryo cup should not be more than 1.6 times that in another control cup.*

Parameter	Details	Remarks *Criteria*
Post-hatch Feeding start date: type/source of feed: amount given: frequency of feeding:	 Day 6, the day following the start of hatch Live brine shrimp nauplii (*Artemia sp.*). As the test progressed, a standard commercial fish food was added to the diet. *Ad libitum* At least three times daily during the week and at least twice daily on weekends. Fish were not fed during the final 24 hours of the test.	Food size and/or quantity were increased during testing on the basis of average fish size.
Stability of chemical in the test system	Stable, as indicated by relatively constant measured concentrations (within 20% among replicate measurements) at all but the lowest treatment level.	
Recovery of chemical: Frequency of measurement: LOD: LOQ:	84-105% of nominal Days 0, 7, 14, 21, 28, and 38 Not reported 1.07 µg ai/L	Based on the results of QC samples in which 20 ml volumes of saltwater were spiked with methyl parathion at 4.62 and 182 µg ai/L and analyzed concurrently with the test samples.
Positive control {if used, indicate the chemical and concentrations}	N/A	
Fertilization success study, if any number of eggs used: on what day the eggs were removed to check the embryonic development:	N/A	
Other parameters, if any	N/A	

2. Observations:

Table 2: Observations

Parameters	Details	Remarks *Criteria*
Parameters measured including the sublethal effects/toxicity symptoms	- Embryo survival - Time to reach ≥95% hatch - Larval survival - Measurement of growth (total length, blotted wet weight) - Clinical signs of toxicity or abnormal behavior	Although dry weights are preferred, wet weight data are acceptable. *Recommended parameters measured include:* *- Number of embryos hatched;* *- Time to hatch;* *- Mortality of embryos, larvae, and Juveniles:* *- Time to swim-up (if appropriate);* *- Measurement of growth;* *- Incidence of pathological or Histological effects;* *- Observations of other effects or clinical signs.*
Observation intervals/dates for: egg mortality: no. of eggs hatched: mortality of fry (e.g.,alevins): swim-up behavior: growth measurements: embryonic development: other sublethal effects	 Daily Daily Daily N/A Day 38 (28 days post-hatch) Not determined Daily	
Water quality was acceptable (Yes/No)	Yes	
Were raw data included?	No. Raw data obtained during daily observations (including survival, time to hatch, and clinical signs of toxicity) were not provided.	Summarized survival data for hatch and post-hatch periods were reported.
Other observations, if any	N/A	

II. RESULTS AND DISCUSSION

A. MORTALITY:

On Day 10, percent egg hatchability averaged 78-88% in all test and control groups, with no treatment-related effect observed. The NOAEC for hatchability was 149 µg ai/L.

On Day 38 (28 days post-hatch), fish survival averaged 87-92% in the controls through 74.2 µg ai/L treatment levels. At the 149 µg ai/L level, post-hatch survival averaged 76%, which was statistically-reduced compared to the pooled controls (p=0.05). The NOAEC for post-hatch survival was 74.2 µg ai/L.

Table 3: Effect of Methyl Parathion on Egg Hatching and Survival at Different Life Stages of Fish.

Treatment, Measured (and Nominal) Concentrations, µg ai/L	Egg hatched/embryo viability			Study Day Treatment Hatch ≥95%	Juvenile-survival on day 38	
	No. of eggs at study initiation	hatch/embryo viability			No. dead	% mortality
		No.	%			
Control (dilution water only)	100	80	80	9	8	10
Solvent control	100	84	84	10	9	11
4.67 (5.0)	100	81	81	9	9	11
8.94 (10)	100	79	79	9	9	11
18.0 (20)	100	88	88	9	10	11
36.2 (40)	100	82	82	9	11	13
74.2 (80)	100	82	82	9	7	8
149 (160)	100	78	78	10	19	24*
NOAEC	149 µg ai/L			149 µg ai/L	74.2 µg ai/L	
EC_{50}	NR			NR	NR	
Positive control, if used mortality: EC_{50}: NOAEC	N/A	N/A		N/A	N/A	

NR – Not reported
* Statistically-significant difference from pooled control using Dunnett's Test (p=0.05).

Table 4: Effect of Methyl Parathion on Growth of Juvenile Fish.

Treatment, Measured (and Nominal) Concentrations, µg ai/L	Swim-up[a]			Growth -length (mm)	Growth-wet weight (mg)
	day x1	day x2	day xn		
Control (dilution water only)	N/A	N/A	N/A	16	0.134
Solvent control	N/A	N/A	N/A	16	0.136
4.67 (5.0)	N/A	N/A	N/A	16	0.134
8.94 (10)	N/A	N/A	N/A	16	0.127
18.0 (20)	N/A	N/A	N/A	15*	0.112*
36.2 (40)	N/A	N/A	N/A	14*	0.106*
74.2 (80)	N/A	N/A	N/A	13*	0.093*
149 (160)[b]	N/A	N/A	N/A	9	0.041
NOAEC	N/A	N/A	N/A	8.94 µg ai/L	8.94 µg ai/L
LOAEC	N/A	N/A	N/A	18.0 µg ai/L	18.0 µg ai/L
EC_{50}	N/A	N/A	N/A	NR	NR
Positive control, if used mortality: EC_{50}: NOAEC	N/A	N/A	N/A	N/A	N/A

[a] Swim-up is generally not applicable for this species.
[b] Treatment was excluded from the statistical analyses due to survival effects.
*Statistically-significant from pooled control using Dunnett's Test (p=0.05).

B. SUB-LETHAL TOXICITY AND OTHER CHRONIC EFFECTS:

No treatment-related effect on the time to hatch was reported. Hatching began in the negative and solvent control groups on days 5 and 7, respectively, and in all test substance groups on days 6-7. The negative control and ≤74.2 µg ai/L groups reached 95% hatch on day 9, and the vehicle control and the 149 µg ai/L group reached 95% hatch on day 10. Hatch was completed (i.e., 100%) in all levels between days 7 and 13. Raw data were not provided. A NOAEC was not reported.

No treatment-related signs of toxicity were observed during the study. Dark discoloration was observed in a single fry each from the negative control, vehicle control, and 4.67 through 36.2 µg ai/L treatment groups. Edema was also observed in one negative control fry. These observations did not follow a dose-dependent response and were not considered to be related to treatment. No behavioral abnormalities were observed during exposure. Raw data were not provided. A NOAEC was not reported.

Statistically-significant reductions (p=0.05) in total length and blotted wet weight were observed at the ≥18.0 µg

ai/L levels compared to the pooled control. The mean standard length was 16 mm in the negative control, solvent control, 4.67, and 8.94 µg ai/L groups, and 15, 14, 13, and 9 mm in the 18.0, 36.2, 74.2, and 149 µg ai/L groups, respectively. Mean blotted wet weight was 0.134 and 0.136 mg in the negative and solvent control levels, respectively, and 0.134, 0.127, 0.112, 0.106, 0.093, and 0.041 mg in the 4.67, 8.94, 18.0, 36.2, 74.2, and 149 µg ai/L levels, respectively. The NOAEC for both growth indicators was 8.94 µg ai/L.

C. REPORTED STATISTICS:

Data that were statistically analyzed included 1) percent egg hatchability, 2) percent fry survival, 3) the mean total length of surviving fish at study termination, and 4) the mean blotted wet weight of surviving fish at study termination. The time to 95% hatch was visually evaluated.

For egg hatchability and fry survival, control data were compared using a two-tailed Fisher's exact test and a two-tailed planned comparison t-test. No significant differences were observed, and the controls were pooled for all subsequent analyses. Data were tested for normality using Shapiro-Wilk's test and for homogeneity of variance using Levene's test. These assumptions were met, and the non-transformed data were subsequently analyzed using ANOVA and a one-tailed Dunnett's test. Data were also analyzed using a Fisher's exact test with a Hochberg adjustment. Test substance treatments with statistically-significant effects on hatchability or fry survival were not included in subsequent growth analyses.

For the growth endpoints, measurements were made on each organism; however, the organisms were not treated as the experimental unit and rather treated as sub-samples measured within the experimental unit. The control data were compared using a two-tailed planned comparison t-test. No significant differences were observed, and the controls were pooled for all subsequent analyses. Growth data were checked for normality using Shapiro-Wilk's test, and for homogeneity of variance using Levene's test. These assumptions were fulfilled, and the non-transformed data were subsequently analyzed using a nested ANOVA procedure, where the treatment means are weighted for number of fish in each chamber, and a one-tailed Dunnett's test, with the alternate hypotheses being the mean for the parameter was reduced in comparison to the control mean.

The NOAEC and LOAEC were based on significance data. The maximum acceptable toxicant concentration (MATC) was calculated as the geometric mean of the overall NOAEC and LOAEC for the most sensitive endpoint. All analyses were performed using SAS software and mean-measured concentrations. LC/EC_{50} values were not determined.

D. VERIFICATION OF STATISTICAL RESULTS:

Statistical Method(s): The endpoints analyzed included percent egg hatchability, percent fry survival (28 days post-hatch), mean standard length (28 days post-hatch) and mean blotted wet weight (28 days post-hatch). Negative and vehicle control data for each endpoint were compared using a Student's t-Test; no statistically significant differences were detected for any endpoint and all subsequent analyses were conducted using the negative control only. Replicate data for each endpoint were tested for normality using Chi-square and Shapiro-Wilk's tests and for homogeneity of variance using Hartley and Bartlett's tests. If the data met these assumptions of ANOVA, the NOAEC and LOAEC values were determined using the parametric Dunnett's and Williams' tests. If the assumptions were not met, the same values were determined using the non-parametric Steels Many-One Rank and Kruskal-Wallis tests. All NOAEC and LOAEC determinations were conducted using Toxstat statistical software. When applicable, EC_{50} values (and 95% C.I.) were determined using the probit method via Nuthatch statistical software. All toxicity values were determined using the time-weighted average concentrations.

Egg Hatchability:
EC_{50}: >148 µg ai/L 95% C.I.: N/A
Probit Slope: N/A
NOAEC: 148 µg ai/L
LOAEC: >148 µg ai/L

Fry Survival:
EC_{50}: >148 µg ai/L 95% C.I.: N/A
Probit Slope: N/A
NOAEC: 74.5 µg ai/L
LOAEC: 148 µg ai/L

Mean Standard Length:
EC_{50}: >148 µg ai/L 95% C.I.: N/A
Probit Slope: 1.51±0.168
NOAEC: 17.7 µg ai/L
LOAEC: 36.0 µg ai/L

Mean Blotted Wet Weight:
EC_{50}: 100 µg ai/L 95% C.I.: 88-120 µg ai/L
Probit Slope: 2.71±0.492
NOAEC: 8.86 µg ai/L
LOAEC: 17.7 µg ai/L

E. STUDY DEFICIENCIES:

There were no major deviations from OPPTS 850.1400 that affected the scientific soundness or acceptability of this study.

F. REVIEWER=S COMMENTS:

The reviewer relied on a non-parametric analysis to determine the NOAEC for length because of identical replicate values for the two lowest treatment levels; as a result, the reviewer's analysis did not detect a significant reduction in length at the17.7 µg ai/L treatment level, while the study author's analysis did. Because the study author's NOAEC level for length is more conservative, it is reported in the Executive Summary and Conclusions sections. Results in both of these sections are expressed based on the time-weighted average concentrations; the study author based toxicity estimates on the mean measured concentrations.

Five days prior to egg collection, approximately 46 female minnow were injected with Human Chorionic Gonadoptropin (HCG) solution (containing 1000 IUs HCG/ml) at a rate of approximately 0.1 ml per fish. These same female fish were injected a second time 2 days prior to egg collection with HCG solution (containing 400 IU HCG/ml) at a rate of approximately 0.1 ml per fish. The injected female sheepshead minnows were used as the source for the unfertilized eggs.

All test solutions were clear and colorless with no visible particulate material, surface film, undissolved test substance, or precipitate for the duration of the definitive test.

In-life dates were September 26 – November 3, 2005.

G. CONCLUSIONS:

This study is scientifically sound and is thus acceptable. Based on treatment-related effects upon mean standard length and mean blotted wet weight at the ≥17.7 µg ai/L levels (the most sensitive endpoints), the NOAEC and LOAEC are 8.86 and 17.7 µg ai/L, respectively.

Egg Hatchability:
EC_{50}: >148 µg ai/L 95% C.I.: N/A
Probit Slope: N/A
NOAEC: 148 µg ai/L
LOAEC: >148 µg ai/L

Fry Survival:
EC_{50}: >148 µg ai/L 95% C.I.: N/A
Probit Slope: N/A
NOAEC: 74.5 µg ai/L
LOAEC: 148 µg ai/L

Mean Standard Length:
EC_{50}: >148 µg ai/L 95% C.I.: N/A
Probit Slope: 1.51±0.168
NOAEC: 8.86 µg ai/L
LOAEC: 17.7 µg ai/L

Mean Blotted Wet Weight:
EC_{50}: 100 µg ai/L 95% C.I.: 88-120 µg ai/L
Probit Slope: 2.71±0.492
NOAEC: 8.86 µg ai/L
LOAEC: 17.7 µg ai/L

Endpoint(s) Affected: Fry Survival and Growth (Length and Wet Weight)
Most Sensitive Endpoint(s): Length and Wet Weight

III. REFERENCES:

U.S. Environmental Protection Agency. 1996. Ecological Effects Test Guidelines, OPPTS 850.1400, Fish Early-Life Stage Toxicity Test, 13 pp.

U.S. Environmental Protection Agency. 1982. Pesticide Assessment Guidelines, Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms, EPA 540/9-82-024, Series 72-4, Fish Early Life-Stage Test.

Mount, D.I., and W.A. Brungs. 1967. A Simplified Dosing Apparatus for Fish Toxicological Studies. Water Res. 1: 21-29.

APPENDIX 1: OUTPUT OF REVIEWER'S STATISTICAL VERIFICATION:

```
% Egg Hatchability, TWA ug ai/L
File: 4201eh        Transform: NO TRANSFORM

      't-test of Solvent and Blank Controls          Ho:GRP1 MEAN = GRP2 MEAN
-------------------------------------------------------------------------------
 GRP1 (SOLVENT CRTL) MEAN =    80.0000     CALCULATED t VALUE =    -0.7385
 GRP2 (BLANK CRTL) MEAN   =    84.0000     DEGREES OF FREEDOM =     6
 DIFFERENCE IN MEANS      =    -4.0000
-------------------------------------------------------------------------------
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

% Egg Hatchability, TWA ug ai/L
File: 4201eh       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     1.876         6.776           10.696          6.776       1.876
OBSERVED     0             8               14              6           0

-------------------------------------------------------------------------------
Calculated Chi-Square goodness of fit test statistic =   5.0826
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.


% Egg Hatchability, TWA ug ai/L
File: 4201eh       Transform: NO TRANSFORMATION

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

D =  712.000

W =    0.970

Critical W (P = 0.05) (n = 28) = 0.924
Critical W (P = 0.01) (n = 28) = 0.896
-------------------------------------------------------------------------------

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


% Egg Hatchability, TWA ug ai/L
File: 4201eh        Transform: NO TRANSFORMATION

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

Calculated H statistic (max Var/min Var) =   14.67
Closest, conservative, Table H statistic =  216.0  (alpha = 0.01)

Used for Table H ==>     R (# groups) =   7,    df (# reps-1) =       3
Actual values    ==>     R (# groups) =   7,    df (# avg reps-1) =   3.00
```

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).

% Egg Hatchability, TWA ug ai/L
File: 4201eh Transform: NO TRANSFORMATION

Bartletts test for homogeneity of variance

Calculated B statistic = 5.56
Table Chi-square value = 16.81 (alpha = 0.01)
Table Chi-square value = 12.59 (alpha = 0.05)

Average df used in calculation ==> df (avg n - 1) = 3.00
Used for Chi-square table value ==> df (#groups-1) = 6

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).

% Egg Hatchability, TWA ug ai/L
File: 4201eh Transform: NO TRANSFORMATION

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	6	254.857	42.476	1.253
Within (Error)	21	712.000	33.905	
Total	27	966.857		

Critical F value = 2.57 (0.05,6,21)
Since F < Critical F FAIL TO REJECT Ho:All groups equal

% Egg Hatchability, TWA ug ai/L
File: 4201eh Transform: NO TRANSFORMATION

DUNNETTS TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	neg control	80.000	80.000		
2	4.57	81.000	81.000	-0.243	
3	8.86	79.000	79.000	0.243	
4	17.7	88.000	88.000	-1.943	
5	36.0	82.000	82.000	-0.486	
6	74.5	82.000	82.000	-0.486	

7	148	78.000	78.000	0.486

Dunnett table value = 2.46 (1 Tailed Value, P=0.05, df=20,6)

% Egg Hatchability, TWA ug ai/L
File: 4201eh Transform: NO TRANSFORMATION

DUNNETTS 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	4.57	4	10.129	12.7	-1.000
3	8.86	4	10.129	12.7	1.000
4	17.7	4	10.129	12.7	-8.000
5	36.0	4	10.129	12.7	-2.000
6	74.5	4	10.129	12.7	-2.000
7	148	4	10.129	12.7	2.000

% Egg Hatchability, TWA ug ai/L
File: 4201eh 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	80.000	80.000	82.000
2	4.57	4	81.000	81.000	82.000
3	8.86	4	79.000	79.000	82.000
4	17.7	4	88.000	88.000	82.000
5	36.0	4	82.000	82.000	82.000
6	74.5	4	82.000	82.000	82.000
7	148	4	78.000	78.000	78.000

% Egg Hatchability, TWA ug ai/L
File: 4201eh 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	82.000				
4.57	82.000	0.486		1.72	k= 1, v=21
8.86	82.000	0.486		1.80	k= 2, v=21
17.7	82.000	0.486		1.83	k= 3, v=21
36.0	82.000	0.486		1.84	k= 4, v=21
74.5	82.000	0.486		1.85	k= 5, v=21
148	78.000	0.486		1.85	k= 6, v=21

s = 5.823
Note: df used for table values are approximate when v > 20.

```
% Fry Survival, Day 38, TWA ug ai/L
File: 4201fs        Transform: NO TRANSFORM

      t-test of Solvent and Blank Controls          Ho:GRP1 MEAN = GRP2 MEAN
-------------------------------------------------------------------------------
 GRP1 (SOLVENT CRTL) MEAN =    89.7500     CALCULATED t VALUE =      0.1573
 GRP2 (BLANK CRTL) MEAN   =    89.0000     DEGREES OF FREEDOM =      6
 DIFFERENCE IN MEANS      =     0.7500
-------------------------------------------------------------------------------
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

% Fry Survival, Day 38, TWA ug ai/L
File: 4201fs       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     1.876         6.776          10.696          6.776      1.876
OBSERVED     0             9               9             10         0

-------------------------------------------------------------------------------
Calculated Chi-Square goodness of fit test statistic =   6.2848
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.


% Fry Survival, Day 38, TWA ug ai/L
File: 4201fs       Transform: NO TRANSFORMATION

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

D = 1288.500

W =    0.979

Critical W (P = 0.05) (n = 28) = 0.924
Critical W (P = 0.01) (n = 28) = 0.896
-------------------------------------------------------------------------------

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


% Fry Survival, Day 38, TWA ug ai/L
File: 4201fs        Transform: NO TRANSFORMATION

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

Calculated H statistic (max Var/min Var) =    6.50
Closest, conservative, Table H statistic =  216.0   (alpha = 0.01)

Used for Table H ==>     R (# groups) =   7,    df (# reps-1) =       3
Actual values    ==>     R (# groups) =   7,    df (# avg reps-1) =   3.00

-------------------------------------------------------------------------------
```

```
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).


% Fry Survival, Day 38, TWA ug ai/L
File: 4201fs       Transform: NO TRANSFORMATION

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

Calculated B statistic =    3.41
Table Chi-square value =   16.81  (alpha = 0.01)
Table Chi-square value =   12.59  (alpha = 0.05)

Average df used in calculation  ==>   df (avg n - 1) =   3.00
Used for Chi-square table value ==>   df (#groups-1) =   6
-------------------------------------------------------------------------------

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).

% Fry Survival, Day 38, TWA ug ai/L
File: 4201fs       Transform: NO TRANSFORMATION

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

SOURCE             DF             SS                MS             F
-------------------------------------------------------------------------------
Between             6            614.929          102.488         1.670

Within (Error)     21           1288.500           61.357
-------------------------------------------------------------------------------
Total              27           1903.429
-------------------------------------------------------------------------------

  Critical F value =   2.57  (0.05,6,21)
  Since  F < Critical F  FAIL TO REJECT  Ho:All groups equal


% Fry Survival, Day 38, TWA ug ai/L
File: 4201fs       Transform: NO TRANSFORMATION

     DUNNETTS TEST   -   TABLE 1 OF 2            Ho:Control<Treatment
-------------------------------------------------------------------------------

                              TRANSFORMED    MEAN CALCULATED IN
GROUP    IDENTIFICATION          MEAN          ORIGINAL UNITS     T STAT  SIG
-----  --------------------    ----------    ------------------   ------  ---
  1           neg control       89.750            89.750
  2                  4.57       89.250            89.250          0.090
  3                  8.86       88.750            88.750          0.181
  4                  17.7       88.750            88.750          0.181
  5                  36.0       86.750            86.750          0.542
  6                  74.5       90.750            90.750         -0.181
  7                   148       76.000            76.000          2.482  *
```

Dunnett table value = 2.46 (1 Tailed Value, P=0.05, df=20,6)

% Fry Survival, Day 38, TWA ug ai/L
File: 4201fs Transform: NO TRANSFORMATION

DUNNETTS 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	4.57	4	13.625	15.2	0.500
3	8.86	4	13.625	15.2	1.000
4	17.7	4	13.625	15.2	1.000
5	36.0	4	13.625	15.2	3.000
6	74.5	4	13.625	15.2	-1.000
7	148	4	13.625	15.2	13.750

% Fry Survival, Day 38, TWA ug ai/L
File: 4201fs 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	89.750	89.750	89.750
2	4.57	4	89.250	89.250	89.250
3	8.86	4	88.750	88.750	88.750
4	17.7	4	88.750	88.750	88.750
5	36.0	4	86.750	86.750	88.750
6	74.5	4	90.750	90.750	88.750
7	148	4	76.000	76.000	76.000

% Fry Survival, Day 38, TWA ug ai/L
File: 4201fs 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	89.750				
4.57	89.250	0.090		1.72	k= 1, v=21
8.86	88.750	0.181		1.80	k= 2, v=21
17.7	88.750	0.181		1.83	k= 3, v=21
36.0	88.750	0.181		1.84	k= 4, v=21
74.5	88.750	0.181		1.85	k= 5, v=21
148	76.000	2.482	*	1.85	k= 6, v=21

s = 7.833
Note: df used for table values are approximate when v > 20.

Mean length (mm), day 38, TWA ug ai/L

```
File: 4201sl        Transform: NO TRANSFORM

       t-test of Solvent and Blank Controls          Ho:GRP1 MEAN = GRP2 MEAN
-------------------------------------------------------------------------------
 GRP1 (SOLVENT CRTL) MEAN =    16.0000     CALCULATED t VALUE =    -0.5222
 GRP2 (BLANK CRTL) MEAN   =    16.2500     DEGREES OF FREEDOM =     6
 DIFFERENCE IN MEANS      =    -0.2500
-------------------------------------------------------------------------------
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

Mean length (mm), day 38, TWA ug ai/L
File: 4201sl       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     1.876          6.776            10.696          6.776       1.876
OBSERVED     0              6                17              5           0

-------------------------------------------------------------------------------
Calculated Chi-Square goodness of fit test statistic =   8.0218
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.


Mean length (mm), day 38, TWA ug ai/L
File: 4201sl       Transform: NO TRANSFORMATION

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

D =    7.250

W =    0.936

Critical W (P = 0.05) (n = 28) = 0.924
Critical W (P = 0.01) (n = 28) = 0.896
-------------------------------------------------------------------------------

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


Mean length (mm), day 38, TWA ug ai/L
File: 4201sl       Transform: NO TRANSFORMATION

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

These two tests can not be performed because at least one group has
zero variance.

Data FAIL to meet homogeneity of variance assumption.
Additional transformations are useless.

-------------------------------------------------------------------------------
```

Mean length (mm), day 38, TWA ug ai/L
File: 4201sl Transform: NO TRANSFORMATION

STEELS MANY-ONE RANK TEST - Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	RANK SUM	CRIT. VALUE	df	SIG
1	neg control	16.000				
2	4.57	16.000	18.00	10.00	4.00	
3	8.86	16.000	18.00	10.00	4.00	
4	17.7	14.750	11.50	10.00	4.00	
5	36.0	13.750	10.00	10.00	4.00	*
6	74.5	12.500	10.00	10.00	4.00	*
7	148	9.250	10.00	10.00	4.00	*

Critical values use k = 6, are 1 tailed, and alpha = 0.05

Mean length (mm), day 38, TWA ug ai/L
File: 4201sl Transform: NO TRANSFORMATION

WILCOXON RANK SUM TEST W/ BONFERRONI ADJUSTMENT - Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	RANK SUM	CRIT. VALUE	REPS	SIG
1	neg control	16.000				
2	4.57	16.000	18.00	None	4	
3	8.86	16.000	18.00	None	4	
4	17.7	14.750	11.50	None	4	
5	36.0	13.750	10.00	None	4	
6	74.5	12.500	10.00	None	4	
7	148	9.250	10.00	None	4	

Critical values use k = 6, are 1 tailed, and alpha = 0.05

Mean length (mm), day 38, TWA ug ai/L
File: 4201sl Transform: NO TRANSFORMATION

KRUSKAL-WALLIS ANOVA BY RANKS - TABLE 1 OF 2

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	RANK SUM
1	neg control	16.000	16.000	88.500
2	4.57	16.000	16.000	90.000
3	8.86	16.000	16.000	90.000
4	17.7	14.750	14.750	58.000
5	36.0	13.750	13.750	42.500
6	74.5	12.500	12.500	27.000
7	148	9.250	9.250	10.000

Calculated H Value = 25.275 Critical H Value Table = 12.590
Since Calc H > Crit H REJECT Ho:All groups are equal.

Mean length (mm), day 38, TWA ug ai/L
File: 4201sl Transform: NO TRANSFORMATION

DUNNS MULTIPLE COMPARISON - KRUSKAL-WALLIS - TABLE 2 OF 2

GROUP	IDENTIFICATION	TRANSFORMED MEAN	ORIGINAL MEAN	GROUP 0 0 0 0 0 0 0 7 6 5 4 2 3 1
7	148	9.250	9.250	\
6	74.5	12.500	12.500	. \
5	36.0	13.750	13.750	. . \
4	17.7	14.750	14.750	. . . \
2	4.57	16.000	16.000	* . . . \
3	8.86	16.000	16.000	* . . . . \
1	neg control	16.000	16.000	* . . . . . \

* = significant difference (p=0.05) . = no significant difference
Table q value (0.05,7) = 3.038 SE = 5.663

Estimates of EC%

Parameter	Estimate	95% Bounds Lower	95% Bounds Upper	Std.Err.	Lower Bound /Estimate
EC5	17.	10.	28.	0.10	0.61
EC10	29.	20.	43.	0.078	0.69
EC25	74.	62.	90.	0.040	0.83
EC50	2.1E+02	1.8E+02	2.5E+02	0.036	0.84

Slope = 1.51 Std.Err. = 0.168

Goodness of fit: p = 0.21 based on DF= 4.0 21.

4201SL : Mean length (mm), day 38, TWA ug ai/L

Observed vs. Predicted Treatment Group Means

Dose	#Reps.	Obs. Mean	Pred. Mean	Obs. -Pred.	Pred. %Control	%Change
0.00	4.00	16.0	16.0	-0.0150	100.	0.00
4.57	4.00	16.0	15.9	0.0832	99.4	0.614
8.86	4.00	16.0	15.7	0.293	98.1	1.92
17.7	4.00	14.8	15.2	-0.416	94.7	5.30
36.0	4.00	13.8	14.0	-0.264	87.5	12.5
74.5	4.00	12.5	12.0	0.493	75.0	25.0
148.	4.00	9.25	9.42	-0.174	58.8	41.2

!!!Warning: EC50 not bracketed by doses evaluated.

Mean Wet Weight (g), day 38, TWA ug ai/L
File: 4201ww Transform: NO TRANSFORM

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

GRP1 (SOLVENT CRTL) MEAN = 0.1363 CALCULATED t VALUE = -0.0921
GRP2 (BLANK CRTL) MEAN = 0.1375 DEGREES OF FREEDOM = 6
DIFFERENCE IN MEANS = -0.0012

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

```
Mean Wet Weight (g), day 38, TWA ug ai/L
File: 4201ww       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     1.876          6.776            10.696          6.776      1.876
OBSERVED     0             10                10              8          0

--------------------------------------------------------------------------------
Calculated Chi-Square goodness of fit test statistic =   5.5524
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.



Mean Wet Weight (g), day 38, TWA ug ai/L
File: 4201ww       Transform: NO TRANSFORMATION

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

D =    0.003

W =    0.963

Critical W (P = 0.05) (n = 28) = 0.924
Critical W (P = 0.01) (n = 28) = 0.896
--------------------------------------------------------------------------------

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



Mean Wet Weight (g), day 38, TWA ug ai/L
File: 4201ww        Transform: NO TRANSFORMATION

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

Calculated H statistic (max Var/min Var) =   11.45
Closest, conservative, Table H statistic =  216.0  (alpha = 0.01)

Used for Table H ==>     R (# groups) =   7,    df (# reps-1) =       3
Actual values    ==>     R (# groups) =   7,    df (# avg reps-1) =   3.00

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

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).



Mean Wet Weight (g), day 38, TWA ug ai/L
File: 4201ww       Transform: NO TRANSFORMATION
```

Bartletts test for homogeneity of variance

Calculated B statistic = 7.25
Table Chi-square value = 16.81 (alpha = 0.01)
Table Chi-square value = 12.59 (alpha = 0.05)

Average df used in calculation ==> df (avg n - 1) = 3.00
Used for Chi-square table value ==> df (#groups-1) = 6

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).

Mean Wet Weight (g), day 38, TWA ug ai/L
File: 4201ww Transform: NO TRANSFORM

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	6	0.0265	0.0044	22.000
Within (Error)	21	0.0033	0.0002	
Total	27	0.0298		

Critical F value = 2.57 (0.05,6,21)
Since F > Critical F REJECT Ho:All groups equal

Mean Wet Weight (g), day 38, TWA ug ai/L
File: 4201ww Transform: NO TRANSFORM

DUNNETTS TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	neg control	0.136	0.136		
2	4.57	0.134	0.134	0.200	
3	8.86	0.128	0.128	0.800	
4	17.7	0.112	0.112	2.475	*
5	36.0	0.107	0.107	2.950	*
6	74.5	0.093	0.093	4.300	*
7	148	0.041	0.041	9.525	*

Dunnett table value = 2.46 (1 Tailed Value, P=0.05, df=20,6)

Mean Wet Weight (g), day 38, TWA ug ai/L
File: 4201ww Transform: NO TRANSFORM

DUNNETTS TEST - TABLE 2 OF 2 Ho:Control<Treatment

	NUM OF	Minimum Sig Diff	% of	DIFFERENCE

GROUP	IDENTIFICATION	REPS	(IN ORIG. UNITS)	CONTROL	FROM CONTROL
1	neg control	4			
2	4.57	4	0.025	18.1	0.002
3	8.86	4	0.025	18.1	0.008
4	17.7	4	0.025	18.1	0.025
5	36.0	4	0.025	18.1	0.030
6	74.5	4	0.025	18.1	0.043
7	148	4	0.025	18.1	0.095

Mean Wet Weight (g), day 38, TWA ug ai/L
File: 4201ww Transform: NO TRANSFORM

WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	neg control	4	0.136	0.136	0.136
2	4.57	4	0.134	0.134	0.134
3	8.86	4	0.128	0.128	0.128
4	17.7	4	0.112	0.112	0.112
5	36.0	4	0.107	0.107	0.107
6	74.5	4	0.093	0.093	0.093
7	148	4	0.041	0.041	0.041

Mean Wet Weight (g), day 38, TWA ug ai/L
File: 4201ww Transform: NO TRANSFORM

WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

IDENTIFICATION	ISOTONIZED MEAN	CALC. WILLIAMS	SIG P=.05	TABLE WILLIAMS	DEGREES OF FREEDOM
neg control	0.136				
4.57	0.134	0.224		1.72	k= 1, v=21
8.86	0.128	0.894		1.80	k= 2, v=21
17.7	0.112	2.767	*	1.83	k= 3, v=21
36.0	0.107	3.298	*	1.84	k= 4, v=21
74.5	0.093	4.808	*	1.85	k= 5, v=21
148	0.041	10.649	*	1.85	k= 6, v=21

s = 0.013
Note: df used for table values are approximate when v > 20.

Estimates of EC%

Parameter	Estimate	95% Bounds Lower	95% Bounds Upper	Std.Err.	Lower Bound /Estimate
EC5	26.	14.	47.	0.13	0.55
EC10	35.	22.	58.	0.10	0.61
EC25	59.	43.	81.	0.066	0.73
EC50	1.0E+02	88.	1.2E+02	0.037	0.84

Slope = 2.71 Std.Err. = 0.492

Goodness of fit: p = 0.094 based on DF= 4.0 21.

4201WW : Mean Wet Weight (g), day 38, TWA ug ai/L

Observed vs. Predicted Treatment Group Means

Dose	#Reps.	Obs. Mean	Pred. Mean	Obs. -Pred.	Pred. %Control	%Change
0.00	4.00	0.136	0.128	0.00839	100.	0.00
4.57	4.00	0.134	0.128	0.00641	100.	0.0110
8.86	4.00	0.128	0.128	0.000621	99.8	0.178
17.7	4.00	0.111	0.126	-0.0141	98.2	1.79
36.0	4.00	0.107	0.115	-0.00786	89.6	10.4
74.5	4.00	0.0932	0.0840	0.00928	65.7	34.3
148.	4.00	0.0410	0.0438	-0.00279	34.2	65.8

APPENDIX 2: COPY OF REVIEWER'S TWA CALCULATIONS:

Nominal Concentration (ug ai/L)	Time (Day)	Measured Concentration (ug ai/L)	TWA (ug ai/L)
5.0	0	5.65	
	7	4.13	
	14	4.70	
	21	4.29	
	28	4.44	
	38	4.83	**4.566**
10	0	9.98	
	7	8.50	
	14	8.90	
	21	8.81	
	28	8.86	
	38	8.61	**8.862**
20	0	19.6	
	7	17.3	
	14	17.7	
	21	16.8	
	28	17.3	
	38	19.2	**17.743**
40	0	36.3	
	7	37.4	
	14	35.0	
	21	35.7	
	28	34.4	
	38	38.2	**35.978**
80	0	69.0	
	7	75.0	
	14	74.2	
	21	77.2	
	28	73.5	
	38	76.1	**74.514**
160	0	149	
	7	151	
	14	145	
	21	151	
	28	143	
	38	155	**148.447**