UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION

MEMORANDUM

Date: 6/29/2015

SUBJECT: Chlorpyrifos: Data Evaluation Records (DERs) for EDSP Tier 1 Assays

PC Code: 059101
Decision No.: 459011
Petition No.: NA
Risk Assessment Type: NA
TXR No.: 0052086
MRID No.: See Table

DP Barcode: D397128
Registration No.: NA
Regulatory Action: NA
Case No.: NA
CAS No.: 2921-88-2
40 CFR: NA

Ver.Apr. 2010

FROM: Greg Akerman, Ph.D.
Immediate Office
Health Effects Division (7509P)

THROUGH: Jess Rowland
Deputy Director
Health Effects Division

TO: Jolene Trujillo
Biologist/Chemical Review Manager
Risk Management and Implementation Branch V
Pesticide Re-evaluation Division (7505P)

I. ACTION REQUESTED

The Pesticide Re-evaluation Division (PRD) of OPP has requested that the Health Effects Division (HED) review the Endocrine Disruptor Screening Program (EDSP) Tier 1 assays submitted in response to the agency's Test Order for chlorpyrifos: Test Order # CON-059101-4.

II. RESPONSE

Attached are the EDSP Tier 1 assay DERs for chlorpyrifos.

III. MRID Table

Chemical: Chlorpyrifos		**PC Code: 059101**
Guideline	**Assay**	**MRID**
890.1100	Amphibian Metamorphosis Assay (Frog)	48615501
890.1150	Androgen Receptor Binding (Rat Prostate)	48615502
890.1200	Aromatase Assay (Human Recombinant)	48615503
890.1250	Estrogen Receptor Binding	48615504
890.1300	Estrogen Receptor Transcriptional Activation (Human Cell Line HeLa-9903)	48615505
890.1350	Fish Short-Term Reproduction	48615506
890.1400	Hershberger (Rat)	48615507
890.1450	Female Pubertal (Rat)	48615508
890.1500	Male Pubertal (Rat)	48615509
890.1550	Steroidogenesis (Human Cell Line – H295R)	48615510
890.1600	Uterotrophic (Rat)	48615511

Data Requirement:	EPA DP Barcode	397139
	OECD Data Point	231
	EPA MRID	48615501
	EPA Guideline	890.1100
		Amphibian Metamorphosis Assay (Frog)

Test Material:	**Chlorpyrifos**		**Purity (%):** 99.8%
Common Name	Chlorpyrifos		
Chemical Name	IUPAC	O,O-Diethyl O-(3,5,6-Trichloro-2-pyridinyl)phosphorothioate	
	CAS Name	Phosphorothioic acid, O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) ester	
	CAS No.	2921-88-2	
	Synonyms	Dursban R	
	EPA PC Code	059101	

Primary Reviewer: John Marton — **Signature:**
Staff Scientist, Cambridge Environmental, Inc. — **Date:** 04/10/2012

Secondary Reviewer: Teri S. Myers — **Signature:**
Senior Scientist, Cambridge Environmental, Inc. — **Date:** 05/31/2012

Primary Reviewer: Amy Blankinship — **Signature:** AMY BLANKINSHIP Digitally signed by AMY BLANKINSHIP DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=AMY BLANKINSHIP, dnQualifier=0000040917 Date: 2015.06.04 12:12:10 -04'00'
USEPA/OCSPP/OPP/EFED/ERB3 — **Date:** 09/12/2012

Additional Reviewer: Catherine Aubee — **Signature:**
USEPA/OCSPP/OPP/EFED/ERB4 — **Date:** 06/03/2015

Final Additional Reviewer: Robin Sternberg — **Signature:** Digitally signed by ROBIN STERNBERG DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=ROBIN STERNBERG, dnQualifier=0000039126 Date: 2015.06.03 12:06:29 -04'00'
USEPA/OCSPP/OPP/EFED/ERB1 — **Date:** 05/28/2015

Date Evaluation Completed: 05/28/2015

CITATION: Coady, K.K., C.M. Lehman, K.L. Hutchinson, T.A. Marino, N. Malowinski, and J. Thomas. 2011. Chlorpyrifos: The Amphibian Metamorphosis Assay Using the African Clawed Frog, *Xenopus laevis*. Unpublished study performed by Toxicology and Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan. Laboratory report number 101127. Study sponsored by Dow AgroSciences LLC, Indianapolis, Indiana. Study completed August 8, 2011.

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Disclaimer: The guideline recommendations in this DER template are offered as a general reference to aid in preparation of the DER. The purpose of these recommendations is not to serve as substitute for the Test Guidelines, nor to provide any guidance on how the study should be conducted.

EXECUTIVE SUMMARY

The 21-day assay of chlorpyrifos on amphibian metamorphosis of the African clawed frog (*Xenopus laevis*) was conducted under flow-through conditions. Amphibian larvae at Nieuwkoop-Faber (NF) stage 51 (80/control and treatment group; 20/replicate) were exposed to negative and solvent (0.1 mL/L dimethylformamide; DMF) controls and chlorpyrifos (99.8% purity) at nominal test chemical concentrations of 0.000310, 0.00125, 0.00500, and 0.0200 mg a.i./L. The 21-day time-weighted average (TWA), measured concentrations were <0.0000537 (<LOQ; controls), 0.000215, 0.000881, 0.00368, and 0.0136 mg a.i./L. The test system was maintained at 21.4 to 22.8°C and a pH of 7.0 to 7.6.

Unless otherwise indicated, all effects are reported based on comparison to the negative control.

There were no treatment-related effects on survival, which ranged from 98.3 to 100% across the controls and all treatment groups. Several tadpoles were observed swimming erratically on Day 8 in the TWA 0.0136 mg a.i./L treatment group; no other behavioral abnormalities were noted.

Chlorpyrifos had no significant effect ($p>0.05$) on any Day 7 growth or development parameters. Chlorpyrifos significantly reduced (Jonckheere-Terpstra; $p<0.05$) Day 21 body wet weight by 23 and 39% at TWA 0.00368 and 0.0136 mg a.i./L, respectively, relative to the negative control. Day 21 snout-vent length (SVL) was also significantly reduced (Jonckheere-Terpstra; $p<0.05$) by 4 to 15% at TWA 0.000881, 0.00368, and 0.0136 mg a.i./L compared to the negative control.

At TWA 0.0136 mg a.i./L, chlorpyrifos significantly delayed (Jonckheere-Terpstra, $p=0.03$) Day 21 NF developmental stage by one stage and significantly reduced (Jonckheere-Terpstra, $p=0.03$) normalized (for snout-vent length) hind-limb length (HLL) by 16.6% when compared to the negative control. No asynchronous development was observed. Mild follicular cell hypertrophy was observed in both controls and all treatment groups, with no apparent treatment-related response.

Concentration-dependent significant reductions ($p<0.05$) of 21-79% in cholinesterase activity from hind limb tissue were observed in all treatment groups on Day 21 compared to the negative control. Cholinesterase

activity was also significantly reduced ($p<0.05$) by 28 and 68% in tail tissue of the TWA 0.00368 and 0.0136 mg a.i./L treatment groups, respectively, on Day 21 relative to the negative control.

The study met all validity and performance criteria with the exception that the coefficient of variation (CV) of the measured concentration for the nominal 0.0200 mg a.i./L treatment group was 22%, exceeding the guideline performance criterion of ≤20%. This deviation did not impact the interpretation of the study.

The assay satisfies the ESP Tier 1 Test Order requirements for an Amphibian Metamorphosis assay (OCSPP Guideline 890.1100).

Results Synopsis:

Test organism NF stage at test initiation: 51
Test organism total length at test initiation (optional): Not reported
Test type: Flow-through

Table 1: Summary of Developmental and Thyroid Pathology/Histopathology Effects[1,2] in the Amphibian Metamorphosis Assay (AMA) with Chlorpyrifos.

Treatment (mg a.i./L) [TWA-measured]	NF Developmental Stage		Hind Limb Length[3]		Asynchronous Development		Thyroid Gross and Histopathology
	Day 7	Day 21	Day 7	Day 21	Day 7	Day 21	Day 21
0.000215	No	No	No	No	No	No	No
0.000881	No	No	No	No	No	No	No
0.00368	No	No	No	No	No	No	No
0.0136	No	Yes	No	Yes	No	No	No

Abbreviations: Diff. Difference. NA Not applicable.

1 A "yes" indicates a significant difference based on comparison to the negative (clean water) control, unless otherwise specified.

2 The criteria for significance are described in the Reviewer's Analysis and Statistical Verification sections of the DER. Conclusions regarding histopathology may be heavily weighted by the expert opinion of a board-certified pathologist.

3 Hind-limb length is normalized to snout-vent length (SVL).

I. MATERIALS AND METHODS

Guideline Followed: This study was conducted following guidelines outlined in: United States Environmental Protection Agency (2009), Endocrine Disruptor Screening Program Test Guidelines OCSPP 890.1100: Amphibian Metamorphosis (Frog), EPA 740-C-09-002, October 2009. The following deviations from 890.1100 were noted:

1. The CV for the measured concentration of the nominal 0.0200 mg a.i./L level was 22% which exceeds the guideline performance criterion of 20%.
2. The storage conditions of the test material were not specified.
3. It was not specified if acclimation conditions were similar to test conditions.
4. The acclimation period for the parental frogs was not specified.
5. No details on the parental feeding regime or parental health were provided.
6. Tadpoles were not selected based on the best single spawn approach.

These deviations do not impact the interpretation of the study.

Compliance: Signed and dated No Data Confidentiality, GLP, and Quality Assurance statements were provided. This study was conducted in compliance with the following Good Laboratory Practice Standards: OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, Number 1, OECD Principles on Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17; and, US Environmental Protection Agency, FIFRA GLPs Title 40 CFR, Part 160, Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Good Laboratory Practice Standards, Final Rule.

A. Test Material Chlorpyrifos (CAS No. 2921-88-2)

Description: Light tan, crystalline solid

OECD recommends describing water solubility, melting/boiling point stability in water and light, pKa, Pow or Kow, vapor pressure of test compound, expiration date.

Lot No./Batch No.: KC28161419, TSN101285 (Lot #)

Purity: 99.8%

Impurities: None Reported

Stability of Compound: The reported TWA concentrations had recoveries of 68% to 74% of nominal and coefficients of variation of 14.5% to 22.1%.

Storage Conditions of Test Chemicals: Stored between 5°C and ambient conditions. No further details were provided.

B. Test Organism

Table 2: General Information About the Test Species and Parental Care.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Species common name:	African clawed frog		*EPA recommends African clawed frog (Xenopus laevis). Western [Africa] clawed frog Silurana (Xenopus) tropicalis may be used as an alternate species*[1]*; however, a list of all of the necessary protocol deviations to accommodate this species is recommended for inclusion in the study report. The guideline recommends that the performance criteria used to support the reliability of the test be identified.*
Species scientific name:	*Xenopus laevis*		
Species strain (if stated):	Not stated		

[1] U.S. Environmental Protection Agency (EPA). (2011). Corrections and Clarifications on Technical Aspects of the Test Guidelines for the Endocrine Disruptor Screening Program Tier 1 Assays (OCSPP Test Guideline Series 890). March 3, 2011. Office of Chemical Safety and Pollution Prevention (OCSPP), Washington, D.C. (http://www.epa.gov/endo/pubs/assayvalidation/clarificationdoc.pdf).

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Were parents maintained as in-house stock?	Yes. Stock culture originally obtained from *Xenopus* Express, Brooksville, Florida.		*EPA recommends that larvae used in the assay be derived from in-house adults.*
Were parental acclimation conditions same as definitive test?	Not specified		
Acclimation period for parental frogs (if applicable):	Not reported		
Details on parental feeding:	Not specified		
Details on parental health:	Not specified		

Table 3: Larval Selection and Care.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Best single spawn?	Not reported	Multiple spawns were available for use. Report indicates that on day of test initiation, tadpoles were removed from their rearing tanks and staged and NF stage 51 tadpoles were selected at this time w/o anesthesia and tadpoles assigned to aquaria in a randomized block design.	*EPA and OECD recommend that the best 2 – 3 individual spawns, with a minimum of 1500 larvae/spawn, be evaluated to identify the best single spawn, and that the larvae selected for testing originate from the best single spawn (i.e., the spawns are not co-mixed)*
Number of spawns evaluated (if applicable):	Not reported		
Number of eggs sampled per spawn:	Not reported		
NF stage at test initiation	NF 51		*EPA recommends that the definitive study be initiated with larvae at Nieuwkoop – Faber (NF) developmental stage 51 (≤17 days post-fertilization).*
Age at test initiation:	13 days post-fertilization (dpf)		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Mean total length at test initiation (if reported):	Not reported		
Range of total length at test initiation (if reported):	Not reported		
Was the optional size selection method used?	No		
Details on larval selection:	Tadpoles were individually staged without anesthesia. No further details on larval selection were provided.		
Loading rate (rearing density):	~4 larvae/L	Holding capacity of 5.2 L in test vessels, each containing 20 tadpoles.	*EPA recommends that rearing density (loading rate) not exceed approximately 10 larvae/L culturing system for flow-through systems or 4 tadpoles/L in static-renewal exposure systems.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Type of food:	Sera Micron®		*EPA recommends Sera Micron® throughout pre-exposure (after NF stage 45/46) and during the entire 21-d definitive study. If another diet is used, the study report should provide analysis of iodide content and potential contaminants, and the diet should demonstrate equal performance to Sera Micron®.*
Source of food:	Sera North America, Montgomeryville, PA		
Iodide concentration in diet (if known):	40 mg/kg	Measured by A & L Great Lakes Laboratories, Inc.	
Frequency of feeding:	2 times/day		*EPA recommends that feeding occur at least twice per day.*
Details on feeding regime:	Feeding regime followed guideline recommendations.		*It is recommended that food rations during the pre-exposure period be increased along with larval growth to approximately 30 mg/larva/day by test initiation. EPA and OECD recommend that food rations increase from 30 mg/larva/day at test initiation (Study Day 0-4) to 80*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
			mg/larva/day in the last week of the test (Study Day 15-21).

C. Exposure System

Table 4: Summary of Information on the Exposure System and Test Vessel Characteristics.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Type of exposure:	Flow-through		*EPA recommends the use of a flow-through system.*
Type of flow-through dilution system (if applicable):	Continuous-flow diluter		*Intermittent flow proportional diluters or continuous flow serial diluters are recommended.*[2]
Flow-through rate (if applicable):	64 mL/min	Complete volume replacement every 1.4 hrs.	*Recommended flow-through rate is 25 mL/min (complete volume replacement ca. every 2.7 hrs).*

[2] Additional guidance for aquatic test design is located in OCSPP Guideline 850.1000, Special Considerations for Conducting Aquatic Laboratory Studies.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Details on toxicant mixing for flow-through systems (if applicable):	For each treatment level, a continuous-flow syringe pump delivery system using gas-tight syringes delivered a concentrated test substance stock solution at a designated rate from a stock vessel to a stainless steel mixing chamber where it was mixed with dilution water.	The diluter system was calibrated prior to test initiation and was monitored at least two times daily throughout the test and flow rates were quantitatively assessed on a daily basis.	*Recommended toxicant mixing for flow-through systems: 1) Mixing chamber is recommended but not required; 2) Aeration is not recommended for mixing; 3) A demonstration that the test solution is completely mixed before introduced into the test system is recommended; 4) The recommended flow splitting accuracy is within 10%.*
Renewal period for static renewal (if applicable):	NA		*If static renewal is used, EPA recommends 24-hr renewal; renewal period is recommended not to exceed 72 hours.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Aeration?	None provided		*EPA recommends maintaining dissolved oxygen concentrations ≥40% air saturation (≥3.5 mg/L). Aeration may be maintained through bubblers. It is recommended to set bubblers at levels that do not cause stress on the tadpoles.*
Source of dilution water:	Dilution water was from Lake Huron, supplied to The Dow Chemical Company by the City of Midland Water Treatment Plant. Water was obtained from the upper Saginaw Bay of Lake Huron of Whitestone Point and was limed and flocculated with ferric chloride. Prior to use,		*EPA recommends natural or reconstituted water; it is recommended that natural water be sterilized with UV and tested for pesticides, heavy metals, and other possible contaminants, including known substrates of the iodine transporter of the thyroid gland (e.g., fluoride, chlorate, perchlorate). OECD accepts any water in which the test species show control survival at least as good as indicated in the test guideline.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
	water was sand-filtered, pH-adjusted with CO_2, carbon-filtered, and UV-irradiated.		
Was dilution water analyzed for pesticides, heavy metals, and other contaminants?	Yes. No pesticides, PCBs, or toxic metals were detected at concentrations considered lethal to *Xenopus laevis*.		
Iodide supplementation in water?	No		*If reconstituted water is used or if background levels of iodide in natural water are less than 0.5 µg/L, iodide supplementation is recommended. This supplementation is in addition to the recommended dietary source of iodide (e.g.. in Sera Micron).*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Test vessel type/materials:	Glass, sealed together with clean silicone adhesive.		*EPA and OECD recommend that water-contact portions of the system not compromise the study (e.g., all glass vessels or glass vessels with stainless steel frames are acceptable examples).*
Test vessel size:	30 x 14.5 x 20 cm; water depth was maintained at 12 cm by a screen covered drain.		
Fill volume:	5.2 L		
Additional details on exposure system:	Diluter and aquaria were cleaned prior to test initiation. All replicate vessels were cleaned daily. Diluter mixing cells and delivery tubing were		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
	cleaned or replaced as needed.		

Table 5: Summary of Water Quality Characteristics in the Test System.

Parameter	Minimum	Maximum	Mean	Measurement Interval	Guideline Recommendations
Hardness (mg/L as $CaCO_3$)	54	70	60.5 [a]	Weekly	*EPA recommends hardness 40 to 48 mg/L as $CaCO_3$.*
pH	7.0	7.6	7.4 [a]	Weekly	*EPA recommends pH 7.5 ± 1, inter-replicate and inter-treatment differentials should not exceed 0.5.*
Dissolved oxygen (mg/L)	6.0	8.4	7.5 [a]	Weekly	*EPA recommends dissolved oxygen (DO) >3.5 mg/L (>40% air saturation). OECD recommends DO concentration >3.5 mg/L (>40% air saturation).*

Parameter	Minimum	Maximum	Mean	Measurement Interval	Guideline Recommendations
Temperature	21.4	22.8	22.3 [a]	Continuous	*EPA recommends temperature 22±1°C; inter-replicate and inter-treatment differentials should not exceed 0.5°C.*
Iodide			<0.01 mg/L	Measured twice per year	*EPA recommends aquatic iodide range 0.5 – 10 µg/L (supplemental iodide should not exceed 2 µg/L).*
Ammonia			<0.1 mg//L	Measured twice per year	*General recommendations for frequency of measurements: EPA recommends that water quality parameters be measured in a control and at one test item concentration at least weekly. In static renewal systems, water quality parameters, including ammonia, should be measured just prior to renewal. In addition, EPA recommends that DO be*
Fluoride			<0.1 mg/L	Measured twice per year	
Perchlorate			<0.2 µg/L	Measured twice per year	
Chlorate			<10 µg/L	Measured twice per year	

Parameter	Minimum	Maximum	Mean	Measurement Interval	Guideline Recommendations
Alkalinity (mg/L as $CaCO_3$)	24	56	40 [a]	Weekly	*measured at each concentration at least weekly and that temperature be measured continuously. OECD recommends that DO and temperature be measured at least weekly and that pH and hardness be measured at least at the beginning and end of the test.*
Conductivity (µmhos/cm)	174.9	199.8	187.6 [a]		

[a] Means were calculated by the reviewer as the average of the minima and maxima for the ranges provided across control and treated levels

D. Study Design and Additional Experimental Conditions

Table 6: Range-Finding Study Conditions (if Applicable).

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Was a range-finder conducted?	Yes		
If yes, what was the method for determining the highest test	Review of existing literature on toxicity of		*EPA recommends that the highest test concentration is either the solubility limit of*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
concentration in the range-finder?	chlorpyrifos to *Xenopus laevis*.		*the test compound, 100 mg/L, or demonstrates adequate evidence of toxicity (e.g., ≤10% mortality), whichever concentration is lowest.*
Species:	*Xenopus laevis*		
Life stage:	>95% NF stage 53		
Test duration:	14 days		
Additional details:	The test was conducted under flow-through conditions using nominal concentrations of 0 (negative and solvent controls), 0.20, 1.00, 5.00, and 25.0 µg a.i./L with two	No significant mortality was observed after 14 days. There was, however, abnormal surfacing among treated tadpoles was transiently noted, and this was particularly evident in the highest treatment group. Tadpoles at the highest treatment group (nominal 25 µg a.i./L; measured 18 µg a.i./L) were smaller with abnormal hind limb	

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
	replicate vessels, each containing 20 tadpoles.	development (edema in the hind limbs and hind limb digits).	

Table 7: Definitive Study Conditions.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Test duration:	21 days		*EPA recommends that the duration of the definitive test be 21 days.*
Method for selecting the highest test concentration in the definitive test:	Based on range-finding test and scientific literature review.		*EPA recommends that the highest test concentration is either the solubility limit of the test compound, 100 mg/L, or demonstrates adequate evidence of toxicity (e.g., ≤10% mortality), whichever concentration is lowest.*
Reference study citation (if applicable):	Henry and Kirk (2001); Richards and Kendall (2002, 2003); Sparling and Fellers		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
	(2007); El-Merhibi *et al.* (2004); Colombo *et al.* (2005)		
Separation of test concentrations:	0.25		*EPA recommends that the maximum concentration separation be 0.1 and the minimum be 0.33.*
Number of test concentrations:	4		*EPA recommends a minimum of 3 concentrations and a control, plus solvent control if appropriate.*
Are nominal concentrations adjusted for purity?	Yes		
Indicate the type of values presented for measured concentrations:	Time-weighted average (TWA)		
Limit of quantification (LOQ):	0.0000537 mg a.i./L		*EPA recommends that for chemical test concentrations below the LOQ, analyses be conducted on the stock solutions.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Level of detection (LOD):	Not Reported		
Frequency of measurement:	Days 0, 1, 4, 7, 10, 11, 12, 14, 14.5, 15, 18, and 21	Aliquots were collected from each replicate test vessel at each sampling interval.	*It is recommended that test item concentration be measured in one tank at each treatment level at test initiation and every week thereafter.*
Number of replicates in control:	4		*EPA recommends 4 replicates.*
Number of replicates in solvent control (if applicable):	4		*EPA and OECD recommend the use of a concurrent solvent control when a solubilizing agent is used. EPA recommends 4 replicates.*
Number of replicates per test item treatment level:	4		*EPA recommends 4 replicates.*
Number of larvae per treatment at test initiation:	80 (equally divided among 4 replicates)		
Was a solvent used?	Yes		
Solvent type (if applicable):	DMF		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Maximum solvent concentration (if applicable):	0.1 mL/L	OCSPP 890.1100 guidance does not specifically state a maximum solvent concentration. However, the solvent concentration was within acceptable limits specified by OECD 231.	*EPA recommends that the solvent not exceed 0.02 ml/L*[3]*. OECD recommends that solvent have no effect on survival nor produce any other adverse effects and that concentration not be greater than 0.1 ml/L*[4]*.*
Was a positive control used?	No		
Positive control (if applicable):	NA		
Positive control concentration(s) (if applicable):	NA		
Photoperiod:	12 hrs light : 12 hrs dark		*EPA recommends photoperiod 12:12 (light:dark).*

[3] Hutchinson TH, Shillabeer N, Winter MJ, Pickford DB (2006). Acute and chronic effects of carrier solvents in aquatic organisms: A critical review. Review. Aquatic Toxicology, 76, pp.69–92.

[4] OECD (2000). Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. Environmental Health and Safety Publications. Series on Testing and Assessment. No. 23. Paris, France.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Light intensity at water's surface:	0.587-0.926 Klux		*EPA recommends light intensity 0.6 – 2 Klux (at water's surface).*
Additional details:		Specific details on test solution appearance did not appear to be reported in the study report.	

Table 8: Summary of Treatment Concentrations in the Amphibian Metamorphosis Assay with Chlorpyrifos.

Treatment ID	Nominal Concentration (mg a.i./L)	Measured Concentration (mg a.i./L)	Mean CV (%)	Details or Remarks	Guideline Recommendations
Negative Control	0	<LOQ	NA		*EPA and OECD recommend that test item concentrations be maintained at a coefficient of variation (CV) ≤20%.*
Solvent Control	0	<LOQ	NA		
Treatment 1	0.000310	0.000215	15.3		
Treatment 2	0.00125	0.000881	15.7		
Treatment 3	0.00500	0.00368	14.5		
Treatment 4	0.0200	0.0136	22.1		

Abbreviations: ^{CV} Coefficient of variation.

LOQ=0.0000537 mg a.i./L

E. Observations

Biological Endpoints: Days 7 and 21- NF stage, asynchronous development, whole body wet weight, hind limb length, normalized hind limb length. Observations for mortality and abnormal behaviors were made daily. Histopathology and cholinesterase inhibition assessment were performed at test termination.

Were raw (individual) data provided? Yes

EPA recommends that observations of mortality and clinical signs occur daily, at a minimum; other observations are recommended as follows: NF developmental stage (Days 7 and 21); any asynchronous development, indicated by tadpoles that cannot be assigned an NF stage (Days 7 and 21); hind limb length (Days 7 and 21); snout-vent length (Days 7 and 21); body weight (test initiation, for optional size-based larval selection); and thyroid gland gross pathology and histopathology (Day 21). Note the histopathology section of the test guideline also includes thyroid gross pathology observations.

II. RESULTS AND DISCUSSION

A. Results

Single mortalities were observed in the solvent control and TWA 0.000215, 0.000881, and 0.0136 mg a.i./L treatment groups (specific details about day of mortality not provided). A second individual in the highest treatment group was accidentally killed during handling and was therefore not included in the reported mortality at this level.

Table 9: Larval Mortality in *Xenopus laevis*.

Treatment (mg a.i./L) [TWA-measured]	Larval Mortality					
	Day 7[1]			Day 21		
	n	Mortality #	Mortality %	n	Mortality #	Mortality %
Negative Control (<LOQ)	--	--	--	80	0	0
Solvent Control (<LOQ)	--	--	--	80	1	1.7
0.000215	--	--	--	80	1	1.7
0.000881	--	--	--	80	1	1.7
0.00368	--	--	--	80	0	0
0.0136	--	--	--	80	1	1.7

Abbreviations: [NA] Not applicable.

LOQ=0.0000537 mg a.i./L

[1] Only total mortality was reported. Therefore, Day 21 mortality represents the cumulative 21-day mortality averages.

Median NF development stage on Day 7 was 53 in the controls and all treatment groups and ranged from 51 to 54. By Day 21, median NF stage was 57 in both controls and ranged from 56 to 58 in the treatment groups, with 10th and 90th percentiles of 56 and 59, respectively. No asynchronous development was observed throughout the test.

Table 10: Larval Development in *Xenopus laevis* – Developmental Stage and Asynchronous Development.

Treatment (mg a.i./L) [TWA-measured]	Developmental Stage					
	Day 7			Day 21		
	n	Median Stage	# Asynchronous	n	Median Stage	# Asynchronous
Negative Control (<LOQ)	4	53	0	4	57	0
Solvent Control (<LOQ)	4	53	0	4	57	0
0.000215	4	53	0	4	57	0
0.000881	4	53	0	4	58	0
0.00368	4	53	0	4	57	0
0.0136	4	53	0	4	56	0

Abbreviations: ^{NA} Not applicable.

LOQ=0.0000537 mg a.i./L

Sample size (n) represents the number of independent replicates.

On Day 7, normalized HLL ranged from 0.10 (0.000881 mg a.i./L) to 0.12 (negative control, 0.000215 mg a.i./L, 0.00368 mg a.i./L). On Day 21, normalized HLL ranged from 0.36 (0.136 mg a.i./L) to 0.047 (0.000881 mg a.i./L).

Table 11: Larval Development in *Xenopus laevis* – Hind Limb Length.

Treatment (mg a.i./L) [TWA-measured]	Hind Limb Length (HLL)							
	Day 7				Day 21			
	n[1]	Mean (mm)	±SD	HLL: SVL[2]	n	Mean (mm)	±SD	HLL: SVL[2]
Negative Control (<LOQ)	4	1.82	0.046	0.12	4	11.9	0.19	0.42
Solvent Control (<LOQ)	4	1.89	0.092	0.11	4	13.0	0.25	0.45
0.000215	4	1.93	0.073	0.12	4	12.8	1.34	0.45
0.000881	4	1.62	0.028	0.10	4	12.9	1.93	0.47
0.00368	4	1.85	0.11	0.12	4	11.5	1.22	0.43
0.0136	4	1.56	0.069	0.11	4	8.87	0.96	0.36

Abbreviations: [NA] Not applicable. [SD] Standard deviation.

LOQ=0.0000537 mg a.i./L

[1] Sample size (n) represents the number of independent replicates.

[2] Summary results for snout-vent length (SVL) are presented in the next table (Table 12).

Day 7 SVL ranged from 14.6 mm (0.0136 mg a.i./L) to 16.3 mm (solvent control), and Day 21 SVl ranged from 24.1 mm (0.0136 mg a.i./L) to 28.5 mm (negative control). Day 7 body weight ranged from 0.274 g (0.0136 mg a.i./L) to 0.0363 g (0.000215 mg a.i./L), and Day 21 body weight ranged from 1.057 g (0.0136 mg a.i./L) to 1.733 g (negative control).

Table 12: Larval Growth in *Xenopus laevis*.

Treatment (mg a.i./L) [TWA -measured]	Snout-Vent Length (SVL)						Body Weight[1]					
	Day 7			Day 21			Day 7			Day 21		
	n	Mean (mm)	±SD	n	Mean (mm)	±SD	n	Mean (g)	±SD	n	Mean (g)	±SD
Negative Control (<LOQ)	4	15.8	0.53	4	28.5	0.43	4	0.320	0.03	4	1.733	0.06
Solvent Control (<LOQ)	4	16.3	0.93	4	28.3	1.5	4	0.362	0.06	4	1.724	0.23
0.000215	4	16.2	0.34	4	28.3	0.82	4	0.363	0.03	4	1.723	0.21
0.000881	4	15.6	0.51	4	27.4	0.97	4	0.317	0.03	4	1.548	0.23
0.00368	4	16.1	0.77	4	26.3	0.90	4	0.345	0.04	4	1.342	0.16
0.0136	4	14.6	0.77	4	24.1	1.31	4	0.274	0.03	4	1.057	0.12

LOQ=0.0000537 mg a.i./L; Abbreviations: NA Not applicable. ND Not determined. SD Standard deviation.

[1] Also referred to as “wet weight” in the test guideline.

The overall size of the thyroid glands of tadpoles exposed to increasing concentrations of chlorpyrifos were comparable to the variability observed in the controls. Further, there was no evidence of glandular atrophy or hypertrophy, or follicular cell hyperplasia in any of the thyroid glands examined across all treatment groups (7-10/group). Mild follicular cell hypertrophy was observed in both controls and all treatment groups. All other histopathological criteria, such as the overall size of the gland, the follicular lumen area, amount and type of colloid, and the follicular cell type and arrangement, of all tadpoles exposed to chlorpyrifos were comparable to those of the controls (data not presented). According to the study report, five juvenile frogs that corresponded to the control median developmental stage were randomly selected from each replicate tank if sufficient numbers allowed. If insufficient numbers of tadpoles, then randomly selected individuals from the next lower and upper developmental stages were alternatively selected to reach a total sample size of five tadpoles/replicate tank.

Table 13: Gross Pathology and Histopathology of the Thyroid Gland in *Xenopus laevis*.

Treatment (mg a.i./L) [TWA-measured]	Diagnostic Observations[1]								
	Severity	Thyroid Gland Hypertrophy		Thyroid Gland Atrophy		Follicular Cell Hypertrophy		Follicular Cell Hyperplasia	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative Control (<LOQ)	0	20	20	20	20	20	11	20	20
	1	20	0	20	0	20	9	20	0
	2	20	0	20	0	20	0	20	0
	3	20	0	20	0	20	0	20	0
Solvent Control (<LOQ)	0	20	20	20	20	20	12	20	20
	1	20	0	20	0	20	8	20	0
	2	20	0	20	0	20	0	20	0
	3	20	0	20	0	20	0	20	0

Treatment (mg a.i./L) [TWA-measured]	Diagnostic Observations[1]								
	Severity	Thyroid Gland Hypertrophy		Thyroid Gland Atrophy		Follicular Cell Hypertrophy		Follicular Cell Hyperplasia	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
0.000215	0	20	20	20	20	20	10	20	20
	1	20	0	20	0	20	10	20	0
	2	20	0	20	0	20	0	20	0
	3	20	0	20	0	20	0	20	0
0.000881	0	20	20	20	20	20	13	20	20
	1	20	0	20	0	20	7	20	0
	2	20	0	20	0	20	0	20	0
	3	20	0	20	0	20	0	20	0
0.00368	0	20	20	20	20	20	11	20	20
	1	20	0	20	0	20	9	20	0
	2	20	0	20	0	20	0	20	0
	3	20	0	20	0	20	0	20	0
0.0136	0	20	20	20	20	20	10	20	20
	1	20	0	20	0	20	10	20	0
	2	20	0	20	0	20	0	20	0
	3	20	0	20	0	20	0	20	0

LOQ=0.0000537 mg a.i./L

[1] Thyroid gland gross pathology and histopathology are graded 0 – 3 based on severity: 0=Not remarkable, 1=Mild, 2=Moderate, 3=Severe. See OECD No. 82 for reference.

Table 14: Additional Thyroid Gland Histopathology Observations in *Xenopus laevis*.

Treatment (mg a.i./L) [TWA-measured]	Additional Qualitative Observations[1]									
	Severity	Follicular Lumen Area (Increase)		Follicular Lumen Area (Decrease)		Follicular Cell Height (Increase)		Follicular Cell Height (Decrease)		Follicular Cell Shape	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative Control (<LOQ)	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Solvent Control (<LOQ)	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
0.000215	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Treatment (mg a.i./L) [TWA-measured]	Additional Qualitative Observations[1]									
	Severity	Follicular Lumen Area (Increase)		Follicular Lumen Area (Decrease)		Follicular Cell Height (Increase)		Follicular Cell Height (Decrease)		Follicular Cell Shape	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
0.000881	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
0.00368	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
0.0136	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Abbreviations: NA Not applicable.

LOQ=0.0000537 mg a.i./L

[1] Thyroid histopathology is graded 0 – 3 based on severity: 0=Not remarkable, 1=Mild, 2=Moderate, 3=Severe. See OECD No. 82 for reference.

On Day 8, several tadpoles in the highest treatment group were observed to be swimming erratically; no other behavioral abnormalities were noted. A few tadpoles were observed with bent tails on Day 21, although these effects were not considered to be treatment-related. No other treatment-related malformations were observed throughout the exposure period.

Table 15: Clinical Signs in *Xenopus laevis*.

Treatment (mg a.i./L) [TWA-measured]	Clinical Signs		
	Type	n	Incidence
Negative Control (<LOQ)	None	80	--
Solvent Control (<LOQ)	None	80	--
0.000215	None	80	--
0.000881	None	80	--
0.00368	None	80	--
0.0136	Several tadpoles swimming erratically on Day 8	80	Not specified

LOQ=0.0000537 mg a.i./L

Cholinesterase activity was measured in the tail and hind limb and results are reported.

Table 16. Mean cholinesterase values ± standard deviation for tail and hind limb tissues of *Xenopus laevis*.

Treatment (mg a.i./L) [TWA-Measured]	Cholinesterase (International Unit/L)			
	n[1]	Tail Tissue	n[1]	Hind Limb Tissue
Negative Control (<LOQ)	19/4	696 ± 148	20/4	1486 ± 410
Solvent Control (<LOQ)	20/4	642 ± 146	20/4	1042 ± 206
0.000215	20/4	745 ± 165	18/4	1170 ± 387
0.000881	20/4	624 ± 138	20/4	825 ± 350
0.00368	20/4	501 ± 194	20/4	717 ± 216
0.0136	20/4	220 ± 104	20/4	319 ± 164

LOQ=0.0000537 mg a.i./L

[1] The study author used individual animals as independent replicates; the reviewer analyzed the data by comparing the mean values for the replicates (n=4).

B. Study Author's Analysis and Conclusions

The appropriate units of statistical analyses were the measures of central tendency from the replicate test vessels. The statistical significance of all tests was judged at the 0.05 significance level, with the exception of the Shapiro-Wilk test, which was judged at the 0.01 significance level. Median values were inspected for each measured endpoint by treatment level to determine if the response was monotonic with increasing concentration. Statistical analyses of the continuous data set, including hind limb length (normalized by SVL), wet weight, and SVL, were analyzed with the Jonckheere-Terpstra test in a step down manner if the data was consistent with a monotone dose-response. If these endpoints were not consistent with a monotone dose-response, the data were assessed for normality using the Shapiro-Wilk's test and homogeneity of variance using Levene's test. When non-normality or heteroskedasticity was observed, normalizing and/or variance stabilizing transformations were applied. If the data were normally distributed with homogenous variances, then a significant treatment effect was determined using Dunnett's test. Hind limb length was normalized by SVL to account for the effects of growth. A significant treatment effect for developmental stage was determined from the step-down application of the multi-quantal Jonckheere-Terpstra test from the 20th to the 80th percentile. For cholinesterase measurements in the tail, the solvent and control controls were not significantly different; however for hind limb, there was a significant difference between the negative and solvent control and treatment groups were compared to the solvent control.

Concentrations of chlorpyrifos used in this study were not lethal to *Xenopus laevis* over the course of the exposure, however, signs of toxicity were apparent from the reduced tadpole wet weight and SVL length observed on both Days 7 and 21 at the two highest treatment concentrations (0.00368 and 0.0136 mg a.i./L). Further, tadpoles at the TWA 0.0136 mg a.i./L treatment group were developmentally delayed with shorter hind limbs relative to controls on Day 21. There were no treatment-related histopathological changes in the thyroid gland in any of the treatment groups. According to the study author, there was no evidence of either increased hypertrophy or hyperplasia in the thyroid gland in response to chlorpyrifos exposure, suggesting that reduced growth and delayed development was likely not associated with altered endocrine activity in the HPT pathway. Further, the reduced cholinesterase activity in both tail and hind limb tissues suggests toxicity among tadpoles.

Since there were no signs of advanced development (as measured by increased developmental stage and/or increased hind limb length) or asynchronous development among chlorpyrifos exposed tadpoles relative to control tadpoles on either day 7 or 21 of the exposure, and since there were no treatment-related histopathological effects in the thyroid glands of chlorpyrifos-exposed tadpoles, the test material is considered "likely thyroid inactive" in the AMA.

C. Reviewer's Analysis and Conclusions

Statistical Methods: Day 21 wet weight and SVL both exhibited a monotonic decreasing trend. Additionally, these data satisfied the assumptions of normality and variance homogeneity, as determined using Shapiro-Wilks and Levene's tests, respectively. As a result, these endpoints were analyzed using the Jonckheere-Terpstra test. The remaining endpoints did not exhibit a monotonic trend and of those, 7-day wet weight, SVL, normalized HLL, and 21-day HLL and normalized HLL satisfied the parametric assumptions; these endpoints were subsequently analyzed using Dunnett's test. Non-monotonic data which failed to satisfy parametric assumptions (i.e., 7- and 21-day development stage and 7-day HLL) were analyzed using the Mann-Whitney U-test. All analyses were conducted using SAS 8.1 and effects were considered statistically significant at $p<0.05$. The reviewer compared all treated levels to the negative control group. The reviewer conducted 2-sided t-tests assuming equal variances to compare the negative and solvent control groups and detected no significant differences between the two ($p>0.05$).

Tail and hind limb cholinesterase data was evaluated using Toxstat v3.5. The treatment groups were compared to the negative control. A statistically significant difference was observed between the negative and solvent control for hind limb values. The reviewer also calculated the mean cholinesterase value for each replicate and used mean replicate data for comparing across treatment groups as oppose to individual animal values as the study author appeared to do. Dunnett's was used to compare treatment groups.

Conclusions:

Day 7 development endpoints (i.e., development stage, normalized HLL, asynchronous) and growth endpoints (*i.e.*, SVL, body weight and HLL) at all treatment levels were comparable to the negative control ($p>0.05$). On Day 21, development was significantly (Jonckheere-Terpstra; $p<0.05$) delayed one NF stage as well as a 17% decrease in normalized hind limb length (Jonckheere-Terpstra; $p<0.05$) at the top concentration, relative to the negative control, while growth was significantly reduced ($p<0.05$) for several parameters. Day 21 SVL was significantly reduced relative to the negative control at the TWA 0.000881-0.0136 mg a.i./L treatment groups (4-15%, $p\leq0.045$), body weight was reduced at the TWA 0.00368 and 0.0136 mg a.i./L treatment groups (23-39%, $p\leq0.004$), and HLL was significantly reduced from the negative control (28%, $p=0.012$) at the 0.0136 mg a.i./L level.

There were no treatment-related effects noted for thyroid pathology. Cholinesterase activity in hind limb tissues (relative to the negative control) was significantly reduced at all treatment groups and in tail tissue (relative to the negative control) at the TWA 0.00368 and 0.0138 mg a.i./L treatment groups (Dunnett's test, $p\leq0.05$).

Table 17: Developmental and Thyroid Gross Pathology/Histopathology Endpoints[1,2] in the AMA with chlorpyrifos.

Treatment (mg a.i./L) [TWA]	NF Developmental Stage				Hind Limb Length[3]				Thyroid Gross and Histopathology
	Day 7		Day 21		Day 7		Day 21		Day 21
	Median	*p*	Median	*p*	% Diff.	*p*	% Diff.	*p*	Treatment-Related Effects? (Yes/No)
Negative Control (<LOQ)	53	NA	57	NA	0	NA	0	NA	No
Solvent Control (<LOQ)	53	0.54	57	0.80	-3.1	0.36	1.00	0.89	No
0.000215	53	0.48	57	0.50	4.4	0.77	4.4	0.61	No
0.000881	53	>0.99	58	0.81	-4.4	0.77	8.5	0.81	No
0.00368	53	0.48	57	0.61	-2.2	0.97	0.6	0.54	No
0.0136	53	>0.99	56	0.03	-4.4	0.77	-16.6	0.03	No
Statistical test	Mann-Whitney U		Jonckheere-Terpstra		Dunnett's		Jonckheere-Terpstra		NA

Abbreviations: [Diff.] Difference. [NA] Not applicable. [NR] Not reported

LOQ=0.0000537 mg a.i./L

1 Unless otherwise indicated, effects are reported based on comparison to the clean water control. Conclusions regarding histopathology may be heavily weighted by the expert opinion of a board-certified pathologist.

2 Unless otherwise specified, effects are considered statistically significant at $p<0.05$.

3 Hind-limb length is normalized to snout-vent length (SVL).

Table 18: Growth Endpoints[1,2] in the AMA with chlorpyrifos.

Treatment (mg a.i./L) [measured]	Snout-Vent Length				Body Weight			
	Day 7		Day 21		Day 7		Day 21	
	% Diff.	*p*	% Diff.	*p*	% Diff.	*p*	% Diff.	*p*
Negative Control (<LOQ)	0	NA	0	NA	0	NA	0	NA
Solvent Control (<LOQ)	3.9	0.33	-0.5	0.85	13.1	0.28	-0.5	0.94
0.000215	2.4	0.79	-0.44	0.33	13.2	0.27	-0.5	0.61
0.000881	-1.2	0.97	-3.7	0.05	-1.1	>0.99	-10.7	0.15
0.00368	2.4	0.80	-7.5	<0.01	7.7	0.69	-22.6	<0.01
0.0136	-7.0	0.07	-15.3	<0.01	-14.5	0.21	-39.0	<0.01
Statistical test	Dunnett's		Jonckheere-Terpstra		Dunnett's		Jonckheere-Terpstra	

Abbreviations: Diff. Difference. NA Not applicable.

LOQ=0.0000537 mg a.i./L

1 Unless otherwise indicated, effects are reported based on comparison to the negative (clean water) control.

2 Unless otherwise specified, effects are considered statistically significant at $p<0.05$.

E. Study Deficiencies

There were deviations from the guideline as noted in Section I. Materials and Methods of the DER. The study met all of the validity and performance criteria with the exception that the coefficient of variation (CV) of the measured concentration for the nominal 0.0200 mg a.i./L treatment group was 22% which exceeds the guideline performance criterion of ≤20%. The study authors addressed this deficiency, attributing it to the limited flow through biofilm-accumulating tubing (as a result of the solvent), as well as the absorptive and lipophilic nature of the test material within the dynamic test system. These deviations and departure from guideline performance criteria did not impact the interpretation of the study.

F. Reviewer's Comments

In general, the reviewer agreed with the study authors' statistical conclusions. The study authors compared treated groups to the pooled control, while the reviewer compared treated groups to only the negative control.

Additionally, the reviewer's interpretation of the 21 day SVL would have been different using historical EFED, as opposed to OCSPP 890.1100 flowchart, methods. Because these data are monotonically decreasing and satisfy parametric assumptions, the suggested use of William's test would have only revealed significant reductions at the top two concentrations, as opposed to the OCSPP 890.1350-recommended Jonckheere-Terpstra test, which revealed significant reductions at the ≥0.000881 mg a.i./L levels.

The daily average %CV of the highest TWA concentration was 22.1%. The study authors reported that the increased variability was associated with biofilm build up in some of the tubing in the system, resulting in some slowed flow rates to the test vessels. These flow rates were quantitatively monitored daily and were promptly readjusted when noted to be outside of the acceptable range. Increased biofilming was observed due to the use of DMF to deliver the test material to the test system. Measures, such as frequent cleaning and swapping out mix cells and tubing, were taken in order to control levels of biofilming during the study.

III. REFERENCES

Busse, J. (2002). Determination of the water solubility of chlorpyrifos. Unpublished laboratory report # FOR010019; DERBI # 112166. January 29, 2002.

Bonfanti, P, Colombo, A., Orsi, F., Nizetto, I., Andriolettic, M., Baccheta, R., Mantecca, P., Fascio, U., Vailati, G., and Vismara, C. (2004). Comparative teratogenicity of Chlorpyrifos and Malathion on Xenopus laevis development. *Aquatic Toxicology*: 70: 189-200.

Colombo, A, Orsi, F., and Bonfanti, P. (2005). Exposure to the organophosphorus pesticide chlorpyrifos inhibits acetylcholinesterase activity and affects muscular integrity in Xenopus laevis larvae. *Chemosphere* 61: 1665-1671.

Ellman, G.L., Courtney, K.D., Andres, V., and Featherstone, R.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology* 7, 88-95.

El-Merhibi, A., Kumar, A., and Smeaton, T. (2004). Role piperonyl butoxide in the toxicity of chlorpyrifos to Ceriodaphnia dubia and Xenopus laevis. *Ecotoxicology and Environmental Safety* 57, 202-212.

Hollander, M. Wolfe, D.A. (1973). Nonparametric Statistical Methods. John Wiley & Sons, New York.

Hunter, P.L., Marshal, R.S., and Padilla, S. (1997). Automated instrument analysis of cholinesterase activity in tissues from carbamate-treated animals: a cautionary note. *Toxicology Methods* 7, 43-53.

Richards, S.M. and Kendall, R.J. (2003). Physical effects of chlorpyrifos on two stages of Xenopus laevis. *Journal of Toxicology and Environmental Health*, Part A, 66, 75-91.

Sparling, D.W. and Fellers, G. (2007). Comparative toxicity of chlorpyrifos, diazinon, malathion and their oxon derivatives to larval Rana boylii. *Environmental Pollution* 147, 535-539.

Vieth, G.D., DeFoe, D.L., and Bergstedt, B.V. (1979). Measuring and estimating the bioconcentration factor of chemicals in fish. *J. Fish Res. Board Can.* 36, 1040.

APPENDIX I. OUTPUT OF REVIEWER'S STATISTICAL VERIFICATION:

STATISTICAL OUTPUT SUMMARY:

Endpoint	Monotonic?	Parametric?	EDSP	EFED	Comments
7-day wet weight	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	EDSP and EFED same conclusions, no effect
7-day median NF stage	No	No	MannWhitney: n.s. p>0.05	MannWhitney: n.s. p>0.05	EDSP and EFED same conclusions, no effect
7-day SN-vent length	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	EDSP and EFED same conclusions, no effect
7-day hind-limb length	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's n.s. p>0.05	EDSP and EFED same conclusions, no effect
7-day normalized HLL	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	EDSP and EFED same conclusions, no effect
21-day stage median	Yes	No	Jonckheere-Terpstra: Dose 4 p=0.03	Jonckheere-Terpstra: Dose 4 p=0.03	EDSP and EFED same conclusions, no effect
21-day wet weight	Yes	Yes	Jonckheere: Dose 3 p=0.004 Dose 4 p<0.001	William's: Dose 3 p=0.003 Dose 4 p<0.001	EDSP and EFED same conclusions; significant reduction at Doses 3 and 4 (p<0.05; 23 and 39% lower than control, respectively)
21-day SN-vent length	Yes	Yes	Jonckheere: Dose 2 p=0.045	William's: Dose 3 p=0.003	EDSP do not have EFED same conclusions; significant reduction at Doses 2, 3, and 4 (p<0.05; 4, 7 and 15%

Endpoint	Monotonic?	Parametric?	EDSP	EFED	Comments
			Dose 3 p=0.002 Dose 4 p<0.001	Dose 4 p<0.001	lower than control, respectively) according to EDSP Jonckheere-Terpstra test. Only doses 3 and 4 were significantly (p>0.05) reduced, according to EFED-recommended William's test.
21-day HLL	No	Yes	Dunnett's: Dose 4 p=0.012	Dunnett's: Dose 4 p=0.012	EDSP and EFED same conclusions; Dose 4 was significantly (p<0.05) reduced 28%, relative to the negative control.
21-day normalized HLL	Yes	Yes	Jonckheere-Terpstra: Dose 4 p = 0.027	Williams: Dose 4 p = 0.020	EDSP and EFED same conclusions, effect at highest treatment level

```
test for amphib metamorph screen study - TEST DATA chlorpyrifos
ANALYSIS RESULTS FOR VARIABLE VAR01  ( 7-d wet weight (g) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.974         0.842          0.531      0.715    USE PARAMETRIC TESTS

*****************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     0.32     0.03       0.02       10.16           0.27,    0.37
  Dose1  4     0.36     0.03       0.01        7.41           0.32,    0.41
  Dose2  4     0.32     0.03       0.01        8.69           0.27,    0.36
  Dose3  4     0.34     0.04       0.02       13.05           0.27,    0.42
  Dose4  4     0.27     0.03       0.02       12.39           0.22,    0.33

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl         0.32     0.28       0.35         .                  .
  Dose1        0.36     0.33       0.39       113.23             -13.23
  Dose2        0.32     0.28       0.34        98.92               1.08
  Dose3        0.35     0.29       0.39       107.73              -7.73
  Dose4        0.26     0.25       0.32        85.51              14.49

*****************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4            15               3.95        0.022

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl     0.32    .         0.34      .         .       .       .       .       .
 Dose1    0.36   0.268      0.34     0.877      .       .       .       .       .
 Dose2    0.32   1.000      0.33     0.789    0.351     .       .       .       .
 Dose3    0.34   0.693      0.33     0.806    0.944   0.762     .       .       .
 Dose4    0.27   0.205      0.27     0.045    0.015   0.411   0.061     .       .

*****************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                9.83       0.043

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend
```

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	0.32	.	.
Dose1	0.36	0.156	0.958
Dose2	0.32	1.000	0.500
Dose3	0.35	0.494	0.610
Dose4	0.26	0.156	0.055

DECREASING TREND TEST SUMMARY	LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
Williams	Dose4
Jonckheere	>highest dose (no sign. differences)

test for amphib metamorph screen study - TEST DATA chlorpyrifos
ANALYSIS RESULTS FOR VARIABLE VAR02 (7-d stage (median))

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.

Shapiro-Wilks Test Stat	Shapiro-Wilks P-value	Levenes Test Stat	Levenes P-value	Conclusion
0.649	<.001	6.750	0.003	USE NON-PARAMETRIC TESTS

****
BASIC SUMMARY STATISTICS

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval
Ctrl	4	53.00	0.00	0.00	0.00	. , .
Dose1	4	53.25	0.50	0.25	0.94	52.45, 54.05
Dose2	4	53.00	0.00	0.00	0.00	. , .
Dose3	4	53.25	0.50	0.25	0.94	52.45, 54.05
Dose4	4	53.00	0.00	0.00	0.00	. , .

Level	Median	Min	Max	%of Control(means)	%Reduction(means)
Ctrl	53.00	53.00	53.00	.	.
Dose1	53.00	53.00	54.00	100.47	-0.47
Dose2	53.00	53.00	53.00	100.00	0.00
Dose3	53.00	53.00	54.00	100.47	-0.47
Dose4	53.00	53.00	53.00	100.00	0.00

****
PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Analysis of Variance (ANOVA) - overall F-test

Numerator df	Denominator df	F-stat	P-value
4	15	0.75	0.573

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5

Ctrl	53.00	.	53.13	.	.	.	.	.	.
Dose1	53.25	0.638	53.13	0.792	.	.	.	.	.
Dose2	53.00	1.000	53.13	0.824	0.795	.	.	.	.
Dose3	53.25	0.638	53.13	0.840	1.000	0.795	.	.	.
Dose4	53.00	1.000	53.00	0.648	0.795	1.000	0.795	.	.

****

NON-PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests

Kruskal-Wallis test - equality among treatment groups

Degrees of Freedom	TestStat	P-value
4	3.17	0.530

MannWhit - testing each trt median signif. different from control

Jonckheere - test assumes dose-response relationship, testing negative trend

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	53.00	.	.
Dose1	53.00	0.478	0.841
Dose2	53.00	1.000	0.500
Dose3	53.00	0.478	0.744
Dose4	53.00	1.000	0.500

DECREASING TREND TEST SUMMARY	LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
Williams	>highest dose (no sign. differences)
Jonckheere	>highest dose (no sign. differences)

test for amphib metamorph screen study - TEST DATA chlorpyrifos

ANALYSIS RESULTS FOR VARIABLE VAR03 (7-d sn-vent length (mm))

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS

Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01

Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05

Use parametric analyses if neither test rejected, otherwise non-parametric analyses.

Shapiro-Wilks Test Stat	Shapiro-Wilks P-value	Levenes Test Stat	Levenes P-value	Conclusion
0.987	0.993	0.543	0.707	USE PARAMETRIC TESTS

****

BASIC SUMMARY STATISTICS

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval	
Ctrl	4	15.76	0.53	0.26	3.34	14.92,	16.59
Dose1	4	16.14	0.34	0.17	2.11	15.60,	16.68
Dose2	4	15.56	0.51	0.25	3.26	14.76,	16.37
Dose3	4	16.13	0.77	0.39	4.80	14.90,	17.36
Dose4	4	14.65	0.77	0.39	5.29	13.42,	15.88

Level	Median	Min	Max	%of Control(means)	%Reduction(means)
Ctrl	15.91	15.04	16.17	.	.
Dose1	16.04	15.87	16.61	102.42	-2.42
Dose2	15.52	14.99	16.21	98.77	1.23

```
  Dose3       16.23    15.10      16.95      102.35              -2.35
  Dose4       14.39    14.08      15.74       92.98               7.02

*****************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               4.01        0.021

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    15.76    .        15.95      .        .       .       .       .       .
 Dose1   16.14   0.788     15.95     0.755     .       .       .       .       .
 Dose2   15.56   0.974     15.84     0.702    0.674    .       .       .       .
 Dose3   16.13   0.804     15.84     0.721    1.000   0.689    .       .       .
 Dose4   14.65   0.067     14.65     0.013    0.025   0.261   0.026    .       .

*****************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 8.33       0.080

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      15.91                    .                        .
  Dose1     16.04                   0.494                    0.807
  Dose2     15.52                   0.678                    0.279
  Dose3     16.23                   0.494                    0.646
  Dose4     14.39                   0.156                    0.072

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  Dose4
   Jonckheere                                >highest dose (no sign. differences)
```

```
test for amphib metamorph screen study - TEST DATA chlorpyrifos
ANALYSIS RESULTS FOR VARIABLE VAR04  ( 7-d hind-limb length (mm) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.894         0.031          0.697      0.606    USE PARAMETRIC TESTS

*****************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     1.79     0.13       0.06        7.10           1.59,    1.99
  Dose1  4     1.93     0.07       0.04        3.79           1.81,    2.05
  Dose2  4     1.68     0.16       0.08        9.40           1.43,    1.93
  Dose3  4     1.77     0.16       0.08        9.27           1.51,    2.03
  Dose4  4     1.60     0.18       0.09       11.42           1.31,    1.89

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl         1.80     1.63       1.94          .                  .
  Dose1        1.94     1.84       2.01       107.85              -7.85
  Dose2        1.61     1.60       1.92        94.01               5.99
  Dose3        1.74     1.61       2.00        98.86               1.14
  Dose4        1.53     1.48       1.87        89.35              10.65

*****************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4            15               2.89        0.059

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl     1.79    .         1.86      .         .       .       .       .       .
 Dose1    1.93   0.482      1.86     0.827      .       .       .       .       .
 Dose2    1.68   0.692      1.73     0.348    0.170     .       .       .       .
 Dose3    1.77   0.999      1.73     0.361    0.544   0.914     .       .       .
 Dose4    1.60   0.239      1.60     0.054    0.040   0.924   0.492     .       .

*****************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                9.07       0.059

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend
```

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	1.80	.	.
Dose1	1.94	0.156	0.958
Dose2	1.61	0.235	0.190
Dose3	1.74	0.678	0.200
Dose4	1.53	0.235	0.020

DECREASING TREND TEST SUMMARY	LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
Williams	>highest dose (no sign. differences)
Jonckheere	Dose4

test for amphib metamorph screen study - TEST DATA chlorpyrifos
ANALYSIS RESULTS FOR VARIABLE VAR05 (7-d norm hind-limb)

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.

Shapiro-Wilks Test Stat	Shapiro-Wilks P-value	Levenes Test Stat	Levenes P-value	Conclusion
0.920	0.101	0.908	0.484	USE PARAMETRIC TESTS

****

BASIC SUMMARY STATISTICS

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval	
Ctrl	4	0.11	0.01	0.00	4.44	0.10,	0.12
Dose1	4	0.12	0.00	0.00	4.26	0.11,	0.13
Dose2	4	0.11	0.01	0.00	8.91	0.09,	0.12
Dose3	4	0.11	0.01	0.00	7.42	0.10,	0.12
Dose4	4	0.11	0.01	0.00	8.91	0.09,	0.12

Level	Median	Min	Max	%of Control(means)	%Reduction(means)
Ctrl	0.11	0.11	0.12	.	.
Dose1	0.12	0.11	0.12	104.44	-4.44
Dose2	0.11	0.10	0.12	95.56	4.44
Dose3	0.11	0.10	0.12	97.78	2.22
Dose4	0.11	0.10	0.12	95.56	4.44

****

PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Analysis of Variance (ANOVA) - overall F-test

Numerator df	Denominator df	F-stat	P-value
4	15	1.17	0.364

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5

Ctrl	0.11	.	0.12	.	.	.	.	.	.
Dose1	0.12	0.772	0.12	0.759	.	.	.	.	.
Dose2	0.11	0.772	0.11	0.322	0.396	.	.	.	.
Dose3	0.11	0.973	0.11	0.333	0.655	0.990	.	.	.
Dose4	0.11	0.772	0.11	0.254	0.396	1.000	0.990	.	.

```
**************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 4.42       0.352

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend
```

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	0.11	.	.
Dose1	0.12	0.285	0.907
Dose2	0.11	0.461	0.263
Dose3	0.11	0.749	0.195
Dose4	0.11	0.461	0.100

```
 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)



test for amphib metamorph screen study - TEST DATA chlorpyrifos
ANALYSIS RESULTS FOR VARIABLE VAR06  ( 21-d stage (median) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.788         <.001          1.350      0.297    USE NON-PARAMETRIC TESTS

**************************************************************************
BASIC SUMMARY STATISTICS
```

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval
Ctrl	4	57.25	0.50	0.25	0.87	56.45, 58.05
Dose1	4	57.25	0.50	0.25	0.87	56.45, 58.05
Dose2	4	57.75	0.96	0.48	1.66	56.23, 59.27
Dose3	4	57.25	0.50	0.25	0.87	56.45, 58.05
Dose4	4	56.25	0.50	0.25	0.89	55.45, 57.05

Level	Median	Min	Max	%of Control(means)	%Reduction(means)
Ctrl	57.00	57.00	58.00	.	.
Dose1	57.00	57.00	58.00	100.00	0.00
Dose2	57.50	57.00	59.00	100.87	-0.87

```
  Dose3       57.00    57.00      58.00      100.00               0.00
  Dose4       56.00    56.00      57.00       98.25               1.75

*****************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               3.13        0.047

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    57.25     .        57.42      .        .       .       .       .       .
 Dose1   57.25   1.000      57.42     0.733     .       .       .       .       .
 Dose2   57.75   0.622      57.42     0.767    0.782    .       .       .       .
 Dose3   57.25   1.000      57.25     0.636    1.000   0.782    .       .       .
 Dose4   56.25   0.114      56.25     0.023    0.203   0.026   0.203    .       .

*****************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 8.86       0.065

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      57.00                    .                        .
  Dose1     57.00                   1.000                    0.500
  Dose2     57.50                   0.526                    0.811
  Dose3     57.00                   1.000                    0.614
  Dose4     56.00                   0.100                    0.034

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  Dose4
   Jonckheere                                Dose4



test for amphib metamorph screen study - TEST DATA chlorpyrifos
ANALYSIS RESULTS FOR VARIABLE VAR07  ( 21-d wet weight (g) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
```

```
      0.957         0.478          2.839      0.062    USE PARAMETRIC TESTS

*************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     1.73     0.06       0.03        3.53           1.64,    1.83
  Dose1  4     1.72     0.21       0.11       12.37           1.38,    2.06
  Dose2  4     1.55     0.23       0.11       14.70           1.19,    1.91
  Dose3  4     1.34     0.16       0.08       12.20           1.08,    1.60
  Dose4  4     1.06     0.12       0.06       11.80           0.86,    1.25

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl         1.74     1.66       1.80          .                  .
  Dose1        1.76     1.44       1.93        99.46               0.54
  Dose2        1.56     1.33       1.75        89.33              10.67
  Dose3        1.27     1.25       1.59        77.45              22.55
  Dose4        1.03     0.94       1.23        60.98              39.02

*************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15              11.37        <.001

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl     1.73    .         1.73      .         .       .       .       .       .
 Dose1    1.72   1.000      1.72     0.550      .       .       .       .       .
 Dose2    1.55   0.376      1.55     0.090    0.598     .       .       .       .
 Dose3    1.34   0.018      1.34     0.003    0.042   0.452     .       .       .
 Dose4    1.06   <.001      1.06     <.001    <.001   0.007   0.173     .       .

*************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                14.21       0.007

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl       1.74                    .                         .
  Dose1      1.76                  0.889                     0.614
  Dose2      1.56                  0.346                     0.153
  Dose3      1.27                  0.067                     0.004
  Dose4      1.03                  0.067                     <.001

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
```

```
   Williams                                    Dose3
   Jonckheere                                  Dose3



test for amphib metamorph screen study - TEST DATA chlorpyrifos
ANALYSIS RESULTS FOR VARIABLE VAR08  ( 21-d sn-vent length (mm) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.969         0.742          0.837      0.523    USE PARAMETRIC TESTS

*****************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    28.45      0.44        0.22          1.56          27.74,   29.16
  Dose1  4    28.33      0.79        0.40          2.80          27.06,   29.59
  Dose2  4    27.40      1.01        0.50          3.69          25.79,   29.01
  Dose3  4    26.33      0.90        0.45          3.43          24.89,   27.76
  Dose4  4    24.10      1.30        0.65          5.38          22.04,   26.16

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl        28.30    28.10      29.10          .                   .
  Dose1       28.50    27.30      29.00        99.56                0.44
  Dose2       27.65    26.10      28.20        96.31                3.69
  Dose3       26.20    25.40      27.50        92.53                7.47
  Dose4       23.80    22.90      25.90        84.71               15.29

*****************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15              14.80        <.001

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    28.45     .       28.45       .         .       .       .       .       .
 Dose1   28.33   0.999     28.33     0.502       .       .       .       .       .
 Dose2   27.40   0.351     27.40     0.082     0.635     .       .       .       .
 Dose3   26.33   0.019     26.33     0.003     0.055   0.501     .       .       .
 Dose4   24.10   <.001     24.10     <.001     <.001   0.001   0.029     .       .

*****************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
```

```
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                14.19       0.007

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      28.30                   .                         .
  Dose1     28.50                 0.779                     0.331
  Dose2     27.65                 0.152                     0.045
  Dose3     26.20                 0.066                     0.002
  Dose4     23.80                 0.066                     <.001

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  Dose3
   Jonckheere                                Dose2



test for amphib metamorph screen study - TEST DATA chlorpyrifos
ANALYSIS RESULTS FOR VARIABLE VAR09  ( 21-d hind-limb length (mm) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.969         0.736          0.310      0.867    USE PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean     StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    12.30      1.33       0.66       10.81          10.19,   14.41
  Dose1  4    12.83      1.35       0.68       10.56          10.67,   14.98
  Dose2  4    12.88      1.93       0.96       14.96           9.81,   15.94
  Dose3  4    11.48      1.25       0.63       10.92           9.48,   13.47
  Dose4  4     8.88      0.92       0.46       10.34           7.41,   10.34

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl        12.45    10.60      13.70         .                    .
  Dose1       12.70    11.30      14.60       104.27               -4.27
  Dose2       13.15    10.30      14.90       104.67               -4.67
  Dose3       11.25    10.20      13.20        93.29                6.71
  Dose4        8.90     7.80       9.90        72.15               27.85

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               5.67        0.005
```

```
Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    12.30     .       12.67       .        .       .       .       .       .
 Dose1   12.83   0.954     12.67     0.730      .       .       .       .       .
 Dose2   12.88   0.938     12.67     0.764    1.000     .       .       .       .
 Dose3   11.48   0.818     11.48     0.275    0.655   0.625     .       .       .
 Dose4    8.88   0.012      8.88     0.002    0.009   0.008   0.113     .       .

*************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                10.55       0.032

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      12.45                   .                         .
  Dose1     12.70                 0.889                     0.614
  Dose2     13.15                 0.889                     0.721
  Dose3     11.25                 0.494                     0.241
  Dose4      8.90                 0.067                     0.004

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  Dose4
   Jonckheere                                Dose4


 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)



test for amphib metamorph screen study - TEST DATA chlorpyrifos
ANALYSIS RESULTS FOR VARIABLE VAR10  ( 21-d norm hind-limb )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.965         0.640          0.481      0.750    USE PARAMETRIC TESTS

*************************************************************************
BASIC SUMMARY STATISTICS
```

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval	
Ctrl	4	0.43	0.05	0.03	12.36	0.35,	0.52
Dose1	4	0.45	0.04	0.02	8.72	0.39,	0.51
Dose2	4	0.47	0.06	0.03	12.14	0.38,	0.56
Dose3	4	0.43	0.03	0.02	7.43	0.38,	0.49
Dose4	4	0.36	0.02	0.01	6.93	0.32,	0.40

Level	Median	Min	Max	%of Control(means)	%Reduction(means)
Ctrl	0.44	0.36	0.49	.	.
Dose1	0.44	0.41	0.51	104.40	-4.40
Dose2	0.48	0.39	0.53	108.51	-8.51
Dose3	0.43	0.40	0.48	100.58	-0.58
Dose4	0.36	0.33	0.38	83.38	16.62

****

PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests

Analysis of Variance (ANOVA) - overall F-test

Numerator df	Denominator df	F-stat	P-value
4	15	3.68	0.028

Dunnett - testing each trt mean signif. different than control

Williams - test assumes dose-response relationship, testing negative trend

Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5
Ctrl	0.43	.	0.45	.	.	.	.	.	.
Dose1	0.45	0.923	0.45	0.807	.	.	.	.	.
Dose2	0.47	0.580	0.45	0.838	0.976	.	.	.	.
Dose3	0.43	1.000	0.43	0.671	0.981	0.792	.	.	.
Dose4	0.36	0.100	0.36	0.020	0.061	0.020	0.159	.	.

****

NON-PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests

Kruskal-Wallis test - equality among treatment groups

Degrees of Freedom	TestStat	P-value
4	8.84	0.065

MannWhit - testing each trt median signif. different from control

Jonckheere - test assumes dose-response relationship, testing negative trend

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	0.44	.	.
Dose1	0.44	0.889	0.614
Dose2	0.48	0.494	0.810
Dose3	0.43	1.000	0.537
Dose4	0.36	0.156	0.027

DECREASING TREND TEST SUMMARY	LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
Williams	Dose4
Jonckheere	Dose4

```
Title:  chlorpyrifos AMA hindlimb cholinesterase
File:      CHLORPAM.HIN             Transform:             NO TRANSFORMATION

                  Shapiro - Wilk's Test for Normality
--------------------------------------------------------------------------------

     D = 510664.2325
     W =   0.9609

     Critical W  = 0.8840 (alpha = 0.01 , N = 24)
              W  = 0.9160 (alpha = 0.05 , N = 24)

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

Data PASS normality test (alpha = 0.01). Continue analysis.



Title:  chlorpyrifos AMA hindlimb cholinesterase
File:      CHLORPAM.HIN             Transform:             NO TRANSFORMATION

                  Levene's Test for Homogeneity of Variance

                               ANOVA Table
--------------------------------------------------------------------------------

   SOURCE             DF               SS                MS            F
--------------------------------------------------------------------------------
   Between             5          75498.4287       15099.6857       2.3654

   Within (Error)     18         114905.3500        6383.6306
--------------------------------------------------------------------------------
   Total              23         190403.7787
--------------------------------------------------------------------------------
                                                              (p-value = 0.0813)

   Critical F  = 4.2479  (alpha = 0.01, df = 5,18)
               = 2.7729  (alpha = 0.05, df = 5,18)

   Since  F < Critical F  FAIL TO REJECT  Ho: All equal (alpha = 0.01)



Title:  chlorpyrifos AMA hindlimb cholinesterase
File:      CHLORPAM.HIN             Transform:             NO TRANSFORMATION

           Summary Statistics on Data              TABLE 1 of 2
--------------------------------------------------------------------------------

        GRP  IDENTIFICATION    N      MIN        MAX        MEAN
        --- ---------------- ---- ---------- ---------- ----------
         1      neg control    4  1171.2000  1693.2000  1485.8500
         2               L1    4  1042.2500  1281.8000  1160.8250
```

3		L2	4	488.6000	1115.8000	825.4000
4		L3	4	555.8000	851.0000	716.5000
5		L4	4	246.2000	394.8000	318.6000

Title: chlorpyrifos AMA hindlimb cholinesterase
File: CHLORPAM.HIN Transform: NO TRANSFORMATION

Summary Statistics on Data TABLE 2 of 2

GRP	IDENTIFICATION	VARIANCE	SD	SEM	C.V. %
1	neg control	56841.6900	238.4150	119.2075	16.0457
2	L1	10599.5475	102.9541	51.4771	8.8690
3	L2	72657.5200	269.5506	134.7753	32.6570
4	L3	17041.9600	130.5449	65.2724	18.2198
5	L4	4923.5467	70.1680	35.0840	22.0239

Title: chlorpyrifos AMA hindlimb cholinesterase
File: CHLORPAM.HIN Transform: NO TRANSFORMATION

ANOVA Table

SOURCE	DF	SS	MS	F
Between	4	3154011.9080	788502.9770	24.3269
Within (Error)	15	486192.7925	32412.8528	
Total	19	3640204.7005		

(p-value = 0.0000)

Critical F = 4.8932 (alpha = 0.01, df = 4,15)
= 3.0556 (alpha = 0.05, df = 4,15)

Since F > Critical F REJECT Ho: All equal (alpha = 0.05)

Title: chlorpyrifos AMA hindlimb cholinesterase
File: CHLORPAM.HIN Transform: NO TRANSFORMATION

Dunnett's Test - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG 0.05

1	neg control	1485.8500	1485.8500		
2	L1	1160.8250	1160.8250	2.5531	*
3	L2	825.4000	825.4000	5.1880	*
4	L3	716.5000	716.5000	6.0434	*
5	L4	318.6000	318.6000	9.1690	*

Dunnett critical value = 2.3600 (1 Tailed, alpha = 0.05, df = 4,15)

Title: chlorpyrifos AMA hindlimb cholinesterase
File: CHLORPAM.HIN Transform: NO TRANSFORMATION

Dunnett's Test - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	MIN SIG DIFF (IN ORIG. UNITS)	% OF CONTROL	DIFFERENCE FROM CONTROL
1	neg control	4			
2	L1	4	300.4385	20.2	325.0250
3	L2	4	300.4385	20.2	660.4500
4	L3	4	300.4385	20.2	769.3500
5	L4	4	300.4385	20.2	1167.2500

Title: chlorpyrifos AMA hindlimb cholinesterase
File: CHLORPAM.HIN Transform: NO TRANSFORMATION

William's Test - TABLE 1 OF 2 Ho: Control<Treatment

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	neg control	4	1485.8500	1485.8500	1485.8500
2	L1	4	1160.8250	1160.8250	1160.8250
3	L2	4	825.4000	825.4000	825.4000
4	L3	4	716.5000	716.5000	716.5000
5	L4	4	318.6000	318.6000	318.6000

Title: chlorpyrifos AMA hindlimb cholinesterase
File: CHLORPAM.HIN Transform: NO TRANSFORMATION

William's Test - TABLE 2 OF 2 Ho: Control<Treatment

IDENTIFICATION	COMPARED MEANS	CALC. WILLIAMS	SIG 0.05	TABLE WILLIAMS	DEGREES OF FREEDOM USED

```
          neg control  1485.8500
                   L1  1160.8250    2.5531      *     1.7500       k= 1, v=15
                   L2   825.4000    5.1880      *     1.8400       k= 2, v=15
                   L3   716.5000    6.0434      *     1.8700       k= 3, v=15
                   L4   318.6000    9.1690      *     1.8800       k= 4, v=15
-------------------------------------------------------------------------------
s = 180.0357



Title:  chlorpyrifos AMA tail cholinesterase
File:      CHLORPAM.TAI             Transform:             NO TRANSFORMATION

                   Shapiro - Wilk's Test for Normality
-------------------------------------------------------------------------------

     D = 80307.8075
     W =   0.9704

     Critical W  = 0.8840 (alpha = 0.01 , N = 24)
              W  = 0.9160 (alpha = 0.05 , N = 24)

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

Data PASS normality test (alpha = 0.01). Continue analysis.



Title:  chlorpyrifos AMA tail cholinesterase
File:      CHLORPAM.TAI             Transform:             NO TRANSFORMATION

                   Levene's Test for Homogeneity of Variance

                               ANOVA Table
-------------------------------------------------------------------------------

   SOURCE             DF               SS                MS           F
-------------------------------------------------------------------------------
   Between             5           6323.7721        1264.7544       0.8498

   Within (Error)     18          26789.4075        1488.3004
-------------------------------------------------------------------------------
   Total              23          33113.1796
-------------------------------------------------------------------------------
                                                     (p-value = 0.5326)

   Critical F  = 4.2479  (alpha = 0.01, df = 5,18)
               = 2.7729  (alpha = 0.05, df = 5,18)

   Since  F < Critical F  FAIL TO REJECT  Ho: All equal (alpha = 0.01)
```

Title: chlorpyrifos AMA tail cholinesterase
File: CHLORPAM.TAI Transform: NO TRANSFORMATION

Summary Statistics on Data TABLE 1 of 2

GRP	IDENTIFICATION	N	MIN	MAX	MEAN
1	neg control	4	641.3000	758.8000	693.7250
2	L1	4	636.8000	825.4000	744.9000
3	L2	4	528.0000	679.2000	623.7500
4	L3	4	427.0000	559.2000	500.9000
5	L4	4	195.2000	279.4000	220.0500

Title: chlorpyrifos AMA tail cholinesterase
File: CHLORPAM.TAI Transform: NO TRANSFORMATION

Summary Statistics on Data TABLE 2 of 2

GRP	IDENTIFICATION	VARIANCE	SD	SEM	C.V. %
1	neg control	2637.2892	51.3545	25.6773	7.4027
2	L1	6300.1733	79.3736	39.6868	10.6556
3	L2	4367.6100	66.0879	33.0439	10.5953
4	L3	3647.0533	60.3908	30.1954	12.0565
5	L4	1578.3567	39.7285	19.8643	18.0543

Title: chlorpyrifos AMA tail cholinesterase
File: CHLORPAM.TAI Transform: NO TRANSFORMATION

ANOVA Table

SOURCE	DF	SS	MS	F
Between	4	700550.5980	175137.6495	47.2566
Within (Error)	15	55591.4475	3706.0965	
Total	19	756142.0455		

(p-value = 0.0000)

Critical F = 4.8932 (alpha = 0.01, df = 4,15)
= 3.0556 (alpha = 0.05, df = 4,15)

Since F > Critical F REJECT Ho: All equal (alpha = 0.05)

Title: chlorpyrifos AMA tail cholinesterase
File: CHLORPAM.TAI Transform: NO TRANSFORMATION

Dunnett's Test - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG 0.05
1	neg control	693.7250	693.7250		
2	L1	744.9000	744.9000	-1.1888	
3	L2	623.7500	623.7500	1.6255	
4	L3	500.9000	500.9000	4.4794	*
5	L4	220.0500	220.0500	11.0037	*

Dunnett critical value = 2.3600 (1 Tailed, alpha = 0.05, df = 4,15)

Title: chlorpyrifos AMA tail cholinesterase
File: CHLORPAM.TAI Transform: NO TRANSFORMATION

Dunnett's Test - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	MIN SIG DIFF (IN ORIG. UNITS)	% OF CONTROL	DIFFERENCE FROM CONTROL
1	neg control	4			
2	L1	4	101.5910	14.6	-51.1750
3	L2	4	101.5910	14.6	69.9750
4	L3	4	101.5910	14.6	192.8250
5	L4	4	101.5910	14.6	473.6750

Title: chlorpyrifos AMA tail cholinesterase
File: CHLORPAM.TAI Transform: NO TRANSFORMATION

William's Test - TABLE 1 OF 2 Ho: Control<Treatment

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	neg control	4	693.7250	693.7250	719.3125
2	L1	4	744.9000	744.9000	719.3125
3	L2	4	623.7500	623.7500	623.7500
4	L3	4	500.9000	500.9000	500.9000
5	L4	4	220.0500	220.0500	220.0500

Title: chlorpyrifos AMA tail cholinesterase
File: CHLORPAM.TAI Transform: NO TRANSFORMATION

William's Test - TABLE 2 OF 2 Ho: Control<Treatment

IDENTIFICATION	COMPARED MEANS	CALC. WILLIAMS	SIG 0.05	TABLE WILLIAMS	DEGREES OF FREEDOM USED
neg control	693.7250				
L1	719.3125	-0.5944		1.7500	k= 1, v=15
L2	623.7500	1.6255		1.8400	k= 2, v=15
L3	500.9000	4.4794	*	1.8700	k= 3, v=15
L4	220.0500	11.0037	*	1.8800	k= 4, v=15

s = 60.8777

WARNING: Procedure has used isotonized means which differ from original (transformed) means.

Day 7

Neg Control

Rep	Wet Weight	NF Stage	SVL	HLL	normHLL
1	0.3177	53.2000	15.6854	1.7834	0.1138
2	0.3314	53.0000	16.1338	1.8132	0.1128
3	0.3546	53.4000	16.1680	1.9368	0.1198
4	0.2771	52.8000	15.0392	1.6276	0.1079
average	**0.3202**	**53.1000**	**15.7566**	**1.7903**	**0.1136**

Solvent Control

Rep	Wet Weight	NF Stage	SVL	HLL	normHLL
1	0.3622	53.2000	16.0096	1.8370	0.1144
2	0.4483	53.4000	17.5528	1.9349	0.1097
3	0.3393	53.2000	16.3892	1.6990	0.1031
4	0.2985	53.0000	15.3358	1.7465	0.1130
average	**0.3621**	**53.2000**	**16.3219**	**1.8044**	**0.1101**
t-test	0.2835	0.5370	0.3305	0.8693	0.3586

Day 21

Neg Control

Rep	Wet Weight	NF Stage	SVL	HLL	normHLL
1	1.6567	56.9333	28.3139	12.0200	0.4240
2	1.7575	57.3333	28.2856	12.9145	0.4573
3	1.7162	57.6000	28.1344	13.6611	0.4846
4	1.8002	56.8667	29.0804	10.5964	0.3611
average	**1.7327**	**57.1833**	**28.4536**	**12.2980**	**0.4317**

Solvent Control

Rep	Wet Weight	NF Stage	SVL	HLL	normHLL
1	1.82572	57.46667	28.9731	13.33419	0.459303
2	1.9811	57.33333	29.85589	13.67534	0.456971
3	1.652133333	57	28.00682	11.72322	0.416714
4	1.437457143	57.14286	26.36856	10.85897	0.411197
average	**1.7241**	**57.2357**	**28.3011**	**12.3979**	**0.4360**
t-test	0.9457	0.8033	0.8508	0.9186	0.8890

hindlimb cholinesterase

group	animal #	rep	A	B	C	D
NC		1	1588	1581	1553	1015
		2	2112	1807	2210	1362
		3	1016	1391	952	1164
		4	2168	1009	1680	1475
		5	1582	1376	1836	840
	mean		1693.2	1432.8	1646.2	1171.2
SC		1	776	988	1022	835
		2	1305	1306	1072	1022
		3	1158	1341	1114	994
		4	980	882	1372	1174
		5	542	1051	1050	860
	mean		952.2	1113.6	1126	977
L1		1	1594	881	1081	1018
		2	499	1040	1223	941
		3	1354	1397	1061	608
		4	1594	1163	658	1602
		5	1368		1972	
	mean		1281.8	1120.25	1199	1042.25
L2		1	824	622	474	510
		2	1245	1117	245	860
		3	881	789	676	980

group	animal #	rep	A	B	C	D
L3		1	754	584	587	849
		2	220	799	556	644
		3	584	1199	937	722
		4	510	881	1065	623
		5	711	792	796	517
	mean		555.8	851	788.2	671
L4		1	142	26	641	245
		2	50	294	351	527
		3	450	188	418	226
		4	354	266	217	224
		5	372	457	347	577
	mean		273.6	246.2	394.8	359.8

	4	1043	1340	492	923
	5	739	1711	556	481
		946.4	1115.8	488.6	750.8

tail cholinesterase

group	animal #	rep A	B	C	D
NC	1	755	804	741	641
	2	663	634	535	839
	3	846	677	797	472
	4	571	620	478	613
	5	959	599	989	
	mean	758.8	666.8	708	641.25
SC	1	599	825	573	676
	2	557	670	641	414
	3	1037	628	503	520
	4	882	691	566	718
	5	670	606	619	443
	mean	749	684	580.4	554.2
L1	1	612	594	722	955
	2	693	665	824	820
	3	1036	467	683	951
	4	789	768	860	520
	5	997	690	771	481

group	animal #	rep A	B	C	D
L3	1	418	248	436	450
	2	517	909	594	421
	3	510	1022	485	492
	4	478	245	662	393
	5	464	276	619	379
	mean	477.4	540	559.2	427
L4	1	276	407	379	273
	2	50	139	177	199
	3	344	259	269	68
	4	174	241	64	266
	5	170	351	125	170
	mean	202.8	279.4	202.8	195.2

	mean		825.4	636.8	772	745.4
L2		1	598	481	789	474
		2	559	778	803	789
		3	545	683	563	535
		4	495	626	502	488
		5	443	676	739	909
			528	648.8	679.2	639

DATA EVALUATION RECORD

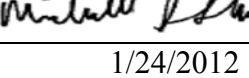
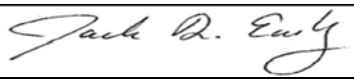
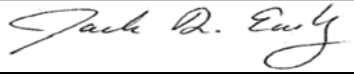
CHLORPYRIFOS

Study Type: OCSPP 890.1150, Androgen Receptor Binding (Rat Prostate Cytosol)

EPA Contract No. EP10H001452
Task Assignment No. 2-14-2012 (MRID 48615502)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CSS-Dynamac Corporation
1910 Sedwick Road,
Building 100, Suite B
Durham, NC 27713

Primary Reviewer Sandra Hastings	Signature: Date:	 01/24/2012
Secondary Reviewer Michelle Sharpe-Kass, M.S.	Signature: Date:	 1/24/2012
Program Manager: Jack D. Early, M.S.	Signature: Date:	 2/01/2012
Quality Assurance: Jack D. Early, M.S.	Signature: Date:	 2/01/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel**.**

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Jessica Ryman, Ph.D., D.A.B.T. **Signature:** (for) 6/15/15
Health Effects Division **Date:**
Secondary Reviewer: Greg Akerman, Ph.D. **Signature:**
Health Effects Division **Date:** 6/15/15

DATA EVALUATION RECORD

STUDY TYPE: Androgen Receptor Binding (Rat Prostate Cytosol); OCSPP 890.1150

PC CODE: 059101 **DP BARCODE:** D397128

TXR#: 0052086 **CAS No.:** 2921-88-2

TEST MATERIAL (PURITY): Chlorpyrifos Technical (99.8%)

SYNONYMS: O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl)ester phosphorothioic acid; Chlorpyrifos-ethyl; Chlorpyriphos

CITATION: LeBaron, M.J., Schisler, M.R., and Visconti, N.R. (2011). Evaluation of Chlorpyrifos In An *In Vitro* Androgen Receptor Binding Assay. The Dow Chemical Company, Toxicology & Environmental Research and Consulting, Midland, MI. Laboratory project study ID 111099, November 1, 2011. MRID 48615502. Unpublished.

SPONSOR: Dow AgroSciences LLC, 9330 Zionsville Rd., Indianapolis, IN

TEST ORDER #: CON-059101-4

EXECUTIVE SUMMARY: In an androgen receptor (AR) binding assay (MRID 48615502), ventral prostate cytosol from Sprague Dawley rats was used as the source of AR. Saturation binding information was provided in the study profile (MRID 48682802) in which the cytosolic AR was characterized by duplicate saturation binding experiments. This study showed that cytosolic AR was present in reasonable numbers and was functioning with appropriate affinity for the radio labeled reference androgen (R1881). The competitive binding experiment was conducted to measure the binding of a single concentration of [^{3}H]-R1881 (1 nM) in the presence of increasing concentrations of chlorpyrifos (logarithmic increase from 10^{-10} to 10^{-3} M). Ethanol was used as the vehicle at a final assay concentration of <3%. The assay included dexamethasone as a weak positive control, and R1881 as the ligand reference standard. Three independent runs were conducted with 3 replicates per concentration per run.

In the saturation binding experiment, the maximum binding capacity (B_{max}) was 3.245 fmol/100 µg protein and the dissociation constant (K_d) was 0.4641 nM. These values were below the range of values from the validation studies (0.685-1.57 nm); however, the results were reproducible. The Scatchard plot indicated a linear response across the concentrations of ligand added. Nonspecific binding as a percent of total binding was less than 20% across the entire

concentration range in the saturation binding assays (range 6.2-19.8%, with the exception of the high concentration (10 nM) in one assay, which was 24.6%).

In the competitive binding experiment, the mean log IC_{50}s for R1881 and the weak positive control (dexamethasone) were -9.0 and -4.5 M, respectively, and the mean relative binding affinity (RBA) for the weak positive control was 0.0034%. The solvent control responses indicated no drift in the study assay. All performance criteria were met, with the exception of the bottom (% binding) of R1881 in Assay #2 which was slightly low (-2.1%), and is considered a minor deviation.

Based on the responses of three independent competitive binding assays, the bottom of the curve for percent of total binding at the 95% confidence interval for chlorpyrifos was between 50 and 75% in all three runs (53.7%, 57.2%, and 59.3% in runs 1, 2, and 3, respectively) with an average of 58.2 ± 1.1%. At the next highest concentration of 10^{-4} M, the mean binding was 74.5 ± 1.0%, which was also in the equivocal range. An IC_{50} and RBA could not be calculated for chlorpyrifos. The Hill slopes for each of the three runs were -1.132, -1.367, and -1.541, suggesting a slightly greater than normal steepness (-1).

Based on the results from the three runs, chlorpyrifos is classified as Equivocal in the Androgen Receptor Binding Assay.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for an Androgen Receptor Binding Assay (OCSPP 890.1150).

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

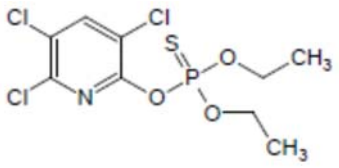
A. MATERIALS

1. Test Facility: The Dow Chemical Company, Toxicology & Environmental Research and Consulting

Location: Midland, MI 48674
Study Director: Schisler, M.R.
Other Personnel: LeBaron, M.J. (Lead Scientist), Visconti, N.R.(Research Biologist), Gollapudi, B.B. (Technical reviewer)
Study Period: June 27, 2011 – July 8, 2011

2. Test substance: Chlorpyrifos

Description: Technical, white solid
Source: Dow AgroSciences LLC
Lot/Batch #: KC28161419, TSN101285
Purity: 99.8%
Solubility: Soluble in ethanol up to 30 mM; 1.05 x 10-3 g/L in water
Volatility: Not reported
Stability: 3.5 years shelf life
Storage conditions: Ambient
CAS #: 2921-88-2
Molecular weight: 350.6
Structure:



3. Non-labeled ligand: R1881

Supplier: Perkin Elmer, Boston, Massachusetts
Catalog and Batch #: Lot # 614156
Purity: >97%
CAS #: 965-93-5

4. Radioactive ligand: [^{3}H]-R1881

Supplier: Perkin-Elmer, Boston, Massachusetts
Catalog and Batch #: NET590250UC, Batch # 614814
Date of production: July 1, 2010
Date of use: June 27, 2011 to July 5, 2011
Radiochemical purity: >97%
Specific activity: 85.1 Ci/mmol
*Information on adjusted specific activity was not available.

Concentration of stock: 1.0 mCi/ml

5. Positive control: Dexamethasone

Supplier: Sigma, St. Louis, Missouri
Catalog and Batch # lot # BCBC9269
Purity: 98.9%
CAS # : 50-02-2

6. Solvent/vehicle control: Ethanol
Justification for choice of solvent: None provided
Final Concentration: <3%

B. METHODS

1. Preparation of Rat Ventral Prostate Cytosol: The rat ventral prostate tissue was purchased from Charles River Laboratories (Wilmington, MA). Male Sprague Dawley rats (number not reported) were castrated at approximately 90 days of age and euthanized approximately 24 hours later. The ventral prostate tissues were collected and stored at approximately -80°C until use, and were processed as a batch and used for multiple studies.

The cytosol was prepared by adding low-salt TEDG buffer [0.01 M Tris, 1 mM sodium molybdate, 1.5 mM EDTA, 10% glycerol and 1 mM phenylmethylsulfonyl fluoride (PMSF) with dithiothreitol (DTT)] at pH 7.4 to the ventral prostate tissues at 10 mL/g of tissue. The tissues were minced, homogenized on ice, and centrifuged for 30 min at 30,000 × *g* at 4°C. The supernatant was collected, pooled from all tissues, aliquoted (amounts not reported) and stored at -80°C until used. Protein concentration of the cytosol prepared for this study was determined to be 6.566 mg/mL using the Pierce BCA method (Thermo Scientific Pierce Research Lab, Rockford, IL).

2. Saturation Radioligand Binding Experiment: A saturation binding experiment measuring total and non-specific binding of [3H]-R1881 was performed to demonstrate that the AR was present in reasonable concentrations and had the appropriate affinity for the R1881 ligand. The conditions for the saturation binding experiment are summarized in Table 1.

TABLE 1. Summary of Conditions for Saturation Binding Experiment [a]

Source of receptor		Rat prostate cytosol
Concentration of radioligand (as serial dilutions)		0.25-10 nM
Concentration of non-labeled ligand (100X [radioligand])		25-1000 nM
Optimization of receptor concentration		Sufficient to bind 8.6-9.0%[b] of radioligand at 0.25 nM
Temperature		~2-8 ºC
Incubation time		~16 hours
Composition of assay buffer (TEDG)	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10%
	Phenylmethylsulfonyl fluoride	1.0 mM
	DTT	1.0 mM

a Data were not included in the study report, but are reported as a separate validation report (MRID 48682802).
b As indicated in the guideline for acceptable assay performance the receptor concentration bound less than 25 to 35% of the radiolabeled R1881.

On the day of the assay, the specific activity of the stock solution [^{3}H]-R1881 was not adjusted for decay over time, and serial dilutions in TEDG buffer were prepared to achieve the final concentrations in cytosol of 0.25, 0.5, 0.7, 1.0, 1.5, 2.5, 5.0, and 10.0 nM to determine total binding. To determine non-specific binding, solutions of non-labeled R1881

were prepared in a similar manner to achieve concentrations that were 100-fold greater than each respective radiolabeled concentration, resulting in final concentrations in cytosol of 25, 50, 70, 100, 150, 250, 500, and 1000 nM. In the absence of cytosol, the radiation found in 7.5, 15, 21, 30, or 45 µL of 10 nM [^{3}H]-R1881 and 7.5, 15, or 30 µL of 100 nM [^{3}H]-R1881 was measured. For each batch of cytosol, the optimal protein concentration was determined by calculating specific binding to differing amounts of protein per tube, using 0.25 nM radiolabeled R1881. The optimal protein concentration was determined to be 1.97 mg protein/assay tube, which resulted in the binding of 8.6-9.0% of the total radioactivity added. As indicated in the guideline for acceptable assay performance the receptor concentration bound less than 25 to 35% of the radiolabeled R1881. Cytosolic protein used in this assay was thawed fresh for this experiment at ~4°C and maintained at ~4°C during the binding assay. Each run contained three concurrent replicates at each concentration, resulting in the 72 samples depicted in Table 2.

TABLE 2. Saturation Binding Experiment Run [a, b]

Total Binding	Non-Specific Binding		Radioligand alone	
Tubes 1-24 [c]	Tubes 25-48 [d]		Tubes 49-72 [e]	
[^{3}H]-R1881 Final conc. (nM)	[^{3}H]-R1881 Final conc. (nM)	R1881 Final conc. (nM)	[^{3}H]-R1881 Initial conc. (nM)	[^{3}H]-R1881 (µL)
0.25	0.25	25	10	7.5
0.50	0.50	50	10	15
0.70	0.70	70	10	21
1.00	1.00	100	10	30
1.50	1.50	150	10	45
2.50	2.50	250	100	7.5
5.00	5.00	500	100	15
10.00	10.00	1000	100	30

a Data were not included in the study report, but are reported as a separate validation report (MRID 48682802).
b Each concentration was run in triplicate for a total of 72 samples.
c Tubes 1-24 contained 50 µL of triamcinolone acetonide and 7.5-45 µL [^{3}H]-R1881. Samples were dried, and 300 µl of prostate cytosol were added.
d Tubes 25-48 contained 50 µL of triamcinolone acetonide and 7.5-45 µL [^{3}H]-R1881. R1881 was added in a 100-fold molar excess of [^{3}H]-R1881 in a volume of 7.5-45 µL. Samples were dried, and 300 µl of prostate cytosol were added.
e Tubes 49-72 contained only 7.5, 15, 21, 30, or 45 µL of 10 nM [^{3}H]-R1881 or 7.5, 15, or 30 µL of 100 nM [^{3}H]-R1881 without cytosol or other components to determine the total counts added.

Following addition of triamcinolone acetonide, [^{3}H]-R1881, and/or R1881, the tubes were dried, dissolved in diluted prostate cytosol (300 µL), and incubated for approximately 16 hours at 2-8°C. Samples were maintained at temperatures of ~4°C except during whole rack vortexing. To separate bound from free R1881, hydroxyapatite (HAP) slurry was added to each tube and vortexed once every 5 minutes for 20 minutes. The samples were then centrifuged, and the supernatant was aspirated and discarded. The samples were washed 3 times in 50 mM TRIS buffer. Following the last wash and decanting of the Tris buffer, pellets were then extracted by addition of 2 ml ethanol. The samples were vortexed 3 times at 5 minute intervals. Samples were maintained on ice at all times between vortexing. Each ethanol supernatant was then decanted into a scintillation vial, and the radiation was quantified by liquid scintillation counting. A total of 4 runs were performed on 2 batches of cytosol with similar results. For the batch of cytosol used for the competitive assay, 2 runs

were performed, which had highly similar binding profiles. Final determination of acceptable AR binding assay performance was primarily based on guideline suggested standards for the competitive binding assay, although the saturation binding parameters were evaluated.

3. Competitive Binding Experiment: A summary of the assay conditions for the competitive binding experiment is included in Table 3.

TABLE 3. Summary of Conditions for Competitive Binding Experiment [a]		
Source of receptor		Rat ventral prostate cytosol
Concentration of radioligand		1 nM
Optimization of receptor concentration		Sufficient to bind 4.3-5.2%[b] of 1.0 nM radioligand
Concentration of test substance (as serial dilutions)		10^{-10} to 10^{-3} M
Incubation Temperature		4-8 ºC
Incubation time		Overnight
Composition of assay buffer	Tris	0.01 M (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10% (v/v)
	Phenylmethylsulfonyl fluoride with DTT	1 mM
	Sodium molybdate	1 mM
	Protease inhibitor	60 µM

a Data were obtained from pages 16-17, 20-21, 40, 42 and 44 of the study report (MRID 48682802).
b As indicated in the test guideline, the receptor concentration bound less than 10-15% of the radiolabeled R1881 (reported in study profile MRID 48682802).

The competitive binding experiment was performed according to the protocol provided in the EPA Test Guidelines OCSPP 890.1150. The competitive binding experiment measures the binding of a single concentration of [^{3}H]-R1881 (specific activity of 85.1 Ci/mmol) to the AR in the presence of increasing concentrations of a test substance.

Ethanol was used as the solvent vehicle and the solubility of the test material in the vehicle and assay buffer was evaluated visually. No precipitation was noted. Results from the saturation binding experiment demonstrated that 6.566 mg/ml or 1.97 mg/assay tube of cytosolic protein contains enough receptor to bind no more than 10-15% of the [$_3$H]-R1881, and that non-specific binding met the guideline recommended value of <20%.

Dilutions of the test substance, reference standard (R1881), weak positive control (dexamethasone), and solvent control (ethanol) were prepared to achieve the concentrations shown in Table 4. Each assay consisted of three independent runs on three different days, and each run contained duplicate blanks, and three replicates at each concentration of the solvent blank, NSB, reference standard, weak positive control, and test chemical resulting in a total of 77 samples per run. In addition, duplicate blanks followed by six replicates [^{3}H]-R1881 only (for total binding calculations) were run the day before each analysis run (the day of preparation of sample tubes).

TABLE 4. Competitor Final Molar (M) Concentrations in Competitive Binding Assay [a b]				
Solvent Control	**Reference standard**	**Weak positive control**	**Test Chemical**	None
Ethanol	R1881	Dexamethasone	Chlorpyrifos	
Tubes 3-5 and 72-74	Tubes 6-23 and 75-77 [c]	Tubes 24-47	Tubes 48-71	Tubes 1-2
	1×10^{-6}	1×10^{-3}	1×10^{-3}	
	1×10^{-7}	1×10^{-4}	1×10^{-4}	
	1×10^{-8}	1×10^{-5}	1×10^{-5}	
	1×10^{-9}	1×10^{-6}	1×10^{-6}	
	1×10^{-10}	1×10^{-7}	1×10^{-7}	
	1×10^{-11}	1×10^{-8}	1×10^{-8}	
	--	1×10^{-9}	1×10^{-9}	
	--	1×10^{-10}	1×10^{-10}	

a Data were obtained from pages 40-45 of the study report.

b Each concentration of each chemical was run in triplicate, plus duplicate blanks for a total of 77 tubes per run. Tubes 3-77 contained 50 µL of triamcinolone acetonide and 30 µL [^{3}H]-R1881. Samples were dried, and 300 µL of prostate cytosol were added. Tubes 3-77 also contained 10 µL of the solvent control, reference standard (non-radiolabeled R1881), weak positive control, or test substance, with the exception of Tubes 6-8 and 75-77 that contained 30 µL of non-radiolabeled R1881 (used to evaluate non-specific binding). Six tubes analyzed the day prior to each run analysis contained only 30 µL of [^{3}H]-R1881 to determine ligand activity.

c Tubes 6-8 and 75-77 were used to evaluate non-specific binding by adding 100x of cold (non-radiolabeled) R1881.

Sample tubes were stored overnight at 4-8°C in the dark to allow the reaction to reach equilibrium, bound R1881 was separated from free R1881 by washing with buffer and extraction with ethanol, followed by scintillation counting of bound [^{3}H]-R1881.

4. Data Analysis: The top and bottom of the curve, Hill slope, inhibition concentration (IC_{50}), and standard deviations were assessed using GraphPad Prism v. 5, and the data were graphed using a "one site binding" non-linear regression.

5. Definitions

a. Classification of test material

If the data fit a 4-parameter nonlinear regression model, the test chemical is classified as:

Binder: The average curve for the test chemical across runs crosses 50% of radioligand bound.

Equivocal: The average lowest portion of curves across runs is between 50% and 75% radioligand binding (*i.e.* radioligand displacement is at least 25% but less than 50%), or the curve falls outside the range for the weak positive control (-0.6 to -1.4).

Non-Binder: The average lowest portion of curves across runs is greater than 75% activity (*i.e.* less than 25% displacement of radioligand), or the data do not fit the model.

Untestable: If the test compound is not soluble above 1×10^{-6} M and the binding curve does not cross 50%, the chemical is judged to be untestable.

b. Descriptors for receptor binding

B_{max}: maximal binding capacity
K_d: dissociation constants
IC_{50}: Concentration of the test substance at which 50% of radioligand is displaced from the AR by the competitor
Relative Binding Affinity (RBA): IC_{50} of R1881 × 100 ÷ IC_{50} of test substance

II. RESULTS

A. <u>SATURATION BINDING EXPERIMENT</u>: Saturation binding experiment parameters are presented in Table 5. The dissociation constant (K_d) for [^{3}H]-R1881 was 0.4641, and the estimated B_{max} (nM) was 0.06392 for the batch of prostate cytosol that was used for this study. The K_d was slightly below the range reported in the EPA validation program (0.685-1.57 nM). Confidence in these numbers is high according to the goodness of fit (R^2 = 0.9871-0.9937) and the small variation among runs.

TABLE 5. Saturation Binding Experiment of R-1881 with Androgen Receptor from Rat Prostate Cytosol[a]

Parameter	Run 1[b]	Run 2[b]	Run 3[b]	Mean Runs 1-2[c]
R^2 (unweighted)	0.9937	0.9871	ND	0.9871-0.9937
B_{max} (nM)	0.06194	0.06590	ND	0.06392
B_{max} (fmol/100µg protein)	3.146	3.343	ND	3.245
K_d (nM)	0.4359	0.4922	ND	0.4641

a Data were not included in the study report, but are reported as a separate validation report.
b Two saturation runs were performed for this batch of cytosol.
c The range of R^2 is reported and the mean is reported for the other parameters. R^2= Goodness of fit for curve calculated for specific binding,
ND not determined

Figure 1 illustrates the non-specific, specific, and total binding curves for [^{3}H]-R1881 to the androgen receptor. The specific binding reached a plateau and the non-specific binding was generally less than 20% of total binding at all concentrations (range 6.2%-19.8%) except the highest concentration in Run 1 (24.6%). All other values indicated acceptable performance of the assay. A Scatchard plot that illustrates the binding of [^{3}H]-R1881 to the androgen receptor is shown in Figure 2. The data fit results in a linear plot.

FIGURE 1. Binding of [^{3}H]-R1881 to the Androgen Receptor during the Saturation Binding Experiment.

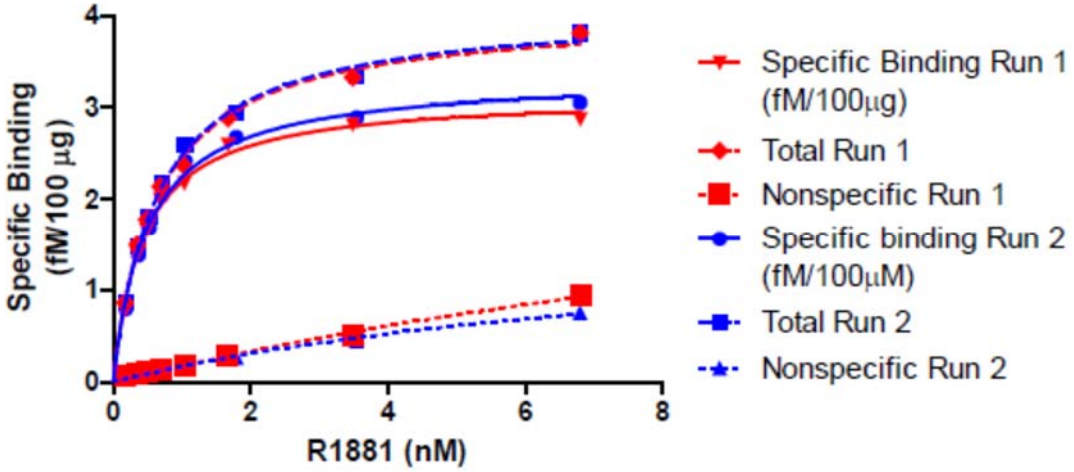
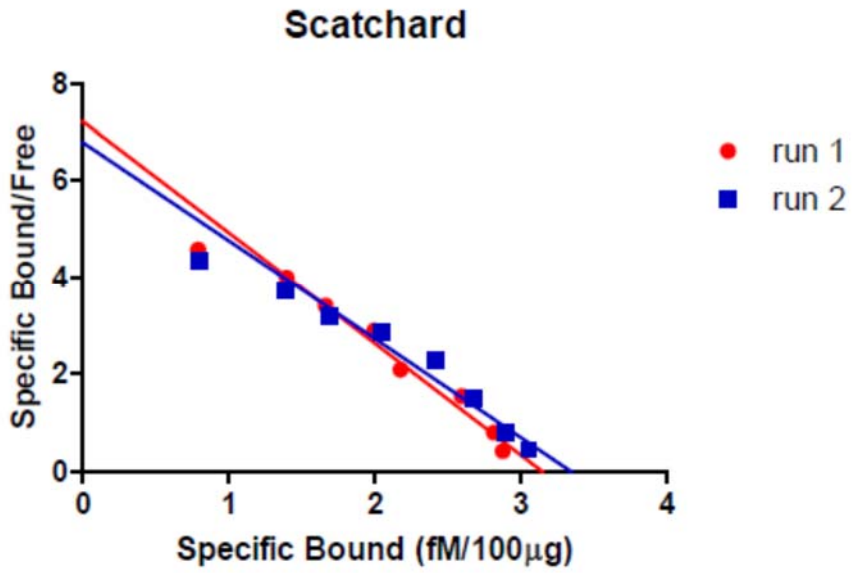


FIGURE 2. Scatchard Plot of the Binding of [3H]-R1881 to the Androgen Receptor.



B. <u>COMPETITIVE BINDING EXPERIMENT</u>: The results from the 3 competitive binding experiments are summarized in Table 6 and shown graphically in Figures 3 and 4. Chlorpyrifos reduced the mean specific binding of the ligand, [3H]-R1881, in all three runs in a concentration dependent manner. At the highest concentration tested (10^{-3}M), chlorpyrifos reduced specific binding of [3H]-R1881 to 56.1, 58.5, and 59.9% in the first, second, and third assays, respectively. Chlorpyrifos is classified as equivocal since the percent [3H]-R1881 binding was between to 50 and 75% at all concentrations of test material in all three runs.

As specific binding was not ≤50% at any chlorpyrifos concentration, an IC_{50} and RBA could not be calculated for chlorpyrifos.

The estimated log IC_{50} for R1881 and the weak positive control (dexamethasone) were -9.0 and -4.5 M, respectively. The mean RBA for the weak positive control was 0.0034%. The solvent control responses indicated no drift in the study assay. The bottom of the curve for percent of total binding at the 95% confidence interval for chlorpyrifos was 53.7%, 57.2%, and 59.3% in runs 1, 2, and 3, respectively with an average of 58.2 ± 1.1 At the next highest concentration of 10^{-4} M, the mean was 74.5 ± 1.0, which was also in the equivocal range. At 10^{-5}, chlorpyrifos was in the non-binding range with an average of 99.l ± 1.0. The Hill slopes for each of the three runs were -1.132, -1.367, and -1.541 suggesting slightly greater than normal steepness (-1).

TABLE 6. Competitive Binding Assay of Chlorpyrifos with AR from Rat Prostate Cytosol [a]

Parameter		Run 1[b]	Run 2[b]	Run 3[b]	Mean[c]
r^2 (unweighted)	R1881	0.9999	1.0000	0.9999	0.9999-1.0000
	Positive control	0.9992	0.9997	0.9985	0.9985-0.9997
	Test substance	0.9980	0.9953	0.9938	0.9938-0.9980
Log IC_{50} (M)	R1881	-8.965	-9.980	-8.981	-8.975
	Positive control	-4.522	-4.431	-4.550	-4.501
	Test substance	NA	NA	NA	NA
IC_{50} (M)	R1881	1.084×10^{-9}	1.048×10^{-9}	1.044×10^{-9}	1.06×10^{-9}
	Positive control	3.006×10^{-5}	3.707×10^{-5}	2.819×10^{-5}	3.15×10^{-5}
	Test substance	NA	NA	NA	NA
RBA (as % IC_{50})[d]	Positive control	0.0036	0.0028	0.0037	0.0034
	Test substance	NA	NA	NA	NA

a Data were obtained from pages 33-34 of the study report.
b The mean is reported for the concurrent replicates within each run.
c The range of R^2 is reported, and the mean is reported for the other parameters. Calculated by the reviewer.
d Calculated by reviewer: [IC_{50} (in M) positive control or chlorpyrifos / IC_{50} (in M) R1881] x 100%
NA Not applicable
r^2 Goodness of fit
RBA (%) relative binding affinity

FIGURE 3. Percentage R1881 Bound to the Androgen Receptor in the Presence of Radioinert R1881, Dexamethasone, and Chlorpyrifos (Assays 1 – 3).

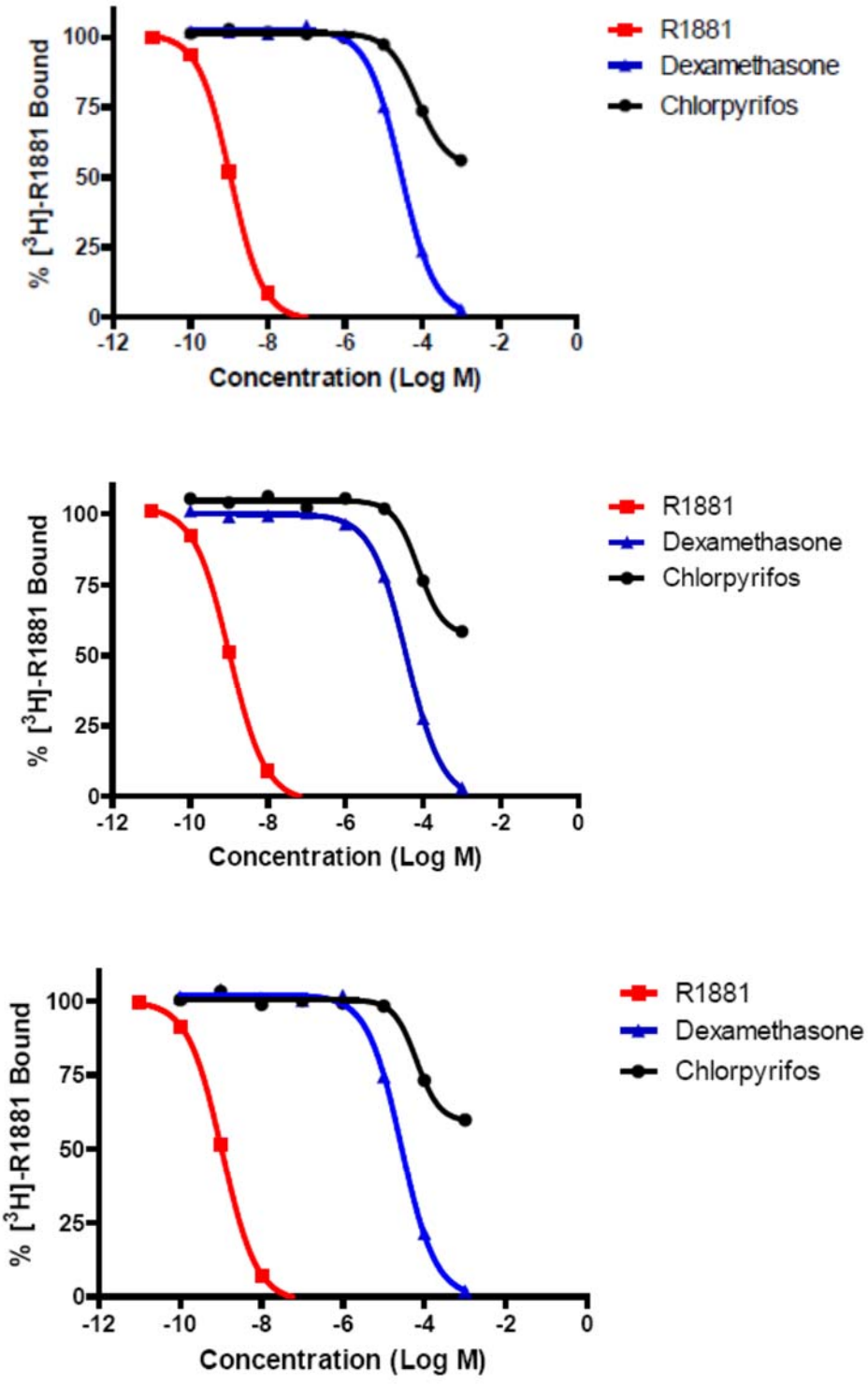
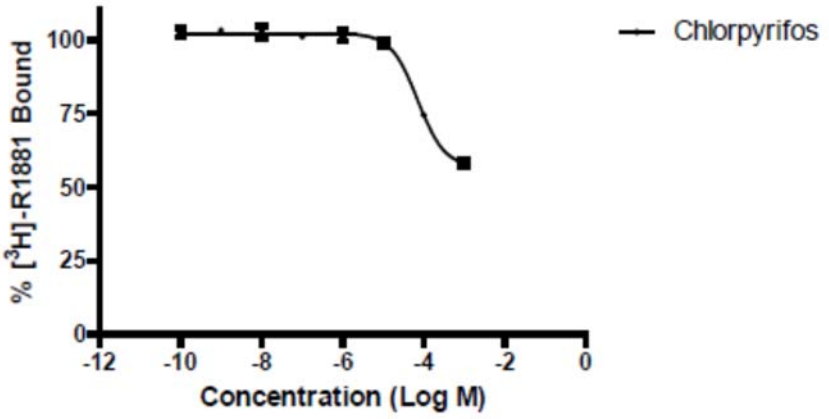


FIGURE 4. Mean of Percentage R1881 Bound to the Androgen Receptor in the Presence of Chlorpyrifos from Three Assays.



C. PERFORMANCE CRITERIA: To ensure that the competitive binding assay was functioning properly, each run was evaluated using the following criteria shown in Table 7.

TABLE 7. Criterion [a]	Tolerance Limit(s) [b]	Value	Yes	No
Ligand depletion is minimal. The recommended ratio of total binding in the absence of competitor to total amount of [^{3}H]-R1881 added per assay tube.	≤15%	4.8-5.5%	X	
Test chemical Top (% binding)	80 to 115	100.3 to 104.7	X	
R1881 fitted curve parameters				
Top (% binding)	82 to 114	99.8 to 102.5	X	
Bottom (% binding)	-2.0 to 2.0	-2.1 to -1.0 [c]		X
Hill Slope	-1.2 to -0.8	-1.0 to -0.9	X	
Weak positive control (dexamethasone) fitted curve parameters				
Top (% binding)	87 to 106	99.9 to 102.7	X	
Bottom (% binding)	-12 to 12	-1.5 to 0.3	X	
Hill Slope	-1.4 to -0.6	-1.0	X	
Saturation Binding Experiment K_d (nM)	0.685-1.57 nM	0.4641		X
Non-specific binding[d] (%)	<20%	7.6	X	

a Data were obtained from pages 33-34, 40, 42 and 44 of the study report.
b These values represent ranges from the validation study.
c In one run the calculated bottom of the curve was slightly low (-2.1%); values were acceptable in the other two runs.
d Values reported for the three NSB tubes at the beginning of the run; does not include the three NSB tubes at the end of the run. Calculated by the reviewer from page 40-44. Avg ethanol (minus background) / Avg NSB (minus background) x 100%.

Additionally, the curve for the reference material showed that increasing concentrations of unlabeled R1881 displaced [^{3}H]-R1881 in a manner consistent with one-site binding, as indicated by a hill slope of -1.0 to -0.9. Examination across the runs indicated consistency of the Hill slope, placement along the X-axis, and top and bottom plateaus.

The percentage of the total specific binding in the solvent controls was approximately 5%. This was within the less than the <20% recommended in the guideline. Ligand depletion

was also minimal (<6%). Sufficient optimization of the number of specific binding sites is supported curves for controls of acceptable steepness, low non-specific binding, and low variability.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATOR'S CONCLUSIONS: Based on the combined results of the three independent assays, chlorpyrifos resulted in partial alterations in the binding of the reference radiolabeled androgen (R1881) at the assay limit concentration (1 mM, 10^{-3} M) and potentially at 10^{-4} M, while no appreciable effect was seen at the lower concentrations (10^{-10} to 10^{-5} M). Under the conditions of the study, chlorpyrifos was determined equivocal for androgen receptor binding, but only at concentrations several orders of magnitude higher than measured blood levels that result in significant brain and blood cholinesterase inhibition in adult female rats (*in vivo*).

B. AGENCY COMMENTS: In the competitive binding experiment, chlorpyrifos reduced the mean specific binding of the radiolabeled ligand in a concentration dependent manner in all three runs. The bottom of the curve for percent of total binding at the 95% confidence interval for chlorpyrifos was 53.7%, 57.2%, and 59.3% in Assays 1, 2, and 3, respectively with an average of 58.2 ± 1.1 At the next highest concentration of 10^{-4} M, the mean was 74.5 ± 1.0, which was also in the equivocal range. At 10^{-5} M, chlorpyrifos was in the non-binding range with an average of 99.1 ± 1.0. The Hill slopes for each of the three runs were -1.132, -1.367, and -1.541 suggesting slightly greater than normal steepness (-1). Chlorpyrifos was classified as equivocal in all three runs. An IC_{50} and RBA could not be calculated for chlorpyrifos.

The mean log IC_{50}s for R1881 and the weak positive control (dexamethasone) were -9.0 and -4.5 M, respectively. The mean RBA for the weak positive control was 0.0034%.

The solvent control responses indicated no drift in the study assay. All performance criteria were met, with the exception of the bottom (% binding) of R1881 in Assay #2 which was slightly low (-2.1%). The reviewers consider these minor deviations and find that the performance criteria were met. Based on the results from the three runs, chlorpyrifos is classified as equivocal in the Androgen Receptor Binding Assay.

C. STUDY DEFICIENCIES: The following deficiencies were noted that are not considered to have had an adverse impact on the results, interpretation or conclusions of this study:

- Information on adjusted specific activity was not available. This information was not considered to adversely impact the study because values calculated from this information (e.g., IC_{50} values) were within expected ranges for controls. Also, changes in specific activity tend to be minor for radionuclides with long half-lives such as ^{3}H.
- Only two saturation binding runs were reported rather than the three runs recommended in the test guideline
- The K_d for saturation binding (0.4641 nM) was less than the recommended range of 0.685- 1.57 nM.

- The Bottom (% binding) for the R1881 was slightly outside of tolerance limits (an upper end of 2.1 instead of 2.0). This minor deviation did not impact the study results as evidenced by expected IC_{50} and Hill Slope values for R1881.

DATA EVALUATION RECORD

CHLORPYRIFOS

Study Type: OCSPP 890.1200; Aromatase Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-14-2012 (MRID 48615503)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CSS-Dynamac Corporation
1910 Sedwick Road,
Building 100, Suite B
Durham, NC 27713

Primary Reviewer	Signature:	Ronnie J. Bever Jr.
Ronnie J. Bever Jr., Ph.D., D.A.B.T.	Date:	1/16/2012
Secondary Reviewer	Signature:	Michael E Viana
Michael E. Viana, Ph.D., D.A.B.T.	Date:	1/26/2012
Program Manager:	Signature:	Jack D. Early
Jack D. Early, M.S.	Date:	2/02/2012
Quality Assurance:	Signature:	Jack D. Early
Jack D. Early, M.S.	Date:	1/02/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel**.**

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Vincent Chen, M.S
Health Effects Division
Signature:
Date: 24 JUN 2015
Secondary Reviewer: Greg Akerman, Ph.D.
Health Effects Division
Signature:
Date: 6/25/15

Template version 08/2011

DATA EVALUATION RECORD

STUDY TYPE: Aromatase (Human Recombinant); OCSPP 890.1200

PC CODE: 059101 **DP BARCODE:** D397128

TXR: 0052086 **CAS:** 2921-88-2

TEST MATERIAL (PURITY): Chlorpyrifos (99.8% a.i.)

SYNONYMS: Chlorpyrifos-ethyl
IUPAC: *O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate
CAS: *O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate

CITATION: Coady, K.K. and Sosinski, L.K. (2011). Evaluation of Chlorpyrifos in the Human Recombinant Aromatase Assay. Toxicology & Environmental Research and Consulting (The Dow Chemical Company [Midland, MI 48674, USA]). Laboratory Study Number: 101142. 21 October 2011. MRID 48615503. Unpublished.

SPONSOR: Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268, USA

TEST ORDER: CON-059101-4

EXECUTIVE SUMMARY: In an *in vitro* aromatase (CYP 19) assay (MRID 48615503), chlorpyrifos (99.8% a.i., Lot # KC28161419, TSN101285) in ethanol was incubated with human recombinant aromatase and tritiated androstenedione ([1β-^{3}H(N)]-androst-4-ene-3,17-dione; [^{3}H]-ASDN) at logarithmic concentrations from 10^{-10} to 10^{-3} M for 15 minutes at 37°C to assess the potential of chlorpyrifos to inhibit aromatase activity.

Aromatase activity was determined by measuring the amount of tritiated water produced at the end of a 15-minute incubation period for each concentration of chemical. Tritiated water was quantified using liquid scintillation counting (LSC). Four independent runs were conducted, and each run included a full activity control, a background activity control, a positive control series (10^{-10} M to 10^{-5} M) using a known inhibitor (4-hydroxyandrostenedione; 4-OH ASDN), and the test chemical series (10^{-10} M to 10^{-3} M) with 3 repetitions per concentration. In Run #1, the average aromatase activity of the full activity controls was 0.0869 nmol·mg-protein^{-1}·min^{-1}, indicating that the microsomal lot used in this particular run (Lot # 85585) had relatively low aromatase activity per mg protein. The report stated that a different lot of microsomes (Lot # 74101) was selected for use in the subsequent runs of the assay. Due to the fact that the full activity levels were lower than the assay performance criteria of 0.1 nmol·mg-protein^{-1}·min^{-1}, Run #1 was not included in the interpretation of the study.

Aromatase activity in the full activity controls ranged from 0.121 to 0.214 nmol·mg-protein^{-1}·min^{-1} for the 3 successful test runs, with a mean and standard deviation of 0.164±0.036 nmol·mg-protein^{-1}·min^{-1}. Activity in the background controls ranged from 2.25 to 2.66% of the full activity controls. The response of the full activity controls and background controls was acceptable for each run.

Results for the positive control were generally within the recommended ranges for the top of the curve, bottom curve, hill slope, log IC_{50}, and coefficient of variation for replicates of each concentration within runs. For 4-OH ASDN, the estimated log IC_{50} averaged −7.17 M, and the Hill slope was −0.97.

For chlorpyrifos, aromatase activity averaged 0.164±0.036 nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration of 10^{-10} M and 0.142±0.030 nmol·mg-protein^{-1}·min^{-1} at the highest tested concentration of 10^{-3} M. The data for chlorpyrifos were modeled; however, the goodness of fit (R^2) was only 0.57–0.77. Because the data failed to model adequately, valid log IC_{50} and Hill slope values could not be determined for chlorpyrifos. The average dose-response curve indicated that chlorpyrifos had no effect on aromatase activity at concentrations of 10^{-10} to 10^{-6} M. However, chlorpyrifos reduced aromatase activity to approximately 93% at 10^{-5} M and to approximately 87% at 10^{-4} M and 10^{-3} M. Aromatase activity was >75% at test concentrations up to 10^{-3} M.

Based on the data from the average response curve, chlorpyrifos is classified as a Non-inhibitor of aromatase activity in this assay.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for an Aromatase assay (OCSPP 890.1200).

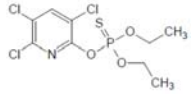
COMPLIANCE: Signed and dated GLP Compliance, Quality Assurance, and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Substance:

Chlorpyrifos

Description:	Not provided
Source:	Dow AgroSciences LLC
Lot # (Expiration Date):	KC28161419, TSN101285 (not reported)
Purity:	99.8%
Vapor Pressure:	1.87×10^{-5} mm Hg at 25°C
Storage Conditions:	5°C to ambient temperature
Stability:	Not reported
Solvent:	Ethanol
Solubility:	2 mg/L water, 79% w/w in isooctane, 43% w/w in methanol Readily soluble in other organic solvents
Highest Concentration Tested:	$10^{-}3$ M
Stock Solution Preparation Methodology:	Dissolved the test material in ethanol.
Molecular Weight:	350.6
CAS #:	2921-88-2
Structure:	

2. Non-Labeled Substrate:

Androstenedione (ASDN)

CAS # :	63-05-8
Source:	Steraloids, Inc. (Cat. # A6030-100)
Batch # (Expiration Date):	L1627 (not reported)
Purity:	98.4%

3. Radiolabeled Substrate:

[1β-^{3}H(N)]-Androst-4-ene-3,17-dione; ([^{3}H]-ASDN)

Source:	Perkin Elmer Life and Analytical Sciences (Cat. # not reported)
Lot # (Expiration Date):	3615304 (not reported)
Radiochemical Purity (Supplier):	>97%
Specific Activity:	20.7 Ci/mmol
Radiochemical Purity (In-lab Determination):	Not reported

4. Positive Control:

4-hydroxyandrostenedione (4-OH ASDN)

CAS #	566-48-3
Source:	Sigma-Aldrich (Cat. # F2552)
Lot # (Expiration Date):	081K2133 (not reported)
Purity:	99.6%

5. Solvent (Vehicle Control):

Ethanol

Source:	Sigma Aldrich (Cat. # E7023)
Lot # (Expiration Date):	04796MK (not reported)
Justification for Choice of Solvent:	Acceptable vehicle by OCSPP guideline 890.1200; Test material wa adequately soluble at specified concentrations
Concentration (% of Total Volume in Assays):	1% v/v

6. Test Microsomes: Human recombinant aromatase (CYP19) microsomes

Source:	BD Biosciences Gentest™ (Woburn, MA; Cat. # 456260)
Lot # (Expiration Date):	74101 and 85585 (not reported)
Protein Concentration:	Not reported, but determined each day of use
Cytochrome C Reductase Activity:	Not reported
Aromatase Activity:	3.4-4.4 pmol/min/pmol P450

B. METHODS

1. Assay Components and Preparations: A mixture of non-labeled and radiolabeled [^{3}H]-ASDN was prepared to result in a 2 µM ASDN solution with a predicted radioactive content of 2.0 µCi/mL.

Test chemical(s) stock solutions were prepared such that the total volume of each test chemical formulation used per assay was no more than 1% v/v of the total assay volume. The report specified that ethanol was chosen because it was mentioned in the guideline as a preferred solvent and the test material was adequately soluble in this solvent at the specified concentration.

A stock solution of the positive control substance, 4-OH ASDN, was formulated in ethanol. Fresh serial dilutions of the stock solution were prepared each time the aromatase inhibition assay was conducted. Dilutions were prepared such that the target concentrations of the positive control substance (10^{-5} to 10^{-10} M; Table 4) were achieved by the addition of 20 µL of the dilution for a final assay volume of 2 mL.

Human recombinant microsomes were purchased from BD Biosciences, and aliquoted into individual vials based on protein content. Microsomes were stored at approximately −80ºC until use.

Other assay components sodium phosphate buffer, propylene glycol, and NADPH are reported in Table 1.

TABLE 1. Assay Components and Conditions

Assay Factor	Values
Sodium phosphate buffer (pH 7.4)	0.1M
Microsomal protein	0.004 mg/mL
NADPH	0.3 mM
[^{3}H]-ASDN	100 nM
Propylene glycol	5%
Temperature	37±1°C
Incubation time	15 min

2. Suitability Assessments: The protein concentration in an aliquot of the microsomes was determined each day of use, and microsomes were diluted with phosphate buffer such that approximately 0.004 mg/ml protein was present in the final reaction solution. Aromatase activity of the microsomes was provided by the vendor as 4.4 and 3.4 pmol/min/pmol P450 for Lot Nos. 74101 and 85585, respectively. The minimum aromatase activity in the full activity control samples was determined to be 0.121 nmol/min/mg protein, which was greater than the minimum acceptable aromatase activity of 0.10 nmol/mg-protein/min.

3. Aromatase Assay: Each assay run contained four tubes for the full enzyme activity and four tubes for the background activity controls. Two tubes of each control were run at the beginning of the assay, and two tubes of each control were run at the end of the assay. A full concentration curve in duplicate for the positive control, and a full concentration curve in triplicate for the test substance were established. The aromatase assay was conducted according to the procedures described in OCSPP 890.1200 (Section h, pp. 9-10).

The amount of ^3H_2O in the aqueous fraction was quantified for each assay tube by LSC, and aromatase activity was reported in units of nmol·mg-protein^{-1}·min^{-1}.

4. Demonstration of Proficiency: No information was provided concerning proficiency testing.

a. Positive Control

(1) Initial Demonstration of Laboratory Proficiency: The positive control data (new/historical data for laboratory) were not reported in regards to the following criteria:

- Mean aromatase activity in the absence of an inhibitor was at least 0.1 nmol/mg-protein/min.
- Mean background control activity was ≤ 15% of the full activity control.
- Coefficient of variation (% CV) for replicates within each sample type and concentration of 4-OH ASDN was <15%.

Performance criteria were reported for the test runs only (Table 2), and served as guidance in identifying runs that provided parameters in the preferred ranges.

(2) Demonstration of Proficiency of New Technician for Conducting Assay (when applicable): Demonstration of proficiency by a new technician, if applicable, was not reported.

TABLE 2. Performance Criteria for the Positive Control[a]

Parameter	Lower Limit Criteria	Upper Limit Criteria	Actual Lower Limit	Actual Upper Limit
Slope	−1.2	−0.8	–1.1	–0.82
Top (%)	90	110	100	107
Bottom (%)	−5	+6	–1.0	1.0
Log IC_{50} (M)	−7.3	−7.0	–7.3	–7.0

a Data were obtained from page 27 of the study report.

b. Proficiency Chemicals: Data were not provided.

TABLE 3. Proficiency Chemicals

Compound	CAS#	Class	Concentrations
Econazole	24169-02-6	Inhibitor	Not reported
Fenarimol	60168-88-9	Inhibitor	Not reported
Nitrofen	1836-75-5	Inhibitor	Not reported
Atrazine	1912-24-9	Non-inhibitor	Not reported

5. Determination of Aromatase Activity with Test Chemical: The response of aromatase activity to the presence of chlorpyrifos at 10^{-10} to 10^{-3} per run, in triplicate, was tested during four independent runs. The report stated that chlorpyrifos was adequately soluble in ethanol at the tested concentrations. In Run #1, the average aromatase activity of the full activity controls was 0.0869 nmol/mg protein/min, indicating that the microsomal lot used in this particular run (Lot #85585) had relatively low aromatase activity per mg protein. The report stated that a different lot of microsomes (Lot # 74101) was selected for use in the subsequent runs of the assay. Due to the fact that the full activity levels were lower than the assay performance criteria of 0.1 nmol/mg protein/min, Run #1 was not included in the interpretation of the study. The full enzymatic activity (≥95% for the means of each run) was obtained at the two lowest concentrations of the test chemical, defining the top of the concentration-response curve.

TABLE 4. Test Chemical Study Design for Each Test Run

Sample Type	Repetitions (Tubes)	Description	Reference or Chemical (M)
Full Activity Control	4	All test components[a] plus solvent vehicle	N/A
Bkgd Activity Control	4	Same as above without NADPH	N/A
4-OH ASDN Conc 1	2	All test components plus 4-OH ASDN	1×10^{-5}
4-OH ASDN Conc 2	2	All test components plus 4-OH ASDN	1×10^{-6}
4-OH ASDN Conc 3	2	All test components plus 4-OH ASDN	$1\times10^{-6.5}$
4-OH ASDN Conc 4	2	All test components plus 4-OH ASDN	1×10^{-7}
4-OH ASDN Conc 5	2	All test components plus 4-OH ASDN	$1\times10^{-7.5}$
4-OH ASDN Conc 6	2	All test components plus 4-OH ASDN	1×10^{-8}
4-OH ASDN Conc 7	2	All test components plus 4-OH ASDN	1×10^{-9}
4-OH ASDN Conc 8	2	All test components plus 4-OH ASDN	1×10^{-10}
Chlorpyrifos Conc 1	3	All test components plus chlorpyrifos	1×10^{-3}
Chlorpyrifos Conc 2	3	All test components plus chlorpyrifos	1×10^{-4}
Chlorpyrifos Conc 3	3	All test components plus chlorpyrifos	1×10^{-5}
Chlorpyrifos Conc 4	3	All test components plus chlorpyrifos	1×10^{-6}
Chlorpyrifos Conc 5	3	All test components plus chlorpyrifos	1×10^{-7}
Chlorpyrifos Conc 6	3	All test components plus chlorpyrifos	1×10^{-8}
Chlorpyrifos Conc 7	3	All test components plus chlorpyrifos	1×10^{-9}
Chlorpyrifos Conc 8	3	All test components plus chlorpyrifos	1×10^{-10}

a The complete assay contained buffer, propylene glycol, microsomal protein, [^{3}H]ASDN, and NADPH.

C. DATA ANALYSIS

1. Raw Data: Raw data were converted to aromatase activity (nmol/mg protein/min) and percent of substrate converted to product for the positive control and test chemical. The following raw data and calculated endpoints for each run were included in the report (Table 5).

TABLE 5. Raw and Calculated Data

Raw/Calculated Data	Included (X)
DPM/mL for each portion of extracted aqueous incubation mixture	X
Average DPM/mL for each aqueous portion (after extraction)	X
Total DPM for each aqueous portion (after extraction)	X
The total DPM present in the assay tube at initiation	X
The percentage of substrate converted to product	X
Total DPM after extraction corrected for background	X
Aromatase activity expressed in nmol/mg protein/min	X
Average aromatase activity in the full activity control tubes	X
Percentage of control activity remaining in the presence of various inhibitor concentrations	X

DPM Disintegrations per minute

2. Statistical Methods: Statistical analyses and graphical displays were conducted using Graph Pad Prism (Version 4.0, La Jolla, CA). Basic statistical analyses were performed on the data, which included means of replicates, standard deviation of the mean, relative standard deviation, and coefficient of variation. The Hill Slope and log IC_{50} values across three independent runs were compared based on a one-way random effects analysis of variance, treating runs as random effects.

The response curve was fitted by nonlinear regression analysis. Model fits were carried out using a 4-parameter regression model. For each run, percent of full activity control were plotted versus logarithm (base 10) of the test chemical concentration or 4-OH ASDN concentration. Each run was plotted with the data's best fit curve. Additionally, the average inhibition response curve across all runs was also plotted.

3. Interpretation of Results: Interpretation of the assay results was based on the average of three runs (Runs #2 – #4), using the categories presented in Table 6.

TABLE 6. Interpretation of Results

Criteria		Interpretation
Data fit 4-parameter nonlinear regression model	Average curve across runs crossed 50% [a]	Inhibitor
	Average lowest portion of curves across runs is between 50% and 75% activity [b]	Equivocal
	Average lowest portion of curves across runs is greater than 75% activity [b]	Non-inhibitor
Data do not fit model	---	

a Ordinarily, an inhibition curve will fall from 90% to 10% over 2 log units with a slope near −1. Unusually steep curves may indicate protein denaturing or solubility issues. If the slope of the curve is steeper than −2.0, the result is classified as equivocal.

b If the test compound was not soluble above 10^{-6} M and the inhibition curve does not cross 50%, the chemical is typically determined to be untestable in the aromatase assay.

II. RESULTS

A. CONTROL ACTIVITY: Aromatase activity in the full activity controls ranged from 0.121 to 0.214 nmol·mg-protein^{-1}·min^{-1} for the 3 successful test runs, with a mean and standard deviation of 0.164±0.036 nmol·mg-protein^{-1}·min^{-1}. Activity in the background controls ranged from 2.25 to 2.66% of the full activity controls. The response of each full activity control within a run was between 94 to 104% of the average full activity.

B. POSITIVE CONTROL: For the positive control substance (4-OH ASDN), aromatase activity averaged 0.169±0.039 nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration (10^{-10} M) and 0.001±0.001 nmol·mg-protein^{-1}·min^{-1} at the highest tested concentration

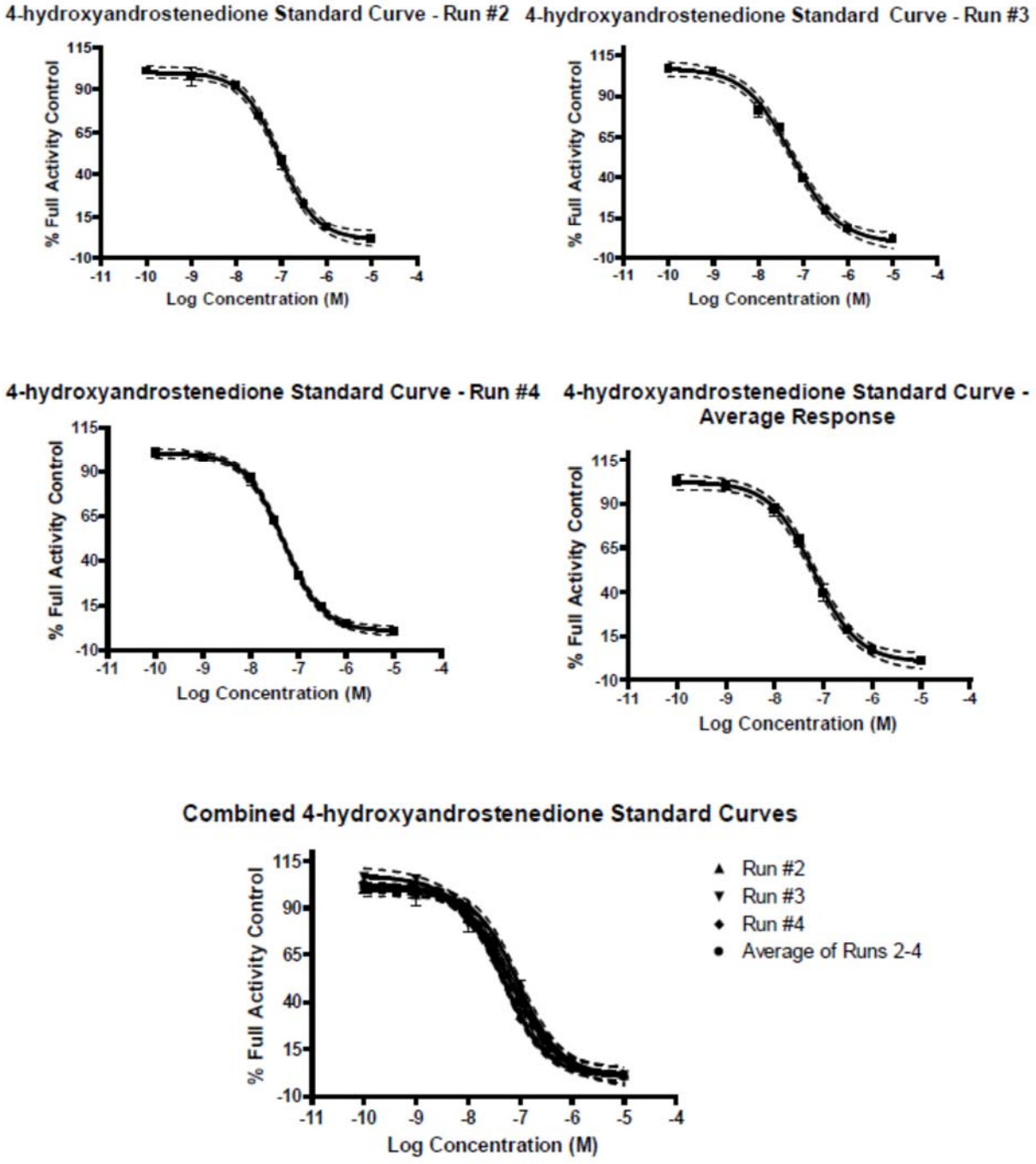
(10^{-5} M). The mean aromatase activity of the positive control (expressed as % full control activity) for each concentration tested across all 3 successful runs is presented in Table 7, along with the overall standard deviation, standard error of the mean, and %CV. Inhibition response curves for the positive control from each run and the average of all runs are shown in Figure 1. These results were within the recommended ranges for the top of the curve, bottom curve, hill slope, log IC_{50}, and %CV for replicates of each concentration within runs (with the exception of Run 3 at 10^{-5} M which was 21%).

TABLE 7. Effect of chlorpyrifos on Aromatase Activity (as Percent of Control) from Independent Runs [a]

Chemical	Conc. Log M	# Runs	Overall Mean	Overall SD	Overall SEM	Overall %CV
4-OH ASDN (positive control)	−5	3	0.98	0.54	0.22	55.1
	−6	3	7.20	1.96	0.80	27.2
	−6.5	3	18.59	3.69	1.51	19.8
	−7	3	39.44	7.82	3.19	19.8
	−7.5	3	69.19	5.92	2.42	8.6
	−8	3	86.45	6.07	2.48	7.0
	−9	3	100.22	5.52	2.26	5.5
	−10	3	102.72	3.54	1.44	3.4
Chlorpyrifos	−3	3	86.94	7.08	2.89	8.1
	−4	3	86.91	6.99	2.85	8.0
	−5	3	92.70	8.56	3.49	9.2
	−6	3	98.88	9.05	3.70	9.2
	−7	3	99.36	4.87	1.99	4.9
	−8	3	99.92	6.48	2.65	6.5
	−9	3	101.16	3.47	1.42	3.4
	−10	3	99.87	4.93	2.01	4.9

a Values were calculated by the reviewers based on data provided on pages 41-54.
SD Standard Deviation
SEM Standard Error of the Mean
CV Coefficient of Variance

FIGURE 1. Inhibition Response Curves for 4-OH ASDN.



C. TEST SUBSTANCE: For chlorpyrifos, aromatase activity averaged 0.164±0.036 nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration of 10^{-10} M and 0.142±0.030 nmol·mg-protein^{-1}·min^{-1} at the highest tested concentration of 10^{-3} M. The mean aromatase activity of chlorpyrifos (expressed as % full control activity) for each concentration tested across all 3 successful runs is provided in Table 7 (presented above), along with the overall standard deviation, standard error of the mean, and %CV. Inhibition response curves for

chlorpyrifos from each run are shown in Figure 2, and the average inhibition response curve across all runs is shown in Figure 3.

FIGURE 2. Inhibition Response Curves for Chlorpyrifos.

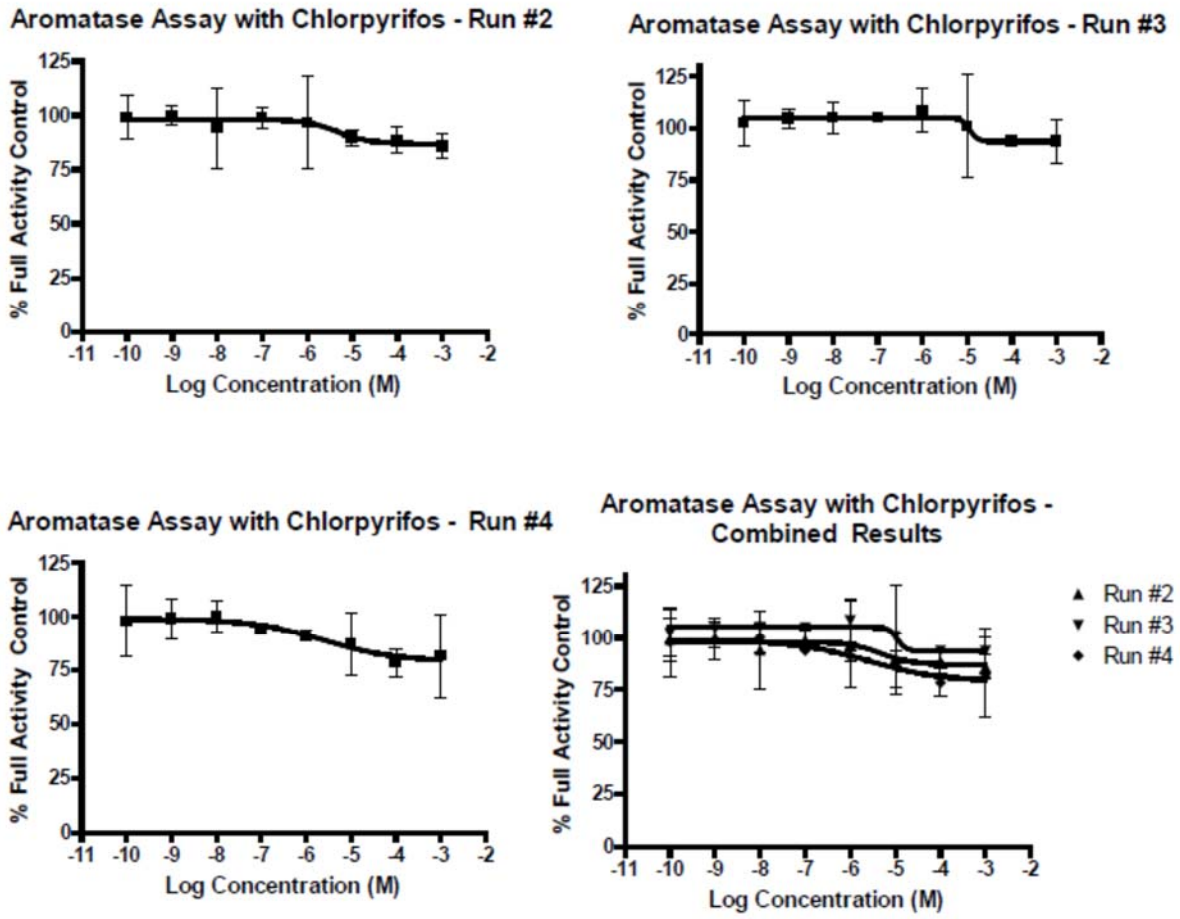
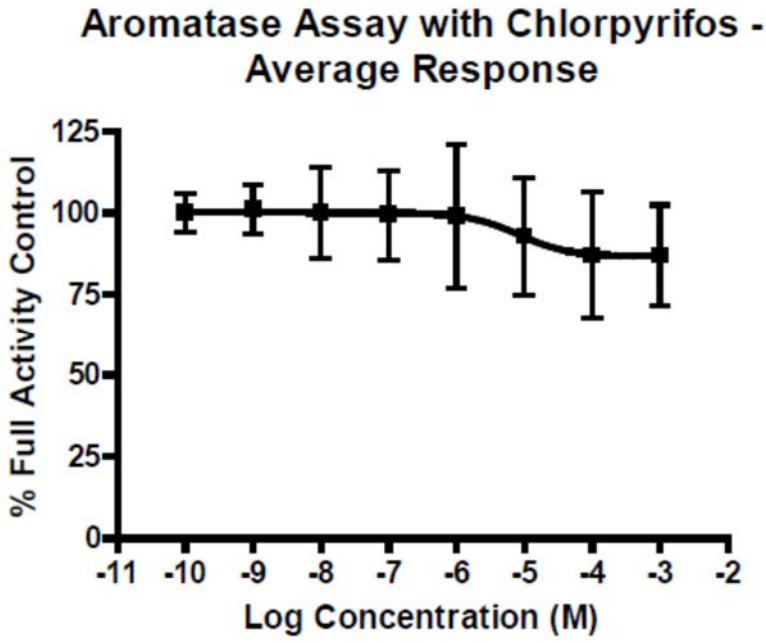


FIGURE 3. Mean Inhibition Response Curve for Chlorpyrifos.



The data for chlorpyrifos were modeled; however, the goodness of fit (R^2) was only 0.57–0.77. In regards to the test material curves, the best fit Hill Slope and log IC_{50} values across three independent runs did not differ significantly based on a one-way random effects analysis of variance ($p = 0.262$ and $p = 0.433$, respectively). The average dose-response curve indicated that the aromatase activity of the test material at concentrations ranging from 10^{-10} M to 10^{-6} M was essentially equivalent to the activity observed in the full activity controls. However, chlorpyrifos reduced aromatase activity, on average, to approximately 93% at 10^{-5} M and 87% at 10^{-4} M and 10^{-3} M. The effect of chlorpyrifos and the positive control on inhibition of aromatase activity is presented in Table 8. Although the study report presented log IC_{50} and Hill slope values for chlorpyrifos, these are not valid values as the chlorpyrifos data did not adequately fit the tested model. For 4-OH ASDN, the estimated log IC_{50} averaged −7.17 M, and the slope was −0.97. The variation in the positive control values was acceptable (<15% CV).

TABLE 8. Effect of Chlorpyrifos and 4-OH ASDN on Aromatase Activity (as Percent of Control) from Independent Runs [a]

Chemical	Run 2	Run 3	Run 4	Mean	SD	%CV
	Log IC_{50} (M)					
Chlorpyrifos [b]	−5.3	−5.0	−5.6	−5.3	0.30	5.66
4-OH ASDN	−7.0	−7.2	−7.3	−7.17	0.15	2.13
	Slope					
Chlorpyrifos [b]	−1.2	−4.6	−0.52	−2.11	2.19	104
4-OH ASDN	−1.0	−1.02	−0.95	−0.97	0.14	14.58

a Data were provided on pages 27-28 of the study report. Mean, SD, and %CV were calculated by the reviewers based on these data.
b Although the report calculated and presented log IC_{50} and Hill Slope values for chlorpyrifos, the reviewers note that the chlorpyrifos data did not fit the 4-parameter regression model.
SD Standard Deviation
CV Coefficient of Variance

As aromatase activity was approximately 87% that of the full activity controls at the highest dose, chlorpyrifos was determined to be a non-inhibitor of aromatase activity in this assay.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATORS' CONCLUSIONS: The results of the human recombinant aromatase assay with chlorpyrifos indicate that under the conditions of this study the test material was classified as a non-inhibitor of aromatase activity.

B. AGENCY COMMENTS: Aromatase activity in the full activity controls ranged from 0.121 to 0.214 nmol·mg-protein^{-1}·min^{-1} for the 3 successful test runs, with a mean and standard deviation of 0.164±0.036 nmol·mg-protein^{-1}·min^{-1}. Activity in the background controls ranged from −0.21 to 0.32% of the overall average of the background adjusted full activity controls. The response of each full activity control within a run was between 94 to 104% of the average full activity.

For the positive control substance (4-OH ASDN), aromatase activity averaged 0.169±0.039 nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration (10^{-10} M) and 0.001±0.001 nmol·mg-protein^{-1}·min^{-1} at the highest tested concentration (10^{-5} M). These results were generally within the recommended ranges for the top of the curve, bottom curve, hill slope, log IC_{50}, and %CV for replicates of each concentration within runs.

For chlorpyrifos, aromatase activity averaged 0.164±0.036 nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration of 10^{-10} M and 0.142±0.030 nmol·mg-protein^{-1}·min^{-1} at the highest tested concentration of 10^{-3} M. Although the chlorpyrifos data were modeled, the data fail to fit the model as the goodness of fit (R^2) values were 0.57–0.77. Therefore, valid log IC_{50} and Hill slope values could not be determined. The average dose-response curve indicated that chlorpyrifos had no effect on aromatase activity at concentrations of 10^{-10} to 10^{-6} M. However, aromatase activity was reduced to approximately 93% at 10^{-5} M, and to approximately 87% at 10^{-4} M and 10^{-3} M.

For 4-OH ASDN, the estimated log IC_{50} averaged −7.17 M, and the slope was −0.97. The variation in the positive control values was acceptable (<15% CV). Aromatase activity was ≥87% at chlorpyrifos concentration up to 10^{-3} M. Based on the data from the average

response curve, chlorpyrifos is classified as a non-inhibitor of aromatase activity in this assay.

C. STUDY DEFICIENCIES: The following deficiencies were noted that are not considered to have had an adverse impact on the results, interpretation or conclusions of this study:

- Proficiency data were not provided.
- Percent of full activity control was not calculated for the positive control and test chemical. The reviewers were able to calculate these values from the supplied data.
- Information on the stability of the test substance in the stock solution was not reported.

DATA EVALUATION RECORD

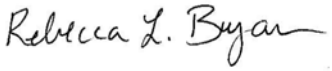
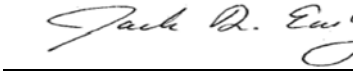
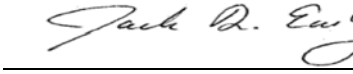
CHLORPYRIFOS

Study Type: OCSPP 890.1250, Estrogen Receptor Binding Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-14-2012 (MRID 48615504)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CSS-Dynamac Corporation
1910 Sedwick Road,
Building 100, Suite B
Durham, NC 27713

Primary Reviewer Michelle Sharpe-Kass, M.S.	Signature: Date:	 2/17/2012
Secondary Reviewer Rebecca L. Bryan, B.S.	Signature: Date:	 2/2/2012
Program Manager: Jack D. Early, M.S.	Signature: Date:	 2/03/2012
Quality Assurance: Jack D. Early, M.S.	Signature: Date:	 2/03/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel**.**

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Jessica Ryman, Ph.D., D.A.B.T **Signature:**
Health Effects Division **Date:**
Secondary Reviewer: Greg Akerman, Ph.D. **Signature:**
Health Effects Division **Date:**

DATA EVALUATION RECORD

STUDY TYPE: Estrogen Receptor Binding Assay Using Rat Uterine Cytosol (ER-RUC); OCSPP 890.1250

PC CODE: 059101 **DP BARCODE:** D397128

TXR#: 0052086 **CAS No.:** 2921-88-2

TEST MATERIAL (PURITY): Chlorpyrifos, (99.8%)

SYNONYMS: O,O-Diethyl O-(3,5,6-trichloro-2-pyridinyl)ester phosphorothioic acid; Chlorpyrifos-ethyl, Chlorpyrifos, Chlorpyriphos

CITATION: LeBaron, M.J., Schisler, M.R., and Visconti, N.R. (2011). Evaluation of chlorpyrifos in an *in vitro* estrogen receptor binding assay. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, MI. Study No.: 111122, October 31, 2011. MRID 48615504. Unpublished.

SPONSOR: Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN

TEST ORDER #: EDSP-059101-30

EXECUTIVE SUMMARY: In an estrogen receptor (ER) binding assay (MRID 48615504), uterine cytosol from Sprague Dawley rats was used as the source of estrogen receptors (ER) to evaluate the potential for chlorpyrifos to displace the bound reference estrogen, radiolabeled [^{3}H]-17β-estradiol from the ER. A saturation binding experiment was conducted prior to the competitive binding experiment to demonstrate that the ER in the uterine cytosol was present in adequate numbers and functioning with the appropriate affinity for the radiolabeled ligand. Saturation binding data were not presented in the study report; however, saturation binding data was presented in the study profile submitted separately by the test order recipient (study profile, MRID 48682804).

The competitive binding experiment was conducted to measure the binding of a single concentration of [^{3}H]-17β-estradiol (1 nM) in the presence of increasing concentrations of chlorpyrifos (logarithmic increase from 10^{-10} to 10^{-3} M). Ethanol was used as test substance vehicle at a final concentration of <3%. The assay included 19-norethindrone as a weak positive control, octyltriethoxysilane as a negative control, and 17-β-estradiol as the natural ligand reference material. Three independent runs were conducted with 3 replicates per concentration per run.

In the saturation binding experiment, the maximum binding capacity (B_{max}) was 59.28 fmol/100 µg protein and the dissociation constant (K_d) was 0.1032 nM. The B_{max} fell within the expected range of 10 to 150 fmol/100 µg protein and the K_d for the run was within the expected range of 0.03 to 1.5 nM. Nonspecific binding as a percent of total binding was 1.7%-8.6% across the entire concentration range in the saturation binding assay. Only a single run of the saturation binding experiment was conducted on the batch of cytosol preparation used for the competitive binding experiment.

In the competitive binding experiment, the bottom of the curve for percent of total binding at the 95% confidence interval for chlorpyrifos was 81.7%, 92.5%, and 77.2% in Assays 1, 2, and 3, respectively. As the minimum binding observed with chlorpyrifos was >75% at concentrations up to 10^{-3} M, an IC_{50} and relative binding affinity (RBA) were not calculated.

The performance criteria were met for chlorpyrifos, and the reference compounds performed as expected in the assay. The negative control, octyltriethoxysilane, had no effect on binding, and the binding curves for 17β-estradiol and 19-norethindrone showed that increasing concentrations of each compound displaced [^{3}H]-17β-estradiol in a manner consistent with one-site binding. The mean log IC_{50} was -5.5 M for 19-norethindrone and -8.9 M for 17β-estradiol, and the mean RBA was 0.034% for 19-noirethindrone compared to the natural ligand.

Based on the results from the three competitive runs, chlorpyrifos is classified as Not Interactive in the Estrogen Receptor Binding Assay.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for an Estrogen Receptor Binding Assay (OCSPP 890.1250).

COMPLIANCE: Signed and dated Confidentiality. GLP and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

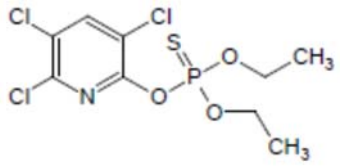
A. MATERIALS

1. Test Facility: Toxicology & Environmental Research and Consulting

Location:	Dow Chemical Company, Midland, MI
Study Director:	M.R. Schisler
Other Personnel:	M.J. LeBaron, Lead Scientist N.R. Visconti, Research Biologist B.B. Gollapudi, Technical Reviewer
Study Period:	September 19 to October 17, 2011

2. Test substance: Chlorpyrifos

Description:	White Solid
Source:	Dow AgroSciences
Lot/Batch #:	KC28161419, TSN101285
Purity:	99.8%
Solubility:	Soluble in ethanol up to 30 mM; 1.05 × 10-3 g/L in water
Volatility:	NR
Stability:	4 year shelf life
Storage conditions:	Ambient
CAS #:	2921-88-2
Molecular weight:	350.6
Structure:	



3. Non-labeled ligand: 17β-estradiol

Supplier:	Sigma, St. Louis, MO
Catalog #	E8875
Batch #:	098K1372
Purity:	100%
CAS #:	50-28-2

4. Radioactive ligand: [^{3}H]-17β-estradiol

Supplier:	Perkin-Elmer, Boston, Massachusets
Catalog #:	NET517001MC
Batch#:	639068
Radiochemical purity:	>97%
Specific activity:	162.91 Ci/mmol *Information on adjusted specific activity was not available.
Concentration of stock:	1.0 mCi/ml

5. Positive control: 19-norethindrone

Supplier:	Sigma, St. Louis, MO
Catalog #	N4128
Batch #:	030M1359
Purity:	99%
CAS # :	68-22-4

6. Negative control: Octyltriethoxysilane

Supplier:	Sigma, St. Louis, MO
Catalog #	440213
Batch #:	72596AMV
Purity:	98.58%
CAS # :	2943-75-1

7. Solvent/vehicle control: Ethanol

Justification for choice of solvent:	None reported.
Final Concentration?	<3%

B. METHODS

1. Preparation of Rat Uterine Cytosol (RUC): Trimmed uterine tissue from 85- to 100-day old female Crl:CD(SD) rats, which were ovariectomized approximately 7-10 days prior to being euthanized, was purchased from Charles River (Wilmington, MA). The uteri were weighed, minced and homogenized in ice-cold TEDG (Tris, EDTA, DTT, glycerol) + PMSF (phenylmethylsulfonyl fluoride) buffer, then centrifuged for 10 min at 2500 × *g* at 4ºC. Supernatant was transferred and centrifuged for 60 minutes at 105,000 × *g*, discarding the resulting pellets. Protein concentration of the cytosol was determined to be 3.573 mg/mL using a protein kit compatible with DTT in the TEDG buffer (e.g., BioRad Protein Assay Kit). Cytosol was divided into aliquots (volume not reported) that were used immediately or stored at -80 ºC for up to 90 days until use.

2. Saturation (radioligand) Binding Experiment: A saturation binding experiment was conducted to measure total and non-specific binding of [^{3}H]-17β-estradiol to demonstrate that the ER in the cytosolic preparation was present in reasonable numbers and had the appropriate affinity for the native ligand. A summary of the conditions for the saturation binding experiment are presented in Table 1.

TABLE 1. Summary of Conditions for Saturation Binding Experiment[a]

Source of receptor		Rat uterine cytosol
Concentration of radioligand (as serial dilutions)		0.03-3.0 nM
Concentration of non-labeled ligand (100X [radioligand])		3.0-300 nM
Concentration of receptor		Sufficient to bind 40.77% of radioligand at 0.03 nM[b]
Temperature		~2-8 ºC
Incubation time		~16 hours
Composition of assay buffer	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10 %
	Phenylmethylsulfonyl fluoride	1 mM
	DTT	1 mM

a Data were not included in the study report, but are reported in the study profile, submitted separately (MRID 48682804).

b This value was slightly higher than the suggested range in the guideline; however, all other values, including minimal ligand depletion, indicated acceptable performance in the assay.

The specific activity of the stock solution [3H]-17β-estradiol was not adjusted for decay over time at the time of the experiment. Serial dilutions in TEDG + PMSF buffer were prepared to achieve the final concentrations of 0.03, 0.06, 0.08, 0.1, 0.3, 0.6, 1, and 3 nM. Solutions of non-labeled 17β-estradiol were prepared in a similar manner to achieve concentrations that were 100-fold greater than each respective radiolabeled concentration to result in final concentrations of 3, 6, 8, 10, 30, 60, 100, and 300 nM. The optimal protein concentration was determined to be 0.1191 mg protein/assay tube, which resulted in the binding of 40.77% of the total radioactivity added. This value was slightly higher than the recommended range in the guideline. Cytosolic protein used in this assay was thawed fresh for this experiment at ~4°C and maintained at ~4°C during the binding assay. Each run contained three concurrent replicates at each concentration, resulting in the 72 samples depicted in Table 2.

TABLE 2. Saturation Binding Experiment Run [a]

Total binding [b]	Non-specific binding [c]	Radioligand alone [d]	Assay Components
Tubes 1-24	Tubes 25-48	Tubes 49-72	
350 µL	300 µL	---	TEDG + PMSF buffer
50 µL	50 µL	50 µL	[3H]-17β-estradiol (8 serial dilutions) [e]
---	50 µL	---	Non-labeled 17β-estradiol (8 serial dilutions, 100x each respective labeled concentration) [f]
100 µL	100 µL	---	Uterine cytosol (diluted to appropriate conc.)
500 µL	500 µL	50 µL	Total volume in each assay tube

a Data were not included in the study report, but are reported in the study profile, submitted separately (MRID 48682804).
b Total binding = [^{3}H]-17β-estradiol bound to ER.
c Non-specific binding = [^{3}H]-17β-estradiol and 100-fold greater non-labeled bound to ER.
d Total [^{3}H]-17β-estradiol alone for dpm determination at each concentration.
e Final concentrations of [^{3}H]-17β-estradiol = 0.03, 0.06, 0.08, 0.1, 0.3, 0.6, 1, and 3 nM.
f Final concentrations of non-labeled 17β-estradiol = 3, 6, 8, 10, 30, 60, 100, and 300 nM.

3. <u>Competitive Binding Experiment</u>: A summary of the experiment conditions for the competitive binding experiment is presented in Table 3.

Tubes were incubated with gentle vortexing for 16 to 20 hours at 4-8 °C. To separate bound from free estradiol, hydroxyapatite (HAP) slurry was added to each tube and vortexed (4 times at 5 minute intervals). Subsequently, the contents of each tube were washed three times as follows: TEDG +PMSF buffer was added, vortexed, centrifuged for 10 min at 1000 x *g*, and the supernatant decanted and discarded. Ethanol was added to the HAP pellet remaining in each tube to extract the bound [^{3}H]-17β-estradiol, followed by vortexing, and centrifugation for 10 min at 1000 x *g*. An aliquot of supernatant was radioassayed by liquid scintillation counting. The temperature was maintained at 4-8ºC throughout the assay prior to extraction with ethanol.

TABLE 3. Summary of Conditions for Competitive Binding Experiment [a]		
Source of receptor		Rat Uterine Cytosol
Concentration of radioligand		1.0 nM
Concentration of receptor		Sufficient to bind 6.21-7.21% of radioligand [b]
Concentration of test substance (as serial dilutions)		10^{-10} to 10^{-3} M
Temperature		4-8 °C
Incubation time		16-20 hours
Composition of assay buffer	Tris	10 mM
	EDTA	1.5 mM
	Glycerol	10%
	Phenylmethylsulfonyl fluoride	1 mM
	DTT	1 mM

a Data were obtained from pages 17, 21 and 22 of the study report.

Solubility of chlorpyrifos in ethanol and assay buffer was evaluated visually and no precipitation was noted. On the day of the assay, the specific activity of the stock solution [^{3}H]-17β-estradiol was not adjusted for decay over time, and diluted in TEDG + PMSF buffer to achieve a final concentration of 1.0 nM.

Serial dilutions of the test substance, positive control (19-norethindrone), negative control (octyltriethoxysilane), and reference material (non-labeled 17β-estradiol) were prepared to achieve the concentrations shown in Table 4. Each assay consisted of three runs, and each run contained three replicates of each test substance at each concentration, resulting in a total of 112 samples.

TABLE 4. Molar (M) concentrations in Competitive Binding Assay Run [a b]			
Chlorpyrifos	Positive control	Negative control	Reference Chemical
	19-norethindrone	Octyltriethoxysilane	Non-labeled 17β-estradiol
Tubes 83-106 [c]	Tubes 35-58 [c]	Tubes 59-82 [c]	Tubes 1-34 and 107-112 [c]
10^{-10}	$10^{-8.5}$	10^{-10}	Solvent control or blank [d]
10^{-9}	$10^{-7.5}$	10^{-9}	10^{-11}
10^{-8}	10^{-7}	10^{-8}	10^{-10}
10^{-7}	$10^{-6.5}$	10^{-7}	$10^{-9.5}$
10^{-6}	10^{-6}	10^{-6}	10^{-9}
10^{-5}	$10^{-5.5}$	10^{-5}	$10^{-8.5}$
10^{-4}	$10^{-4.5}$	10^{-4}	10^{-8}
10^{-3}	10^{-4}	10^{-3}	10^{-7}

a Data were obtained from pages 41-42 of the study report.
b Each tube contains: 10µL of either the test substance, positive control, negative control, solvent control, or non-labeled 17β-estradiol; 390 µL of TEDG + PMSF buffer with [^{3}H]-17β-estradiol; and 100 µL of uterine cytosol (with ER), for a total of 500 µL.
c Each concentration of each chemical was run in triplicate, for a total of 112 tubes per run.
d Solvent is ethanol

C. <u>DATA ANALYSIS</u>: For the competitive binding assay, GraphPad Prism was used to generate nonlinear regression used to fit a curve (for 17β-estradiol, the positive control, and

the test substance) to the Hill equation formula which incorporated IC_{50} as a parameter to be estimated. For parameters reported from the competitive binding experiment (log IC_{50} and RBA), mean and standard deviation were calculated for each run and mean and standard error were calculated for the composite three runs.

1. Definitions

a. Classification of test material: Classification of the test material is based on the average of three runs. Each run was first individually classified as follows:

Interactive = lowest point on the fitted curve within the range of the data is less than 50% (i.e., >50% of the radiolabeled estradiol has been displaced from the ER).

Not interactive = there are usable data points at or above 10^{-6} M and either the lowest point on the fitted response curve within the range of the data is above 75% (i.e., <25% of the radiolabeled estradiol has been displaced from the ER) or a binding curve cannot be fitted and the lowest average percent binding among concentration groups in the data is above 75%.

Equivocal up to the limit of concentrations tested = there are no data points at or above a test chemical concentration of 10^{-6}M and either a binding curve can be fit but ≤50% of the radiolabeled estradiol has been displaced from the ER or a binding curve cannot be fit and the lowest average percent binding among concentration groups in the data is >50%.

Equivocal = A run is classified as equivocal if it does not fall into any of the categories above.

The categorical classification of each run was assigned a numerical value as follows:

Run Classification	Numerical Value
Interactive	2
Equivocal	1
Not interactive	0
Equivocal up to the limit of concentrations tested	"missing"

The values for each run were then averaged across runs and the chemical classified using the following ranges:

Test Material Classification	Numerical Range
Interactive	average ≥1.5
Equivocal	0.5≥ average <1.5
Not interactive	average <0.5
Equivocal up to the limit of concentrations tested	"missing"

b. Descriptors for receptor binding:

B_{max}: maximum specific binding number (fmol ER/100 µg cytosol protein) measures the concentration of active receptor sites

K_d: dissociation constant (nM), measures the affinity of the receptor for its natural ligand
IC_{50}: concentration of the test substance at which 50% of the radioligand is displaced from the receptor
Relative Binding Affinity (RBA %): IC_{50} of 17β-estradiol ÷ IC_{50} of test substance × 100

II. RESULTS

A. <u>SATURATION BINDING EXPERIMENT:</u> Saturation Binding Experiment parameters are presented in Table 5. The Kd for [3H]-17β-estradiol was 0.1032 nM and the Bmax (nM) was 0.07097 for the prepared rat uterine cytosol used in these experiments. The Kd for the run was within the expected range of 0.03 to 1.5 nM. The Bmax was also within the expected range of 10-150 fmol/100 µg protein.

TABLE 5. Saturation Binding Experiment of 17β-estradiol with Estrogen Receptor from Rat Uterine Cytosol [a]

Parameter	Run 1	Run 2	Run 3	Mean ± SE
r^2 (unweighted)	0.9617	N/A	N/A	N/A
B_{max} (nM)	0.07097	N/A	N/A	N/A
B_{max} (fmol/100 µg protein)	59.28	N/A	N/A	N/A
K_d (nM)	0.1032	N/A	N/A	N/A

a Data were not included in the study report, but are reported in the study profile, submitted separately (MRID 48675404).
b Only a single run of the Saturation Binding Experiment was conducted on the batch of cytosol used for this competitive binding experiment.
r^2 Goodness of fit for curve calculated for specific binding

Specific, non-specific, and total binding curves for [3H]-17β-estradiol to the estrogen receptor are presented in Figure 1 below. The specific binding reached a plateau, and non-specific binding was less than 20% of total binding at all concentrations (range 1.7%-8.6%). Figure 2 is a Scatchard plot that illustrates the binding of [3H]-17β-estradiol to the estrogen receptor. The data fit results in a linear plot.

FIGURE 1. Binding of [3H]-17β-estradiol to the ER during the Saturation Binding Experiment.

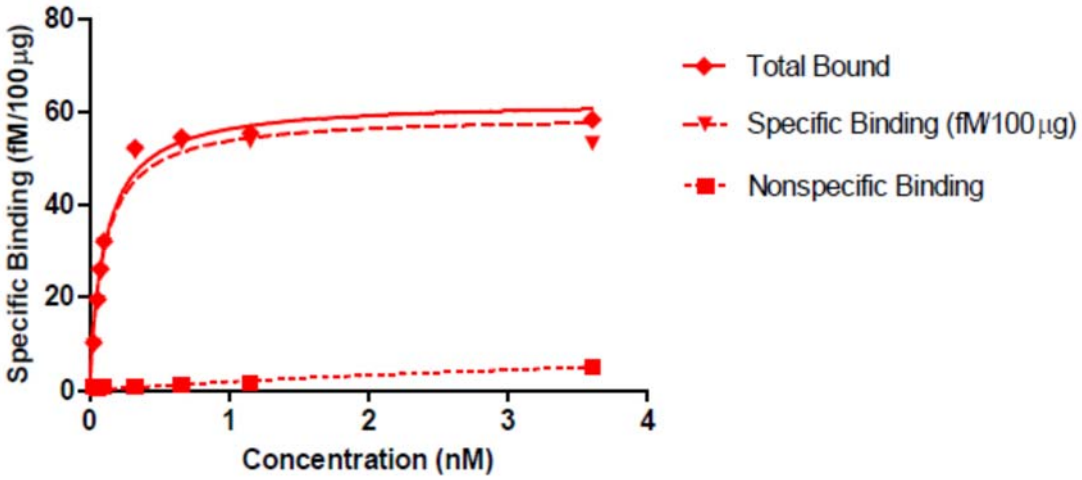
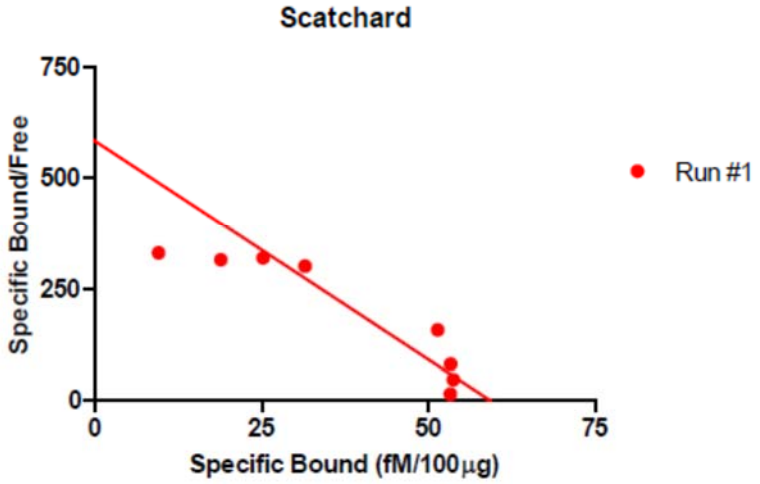


FIGURE 2. Scatchard Plot of the Binding of [3H]-17β-estradiol to the ER during the Saturation Binding Experiment.



B. COMPETITIVE BINDING EXPERIMENT The non-specific binding was less than 3.5% in all assays, and was calculated by reviewers for each assay by dividing the average ethanol concentration (minus background) by the average non-specific binding (on pages 41-45 of the study report) and multiplying by 100%. Non-specific binding was considered well below the recommended maximum limit of 50%. Ligand depletion was stated in the study report data tables on pages 41, 43, and 45 and was ≤7.2%. This was within the recommended tolerance limit of 15%.

The results from the three competitive binding experiments are summarized in Table 6 and presented graphically in Figures 3-5. The bottom of the curve for percent of total binding at the 95% confidence interval for chlorpyrifos was 81.7%, 92.5%, and 77.2% in Assays 1, 2, and 3, respectively. Chlorpyrifos was considered not interactive with the estrogen receptor.

An IC_{50} and RBA were not calculated for chlorpyrifos as the minimum percent binding was ≥50%.

The reference compounds performed as expected in the assay. Octyltriethoxysilane had no effect on binding of the radiolabeled ligand, and 17β-estradiol and 19-norethindrone showed competitive binding at the expected concentrations. The mean log IC_{50} for 19-norethindrone was -5.5 M and the mean log IC_{50} for 17β-estradiol was -8.9 M. The mean RBA was 0.034% for 19-noirethindrone.

Confidence in these numbers is high due to the small variation. The solvent control responses indicated no drift in the study assay, and additional runs were unnecessary. As chlorpyrifos displaced <25% of the radiolabeled estradiol from the ER at concentrations up to 10^{-3} M in all three runs, it is classified as not interactive (0) in this assay (Table 7).

TABLE 6. Competitive Binding Assay of Chlorpyrifos with Estrogen Receptor from Rat Uterine Cytosol [a]

Parameter		Run 1 [b]	Run 2 [b]	Run 3 [b]	Mean ± SE
r^2 (unweighted),	17β-estradiol	0.9995	0.9977	0.9998	NA
	19-norethindrone	0.9962	0.9985	0.9997	NA
	Chlorpyrifos	0.9960	0.9767	0.9955	NA
Log IC_{50} (M),	17β-estradiol	-8.961	-8.939	-8.929	-8.943
	19-norethindrone	-5.534	-5.442	-5.445	-5.473
	Chlorpyrifos	NA	NA	NA	NA
IC_{50} (M),	17β-estradiol	1.095×10^{-9}	1.151×10^{-9}	1.177×10^{-9}	1.140×10^{-9}
	19-norethindrone	2.923×10^{-6}	3.614×10^{-6}	3.586×10^{-6}	3.361×10^{-6}
	Chlorpyrifos	NA	NA	NA	NA
RBA (as % IC_{50}) [c],	19-norethindrone	0.037	0.032	0.033	0.034
	Chlorpyrifos	NA	NA	NA	NA

a Data were obtained from pages 34-35 of the study report.
b The mean and standard deviation are reported for the concurrent replicates within each run.
c Calculated by reviewer: [IC_{50} (in M) positive control or chlorpyrifos / IC_{50} (in M) R1881] x 100%
NA Not applicable
r^2 Goodness of fit
RBA (%) Relative binding affinity

TABLE 7. Binding Classification of Chlorpyrifos with Estrogen Receptor [a]					
Run	1	2	3	Mean [c]	Binding Classification [d]
Classification category value [b]	0	0	0	0	Not interactive

a Data were obtained from page 35 of the study report.
b Classification category value: Interactive = 2; Equivocal = 1; Not interactive = 0; Equivocal up to the limit of concentrations tested ("missing", i.e., not included in calculation of mean).
c Mean of three runs expressed to the tenths place
d Interactive = mean ≥1.5; Equivocal = 0.5≤ mean <1.5; Not interactive = mean <0.5

FIGURE 3. Percentage E2 Bound to the Estrogen Receptor in the Presence of Unlabeled E2, 19-Norethindrone or Octyltriethoxysilane. Run 1

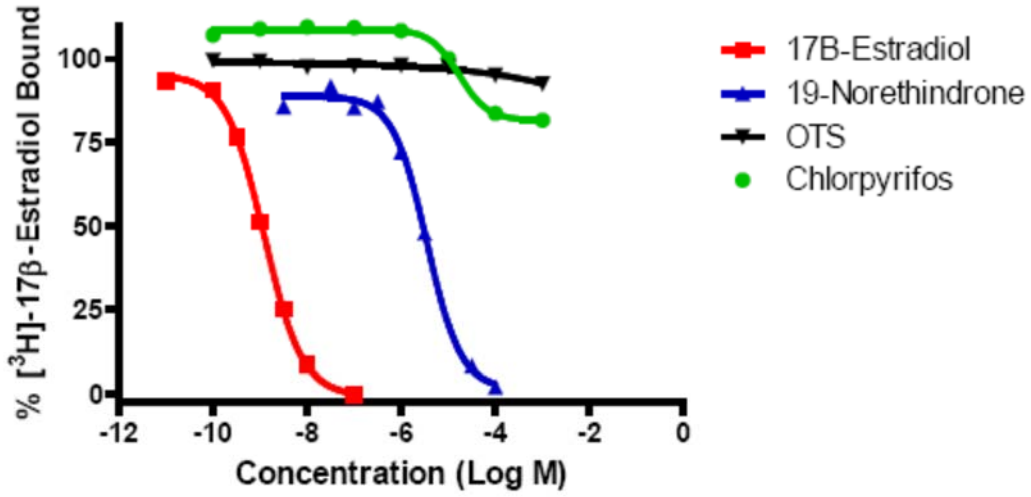


FIGURE 4. Percentage E2 Bound to the Estrogen Receptor in the Presence of Unlabeled E2, 19-Norethindrone or Octyltriethoxysilane. Run 2

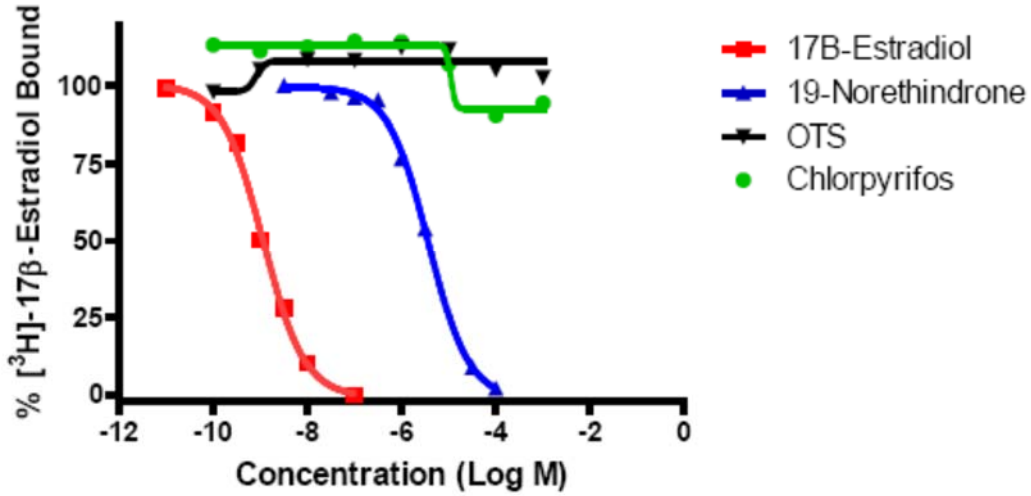
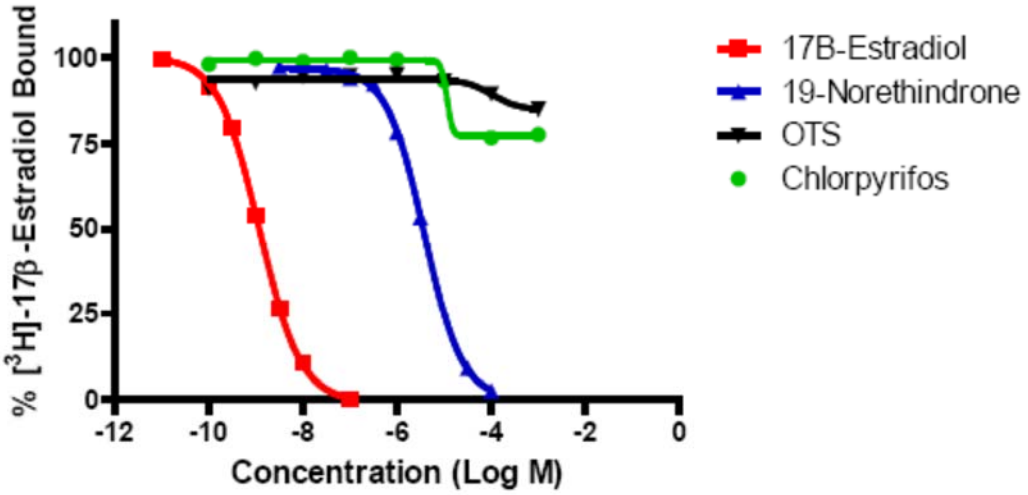


FIGURE 5. Percentage E2 Bound to the Estrogen Receptor in the Presence of Unlabeled E2, 19-Norethindrone or Octyltriethoxysilane. Run 3



C. **PERFORMANCE CRITERIA:** To ensure that the competitive binding assay functioned properly, each run was evaluated using the following criteria:

TABLE 8. Criterion [a]	Tolerance Limit(s)	Value	Yes	No
17β-estradiol fitted curve parameters				
Log_e residual SD	≤2.35	-0.15 to 0.24	X	
Top (% binding) [b]	94 to 111	95-101	X	
Bottom (% binding)	-4 to 1	-0.7 to -0.9	X	
Hill Slope ($log_{10}(M)^{-1}$)	-1.1 to -0.7	-1.0 to -1.1	X	
Weak Positive control (19-norethindrone) fitted curve parameters				
Log_e residual SD	NA	-0.02 to 1.1	X	
Top (% binding) [b]	NA	89-100	X	
Bottom (% binding)	NA	-1.3 to 1.7	X	
Hill Slope ($log_{10}(M)^{-1}$)	NA	-1.0 to -1.2	X	
Solvent concentration				
Ethanol	≤3%	<3%	X	
Negative control (octyltriethoxysilane) does not displace more than 25% of [^{3}H]-17β-estradiol from the ER on average across all concentrations	≤25%	<16%	X	

a Data were obtained from page 34 of the study report.
b If the top plateau for estradiol is significantly above the upper performance criterion, then curves for all chemicals in the run may be normalized using binding of estradiol at the lowest concentration in the reference curve as 100%.
NA Not applicable

Additionally, the curve for the reference material showed that increasing concentrations of unlabeled 17β-estradiol displaced [^{3}H]-17β-estradiol in a manner consistent with one-site binding, as indicated by a hill slope of -1.0 to -1.1.

Chlorpyrifos was tested over a concentration range that fully defined the top of the curve. The percent binding at this top plateau (99.2-113.3%) was within 25 percentage points of

the lowest concentration of the estradiol standard 93.5-99.6%. Examination across the runs indicated consistency of the Hill slope, placement along the X-axis, and top and bottom plateaus.

The percentage of the total specific binding in the solvent controls was approximately 7%. This was within the less than the ≤10-15% recommended in the guideline. Ligand depletion was also minimal. Sufficient optimization of the number of specific binding sites is supported curves for controls of acceptable steepness, low non-specific binding, and low variability.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATOR'S CONCLUSIONS: Based on the combined responses in each of three independent ER binding assays, it was determined that chlorpyrifos had no appreciable effect in the binding of the radiolabeled reference estrogen ([^{3}H]-17β-estradiol) at any concentration tested (up to 10^{-3}M). The results of the *in vitro* ER binding assay using rat uterine cytosol indicate that, under the conditions of this study, chlorpyrifos was not interactive for ER binding at concentrations up to 10^{-3} M.

B. AGENCY COMMENTS: The results of the saturation binding experiment were not reported in the study report; however, summary saturation data including graphs showing specific, non-specific, and total binding curves for [3H]-17β-estradiol to the estrogen receptor were reported in a study profile (MRID 48675401). The maximum binding capacity (B_{max}) was 59.28 fmol/100 µg protein and the dissociation constant (K_d) was 0.1032 nM. The B_{max} fell within the expected range of 10 to 150 fmol/100 µg protein and the K_d for the run was within the expected range of 0.03 to 1.5 nM. Only one saturation binding run was performed instead of the recommended three runs. For the competitive binding experiment, the bottom of the curve for percent of total binding at the 95% confidence interval for chlorpyrifos was 81.7%, 92.5%, and 77.2% in Assays 1, 2, and 3, respectively. Chlorpyrifos was considered not interactive with the estrogen receptor. As the minimum binding observed with chlorpyrifos was >75% at concentrations up to 10^{-3} M, an IC_{50} and RBA were not calculated.

The performance criteria were met for chlorpyrifos, and the reference compounds performed as expected in the assay. The negative control, octyltriethoxysilane, had no effect on binding, and the binding curves for 17β-estradiol and 19-norethindrone showed that increasing concentrations of each compound displaced [^{3}H]-17β-estradiol in a manner consistent with one-site binding. The mean log IC_{50} was -5.5 M for 19-norethindrone and -8.9 M for 17β-estradiol, and the mean RBA was 0.034% for 19-norethindrone compared to the natural ligand.

C. STUDY DEFICIENCIES: The following deficiencies were noted that are not considered to have had an adverse impact on the results, interpretation or conclusions of this study:

- Only a single run of the saturation binding experiment was performed on the cytosol preparation used for the competitive binding experiment instead of the recommended three runs/cytosol preparations.

DATA EVALUATION RECORD

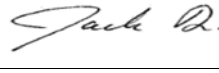
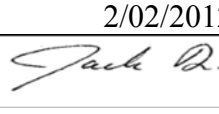
CHLORPYRIFOS

Study Type: OCSPP 890.1300, Estrogen Receptor Transcriptional Activation

EPA Contract No. EP10H001452
Task Assignment No. 2-14-2012 (MRID 48615505)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CSS-Dynamac Corporation
1910 Sedwick Road,
Building 100, Suite B
Durham, NC 27713

Primary Reviewer Michelle Sharpe-Kass, M.S.	Signature: Date:	 2/17/2012
Secondary Reviewer Michael E. Viana, Ph.D., D.A.B.T.	Signature: Date:	 2/01/2012
Program Manager: Jack D. Early, M.S.	Signature: Date:	 2/02/2012
Quality Assurance: Jack D. Early, M.S.	Signature: Date:	 2/02/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel.

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Sheila Healy, PhD **Signature:**
Health Effects Division **Date:** 6/15/15
Secondary Reviewer: Minerva Mercado, PhD, DABT **Signature:**
Health Effects Division **Date:** 6-19-15

DATA EVALUATION RECORD

STUDY TYPE: Estrogen Receptor Transcriptional Activation (Human cell Line, HeLa-9903); OCSPP 890.1300; OECD 455.

PC CODE: 059101 **DP BARCODE:** D397128

TXR#: 0052086 **CAS No.:** 2921-88-2

TEST MATERIAL (PURITY): Chlorpyrifos, 99.8% a.i.

SYNONYMS: *O,O*-Diethyl *O*-(3,5,6-trichloro-2-pyridinyl)ester phosphorothioic acid; Chlorpyrifos-ethyl, Chlorpyrifos, Chlorpyriphos

CITATION: LeBaron, M.J, Kan, H.L. (2011) Evaluation of chlorpyrifos in an *in vitro* estrogen receptor transcriptional activation assay in human cell line hERα-HELA-9903. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, MI. Laboratory Project Study ID: 101190, October 27, 2011. MRID 48615505. Unpublished.

SPONSOR: Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN

TEST ORDER #: CON-059101-4

EXECUTIVE SUMMARY: In an estrogen receptor transcriptional activation assay (MRID 48615505), hERα-HELA-9903 cells cultured *in vitro* were exposed to chlorpyrifos (99.8% a.i., Lot# KC28161419) at logarithmically increasing concentrations from 10^{-10} to 10^{-4} M in DMSO (final concentration 0.1%) for 24 hours. The experiments were performed using 96-well plates and each chlorpyrifos concentration was tested in triplicate (3 wells/plate). Cells were exposed to the test agents for 24 hours to induce reporter (luciferase) gene products. Luciferase expression in response to activation of the estrogen receptor by chlorpyrifos was measured upon addition of a luciferase substrate and detection with a luminometer with acceptable sensitivity.

Chlorpyrifos was tested up to the limit of solubility, 10^{-4} M.

The mean RPC_{Max} for chlorpyrifos was 25.4% in the first run, 10.2% in the second run and 19.5% in the third run, and the associated PC_{Max} was 10^{-4} M for the first and third run, and 10^{-5} M for the second run. Acceptance criteria were met for all reference chemicals, and the assay displayed slightly increased sensitivity to very weak agonists like 17α-methyltestosterone. This does not negatively impact the validity of the study.

Because the $RPC_{Max} > PC_{10}$ in all three assay runs, chlorpyrifos was considered positive for estrogen receptor transcriptional activation in this test system.

This assay **satisfies** the EDSP Tier 1 Test Order requirement for an Estrogen Receptor Transcriptional Activation assay (OCSPP 890.1300).

COMPLIANCE: Signed and dated GLP, Quality Assurance and Data Confidentiality statements were provided.

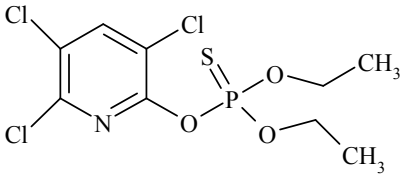
I. MATERIALS AND METHODS

A. MATERIALS

1. Test Substance: Chlorpyrifos

Description: White solid, MW 350.6
Source: Dow AgroSciences LLC
Lot/Batch #: KC28161419
Purity: 99.8%
Solubility: DMSO up to 0.1M; 1.05 x 10^{-3} g/L in water
Volatility: Not reported
Stability: 3.5 year shelf life
Storage conditions: Ambient
CAS #: 2921-88-2

Structure:



2. Reference substances

17β-estradiol (strong estrogen; positive control)

Supplier: Sigma, St. Louis, MO
Catalog # and Batch #: E-8875, Lot# 079K0131
Purity: 100%
CAS #: 50-28-2

17α-estradiol (weak estrogen)

Supplier: Sigma, St. Louis, MO
Catalog # and Batch #: E-8750, Lot # 029K4116
Purity: ≥99.5%
CAS # : 57-91-0

17α-methyltestosterone (very weak agonist)

Supplier: Sigma, St. Louis, MO
Catalog # and Batch #: M-7252, Lot# 060M1543V
Purity: 99%
CAS # : 58-18-4

Corticosterone (negative compound)

Supplier: Sigma, St. Louis, MO
Catalog # and Batch #: C-2505, Lot # 010M2010
Purity: 100%
CAS # : 50-22-6

3. Vehicle(s)

Solvent:	DMSO, Sigma-Aldrich, Cat. # 276855, Lot # 36296DM and 68996LMV
Solvent control (final concentration):	0.1%

B. METHODS

1. Cell Culture: Stably-transfected hERα-HeLa-9903 cells were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank and were verified to be free of mycoplasma infection by ATCC laboratory. Cells were maintained in Eagles Minimum Essential Medium (EMEM) without phenol red, supplemented with kanamycin (60 mg/L) and 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS; in-house prep, Hyclone Laboratories, Inc. Logan, Utah; Lot# DCC-FBS 062410 and 040111), in an incubator under 5% CO_2 at 37ºC. Upon reaching 75-90% confluence, cells were subcultured no more than 40 times prior to exposure to the test material. DCC-FBS was prepared according to the protocol provided in the Guideline. It was not reported if testing was conducted to ensure that all hormones were stripped.

2. Transcriptional Activation Assays: For each test, cells were plated in a 96-well microplate at a density of 1.0-1.5 × 10^4 cells/100 µL medium/well and allowed to attach for 3 hours. The growth media was replaced with media containing serial log dilutions of chlorpyrifos in DMSO. Cells were incubated for 24 hours at 37±1 °C. The final concentration of DMSO in the assay was 0.1%. Cytotoxicity was determined microscopically.

Transcriptional activation of the estrogen receptor was determined using a commercial luciferase assay (Promega Luciferase Assay System, Cat. # E1501, Madison, WI). Chemiluminescence was measured immediately after adding luciferase agents in a Packard Top Count NXT luminescence counter. This luminometer enables linear responses in 100 to 20 million counts per second in glow type luminescence performance, and has a detection limit of ~5 cells in 100 µl medium.

a. Preliminary Test: A preliminary test evaluating concentrations ranging from 10^{-10} M to 10^{-3} M was conducted to determine the appropriate concentration range and to determine concentrations resulting in insolubility and/or cytotoxicity.

b. Proficiency Chemicals: It was stated that laboratory validation assays with 10 proficiency chemicals were performed to confirm the responsiveness of the ER transcriptional activation assay. These non-GLP, unpublished experimental results demonstrated laboratory proficiency and assay validation and were included in a separate report; however, this report was not provided with this study.

c. Reference Chemicals: To ensure the stability of the response from the cell line, six concentrations of each of the following reference chemicals were included in each plate in the current assay, along with the test chemical:

Reference Chemical	CAS No.	Concentration Range	Class
17β-estradiol	50-28-2	10^{-14} to 10^{-8}	Strong estrogen
17α-estradiol	57-91-0	10^{-12} to 10^{-6}	Weak estrogen
Corticosterone	50-22-6	10^{-10} to 10^{-4}	Negative compound
17α-methyltestosterone	58-18-4	10^{-11} to 10^{-5}	Very weak agonist

3. Data analysis: To obtain the relative transcriptional activity to the 1 nM E2 positive control (PC), the luminescence signals from the concurrent plate were analyzed by subtracting the mean value of the vehicle control from each well value to normalize the data; each normalized value was then divided by the mean value of the normalized PC. The resulting value was multiplied by 100 in order to express relative transcriptional activity as a percentage of the PC. Graph Pad Prism v. 5.0 (GraphPad Software, Inc., La Jolla, CA) was used to calculate the EC_{50}, PC_{10}, PC_{50}, RPC_{Max}, and PC_{Max} for chlorpyrifos when applicable. The test material was defined as positive for inducing estrogen receptor transcriptional activation if the $RPC_{Max} \geq PC_{10}$ in at least 2 of 2 (or 2 of 3) runs. The Log EC_{50} and Hill slope values were calculated only if a positive response was observed. Coefficients of variation (%CV) were calculated for the luminescence data triplicates. Concentrations showing >20% cytotoxicity or evidence of insolubility were excluded from analyses.

4. Definitions

EC_{50} = concentration of agonist that induces a response halfway between the baseline (bottom) and maximum (top) response

PC_{10} = concentration of a test chemical at which the response is 10% of the response induced by the positive control (E2 at 1 nM) in each plate

PC_{50} = concentration of a test chemical at which the response is 50% of the response induced by the positive control (E2 at 1 nM) in each plate

RPC_{Max} = maximum level of response induced by a test chemical, expressed as a percentage of the response induced by the positive control (1 nM E2) on the same plate

PC_{Max} = concentration of a test chemical inducing the RPC_{Max}

II. RESULTS

A. PRELIMINARY TEST: A preliminary test evaluating chlorpyrifos concentrations ranging from 10^{-10} to 10^{-3} M was conducted to determine the appropriate concentration range and to determine concentrations resulting in insolubility and/or cytotoxicity (Table 1). Precipitation was noted in the treatment medium at 10^{-3} M. Based on these results, concentrations of 10^{-10} M to 10^{-4} M were selected for the assay.

TABLE 1. Preliminary Test for Solubility, Cytotoxicity, and Concentration-Selection for Chlorpyrifos [a]

Concentration (M)	% Viability	Comments
10^{-3}	--	Precipitation noted in the treatment medium
10^{-4}	91.8	
10^{-5}	96.2	
10^{-6}	101.0	
10^{-7}	93.3	
10^{-8}	95.1	
10^{-9}	98.2	
10^{-10}	99.9	
E2 1nM	100.8	
VC [b]	100.0	

a Data were obtained from page 47 of the study report.
b VC = Vehicle control

B. POSITIVE AND NEGATIVE REFERENCE CHEMICALS

1. Proficiency Chemicals: It was stated that laboratory validation assays with 10 proficiency chemicals; however, these date were included in a separate report (not provided).

TABLE 2. Proficiency Chemicals [a]

Compound	Expected Response	Lab Response
Diethylstilbestrol	Positive	Not reported
17α-Ethynyl estradiol	Positive	Not reported
Hexestrol	Positive	Not reported
Genistein	Positive	Not reported
Estrone	Positive	Not reported
Butyl paraben	Positive	Not reported
1, 3, 5-Tris(4-hydroxyphenyl)benzene	Positive	Not reported
Dibutyl phthalate	Negative	Not reported
Atrazine	Negative	Not reported
Corticosterone	Negative	Not reported

2. Reference Chemicals: Values derived from the concentration response curve (*e.g.*, log PC_{50}, log PC_{10}, log EC_{50}, and Hill slope) for the four concurrently run reference materials are included in Table 3. In the first and third test, all acceptance criteria were met for 17β-estradiol, 17α-estradiol and corticosterone. For the second run, the Hill slope was less than the validated range for both 17β-estradiol and 17α-estradiol. In all three runs, the responsiveness of 17α-methyltestosterone was greater than the expected limits.

TABLE 3. Performance Criteria for Reference Chemicals[a]						
Reference Chemical Parameter	Acceptable Range	Values			Acceptable	
		Run 1	Run 2	Run 3	Yes	No
17β-estradiol						
Log PC_{50}	−11.4 to −10.1	−11.2	−10.6	−10.7	X	
Log PC_{10}	<−11	−12.9	−12.5	−12.5	X	
Log EC_{50}	−11.3 to −10.1	−11.1	−10.2	−10.7	X	
Hill Slope	0.7 to 1.5	0.7	0.4	0.7	X	
Test range	10^{-14} to 10^{-8} M	10^{-14} to 10^{-8} M	10^{-14} to 10^{-8} M	10^{-14} to 10^{-8} M	X	
17α-estradiol						
Log PC_{50}	−9.6 to −8.1	−9.4	−9.3	−9.0	X	
Log PC_{10}	−10.7 to −9.3	−10.6	−10.5	−10.6	X	
Log EC_{50}	−9.6 to −8.4	−8.9	−8.9	−8.9	X	
Hill Slope	0.9 to 2.0	1.1	0.6	0.9	X	
Test range	10^{-12} to 10^{-6} M	10^{-12} to 10^{-6} M	10^{-12} to 10^{-6} M	10^{-12} to 10^{-6} M	X	
Corticosterone						
Test range	10^{-10} to 10^{-4} M	10^{-10} to 10^{-4} M	10^{-10} to 10^{-4} M	10^{-10} to 10^{-4} M	X	
17α-methyltestosterone						
Log PC_{50}	−6.0 to −5.1	−7.6	−6.6	−6.1	X	
Log PC_{10}	−8.0 to −6.2	−8.8	−8.9	−8.8	X	
Test range	10^{-11} to 10^{-5} M	10^{-11} to 10^{-5} M	10^{-11} to 10^{-5} M	10^{-11} to 10^{-5} M	X	

a Data were obtained from page 28 of the study report.

C. DEFINITIVE ASSAY

1. Vehicle and Positive Controls: Data for the vehicle and positive controls are included in Table 4 (expressed as arbitrary light units).

TABLE 4. Transcriptional Activation (TA) Response of Vehicle and Positive Control [a]					
Sample	Vehicle Control		Positive Control [b]		
Runs	Mean	SD	Mean	SD	Fold Induction [c]
1	2241	346	19757	905	8.8
2	1338	246	9427	1134	7.0
3	4151	612	29921	1416	7.5

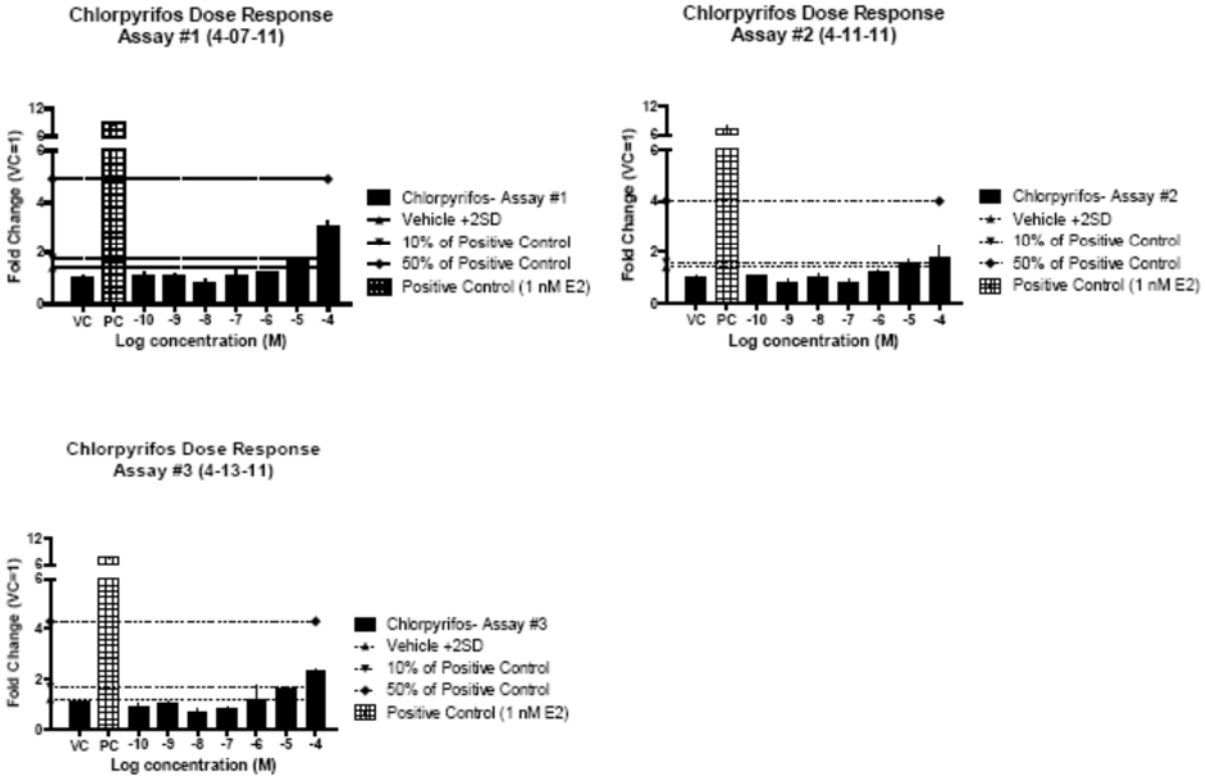
a Data were obtained from page 49 of the study report.
b Positive control was 17β-estradiol (E2) at 1 nM.
c Fold-induction = (mean TA of PC)/(mean TA of VC)

2. Test Material: Relative (to the PC) transcriptional activation at each concentration of the test chemical during the three assay runs is presented in Table 5. The concentration-response curves depicting fold induction of relative transcriptional activation is presented in Figure 1 below. The mean RPC_{Max} for chlorpyrifos was 25.4% in the first run, 10.2% in the second run and 19.5% in the third run, and the associated PC_{Max} was 10^{-4} for the first and third run, and 10^{-5} for the second run. Because the $RPC_{Max} > PC_{10}$ in all three assay runs, chlorpyrifos was considered positive for estrogen receptor transcriptional activation in this test system. A PC_{50} could not be calculated.

TABLE 5. Relative Transcriptional Activation (RTA) of Chlorpyrifos[a]						
Parameter	**RTA (mean ± SD); % of Positive Control (PC)**					
	Run 1		**Run 2**		**Run 3**	
Conc. (M)	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
10^{-4}	25.4	4.8	13.4	0.7	19.5	2.8
10^{-5}	8.7	1.1	10.2	3.2	9.8	2.0
10^{-6}	2.4	0.6	2.4	1.8	3.4	3.2
10^{-7}	1.0	3.4	−2.7	2.6	−2.7	3.5
10^{-8}	−2.6	4.0	−0.6	3.7	−4.3	3.8
10^{-9}	1.0	1.1	−3.4	4.6	−0.8	2.6
10^{-10}	1.6	3.0	1.3	0.7	−2.3	2.7
Log EC_{50}	−3.9		−5.5		−4.8	
Hill Slope	0.6		1.1		0.7	
RPC_{Max}	25.4		10.2*		19.5	
PC_{Max}	10^{-4}		10^{-5}		10^{-4}	
PC_{50}	NA		NA		NA	
PC_{10}	$10^{-4.9}$		10^{-5}		10^{-5}	

a Data were obtained from page 50 of the study report.
* Cells treated with 10^{-4} M chlorpyrifos showed a higher than 20% cytotoxicity, so the RPC_{Max} was observed at 10^{-5} M.
NA = Not Applicable

FIGURE 1. Fold Induction of Relative Transcription Activation (RTA) of Chlorpyrifos Compared to the Positive Control.



Figures were obtained from page 30 of the study report.
VC= Vehicle Control
PC= Positive Control (1 nM E2)

3. Performance Criteria: The laboratory proficiency assays using the required reference compounds were not included in the study report and were not available to the reviewer. Acceptance criteria were generally met for 17β-estradiol, 17α-estradiol and corticosterone. For the second run, the Hill slope was less than the acceptable range for both 17β-estradiol and 17α-estradiol. In all three runs, the responsiveness of 17α-methyltestosterone was greater than the validated range. Although this is outside the acceptable limits, it indicates an increased sensitivity to weak agonists, and therefore, does not impact the validity of the study. Mean Luciferase activity was greater than 4-fold that of the mean vehicle control on each plate, and the fold-induction corresponding to the PC_{10} of the concurrent PC was greater than 1+2 standard deviations of the fold-induction value of the concurrent VC, as expected. Variability was minimal and the results were reproducible, indicating a reliable PC_{10}.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATORS' CONCLUSIONS: Based on the combined responses in each of three independent estrogen receptor transactivation assays, it was determined that chlorpyrifos treatment resulted in a weak ER-mediated transcriptional activation at the highest acceptable concentration tested (100 μM, 10^{-4} M). The results of the *in vitro* estrogen receptor transcriptional activation assay using the stably transfected human hERα-HeLa-9903 cell line indicate that, under the conditions of this study, chlorpyrifos slightly increased estrogen receptor-mediated transactivation, but only at *in vitro* concentrations significantly higher than *in vivo* blood levels that markedly inhibit brain and red blood cell cholinesterase activity in adult female rats.

B. AGENCY COMMENTS: Chlorpyrifos was tested up to the limit of solubility, 10^{-4} M. Acceptance criteria were met for all reference chemicals, and displayed slightly increased sensitivity of the system to very weak agonists like 17α-methyltestosterone. This does not negatively impact the validity of the study.

The mean RPC_{Max} for Chlorpyrifos was 25.4% in the first run, 10.2% in the second run and 19.5% in the third run, and the associated PC_{Max} was 10^{-4} M for the first and third run, and 10^{-5} M for the second run. Because the $RPC_{Max} > PC_{10}$ in all three assay runs, chlorpyrifos was considered positive for estrogen receptor transcriptional activation in this test system. A PC_{50} could not be calculated.

C. STUDY DEFICIENCIES: The following deficiency was noted:

- It was stated that laboratory validation assays with 10 proficiency chemicals were performed to confirm the responsiveness of the ER transcriptional activation assay. However, results from these non-GLP, unpublished proficiency tests were not provided with this study.

Data Requirement:

EPA DP Barcode	
OECD Data Point	
EPA MRID	48615506
EPA Guideline	890.1350, Fish Short-Term Reproduction Assay

Test material: **Chlorpyrifos** **Purity:** 99.8%

Common name

Chemical name: IUPAC: O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate.

CAS name: O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate.

CAS No.: 2921-88-2

Synonyms:

EPA PC Code: 059101

Primary Reviewer: Joan Gaidos **Signature:**

Senior Scientist, Cambridge Environmental, Inc. **Date:** 05/14/2012

Secondary Reviewer: Teri S. Myers **Signature:**

Senior Scientist, Cambridge Environmental, Inc **Date:** 06/01/2012

Primary Reviewer: Patience Browne **Signature:** Digitally signed by PATIENCE BROWNE DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=PATIENCE BROWNE, dnQualifier=0000048202 Date: 2015.06.03 16:20:08 -04'00'

USEPA/OCSPP/OSCP **Date:** 06/25/2012

Additional Reviewer: Amy Blankinship **Signature:** AMY BLANKINSHIP Digitally signed by AMY BLANKINSHIP DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=AMY BLANKINSHIP, dnQualifier=0000040917 Date: 2015.06.05 10:06:13 -04'00'

USEPA/OCSPP/OPP/EFED/ERB3 **Date:**

Final Additional Reviewer: Robin Sternberg **Signature:** Digitally signed by ROBIN STERNBERG DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=ROBIN STERNBERG, dnQualifier=0000039126 Date: 2015.06.03 15:43:23 -04'00'

USEPA/OCSPP/OPP/EFED/ERB1 **Date:** 05/28/2015

Date Evaluation Completed: 05/28/2015

CITATION: Currie, R.J., D.W. Louch, K.K. Coady, J.A. Fiting, T.A. Marino, A.W. Perala, L.K. Sosinski, J. Thomas. 2011. Chlorpyrifos: A Fish Short-term Reproduction Assay with the Fathead Minnow, *Pimephales promelas*. Unpublished study performed by Toxicology & Environmental Research Consulting, The Dow Chemical Company, Midland, Michigan. Lab Study No.: 101123. Study sponsored by Dow AgroSciences LLC, Indianapolis, Indiana. Study completed October 24, 2011.

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Disclaimer: The guideline recommendations in this DER template are offered as a general reference to aid in preparation of the DER. The purpose of these recommendations is not to serve as substitute for the Test Guidelines, nor to provide any guidance on how the study should be conducted.

EXECUTIVE SUMMARY:

The 21-day short-term reproduction assay of chlorpyrifos technical with fathead minnows (*Pimephales promelas)* was conducted under flow-through conditions. Adult fish (16 spawning groups; 2 males and 4 females in each group; 1 group per replicate tank and 4 replicates per treatment level; 6 months old) were exposed to chlorpyrifos (99.8% purity) at nominal concentrations of 0 (negative control), 0.0002, 0.00064, and 0.002 mg a.i./L; time-weighted average (TWA), measured concentrations were <0.0000312 (<LOQ), 0.000251, 0.000812, and 0.00302 mg a.i./L. The test system was maintained at 24.8 to 25.3°C and a pH of 7.3 to 7.8.

There was no significant effect ($p>0.05$) on fish mortality; overall mean survival values were 83.3, 91.6, 87.5 and 91.6% in the negative control, low, mid, and high treatment groups, respectively. Clinical signs of toxicity were observed in both the negative control and treatment groups with similar lesions (*e.g.*, injury to eyes, body) and incident rates. Male body weights were significantly reduced ($p<0.05$) by 17% in the high treatment group relative to the negative control.

Spawning in the negative control occurred at least every four days, and fecundity averaged 34.2 eggs/female/day; fertilization success in the negative control was 98.9%. There were significant reductions (Jonckheere-Terpstra; $p<0.05$) in fecundity of 52 to 71% at all treatment levels compared to the negative control. Fertility was not significantly different ($p>0.05$) for the treatment groups compared to the negative control.

There were no significant differences ($p>0.05$) in male tubercle scores or male or female gonado-somatic index (GSI) compared to the negative control. Tubercles were not observed in females. Plasma vitellogenin, testosterone, and estradiol for the treatment groups were not significantly different ($p>0.05$) when compared to the negative control.

Although not analyzed statistically, there was a marginally higher incidence of oocyte atresia (all severities) in treated females compared to negative control females; however, this may have been due to granulomatous inflammation and was interpreted by the study authors as a spontaneous alteration not associated with exposure to chlorpyrifos due to the lack of a treatment-related response and bacterial etiology. There were also incidences of egg debris in the oviduct of females in the negative control and treatment groups. Similarly, incidences of

granulomatous inflammation that were also attributed to an infectious agent were observed in the negative control and treatments groups. Overall, these histopathological findings were not attributed to chlorpyrifos exposure.

The study authors' analysis detected significant decreases of cholinesterase activity in brain tissue of females at all treatment levels (40 to 92%) and in brain tissue of males at the mid and high treatment levels (72% and 90%, respectively). While not significant, a 65% decrease in cholinesterase activity in males was observed at the low treatment level compared to the negative control.

All performance and validity criteria were met with the exception that average negative control survival was 83.3% for the combined sexes which is less than the guideline criterion of ≥90%. This deviation did not impact the interpretation of the study.

This assay **satisfies** the EDSP Tier 1 Test Order requirement for a Fish Short-Term Reproduction Assay (OCSPP Guideline 890.1350).

Results Synopsis

Test Organism age at test initiation: *ca*. 6 months
Mean body weight at test initiation (if measured): Not reported.
Mean length at test initiation (if measured): Not reported.

Test Type (Flow-through, Static, Static Renewal): Flow-through

Table 1: Summary of Reproductive and HPG Effects[1,2] in the Fish Short-Term Reproduction Assay (FSTRA) with Chlorpyrifos.[3]

Treatment (mg a.i./L) [TWA-measured]	Fecundity	Fert. Success	Tubercle Score		GSI		Gonadal Histo.		Plasma VTG		Plasma T		Plasma E2	
			M	F	M	F	M	F	M	F	M	F	M	F
0.000251	Yes	No	No	No	No	No	No	No	No	No	No	NA	No	NA
0.000812	Yes	No	No	No	No	No	No	No	No	No	No	NA	No	NA
0.00302	Yes	No	No	No	No	No	No	No	No	No	No	NA	No	NA

Abbreviations: Conc. Concentration. Diff. Difference. E2 17β-estradiol. F Female. Fert. Fertilization. GSI Gonado-Somatic Index. Histo. Histopathology. M Male. NA Not applicable. T Testosterone. VTG Vitellogenin.

1 A "yes" indicates a significant difference based on comparison to the negative (clean water) control, unless otherwise specified.

2 The criteria for significance are described in the Reviewer's Analysis and Statistical Verification sections of the DER. Conclusions regarding histopathology may be heavily weighted by the expert opinion of a board-certified pathologist.

3 Median male cholinesterase values showed a statistically significant monotonic decrease at 0.000812 and 0.00302 mg a.i./L compared to the negative control ($p<0.05$; Jonckheere-Terpstra test). Mean female cholinesterase values also showed a statistically significant monotonic decrease at all treatment levels compared to the negative control ($p<0.05$; Jonckheere-Terpstra test).

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: This study was conducted according to the U.S. EPA OCSPP 890.1350: "Fish Short-Term Reproductive Assay" and OECD 229 (2009). The following deviations were noted:

1. The survival validity criterion was not met because average male and female fish survival was less than 90% in the negative control.
2. The total organic carbon content of the dilution water, residual chlorine, and unionized ammonia levels were not reported.
3. Analytical verification of the test solutions at Days 0, 1, 4, 7, 11, 14, 18 and 21 yielded recoveries ranging from 90.2 to 191% of nominal concentrations. The %CV of some replicate chambers exceeded 20% (ranging from 20.1 to 25%) over the course of the 21 day study, but the daily averages for each level were maintained satisfactorily below 20% (*i.e.*, 16.3-19.9%).

These deficiencies/deviations do not have an impact on the interpretation of the study.

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 by the U.S. EPA (40 CFR Parts 160), and OECD Principles of GLP [ENV/MC/CHEM(98)17].

A. TEST MATERIAL: **Chlorpyrifos**

Description: Light tan, crystalline solid; stability under normal storage conditions not reported. Expiration date not reported. Water solubility 1.05 mg/L at 25°C. Log_{ow} of 4.82.

OECD recommends describing water solubility, melting/boiling point stability in water and light, pKa, Pow or Kow, vapor pressure of test compound, expiration date.

Lot No./Batch No. : KC28161419, TSN101285 (Lot No.)

Purity: 99.8%

Impurities: Not reported

Stability of Compound: Analytical verification of diluter stock fortified with 0.05, 0.16 and 0.5 mg a.i./L yielded recoveries ranging from 54.9 to 67.5% of nominal concentrations. Recoveries of toluene extracts from laboratory dilution water spiked with chlorpyrifos at 0.0002, 0.00205 and 0.50 mg a.i./L were 77.8 to 94.2%. Analytical verification on the test solutions on Days 0, 1, 5, 7, 11, 14, 18 and 21 of the definitive test yielded recoveries ranging from 90.2 to 191% of nominal concentrations. The coefficient of variations ranged from 12.9 to 25.07%, however, the mean CVs were <20% for all concentrations of chlorpyrifos.

Storage Conditions of Test Chemicals: Not reported

B. Test organism:

Table 2: General Information About the Test Species and Acclimation.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Species common name:	Fathead Minnow		*EPA recommends fathead minnow (Pimephales promelas).*
Species scientific name:	*Pimephales promelas*		
Species strain (if stated):	Not reported		
Were fish obtained from a single laboratory stock?	Yes	New England bioassay, Manchester, Connecticut.	*EPA recommends that fish be from a single laboratory stock.*
Were acclimation conditions same as definitive test?	Yes		*EPA recommends that fish be acclimated under water quality and illumination conditions that are similar to the definitive test.*
Acclimation period:	*Ca.* 3 weeks		*EPA recommends a minimum two-week acclimation period. Note that the acclimation period is different from the subsequent, in situ pre-exposure phase.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Details on health:		Mortalities <5% during 7 days prior to pre-exposure period. Fish did not receive any treatment for disease during acclimation period. Fish with behavioral abnormalities or clinical signs were not used in the study.	*EPA recommends that mortality during the 7 days prior to the pre-exposure phase be less than 5% of the culture population. If mortality during these 7 days is greater than 10%, EPA recommends that the fish be rejected. If mortality is between 5-10%, EPA recommends that fish be held another 7 days. If mortalities greater than 5% occur during this extended acclimation period, EPA recommends that the fish not be used.*
Type of food:	Frozen brine shrimp		*EPA recommends that fish be fed frozen brine shrimp twice per day to promote active reproduction and maintain body condition.*
Source of food:	Brine Shrimp Direct, Ogden Utah		
Frequency of feeding:	Frozen shrimp: 2 times/day		
Details on feeding:		2.5-3.0 mL frozen (thawed) brine shrimp/vessel.	

Table 3: Fish Selection and Pre-Exposure Performance.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Age at test initiation:	6 months		*EPA recommends reproductively mature (sexually dimorphic) fish, 4.5 - 6 months old.*
Mean weight of males at test initiation (if determined):	3.5 ± 0.4 g	Based on 60 fish used to stock aquaria for pre-exposure phase.	*EPA recommends that a subsample of fish be weighed before the test to estimate the mean weight for each sex. It is recommended that the individual weight of each fish selected for the test be within ±20% of the estimated mean for each sex.*
Range of individual weights (males) at test initiation (if determined):	± 20%	Individual weights within ± 20% of the estimated mean.	
Mean weight of females at test initiation (if determined):	1.7 ± 0.2 g	Based on 120 fish used to stock aquaria for pre-exposure phase.	
Range of individual weights (females) at test initiation (if determined):	± 20%	Individual weights within ± 20% of the estimated mean.	
Mean length of males at test initiation (if determined):	Not reported		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Mean length of females at test initiation (if determined):	Not reported		
Duration of pre-exposure phase:	15 days		*EPA recommends a minimum of 14 days.*
Were pre-exposure conditions identical to the definitive test?	Yes		*EPA recommends that pre-exposure conditions, including temperature, photoperiod, feeding, etc., be identical to definitive test conditions.*
Number of pre-exposure tanks:	30 tanks		*EPA recommends that additional tanks set up at the beginning of pre-exposure will ensure that sufficient replicates with the correct sex ratio are available for the definitive test.*
Number of males per tank:	2 males/tank		
Number of females per tank:	4 females/tank		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Pre-exposure fecundity:	≥ 15 eggs/female/ reproductive day/ replicate		*EPA recommends that pre-exposure fecundity in each replicate (tank) selected for use in the definitive test be at least 15 eggs/female/reproductive day/replicate during the 7 days prior to the definitive test.*
Number of spawns during pre-exposure:	≥ 2 times in 7 days	Spawning occurred at least every 3 days.	*EPA recommends that spawning occur at least twice in the 7 days prior to the definitive test.*
Details on pre-exposure:		None	

C. Exposure System

Table 4: Summary of Information on the Exposure System and Test Vessel Characteristics.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Type of exposure:	Flow-through		*EPA recommends the use of a flow-through system. As noted in the Corrections and Clarifications document[1], the use of a static renewal system is not recommended for this assay.*
Type of flow-through dilution system:	Continuous flow proportional diluter		*Intermittent flow proportional diluters or continuous flow serial diluters are recommended.[2]*
Flow-through rate:	Exposure: 90 ±9 mL/min	Initial flow: 45 ± 5 mL/min Equilibrium phase (prior to addition of fish): 90 ± 9 mL/min.	*Recommended flow-through rate is 45 mL/min (2.7 L/hr), or at least 6 total volume exchanges per day.*

[1] U.S. Environmental Protection Agency (EPA). (2011). Corrections and Clarifications on Technical Aspects of the Test Guidelines for the Endocrine Disruptor Screening Program Tier 1 Assays (OCSPP Test Guideline Series 890). March 3, 2011. Office of Chemical Safety and Pollution Prevention (OCSPP), Washington, D.C. (http://www.epa.gov/endo/pubs/assayvalidation/clarificationdoc.pdf).

[2] Additional guidance for aquatic test design is located in OCSPP Guideline 850.1000, Special Considerations for Conducting Aquatic Laboratory Studies.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Details on toxicant mixing for flow-through systems:		Solution from mixing chamber split equally among replicate test vessels. Treatment recoveries prior to test initiation were not reported. The flow splitting accuracy was not reported.	*Recommended toxicant mixing for flow-through systems: 1) Mixing chamber is recommended but not required; 2) Aeration is not recommended for mixing; 3) A demonstration that the test solution is completely mixed before introduced into the test system is recommended; 4) The recommended flow splitting accuracy is within 10%.*
Aeration?	No		*EPA recommends aeration if dissolved oxygen reaches $\leq$4.9 mg/L ($\leq$ 60% saturation).*
Source of dilution water:	Lake Huron water	Dilution water was limed and flocculated and pumped to the laboratory prior to municipal treatment. Prior to use, the water was sand-filtered, pH-adjusted (gaseous CO_2), carbon-filtered and UV-irradiated.	*EPA recommends natural or reconstituted water; it is recommended that natural water 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 indicated in the test guideline.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Was dilution water analyzed for pesticides, heavy metals, and other contaminants?	Yes		
Test vessel type/materials:	Glass aquaria with stainless steel screen drains		*EPA and OECD recommend that water-contact portions of the system not compromise the study (e.g., all glass vessels or glass vessels with stainless steel frames are acceptable examples).*
Test vessel size:	20 cm wide x 39 cm long x 25 cm high; solution depth 13 cm.		*EPA recommends the use of 18 L test chambers (e.g., 40 x 20 x 20 cm).*
Fill volume:	10 L		*EPA recommends 10 L solution per tank.*
Spawning substrate material:	PVC pipe cut and sectioned lengthwise and inverted to form a semicircular arch and positioned on stainless steel trays.		*EPA recommends that each tank contain three semi-circular spawning substrates, e.g., aged PVC pipe, 10 - 20 cm in length, split lengthwise.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Spawning substrate size:	9 cm long and 10 cm diameter positioned on 13 cm long stainless steel tray.		
Additional details on exposure system:		None.	

Table 5: Summary of Water Quality Characteristics in the Test System.

Parameter	Minimum	Maximum	Mean	Measurement Interval	Guideline Recommendations
Temperature (°C)	24.8	25.3	25 ± 1°C	Continuous	*EPA recommends temperature 25±1°C; inter-replicate and inter-treatment differentials should not exceed 1°C.*
pH	7.3	7.8	7.6[1]	Weekly	*EPA recommends pH 6.5 to 9.0.*
Dissolved oxygen (mg/L)	7.0	9.1	8.1[1]	Weekly	*EPA recommends dissolved oxygen (DO) ≥4.9 mg/L (>60% air saturation)*
Total alkalinity (mg/L as $CaCO_3$)	34	42	38[1]	Weekly	*EPA recommends total alkalinity > 20 mg/L as $CaCO_3$.*

Parameter	Minimum	Maximum	Mean	Measurement Interval	Guideline Recommendations
Hardness (mg/L as $CaCO_3$)	64	74	69[1]	Weekly	
Total organic carbon (mg/L)	Not reported	Not reported	Not reported	Not reported	*EPA recommends that total organic carbon in dilution water be ≤ 2 mg/L.*
Unionized ammonia (µg/L)	Not reported	Not reported	<100 (as N)	Once	*EPA recommends that unionized ammonia in the dilution water be ≤ 1 µg/L.*
Residual chlorine (µg/L)	Not reported	Not reported	<20	Once	*EPA recommends that residual chlorine in dilution water be < 10 µg/L.*
Other	None	None	None	None	*General recommendations for frequency of measurements: EPA recommends that temperature, pH, and dissolved oxygen be measured in all test tanks at least weekly and that hardness and alkalinity be measured in controls and in one tank at the highest test concentration at least weekly. In addition, continuous temperature monitoring of at least one tank is encouraged.*

Abbreviations: NA Not applicable.

1 Means were calculated by the reviewer as the average of the minima and maxima for the ranges provided across control and treated levels

D. Study Design and Additional Experimental Conditions

Table 6: Range-Finding Study Conditions (if Applicable).

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Was a range-finder conducted?	Yes		*EPA recommends conducting a range-finder if 96-hour LC_{50} data for the fathead minnow are unavailable.*
If yes, what was the method for determining the highest test concentration in the range-finder?	0.05 mg/L	The highest test concentration based on toxicity data from other fish studies. Nominal range-finding test concentrations were 0 (negative control), 0 (solvent control), 0.0004, 0.002, 0.010, and 0.050 mg a.i./L.	*EPA recommends that the highest test concentration be selected based on toxicity data for other fish studies or species, if available. Otherwise, either the solubility limit of the test compound or 100 mg/L (whichever is lower) is appropriate.*
Species:	*Pimephales promelas*	Fathead minnows	
Life stage:	6 months		*EPA recommends that range-finding tests be performed with fish of similar age and size to those that would be utilized in the test.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Test duration:	14 days		*EPA recommends a 96-hour exposure.*
Additional details:		Stock solutions for the range-finding study were made in acetone (≤0.05 mL/L) in lab dilution water. Because this treatment level was well below the solubility limit of chlorpyrifos (1.05 mg a.i./L), a solvent was not necessary in the definitive test.	*EPA recommends conducting a range-finder with five test concentrations plus a control (six total treatment levels), with four females and two males per exposure tank (36 fish total). The number of mortalities that occur may be used to develop a concentration-response curve.* *Based upon the results, the highest concentration that does not result in increased mortality or signs of overt morbidity compared to controls, or 1/3 the derived 96-hr $LC_{50,}$ may be selected as the highest exposure concentration in the 21-day test.*

Table 7: Definitive Study Conditions.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Test duration:	21 days		*EPA recommends that the duration of the definitive test be 21 days.*
Method for selecting the highest test concentration in the definitive test:	Highest definitive test concentration of 0.002 mg a.i./L.	The highest nominal test concentration was based on the estimated maximum tolerated concentration (MTC), which was estimated to be 0.002 mg a.i./L based on the lack of treatment effects in the range-finding study.	*EPA recommends that the highest test concentration is either the solubility limit of the test compound, 100 mg/L, or demonstrates adequate evidence of toxicity (e.g., 1/3 the 96-hour LC_{50}), whichever concentration is lowest.*
Reference study citation (if applicable):		Geiger et al, 1988; Holcombe et al, 1982; Phipps and Holcombe, 1985; Jarvinen and Tanner, 1982; Mehler et al, 2008; Jarvinen et al, 1988; Jarvinen et al, 1983; Mayes et al, 1993.	
Separation of test concentrations:	0 (negative control), 0.0002, 0.00064, and 0.002 mg a.i./L		*EPA suggests that a concentration separation of between 0.33 (or three-fold) and 0.1 (or ten-fold) is scientifically acceptable*[1].

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Number of test concentrations:	4		*EPA recommends a minimum of 3 concentrations and a control, plus solvent control if appropriate.*
Are nominal concentrations adjusted for purity?	Yes		
Indicate the type of values presented for measured concentrations:	Time-weighted mean		
Limit of quantification (LOQ):	0.0000312 mg a.i./L		*EPA recommends that for chemical test concentrations below the LOQ, analyses be conducted on the stock solutions.*
Level of detection (LOD):	Not reported		
Frequency of measurement:	0, 1, 5, 7, 11, 14, 18 and 21 days		*It is recommended that test item concentration be measured prior to the addition of fish in all tanks and at least weekly thereafter in two replicates per treatment level.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Was the randomized complete block design used?	Yes		*EPA recommends that all fish be randomly assigned to tanks during pre-exposure. Tanks are then ranked according to pre-exposure fecundity, and the tanks with the highest fecundity are randomly assigned to a definitive test treatment and block first. Each block contains one replicate of each treatment, including controls.*
Number of replicates in control:	4		*EPA recommends 4 replicates.*
Number of replicates in solvent control (if applicable):	NA		*EPA recommends the use of a concurrent solvent control when a solubilizing agent is used. EPA recommends 4 replicates.*
Number of replicates per test item treatment level:	4		*EPA recommends 4 replicates.*
Number of male fish per replicate at test initiation:	2		*EPA recommends 2 males per replicate.*
Number of female fish per replicate at test initiation:	4		*EPA recommends 4 females per replicate.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Was a solvent used?	No		
Solvent type (if applicable):	NA		
Maximum solvent concentration (if applicable):	NA		*EPA recommends that the solvent not exceed 0.02 ml/L*[3]*. OECD recommends that solvent have no effect on survival nor produce any other adverse effects and that concentration not be greater than 0.1 ml/L*[4]*.*
Was a positive control used?	No		
Positive control (if applicable):	NA		
Positive control concentration(s) (if applicable):	NA		
Photoperiod:	16 hrs light: 8 hrs dark		*EPA recommends photoperiod 16:8 (light:dark).*

[3] Hutchinson TH, Shillabeer N, Winter MJ, Pickford DB (2006). Acute and chronic effects of carrier solvents in aquatic organisms: A critical review. Review. Aquatic Toxicology, 76, pp.69–92.

[4] OECD (2000). Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. Environmental Health and Safety Publications. Series on Testing and Assessment. No. 23. Paris, France.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Light intensity at water's surface:	764 to 894 lux		*EPA recommends light intensity 540 – 1080 lux (at water's surface).*
Additional details:		Specific details about test solution appearance did not appear to be reported in study report.	

Table 8: Summary of Treatment Concentrations in the Fish Short-Term Reproduction Assay with Chlorpyrifos.

Treatment ID	Nominal Concentration (mg a.i./L)	Time-Weighted Average, Measured Concentration (mg a.i./L)	Mean CV (%)	Details or Remarks	Guideline Recommendations
Negative control	0	<LOQ	NA		*EPA recommends that test item concentrations be maintained at a coefficient of variation (CV) ≤20%.*
Treatment 1	0.0002	0.000251	19.9		
Treatment 2	0.00064	0.000812	19.6		
Treatment 3	0.002	0.00302	16.3		

Abbreviations: [CV] Coefficient of variation. [NA] Not applicable

LOQ=0.0000312 mg a.i./L.

E. Observations

Biological Endpoints: Survival, fecundity, fertilization success, and clinical signs were observed daily. At test termination (Day 21), secondary sex characterization (body color, pattern, body shape), body weight, length, tubercle score, gonadal staging and histopathology, plasma vitellogenin, cholinesterase from brain tissue, and male and female plasma sex steroids (testosterone and 17β-estradiol) were evaluated.

Were raw (individual) data provided? No

EPA recommends that observations of survival, fecundity, fertilization success, secondary sex characteristics, and other clinical signs occur at least daily. At test termination (Day 21), additional observations include body weight and length, nuptial tubercle score, gonadal staging and histopathology, plasma vitellogenin, and plasma sex steroids (testosterone and 17β-estradiol, if measured). Gonado-somatic index (GSI) is calculated using a ratio of gonad weight to body weight (gonad weight to the nearest 0.1 mg / body weight in mg x 100) at test termination.

Clinical signs of overt toxicity may include (but are not limited to) hemorrhage, cessation of feeding, and other abnormal behavior.

II. RESULTS AND DISCUSSION

A. Results

Overall mean survival values were 83.3, 91.6, 87.5 and 91.6% in the TWA-measured 0 (negative control), 0.000251, 0.000812, and 0.00302 mg a.i./L treatment levels, respectively.

Table 9: Adult Fish Survival in Fathead Minnow (*Pimephales promelas*).

Treatment (mg a.i./L) [TWA-measured]	Males			Females		
	n	# Surviving	% Survival	n	# Surviving	% Survival
Negative control (<LOQ)	8	7	87.5	16	13	81.2
0.000251	8	7	87.5	16	15	93.7
0.000812	8	6	75.0	16	15	93.7
0.00302	8	7	87.5	16	15	93.7

Abbreviations: [NA] Not applicable.

LOQ=0.0000312 mg a.i./L.

Mean male body weight values were 4.14, 3.93, 3.54 and 3.42 g, and female body weight values were 1.82, 1.91, 1.76 and 1.81 g in the TWA-measured 0 (negative control), 0.000251, 0.000812, and 0.00302 mg a.i./L treatment levels, respectively. Mean male body length values were 54.9, 55.1, 53.4 and 52.6 mm, and female body length values were 43.5, 43.5, 43.0 and 43.7 mm in the TWA-measured 0 (negative control), 0.000251, 0.000812, and 0.00302 mg a.i./L treatment levels, respectively.

Table 10: Size at Test Termination in Fathead Minnow (*Pimephales promelas*).

Treatment (mg a.i./L) [TWA-measured]	Body Weight						Length					
	Males			Females			Males			Females		
	n	Mean (g)	±SD	n	Mean (g)	±SD	n	Mean (mm)	±SD	n	Mean (mm)	±SD
Negative control (<LOQ)	4	4.14	0.563	4	1.82	0.162	4	54.9	2.81	4	43.5	1.31
0.000251	4	3.93	0.568	4	1.91	0.145	4	55.1	2.85	4	43.5	0.38
0.000812	4	3.54	0.740	4	1.76	0.034	4	53.4	3.38	4	43.0	0.30
0.00302	4	3.42	0.406	4	1.81	0.176	4	52.6	2.04	4	43.7	0.96

Abbreviations: [NA] Not applicable. [ND] Not determined. [SD] Standard deviation.

LOQ=0.0000312 mg a.i./L.

Mean fecundity values were 34.3, 16.5, 12.6 and 9.8, and fertilization success was 98.9, 97.6, 98.7 and 97.6% in the TWA-measured 0 (negative control), 0.000251, 0.000812, and 0.00302 mg a.i./L treatment levels, respectively.

Table 11: Fecundity and Fertilization Success in Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [TWA-measured]	Fecundity[1]		Fertilization Success (%)[2]	
	Mean	± SD	Mean	± SD
Negative control (<LOQ)	34.2	3.05	98.9	0.54
0.000251	16.5	4.52	97.6	1.53
0.000812	12.6	3.16	98.7	0.99
0.00302	9.8	5.29	97.6	1.69

Abbreviations: [NA] Not applicable. [ND] Not determined.

LOQ=0.0000312 mg a.i./L.

[1] Fecundity is calculated as the number of eggs per surviving female per reproductive day per replicate.

[2] Fertilization success (%) is calculated as the number of embryos divided by the number of eggs, multiplied by 100.

Median male tubercle scores were 21, 24, 19, and 20 in the TWA-measured 0 (negative control), 0.000251, 0.000812, and 0.00302 mg a.i./L treatment levels, respectively. None of the surviving females were found to have tubercles.

Table 12: Nuptial Tubercle Score in Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [TWA-measured]	Males		Females	
	n	Median Tubercle Score	n	Median Tubercle Score
Negative control (<LOQ)	4	21	4	0
0.000251	4	24	4	0
0.000812	4	19	4	0
0.00302	4	20	4	0

Abbreviations: [NA] Not applicable. [ND] Not determined. [SD] Standard deviation.

LOQ=0.0000312 mg a.i./L.

Mean male GSI was 1.16, 1.22, 1.32 and 1.06, and mean female GSI was 15.25, 15.20, 15.00 and 15.78 in the mean-measured 0 (negative control), 0.000251, 0.000812, and 0.00302 mg a.i./L treatment levels, respectively.

Table 13: Gonado-Somatic Index (GSI) in Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [TWA-measured]	Males			Females		
	n	Mean GSI[1] (%)	±SD	n	Mean GSI[1] (%)	±SD
Negative control (<LOQ)	4	1.16	0.115	4	15.25	2.255
0.000251	4	1.22	0.221	4	15.20	3.570
0.000812	4	1.32	0.275	4	15.00	1.439
0.00302	4	1.06	0.194	4	15.78	3.225

Abbreviations: NA Not applicable.

LOQ=0.0000312 mg a.i./L.

[1] Gonado-somatic index (%) is calculated as gonad weight (to the nearest 0.1 mg) / body weight (mg) x 100.

Median male gonadal stage was 2, 2, 2, 2, and 2, and median female gonadal stage was 3, 3, 3, 3, and 3 in the TWA-measured 0 (negative control), 0.000251, 0.000812, and 0.00302 mg a.i./L treatment levels, respectively.

Table 14: Gonadal Staging in Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [TWA-measured]	Males		Females	
	n	Median Stage[1]	n	Median Stage[2]
Negative control (<LOQ)	7	2	13	3
0.000251	7	2	15	3
0.000812	6	2	15	3
0.00302	7	2	15	3

Abbreviations: [J] Juvenile. [NA] Not applicable. [ND] Not determined. [UTS] Unable to stage.

LOQ=0.0000312 mg a.i./L.

[1] The guideline recommends the following gonadal staging scale for male fathead minnow: 0=undeveloped, 1=early spermatogenic, 2=mid-spermatogenic, 3=late spermatogenic, 4=spent.

[2] The guideline recommends the following gonadal staging scale for female fathead minnow: 0=undeveloped, 1=early development, 2=mid-development, 3=late development, 4=late development/hydrated, 5=post-ovulatory.

In the study report, specific details (incidence and severity) were reported only for the findings where there were reported changes. However, in the report, according to the study authors, other potential histologic changes such as proportion of spermatogonia, presence of testis-ova, testicular degenerative changes, Leydig cell hyperplasia/hypertrophy, presence of vascular and/or interstitial proteinaceous fluid, asynchronous gonad development, and altered proportions of spermatocytes or spermatids were evaluated in males. In females, the extent of oocyte atresia, perifollicular cell hyperplasia/hypertrophy, yolk formation, interstitial fibrosis, inflammatory changes and post-ovulatory follicles were also evaluated. There was one finding of duct mineralization and 14 findings of multifocal granulomatous inflammation (minimal to moderate) across all treatment levels.

In females, there were 4 minimal findings of aggregation of macrophages, and 38 findings of minimal to severe multifocal granulomatous inflammation across all treatment levels, however no apparent concentration-response was noted. Eight to 13 findings of increased oocyte atresia were present, 8 in the negative control and 11 at 0.000251 mg a.i./L and 13 in each of the 0.000812 and 0.00302 mg a.i./L treatment groups. There was one incidence (minimal) of egg debris in the oviduct in the negative control, 2 incidences (mild) at 0.000251 and 0.000812 mg a.i./L treatments, and 4 incidents (mild to severe) at 0.00302 mg a.i./L.

Although not analyzed statistically, there was a marginally higher incidence of oocyte atresia (all severities) in treated females compared to the control fish; however, this may have been due to granulomatous inflammation and was interpreted by the study authors as a spontaneous alteration not associated with exposure to chlorpyrifos due to the lack of a treatment-related response and bacterial etiology. There was also incidence of egg debris in the oviduct in females in the negative control and treatment groups. Similarly, incidences of granulomatous inflammation, which were also attributed to an infectious agent, were observed in the negative control and treatment groups. Overall, the observed findings were not attributed to chlorpyrifos exposure.

Table 15a: Gonadal Histopathology in Male Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [TWA-measured]	Diagnostic Observations[1]											
	Severity	Increased Proportion of Spermatogonia		Presence of Testis-Ova		Increased Testicular Degeneration		Interstitial Cell Fibrosis		Germinal Epithelium, Atrophy	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative control (<LOQ)	0	7	NA	7	NA	7	NA	7	NA	7	NA
	1	7	NA	7	NA	7	NA	7	NA	7	NA
	2	7	NA	7	NA	7	NA	7	NA	7	NA
	3	7	NA	7	NA	7	NA	7	NA	7	NA
	4	7	NA	7	NA	7	NA	7	NA	7	NA
0.000251	0	7	NA	7	NA	7	NA	7	NA	7	NA
	1	7	NA	7	NA	7	NA	7	NA	7	NA
	2	7	NA	7	NA	7	NA	7	NA	7	NA
	3	7	NA	7	NA	7	NA	7	NA	7	NA
	4	7	NA	7	NA	7	NA	7	NA	7	NA
0.000812	0	6	NA	6	NA	6	NA	6	NA	6	NA
	1	6	NA	6	NA	6	NA	6	NA	6	NA
	2	6	NA	6	NA	6	NA	6	NA	6	NA
	3	6	NA	6	NA	6	NA	6	NA	6	NA
	4	6	NA	6	NA	6	NA	6	NA	6	NA
0.00302	0	7	NA	7	NA	7	NA	7	NA	7	NA
	1	7	NA	7	NA	7	NA	7	NA	7	NA
	2	7	NA	7	NA	7	NA	7	NA	7	NA
	3	7	NA	7	NA	7	NA	7	NA	7	NA
	4	7	NA	7	NA	7	NA	7	NA	7	NA

Abbreviation: NA Not applicable.

LOQ=0.0000312 mg a.i./L.

[1] Gonadal histopathology diagnostic observations are graded 0 – 4 based on severity: 0=Not remarkable, 1=Minimal, 2=Mild, 3=Moderate, 4=Severe. See Appendix E of the test guideline for reference.

Table 15b: Gonadal Histopathology in Male Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [TWA-measured]	Diagnostic Observations[1]				
	Severity	Duct Mineralizaton		Aggregates of Histiocytic Duct Cells	
		n	Incidence	n	Incidence
Negative control (<LOQ)	0	7	7	7	NA
	1	7	0	7	NA
	2	7	0	7	NA
	3	7	0	7	NA
	4	7	0	7	NA
0.000251	0	7	6	7	NA
	1	7	0	7	NA
	2	7	1	7	NA
	3	7	0	7	NA
	4	7	0	7	NA
0.000812	0	6	6	6	NA
	1	6	0	6	NA
	2	6	0	6	NA
	3	6	0	6	NA
	4	6	0	6	NA
0.00302	0	7	7	7	NA
	1	7	0	7	NA
	2	7	0	7	NA
	3	7	0	7	NA
	4	7	0	7	NA

Abbreviation: [NA] Not applicable.

LOQ=0.0000312 mg a.i./L.

[1] Gonadal histopathology diagnostic observations are graded 0 – 4 based on severity: 0=Not remarkable, 1=Minimal, 2=Mild, 3=Moderate, 4=Severe. See Appendix E of the test guideline for reference.

Table 16: Additional Gonadal Histopathology Observations in Male Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [TWA-measured]	Additional Diagnostic Observations[1]										
	Severity	Decreased Proportion of Spermatogonia		Increased Vascular or Interstitial Proteinaceous Fluid		Asynchronous Gonad Development		Altered Proportions of Spermatocytes or Spermatids		Granulomatous Inflammation	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative control (<LOQ)	0	7	NA	7	NA	7	NA	7	NA	7	5
	1	7	NA	7	NA	7	NA	7	NA	7	0
	2	7	NA	7	NA	7	NA	7	NA	7	2
	3	7	NA	7	NA	7	NA	7	NA	7	0
	4	7	NA	7	NA	7	NA	7	NA	7	0
0.000251	0	7	NA	7	NA	7	NA	7	NA	7	2
	1	7	NA	7	NA	7	NA	7	NA	7	1
	2	7	NA	7	NA	7	NA	7	NA	7	3
	3	7	NA	7	NA	7	NA	7	NA	7	1
	4	7	NA	7	NA	7	NA	7	NA	7	0

Treatment (mg a.i./L) [TWA-measured]	Severity	Additional Diagnostic Observations[1]									
		Decreased Proportion of Spermatogonia		Increased Vascular or Interstitial Proteinaceous Fluid		Asynchronous Gonad Development		Altered Proportions of Spermatocytes or Spermatids		Granulomatous Inflammation	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
0.000812	0	6	NA	6	NA	6	NA	6	NA	6	2
	1	6	NA	6	NA	6	NA	6	NA	6	3
	2	6	NA	6	NA	6	NA	6	NA	6	0
	3	6	NA	6	NA	6	NA	6	NA	6	1
	4	6	NA	6	NA	6	NA	6	NA	6	0
0.00302	0	7	NA	7	NA	7	NA	7	NA	7	4
	1	7	NA	7	NA	7	NA	7	NA	7	1
	2	7	NA	7	NA	7	NA	7	NA	7	2
	3	7	NA	7	NA	7	NA	7	NA	7	0
	4	7	NA	7	NA	7	NA	7	NA	7	0

Abbreviation: NA Not applicable.

LOQ=0.0000312 mg a.i./L.

[1] Gonadal histopathology diagnostic observations are graded 0 – 4 based on severity: 0=Not remarkable, 1=Minimal, 2=Mild, 3=Moderate, 4=Severe. See Appendix E of the test guideline for reference.

Table 17a: Gonadal Histopathology in Female Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [TWA-measured]	Additional Diagnostic Observations[1]								
	Severity	Increased Oocyte Atresia		Perifollicular Cell Hyperplasia/ Hypertrophy		Decreased Yolk Formation		Infection, Microsporidia	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative control (<LOQ)	0	13	5	13	NA	13	NA	13	NA
	1	13	6	13	NA	13	NA	13	NA
	2	13	0	13	NA	13	NA	13	NA
	3	13	1	13	NA	13	NA	13	NA
	4	13	1	13	NA	13	NA	13	NA
0.000251	0	15	4	15	NA	15	NA	15	NA
	1	15	4	15	NA	15	NA	15	NA
	2	15	1	15	NA	15	NA	15	NA
	3	15	2	15	NA	15	NA	15	NA
	4	15	4	15	NA	15	NA	15	NA
0.000812	0	15	2	15	NA	15	NA	15	NA
	1	15	8	15	NA	15	NA	15	NA
	2	15	1	15	NA	15	NA	15	NA
	3	15	1	15	NA	15	NA	15	NA
	4	15	3	15	NA	15	NA	15	NA
0.00302	0	15	2	15	NA	15	NA	15	NA
	1	15	5	15	NA	15	NA	15	NA
	2	15	3	15	NA	15	NA	15	NA
	3	15	2	15	NA	15	NA	15	NA
	4	15	3	15	NA	15	NA	15	NA

Abbreviation: [NA] Not applicable.

LOQ=0.0000312 mg a.i./L.

[1] Gonadal histopathology diagnostic observations are graded 0 – 4 based on severity: 0=Not remarkable, 1=Minimal, 2=Mild, 3=Moderate, 4=Severe. See Appendix E of the test guideline for reference.

Table 17b: Gonadal Histopathology in Female Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [TWA-measured]	Diagnostic Observations[1]				
	Severity	Subacute Multifocal Inflammation		Aggregates of Macrophages	
		n	Incidence	n	Incidence
Negative control (<LOQ)	0	13	NA	13	12
	1	13	NA	13	1
	2	13	NA	13	0
	3	13	NA	13	0
	4	13	NA	13	0
0.000251	0	15	NA	15	15
	1	15	NA	15	0
	2	15	NA	15	0
	3	15	NA	15	0
	4	15	NA	15	0
0.000812	0	15	NA	15	15
	1	15	NA	15	0
	2	15	NA	15	0
	3	15	NA	15	0
	4	15	NA	15	0
0.00302	0	15	NA	15	12
	1	15	NA	15	3
	2	15	NA	15	0
	3	15	NA	15	0
	4	15	NA	15	0

Abbreviation: [NA] Not applicable.

LOQ=0.0000312 mg a.i./L.

[1] Gonadal histopathology diagnostic observations are graded 0 – 4 based on severity: 0=Not remarkable, 1=Minimal, 2=Mild, 3=Moderate, 4=Severe. See Appendix E of the test guideline for reference.

Table 18: Additional Gonadal Histopathology Observations in Female Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [TWA-measured]	Additional Diagnostic Observations[1]								
	Severity	Interstitial Fibrosis		Egg Debris in Oviduct		Granulomatous Inflammation		Decreased Post-Ovulatory Follicles	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative control (<LOQ)	0	13	NA	13	12	13	6	13	NA
	1	13	NA	13	1	13	4	13	NA
	2	13	NA	13	0	13	2	13	NA
	3	13	NA	13	0	13	1	13	NA
	4	13	NA	13	0	13	0	13	NA
0.000251	0	15	NA	15	13	15	4	15	NA
	1	15	NA	15	0	15	3	15	NA
	2	15	NA	15	2	15	3	15	NA
	3	15	NA	15	0	15	4	15	NA
	4	15	NA	15	0	15	1	15	NA
0.000812	0	15	NA	15	0	15	2	15	NA
	1	15	NA	15	13	15	4	15	NA
	2	15	NA	15	2	15	6	15	NA
	3	15	NA	15	0	15	3	15	NA
	4	15	NA	15	0	15	0	15	NA
0.00302	0	15	NA	15	0	15	8	15	NA
	1	15	NA	15	11	15	2	15	NA
	2	15	NA	15	1	15	3	15	NA
	3	15	NA	15	1	15	2	15	NA
	4	15	NA	15	2	15	0	15	NA

Abbreviation: [NA] Not applicable.

LOQ=0.0000312 mg a.i./L.

[1] Gonadal histopathology diagnostic observations are graded 0 – 4 based on severity: 0=Not remarkable, 1=Minimal, 2=Mild, 3=Moderate, 4=Severe. See Appendix E of the test guideline for reference.

Mean male VTG was 650, 964, 2850 and 287 ng/mL, and female VTG was $67.2x10^6$, $48.8x10^6$, $59.9x10^6$ and $38.8x10^6$ ng/mL in the mean-measured 0 (negative control), 0.000251, 0.000812, and 0.00302 mg a.i./L treatment levels, respectively.

Table 19a: Plasma Vitellogenin in Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [TWA-measured]	Plasma Vitellogenin (VTG)					
	Males			Females		
	n	Mean (ng/mL plasma)	±SD	n	Mean (ng/mL plasma)	±SD
Negative control (<LOQ)	4	650	846	4	67.2×10^6	47.3×10^6
0.000251	4	964	809	4	48.8×10^6	23.4×10^6
0.000812	4	2850	5040	4	59.9×10^6	19.0×10^6
0.00302	4	287	255	4	38.8×10^6	10.3×10^6

Abbreviations: [NA] Not applicable. [ND] Not determined. [SD] Standard deviation.

LOQ=0.0000312 mg a.i./L.

Mean male brain cholinesterase was 5702.4, 3711.9, 1614.8 and 543.25 U/L, and female cholinesterase was 3893.6, 2332.5, 1038.4 and 307.69 U/L in the mean-measured 0 (control), 0.000251, 0.000812, and 0.00302 mg a.i./L treatment levels, respectively.

Table 19b: Cholinesterase in Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [TWA-measured]	Cholinesterase (brain) (CHOL)					
	Males			Females		
	n	Mean (Median) (U/L)	±SD	n	Mean (Median) (U/L)	±SD
Negative control (<LOQ)	4	5702.4 (5662.3)	1518.27	4	3893.6 (4613.0)	936.75
0.000251	4	3711.9 (3417.3)	1099.35	4	2332.5 (2186.5)	302.99
0.000812	4	1614.8 (832.50)	1848.33	4	1038.4 (945.75)	110.50
0.00302	4	543.25 (549.50)	200.30	4	307.69 (275.50)	83.95

Abbreviations: NA Not applicable. ND Not determined. SD Standard deviation.

LOQ=0.0000312 mg a.i./L.

U/L = International unit

Mean male plasma testosterone values were 4.13, 4.02, 2.34 and 4.22 ng/L, and mean male plasma 17β-estradiol was 0.125, 0.125, 3.54 and 0.184 ng/mL in the mean-measured 0 (negative control), 0.000251, 0.000812, and 0.00302 mg a.i./L treatment levels, respectively. Mean female plasma testosterone values were 2.97, 3.59, 3.46 and 5.82 ng/L, and mean female plasma 17β-estradiol was 9.89, 8.83, 6.72 and 8.67 ng/mL in the mean-measured 0 (negative control), 0.000251, 0.000812, and 0.00302 mg a.i./L treatment levels, respectively. The relatively larger mean for male 17β-estradiol in the 0.000812 mg a.i./L treatment group was due to one fish in which the measured concentration was 13.6 ng/mL; all other male fish in this treatment available for E2 measurement had values below the LOQ, except for one fish which had a reported value of 0.513 ng/mL. In addition, the plasma testosterone levels for females in one replicate (comprised of two values) of the high concentration was substantially higher, contributing to a 95% increase in testosterone for this treatment compared to the negative control.

Table 20: Plasma Sex Steroids in Fathead Minnow (*Pimephales promelas*).

Treatment (mg a.i./L) [TWA-measured]	Plasma Testosterone (T)						Plasma 17β-estradiol (E2)					
	Males			Females			Males			Females		
	n	Mean (ng/mL plasma)	±SD	n	Mean (ng/mL plasma)	±SD	n	Mean (ng/mL plasma)	±SD	n	Mean (ng/mL plasma)	±SD
Negative control (<LOQ)	4	4.13	0.663	4	2.97	1.867	4	0.125	0	4	9.89	3.864
0.000251	4	4.02	1.400	4	3.59	2.085	4	0.125	0	4	8.83	4.024
0.000812	4	2.34	1.406	4	3.46	2.005	4	3.54	6.706	4	6.72	2.798
0.00302	4	4.22	2.103	4	5.82	5.790	4	0.184	0.072	4	8.67	6.127

Abbreviations: [NA] Not applicable. [ND] Not determined. [SD] Standard deviation.

LOQ=0.0000312 mg a.i./L.

Throughout the study, observations of clinical signs of toxicity were reported in the negative control and treatment groups including injuries to eyes, scraped sides and hemorrhaging, and discoloration/loss of dark coloration. Taking into account the mortality observed, the number of incidences and the types of clinical signs were comparable across treatments.

Table 21: Secondary Sex Characteristics and Clinical Signs in Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [TWA-measured]	Secondary Sex Characteristics and Clinical Signs					
	Males			Females		
	Type	n	Incidence	Type	n	Incidence
Negative control (<LOQ)	Injured caudal fin, died	8	1	1 Injured right eye; 1 injury on under belly; 1 skin discoloration (yellow), died; 1 lethargic, died	16	4
0.000251	None reported	8	0	1 vertical banding/dark coloration;1 bulging eye; 1 dark coloration; 1 injured eye, scales missing	16	4
0.000812	1 loss of equilibrium, died; 1 bent tail, thin, pale skin	8	2	1 bulging and missing eye, died; 1 bleeding mouth, side scrapes	16	2
0.00302	1 injured eye, scales missing, died; 2 no dark coloration; 1 injured/bloody snout, fatpad absent	8	4	1 bloated, died; 1 bloated; 1 side scrapes	16	3

LOQ=0.0000312 mg a.i./L.

B. Study Author's Analysis and Conclusions

The study authors analyzed survival, weight, length, tubercle score, GSI, fertility, fecundity, VTG, cholinesterase, testosterone and estradiol. Data were gender specific and analyzed in comparison to the negative control.

Descriptive statistics (mean, standard deviation, etc.) were determined for each endpoint. All analysis was based on $p<0.05$ unless otherwise noted (statistical program used was not reported). The NOAEC for survival was determined using Cochran-Armitage Trend test (with Yate's Continuity Correction). The NOAEC for length, weight, fertility, GSI, VTG, male and female testosterone, and female estradiol was determined by ANOVA and one-tailed Dunnett's test. The NOAEC for male estradiol was determined using a Mann-Whitney-Wilcoxon U test (with Bonferroni-Holm adjustment). The NOAEC for tubercle score, fecundity, and male and female cholinesterase were determined by ANOVA and Jonckheere-Terpstra test. Prior to Dunnett's, data were analyzed by Shapiro-Wilk's test and Levene's to test for normality and homogeneity of variance, respectively, over treatments. If normality or homogeneity were indicated ($P>0.01$), a parametric analysis was performed. If non-normality or unequal variance were indicated ($p<0.01$), a non-parametric analysis was performed on the ranks of the data. These methods appear to be consistent with the methods recommended in the guideline.

Adult survival (range 87.5-91.6%) was not significantly different compared to the controls (83.3%); however control survival did not meet OCSPP 890.1350 guideline requirements of ≥90%. There was a statistically significant decrease in mean fecundity compared to the controls at all treatment levels ($p<0.05$; Jonckheere-Terpstra test). There was also a statistically significant decrease in male cholinesterase at the 0.000812 and 0.00302 mg a.i./L treatment groups and in females at all treatment levels compared to the control ($p<0.05$; Jonckheere-Terpstra test). There were no significant effects detected by the study authors for any other endpoints. Although not analyzed statistically, there was a marginally higher incidence of oocyte atresia (all severities) in treated females compared to the controls; however, this may have been due to granulomatous inflammation and was interpreted as a spontaneous alteration not associated with exposure to chlorpyrifos due to the lack of a concentration-response relationship and bacterial etiology. The study authors also noted that there

was an increase in the prevalence and severity of egg debris in the oviduct with increasing treatment, suggesting that there were chlorpyrifos-induced effects on histopathology in females.

C. Reviewer's Analysis and Conclusions

Statistical Methods: The reviewer analyzed combined sex and male survival (mortality) data using Fisher's Exact test; for females and combined sexes, survival in the treated conditions exceeded that in the control. Female weight and length, mean vitellogenin (VTG), gonadal somatic index (GSI), male tubercle score, fertility, male and female testosterone, and male and female estradiol data were not consistent with a monotonic concentration-response. Cholinesterase was not statistically analyzed. All data were tested for normality using Shapiro-Wilks test and for homogeneity of variance using Levene's test (SAS 8.1). Data which met the assumptions of normality and homogeneity of variances were then analyzed using the parametric Dunnett's test, while those that did not satisfy parametric assumptions (i.e., male VTG, male tubercle score, female testosterone, and male estradiol) were analyzed using the non-parametric Mann-Whitney U test.

Male body weights, male lengths, and fecundity exhibited decreasing monotonic trends and satisfied the parametric assumptions so these endpoints were analyzed using the Jonckheere-Terpstra test. Histopatholgy and gonadal staging were visually assessed for effects, along with secondary sex characteristics. Unless otherwise indicated, effects were considered statistically significant at $p<0.05$.

Conclusions:

Fecundity was significantly reduced by 52 to 71% at all treatment levels compared to the negative control group ($p<0.05$). A significant reduction of 17% in male body weights at the 0.00302 mg a.i./L treatment level (Jonckheere-Terpstra; $p<0.05$) was observed. Significant treatment-related inhibition of cholinesterase was observed at all treatment levels in females (40 to 92% of control) and in the mid and high treatment males (72% and 90% of control, respectively), with a 65% decrease at the lowest concentration for males which was not statistically significant.

There were no significant differences ($p>0.05$) in male tubercle scores (tubercles were not observed in females) or in male or female GSI compared to the negative control. Plasma vitellogenin, testosterone, and estradiol endpoints for the chlorpyrifos treated groups were not significantly different ($p>0.05$) when compared to the negative control. The measured plasma estradiol in one male and vitellogenin concentration for two males (in which one was the same fish as for estradiol) in the 0.000812 mg a.i./L group was substantially higher than the controls which contributed to a 2700 and 4000% increase for estradiol and vitellogenin for this treatment compared to the negative control. In addition, the plasma testosterone levels for females in one replicate (comprised of two values) of the highest concentration was substantially higher contributing to a 95% increase in testosterone for this treatment compared to the negative control.

Table 22: Reproductive and HPG Endpoints[1,2] for Male Fathead Minnow (*Pimephales promelas)* in the FSTRA with Chlorpyrifos.

Treatment (mg a.i./L) [TWA-measured]	Tubercle Score		GSI		Gonadal Staging and Histo.	Plasma VTG		Plasma T		Plasma E2	
	Median	p	% Diff.	p	Effect? (Yes/No)	% Diff.	p	% Diff.	p	% Diff.	p
Negative control (<LOQ)	21	NA	0	NA	No	0	NA	0	NA	0	NA
0.000251	24	0.24	4.73	0.97	No	48.5	0.35	-2.73	0.99	0.00	>0.99
0.000812	19	0.34	13.3	0.61	No	4093	0.35	-43.3	0.26	2733	0.23
0.00302	20	>0.99	-8.39	0.85	No	-55.8	0.89	2.24	0.99	46.8	0.23
Statistical Test	Mann-Whitney U		Dunnett's		NA	Mann-Whitney U		Dunnett's		Mann-Whitney U	

Abbreviations: [Conc.] Concentration. [Diff.] Difference. [E2] 17β-estradiol. [GSI] Gonado-Somatic Index. [Histo.] Histopathology. [NA] Not applicable. [T] Testosterone. [VTG] Vitellogenin.

LOQ=0.0000312 mg a.i./L.

[1] Unless otherwise indicated, effects and percent (%) differences are reported based on comparison to the negative (clean water) control. Conclusions regarding histopathology may be heavily weighted by the expert opinion of a board-certified pathologist.

[2] Unless otherwise specified, effects are considered statistically significant at $p<0.05$.

Table 23: Reproductive and HPG Endpoints[1,2] for Female Fathead Minnow (*Pimephales promelas)* in the FSTRA with Chlorpyrifos.

Treatment (mg a.i./L) [TWA-measured]	Fecundity		Fert. Success		Tubercle Score		GSI		Gonadal Staging and Histo.	Plasma VTG		Plasma T		Plasma E2	
	% Diff.	p	% Diff.	p	Median	p	% Diff.	p	Effect? (Yes/No)	% Diff.	p	% Diff.	p	% Diff.	p
Negative control (<LOQ)	0	NA	0	NA	0	NA	0	NA	No	0	NA	0	NA	0	NA
0.000251	-51.7	0.010	-1.31	0.37	0	NA	-0.36	>0.99	No	-27.3	0.70	20.8	0.89	-10.6	0.97
0.000812	-63.3	0.002	-0.23	0.99	0	NA	-1.61	0.99	No	-10.8	0.97	16.5	0.89	-32.1	0.60
0.00302	-71.4	<0.001	-1.29	0.39	0	NA	3.46	0.99	No	-42.2	0.39	95.6	0.41	-12.4	0.96
Statistical Test	Jonckheere-Terpstra		Dunnett's		None		Dunnett's		NA	Dunnett's		Mann-Whitney U		Dunnett's	

Abbreviations: Conc. Concentration. Diff. Difference. E2 17β-estradiol. Fert. Fertilization. GSI Gonado-Somatic Index. Histo. Histopathology.

NA Not applicable. T Testosterone. VTG Vitellogenin.

LOQ=0.0000312 mg a.i./L.

1 Unless otherwise indicated, effects and percent (%) differences are reported based on comparison to the negative (clean water) control. Conclusions regarding histopathology may be heavily weighted by the expert opinion of a board-certified pathologist.

2 Unless otherwise specified, effects are considered statistically significant at $p<0.05$.

Table 24: Growth Endpoints[1,2] in the Fish Short-Term Reproduction Assay (FSTRA) with Chlorpyrifos.

Treatment (mg a.i./L) [TWA-measured]	Body Weight				Length			
	Males		Females		Males		Females	
	% Diff.	p	% Diff.	p	% Diff.	p	% Diff.	p
Negative control (<LOQ)	0	NA	0	NA	0	NA	0	NA
0.000251	-4.95	0.39	4.94	0.70	0.36	0.50	-0.06	>0.99
0.000812	-14.4	0.12	-3.29	0.88	-2.73	0.17	-1.15	0.74
0.00302	-17.5	**0.046**	-0.55	0.99	-4.19	0.062	0.40	0.98
Statistical Test	Jonckheere-Terpstra		Dunnett's		Jonckheere-Terpstra		Dunnett's	

Abbreviations: [Diff.] Difference. [NA] Not applicable. [ND] Not determined.

LOQ=0.0000312 mg a.i./L.

1 Unless otherwise indicated, percent (%) differences are reported based on comparison to the negative (clean water) control.

2 Unless otherwise specified, effects are considered statistically significant at $p<0.05$.

E. Study Deficiencies

There were deviations from the guideline as noted in Section I. Materials and Methods of the DER. All performance and validity criteria were met with the exception that average negative control survival was 83.3% for the combined sexes which is less than the guideline criterion of ≥90%. In the negative control group, average female survival was 81.2% and average male survival was 87.5%. These deviations did not impact the interpretation of the study.

F. Reviewer's Comments

The reviewer's and the study authors' results were in agreement regarding effects on fecundity; however conclusions regarding male body weight differed, and the reviewer did not analyze male and female cholinesterase. The study author reported no significant decrease in male body weight and significant concentration-dependent decrease in mean female cholinesterase of 40 to 92% at all treatment levels and mean male cholinesterase of 72% and 90% in the 0.000812 and 0.00302 mg a.i./L treatment groups, respectively. The reviewer's conclusions using the OCSPP 890.1350 EDSP flowchart are presented in the Executive Summary and Conclusions sections of this DER. For endpoints exhibiting a monotonic trend and satisfying parametric assumptions, historical EFED methods suggest that conclusions be made using the results of William's test. If the reviewer had relied on this test, male body weight would not have been significantly different from the control at the highest treatment level ($p>0.05$).

Residual chlorine in dilution water was <20 μg/L, however, EPA recommends that residual chlorine in dilution water be <10 μg/L. Ammonia (as N) in dilution water was 100 μg/L, however, EPA recommends that unionized ammonia in the dilution water be $\leq$ 1 μg/L. Additionally, the TOC of the dilution water was not reported.

Analytical verification of the test solutions at Days 0, 1, 4, 7, 11, 14, 18 and 21 yielded recoveries ranging from 90.2 to 191% of nominal concentrations. The study authors and reviewer based toxicity calculations on the TWA-measured concentrations. The %CV of some replicate chambers exceeded 20% (ranging from 20.1 to 25%) over the course of the 21 day study, but the daily averages for each level were maintained satisfactorily below 20% (i.e., 16.3-19.9%).

III. REFERENCES

Busse, J. (2002). Determination of the water solubility of chlorpyrifos. Unpublished laboratory report #Dow AgroSciences Report No. FOR01019; DERBI # 112166. January 29, 2002.

Ellman, G.L., Courtney, K.D., Andres, Jr. V., and Featherstone, R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 1961, 7, 88-95.

Geiger, D. L., Call, D. J., and Brooks, L. T. (1988). Acute toxicities of organic chemicals to fathead minnows (Pimephales promelas). Ctr. for Lake Superior Environ. Stud., 4, Univ. of Wisconsin-Superior, Superior, WI :355.

Holcombe, G. W., Phipps, G. L., and Tanner, D. K. (1982). The acute toxicity of kelthane, dursban, disulfoton, pydrin, and permethrin to fathead minnows (Pimephales promelas) and rainbow trout (Salmo gairdneri). Environ. Pollut. Ser. A. 29, 167-178.

Hollander, M. and D. A. Wolfe. (1973). Nonparametric Statistical Methods. John Wiley & Sons, Inc. New York. Pp 120-123.

Hunter, P.L., Marshal, R.S., and Padilla, S. Automated instrument analysis of cholinesterase activity in tissues from carbamate-treated animals: a cautionary note. Toxicology Methods 1997, 7, 43-53.

Jarvinen, A. W., Tanner, D. K., and Kline, E. R. (1988). Toxicity of chlorpyrifos, endrin, or fenvalerate to fathead minnows following episodic or continuous exposure. Ecotoxical. Environ. Saf. 15, 78-95 (1988).

Jarvinen, A. W., Nordling, B. R., and Henry, M. E. (1983). Chronic toxicity of Dursban (chlorpyrifos) to the fathead minnow (Pimephales promelas) and the resultant acetylcholinesterase inhibition. Ecotoxical. Environ. Saf. 7, 423-434.

Jarvinen, A. W. and Tanner, D. K. (1982). Toxicity of selected controlled release and corresponding unformulated technical grade pesticides to the fathead minnow (Pimephales promelas). Environ. Pollut. 27, 179-195.

Koors, B. (2011). Determination of purity and/or identity of Chlorpyrifos, Lot # KC28161419, TSN101285, for use in a study. FA&PC Number 11-285443. Dow AgroSciences LLC, Indianapolis, Indiana.

Mayes, M. A., Weinberg, J. T., Rick, D. L., and Martin, M. D. (1993). Chlorpyrifos: A life-cycle toxicity test with the fathead minnow, Pimephales promelas Rafinesque. Unpublished report of The Dow Chemical Company. DR-0043-4946.

Mehler, W. T., Schuler, L. J. and Lydy, M. J. (2008). Examining the joint toxicity of chlorpyrifos and atrazine in the aquatic species: Lepomis macrochirus, Pimephales promelas and Chironomus tentans. Environ. Pollut. 152, 217-224.

Phipps, G. L., and Holcombe, G. W. (1985). A method for aquatic multiple species toxicant testing: Acute toxicity of 10 chemicals to 5 vertebrates and 2 invertebrates; Toxicity of sevin (carbaryl) to Chinook salmon. Environ. Pollut. Ser. A. 38, 141-157.

USEPA (2002a). A Short-term Test Method for Assessing the Reproductive Toxicity of Endocrine-Disrupting Chemicals Using the Fathead Minnow (Pimephales promelas). EPA/600/R-01/067.

USEPA (2002b). Interim Reregistration Eligibility Decision for Chlorpyrifos. EPA 738-R-01-007.

Vieth, G. D., DeFoe, D. L., and Bergstedt, B.V. (1979). Measuring and estimating the bioconcentration factor of chemicals in fish. J. Fish Res. Board Can 36, 1040.

APPENDIX I. OUTPUT OF REVIEWER'S STATISTICAL VERIFICATION:

Endpoint	Monotonic?	Parametric?	890.1350	EFED	Comments
Female body weight	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
Male body weight	Yes, decreasing	Yes	Jonckheere: Dose 3 p=0.046	William's: n.s. p>0.05	890.1350 and EFED conclusions differ. 890.1350 method suggestion of Jonckheere-Terpstra indicates a significant 17% reduction at the 0.00302 mg a.i./L level, while historical EFED method using William's test does not detect an effect (p>0.05).
Female body length	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
Male body length	Yes, decreasing	Yes	Jonckheere: n.s. p>0.05	William's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
Female VTG	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
Male VTG	No	No	Mann-Whitney: n.s. p>0.05	Mann-Whitney: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
Female GSI	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
Male GSI	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect

Endpoint	Monotonic?	Parametric?	890.1350	EFED	Comments
Female tubercle score	NA	NA	NA	NA	No score
Male tubercle score	No	No	Mann-Whitney: n.s. p>0.05	Mann-Whitney: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
Fecundity	Yes	Yes	Jonckheere: Dose 1 p=0.010 Dose 2 p=0.002 Dose 3 p<0.001	William's: Dose 1 p<0.001 Dose 2 p<0.001 Dose 3 p<0.001	890.1350 and EFED same conclusions, significant (p<0.05; at all levels) monotonic decrease, ranging from 52 to 71% lower than negative control.
Fertility	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
F testosterone	No	No	Mann-Whitney: n.s. p>0.05	Mann-Whitney: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
M testosterone	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
F estradiol	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
M estradiol	No	No	Mann-Whitney: n.s. p>0.05	Mann-Whitney: n.s. p>0.05	890.1350 and EFED same conclusions, no effect

059101 Chlorpyrifos 890.1350 48615506
ANALYSIS RESULTS FOR VARIABLE VAR01 (F body weight (g))

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.

Shapiro-Wilks Test Stat	Shapiro-Wilks P-value	Levenes Test Stat	Levenes P-value	Conclusion
0.937	0.316	2.776	0.087	USE PARAMETRIC TESTS

****
BASIC SUMMARY STATISTICS

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval
Ctrl	4	1.82	0.16	0.08	8.90	1.56, 2.08
Dose1	4	1.91	0.14	0.07	7.55	1.68, 2.14
Dose2	4	1.76	0.04	0.02	1.99	1.71, 1.82
Dose3	4	1.81	0.17	0.09	9.63	1.53, 2.09

Level	Median	Min	Max	%of Negative control(means)	%Reduction(means)
Ctrl	1.81	1.68	2.00	.	.
Dose1	1.87	1.79	2.12	104.94	-4.94
Dose2	1.77	1.72	1.80	96.71	3.29
Dose3	1.79	1.64	2.04	99.45	0.55

****
PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Analysis of Variance (ANOVA) - overall F-test

Numerator df	Denominator df	F-stat	P-value
3	12	0.79	0.522

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5
Ctrl	1.82	.	1.87	.	.	.	.	.	.
Dose1	1.91	0.698	1.87	0.757	.	.	.	.	.
Dose2	1.76	0.877	1.79	0.462	0.461	.	.	.	.
Dose3	1.81	0.999	1.79	0.478	0.748	0.957	.	.	.

****
NON-PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Kruskal-Wallis test - equality among treatment groups

Degrees of Freedom	TestStat	P-value
3	2.49	0.476

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	1.81	.	.
Dose1	1.87	0.678	0.718
Dose2	1.77	1.000	0.232
Dose3	1.79	1.000	0.227

DECREASING TREND TEST SUMMARY	LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
Williams	>highest dose (no sign. differences)
Jonckheere	>highest dose (no sign. differences)

```
************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12              0.79        0.522

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    -1.82    .        -1.82      .        .       .       .       .       .
 Dose1   -1.91   0.698     -1.83     0.555     .       .       .       .       .
 Dose2   -1.76   0.877     -1.83     0.589    0.461    .       .       .       .
 Dose3   -1.81   0.999     -1.83     0.607    0.748   0.957    .       .       .

************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                2.49       0.476

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      -1.81                    .                         .
  Dose1     -1.87                   0.678                     0.282
  Dose2     -1.77                   1.000                     0.768
  Dose3     -1.79                   1.000                     0.773

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

059101 Chlorpyrifos 890.1350 48615506
ANALYSIS RESULTS FOR VARIABLE VAR02  ( M body weight (g) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.959         0.635          0.334      0.801    USE PARAMETRIC TESTS

************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     4.14     0.57       0.28       13.65          3.24,    5.04
  Dose1  4     3.94     0.57       0.28       14.40          3.03,    4.84
  Dose2  4     3.54     0.74       0.37       20.87          2.37,    4.72
  Dose3  4     3.42     0.40       0.20       11.84          2.77,    4.06

 Level       Median     Min       Max    %of Negative control(means)   %Reduction(means)
  Ctrl         4.19     3.41       4.78          .                  .
  Dose1        4.01     3.27       4.45        95.05               4.95
  Dose2        3.35     2.88       4.60        85.57              14.43
```

```
  Dose3        3.41     3.04       3.82       82.55              17.45

************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12               1.34        0.307

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl     4.14    .         4.14      .        .       .       .       .       .
 Dose1    3.94   0.924      3.94     0.373     .       .       .       .       .
 Dose2    3.54   0.368      3.54     0.108    0.776    .       .       .       .
 Dose3    3.42   0.236      3.42     0.066    0.604   0.990    .       .       .

************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                3.51       0.320

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl       4.19                    .                        .
  Dose1      4.01                   0.889                    0.386
  Dose2      3.35                   0.235                    0.121
  Dose3      3.41                   0.156                    0.046

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                Dose3

************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12               1.34        0.307

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    -4.14    .        -3.76      .        .       .       .       .       .
 Dose1   -3.94   0.924     -3.76     0.884     .       .       .       .       .
 Dose2   -3.54   0.368     -3.76     0.907    0.776    .       .       .       .
 Dose3   -3.42   0.236     -3.76     0.919    0.604   0.990    .       .       .

************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
```

```
          3                 3.51       0.320

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      -4.19                    .                          .
  Dose1     -4.01                  0.889                      0.614
  Dose2     -3.35                  0.235                      0.879
  Dose3     -3.41                  0.156                      0.954

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)

059101 Chlorpyrifos 890.1350 48615506
ANALYSIS RESULTS FOR VARIABLE VAR03  ( F body length (mm) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.863         0.021          2.292      0.130    USE PARAMETRIC TESTS

*****************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    43.53     1.27       0.63        2.91          41.51,   45.54
  Dose1  4    43.50     0.39       0.20        0.90          42.88,   44.12
  Dose2  4    43.03     0.30       0.15        0.69          42.55,   43.50
  Dose3  4    43.70     0.98       0.49        2.23          42.15,   45.25

 Level       Median     Min       Max    %of Negative control(means)   %Reduction(means)
  Ctrl        43.00    42.70     45.40          .                          .
  Dose1       43.45    43.10     44.00        99.94                      0.06
  Dose2       43.00    42.70     43.40        98.85                      1.15
  Dose3       43.40    42.90     45.10       100.40                     -0.40

*****************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3            12               0.48        0.704

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    43.53     .       43.53       .        .       .       .       .       .
 Dose1   43.50   1.000     43.50     0.565      .       .       .       .       .
 Dose2   43.03   0.738     43.36     0.497    0.852     .       .       .       .
 Dose3   43.70   0.982     43.36     0.513    0.986   0.673     .       .       .

*****************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
```

```
     Degrees of Freedom   TestStat    P-value
           3                 2.87       0.412

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value          Jonckheere p-value
  Ctrl      43.00                   .                        .
  Dose1     43.45                0.494                    0.807
  Dose2     43.00                1.000                    0.330
  Dose3     43.40                0.580                    0.629

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

*****************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12               0.48        0.704

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl   -43.53    .       -43.35      .        .       .       .       .       .
 Dose1  -43.50   1.000    -43.35     0.702     .       .       .       .       .
 Dose2  -43.03   0.738    -43.35     0.736    0.852    .       .       .       .
 Dose3  -43.70   0.982    -43.70     0.504    0.986   0.673    .       .       .

*****************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                 2.87       0.412

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value          Jonckheere p-value
  Ctrl     -43.00                   .                        .
  Dose1    -43.45                0.494                    0.193
  Dose2    -43.00                1.000                    0.670
  Dose3    -43.40                0.580                    0.371

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

059101 Chlorpyrifos 890.1350 48615506
ANALYSIS RESULTS FOR VARIABLE VAR04  ( M body length (mm) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
```

```
    Test Stat       P-value       Test Stat   P-value
      0.909          0.113          1.059      0.402    USE PARAMETRIC TESTS

****************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    54.95     2.79        1.39        5.07          50.51,   59.39
  Dose1  4    55.15     2.84        1.42        5.15          50.63,   59.67
  Dose2  4    53.45     3.36        1.68        6.29          48.10,   58.80
  Dose3  4    52.65     2.01        1.01        3.83          49.45,   55.85

 Level       Median     Min       Max    %of Negative control(means)   %Reduction(means)
  Ctrl        55.20    51.40      58.00          .                          .
  Dose1       55.80    51.30      57.70       100.36                     -0.36
  Dose2       53.35    50.40      56.70        97.27                      2.73
  Dose3       52.75    50.10      55.00        95.81                      4.19

****************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12               0.74        0.547

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    54.95    .        55.05      .        .       .       .       .       .
 Dose1   55.15   0.999     55.05     0.605     .       .       .       .       .
 Dose2   53.45   0.791     53.45     0.295    0.825    .       .       .       .
 Dose3   52.65   0.532     52.65     0.175    0.600   0.977    .       .       .

****************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                2.50       0.475

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value          Jonckheere p-value
  Ctrl      55.20                   .                        .
  Dose1     55.80                  1.000                    0.500
  Dose2     53.35                  0.580                    0.170
  Dose3     52.75                  0.346                    0.062

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)

****************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12               0.74        0.547

Dunnett - testing each trt mean signif. different than control
```

Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5
Ctrl	-54.95	.	-54.05	.	.	.	.	.	.
Dose1	-55.15	0.999	-54.05	0.758	.	.	.	.	.
Dose2	-53.45	0.791	-54.05	0.791	0.825	.	.	.	.
Dose3	-52.65	0.532	-54.05	0.809	0.600	0.977	.	.	.

****
NON-PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Kruskal-Wallis test - equality among treatment groups

Degrees of Freedom	TestStat	P-value
3	2.50	0.475

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	-55.20	.	.
Dose1	-55.80	1.000	0.500
Dose2	-53.35	0.580	0.830
Dose3	-52.75	0.346	0.938

INCREASING TREND TEST SUMMARY	LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
Williams	>highest dose (no sign. differences)
Jonckheere	>highest dose (no sign. differences)

059101 Chlorpyrifos 890.1350 48615506
ANALYSIS RESULTS FOR VARIABLE VAR05 (F vitellogenin (ng/mL))

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.

Shapiro-Wilks Test Stat	Shapiro-Wilks P-value	Levenes Test Stat	Levenes P-value	Conclusion
0.941	0.362	2.504	0.109	USE PARAMETRIC TESTS

****
BASIC SUMMARY STATISTICS

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval
Ctrl	4	67.16	47.33	23.66	70.47	-8.15, 142.47
Dose1	4	48.85	23.36	11.68	47.82	11.68, 86.01
Dose2	4	59.91	18.95	9.48	31.63	29.76, 90.07
Dose3	4	38.80	10.31	5.15	26.57	22.39, 55.20

Level	Median	Min	Max	%of Negative control(means)	%Reduction(means)
Ctrl	54.77	26.44	132.66	.	.
Dose1	45.23	27.85	77.09	72.74	27.26
Dose2	59.41	43.10	77.73	89.21	10.79
Dose3	41.52	25.08	47.08	57.77	42.23

****
PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Analysis of Variance (ANOVA) - overall F-test

Numerator df	Denominator df	F-stat	P-value
3	12	0.76	0.536

```
Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    67.16    .        67.16      .        .       .       .       .       .
 Dose1   48.85   0.697     54.38     0.321     .       .       .       .       .
 Dose2   59.91   0.969     54.38     0.343    0.945    .       .       .       .
 Dose3   38.80   0.392     38.80     0.120    0.958   0.726    .       .       .

*************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                1.88       0.599

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value          Jonckheere p-value
  Ctrl      54.77                   .                        .
  Dose1     45.23                  0.889                    0.386
  Dose2     59.41                  0.678                    0.721
  Dose3     41.52                  0.494                    0.320

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

*************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12               0.76        0.536

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl   -67.16    .       -53.68      .        .       .       .       .       .
 Dose1  -48.85   0.697    -53.68     0.823     .       .       .       .       .
 Dose2  -59.91   0.969    -53.68     0.852    0.945    .       .       .       .
 Dose3  -38.80   0.392    -53.68     0.867    0.958   0.726    .       .       .

*************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                1.88       0.599

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value          Jonckheere p-value
  Ctrl     -54.77                  .                        .
  Dose1    -45.23                 0.889                    0.614
```

```
  Dose2    -59.41                   0.678                   0.279
  Dose3    -41.52                   0.494                   0.680

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)

059101 Chlorpyrifos 890.1350 48615506
ANALYSIS RESULTS FOR VARIABLE VAR06  ( M vitellogenin (ng/mL) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.600         <.001          8.422      0.003    USE NON-PARAMETRIC TESTS

*****************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4   649.25   845.12      422.56      130.17        -695.53, 1994.03
  Dose1  4   964.25   810.24      405.12       84.03        -325.02, 2253.52
  Dose2  4 27223.75 47083.73    23541.87      172.95       -47697.0,102144.5
  Dose3  4   287.13   254.85      127.43       88.76        -118.41,  692.66

 Level       Median     Min       Max    %of Negative control(means)   %Reduction(means)
  Ctrl       287.00   113.00    1910.00          .                .
  Dose1      739.00   259.00    2120.00       148.52           -48.52
  Dose2     5628.50   138.00   97500.00      4193.11          -4093.11
  Dose3      237.00    48.50     626.00        44.22            55.78

*****************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12               1.28        0.327

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl   649.25    .      9612.42      .        .       .       .       .       .
 Dose1  964.25   1.000   9612.42     0.785     .       .       .       .       .
 Dose227223.75   0.301   9612.42     0.816    0.426    .       .       .       .
 Dose3  287.13   1.000    287.13     0.627    1.000   0.406    .       .       .

*****************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                 3.75       0.290

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value          Jonckheere p-value
  Ctrl     287.00                   .                        .
```

```
  Dose1    739.00                    0.346                    0.876
  Dose2   5628.50                    0.346                    0.928
  Dose3    237.00                    0.889                    0.463

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

*****************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12               1.28        0.327

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl  -649.25     .      -649.25      .        .       .       .       .       .
 Dose1 -964.25   1.000    -964.25     0.575     .       .       .       .       .
 Dose2-27223.8   0.301   -13755.4     0.285    0.426    .       .       .       .
 Dose3 -287.13   1.000   -13755.4     0.295    1.000   0.406    .       .       .

*****************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                3.75       0.290

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value          Jonckheere p-value
  Ctrl    -287.00                   .                        .
  Dose1   -739.00                  0.346                    0.124
  Dose2  -5628.50                  0.346                    0.072
  Dose3   -237.00                  0.889                    0.537

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

059101 Chlorpyrifos 890.1350 48615506
ANALYSIS RESULTS FOR VARIABLE VAR07  ( F GSI )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.940         0.351          0.765      0.535    USE PARAMETRIC TESTS

*****************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    15.25     2.26       1.13       14.80          11.66,   18.84
  Dose1  4    15.20     3.57       1.78       23.48           9.52,   20.88
```

```
  Dose2  4    15.01     1.44       0.72        9.58          12.72,   17.30
  Dose3  4    15.78     3.22       1.61       20.43          10.65,   20.91

 Level        Median     Min       Max    %of Negative control(means)   %Reduction(means)
  Ctrl         15.33    12.54     17.82          .                .
  Dose1        14.08    12.54     20.10        99.64             0.36
  Dose2        14.95    13.53     16.60        98.39             1.61
  Dose3        15.26    12.48     20.12       103.46            -3.46

*************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12               0.06        0.981

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    15.25    .        15.31      .        .       .       .       .       .
 Dose1   15.20   1.000     15.31     0.596     .       .       .       .       .
 Dose2   15.01   0.999     15.31     0.630    1.000    .       .       .       .
 Dose3   15.78   0.986     15.31     0.649    0.990   0.978    .       .       .

*************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                0.20       0.977

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value          Jonckheere p-value
  Ctrl      15.33                   .                        .
  Dose1     14.08                  1.000                    0.442
  Dose2     14.95                  0.889                    0.529
  Dose3     15.26                  1.000                    0.592

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)

*************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12               0.06        0.981

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl   -15.25    .       -15.15      .        .       .       .       .       .
 Dose1  -15.20   1.000     -15.15     0.605     .       .       .       .       .
```

```
 Dose2  -15.01   0.999    -15.15     0.640    1.000    .       .       .       .
 Dose3  -15.78   0.986    -15.78     0.515    0.990   0.978    .       .       .

*************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                 0.20       0.977

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl     -15.33                   .                         .
  Dose1    -14.08                 1.000                     0.558
  Dose2    -14.95                 0.889                     0.471
  Dose3    -15.26                 1.000                     0.408

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

059101 Chlorpyrifos 890.1350 48615506
ANALYSIS RESULTS FOR VARIABLE VAR08  ( M GSI )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.965         0.748          0.644      0.601    USE PARAMETRIC TESTS

*************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     1.16     0.12       0.06       10.07          0.98,    1.35
  Dose1  4     1.22     0.22       0.11       18.02          0.87,    1.57
  Dose2  4     1.32     0.27       0.14       20.85          0.88,    1.75
  Dose3  4     1.07     0.19       0.10       17.96          0.76,    1.37

 Level       Median     Min       Max    %of Negative control(means)   %Reduction(means)
  Ctrl         1.18     1.01      1.29          .                .
  Dose1        1.26     0.96      1.40       104.73            -4.73
  Dose2        1.30     1.00      1.67       113.33           -13.33
  Dose3        1.14     0.79      1.20        91.61             8.39

*************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12               1.02        0.416

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl     1.16    .         1.23      .        .       .       .       .       .
```

```
 Dose1    1.22   0.965      1.23     0.764      .       .       .       .       .
 Dose2    1.32   0.605      1.23     0.797    0.903     .       .       .       .
 Dose3    1.07   0.847      1.07     0.344    0.733   0.359     .       .       .

************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
      Degrees of Freedom   TestStat    P-value
            3                2.24       0.524

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl       1.18                   .                         .
  Dose1      1.26                 0.889                     0.614
  Dose2      1.30                 0.494                     0.768
  Dose3      1.14                 0.580                     0.303

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
            3           12               1.02        0.416

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    -1.16    .        -1.16      .        .       .       .       .       .
 Dose1   -1.22   0.965     -1.20     0.475     .       .       .       .       .
 Dose2   -1.32   0.605     -1.20     0.506    0.903    .       .       .       .
 Dose3   -1.07   0.847     -1.20     0.523    0.733   0.359    .       .       .

************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
      Degrees of Freedom   TestStat    P-value
            3                2.24       0.524

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      -1.18                  .                         .
  Dose1     -1.26                0.889                     0.386
  Dose2     -1.30                0.494                     0.232
  Dose3     -1.14                0.580                     0.697

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

059101 Chlorpyrifos 890.1350 48615506
```

ANALYSIS RESULTS FOR VARIABLE VAR09 (F tubercle score (median))

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.

Shapiro-Wilks Test Stat	Shapiro-Wilks P-value	Levenes Test Stat	Levenes P-value	Conclusion
.	.	.	.	NO DATA FOR TEST

BASIC SUMMARY STATISTICS

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval
Ctrl	4	0.00	0.00	0.00	.	. , .
Dose1	4	0.00	0.00	0.00	.	. , .
Dose2	4	0.00	0.00	0.00	.	. , .
Dose3	4	0.00	0.00	0.00	.	. , .

Level	Median	Min	Max	%of Negative control(means)	%Reduction(means)
Ctrl	0.00	0.00	0.00	.	
Dose1	0.00	0.00	0.00	.	
Dose2	0.00	0.00	0.00	.	
Dose3	0.00	0.00	0.00	.	

PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Analysis of Variance (ANOVA) - overall F-test

Numerator df	Denominator df	F-stat	P-value
.	1	.	.

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5
Ctrl	0.00	.	.	.	.	.	.	.	.
Dose1	0.00	.	.	.	.	.	.	.	.
Dose2	0.00	.	.	.	.	.	.	.	.
Dose3	0.00	.	.	.	.	.	.	.	.

NON-PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Kruskal-Wallis test - equality among treatment groups

Degrees of Freedom	TestStat	P-value
3	0.00	1.000

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	0.00	.	.
Dose1	0.00	1.000	.
Dose2	0.00	1.000	.
Dose3	0.00	1.000	.

DECREASING TREND TEST SUMMARY	LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
Williams	Dose1
Jonckheere	Dose1

```
************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
 .         .      .    1     .        .        .       .       .       .       .


Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl     0.00    .          .        .        .       .       .       .       .
 Dose1    0.00    .          .        .        .       .       .       .       .
 Dose2    0.00    .          .        .        .       .       .       .       .
 Dose3    0.00    .          .        .        .       .       .       .       .

************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                 0.00       1.000

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl       0.00                   .                        .
  Dose1      0.00                 1.000                      .
  Dose2      0.00                 1.000                      .
  Dose3      0.00                 1.000                      .

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
   Williams                                  Dose1
   Jonckheere                                Dose1

059101 Chlorpyrifos 890.1350 48615506
ANALYSIS RESULTS FOR VARIABLE VAR10  ( M tubercle score (median) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.984         0.988          3.861      0.038    USE NON-PARAMETRIC TESTS

************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    20.38     1.49       0.75        7.33          18.00,   22.75
  Dose1  4    22.88     2.39       1.20       10.46          19.07,   26.68
  Dose2  4    19.25     1.32       0.66        6.87          17.15,   21.35
  Dose3  4    20.13     3.75       1.88       18.63          14.16,   26.09

 Level       Median     Min       Max    %of Negative control(means)   %Reduction(means)
  Ctrl        20.50    18.50      22.00          .                 .
  Dose1       23.50    19.50      25.00       112.27            -12.27
```

```
  Dose2       19.00    18.00      21.00       94.48              5.52
  Dose3       20.25    16.00      24.00       98.77              1.23

************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12               1.63        0.235

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    20.38    .        21.63      .        .       .       .       .       .
 Dose1   22.88   0.370     21.63     0.838     .       .       .       .       .
 Dose2   19.25   0.852     19.69     0.442    0.207    .       .       .       .
 Dose3   20.13   0.998     19.69     0.457    0.417   0.956    .       .       .

************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                4.38       0.223

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      20.50                   .                         .
  Dose1     23.50                  0.235                     0.926
  Dose2     19.00                  0.341                     0.209
  Dose3     20.25                  1.000                     0.186

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12               1.63        0.235

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl   -20.38    .       -20.38      .        .       .       .       .       .
 Dose1  -22.88   0.370    -20.75     0.491     .       .       .       .       .
 Dose2  -19.25   0.852    -20.75     0.522    0.207    .       .       .       .
 Dose3  -20.13   0.998    -20.75     0.539    0.417   0.956    .       .       .

************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
```

```
     Degrees of Freedom   TestStat    P-value
           3                 4.38       0.223

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value          Jonckheere p-value
  Ctrl     -20.50                   .                        .
  Dose1    -23.50                 0.235                    0.074
  Dose2    -19.00                 0.341                    0.791
  Dose3    -20.25                 1.000                    0.814

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
   Williams                              >highest dose (no sign. differences)
   Jonckheere                            >highest dose (no sign. differences)

059101 Chlorpyrifos 890.1350 48615506
ANALYSIS RESULTS FOR VARIABLE VAR11  ( fecundity )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.935         0.289          0.805      0.515    USE PARAMETRIC TESTS

*****************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    34.20     3.05       1.53        8.93          29.34,   39.06
  Dose1  4    16.53     4.52       2.26       27.33           9.34,   23.71
  Dose2  4    12.55     3.16       1.58       25.15           7.53,   17.57
  Dose3  4     9.78     5.29       2.65       54.15           1.35,   18.20

 Level       Median     Min       Max    %of Negative control(means)   %Reduction(means)
  Ctrl        34.75    30.00     37.30         .                  .
  Dose1       16.45    12.20     21.00       48.32              51.68
  Dose2       12.40     9.60     15.80       36.70              63.30
  Dose3       11.00     2.40     14.70       28.58              71.42

*****************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12              28.49       <.001

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    34.20    .        34.20      .        .       .       .       .       .
 Dose1   16.53   <.001     16.53     <.001     .       .       .       .       .
 Dose2   12.55   <.001     12.55     <.001    0.542    .       .       .       .
 Dose3    9.78   <.001      9.78     <.001    0.148   0.777    .       .       .

*****************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
```

```
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                10.41       0.015

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      34.75                   .                          .
  Dose1     16.45                 0.067                      0.010
  Dose2     12.40                 0.067                      0.002
  Dose3     11.00                 0.067                      <.001

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  Dose1
   Jonckheere                                Dose1

*************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12              28.49        <.001

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl   -34.20    .       -18.26      .        .       .       .       .       .
 Dose1  -16.53   <.001    -18.26     1.000     .       .       .       .       .
 Dose2  -12.55   <.001    -18.26     1.000    0.542    .       .       .       .
 Dose3   -9.78   <.001    -18.26     1.000    0.148   0.777    .       .       .

*************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                10.41       0.015

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl     -34.75                  .                          .
  Dose1    -16.45                0.067                      0.990
  Dose2    -12.40                0.067                      0.998
  Dose3    -11.00                0.067                      1.000

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

059101 Chlorpyrifos 890.1350 48615506
ANALYSIS RESULTS FOR VARIABLE VAR12  ( fertility )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
```

Shapiro-Wilks Test Stat	Shapiro-Wilks P-value	Levenes Test Stat	Levenes P-value	Conclusion
0.982	0.975	1.009	0.423	USE PARAMETRIC TESTS

****

BASIC SUMMARY STATISTICS

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval
Ctrl	4	98.90	0.54	0.27	0.54	98.05, 99.75
Dose1	4	97.60	1.53	0.76	1.57	95.17, 100.03
Dose2	4	98.68	0.99	0.50	1.00	97.10, 100.25
Dose3	4	97.63	1.69	0.85	1.74	94.93, 100.32

Level	Median	Min	Max	%of Negative control(means)	%Reduction(means)
Ctrl	99.15	98.10	99.20	.	.
Dose1	97.95	95.50	99.00	98.69	1.31
Dose2	98.55	97.60	100.00	99.77	0.23
Dose3	97.25	96.00	100.00	98.71	1.29

****

PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests

Analysis of Variance (ANOVA) - overall F-test

Numerator df	Denominator df	F-stat	P-value
3	12	1.16	0.366

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5
Ctrl	98.90	.	98.90	.	.	.	.	.	.
Dose1	97.60	0.373	98.14	0.247	.	.	.	.	.
Dose2	98.68	0.989	98.14	0.264	0.641	.	.	.	.
Dose3	97.63	0.387	97.63	0.118	1.000	0.658	.	.	.

****

NON-PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests

Kruskal-Wallis test - equality among treatment groups

Degrees of Freedom	TestStat	P-value
3	3.95	0.266

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	99.15	.	.
Dose1	97.95	0.154	0.041
Dose2	98.55	0.676	0.279
Dose3	97.25	0.343	0.103

DECREASING TREND TEST SUMMARY	LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
Williams	>highest dose (no sign. differences)
Jonckheere	>highest dose (no sign. differences)

****

PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests

Analysis of Variance (ANOVA) - overall F-test

Numerator df	Denominator df	F-stat	P-value
3	12	1.16	0.366

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5
Ctrl	-98.90	.	-98.20	.	.	.	.	.	.
Dose1	-97.60	0.373	-98.20	0.851	.	.	.	.	.
Dose2	-98.68	0.989	-98.20	0.878	0.641	.	.	.	.
Dose3	-97.63	0.387	-98.20	0.891	1.000	0.658	.	.	.

****
NON-PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Kruskal-Wallis test - equality among treatment groups

Degrees of Freedom	TestStat	P-value
3	3.95	0.266

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	-99.15	.	.
Dose1	-97.95	0.154	0.959
Dose2	-98.55	0.676	0.721
Dose3	-97.25	0.343	0.897

INCREASING TREND TEST SUMMARY	LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
Williams	>highest dose (no sign. differences)
Jonckheere	>highest dose (no sign. differences)

059101 Chlorpyrifos 890.1350 48615506
ANALYSIS RESULTS FOR VARIABLE VAR13 (F testosterone (ng/mL))

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.

Shapiro-Wilks Test Stat	Shapiro-Wilks P-value	Levenes Test Stat	Levenes P-value	Conclusion
0.930	0.275	4.365	0.030	USE NON-PARAMETRIC TESTS

****
BASIC SUMMARY STATISTICS

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval
Ctrl	4	2.98	1.86	0.93	62.66	0.01, 5.94
Dose1	4	3.59	2.08	1.04	58.01	0.28, 6.91
Dose2	4	3.47	2.01	1.00	57.90	0.27, 6.66
Dose3	3	5.82	5.79	3.34	99.45	-8.56, 20.20

Level	Median	Min	Max	%of Negative control(means)	%Reduction(means)
Ctrl	2.29	1.61	5.72	.	.
Dose1	4.15	0.64	5.43	120.78	-20.78
Dose2	4.05	0.57	5.19	116.47	-16.47
Dose3	2.67	2.29	12.50	195.63	-95.63

****
PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Analysis of Variance (ANOVA) - overall F-test

Numerator df	Denominator df	F-stat	P-value
3	11	0.56	0.655

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5
Ctrl	2.98	.	3.84	.	.	.	.	.	.
Dose1	3.59	0.984	3.84	0.739	.	.	.	.	.
Dose2	3.47	0.992	3.84	0.773	1.000	.	.	.	.
Dose3	5.82	0.506	3.84	0.781	0.777	0.747	.	.	.

****
NON-PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Kruskal-Wallis test - equality among treatment groups

Degrees of Freedom	TestStat	P-value
3	0.51	0.916

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	2.29	.	.
Dose1	4.15	0.889	0.614
Dose2	4.05	0.889	0.558
Dose3	2.67	0.411	0.660

DECREASING TREND TEST SUMMARY	LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
Williams	>highest dose (no sign. differences)
Jonckheere	>highest dose (no sign. differences)

****
PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Analysis of Variance (ANOVA) - overall F-test

Numerator df	Denominator df	F-stat	P-value
3	11	0.56	0.655

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5
Ctrl	-2.98	.	-2.98	.	.	.	.	.	.
Dose1	-3.59	0.984	-3.53	0.474	.	.	.	.	.
Dose2	-3.47	0.992	-3.53	0.505	1.000	.	.	.	.
Dose3	-5.82	0.506	-5.82	0.162	0.777	0.747	.	.	.

****
NON-PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Kruskal-Wallis test - equality among treatment groups

Degrees of Freedom	TestStat	P-value
3	0.51	0.916

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	-2.29	.	.

```
  Dose1     -4.15                  0.889                  0.386
  Dose2     -4.05                  0.889                  0.442
  Dose3     -2.67                  0.411                  0.340

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)

059101 Chlorpyrifos 890.1350 48615506
ANALYSIS RESULTS FOR VARIABLE VAR14  ( M testosterone (ng/mL) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.940         0.351          1.114      0.382    USE PARAMETRIC TESTS

****************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     4.13     0.66       0.33       16.09           3.07,    5.18
  Dose1  4     4.02     1.40       0.70       34.87           1.79,    6.24
  Dose2  4     2.34     1.41       0.70       60.15           0.10,    4.58
  Dose3  4     4.22     2.10       1.05       49.82           0.87,    7.57

 Level       Median     Min       Max    %of Negative control(means)   %Reduction(means)
  Ctrl         4.31     3.23       4.66          .                 .
  Dose1        4.43     2.00       5.21       97.27              2.73
  Dose2        2.31     0.85       3.90       56.72             43.28
  Dose3        4.70     1.26       6.22      102.24             -2.24

****************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12               1.45        0.277

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl     4.13    .         4.13      .        .       .       .       .       .
 Dose1    4.02   0.999      4.02     0.538     .       .       .       .       .
 Dose2    2.34   0.257      3.28     0.278    0.417    .       .       .       .
 Dose3    4.22   0.999      3.28     0.288    0.997   0.324    .       .       .

****************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                4.83       0.185

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value          Jonckheere p-value
```

```
  Ctrl       4.31                    .                       .
  Dose1      4.43                1.000                   0.500
  Dose2      2.31                0.103                   0.029
  Dose3      4.70                0.678                   0.320

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)

*****************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12               1.45        0.277

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    -4.13    .        -3.49      .        .       .       .       .       .
 Dose1   -4.02   0.999     -3.49     0.804     .       .       .       .       .
 Dose2   -2.34   0.257     -3.49     0.835    0.417    .       .       .       .
 Dose3   -4.22   0.999     -4.22     0.598    0.997   0.324    .       .       .

*****************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                4.83       0.185

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      -4.31                    .                       .
  Dose1     -4.43                1.000                   0.500
  Dose2     -2.31                0.103                   0.971
  Dose3     -4.70                0.678                   0.680

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)

059101 Chlorpyrifos 890.1350 48615506
ANALYSIS RESULTS FOR VARIABLE VAR15  ( F 17b-estradiol (ng/mL) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.909         0.132          1.398      0.295    USE PARAMETRIC TESTS

*****************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     9.89     3.87       1.93       39.09           3.74,   16.04
```

```
 Dose1  4     8.84     4.03       2.02       45.64          2.42,   15.26
 Dose2  4     6.72     2.80       1.40       41.64          2.27,   11.17
 Dose3  3     8.67     6.13       3.54       70.70         -6.55,   23.89

Level       Median     Min       Max    %of Negative control(means)   %Reduction(means)
 Ctrl         9.86     5.45      14.40             .                          .
 Dose1        9.79     3.68      12.10           89.38                      10.62
 Dose2        6.70     3.53       9.94           67.92                      32.08
 Dose3       11.90     1.60      12.50           87.63                      12.37

***************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           11               0.40        0.756

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl     9.89    .         9.89      .        .       .       .       .       .
 Dose1    8.84   0.970      8.84     0.432     .       .       .       .       .
 Dose2    6.72   0.597      7.55     0.285    0.888    .       .       .       .
 Dose3    8.67   0.963      7.55     0.317    1.000   0.927    .       .       .

***************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                1.44       0.696

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value          Jonckheere p-value
  Ctrl       9.86                   .                        .
  Dose1      9.79                  0.889                    0.386
  Dose2      6.70                  0.346                    0.121
  Dose3     11.90                  1.000                    0.269

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)

***************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           11               0.40        0.756

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    -9.89    .        -8.48      .        .       .       .       .       .
```

Dose1	-8.84	0.970	-8.48	0.764	.	.	.	.	.
Dose2	-6.72	0.597	-8.48	0.797	0.888	.	.	.	.
Dose3	-8.67	0.963	-8.67	0.785	1.000	0.927	.	.	.

****
NON-PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Kruskal-Wallis test - equality among treatment groups

Degrees of Freedom	TestStat	P-value
3	1.44	0.696

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	-9.86	.	.
Dose1	-9.79	0.889	0.614
Dose2	-6.70	0.346	0.879
Dose3	-11.90	1.000	0.731

INCREASING TREND TEST SUMMARY	LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
Williams	>highest dose (no sign. differences)
Jonckheere	>highest dose (no sign. differences)

059101 Chlorpyrifos 890.1350 48615506
ANALYSIS RESULTS FOR VARIABLE VAR16 (M 17b-estradiol (ng/mL))

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.

Shapiro-Wilks Test Stat	Shapiro-Wilks P-value	Levenes Test Stat	Levenes P-value	Conclusion
0.575	<.001	8.924	0.002	USE NON-PARAMETRIC TESTS

****
BASIC SUMMARY STATISTICS

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval
Ctrl	4	0.13	0.00	0.00	0.00	. , .
Dose1	4	0.13	0.00	0.00	0.00	. , .
Dose2	4	3.54	6.71	3.35	189.31	-7.13, 14.21
Dose3	4	0.18	0.07	0.04	39.31	0.07, 0.30

Level	Median	Min	Max	%of Negative control(means)	%Reduction(means)
Ctrl	0.13	0.13	0.13	.	.
Dose1	0.13	0.13	0.13	100.00	0.00
Dose2	0.22	0.13	13.60	2833.80	-2733.80
Dose3	0.17	0.13	0.27	146.80	-46.80

****
PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Analysis of Variance (ANOVA) - overall F-test

Numerator df	Denominator df	F-stat	P-value
3	12	1.03	0.415

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5

```
 Ctrl     0.13    .         1.26      .        .       .       .       .       .
 Dose1    0.13   1.000      1.26     0.766     .       .       .       .       .
 Dose2    3.54   0.375      1.26     0.799    0.499    .       .       .       .
 Dose3    0.18   1.000      0.18     0.647    1.000   0.513    .       .       .

************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                5.03       0.170

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl       0.13                   .                         .
  Dose1      0.13                 1.000                       .
  Dose2      0.22                 0.227                     0.965
  Dose3      0.17                 0.227                     0.958

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)

************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           3           12               1.03        0.415

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    -0.13    .        -0.13      .        .       .       .       .       .
 Dose1   -0.13   1.000     -0.13     0.583     .       .       .       .       .
 Dose2   -3.54   0.375     -1.86     0.305    0.499    .       .       .       .
 Dose3   -0.18   1.000     -1.86     0.316    1.000   0.513    .       .       .

************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           3                5.03       0.170

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      -0.13                   .                         .
  Dose1     -0.13                 1.000                       .
  Dose2     -0.22                 0.227                     0.035
  Dose3     -0.17                 0.227                     0.042

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              Dose1
```

Overall Survival

Fisher's Exact Test

IDENTIFICATION	NUMBER OF DEAD	NUMBER OF ALIVE	TOTAL ANIMALS
CONTROL	4	20	24
0.000251	2	22	24
TOTAL	6	42	48

Critical Fisher's value (24,24,4) (alpha=0.05) is negative. b value is 2.
no significant difference

Fisher's Exact Test

IDENTIFICATION	NUMBER OF DEAD	NUMBER OF ALIVE	TOTAL ANIMALS
CONTROL	4	20	24
0.000812	3	21	24
TOTAL	7	41	48

Critical Fisher's value (24,24,4) (alpha=0.05) is negative. b value is 3.
no significant difference

Fisher's Exact Test

IDENTIFICATION	NUMBER OF DEAD	NUMBER OF ALIVE	NUMBER OF TOTAL ANIMALS
CONTROL	4	20	24
0.00302	2	22	24
TOTAL	6	42	48

Critical Fisher's value (24,24,4) (alpha=0.05) is negative. b value is 2.
no significant difference

Summary of Fisher's Exact Tests

GROUP	IDENTIFICATION	NUMBER EXPOSED	NUMBER DEAD	SIG 0.05
	CONTROL	24	4	
1	0.000251	24	2	
2	0.000812	24	3	
3	0.00302	24	2	

Male Survival

Fisher's Exact Test

IDENTIFICATION	NUMBER OF ALIVE	NUMBER OF DEAD	NUMBER OF TOTAL ANIMALS
CONTROL	7	1	8
0.000251	7	1	8
TOTAL	14	2	16

Critical Fisher's value (8,8,7) (alpha=0.05) is 2.0. b value is 7.
Since b is greater than 2.0 there is no significant difference
between CONTROL and TREATMENT at the 0.05 level.

Fisher's Exact Test

IDENTIFICATION	NUMBER OF ALIVE	NUMBER OF DEAD	TOTAL ANIMALS
CONTROL	7	1	8
0.000812	6	2	8
TOTAL	13	3	16

Critical Fisher's value (8,8,7) (alpha=0.05) is 2.0. b value is 6.
Since b is greater than 2.0 there is no significant difference
between CONTROL and TREATMENT at the 0.05 level.

Fisher's Exact Test

IDENTIFICATION	NUMBER OF ALIVE	NUMBER OF DEAD	TOTAL ANIMALS
CONTROL	7	1	8
0.00302	7	1	8
TOTAL	14	2	16

Critical Fisher's value (8,8,7) (alpha=0.05) is 2.0. b value is 7.
Since b is greater than 2.0 there is no significant difference
between CONTROL and TREATMENT at the 0.05 level.

Summary of Fisher's Exact Tests

GROUP	IDENTIFICATION	NUMBER EXPOSED	NUMBER DEAD	SIG 0.05
	CONTROL	8	1	
1	0.000251	8	1	
2	0.000812	8	2	
3	0.00302	8	1	

DATA EVALUATION RECORD

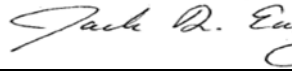
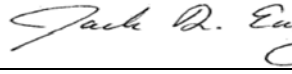
CHLORPYRIFOS

Study Type: OCSPP 890.1400, *In vivo* Hershberger Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-14-2012 (MRID 48615507)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CSS-Dynamac Corporation
1910 Sedwick Road,
Building 100, Suite B
Durham, NC 27713

Primary Reviewer Kelly Luck, M.S.	Signature: Date:	 01/20/2012
Secondary Reviewer Michael E. Viana, Ph.D., D.A.B.T.	Signature: Date:	 01/30/2012
Program Manager: Jack D. Early, M.S.	Signature: Date:	 2/02/2012
Quality Assurance: Jack D. Early, M.S.	Signature: Date:	 2/02/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel**.**

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Greg Akerman, Ph.D. **Signature:**
Health Effects Division **Date:** 5/28/13
Secondary Reviewer: John Liccione, Ph.D **Signature:**
Health Effects Division **Date:** 6/5/13

Template version 10/2011

DATA EVALUATION RECORD

STUDY TYPE: *In Vivo* Hershberger Assay (Rat); OCSPP 890.1400; OECD 441

PC CODE: 059101 **DP BARCODE:** D397128

TXR#: 0052086 **CAS#:** 2921-88-2

TEST MATERIAL (PURITY): Chlorpyrifos (99.8% a.i.)

SYNONYMS: Chlorpyrifos-ethyl; Chlorpyriphos; Chlorpyrifos; *O,O*-Diethyl *O*-(3,5,6-trichloro-2-pyridinyl)ester phosphorothioic acid

CITATION: Marty, M.S. and Marshall, V.A. (2011) Chlorpyrifos: Hershberger Assay in Castrated Male CrL:CD(SD) Rats. Toxicology & Environmental Research and Consulting, Dow Chemical Company, Midland, MI. Laboratory Project Study ID: 101152, October 25, 2011. MRID 48615507. Unpublished.

SPONSOR: Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN

TEST ORDER #: CON-059101-4

EXECUTIVE SUMMARY: In a Hershberger assay (MRID 48615507) screening for androgenic activity, chlorpyrifos (99.8% a.i., lot# KC28161419) in corn oil was administered daily via oral gavage to seven 55-day old, castrated male Sprague Dawley rats at dose levels of 0 (vehicle), 1, 6, or 12 mg/kg/day. An androgenic positive control group consisted of seven castrated rats exposed to 0.4 mg/kg/day of testosterone propionate (TP) by subcutaneous (s.c.) injection.

To screen for potential anti-androgenic activity, chlorpyrifos (99.8%, lot# KC28161419) in corn oil was administered daily via oral gavage to seven 55-day old, castrated male Sprague Dawley rats at dose levels of 0 (vehicle), 1, 6, or 12 mg/kg/day in conjunction with a daily dose of reference androgen TP at 0.4 mg/kg/day by s.c. injection. The anti-androgenic positive control group consisted of seven castrated rats exposed to 0.4 mg/kg/day TP and 3 mg/kg/day flutamide (FT). TP alone was used as the anti-androgenic negative control.

For both components of the assay, body weights were determined daily, and food consumption was measured at 3- to 4-day intervals during dosing. The animals were dosed for 10 consecutive days and terminated approximately 24 hours after the final dose administration. At necropsy, the five androgen-dependent tissues, liver, adrenals, and kidneys were collected and weighed. Brain and red blood cell (RBC) samples were also collected for determination of cholinesterase (ChE) activity.

All animals survived until scheduled termination. There were no clinical signs of toxicity and no treatment-related gross pathological findings.

In the androgen agonist assay, there were no significant effects on body weights or body weight gains in the chlorpyrifos treated groups. Rats dosed with TP (positive control) had increased ($p<0.05$) terminal body weights (↑6%), resulting in increased body weight gains (↑29%) compared to the controls. Food consumption for Days 4-7 was increased ($p<0.05$) by 7% in the 12 mg/kg/day group; this followed a period of decreased body weight gain (not statistically significant, NS) in this group, and thus reduced the effects on body weight gain. Food consumption for Days 7-11 was decreased ($p<0.05$) in the 6 mg/kg/day group (↓5%); however, this change was not considered biologically meaningful because it was not dose related.

Kidney and liver weights in all chlorpyrifos groups were comparable to vehicle controls. Rats in the 12 mg/kg/day group had increased ($p<0.05$) adrenal glands weights (↑38%); adrenal glands weights in the 1 and 6 mg/kg/day dose groups were comparable to the controls. ChE activity in RBC was significantly decreased ($p<0.05$) at 1 mg/kg/day (90% inhibition) and below the level of detection at 6 and 12 mg/kg/day. Brain ChE activity levels were significantly decreased ($p<0.05$) at 6 and 12 mg/kg/day (64% and 79% inhibition). There were no increases in accessory sex organ weights at any dose in the chlorpyrifos treated animals. Rats in the positive control (TP) group responded properly with accessory sex organ weight increases ($p<0.05$) in all five target tissues.

In the anti-androgen assay, there were no effects on body weights, body weight gains, or food consumption in the chlorpyrifos + TP treated groups when compared to the TP control. Rats in the 6 and 12 mg/kg/day had increased ($p<0.05$) adrenal glands weights (↑15% and ↑20%) compared to controls. Adrenal weights in the 1 mg/kg/day group, and kidney and liver weights in all chlorpyrifos treatment groups were comparable to controls. RBC ChE activity levels were significantly decreased ($p<0.05$) at 1 mg/kg/day (91% inhibition) and were below the level of detection at 6 and 12 mg/kg/day. Brain ChE activity levels were significantly decreased ($p<0.05$) at 6 and 12 mg/kg/day (60% and 76% inhibition). Rats in the 6 and 12 mg/kg/day groups had decreased ($p<0.05$) glans penis weights (↓6% and ↓8%) compared to controls; glans penis weights in the 1 mg/kg/day group were comparable to controls. The weights of the four other accessory sex organs in the chlorpyrifos treatment groups were comparable to vehicle controls. Rats in the positive control (TP + FT) group responded appropriately with significant decreases in all five of the target accessory sex organ weights. All CV values were less than the maximum recommended values for each organ, with the exception of levator ani-bulbocavernosus (LABC) in the 12 mg/kg/day group (33% CV compared to maximum recommended CV of 20%).

The doses tested were judged to be adequate based on the observed RBC and brain cholinesterase activity.

Statistically significant changes were not seen in two or more of the five androgen sensitive tissue weights. Chlorpyrifos was negative for androgenicity and anti-androgenicity in the Hershberger assay.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for a Hershberger assay (OCSPP 890.1400).

COMPLIANCE: Signed and dated GLP Compliance and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Facility:

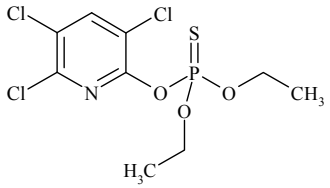
Dow Chemical Company, Toxicology & Environmental Research and Consulting

Location: Midland, MI
Study Director: V.A. Marshall
Other Personnel: M.S. Marty (Lead Scientist)
Study Period: January 3-October 25, 2011

2. Test Substance:

Chlorpyrifos

Description: Crystalline solid, light tan; Molecular weight = 350.6 g/mol
Source: Dow AgroSciences LLC (Indianapolis, IN))
Lot #: KC28161419 (TSN101285)
Purity: 99.8%
Stability: Stable in corn oil for up to 12 days (temperature not reported)
CAS #: 2921-88-2
Structure:



3. Reference Androgen:

Testosterone propionate (TP)

Supplier: Sigma-Aldrich (St. Louis, MO)
Lot #: 048K1328 (expiration date not provided)
Purity: ≥97%
CAS #: 57-85-2

4. Reference Anti-androgen:

Flutamide (FT)

Supplier: Sigma-Aldrich (St. Louis, MO)
Lot #: 099K1112 (expiration date not provided)
Purity: >99 %
CAS #: 1311-84-7

5. Solvent/Vehicle Control:

Corn Oil

Supplier: Sigma-Aldrich (St. Louis, MO)
Lot/Batch #: Not provided
Rationale (if other than water): Selected due to the solubility properties of the test substance
Final concentration: 4 ml/kg bw (chlorpyrifos and flutamide); 0.5 ml/kg bw (s.c. TP)

6. Test Animals:

Species:	Rat (males only)
Strain:	Sprague Dawley (Crl:CD[SD])
Age/weight at dose initiation:	Post-natal day (PND) 55/ 235.0 – 298.7 g
Source:	Charles River Laboratories (Portage, MI)
Housing:	Rats were housed 2-3 per cage in stainless steel cages with wire mesh floors suspended above absorbent paper; non-woven gauze was placed in the cages as a cushion from the flooring.
Diet:	LabDiet Certified Rodent Diet #5002 (PMI Nutrition International, St. Louis, MO), *ad libitum* Phytoestrogen content was not reported
Water:	Tap water, *ad libitum*
Environmental conditions:	**Temperature:** 22 ± 3 ºC **Humidity:** 40-70% **Air changes:** 12-15/hr **Photoperiod:** 12 hrs light/12 hrs dark
Acclimation period:	Rats were castrated at the supplier at PND 45 and received at the study facility at PND 49-50; The post-castration acclimation period was 10 days (5-6 days at study facility)

B. STUDY DESIGN

1. In-life dates: Start: January 9-10, 2011 End: January 20-21, 2011

2. Study Design: In a Hershberger Assay conducted to screen for potential androgenic activity, the test substance was administered daily via oral gavage to castrated male rats. Positive androgenic activity is defined as a significant increase in two or more target organ weights compared to the vehicle control. Additionally, in a Hershberger Assay conducted to screen for the potential anti-androgenic activity, the test substance was administered daily via oral gavage to castrated male rats in conjunction with a daily dose of TP (0.4 mg/kg/day) by s.c. injection. Anti-androgenic activity is indicated by a statistically significant decrease in two or more target organ weights of the treated groups (test substance + TP) compared to the TP-only control group. For both assays, the animals were dosed for 10 consecutive days and necropsied approximately 24 hours after the final dose administration for organ weight measurements. In addition to the required endpoints, Approximately 6-7 hours after the last dose on Day 10, a blood sample was collected (via jugular vein) for RBC cholinesterase (ChE) measurements. This time point was selected for peak RBC ChE inhibition. At necropsy, brain samples were collected for brain ChE measurements. Liver, kidney and adrenal weights were also collected to assess toxicity.

3. Study Schedule: Sexually mature male rats were castrated on PND 45 by the supplier (Charles River Laboratories) according to standard procedures and allowed 10 days for recovery and regression of accessory sex organ weights prior to initiation of dosing. The dose administration period was from PND 55 through PND 64. Rats were euthanized on PND 65 approximately 24 hours after the last dose and necropsied for organ weight measurements. .

4. Animal Assignment: Animals were randomly assigned, stratified by body weight, to the test groups noted in Table 1. The study authors stated that animal assignment was conducted using a computer program designed to increase the probability of uniform group

mean weights and standard deviations at the start of study. However, the study authors did not report whether the body weight of each animal was within ±20% of the overall mean.

TABLE 1. Study Design [a]		
Test group	**Dose (mg/kg/day)**	**# of Males**
Androgen Agonist Assay		
Vehicle control (negative control)	0	7
Low	1	7
Mid	6	7
High	12	7
Testosterone propionate (TP), positive control[b]	0.4	7
Anti-Androgen Assay		
Vehicle control (+TP)[b,c]	0	7
Low (+TP[c])	1	7
Mid (+TP[c])	6	7
High (+TP[c])	12	7
Flutamide + TP[b], positive control	3	7

a Data were obtained from Table 1 on page 18 of the study report.
b Same animals dosed for both androgen agonist (served as positive control) and anti-androgen (served as vehicle control) assays. This did not affect the outcome or conclusions of this study.
c TP administered once daily by s.c. on all test days at 0.4 mg/kg/day.

5. **Dose Selection Rationale:** The dose levels were selected based on the results from a probe study[1] in which male and female rats were administered the chlorpyrifos in corn oil via gavage at doses of 0, 1, 2, 4, or 8 mg/kg/day for 15 days. Decreased body weight gains were observed in females at the 4 and 8 mg/kg/day dose groups. There were no treatment-related differences in clinical chemistry parameters in males or females with the exception of a decrease in alanine aminotransferase levels in females at 4 and 8 mg/kg/day chlorpyrifos. There were no effects on liver or kidney weights in male or female rats, although relative adrenal weights were increased at 4 and 8 mg/kg/day in females only. There was significant inhibition ChE level in RBC at all doses of chlorpyrifos in both males and females. RBC ChE was inhibited by 75-81% at 1 mg/kg/day and ≥ 95% at doses greater than 2 mg/kg/day. Males and females had significant decreases in brain ChE at doses ≥2 mg/kg/day. It was stated that administration of 12 mg/kg/day was expected to produce some systemic toxicity and substantial inhibition of both RBC and brain ChE without causing death or severe suffering, and that the lower dose levels were chosen to establish a dose response for any observed effects.

6. **(a) Dose Preparation:** Dose formulations were prepared by mixing appropriate amounts of test substance with corn oil. Chlorpyrifos dosing solutions were reportedly prepared in accordance with established stability limits. Dose volumes were adjusted daily based on individual body weight measurements. Prior to dose administration, samples of chlorpyrifos dose formulations from all three dose levels were analyzed for achieved concentration and

[1] Marty, M. S. and Marshall, V. A. (In progress). Chlorpyrifos: Hershberger, Uterotrophic, and Pubertal Assay Probe Study in Crl:CD(SD) Rats. Report of Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan.

samples from the low and high dose formulations were tested for homogeneity; samples were taken from the top, middle, and bottom of the container after stirring overnight. In a previous study,[2] chlorpyrifos was determined to be stable in corn oil for up to 12 days at concentrations ranging 0.00356-9.985 mg/mL (temperature not specified).

(**b) Dose Analysis**

Results

Homogeneity (%RSD): 0.5-0.8%

Stability (% of Day 0): Not provided

Concentration (% of nominal): 95.6-99.1%

The analytical data indicated that the mixing procedure was adequate and that the variation between nominal and actual dosage to the animals was acceptable.

7. Dosage administration: Test formulations were administered to the animals daily via oral gavage (dose volume 4 mL/kg bw) for 10 days. TP was given via subcutaneous (s.c.) injection (dose volume 0.5 mL/kg bw), and FT was administered via oral gavage (dose volume 4 mL/kg bw).

8. Statistics: Body weights, body weight gains, absolute organ weights, feed consumption, and ChE activity were first analyzed by Bartlett's test for equality of variance. Depending on the results of Bartlett's test, a parametric or non-parametric ANOVA was performed. For the accessory sex organs, if the ANOVA was significant, analysis using one-sided Dunnett's test (upper for androgenicity and lower for ant-androgenicity) was performed. For body weights, body weight gains, absolute organ weights, feed consumption, and ChE activity, if the ANOVA was significant, a one-sided Dunnett's test or the Wilcoxon Rank-Sum test, with Bonferroni's correction, was performed. Because there were no significant changes in terminal body weights in this study, the potential effects of body weight on organ weights were not considered; a previous feed restriction study[3] had established that most organ weights in the Hershberger assay were relatively insensitive to body weight changes. Significance was denoted at $p \leq 0.05$. The statistical analyses were considered to be adequate.

C. METHODS

1. Clinical Examinations: Cage-side examinations for mortality, moribundity, and clinical abnormalities were conducted twice daily. During dosing, hand-held examinations were conducted daily (at approximately six hours after dosing, the anticipated time of peak

[2] Marty, M. S. and Andrus, A. K. (2010). Comparison of cholinesterase (ChE) inhibition in young adult and preweanling CD rats after acute and repeated chlorpyrifos or chlorpyrifos-oxon exposures. Report of Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan.
[3] Marty, M. S., Johnson, K. A., and Carney E. W. (2003). Effect of Feed Restriction on Hershberger and Pubertal Male Assay Endpoints. Birth Defects Res B 68, 363-374.

effects) for clinical signs of toxicity and alterations in behavior or demeanor; the final examination was conducted prior to necropsy.

2. **Body Weight:** Animals were weighed at randomization and daily throughout the dosing period. Statistical analyses of body weights were conducted using data collected on Days 1, 4, 7, and 11 (terminal body weight).

3. **Food Consumption:** Food consumption was measured for each cage by subtracting the amount of food remaining in the cage from the amount supplied. Food consumption was measured for the following intervals: Days 1 to 4, Days 4 to 7, and Days 7 to 11. Values were reported as group mean daily food consumption (g/animal/day).

4. **Serum Hormone Measurements (Optional):** At study termination, each animal was anesthetized with isofluorane/oxygen and blood was collected via cardiac puncture for potential serum hormone analyses; however, serum hormone measurements were not conducted.

5. **Dissection and Measurement of Tissue and Organ Weights:** On PND 65 (approximately 24 hours after the final administration of the test substance), all surviving animals were anesthetized and blood samples were collected by cardiac puncture. The rats were then euthanized by decapitation and subjected to a gross necropsy. The brain was removed, rinsed with saline, and blotted dry. The five mandatory androgen-dependent organs (ventral prostate, seminal vesicles, LABC, Cowper's gland, and glans penis) were excised, trimmed of fat and connective tissue, and weighed according to the standard operating procedures detailed in the U.S. EPA Guideline (OCSPP 890.1400). Additionally, the liver, kidneys, and adrenals were weighed. Portions of the liver were preserved in neutral, phosphate-buffered 10% formalin for possible histological evaluation, and a separate portion was frozen for possible evaluation of liver enzyme induction.

   On the day of necropsy, rats were examined for preputial separation.

   In addition to the terminal blood samples, blood samples were collected from the jugular veins of unanesthetized rats (control and chlorpyrifos-treated) approximately 6-7 after dosing. All blood samples were centrifuged and packed RBCs were collected, diluted in 1% Triton X-100, and stored at -80 °C. Brain samples were dissected into right and left hemispheres and the right hemisphere was weighed. Both hemispheres were stored at -80 °C. RBC and right hemisphere samples were sent to WIL Research Laboratories (Ashland, OH) for ChE analyses.

6. **Assessment of RBC ChE Activity:** Blood was collected for RBC ChE activity from control and treated animals. Blood samples were 6-7 h following the final dose by rapidly collecting blood via the jugular vein. Terminal blood samples were collected from anesthetized animals at necropsy. RBC samples were collected from all control and chlorpyrifos-treated animals. Blood samples were placed on ice, and centrifuged to harvest the packed RBC. RBCs were diluted 1:20 in 1% Triton X-100 and shipped to contract research laboratory for ChE analyses (WIL Research Laboratories, Ashland, OH). RBC ChE activity was determined using an assay based on the modified Ellman reaction.

7. Assessment of Brain ChE Activity: Following decapitation at necropsy, the brain cavity for each animal (flutamide treated animals were not analyzed for brain ChE activity) was opened and the brain was removed, rinsed with saline and blotted. The brain was dissected into right and left hemisphere. The weight was recorded for the right hemisphere. Both hemispheres were quick frozen in liquid nitrogen and stored at -80° C for brain ChE activity. The right hemispheres were shipped to a contract research laboratory for ChE analyses (WIL Research Laboratories, Ashland, OH). Brain ChE activity was measured using an assay based on the modified Ellman reaction.

II. RESULTS

A. OBSERVATIONS

1. Mortality: All animals survived until scheduled termination.

2. Clinical signs of toxicity: No clinical signs of toxicity were observed in any of the dose groups.

B. BODY WEIGHT AND WEIGHT GAIN: Selected body weight and body weight gain data for the androgen agonist assay are presented in Table 2. At 12 mg/kg/day, body weight gains during Days 1-4 were decreased ($p<0.05$) by 27%. As overall (Days 1-11) body weight gains were similar to controls, this single decrease was not considered adverse. Body weights and body weights gains in the 1 and 6 mg/kg/day treatment groups were comparable to controls throughout the duration of the study. Terminal body weights were significantly increased ($p<0.05$) in rats dosed with 0.4 mg/kg/day TP (↑6%), resulting in increased body weight gains (↑29%) compared to the controls.

TABLE 2. Selected Group Mean Body Weights and Cumulative Body Weight Gains (g) in the Androgen Agonist Assay [a]

Study Day	Dose (mg/kg/day)														
	Vehicle Control			TP (0.4)			Chlorpyrifos (1)			Chlorpyrifos (6)			Chlorpyrifos (12)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
1	7	264.5	15.6	7	265.9	10.2	7	263.7	16.2	7	267.9	18.8	7	270.0	11.7
4	7	287.8	17.2	7	292.7	9.6	7	288.6	19.0	7	288.9	19.7	7	286.9	11.5
7	7	307.3	17.4	7	320.3	10.2	7	310.2	19.1	7	308.9	21.1	7	309.6	14.5
11	7	327.7	19.0	7	347.5* (↑6)	12.0	7	331.4	23.0	7	330.8	27.0	7	328.0	12.6
Body Weight Gain (Days 1-11)	7	63.2	5.2	7	81.6* (↑29)	7.4	7	67.7	9.8	7	62.9	12.0	7	58.1	8.0

a Data were obtained from Table 4 on pages 39 and 40 of the study report. Percent differences from controls were calculated by the reviewers and included in parentheses.
N Number of animals in the group
SD Standard Deviation
* Significantly different from controls at $p<0.05$

Selected body weight and body weight gain data for the anti-androgen assay are presented in Table 3. Body weights and body weights gains for all chlorpyrifos-treated groups and the

positive control group were comparable to the vehicle control throughout the duration of the study.

TABLE 3. Selected Group Mean Body Weights and Cumulative Body Weight Gains (g) in the Anti-Androgen Assay [a]

Study Day	Dose (mg/kg/day)														
	Vehicle Control + TP (0.4)			FT (3) + TP (0.4) Positive Control			Chlorpyrifos (1) + TP (0.4)			Chlorpyrifos (6) + TP (0.4)			Chlorpyrifos (12) + TP (0.4)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
1	7	265.9	10.2	7	265.9	9.7	7	266.6	15.8	7	267.5	11.1	7	266.8	12.8
4	7	292.7	9.6	7	290.4	12.1	7	293.5	17.2	7	293.6	14.1	7	289.8	15.0
7	7	320.3	10.2	7	315.6	11.1	7	319.9	17.7	7	320.9	16.3	7	314.1	14.7
11	7	347.5	12.0	7	346.2	11.1	7	347.0	20.3	7	347.2	17.4	7	339.6	16.7
Body Weight Gain (Days 1-11)	7	81.6	7.4	7	80.4	3.5	7	80.3	8.7	7	79.7	9.3	7	72.8	9.2

a Data were obtained from Table 4 on pages 41 and 42 of the study report.
N Number of animals in the group
SD Standard Deviation

C. <u>FOOD CONSUMPTION</u>: Food consumption data for the androgen agonist assay and anti-androgen assay are presented in Tables 4 and 5, respectively. For the androgen agonist assay, food consumption on Days 4-7 was increased ($p<0.05$) by 7% in the 12 mg/kg/day group; however, this minor change was not considered adverse. Food consumption on Days 7-11 was decreased ($p<0.05$) in the 6 mg/kg/day group (↓5%); because it was not dose related, it was not considered biologically meaningful. Food consumption in the 1 mg/kg/day group was comparable to the vehicle control over the duration of the study. Food consumption on Days 4-7 and 7-11 was increased ($p<0.05$) in the positive control group (↑9% and ↑10%, respectively) compared to the vehicle control group.

TABLE 4. Food Consumption (g/animal/day) in the Androgen Agonist Assay[a]

Study Days	Dose (mg/kg/day)														
	Vehicle Control			TP (0.4)			Chlorpyrifos (1)			Chlorpyrifos (6)			Chlorpyrifos (12)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
1-4	7	21.4	0.8	7	21.2	1.1	7	21.1	1.2	7	20.3	0.6	7	21.3	0.5
4-7	7	21.0	1.0	7	22.8* (↑9)	1.0	7	21.2	0.8	7	20.6	0.8	7	22.5* (↑7)	0.9
7-11	7	20.9	1.0	7	23.0* (↑10)	0.5	7	20.7	0.4	7	19.9* (↓5)	0.7	7	21.1	0.6

a Data were obtained from Table 5 on pages 43 and 44 of the study report. Percent differences from controls were calculated by the reviewers and included in parentheses.
N Number of animals in the group
SD Standard Deviation
* Significantly different from controls at $p<0.05$

In the anti-androgen assay, food consumption in all chlorpyrifos-treated groups was comparable to the vehicle control. Food consumption was increased ($p<0.05$) by 5% on Days 4-7 in the positive control group compared to the vehicle controls.

TABLE 5. Food Consumption (g/animal/day) in the Anti-Androgen Assay [a]

Study Days	Dose (mg/kg/day)														
	Vehicle Control + TP (0.4)			FT (3) + TP (0.4) Positive Control			Chlorpyrifos (1) + TP (0.4)			Chlorpyrifos (6) + TP (0.4)			Chlorpyrifos (12) + TP (0.4)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
1-4	7	21.2	1.1	7	20.4	0.2	7	21.2	1.0	7	21.5	0.4	7	21.9	1.1
4-7	7	22.8	1.0	7	21.5* (↑5)	0.6	7	22.5	1.1	7	23.6	0.5	7	22.5	1.1
7-11	7	23.0	0.5	7	22.3	0.8	7	22.4	1.0	7	23.1	0.5	7	23.4	1.2

a Data were obtained from Table on pages 45 and 46 of the study report. Percent differences from controls were calculated by the reviewers and included in parentheses.
N Number of animals in the group
SD Standard Deviation
* Significantly different from controls at $p<0.05$

D. SERUM HORMONE CONCENTRATIONS (OPTIONAL): Serum hormone concentrations were not determined.

E. ORGAN WEIGHTS: Accessory sex organ, adrenal, kidney, and liver weights for the androgen agonist assay are presented in Table 6. Kidney and liver weights in all chlorpyrifos treatment groups were comparable to vehicle controls. Rats in the 12 mg/kg/day group had increased ($p<0.05$) adrenal glands weights (↑38%); adrenal glands weights in the 1 and 6 mg/kg/day dose groups were comparable to the controls. There were no significant increases in accessory sex organ weights at any dose of chlorpyrifos.

Rats in the positive control (TP) group had accessory sex organ weight increases ($p<0.05$) as follows: 1221% in seminal vesicles; 843% in ventral prostate; 143% in LABC; 678% in Cowper's glands; and 72% in glans penis. Adrenal glands, kidney, and liver weights in the positive control group were comparable to the vehicle control.

The coefficients of variance (CVs) for the accessory sex organs in the control and high dose (12 mg/kg/day) groups were compared to the performance criteria in the Guideline; all CV values were less than the maximum recommended values for each organ as stated in the test guideline.

TABLE 6. Accessory Sex Organ Weights (mg), Adrenal Glands Weights (mg), and Kidney and Liver Weights (g) from the Androgen Agonist Assay in Sprague Dawley Rats [a]

Organ	Dose (mg/kg/day)																
	Vehicle control				Chlorpyrifos (1)			Chlorpyrifos (6)			Chlorpyrifos (12)				TP (0.4)		
	N	Mean	SD	CV (%)	N	Mean	SD	N	Mean	SD	N	Mean	SD	CV (%)	N	Mean	SD
Seminal vesicles	7	41.0	9.8	24	7	45.4	9.9	7	46.7	8.0	7	47.0	5.8	12	7	541.5* (↑1221)	55.1
Ventral prostate	7	19.4	5.9	30	7	25.2	8.3	7	22.1	3.3	7	24.5	4.2	17	7	183.0* (↑843)	46.9
LABC	6[b]	119.1	11.8	10	7	135.1	19.4	7	144.1	36.8	6[b]	112.7	16.6	15	6[b]	289.8* (↑143)	45.6
Cowper's glands	7	4.9	1.1	22	7	5.9	1.1	7	6.1	1.6	7	6.5	1.9	29	7	38.1* (↑678)	4.4
Glans penis	7	53.0	8.7	16	7	49.9	7.0	7	48.7	5.7	7	51.7	6.5	13	7	91.2* (↑72)	6.0
Adrenal glands	7	60.4	10.5	NA	7	68.2	8.3	7	66.5	6.1	7	83.5* (↑38)	10.8	NA	7	59.2	3.2
Kidneys	7	2.03	0.17	NA	7	2.07	0.18	7	2.10	0.19	7	2.10	0.06	NA	7	2.16	0.14
Liver	7	12.8	1.2	NA	7	12.9	1.1	7	12.5	1.4	7	13.0	0.4	NA	7	13.2	1.2

a Data were obtained from Tables 8 and 9 on pages 51, 52, and 55 of the study report. Percent differences from controls were calculated by the reviewers and included in parentheses.
b One sample excluded due to weighing error at necropsy.
N Number of animals in the group
SD Standard Deviation
CV Coefficient of Variation
* Significantly different from controls at $p<0.05$
NA Not applicable

Accessory sex organ weights and liver weights for the anti-androgen agonist assay are presented in Table 7. Rats in the 6 and 12 mg/kg/day had increased ($p<0.05$) adrenal glands weights (↑15% and ↑20%, respectively). No treatment related effects on kidney or liver weights were observed at any dose.

Statistically significant decreases in glans penis weights were observed at 6 and 12 mg/kg/day chlorpyrifos when co-administered TP. No significant decreases were seen in the other four target accessory sex tissues.

Rats in the positive control (FT) group had accessory sex organ weight decreases ($p<0.05$) as follows: 81% in seminal vesicles; 76% in ventral prostate; 36% in LABC; 64% in Cowper's glands; and 29% in glans penis. Rats in the positive control group also had increased ($p<0.05$) adrenal glands weights (↑9) compared to the negative control; kidney and liver weights in this group were comparable to the negative control.

The CVs for the accessory sex organs in the control and high-dose groups were compared to the performance criteria in the Guideline; all CV values were less than the maximum recommended values for each organ, with the exception of LABC in the 12 mg/kg/day group (33% CV compared to maximum permissible CV of 20%).

TABLE 7. Accessory Sex Organ Weights (mg), Adrenal Glands Weights (mg), and Kidney and Liver Weights (g) from the Anti-Androgen Assay in Sprague Dawley Rats [a]

Organ	Dose (mg/kg/day)																
	Vehicle Control + TP (0.4)				Chlorpyrifos (1) + TP (0.4)			Chlorpyrifos (6) + TP (0.4)			Chlorpyrifos (12) + TP (0.4)				FT (3) + TP (0.4) Positive Control		
	N	Mean	SD	CV (%)	N	Mean	SD	N	Mean	SD	N	Mean	SD	CV (%)	N	Mean	SD
Seminal vesicles	7	541.5	55.1	10	7	564.2	170.9	7	423.7	107.8	7	434.1	127.2	29	7	100.8* (↓81)	27.8
Ventral prostate	7	183.0	46.9	26	7	209.6	49.5	7	138.1	35.1	7	167.5	30.0	18	7	43.4* (↓76)	10.1
LABC	6[b]	289.8	45.6	16	7	280.8	29.5	7	265.8	66.3	6[b]	263.6	87.3	33	7	185.4* (↓36)	56.2
Cowper's glands	7	38.1	4.4	12	6[c]	38.9	8.8	7	33.5	12.7	7	32.4	3.8	12	7	13.7* (↓64)	3.1
Glans penis	7	91.2	6.0	7	7	90.8	3.6	7	85.8* (↓6)	4.5	7	84.1* (↓8)	3.3	4	7	65.2* (↓29)	7.5
Adrenal glands	7	59.2	3.2	NA	7	61.1	4.5	7	68.2* (↑15)	8.4	7	70.8* (↑20)	8.0	NA	7	64.8* (↑9)	4.5
Kidneys	7	2.16	0.14	NA	7	2.18	0.13	7	2.19	0.21	7	2.24	0.12	NA	7	2.22	0.19
Liver	7	13.2	1.2	NA	7	14.1	1.0	7	14.5	1.5	7	13.7	1.1	NA	7	14.1	0.5

a Data were obtained from Tables 8 and 10 on pages 53, 54, and 56 of the study report. Percent differences from controls were calculated by the reviewers and included in parentheses.
b One sample excluded due to weighing error at necropsy.
c One sample excluded because fluids were lost at necropsy.
N Number of animals in the group
SD Standard Deviation
CV Coefficient of Variation
* Significantly different from controls at $p<0.05$
NA Not applicable

It was reported that preputial separation occurred in all test animals prior to scheduled necropsy. There were no treatment-related gross observations at necropsy in rats from any of the dose groups, including all positive controls. No histological evaluations or evaluation of liver enzyme induction were conducted.

F. <u>CHOLINESTERASE ACTIVITY</u>: Cholinesterase activity data for the androgen agonist and anti-androgen assays are presented in Tables 8 and 9. For both assays, RBC ChE levels were significantly decreased ($p<0.05$) at 1 mg/kg/day (90-91% inhibition) and below the level of detection at 6 and 12 mg/kg/day. Brain ChE levels were significantly decreased ($p<0.05$) at 6 and 12 mg/kg/day (60-64% and 76-79% inhibition, respectively). Brain ChE levels were comparable to the controls at 1 mg/kg/day in both assays.

TABLE 8. Cholinesterase Activity from the Androgen Agonist Assay in Sprague Dawley Rats[a]

Parameter	Dose (mg/kg/day)											
	Vehicle control			Chlorpyrifos (1)			Chlorpyrifos (6)			Chlorpyrifos (12)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
RBC, U/L cholinesterase	7	4307	1095	7	438* (↓90)	530	7	10 [b,*]	0.0	7	10 [b,*]	0.0
Brain, U/L cholinesterase	7	51928	2166	7	49727	1706	7	18731* (↓64)	1425	7	10836* (↓79)	1505

a Data were obtained from Table 6 and Table S1, Appendix 2, on pages 47 and 184 of the study report. Percent differences from controls were calculated by the reviewers and included in parentheses.
b Cholinesterase values below instrument range were assigned a value of 10 U/L (half the lower limit of quantitation) for statistical analysis and reporting.
N Number of animals in the group
SD Standard deviation
* Significantly different from controls at $p<0.05$.

TABLE 9. Cholinesterase Activity from the Anti-Androgen Assay in Sprague Dawley Rats[a]

Parameter	Dose (mg/kg/day)											
	Vehicle control			Chlorpyrifos (1)			Chlorpyrifos (6)			Chlorpyrifos (12)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
RBC, U/L cholinesterase	7	4343	676	7	395* (↓91)	341	7	10 [b,*]	0.0	7	10 [b,*]	0.0
Brain, U/L cholinesterase	7	52519	1189	7	53620	4254	7	20778* (↓60)	2868	7	12576* (↓76)	2000

a Data were obtained from Table 6 and Table S1, Appendix 2, on pages 48 and 184 of the study report. Percent differences from controls were calculated by the reviewers and included in parentheses.
b Cholinesterase values below instrument range were assigned a value of 10 U/L (half the lower limit of quantitation) for statistical analysis and reporting.
N Number of animals in the group
SD Standard deviation
* Significantly different from controls at $p<0.05$.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATOR'S CONCLUSIONS: There were no clinical signs associated with chlorpyrifos treatment at doses ≤ 12 mg/kg/day. Body weights were not significantly affected at any dose level and body weight gains were not affected at ≤ 6 mg/kg/day chlorpyrifos. There were decreases in body weight gains (15-28%) from Days 1-4 at 12 mg/kg/day chlorpyrifos in the presence or absence of TP; however, this effect was not sustained as body weight gains were decreased by ≤ 11% in these groups over the entire dosing period. The improvement in body weight gains was attributed to increases in feed consumption in these animals. All study animals completed preputial separation prior to necropsy. At necropsy, there were no gross lesions attributed to treatment with chlorpyrifos. There were significant, treatment-related increases in absolute adrenal weights at 6 and/or 12 mg/kg/day chlorpyrifos with and without TP. Accessory sex organ weights were not significantly altered at any dose of chlorpyrifos in the androgenic portion of the assay. There was a small, but significant, decrease in glans penis weights at ≥ 6 mg/kg/day chlorpyrifos with TP; however, there were no significant decreases in any other accessory sex organ weights at ≤ 12 mg/kg/day chlorpyrifos in the anti-androgenic portion of the assay. There were no effects on liver or kidney weights at any dose of chlorpyrifos. In both the presence and absence of TP, RBC ChE activity was significantly decreased at all doses of chlorpyrifos and brain ChE activity was significantly decreased at ≥ 6 mg/kg/day

chlorpyrifos. The positive and negative control compounds produced the expected responses: the positive control for androgenicity (0.4 mg/kg/day TP) significantly increased body weight gains and weights in all five accessory sex organs, whereas the positive anti-androgen control (0.4 mg/kg/day TP plus 3 mg/kg/day flutamide) significantly mitigated TP-induced weight increases in all five accessory sex organs and increased adrenal weights. This assay met the CV performance criteria outlined in the corresponding test guidelines. Thus the assay CV values, coupled with a positive androgenic response for TP and a positive anti-androgenic response for flutamide, indicate that the Hershberger assay had appropriate sensitivity to detect androgenic/anti-androgenic effects if these effects had occurred.

Based on the lack of statistically significant, treatment-related changes in two accessory sex organ weights, chlorpyrifos at doses ≤ 12 mg/kg/day was deemed negative for both androgenic and anti-androgenic activity in the Hershberger assay. ChE activity, which was significantly inhibited in RBCs at ≥ 1 mg/kg/day and in brain at ≥ 6 mg/kg/day chlorpyrifos, remains a highly sensitive endpoint to detect chlorpyrifos exposure and toxicity.

B. AGENCY COMMENTS: All animals survived until scheduled termination. There were no clinical signs of toxicity and no treatment-related gross pathological findings.

In the androgen agonist assay, there were no significant effects on body weights or body weight gains in the 1, 6, or 12 mg/kg/day treatment groups. Food consumption on days 4-7 was increased ($p<0.05$) by 7% in the 12 mg/kg/day group; this followed a period of decreased body weight gain (NS) in this group, and thus reduced the effects on body weight gain. Food consumption on Days 7-11 was decreased ($p<0.05$) in the 6 mg/kg/day group (↓5%), but it was not considered biologically meaningful as it was not dose related. Kidney and liver weights were comparable to vehicle controls. Rats in the 12 mg/kg/day group had increased ($p<0.05$) adrenal glands weights (↑38%); adrenal glands weights in the 1 and 6 mg/kg/day dose groups were comparable to the controls. There was no increase in accessory sex organ weights at any dose in the chlorpyrifos treated animals. Rats in the positive control (TP) group responded appropriately with significant increases in all five of the target accessory sex organ weights. The performance criteria indicated that the assay was performing as expected. The RBC ChE levels were significantly decreased ($p<0.05$) at 1 mg/kg/day (90% inhibition), and fell below the level of detection at 6 and 12 mg/kg/day. Brain ChE levels were significantly decreased ($p<0.05$) at 6 and 12 mg/kg/day (64% and 79% inhibition).

In the anti-androgen assay, there were no effects on body weights, body weight gains, or food consumption in chlorpyrifos treated groups. Rats in the 6 and 12 mg/kg/day groups had increased ($p<0.05$) adrenal glands weights (↑15% and ↑20%, respectively). Adrenal weights in the 1 mg/kg/day group, and kidney and liver weights in all chlorpyrifos treatment groups were comparable to controls. RBC ChE levels were significantly decreased ($p<0.05$) at 1 mg/kg/day (91% inhibition) and below the level of detection at 6 and 12 mg/kg/day. Brain ChE levels were significantly decreased ($p<0.05$) at 6 and 12 mg/kg/day (60% and 76% inhibition). Rats in the 6 and 12 mg/kg/day groups had decreased glans penis weights (↓6% and ↓8%, respectively); glans penis weights in the 1 mg/kg/day group were comparable to controls. There were no significant changes in organ weights in the

remaining four target accessory sex organs. Rats in the positive control (TP) group responded appropriately with significant decreases in all five of the target accessory sex organ weights. All CV values were less than the maximum recommended values for each organ, with the exception of LABC in the 12 mg/kg/day group (33% CV compared to maximum recommended CV of 20%). The performance criteria indicated that the assay was performing as expected.

No statistically significant changes were seen in two or more of the five androgen responsive tissue weights. Chlorpyrifos was negative for androgenicity and anti-androgenicity in the Hershberger assay.

C. STUDY DEFICIENCIES: The following deficiency was noted that did not have an adverse effect on the results, interpretation or conclusions of this study:

- LABC in the 12 mg/kg/day group exceeded the recommended performance criteria value (33% CV compared to maximum recommended CV of 20%).

DATA EVALUATION RECORD

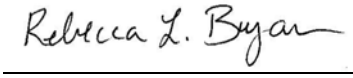
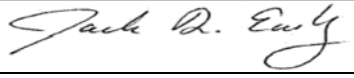
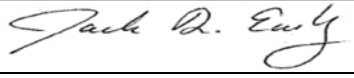
CHLORPYRIFOS

Study Type: OCSPP 890.1450, Female Pubertal Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-14-2012 (MRID 48615508)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CSS-Dynamac Corporation
1910 Sedwick Road,
Building 100, Suite B
Durham, NC 27713

Primary Reviewer	Signature:	
Kelly A. Luck, M.Sc.	Date:	01/26/2012
Secondary Reviewer	Signature:	
Rebecca L. Bryan, B.S.	Date:	2/03/2012
Program Manager:	Signature:	
Jack D. Early, M.S.	Date:	2/07/2012
Quality Assurance:	Signature:	
Jack D. Early, M.S.	Date:	2/07/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel**.**

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: John Liccione, Ph.D. **Signature:**
Health Effects Division **Date:** 5-21-15
Secondary Reviewer: Elizabeth Mendez, Ph.D. **Signature:**
Health Effects Division **Date:** 6/9/15

DATA EVALUATION RECORD

STUDY TYPE: Female Pubertal Assay; OCSPP 890.1450; OECD None.

PC CODE: 059101 **DP BARCODE:** D397128

TXR#: 0052086 **CAS#:** 2921-88-2

TEST MATERIAL (PURITY): Chlorpyrifos (99.8%)

SYNONYMS: Chlorpyrifos-ethyl; Chlorpyriphos; O,O-Diethyl O-(3,5,6-trichloro-2-pyridinyl)ester phosphorothioic acid

CITATION: Marty, M.S., Zablotny, C.L; and Stebbins, K.E (2011). Pubertal Development And Thyroid Function In Intact Juvenile/Peripubertal Female CrL:CD(SD) Rats. Dow Chemical Company, Midland, MI. Laboratory Project Study ID: 101176, November 1, 2011. MRID 48615508. Unpublished.

SPONSOR: Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN

TEST ORDER #: CON-059101-4

EXECUTIVE SUMMARY: In a Female Pubertal Assay (MRID 46815508), 16 Sprague Dawley [Crl:CD(SD)] rats/dose group were treated daily via oral gavage with chlorpyrifos (99.8% a.i., lot# KC28161419, TSN101285) in corn oil at doses of 0, 0.5, 1.0, or 2.0 mg/kg/day from post-natal day (PND) 22 to 42. Animals were examined for vaginal opening (VO) daily beginning on PND 22, and the age and weight at day of attainment was recorded. Following sacrifice on PND 42, total thyroxine (T_4) and thyroid stimulating hormone (TSH) levels were determined using chemiluminescent immunoassay and radioimmunoassay, respectively. Weights were recorded for the liver, kidneys, urogenital organs, pituitary, thyroid and adrenal glands and microscopic examinations were performed on the ovaries, uterus, thyroid, and kidneys. Cholinesterase (ChE) activity was determined in red blood cells (RBC) and the brain.

There were no effects of treatment on mortality, clinical signs of toxicity, body weights, body weight gains, age of attainment of VO, body weight at VO, mean age at first estrus, mean cycle length, percent cycling, percent regular cycling, organ weights, serum T_4 and TSH levels, clinical chemistry parameters, or gross or microscopic pathology.

RBC ChE activity was decreased ($p<0.05$) compared to the control at 0.5 (↓19%) and 1.0 (↓88%) mg/kg/day, and completely inhibited at 2 mg/kg/day. Brain ChE activity was decreased ($p<0.05$) at 2 mg/kg/day (↓22%) compared to the control, but not affected at 0.5 or 1.0 mg/kg/day. The

doses tested were considered adequate based on the observed RBC and brain cholinesterase activity.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for a Female Pubertal Assay (OCSPP 890.1450).

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

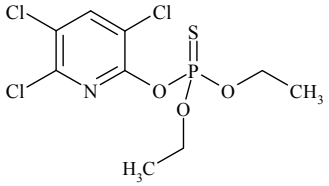
I. MATERIALS AND METHODS

A. MATERIALS

1. Test Facility: Dow Chemical Company, Toxicology & Environmental Research and Consulting

Location:	Midland, MI
Study Director:	C.L. Zablotny
Other Personnel:	M.S. Marty (Lead Scientist); K.E. Stebbins (Pathologist)
Study Period:	June 9 - November 1, 2011

2. Test Substance: Chlorpyrifos

Description:	Molecular weight = 350.6 g/mol
Source:	Dow AgroSciences LLC (Indianapolis, IN)
Lot/Batch #:	KC28161419, TSN101285
Purity:	99.8%
Stability:	Stable in corn oil for up to 12 days at concentrations up to 10 mg/mL and for 42 days at concentrations up to 1 mg/mL; temperature of stability determination not reported
CAS #:	2921-88-2
Structure:	

3. Vehicle: Corn oil

4. Test Animals:

Species:	Rat	
Strain:	Sprague Dawley [Crl:CD(SD)]	
Age/Weight at Study Initiation:	PND 22/40.9 - 56.8 g females only	
Source:	Charles River Laboratories (Portage, MI)	
Housing:	Female weanlings were housed 2 per cage in plastic solid bottom cages with heat-treated laboratory grade wood shavings.	
Diet:	Teklad Diet #2016 (Harlan Laboratories, Inc., Indianapolis, IN), *ad libitum* Total genistein equivalents < 325 µg/g diet	
Water:	Deionized water, *ad libitum*	
Environmental Conditions:	**Temperature:**	22 ± 3°C
	Humidity:	40-70%
	Air changes:	12-15/hr
	Photoperiod:	12 hrs light/ 12 hrs dark

B. STUDY DESIGN

1. In-Life Dates: Start: June 10, 2011 End: July 1, 2011

2. Mating: Time-mated pregnant female rats were received from the supplier on gestation day (GD) 7-10 and then ordered to reach GD 21 on the same day. Litters born on either GD 21 or 22 were used. Litters were culled to 10 pups on PND 4, to five males and five females whenever possible.

3. Animal Assignment: Following weaning on PND 21, female pups were implanted with transponders, weighed, and ranked by body weight. Animals were assigned to the test groups noted in Table 1 using a computer program designed to increase the probability of uniform group mean weights and standard deviations. Whenever possible, four females were selected from each litter, and one female per litter was assigned to each dose group. Littermates were not assigned to the same treatment group.

TABLE 1. Study Design [a]

Test group	Dose (mg/kg/day)	# of Females
Control	0	16
Low	0.5	16
Mid	1.0	16
High	2.0	16

a Data were obtained from page 18 of the study report.

4. Dose Selection Rationale: The dose levels were selected based on the results from a probe study[1] in which male and female rats were administered the test substance in corn oil via gavage at doses of 0, 1, 2, 4, or 8 mg/kg/day for 15 days for PND 30-44 (males) or PND 22-36 (females). Significant decreases in terminal body weights and body weight gains were observed in females at the 4 and 8 mg/kg/day dose groups. Males and females had significant decreases (≥65%) in brain ChE activity at 4 and 8 mg/kg/day, with significant decreases (15%) in the 2 mg/kg/day dose group; red blood cell ChE was 95% inhibited at this dose level. The high dose level of 2 mg/kg/day was expected to be an adequate high dose level based on the ChE inhibition in the probe study.

5. Dose Preparation and Analysis: Dose formulations were prepared by mixing appropriate amounts of test substance with corn oil. Dosing solutions were reportedly prepared periodically during the study based on stability data. In previous studies, chlorpyrifos was reportedly determined to be stable in corn oil for up to 12 days at concentrations ranging 0.00356-9.985 mg/mL,[2] and for up to 42 days at concentrations ranging 0.06-1 mg/mL[3]; the temperatures at which these stability determinations were conducted was not provided. Prior to dose administration, samples of chlorpyrifos dose formulations from all three dose levels were analyzed for achieved concentration and homogeneity; samples were taken from the top, middle, and bottom of the container after stirring.

Results of Dose Analysis

Homogeneity (%RSD): 0.6-1.7% (top, middle, and bottom)

Concentration (% of nominal): 97.7-100.3%

[1] Marty, M. S. and Marshall, V. A. (In progress). Chlorpyrifos: Hershberger, Uterotrophic, and Pubertal Assay Probe Study in Crl:CD(SD) Rats. Report of Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan.
[2] Marty, M. S. and Andrus, A. K. (2010). Comparison of cholinesterase (ChE) inhibition in young adult and preweanling CD rats after acute and repeated chlorpyrifos or chlorpyrifos-oxon exposures. Report of Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan.
[3] Hoberman, A. M. (1998). Developmental neurotoxicity study of chlorpyrifos administered orally via gavage to Crl:CD®BR VAF/Plus® presumed pregnant rats. Report of Toxicology and Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan.

The analytical data indicated that the mixing procedure was adequate and that the variation between nominal and actual dosage to the animals was acceptable. The referenced stability studies should be submitted for verification of stability findings.

6. **Dosage Administration:** All doses were administered once daily by gavage, from PND 22 through PND 42, in a volume of 4 mL/kg of body weight. Dosing was performed at approximately the same time each day (time of dosing not reported).

7. **Statistics:** Continuous variables were analyzed by Bartlett's test for equality of variance. Depending on the results of Bartlett's test, variables were transformed prior to statistical analysis. If the Bartlett's test was significant, a Kruskal-Wallis test was conducted; if this test was significant, a Wilcoxon rank-sum test was performed, with a Bonferroni-Holm adjustment for multiple comparisons to the control. Initial body weight, body weight gain, adjusted body weight, clinical chemistries, mean cycle length, age at VO, body weight at VO, age at first vaginal estrus, organ weights, organ-to-body weight ratios (liver, kidney, adrenal and pituitary weights), ChE activity, and serum hormones were analyzed with a two-way analysis of variance (ANOVA) with block and treatment as main effects. When significant dose effects were determined in the two-way ANOVA, individual dose groups were compared to controls using Dunnett's test. Age and body weight at VO and organ weights (thyroid, ovaries, and uterus) were analyzed with analysis of covariance (ANCOVA) with body weight on PND 22 as the covariate. When the dose effect was significant, a Dunnett's correction was used to determine differences. When the ANCOVA was not statistically significant, a Dunnett's test also was performed. Globulin and albumin/globulin ratio, which were calculated values, were not statistically analyzed. A chi-square analysis was used to analyze cycling status and percent cycling regularly. When the chi-square statistic was significant, it was followed by pairwise comparisons to the control group via a Fisher's exact test with a Bonferroni-Holm adjustment. Significance was denoted at $p \leq 0.05$. The statistical analyses were considered to be adequate.

C. METHODS

1. **Mortality and Clinical Examinations:** Cage-side examinations of all animals were conducted at least twice daily for mortality, moribundity, and significant clinical abnormalities. Hand-held clinical examinations were conducted each dosing day following dosing.

2. **Body Weight:** All animals were weighed on PND 21 (day of randomization) and daily prior to dosing during PND 22-42.

3. **Vaginal Opening:** Beginning on PND 22, all animals were examined daily for onset of VO. Age and weight on the day of completion of VO were recorded.

4. **Estrous Cyclicity:** Beginning on the day of VO, up to and including the day of necropsy, daily vaginal lavage samples were obtained to determine the age of first estrus and to evaluate estrous cycle pattern. The mean age at first vaginal estrus, the mean cycle length for each group, the percent of each group cycling, the percent of each group cycling regularly, and the stage of the cycle at the time of necropsy were reported.

5. Sacrifice and Pathology: On the day before termination, rats were transferred to a holding area to avoid cage transfer on the day of necropsy. On the day of termination, rats were removed one at a time to a separate room for euthanasia and terminal procedures. Approximately two hours after the last dose on PND 42, all surviving animals were anesthetized by isoflurane inhalation and blood was collected by cardiac puncture. Animals were euthanized beginning at least 2 hours following dose administration that day, and all sacrifices were completed by 1300 hours. It was reported that blood samples were generally collected within 3 minutes of animal being removed from its cage, and that animals that did not reach a sufficient level of anesthesia for exsanguination within 2 minutes were marked as deviations. Animals were then euthanized by decapitation. It was stated that necropsies were completed before 1300 hours. Blood samples were transferred to separate tubes for clinical chemistry analyses, serum hormone analyses, and RBC for ChE activity; samples were generally kept on ice or refrigerated during necropsy. Samples for serum hormone analyses were centrifuged as soon as possible and then stored at -80°C until shipment to WIL Research Laboratories (Ashland OH) for analysis. Samples for RBC ChE activity determination were centrifuged and RBC were collected, diluted in 1% Triton X-100, and stored at -80°C until shipment to WIL Research Laboratories for analysis. Historical control data were not provided.

a. Hormone Analysis: Total thyroxine (T_4) and thyroid stimulating hormone (TSH) levels were determined using chemiluminescent immunoassay and radioimmunoassay procedures, respectively.

b. Clinical Chemistry: The following CHECKED (X) parameters were examined.

X	ELECTROLYTES	X	OTHER
X	Calcium	X	Albumin
X	Chloride	X	Creatinine*
	Magnesium	X	Urea nitrogen*
X	Phosphorus	X	Total cholesterol
X	Potassium	X	Globulins
X	Sodium		Glucose
	ENZYMES	X	Total bilirubin
X	Alkaline phosphatase (ALK)	X	Total protein
X	Cholinesterase (ChE)		Triglycerides
	Creatine phosphokinase		Serum protein electrophoresis
	Lactic acid dehydrogenase (LDH)	X	Albumin/globulin ratio (calculated)
X	Alanine aminotransferase (ALT/also SGPT)		
X	Aspartate aminotransferase (AST/also SGOT)		
	Sorbitol dehydrogenase		
X	Gamma glutamyl transferase (GGT)		
	Glutamate dehydrogenase		

* Recommended for the pubertal assay in female rats based on guideline 890.1450.

c. Organ Weights and Histopathology: The following CHECKED (X) tissues were collected and weighed. The (XX) organs, in addition, were subjected to histological examination.

X	UROGENITAL	X	OTHER
XX	Ovaries (paired, without oviducts)*+	XX	Thyroid*+
XX	Uterus*+	X	Liver*
XX	Kidneys (paired)*+	X	Adrenals (paired)*
		X	Pituitary*

* Weights required based on guideline 890.1450
\+ Histopathological examination required based on guideline 890.1450

All organs collected, except the thyroid/trachea and pituitary, were weighed prior to fixation. Paired organs (kidneys, adrenals, and ovaries) were weighed together. The uterus and cervix were separated from the vagina and weighed. The uterus was weighed again following removal of the fluid in the lumen (blotted weight).

The kidneys, thyroid (with attached trachea), ovaries (right) and uterus were fixed in 10% buffered formalin for at least 24 hrs and rinsed in graded ethanol solutions prior to embedding. Following fixation, the thyroid was dissected from the trachea. All collected tissues were routinely processed into paraffin blocks, sectioned, stained with hematoxylin and eosin, and examined microscopically.

Thyroid sections were subjectively evaluated for follicular cell height and colloid area using a five point grading scale (1 = shortest/smallest; 5 = tallest/largest), and any abnormalities/lesions noted. At least two sections from each of the two lobes of the thyroid were examined. Evaluation of the ovary included a qualitative evaluation of follicular development including the presence or absence of primary, atretic, and tertiary/antral follicles, presence or absence of corpora lutea, and changes in corpus luteum development, in addition to any abnormalities/lesions, such as ovarian atrophy. Five sections of the right ovary were evaluated with sampling conducted in a manner that provided a good overall assessment of the ovarian tissue. The uterus evaluation included an assessment of uterine hypertrophy or atrophy as characterized by changes in uterine horn diameter and myometrial, stromal, or endometrial gland development. The histological assessment of the ovary and uterus took into account the stage of the estrous cycle of the female at the time of necropsy.

II. RESULTS

A. Mortality: All animals survived until scheduled termination.

B. Clinical Signs of Toxicity: No clinical signs of toxicity were observed in animals for any dose groups.

C. General Growth and Vaginal Opening: Body weights, body weight gains, age of attainment of VO and weight at day of attainment are presented in Table 2. Body weights and body weight gains were unaffected by treatment. Age and body weight at VO were similar across all groups. All animals achieved VO by PND 42.

TABLE 2. General Growth and Vaginal Opening (VO)[a].

Parameter Evaluated		Vehicle Control				0.5 mg/kg/day				1.0 mg/kg /day				2.0 mg/kg/day			
		N	Mean	SD/ SE	CV (%)	N	Mean	SD/ SE	CV (%)	N	Mean	SD/ SE	CV (%)	N	Mean	SD/ SE	CV (%)
Initial body weight (PND 22; g)	U	16	49.3	4.5	9.1	16	49.9	4.1	8.3	16	50.2	3.2	6.4	16	50.1	4.3	8.5
	A	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Body weight at VO (g)	U	16	115.8	16.7	14.4	16	111.4	15.2	13.6	16	109.9	14.1	12.8	16	115.9	16.3	14.1
	A	16	116.5	3.8	NA	16	111.4	3.7	NA	16	109.5	3.8	NA	16	115.6	3.7	NA
Final body weight (g)	U	16	151.0	12.5	8.2	16	152.4	15.3	10.0	16	149.9	14.5	9.7	16	153.2	15.2	9.9
	A	16	152.1	3.1	NA	16	152.4	3.1	NA	16	149.3	3.1	NA	16	152.8	3.1	NA
Final body weight (% of control)	U	NA	NA	NA	NA	NA	100.9	10.1	10.0	NA	99.3	9.6	9.7	NA	101.4	10.1	9.9
	A	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Body weight gain (final – initial; g)	U	16	101.7	11.5	11.3	16	102.5	13.2	12.9	16	99.7	13.0	13.0	16	103.1	13.5	13.1
	A	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Age at VO (PND)	U	16	35.4	2.3	6.5	16	34.4	2.2	6.3	16	34.3	1.9	5.5	16	35.3	1.9	5.3
	A	16	35.4	0.5	NA	16	34.4	0.5	NA	16	34.3	0.5	NA	16	35.3	0.5	NA
Proportion unopened (#/N)		0/16	NA			0/16	NA			0/16	NA			0/16	NA		

a Data were obtained from Table 7 on page 49 of the study report.
U = Unadjusted for body weight on PND 22
A = Adjusted for body weight on PND 22
N = Number of animals examined
SD = Standard deviation; reported for unadjusted and relative values
SE = Standard error of the mean; reported for adjusted values.
CV = Coefficient of Variation
NA = Not applicable

D. Organ Weights: Organ weights at necropsy are presented in Table 3. There were no significant ($p<0.05$) differences in absolute, adjusted, or relative organ weights in any dose groups.

TABLE 3. Organ Weights at Necropsy[a]

Organ		Vehicle Control				0.5 mg/kg/day				1.0 mg/kg/day				2.0 mg/kg/day			
		N	Mean	SD/SE	CV (%)	N	Mean	SD/SE	CV (%)	N	Mean	SD/SE	CV (%)	N	Mean	SD/SE	CV (%)
Liver (g)	U	16	6.94	0.85	12.17	16	7.07	0.87	12.36	16	6.83	0.82	11.97	16	7.11	0.99	13.99
	A	16	6.99	0.207	NA	16	7.07	0.206	NA	16	6.80	0.207	NA	16	7.09	0.206	NA
	R	16	4.59	0.27	5.96	16	4.64	0.25	5.45	16	4.55	0.21	4.54	16	4.63	0.30	6.47
Kidneys (g)	U	16	1.22	0.12	10.12	16	1.25	0.12	9.50	16	1.23	0.11	8.79	16	1.24	0.11	8.90
	A	16	1.23	0.026	NA	16	1.25	0.026	NA	16	1.23	0.026	NA	16	1.23	0.026	NA
	R	16	0.81	0.06	7.20	16	0.82	0.05	6.04	16	0.83	0.05	5.77	16	0.81	0.06	7.30
Pituitary (mg)	U	16	7.6	0.9	12.4	16	7.8	1.2	15.9	16	7.9	1.3	17.0	16	7.7	0.9	11.8
	A	16	7.6	0.26	NA	16	7.8	0.26	NA	16	7.8	0.26	NA	16	7.7	0.26	NA
	R	16	5.0	0.5	10.3	16	5.1	0.6	11.9	16	5.2	0.6	12.1	16	5.0	0.5	9.6
Adrenals (mg)	U	16	31.6	5.4	17.0	16	33.6	4.3	12.8	16	34.6	6.9	20.0	16	32.9	4.1	12.6
	A	16	31.8	1.31	NA	16	33.6	1.31	NA	16	34.5	1.31	NA	16	32.8	1.31	NA
	R	16	21.0	3.7	17.6	16	22.1	2.5	11.4	16	23.0	3.6	15.7	16	21.6	3.4	15.8
Ovaries (mg)	U	16	54.1	7.1	13.2	16	55.8	7.3	13.0	16	55.2	8.8	16.0	16	55.7	7.2	13.0
	A	16	54.2	1.93	NA	16	55.8	1.93	NA	16	55.2	1.93	NA	16	55.7	1.93	NA
Uterus, wet (mg)	U	16	307.8	136.2	44.3	16	307.2	140.7	45.8	16	364.2	166.2	45.6	16	312.3	138.2	44.2
	A	16	311.8	36.17	NA	16	307.1	36.07	NA	16	361.7	36.11	NA	16	310.9	36.09	NA
Uterus, blotted (mg)	U	16	273.2	67.5	24.7	16	270.4	68.2	25.2	16	300.9	71.3	23.7	16	272.6	64.3	23.6
	A	16	275.1	16.83	NA	16	270.4	16.79	NA	16	299.7	16.80	NA	16	271.9	16.79	NA
Thyroid, fixed (mg)	U	16	8.9	1.6	17.8	15	8.2	1.3	15.8	15	9.0	2.1	23.7	16	8.9	1.5	16.8
	A	16	8.9	0.40	NA	15	8.2	0.41	NA	15	8.9	0.41	NA	16	8.8	0.40	NA

a Data were obtained from Table 10 on page 53 of the study report.
U = Unadjusted for body weight on PND 22
A = Adjusted for body weight on PND 22
N = Number of animals examined
SD = Standard Deviation
CV = Coefficient of Variation
R = Organ-to-body weight ratio (relative to body weight)

E. Estrous Cyclicity: Estrous cycle data are provided in Table 4. There were no significant differences in mean age at first estrus in any dose group. The study authors reported that using both two-way ANOVA and ANCOVA analysis, there was a significant difference in mean cycle length (first day of estrus to the next first day of estrus); however, in pair-wise comparisons, a difference was not statistically identified by either Dunnett's test or least square means. Mean estrous cycle length may have been identified in the overall analyses due to the increase in the 0.5 mg/kg/day group (5.0 vs. 4.8 days in the control group) followed by a decrease in mean cycle length in the 1.0 and 2.0 mg/kg/day groups (4.5 and 4.4 days, respectively). It was concluded that these minor differences in estrous cycle length were not biologically meaningful and deemed unrelated to treatment. The percent cycling and percent of regularly cycling rats were similar across all groups; the percent of cycling rats at 2.0 mg/kg/day was slightly greater than the control but this difference was not statistically significant. There were no treatment-related differences in estrus stage at necropsy. It was noted that interpretation of estrous cycle data was hindered by the limited length of sampling (7 days after VO).

TABLE 4. Estrous Cyclicity[a]											
								Cycle Status at Necropsy (# Females)			
Dose Level (mg/kg/day)	N	Mean Age at First Vaginal Estrus (PND)	N	Mean Cycle Length (days)	N	Cycling (%)	Regularly Cycling (%)	Diestrus	Proestrus	Estrus	Not Cycling
Vehicle	16	36.6	10	4.8	16	100	81	9	0	7	0
0.5	16	35.9	12	5.0	16	100	81	12	0	4	0
1.0	16	35.3	12	4.5	16	94	94	10	0	6	0
2.0	16	36.2	14	4.4	16	100	94	10	0	6	0

a Data were obtained from Table 8 on page 50 of the study report.
N = Number of animals examined

F. <u>Clinical Chemistry and Hormone Levels</u>: Mean hormone levels, ChE activity, and clinical chemistry parameters are presented in Table 5. There were no treatment-related effects on levels of serum T_4, TSH, electrolytes, or clinical chemistry parameters in any dose group. ChE activity in RBC was significantly decreased ($p<0.05$) compared to the control at 0.5 (19% inhibition) and 1.0 (88% inhibition) mg/kg/day, and completely inhibited at 2 mg/kg/day. ChE activity in the brain was significantly decreased ($p<0.05$) at 2 mg/kg/day (22% inhibition) compared to the control, but not significantly affected at 0.5 or 1.0 mg/kg/day. It was noted that the time of collection of blood and brain samples for ChE measurements, 2 hours after dosing, was earlier than the expected time of peak ChE inhibition (6 hours after dosing).

TABLE 5. Hormone Levels, Cholinesterase Activity, and Clinical Chemistry [a]																
Parameter Evaluated	Vehicle Control				0.5 mg/kg/day				1.0 mg/kg/day				2.0 mg/kg/day			
	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Hormones																
Serum T_4, Total (µg/dL)	16	4.01	0.71	17.69	16	3.72	0.55	14.70	16	3.98	0.87	21.89	16	3.83	0.68	17.88
Serum TSH (ng/mL)	16	4.95	2.05	41.34	16	4.63	2.35	50.89	16	4.34	2.26	52.14	16	4.29	2.41	56.22
Cholinesterase																
RBC (U/L)	16	4135	647	16	16	3341* (↓19)	1750	52	16	489* (↓88)	521	106	16	12* (↓100)	7	60
Brain (U/L)	16	50502	1705	3	16	50543	1488	3	16	49671	1353	3	16	39456* (↓22)	3904	10
Clinical Chemistry																
Creatinine (µmol/L)	16	0.1[b]	0.0	0.0	16	0.1[b,c]	0.0	23.5	16	0.1[b,c]	0.0	23.5	16	0.1[b,c]	0.0	23.5
Serum urea nitrogen (mg/dL)	16	12	2	15	16	12	2	18	16	11	2	17	16	12	2	20
Alkaline phosphatase (U/L)	16	322	65	20	16	303	50	16	16	297	38	13	16	296	30	10
ALT (U/L)	16	53	8	16	16	55	20	36	16	55	14	26	16	53	8	15
AST (U/L)	16	100	21	21	16	144	123	85	16	115	47	40	16	107	17	16
GGT (U/L)	16	1.5[b]	0.0	0.00	16	1.5[b]	0.0	0.00	16	1.5[b]	0.0	0.00	16	1.5[b]	0.0	0.00
Albumin (g/dL)	16	4.0	0.1	3.0	16	4.1	0.2	4.4	16	4.0	0.3	7.1	16	3.9	0.1	3.6
A/G Ratio	16	2.8	0.4	12.8	16	3.0	0.3	10.4	16	3.0	0.5	16.2	16	2.9	0.4	12.9

TABLE 5. Hormone Levels, Cholinesterase Activity, and Clinical Chemistry [a]																
Parameter Evaluated	Vehicle Control				0.5 mg/kg/day				1.0 mg/kg/day				2.0 mg/kg/day			
	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Cholesterol (mg/dL)	16	100	12	12	16	94	10	11	16	95	14	14	16	93	12	13
Globulin (g/dL)	16	1.4	0.2	10.7	16	1.4	0.1	8.6	16	1.4	0.1	10.3	16	1.4	0.1	10.9
Total bilirubin (mg/dL)	16	0.05[b,c]	0.01	23.53	16	0.05[b]	0.00	0.00	16	0.05[b]	0.00	0.00	16	0.05[b]	0.00	0.00
Total protein (g/dL)	16	5.4	0.1	2.2	16	5.4	0.2	3.8	16	5.4	0.3	5.0	16	5.3	0.2	3.0
Sodium (mmol/L)	16	140	2	2	16	141	1	1	16	141	2	1	16	141	1	1
Potassium (mmol/L)	16	6.0	0.7	11.1	16	5.6	0.5	8.6	16	5.7	0.9	15.0	16	5.8	0.5	9.0
Chloride (mmol/L)	16	101	2	2	16	102	2	2	16	102	2	2	16	102	1	1
Calcium (mg/dL)	16	11.2	0.3	3.1	16	11.3	0.5	4.2	16	11.2	0.3	2.7	16	11.2	0.3	2.8
Phosphorus (mg/dL)	16	9.2	1.1	12.3	16	8.4	1.0	11.4	16	8.6	1.1	12.4	16	8.9	0.9	10.2

a Data were obtained from Table 9 on page 51 of the study report. Percent differences from controls were calculated by the reviewers and included in parentheses.
b Values below the detection limit were assigned a value of one-half the detection limit (0.1 µmol/L for creatinine; 1.5 U/L for GGT; 0.05 mg/dL for total bilirubin) for statistical analysis and reporting.
c Includes one result at the limit of detection, with the remainder below the limit of detection.
N = Number of animals examined
SD = Standard Deviation
CV = Coefficient of Variation
ALT = Alanine aminotransferase
AST = Aspartate aminotransferase
GGT = Gamma glutamyl transferase
* Significantly different from controls at $p<0.05$.

G. <u>Histopathology</u>: The incidences of histopathological findings of the thyroid gland are presented below in Table 6. There were no significant differences in colloid area or follicular cell height between the control and the 2 mg/kg/day dose group. The thyroid glands from rats in the 0.5 and 1.0 mg/kg/day dose groups were not examined.

TABLE 6. Incidence of Histopathological Findings of the Thyroid Gland [a]						
Treatment Groups	Parameter Evaluated					
	Colloid Area			Follicular Cell Height		
	Grade	Incidence		Grade	Incidence	
		Observed	Examined		Observed	Examined
Vehicle Control	1	0	16	1	8	16
	2	0	16	2	6	16
	3	2	16	3	2	16
	4	6	16	4	0	16
	5	8	16	5	0	16
2.0 mg/kg/day	1	0	16	1	8	16
	2	0	16	2	7	16
	3	1	16	3	1	16
	4	7	16	4	0	16
	5	8	16	5	0	16

a Data were obtained from Table 12 on page 59 of the study report.
b Thyroid histopathology is graded 1-5. Colloid area: 1 = most colloid, 5 = least colloid. Follicular Cell Height: 1 = lowest, 5 = highest.
O = Number Observed
E = Number Examined
NA = Not applicable

The incidence of histopathological findings of the ovaries, uterus, and kidneys are presented in Table 7. There were no treatment-related findings for the ovaries, uterus, or kidneys. The ovaries, uterus, and kidneys from rats in the 0.5 and 1.0 mg/kg/day dose groups were not examined.

TABLE 7. Incidence of Histopathological Lesions of the Ovaries, Uterus and Kidney[a]								
Findings	Dose Level (# mg/kg bw/day)							
	Vehicle Control		0.5		1.0		2.0	
	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined
Ovaries								
Within Normal Limits	16	16	---	0	---	0	16	16
Uterus								
Within Normal Limits	16	16	---	0	---	0	16	16
Kidney								
Within Normal Limits	6	16	---	0	---	0	8	16
Cyst; cortex; focal	1	16	---	0	---	0	2	16
Degeneration; tubule; focal (very slight)	6	16	---	0	---	0	4	16
Degeneration; tubule; multifocal (very slight)	3	16	---	0	---	0	4	16
Inflammation; chronic; interstitium; focal (very slight)	1	16	---	0	---	0	1	16

a Data were obtained from Table 13 on page 60 of the study report.

III. DISCUSSION AND CONCLUSIONS

A. <u>INVESTIGATORS' CONCLUSIONS</u>: No treatment-related effects were observed on mortality, clinical signs, body weights, body weight gains, age or weight at attainment of

VO, estrous cycle (length, age at first estrus, percent cycling, and percent regularly cycling animals), hormone (serum T_4, serum TSH) levels, organ weights, and gross or histopathology parameters at any dose. There was significant treatment-related inhibition of RBC ChE activity by 19%, 88%, and >99% at 0.5, 1.0 and 2.0 mg/kg/day chlorpyrifos, respectively. Brain ChE activity was also significantly inhibited (22%) at 2.0 mg/kg/day chlorpyrifos. The RBC and brain ChE inhibition indicated that the animals were sufficiently challenged with the high dose of chlorpyrifos.

B. AGENCY COMMENTS: Chlorpyrifos was tested up to 2 mg/kg/day. There were no effects of treatment on mortality, clinical signs of toxicity, body weights, body weight gains, age of attainment of VO, body weight at VO, mean age at first estrus, mean cycle length, percent cycling, percent regular cycling, organ weights, serum T_4 and TSH levels, clinical chemistry parameters, or gross or microscopic pathology.

RBC ChE activity was significantly decreased ($p<0.05$) compared to the control at 0.5 (19% inhibition) and 1.0 (88% inhibition) mg/kg/day, and completely inhibited at 2 mg/kg/day. Brain ChE activity was significantly decreased ($p<0.05$) at 2 mg/kg/day (22% inhibition) compared to the control, but not significantly affected at 0.5 or 1.0 mg/kg/day.

C. STUDY DEFICIENCIES: The following minor deficiencies were noted that are not considered to have had an adverse impact on the results, interpretation or conclusions of this study:

- Control CV of mean weight at VO (14.37%) was greater than the performance criteria maximum of 13.97%
- Control mean adrenal weight (31.6 mg) was below the performance criteria acceptable range (38.34-48.84 mg)

DATA EVALUATION RECORD

CHLORPYRIFOS

Study Type: OCSPP 890.1500, Male Pubertal Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-14-2012 (MRID 48615509)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CSS-Dynamac Corporation
1910 Sedwick Road,
Building 100, Suite B
Durham, NC 27713

Primary Reviewer:	Signature:	David A. McEwen
David A. McEwen, B.S.	Date:	1/27/2012
Secondary Reviewer:	Signature:	Michael E Viana
Michael E. Viana, Ph.D., D.A.B.T.	Date:	1/31/2012
Program Manager:	Signature:	Jack D. Early
Jack D. Early, M.S.	Date:	2/02/2012
Quality Assurance:	Signature:	Jack D. Early
Jack D. Early, M.S.	Date:	2/02/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel**.**

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: P V Shah, Ph.D
Health Effects Division
Secondary Reviewer: John Liccione, Ph.D.
Health Effects Division

Signature: P.V. Shah
Date: 5/27/2015
Signature:
Date: 6/8/15

DATA EVALUATION RECORD

STUDY TYPE: Male Pubertal Assay; OCSPP 890.1500; OECD None.

PC CODE: 059101 **DP BARCODE:** D397128

TXR#: 0052086 **CAS No:** 2921-88-2

TEST MATERIAL (PURITY): Chlorpyrifos (99.8% a.i.)

SYNONYMS: *O,O*-Diethyl *O*-(3,5,6-trichloro-2-pyridinyl)ester phosphorothioic acid

CITATION: Marty, M.S., Andrus, A.K., and Hukkanen, R.R. (2011) Chlorpyrifos: Pubertal development and thyroid function in intact juvenile/peripubertal male Crl:CD(SD) rats. Toxicology & Environmental Research and Consulting, Dow Chemical Co., Midland, MI. Laboratory Study ID: 111077, October 28, 2011. MRID 48615509. Unpublished.

SPONSOR: Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN

TEST ORDER #: CON-059101-4

EXECUTIVE SUMMARY: In a male pubertal assay (MRID 48615509), 16 Sprague-Dawley (Crl:CD[SD]) rats/dose group were treated daily via oral gavage (4 mL/kg) with chlorpyrifos (99.8% a.i., Lot # KC28161419, TSN101285) in corn oil at doses of 0, 0.5, 1.0, or 2.0 mg/kg/day from post-natal day (PND) 23 to 53. Animals were examined for preputial separation (PPS) daily beginning on PND 30, and the age and weight at day of attainment was recorded. Following sacrifice on PND 53, total serum testosterone, thyroxine (T_4), and thyroid stimulating hormone (TSH) levels were analyzed using radioimmunoassays (TSH) or chemiluminescent assays (testosterone and T_4). Additionally, standard clinical chemistry parameters were evaluated, along with red blood cell (RBC) and brain cholinesterase (ChE) activities. Weights were recorded for the liver, kidneys, urogenital organs, pituitary, thyroid and adrenal glands, and microscopic examinations were performed on the testes, epididymides, thyroid, and kidneys.

At chlorpyrifos doses up to 2 mg/kg/day, no treatment-related effects were observed on mortality, clinical signs, body weights, body weight gains, age or weight at attainment of PPS, serum hormone (T_4, TSH, or testosterone) levels, organ weights, and gross or histopathological parameters. RBC ChE activity was dose-dependently inhibited ($p<0.05$) between 34-100% at all doses of chlorpyrifos. Brain ChE activity was also inhibited ($p<0.05$) by 23% at 2 mg/kg/day. The doses tested were judged to be adequate based on the observed RBC and brain cholinesterase activity.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for a Male Pubertal Assay (OCSPP 890.1500).

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

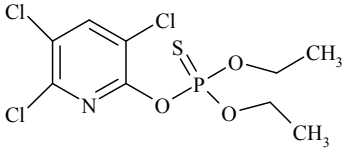
I. MATERIALS AND METHODS

A. MATERIALS

1. Test Facility: Toxicology & Environmental Research and Consulting, The Dow Chemical Company

Location:	Midland, MI
Study Directors:	A.K. Andrus, M.S.
Other Personnel:	M.S. Marty, Ph.D. (Lead scientist) and R.R. Hukkanen, D.V.M.
Study Period:	June 7, 2011 to October 28, 2011

2. Test Substance: Chlorpyrifos

Description:	Not reported
Source:	Dow AgroSciences LLC, Indianapolis, IN
Lot #:	KC28161419
Purity:	99.8% a.i.
Stability:	It was stated that the test material was previously shown to be stable in corn oil for up to 12 days at concentrations bracketing those used in the current study (Marty and Andrus, 2010)
CAS #:	2921-88-2
Structure:	

3. Vehicle: Corn oil

4. Test Animals:

Species:	Rat (males only)	
Strain:	Sprague-Dawley (Crl:CD[SD])	
Age/Mean weight at study initiation:	PND 23 / 55.7-56.8 g	
Source:	Charles River Laboratories, Portage, MI	
Housing:	After weaning, 2 males/cage were housed in plastic solid-bottom cages with heat-treated aspen wood shavings.	
Diet:	Teklad Diet #2016 (Harlan Laboratories, Inc., Indianapolis, IN), *ad libitum*. It was stated that the genistein-equivalent content was <325 µg/g diet.	
Water:	Deionized water, *ad libitum*	
Environmental Conditions:	**Temperature:**	22±1°C
	Humidity:	40-70%
	Air changes:	12-15 times/hr
	Photoperiod:	12 h light / 12 h dark

B. STUDY DESIGN

1. In-Life Dates: Start: June 11, 2011 End: July 12, 2011

2. Mating: Time-mated pregnant dams (8-12 weeks old) were received from the supplier on gestation day (GD) 7 to 10, and were allowed to deliver natural litters at the test facility. Litters were culled to 10 pups/litter on post-natal day (PND) 4 with 5 pups/sex, whenever possible.

3. Animal Assignment: Following weaning on PND 21, male pups from acceptable litters were stratified by body weight and randomly assigned to the test groups in Table 1. Whenever possible, four males were selected from each litter (one male/litter assigned to each dose group), and no group contained littermates.

TABLE 1. Study Design [a]

Test group	Dose (mg/kg/day)	# of Males
Control	0	16
Low	0.5	16
Mid	1	16
High	2	16

a Data were obtained from page 20 of the study report.

4. Dose Selection Rationale: It was stated that the doses were selected based on the results of a recently performed probe assay (Marty and Marshall, In Progress[1]) in which rats were administered chlorpyrifos in corn oil by oral gavage at doses of 0, 1, 2, 4, or 8 mg/kg/day for 15 days from PND 30-44 in males. No adverse effects were observed on mortality, clinical signs, body weights, food consumption, clinical chemistry parameters, or organ weights at any dose in males. However, RBC ChE activity was significantly decreased at ≥1 mg/kg/day and brain ChE was decreased at doses ≥2 mg/kg/day. Therefore, 2 mg/kg/day was selected as the high dose for this assay.

5. Dose Preparation and Analysis: The test material was mixed in corn oil such that a dose volume of 4 mL/kg body weight yielded the target dose. Dose volumes were adjusted using the most current body weight. Test formulations were prepared periodically throughout the study (frequency not reported) based on stability data. Concentration and homogeneity analyses were performed on all dose levels from the first mix prior to initiation of dosing. It was reported that chlorpyrifos was previously determined to be stable in corn oil for up to 12 days at concentrations ranging from 0.00356 to 9.985 mg/mL (Marty and Andrus, 2010[2]), which bracketed those used in the current study. However, data were not provided.

Results

Concentration (% of nominal): 97.7 to 100.3%

Homogeneity (% RSD): 0.6 to 1.7%

The analytical data indicated that the variation between nominal and actual dosage to the animals was acceptable.

6. Dosage Administration: The dose formulations were administered once daily by oral gavage at a dose volume of 4 mL/kg of body weight from PND 23 through PND 53.

[1] Marty, M.S., and Marshall, V.A. (In Progress) Chlorpyrifos: Hershberger, uterotrophic, and pubertal assay probe study in Crl:CD(SD) rats. Report of Toxicology & Environmental Research And Consulting, The Dow Chemical Company, Midland, MI.

[2] Marty, M.S., and Andrus, A.K. (2010) Comparison of cholinesterase inhibition in young adult and preweanling CD rats after acute and repeated chlorpyrifos or chlorpyrifos-oxon exposures. Report of Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, MI.

7. **Statistics:** Continuous data were first analyzed by Bartlett's test for homogeneity of variance (p=0.01). Based on the results of Bartlett's test, data may have been transformed (log, inverse, or square root) prior to statistical analysis as described below. Statistical outliers were identified by a sequential test.

Parameter	Procedure
Body weight Body weight gain Adjusted body weight Clinical chemistry parameters RBC and Brain ChE activity Age and body weight at preputial separation Absolute organ weights Relative (to body) organ weights (liver, adrenal, kidney and pituitary) Serum hormones	If Bartlett's test was not significant at the 1% level, parametric methods were applied. Data were analyzed using a two-way analysis of variance (ANOVA) with block and treatment as main effects. If the ANOVA was significant at the 5% level, individual dose groups were compared to the controls using Dunnett's test. If Bartlett's test was significant, logarithmic, inverse, and square-root transformations were tried. If Bartlett's test was still significant, non-parametric methods were applied. Data were analyzed using the Kruskal-Wallis test. If the Kruskal-Wallis test was significant, a Wilcoxon rank-sum test with a Bonferroni-Holm adjustment for multiple comparisons was used.
Age at preputial separation Weight at preputial separation Organ weights	Data were subjected to analysis of covariance (ANCOVA) with body weight at PND 23 as the covariate. If the ANCOVA was significant at the 5% level, least square means with a Dunnett's correction was used. If the ANCOVA was not significant, a Dunnett's test was performed.

It was stated that outliers and questionable data points were excluded only for documented scientifically sound reasons. Significance was denoted at 5%, 1% and 0.1% levels. The statistical analyses were considered appropriate.

C. METHODS

1. **Mortality and Clinical Examinations:** Beginning on PND 23, males were observed twice daily for mortality, morbidity, and clinical signs of toxicity, and given a detailed physical examination daily following dosing.

2. **Body Weight:** All males were weighed on PND 21 (day of randomization) and daily prior to dosing and during PND 23-53. However, body weights were only reported for PND 21, 23, 26, 30, 37, 42, 45, and 53. Body weight gains were reported for the associated body weight intervals beginning on PND 23-26 and including the overall dosing period (PND 23-53).

3. **Food Consumption:** Food consumption data were not reported.

4. **Preputial Separation (PPS):** Beginning on PND 30, all males were examined daily following dosing for onset of PPS. Age and weight on the day of completion of PPS were recorded.

5. **Sacrifice and Pathology:** All males were anesthetized by isoflurane inhalation and blood was collected for hormone and clinical chemistry analyses on PND 53 approximately 2 hours post-dosing. The animals were then euthanized by exsanguination followed by

decapitation. It was stated that the necropsies were completed by 1300 hours. The animals were not fasted overnight prior to sacrifice. Blood was collected via cardiac puncture and was transferred into separate tubes for clinical chemistry analysis, serum hormone analysis, and RBC ChE activity.

a. **Hormone Analysis:** Total serum testosterone and T_4 were analyzed by chemiluminescent assays, and TSH levels were analyzed using an unspecified radioimmunoassay.

b. **Clinical Chemistry:** The following CHECKED (X) parameters were examined.

	ELECTROLYTES		OTHER
X	Calcium	X	Albumin
X	Chloride	X	Creatinine*
	Magnesium	X	Urea nitrogen*
X	Phosphorus	X	Total cholesterol
X	Potassium	X	Globulin (calculated)
X	Sodium		Glucose
	ENZYMES	X	Total bilirubin
X	Alkaline phosphatase (ALP)	X	Total protein
X	Cholinesterase (ChE)		Triglycerides
	Creatine phosphokinase	X	Albumin/globulin ratio (calculated)
	Lactic acid dehydrogenase (LDH)		
X	Alanine aminotransferase (ALT/also SGPT)		
X	Aspartate aminotransferase (AST/also SGOT)		
	Sorbitol dehydrogenase		
X	Gamma glutamyl transferase (GGT)		
	Glutamate dehydrogenase		

* Recommended for the pubertal assay in male rats based on guideline 890.1500.

c. **RBC and Brain ChE Activity:** RBC and brain ChE activities were determined using an assay based on a modification of the Ellman reaction (Ellman *et al*., 1961; Hunter *et al*., 1997).

d. **Organ Weights and Histopathology:** The following CHECKED (X) tissues were collected and weighed. The (XX) organs, in addition, were subjected to histological examination.

	UROGENITAL		OTHER
XX	Testes (left and right separately)*+	XX	Thyroid a*+
XX	Epididymides (left and right separately)*+	X	Liver*
X	Seminal vesicle plus coagulating glands (with and without fluid)*	X	Adrenals (paired)*
X	Ventral prostate*	X	Pituitary*
X	Dorsolateral prostate*		
X	Levator ani/bulbocavernosus (LABC) muscle complex*		
XX	Kidneys (paired)*+		
X	Gross lesions and masses b		

a Thyroid and parathyroids were collected and weighed together
b Gross lesions and masses were collected but not weighed.
* Weights required based on guideline 890.1500.
+ Histopathological examination required based on guideline 890.1500.

All collected organs, except the thyroid and pituitary, were weighed prior to fixation. The left and right testes and epididymides were weighed separately. The seminal vesicle plus coagulating glands were weighed with and without fluid. Remaining paired organs (kidneys and adrenals) were weighed together. The testes and epididymides were initially fixed in Bouin's fixative (24-72 hrs) and retained in 70% ethanol until embedded in paraffin. The recommended fixation time in Bouin's fixative is no more than 24 hrs; however, it was stated that the preservation time in Bouin's fixative (from 24 to 72 hrs) has been evaluated in this laboratory and shown not to impact tissue quality for histopathological evaluation. All other tissues were fixed in 10% neutral buffered formalin.

Testes and epididymides were evaluated as required by the EPA's Health Effects Test Guideline OCSPP 870.3800: Reproduction and Fertility Effects. In the testes, these evaluations were conducted in order to identify treatment-related effects such as retained spermatids, missing germ cell layers or types, multinucleated giant cells, or sloughing of spermatogenic cells into the lumen. Examination of the intact epididymis included the caput, corpus, and cauda, accomplished by evaluation of a longitudinal section, and was conducted in order to identify such lesions as sperm granulomas, leukocytic infiltration (inflammation), aberrant cell types within the lumen, or the absence of clear cells in the cauda epididymal epithelium.

Thyroid sections were subjectively evaluated for follicular cell height and colloid area using a five point grading scale (1 = shortest; 5 = tallest/largest) (Capen and Martin, 1989), and any abnormalities/lesions were noted. At least two sections from each of the two lobes of the thyroid were examined.

All collected tissues were routinely processed into paraffin blocks, sectioned, stained with hematoxylin and eosin (except the testes which were stained with modified periodic acid-Sciffs and eosin), and examined microscopically.

II. RESULTS

A. Mortality: All animals survived until scheduled termination.

B. Clinical Signs of Toxicity: No clinical signs of toxicity were observed in animals for any dose group.

C. General Growth and Preputial Separation: Body weights, body weight gains, age of attainment of PPS, weight at PPS, and proportion/incidence of unseparated are presented in Table 2.

No treatment-related effects on age or weight at attainment of PPS were observed at any dose of chlorpyrifos. The ages and weights at attainment of PPS at all doses were within the acceptable range of the performance criteria provided in the Guideline (OCSPP 890.1500).

No treatment-related effects on body weight or body weight gain were observed in any chlorpyrifos group compared to controls.

TABLE 2. General Growth and Preputial Separation (PPS) [a]

Parameter Evaluated		Vehicle Control				0.5 mg/kg/day				1 mg/kg/day				2 mg/kg/day			
		# of Males	Mean	SD	CV (%)	# of Males	Mean	SD	CV (%)	# of Males	Mean	SD	CV (%)	# of Males	Mean	SD	CV (%)
Initial body weight (PND 23; g)	U	16	55.7	5.31	9.53	16	56.6	4.62	8.16	16	56.0	4.9	8.75	16	56.8	5.83	10.27
Body weight at PPS (g)	U	16	199	32	16.08	15	201.9	25.85	12.8	16	200	21.87	10.93	16	204.4	22.66	11.08
	A	16	200.4	5.71	NA	15	200.8	5.89	NA	16	200.8	5.7	NA	16	203.3	5.7	NA
Final body weight (g)	U	16	274.3	33.47	12.2	16	272.4	31.84	11.69	16	270.1	27.68	10.25	16	280.2	25.5	9.1
	A	16	276.7	5.31	NA	16	271	5.3	NA	16	271.2	5.3	NA	16	278.1	5.31	NA
Final body weight (% of control)	U	NA	NA	NA	NA	16	99.3	11.6	11.7	16	98.5	10.1	10.2	16	102.1	9.3	9.1
	A	NA	NA	NA	NA	---	---	---	---	---	---	---	---	---	---	---	---
Body weight gain (final – initial; g)	U	16	218.6	29.65	13.56	16	215.8	28.3	13.12	16	214.1	24.87	11.62	16	223.4	21.99	9.84
	A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Age at PPS (PND)	U	16	43.8	1.4	3.1	15	44.2	1.3	2.9	16	44.2	1.6	3.5	16	43.8	2.1	4.8
	A	16	43.7	0.37	NA	15	44.3	0.39	NA	16	44.2	0.37	NA	16	43.8	0.37	NA
Proportion unseparated (#/N)		0/16				0/15				0/16				0/16			

a Data were obtained from page 48 of the study report.
U Unadjusted for body weight on PND 23
A Adjusted for body weight on PND 23
SD Standard Deviation
CV Coefficient of Variation
NA Not applicable
--- Not required as part of the test guideline

D. Food Consumption: Food consumption data were not reported.

E. Organ Weights: Organ weights at necropsy are presented in Table 3. No treatment-related organ weight effects were observed at any dose of chlorpyrifos compared to controls. At 1 mg/kg/day, the seminal vesicle + coagulating gland with fluid and without fluid weights were increased ($p<0.05$) by 15 and 16%, respectively. However, these findings were not considered to be related to treatment as they were within the expected range of normal biological variability and were not dose dependent.

The unadjusted values for all organ weights in the control group were within the acceptable range of the performance criteria provided in the Guideline (OCSPP 890.1500), with the exception of the mean thyroid weight (10.8 mg compared with 14 mg as the lowest acceptable value), and kidney weight (1.86 g compared with 2.242 g as the lowest acceptable value). It was stated that the mean thyroid and kidney weights were consistent with historical control data generated in this laboratory (data not provided), which indicates consistency in the dissection of these tissues; therefore, these differences were unlikely to affect the study outcome. With respect to CV values, several parameters had slightly elevated CVs relative to the performance criteria, including seminal vesicle weight, body weight at PPS, final body weight and liver weight. Generally, the CV values in the current study were only slightly higher than the acceptable ranges; therefore, these differences have little or no impact on the outcome of the study.

TABLE 3. Organ Weights at Necropsy [a]

Organ		Vehicle Control				0.5 mg/kg/day				1 mg/kg/day				2 mg/kg/day			
		# of Males	Mean	SD	CV (%)	# of Males	Mean	SD	CV (%)	# of Males	Mean	SD	CV (%)	# of Males	Mean	SD	CV (%)
Liver (g)	U	16	12.4	1.94	15.63	16	12.43	1.89	15.18	16	12.11	1.44	11.87	16	12.88	1.5	11.67
	A	16	12.5	0.368	NA	16	12.37	0.367	NA	16	12.16	0.367	NA	16	12.79	0.368	NA
	R	16	4.51	0.32	7.13	16	4.55	0.31	6.79	16	4.48	0.3	6.69	16	4.59	0.29	6.23
Kidneys (g)	U	16	1.86	0.24	12.89	16	1.89	0.24	12.74	16	1.89	0.14	7.27	16	1.96	0.22	11.27
	A	16	1.88	0.037	NA	16	1.88	0.037	NA	16	1.89	0.037	NA	16	1.94	0.037	NA
	R	16	0.68	0.04	5.54	16	0.69	0.04	6.37	16	0.7	0.04	5.98	16	0.7	0.04	5.42
Pituitary (mg)	U	15	9.2	1.4	15.4	16	9.3	1.3	14	16	9.2	1	10.9	16	8.9	0.8	9.3
	A	15	9.2	0.26	NA	16	9.3	0.25	NA	16	9.3	0.25	NA	16	8.9	0.25	NA
	R	15	3.3	0.3	10.2	16	3.4	0.3	8.6	16	3.4	0.4	12.1	16	3.2	0.3	10.1
Adrenals (mg)	U	15	42.5	8.1	19	16	42.1	6.6	15.7	16	44.0	8.4	19	16	42.2	6.4	15.1
	A	15	42.9	1.73	NA	16	41.9	1.67	NA	16	44.2	1.67	NA	16	41.9	1.67	NA
	R	15	15.5	2.1	13.4	16	15.5	2.5	15.8	16	16.3	2.9	18	16	15.1	1.8	11.7
Thyroid, fixed (mg)	U	16	10.8	1.9	17.8	16	12.1	2.7	22	16	10.9	1.9	17.4	16	11.5	2	17.5
	A	16	10.8	0.54	NA	16	12.1	0.54	NA	16	10.9	0.54	NA	16	11.5	0.54	NA
Seminal vesicle + coagulating gland, with fluid (mg)	U	15	431.1	101.9	23.6	16	475.7	105.3	22.1	16	503.5	136	27	16	464.2	96.2	20.7
	A	15	441.7	21.15	NA	16	470.2	20.44	NA	16	507.0* (↑15)	20.43	NA	16	456.2	20.45	NA
Seminal vesicle + coagulating gland, without fluid (mg)	U	15	219.8	37.7	17.1	16	254.2	50.2	19.8	16	259.8	71.7	27.6	16	244.5	44.7	18.3
	A	15	224.7	10.31	NA	16	251.6	9.97	NA	16	261.5* (↑16)	9.96	NA	16	240.8	9.98	NA
Ventral prostate (mg)	U	15	193.5	29.2	15.1	16	204.2	39.5	19.3	16	202.3	56.2	27.8	16	187.4	39.2	20.9
	A	15	196.3	9.72	NA	16	202.7	9.39	NA	16	203.2	9.39	NA	16	185.3	9.4	NA
Dorsolateral prostate (mg)	U	16	164.0	38.7	23.6	16	160.1	29.5	18.4	16	167.6	31.9	19	16	164.9	26.6	16.2
	A	16	165.4	7.39	NA	16	159.2	7.39	NA	16	168.3	7.38	NA	16	163.6	7.39	NA
LABC (mg)	U	16	451.4	79.2	17.5	16	462.4	78.7	17.0	16	459.2	80.3	17.5	16	452.8	60.7	13.4
	A	16	456.6	14.78	NA	16	459.3	14.76	NA	16	461.7	14.76	NA	16	448.2	14.77	NA
Epididymis, left (mg)	U	16	203.8	24.7	12.1	16	209.8	27.9	13.3	16	214.7	20.9	9.7	16	209.9	28.5	13.6
	A	16	205.2	5.53	N	16	208.9	5.52	NA	16	215.4	5.52	NA	16	208.6	5.53	NA
Epididymis, right (mg)	U	16	211.6	29.6	14.0	16	217.9	26	11.9	16	224.3	19.2	8.6	16	210.3	28.5	13.6
	A	16	213.4	5.24	NA	16	216.9	5.23	NA	16	225.2	5.23	NA	16	208.7	5.23	NA

TABLE 3. Organ Weights at Necropsy [a]

Organ		Vehicle Control				0.5 mg/kg/day				1 mg/kg/day				2 mg/kg/day			
		# of Males	Mean	SD	CV (%)	# of Males	Mean	SD	CV (%)	# of Males	Mean	SD	CV (%)	# of Males	Mean	SD	CV (%)
Testis, left (mg)	U	16	1416	157.4	11.1	16	1415	107.2	7.6	16	1422	73.0	5.1	16	1420	92.3	6.5
	A	16	1421	25.6	NA	16	1412	25.57	NA	16	1425	25.56	NA	16	1415	25.59	NA
Testis, right (mg)	U	16	1416	143.0	10.1	16	1418	109.1	7.7	16	1436	75.3	5.2	16	1425	99.9	7
	A	16	1421	25.52	NA	16	1415	25.49	NA	16	1438	25.49	NA	16	1420	25.51	NA

a Data were obtained from Table 9 on pages 52 and 53 of the study report. Percent difference from controls (calculated by reviewers) is presented parenthetically.
U Unadjusted for body weight on PND 23
A Adjusted for body weight on PND 23
R Organ-to-body weight ratio (relative to body weight)
SD Standard Deviation
CV Coefficient of Variation
* Significantly different by Dunnett's test (alpha = 0.05) after a non-significant ANCOVA analysis.

F. Clinical Chemistry and Hormone Levels: Mean hormone levels are presented in Table 4. No treatment-related effects on serum T_4, serum TSH, or testosterone levels were observed at any dose of chlorpyrifos compared to controls. RBC ChE activity was dose-dependently inhibited ($p<0.05$) between 34-100% at all doses of chlorpyrifos. Brain ChE activity was inhibited ($p<0.05$) by 23% at 2 mg/kg/day. No statistically significant differences from controls were noted in any other clinical chemistry parameter. The hormone values for the control group were within the acceptable range of the performance criteria provided in the Guideline (OCSPP 890.1500).

TABLE 4. Hormone Levels and Clinical Chemistry [a]

Parameter Evaluated	Vehicle Control				0.5 mg/kg/day				1 mg/kg/day				2 mg/kg/day			
	# of Males	Mean	SD	CV (%)	# of Males	Mean	SD	CV (%)	# of Males	Mean	SD	CV (%)	# of Males	Mean	SD	CV (%)
Hormones																
Serum T_4, Total (µg/dL)	16	4.78	0.543	11.35	16	4.98	0.76	15.25	16	4.97	0.477	9.6	16	4.55	0.517	11.36
Serum TSH (ng/mL)	16	8.16	4.105	50.29	16	6.86	5.091	74.25	16	8.41	4.27	50.8	16	5.47	3.621	66.21
Serum testosterone (ng/mL)	16	2.67	1.388	51.95	16	3.03	1.98	65.23	16	3.67	2.802	76.39	16	2.61	1.437	55.04
Cholinesterase																
RBC (U/L)	15	3965	1570.1	39.6	16	2625* (↓34)	862.5	32.86	16	554* (↓86)	617.5	111.46	16	10*[b]	0	0
Brain (U/L)	16	50078	990.4	1.98	16	50077	2026.5	4.05	16	50067	1139	2.27	16	38795* (23)	3710.7	9.56

a Data were obtained from Table 8 on page 49 of the study report. Percent ChE inhibition (calculated by reviewers) is presented parenthetically.
b RBC cholinesterase levels below the limit of quantitation (LOQ = 20 U/L) were expressed as 10 U/L for reporting purposes.
SD Standard Deviation
CV Coefficient of Variation
* Significantly different from controls at $p<0.05$.

G. Gross pathology: There were no effects of treatment observed at necropsy.

H. Histopathology: The incidences of histopathological findings of the thyroid gland are presented below in Table 5. All thyroid glands examined in the 2 mg/kg/day group were considered within normal limits of control tissues.

There were no treatment-related histopathological findings noted in the testes, epididymides or kidneys at any dose.

TABLE 5. Incidence of Histopathological Lesions of the Thyroid Gland [a]

Treatment Groups	Parameter Evaluated					
	Colloid Area			Follicular Cell Height		
	Grade [b]	Incidence		Grade [b]	Incidence	
		O	E		O	E
Vehicle control	1	0	16	1	1	16
	2	0	16	2	12	16
	3	3	16	3	3	16
	4	12	16	4	0	16
	5	1	16	5	0	16
2 mg/kg/day	1	0	16	1	0	16
	2	1	16	2	11	16
	3	4	16	3	4	16
	4	11	16	4	1	16
	5	0	16	5	0	16

a Data were obtained from Table 11 on page 59 of the study report.
b Thyroid histopathology is graded 1 – 5; follicular cell height, 1 = lowest, 5 = highest, and colloid area, 1 = most colloid, 5 = least colloid. See OECD No. 82 for reference.
O No. Observed
E No. Examined

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATOR'S CONCLUSIONS: The Investigators concluded that based on the lack of treatment-related changes in puberty onset, endocrine-sensitive organ weights, serum testosterone, T_4 and TSH levels, and testicular, epididymal and thyroid histopathology, there was no evidence of endocrine activity for chlorpyrifos in the male pubertal assay at doses up to and including 2.0 mg/kg/day, the highest dose level tested. ChE activity, which was significantly inhibited in RBCs at all dose levels and in brain at 2.0 mg/kg/day, remains a highly sensitive endpoint to detect chlorpyrifos exposure and toxicity.

B. AGENCY COMMENTS: Chlorpyrifos was tested up to 2 mg/kg/day. No treatment-related effects were observed on mortality, clinical signs, body weights, body weight gains, age or weight at attainment of PPS, hormone (serum T_4, serum TSH, or testosterone) levels, organ weights, and gross or histopathology parameters at any dose.

RBC ChE activity was dose-dependently inhibited ($p<0.05$) between 34-100% at all doses of chlorpyrifos. Brain ChE activity was inhibited ($p<0.05$) by 23% at 2 mg/kg/day.

C. STUDY DEFICIENCIES: The following deficiencies were noted that are not considered to have had an adverse impact on the results, interpretation or conclusions of this study:

- Several parameters had slightly elevated CVs relative to the performance criteria, including seminal vesicle weight, body weight at PPS, final body weight and liver weight.

DATA EVALUATION RECORD

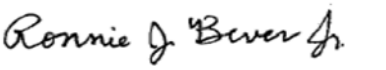
CHLORPYRIFOS

Study Type: OCSPP 890.1550, Steroidogenesis Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-14-2012 (MRID 48615510)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CSS-Dynamac Corporation
1910 Sedwick Road,
Building 100, Suite B
Durham, NC 27713

Primary Reviewer Michelle Sharpe-Kass, M.S.	Signature: Date:	 2/03/2012
Secondary Reviewer Ronnie J. Bever Jr., Ph.D., D.A.B.T.	Signature: Date:	 2/06/2012
Program Manager: Jack D. Early, M.S.	Signature: Date:	 2/08/2012
Quality Assurance: Jack D. Early, M.S.	Signature: Date:	 2/08/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel**.**

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Gregory Akerman **Signature:**
Health Effects Division **Date:**
Secondary Reviewer: John Liccione, Ph.D. **Signature:**
Health Effects Division **Date:** 5-21-15

DATA EVALUATION RECORD

STUDY TYPE: Steroidogenesis Assay (H295R Cells); OCSPP 890.1550

PC CODE: 059101 **DP BARCODE:** D397128

TXR#: 0052086 **CAS#:** 2921-88-2

TEST MATERIAL (PURITY): Chlorpyrifos (99.8% a.i.)

SYNONYMS: Chlorpyrifos-ethyl; Chlorpyrifos; Chlorpyriphos; O,O-Diethyl O-(3,5,6-trichloro-2-pyridinyl)phosphorothioate

CITATION: Le Baron, M.J.; Kan, H.L.; Perala, A.W. (2011). Evaluation of chlorpyrifos in the *in vitro* steroidogenesis assay. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, MI. Laboratory Report No.: 101189, Oct 24, 2011. MRID 48615510. Unpublished.

SPONSOR: Dow AgroSciences, LLC, Indianapolis IN

TEST ORDER #: CON-059101-4

EXECUTIVE SUMMARY: In a steroidogenesis assay (MRID 48615510), H295R cells cultured *in vitro* in 24-well plates were incubated with chlorpyrifos (99.8% purity, Lot # KC28131419, TSN101285 at concentrations of 100, 10, 1, 0.1, 0.01, 0.001, and 0.0001 µM in triplicate for 48 hours. Dimethyl sulfoxide (DMSO) was used as a vehicle, at a final concentration of 0.1%.

Testosterone and estradiol levels were measured using LC/APPI-MS/MS. Three independent experiments were performed. A Quality Control (QC) plate was run concurrently with each independent run of a test chemical plate to demonstrate that the assay responded properly to positive control agents at two concentration levels. The positive controls included the known inhibitor (prochloraz) and inducer (forskolin) of estradiol and testosterone production.

Guideline recommendations were met including: lack of cytotoxicity, adequate production of testosterone and estradiol, acceptable reproducibility (low %CV), and appropriate induction and inhibition with positive controls.

Both testosterone and estradiol concentrations were affected by chlorpyrifos. In each of the three independent runs of the assay at 10 and 100 µM, statistically significant inhibition of testosterone was observed, and statistically significant increases of estradiol were observed. The average decrease in testosterone concentration was 0.6-fold at 10 µM and 0.4-fold at 100 µM. The

average increase in estradiol concentration was 2.3-fold at 10 μM and 2.4-fold at 100 μM. No changes in hormone production were noted at ≤ 1 μM of chlorpyrifos.

Based on the hormone responses in each of the three independent runs, chlorpyrifos treatment resulted in statistically significant and reproducible decreases in testosterone production and increases in estradiol production.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for a Steroidogenesis assay (OCSPP 890.1550).

COMPLIANCE: Signed and dated GLP Compliance, Quality Assurance, and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS

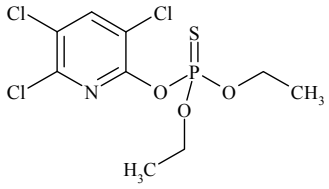
A. MATERIALS

1. Test Facility: Toxicology & Environmental Research and Consulting

Location: Midland, MI
Study Director: Kan, H.L
Other Personnel: LeBaron, M.J., Lead Scientist
Perala, A.W., Analytical Chemist
Gollapudi, B.B., Technical reviewer
Study Period: 2-1-2011 to 2-8-2011

2. Test Substance: Chlorpyrifos

Description: White solid
Lot # (expiration date): KC28161419, TSN101285 (Not provided)
Purity: 99.8% a.i.
Solubility (in solvent): Soluble in DMSO up to 0.1 M
Volatility: Not provided
Stability: Not provided
Storage conditions: Ambient
CAS #: 2921-88-2
Molecular weight: 350.6
Structure:



3. Positive Control: Forskolin

Description (molecular weight): White powder (410.5)
Source: Sigma-Aldrich (St Louis, MO)
Lot #: (expiration date): 097D50653 (not provided)
Purity: 99%
Solubility (in solvent): Soluble in DMSO up to 0.01 M
Storage conditions: Ambient
CAS #: 66575-29-9

4. Negative Control: Prochloraz

Description (molecular weight): White, Beige Powder (376.67)
Source: Sigma-Aldrich (St Louis, MO)
Lot #: (expiration date): SZE6220X (not provided)
Purity: 99.1%
Solubility (in solvent): Soluble in DMSO up to 0.01M
Storage conditions: Ambient
CAS #: 67747-09-5

5. Solvent/Vehicle Control: Dimethyl Sulfoxide (DMSO)

Description (molecular weight):	Clear liquid (78.13)
Source:	Sigma-Aldrich (St. Louis, MO) (Cat. # 276855; D5879)
Lot # (expiration date):	68996LMV:108K0186 (not provided)
Purity:	99.9–100%
Storage conditions:	Ambient
CAS #:	67-68-5
Justification for choice of solvent:	Not provided
Final concentration: (% volume in assay)	0.1%

6. Stock Medium: Dulbecco's modified Eagle's medium/F12 Ham nutrient mixture

Source:	Sigma-Aldrich (St. Louis, MO) (Cat. # D-6434)
Lot #: (expiration date):	RNBB 2720 (not provided)
Sodium bicarbonate:	A component of DMEM: F12 Ham media
Nu-Serum:	BD-Biosciences (Palo Alto, CA); Catalog # 355100; Lot # 81515; tested for background hormone concentrations by performing laboratory
ITS+ premix:	BD-Biosciences (Palo Alto, CA); Catalog # 354352; Lot # 84337
Other components:	2.5 mM L-glutamine, 25 IU/mL penicillin, 25 µg/mL streptomycin (GIBCO, Grand Island, NY)

7. Test Cells: H295R human adrenocortical carcinoma cells (ATCC CLR-2128; lot # not reported) at passage 7.5 – 8.5 were incubated in the stock medium. Incubation conditions were at 5% CO_2 and approximately 37°C.

The following performance criteria were met (indicated by an "x"):

x	Cell passage identifier. Cell Passage #: 7.5 – 8.5
x	Cells frozen down at passage 5
x	Frozen cells cultured for at least 4 additional passages
x	Total number of passages does not exceed 10

B. METHODS

1. Pre-Test Information:

a. Hormone Assay Interference Test: A hormone assay interference test was not performed.

b. Hormone Extraction: See Section on "Hormone Measurement System."

c. Laboratory Proficiency Test: No laboratory proficiency test data were provided.

2. Test Solutions: Chlorpyrifos was dissolved in DMSO to make stock solutions from 10^{-7} to 10^{-1} M. Stock solutions were then diluted 1:1000 in the final treatment medium. No information was provided on the creation of stock solutions for forskolin and prochloraz. When added to the cell culture plates, the final concentration for forskolin was 1 or 10 µM and the final concentration for prochloraz was 0.1 or 1 µM. The final concentration of DMSO in the medium was 0.1%. No precipitation was reported.

3. **Cell Plating and Preincubation:** H295R cells (ATCC CLR-2128) were grown for five passages, frozen in liquid nitrogen, then thawed and cultured for four additional passages. The cells were then seeded in 24-well plates at a concentration of 200,000–300,000 cells/mL, yielding approximately 50-60% confluency at 24 hours. The seeded plates were incubated for 24 hours at 37 °C in a 5% CO_2 atmosphere. The cells were checked microscopically for good attachment and proper morphology.

4. **Exposure:** The medium was removed and replaced with medium containing chlorpyrifos at the appropriate concentration (or only 0.1% DMSO) in triplicate according to the schematic presented in Table 1.

TABLE 1. Dosing Schematic for the Exposure of H295R Cells to Chlorpyrifos (Final Concentrations in µM)[a]

	1	2	3	4	5	6
A	DMSO	DMSO	DMSO	0.1	0.1	0.1
B	100	100	100	0.01	0.01	0.01
C	10	10	10	0.001	0.001	0.001
D	1	1	1	0.0001	0.0001	0.0001

a Data were obtained from page 17 of the study report.

A concurrent QC plate was included with each of the three independent runs of the test chemical plates to demonstrate the assay's response to forskolin (an inducer of testosterone and estradiol production) and prochloraz (an inhibitor of testosterone and estradiol production). The QC plate was prepared and dosed in the same manner with either forskolin or prochloraz according to the schematic presented in Table 2.

TABLE 2. Dosing Schematic for the QC Plate for Positive Controls (Final Concentrations in µM)[a]

	1	2	3	4	5	6
A	Blank[b]	Blank	Blank	Blank + MeOH[c]	Blank + MeOH	Blank + MeOH
B	DMSO	DMSO	DMSO	DMSO + MeOH	DMSO + MeOH	DMSO + MeOH
C	Forskolin 1µM	Forskolin 1µM	Forskolin 1µM	Prochloraz 0.1µM	Prochloraz 0.1µM	Prochloraz 0.1µM
D	Forskolin 10µM	Forskolin 10µM	Forskolin 10µM	Prochloraz 1µM	Prochloraz 1µM	Prochloraz 1µM

a Data were obtained from page 16 of the study report.
b Blank wells received medium only.
c MeOH = 70% methanol was added to these wells for 30 minutes at room temperature following medium removal.

Following dosing, the plates were incubated for 48 hours under the conditions previously described. The medium from each well was removed, split into two equal volume aliquots, and frozen at −80 °C until hormone measurements.

5. **Cell Viability/Cytotoxicity Assay:** Cell viability was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Madison Wisconsin, Catalog # G3580) immediately after removal of the culture medium. The kit was used per manufacturer's instruction. The assay kit is a colorimetric modified MTT cell viability assay.

6. **Hormone Measurement System:** Testosterone and estradiol were extracted from H295R supplemented medium by liquid-liquid extraction using methylene chloride after the

addition of internal standards for testosterone and estradiol. Extracts were derivatized by adding sodium bicarbonate buffer followed by a dansyl chloride solution. The samples were analyzed by a validated LC/APPI-MS/MS procedure. The lower limit of quantification (LLQ) was 10 pg/mL for estrogen and 25 pg/mL for testosterone. The levels of testosterone and estradiol were quantified using internal standard calibration. No study samples were re-analyzed during this study.

The following performance criteria were met (indicated by an "x"):

x	Method detection limit (100 pg/mL testosterone; 10 pg/mL estradiol)
x	Spiked sample recovery acceptable for two concentrations of testosterone and estradiol (mean measured amount from triplicate samples within 30% of nominal concentration)
NA	Hormone cross-reactivity (antibody-based assays only; ≤30% of basal production of the respective hormone)
x	Solvent control within 75% range below maximum response on standard curve
NA	Test compound tested for interference with measurement system

C. DATA ANALYSIS: Mean values (pg/mL) and standard deviations for testosterone and estradiol were calculated for each concentration of chlorpyrifos, reference chemical, solvent control (SC), blank, and background wells. Relative changes in testosterone and estradiol production were calculated using the equation below:

Relative change = (hormone concentration in each well) ÷ (mean SC hormone concentration).

Homogeneity of variance was evaluated by Bartlett's test and normality by Shapiro-Wilk's test at alpha = 0.01. If the data were not homogeneous or normally distributed, then the data were transformed to approximate homogeneity or a normal distribution. If the data were homogeneous and approximately normally distributed, differences between chemical treatments and SC were analyzed using a parametric analysis of variance followed by Dunnett's test, if significant. If the data were not homogeneous or normally distributed, a non-parametric test was used (Kruskal Wallis) and if significant, was followed by the Wilcoxon rank sum test with a Bonferroni-Holm correction. Differences were considered significant at $p \leq 0.05$. Software used for statistical analysis was not reported. The reviewers consider these analyses acceptable.

II. RESULTS

A. TEST COMPOUND: Precipitation of the test compound was not reported for chlorpyrifos. The %CVs for solvent control replicate wells for testosterone within a plate based on absolute concentrations were 2.01-5.38%. The %CVs for solvent control replicate wells for estradiol within a plate based on absolute concentrations were 2.53-4.47%. The between plate %CV for solvent controls based on absolute concentrations was 1.0% for testosterone and 17% for estradiol. These values were below the maximum guideline recommended level of 30%.

Both testosterone and estradiol concentration were affected by chlorpyrifos. In each of the three independent runs of the assay, statistically significant inhibition of testosterone was observed, and statistically significant increases of estradiol were observed (Table 3). The average fold decrease in testosterone concentration was 0.6 at 10 µM and 0.4 at 100 µM.

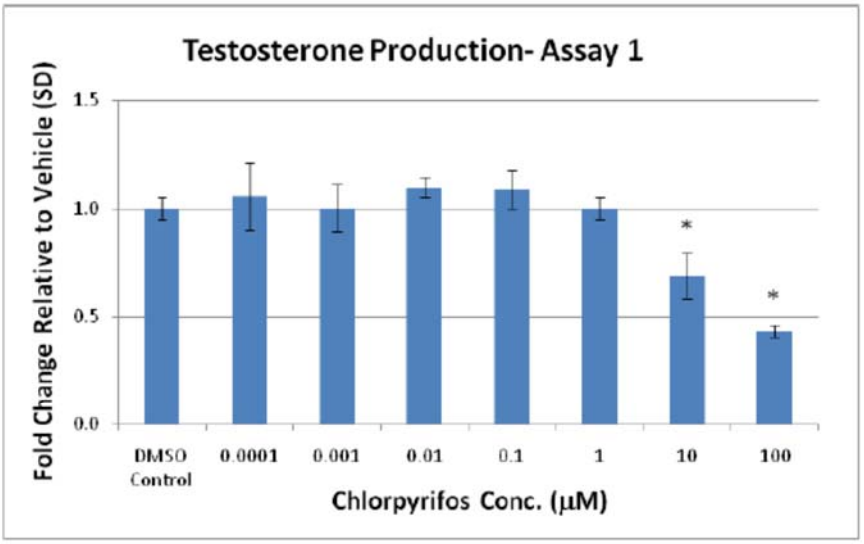
The average fold increase in estradiol concentration was 2.3 at 10 µM and 2.4 at 100 µM. The change in hormone concentrations was statistically significant for all three runs of the assay at 10 and 100 µM chlorpyrifos. Testosterone and estradiol levels were unaffected at concentrations of ≤1 µM chlorpyrifos. The changes in testosterone and estradiol concentrations are shown graphically in Figures 1 and 2, respectively.

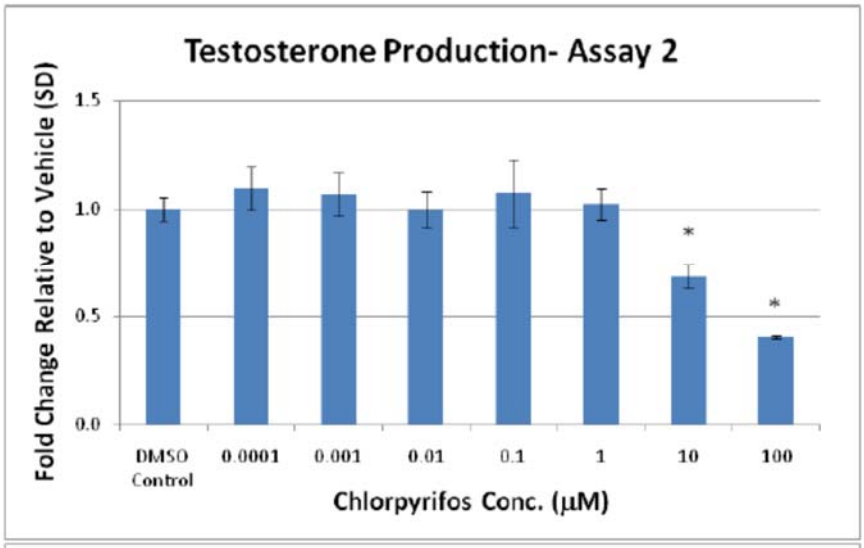
TABLE 3. Mean (±SD) Hormone Concentrations Following Treatment with Chlorpyrifos for 48 Hours.[a]

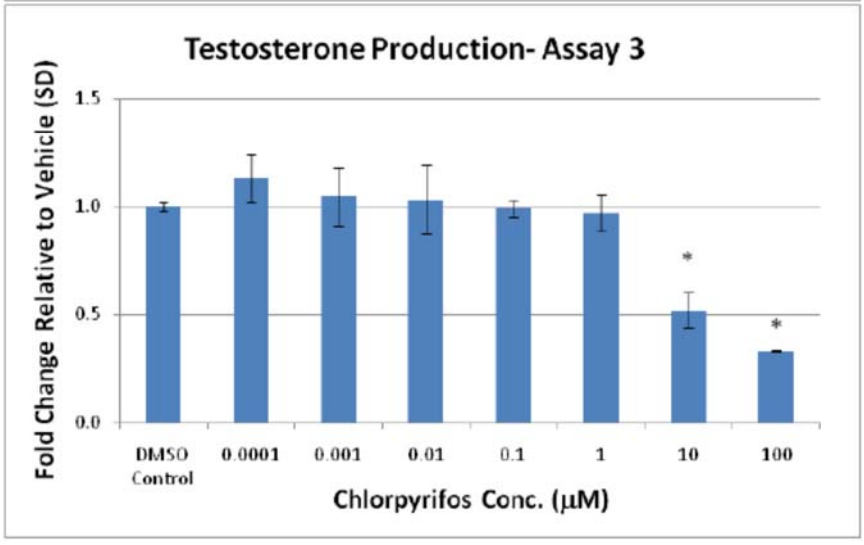
Nominal Concentration (µM)	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Mean	± SD	Statistical Significance
	Testosterone (pg/mL)			Fold Difference					
DMSO	525.0	536.0	531.3	1.0	1.0	1.0	1.0	0.0	
0.0001	554.3	586.3	600.0	1.1	1.1	1.1	1.1	0.0	None
0.001	526.0	573.0	555.3	1.0	1.1	1.0	1.0	0.0	None
0.01	576.0	535.0	547.7	1.1	1.0	1.0	1.0	0.1	None
0.1	571.3	575.7	526.7	1.1	1.1	1.10	1.1	0.1	None
1	526.0	548.7	514.7	1.0	1.0	1.0	1.0	0.0	None
10	362.7	367.3	276.3	0.7	0.7	0.5	0.3	0.1	Trials 1, 2, and 3
100	222.7	217.3	176.0	0.4	0.4	0.3	0.4	0.1	Trials 1, 2, and 3
	Estradiol (pg/mL)			Fold Difference					
DMSO	49.8	54.2	68.5	1.0	1.0	1.0	1.0	0.0	
0.0001	43.3	48.3	56.3	0.9	0.9	0.8	0.9	0.0	None
0.001	45.0	47.5	56.8	0.9	0.9	0.8	0.9	0.0	None
0.01	45.4	48.2	59.1	0.9	0.9	0.9	0.9	0.0	None
0.1	45.3	49.3	60.1	0.9	0.9	0.9	0.9	0.0	None
1	53.8	54.4	65.3	1.1	1.0	1.0	1.0	0.1	None
10	116.7	130.0	150.7	2.3	2.4	2.2	2.3	0.1	Trials 1, 2, and 3
100	135.0	127.0	150.3	2.7	2.3	2.2	2.4	0.3	Trials 1, 2, and 3

a Data were obtained from page 30 of the study report.

FIGURE 1. Change in Testosterone Production Relative to Chlorpyrifos Concentration.

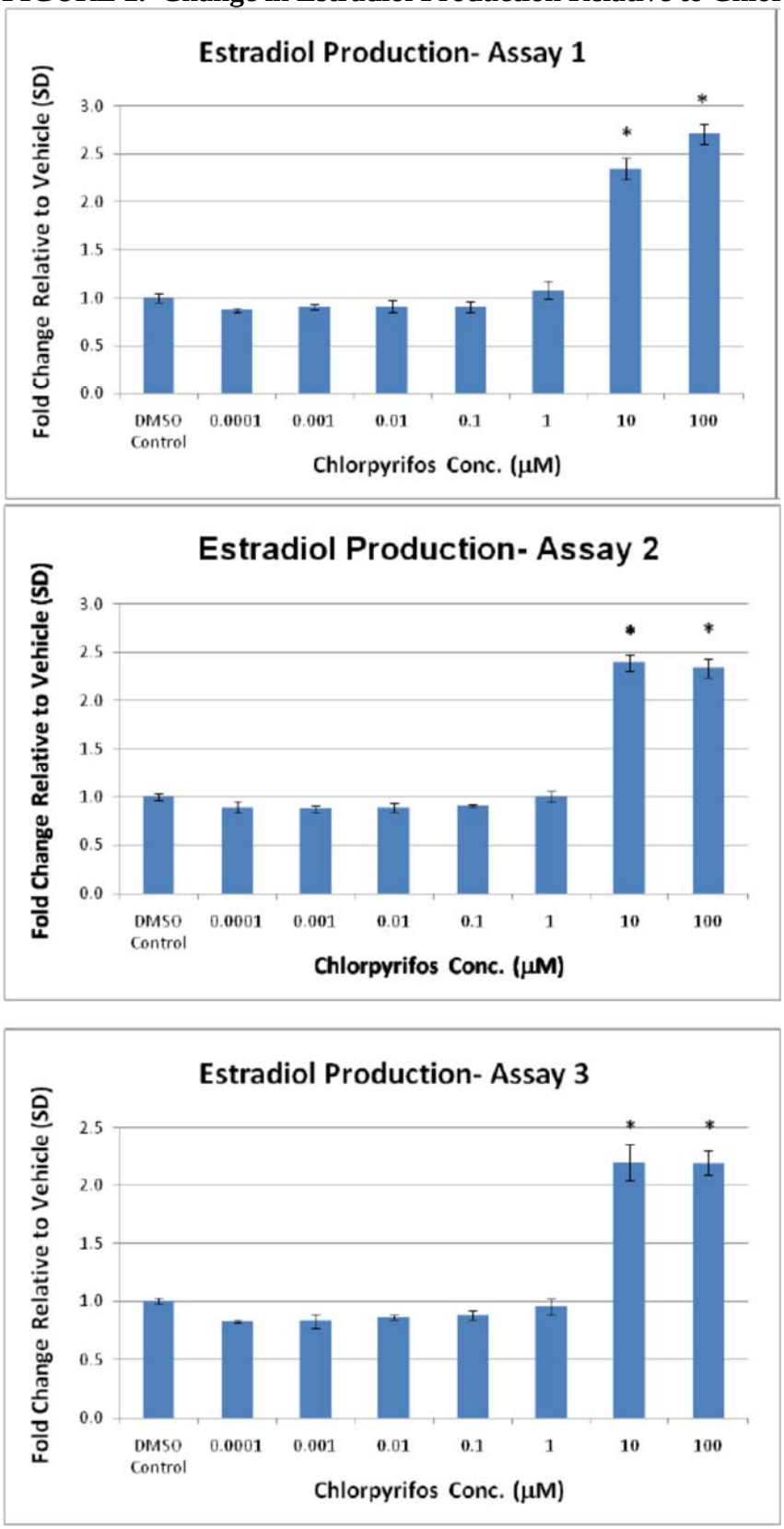






* Significantly different from the solvent control at $p \leq 0.05$.

FIGURE 2. Change in Estradiol Production Relative to Chlorpyrifos Concentration.



* Significantly different from the solvent control at $p \leq 0.05$.

B. CYTOTOXICITY: Less than 5% cytotoxicity was noted in the reference and chlorpyrifos-treated wells (106.7-130.2% cytotoxicity, unrelated to dose), except in the methanol-treated wells. Data are summarized in Table 4.

TABLE 4. Mean (±SD) MTT Cell Viability Results after Treatment with Forskolin, Prochloraz, or Chlorpyrifos for 48 Hours. [a]

Compound	Concen. (µM)	Cell Viability – Trial 1		Cell Viability – Trial 2		Cell Viability – Trial 3	
		Mean	SD	Mean	SD	Mean	SD
DMSO Control	NA	100.0	NR	100.0	NR	100.0	NR
Media	NA	84.2	NR	96.1	NR	92.0	NR
DMSO + Methanol	NA	27.1	NR	32.0	NR	31.9	NR
Media +Methanol	NA	27.4	NR	29.3	NR	29.7	NR
Forskolin	1 [b]	108.3	NR	110.6	NR	125.7	NR
Forskolin	10	115.6	NR	111.1	NR	127.1	NR
Prochloraz	0.1	103.9	NR	108.6	NR	106.4	NR
Prochloraz	1	102.3	NR	107.7	NR	104.9	NR
DMSO Control	NA	100.0	NR	100.0	NR	100.0	NR
Chlorpyrifos	0.0001	118.9	NR	111.8	NR	130.2	NR
Chlorpyrifos	0.001	107.5	NR	111.6	NR	129.0	NR
Chlorpyrifos	0.01	116.0	NR	110.8	NR	125.5	NR
Chlorpyrifos	0.1	109.4	NR	112.1	NR	109.3	NR
Chlorpyrifos	1	113.7	NR	112.6	NR	112.9	NR
Chlorpyrifos	10	106.7	NR	108.9	NR	117.7	NR
Chlorpyrifos	100	110.2	NR	113.2	NR	118.7	NR

a Data were obtained from page 42 of the study report.
NA = Not applicable
NR = Not reported

C. QC PLATE: The minimum basal hormone production levels (500 pg/mL for testosterone, 40 pg/mL for estradiol) were generally met in both blank and SC wells (Table 5). There were slight departures in one testosterone SC well (470 pg/mL) and in one estradiol SC well (35.5 pg/mL). Compared to SC, 10 µM forskolin on average induced testosterone by 2.9-fold and estradiol by 17.3-fold. Compared to SC, 1 µM prochloraz on average inhibited the synthesis of testosterone to 0.3-fold and estradiol to 0.5-fold. Thus, the guideline requirements were met indicating that the assay was sensitive to induction and inhibition of testosterone and estradiol.

The variability (%CV) between the runs (calculated by the reviewer) based on the absolute hormone concentrations in the SC were 13% for testosterone and 19% for estradiol, and were within the recommended limit of ≤30%. The %CVs within each run for the QC plates were 2.0-7.2% for testosterone and 2.9-4.9% for estradiol.

TABLE 5. Hormone Concentrations Following Treatment with Forskolin or Prochloraz for 48 Hours.[a]									
Compound	Conc. (µM)	Trial 1	Trial 2	Trial 3	Trial 1	Trail 2	Trial 3	Mean	± SD
		Testosterone (pg/mL)			Fold Difference				
Background	NA	NR	NR	NR	NR	NR	NR	NR	NR
Blank	NA	773	614	693	--	--	--	--	--
DMSO	NA	563	470	548	1.0	1.0	1.0	1.0	0.0
Forskolin	1	1130	969	1043	2.0	2.1	1.9	2.0	0.1
Forskolin	10	1750	1360	1497	3.1	2.9	2.7	2.9	0.2
Prochloraz	0.1	393	346	357	0.7	0.7	0.7	0.7	0.0
Prochloraz	1	183	133	122	0.3	0.3	0.2	0.3	0.1
		Estradiol (pg/mL)			Fold Difference				
Background	NA	NR	NR	NR	NR	NR	NR	NR	NR
Blank	NA	45.0	57.2	61.1	--	--	--	--	--
DMSO	NA	35.5	46.8	57.1	1.0	1.0	1.0	1.0	0.0
Forskolin	1	390	430	473	11.0	9.2	8.3	9.5	1.4
Forskolin	10	730	793	818	20.6	16.8	14.3	17.3	3.2
Prochloraz	0.1	28.5	35.5	39.1	0.8	0.8	0.7	0.7	0.1
Prochloraz	1	20.6	22.7	23.6	0.6	0.5	0.4	0.5	0.1

a Data were obtained from page 31 of the study report.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATOR'S CONCLUSIONS: Based on the combined hormone responses to chlorpyrifos in each of three independent H295R steroidogenesis assays, it was determined that high-dose chlorpyrifos administration resulted in a statistically significant increase in estradiol production and decrease in testosterone production. Thus, under the conditions of this study, chlorpyrifos was considered to alter steroidogenesis, but only at concentrations several orders of magnitude higher than measured blood levels that result in significant brain and red blood cell cholinesterase inhibition in adult female rats.

B. AGENCY COMMENTS: All guideline acceptability recommendations and requirements were met, including lack of cytotoxicity, adequate production of testosterone and estradiol, acceptable reproducibility (low %CV), and appropriate induction and inhibition with positive controls.

Both testosterone and estradiol production were affected by chlorpyrifos at concentrations of ≥10 µM. In each of the three independent runs of the assay at 10 and 100 µM, statistically significant inhibition of testosterone was observed, and statistically significant increases of estradiol were observed. The average fold decrease in testosterone concentration was 0.6 at 10 µM and 0.4 at 100 µM. The average fold increase in estradiol concentration was 2.3 at 10 µM and 2.4 at 100 µM. No changes in hormone production were noted at ≤ 1 µM of chlorpyrifos. Based on the hormone responses in each of the three independent runs, chlorpyrifos treatment resulted in statistically significant changes in testosterone and estradiol levels.

C. STUDY DEFICIENCIES: The following deficiencies were noted that are not considered to have had an adverse impact on the results, interpretation or conclusions of this study:

- The authors reported representative recovery of testosterone and estradiol supplemented media. These values were generally acceptable (>70%). However, the recovery of 25

and 50 ppt testosterone was low (47-65%), while recovery was 79% at 10 ppt. An explanation was not provided.

- %CV was not reported, but was calculated from the data presented.

DATA EVALUATION RECORD

CHLORPYRIFOS

Study Type: OCSPP 890.1600, *In vivo* Uterotrophic Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-14-2012 (MRID 48615511)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CSS-Dynamac Corporation
1910 Sedwick Road,
Building 100, Suite B
Durham, NC 27713

Primary Reviewer	Signature:	Rebecca L. Bryan
Rebecca L. Bryan, B.S.	Date:	1/23/2012
Secondary Reviewer	Signature:	David A. McEwen
David A. McEwen, B.S.	Date:	1/30/2012
Program Manager:	Signature:	Jack D. Early
Jack D. Early, M.S.	Date:	2/02/2012
Quality Assurance:	Signature:	Jack D. Early
Jack D. Early, M.S.	Date:	2/02/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel**.**

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Sheila Healy, Ph.D. **Signature:**
Health Effects Division **Date:** 6/5/15
Secondary Reviewer: Jess Rowland **Signature:**
Health Effects Division **Date:** 6/5/15

DATA EVALUATION RECORD

STUDY TYPE: Uterotrophic Assay (Rat); OCSPP 890.1600; OECD 440

PC CODE: 059101 **DP BARCODE:** D397128

TXR#: 0052086 **CAS#:** 2921-88-2

TEST MATERIAL (PURITY): Chlorpyrifos (99.8% a.i.)

SYNONYMS: Chlorpyrifos-ethyl; Chlorpyriphos; O,O-Diethyl O-(3,5,6-trichloro-2-pyridinyl)ester phosphorothioic acid

CITATION: Marty, M.S., Brooks, K.J. and Jeong, Y.C. (2011). Chlorpyrifos: Uterotrophic assay in the immature female CrL:CD(SD) rat. Dow Chemical Company, Midland, MI. Laboratory Project Study ID: 111008, October 10, 2011. MRID 48615511. Unpublished.

SPONSOR: Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN

TEST ORDER #: CON-059101-4

EXECUTIVE SUMMARY: In a uterotrophic assay (MRID 48615511) conducted to screen for potential estrogenic activity, chlorpyrifos (99.8% a.i., lot # KC28161419) in corn oil was administered daily via oral gavage to groups of 6 immature female, Sprague-Dawley rats at dose levels of 0 (vehicle), 0.5, 1.5, or 4 mg/kg/day on post-natal days (PND) 19−21. A positive control group was treated with 17α-ethynyl estradiol (EE) in corn oil by gavage at a dose level of 10 µg/kg/day. All animals were terminated and necropsied approximately 24 hours after the final dose on PND 22 to determine wet and blotted uterine weights.

All animals survived until scheduled termination. No clinical signs of toxicity were observed in any animals for any chlorpyrifos treated groups. No precocious vaginal opening was observed in treated females. Body weights and overall body weight gains (Days 1-4) in the chlorpyrifos treated groups were comparable to the controls throughout the study. Uterine weights in the chlorpyrifos treated groups were also comparable to the controls. Absolute wet and blotted uterus weights for the positive EE group were increased ($p<0.05$) by 528% and 408%, respectively, as expected.

The dose levels tested in this study are adequate based on the results of a probe study which showed significant decreases in brain ChE at doses ≥2 mg/kg/day.

No statistically significant changes were seen in uterine weight in this assay. Chlorpyrifos was negative in the uterotrophic assay.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for a uterotrophic assay (OCSPP 890.1600).

COMPLIANCE: Signed and dated Data Confidentiality, GLP Compliance, Flagging, and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

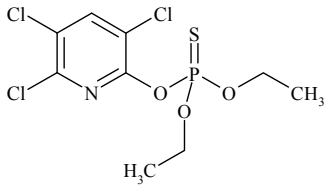
A. MATERIALS

1. Test Facility: Dow Chemical Company, Toxicology & Environmental Research and Consulting

Location: Midland, MI
Study Director: K.J. Brooks
Other Personnel: M.S. Marty (Lead Scientist)
Study Period: January 28, 2011-February 4, 2011

2. Test Substance: Chlorpyrifos

Description: Molecular weight = 350.6 g/mol
Source: Dow AgroSciences LLC (Indianapolis, IN)
Lot #: KC28161419, TSN101285
Purity: 99.8%
Stability: Stable in corn oil for up to 12 days; temperature of stability determination not reported
CAS #: 2921-88-2
Structure:



3. Reference Estrogen: 17α-ethynyl estradiol (EE)

Supplier: Sigma Aldrich (St. Louis, MO)
Lot #: 090m1241v
Purity: 98%
CAS # : 57-63-6

4. Solvent/Vehicle Control: Corn Oil

Supplier: Sigma Aldrich (St. Louis, MO)
Lot #: 0MKBD6671V
Rationale (if other than water): Selected due to the solubility properties of the test substance
Final concentration: Not applicable

5. Test Animals:

Species: Rats (immature, female only)
Strain: Sprague-Dawley (Crl:CD[SD])
Age/weight at dose initiation: Post-natal day (PND) 19; 40.7-52.9 g
Source: Charles River Laboratories (Portage, MI)
Housing: Rats were housed 6 per cage in solid bottom cages with paper pulp bedding with low phytoestrogen content (7089 Tekland Diamond Soft bedding, Harlan Laboratories, Inc., Indianapolis, IN).
Diet: Teklad Diet #2016 (Harlan Laboratories, Inc., Indianapolis, IN), *ad libitum* (low phytoestrogen rodent diet, total genistein equivalents <325 µg/g).
Water: Tap water, *ad libitum*
Environmental conditions:
Temperature: 22 ± 3 ºC
Humidity: 40-70%
Air changes: 12-15 times/hour (average)
Photoperiod: 12 hrs light/12 hrs dark
Acclimation period: 8 days; immature females housed with their dam.

B. METHODS

1. In-Life Dates: Dates not specified.

2. Study Design: Following an 8-day acclimation period, immature, intact female rats were administered the test substance from PND 19-21. Rats were euthanized approximately 24 hours after the last dose and necropsied for uterine weight measurements. The pups were housed with their dam prior to weaning on PND 18.

3. Animal Assignment: Animals were randomly assigned, stratified by body weight, to the test groups noted in Table 1 using a computer program designed to increase the probability of uniform group mean weights and standard deviations on Day 1. Statistical analysis indicated that there were no significant differences in group means at study initiation. It was not stated if the body weight of each animal was within 20% of the overall mean.

TABLE 1. Study Design [a]

Test Group	Dose (mg/kg/day)	# of Females [b]
Estrogen Agonist Assay		
Vehicle Control	0	6
Low	0.5	6
Mid	1.5	6
High	4	6
17α-ethynyl estradiol (EE), Reference Estrogen	10 µg/kg/day	6

a Data were obtained from Text Table 1 on page 16 of the study report.

5. Dose Selection Rationale: The dose levels were selected based on the results from a probe study[1] in which male and female rats were administered the test substance in corn oil via gavage at doses of 0, 1, 2, 4, or 8 mg/kg/day for 15 days. Decreased body weight gains were observed in females at the 4 and 8 mg/kg/day dose groups. There were no treatment-related differences in clinical chemistry parameters in males or females with the exception of a decrease in alanine aminotransferase levels in females at 4 and 8 mg/kg/day chlorpyrifos. There were no effects on liver or kidney weights in male or female rats, although relative adrenal weights were increased at 4 and 8 mg/kg/day in females only. There was significant inhibition of RBC ChE at all doses of chlorpyrifos in both males and females. Red blood cell (RBC) cholinesterase (ChE) activity was inhibited by 75-81% at 1 mg/kg/day and ≥ 95% at doses greater than 2 mg/kg/day. Males and females had significant decreases in brain ChE at doses ≥2 mg/kg/day. Based on these results, the dose levels selected for this study were ≤ 4 mg/kg/day.

[1] Marty, M. S. and Marshall, V. A. (In progress). Chlorpyrifos: Hershberger, Uterotrophic, and Pubertal Assay Probe Study in Crl:CD(SD) Rats. Report of Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan.

6. **(a) Dose Preparation:** Dose formulations were prepared by mixing appropriate amounts of test substance with corn oil. Chlorpyrifos dosing solutions were reportedly prepared in accordance with established stability limits. Dose volumes were adjusted daily based on individual body weight measurements. Prior to dose administration, samples of chlorpyrifos dose formulations from all three dose levels were analyzed for achieved concentration and samples from the low and high dose formulations were tested for homogeneity; samples were taken from the top, middle, and bottom of the container after stirring overnight. In a previous study,[2] chlorpyrifos was determined to be stable in corn oil for up to 12 days at concentrations ranging 0.00356-9.985 mg/mL (temperature not specified).

Results of Dose Analysis

Homogeneity (%RSD): 1.9-4.6% (top, middle, and bottom)

Stability: Stable in corn oil for up to 12 days; temperature of stability determination not reported (Dow Chemical Company, 2010)

Concentration (% of nominal): 102.0-103.9%

The analytical data indicated that the mixing procedure was adequate and that the variation between nominal and actual dosage to the animals was acceptable.

6. **Dosage Administration:** Animals were administered the chlorpyrifos formulations, positive control (EE) or vehicle control daily via gavage for three consecutive days in a dose volume of 4 mL/kg body weight. Dose volumes were adjusted daily based on the concurrent body weight measurement.

7. **Statistics:** Body weights and body weight gains were analyzed by a forced parametric test. The blotted and wet uterine weights were first analyzed by Bartlett's test for homogeneity of variance. Analysis of covariance (ANCOVA) was performed with terminal body weight as the covariate. If the ANCOVA was significant ($p \leq 0.05$), either the least square means with Dunnett's correction or the Wilcoxon Rank-Sum test was performed, as appropriate. Significance was denoted at $p \leq 0.05$. The statistical analyses were considered adequate.

C. METHODS

1. **Clinical Examinations:** Cage-side checks for mortality, moribundity and clinical signs of toxicity were conducted at least twice daily.

On PND 22 prior to necropsy, all animals were examined for vaginal patency.

[2] Marty, M. S. and Andrus, A. K. (2010). Comparison of cholinesterase (ChE) inhibition in young adult and preweanling CD rats after acute and repeated chlorpyrifos or chlorpyrifos-oxon exposures. Report of Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan.

2. **Body Weight:** Animals were weighed at randomization, daily throughout the dosing period and at termination.

3. **Food Consumption (Optional):** Food consumption was not measured.

4. **Necropsy and Measurement of Uterine Weight:** On PND 22 (approximately 24 hours after final administration of the test substance), all surviving animals were anesthetized by isoflurane, euthanized by cervical dislocation, and subjected to a gross necropsy. Dissection of the uterus was performed according to the U.S. EPA Guideline. Briefly, the vagina was removed just below the cervix in order to retain the luminal fluid in the uterus. The "wet" uterus (i.e., containing the luminal fluid) was weighed. Subsequently, the uterine horns were cut longitudinally and gently blotted with moist filter paper to remove the luminal fluid while preventing desiccation and the blotted uterus was weighed. After weighing, the uteri were fixed in 10% neutral phosphate-buffered formalin for potential future examination.

   The blood and brain samples for ChE activity assessment were collected on PND 22 from the vehicle control and chlorpyrifos-treated animals. Blood samples were collected from heart nick, stored on ice, and centrifuged, and the resulting RBC samples were collected and diluted in 1% Triton X-100. The brain samples were collected, dissected into right and left hemispheres, the right hemisphere was weighed, and both hemispheres were quick frozen in liquid nitrogen. The RBC and brain samples were stored frozen at -80 °C.

5. **Microscopic Examination (Optional):** Microscopic examinations were not conducted.

II. RESULTS

A. OBSERVATIONS

1. **Mortality:** All animals survived until scheduled termination.

2. **Clinical Signs of Toxicity:** No clinical signs of toxicity were observed in animals for any dose groups.

   No precocious vaginal opening was observed in test animals.

B. BODY WEIGHT AND WEIGHT GAIN: Body weight and body weight gain data are presented in Table 2. Body weights and overall body weight gains in the chlorpyrifos treated groups and the positive control group were comparable to the control group throughout the study.

TABLE 2. Group Body Weights and Cumulative Body Weight Gains (g) in the Estrogen Agonist Assay [a]

Study Day #	Dose Group (mg/kg/day)														
	Vehicle Control			Chlorpyrifos (0.5)			Chlorpyrifos (1.5)			Chlorpyrifos (4)			Estrogen, EE (10 µg/kg/day)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
1	6	45.4	3.3	6	46.7	3.6	6	46.4	3.6	6	46.8	3.7	6	46.2	3.7
2	6	49.4	3.7	6	49.9	4.7	6	49.9	3.8	6	49.2	3.9	6	49.4	4.0
3	6	54.0	3.6	6	53.9	4.8	6	54.8	4.4	6	52.6	4.1	6	53.3	4.7
4	6	57.8	3.8	6	58.5	5.5	6	58.4	5.1	6	56.4	3.8	6	57.0	5.2
BWG Days 1-4	6	12.4	1.2	6	11.8	2.4	6	12.0	2.3	6	9.6	0.3	6	10.9	2.0

a Data were obtained from Table 3 on page 31 of the study report.
N No. of animals in the group
SD Standard Deviation

C. FOOD CONSUMPTION (Optional): Food consumption was not measured.

D. PATHOLOGY

1. Uterine and Liver Weights: Uterine weight data are presented in Table 3. Uterine weights in the chlorpyrifos treated groups were comparable to the controls.

Absolute wet and blotted uterus weights for the positive EE group were increased ($p<0.05$) by 528% and 408%, respectively. The positive controls elicited the expected response.

TABLE 3. Uterine Weights (mg) from Estrogen Agonist Assay in SD Rats [a]

Parameter	Dose Group (mg/kg/day)														
	Vehicle Control			Chlorpyrifos (0.5)			Chlorpyrifos (1.5)			Chlorpyrifos (4)			Estrogen, EE (10 µg/kg/day)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
Terminal BW (g)	6	57.8	3.8	6	58.5	5.5	6	58.4	5.1	6	56.4	3.8	6	57.0	5.2
Wet, absolute (g)	5 [b]	0.0276	0.0039	6	0.0266	0.0039	6	0.0266	0.0021	6	0.0277	0.0023	6	0.1732 * (↑528)	0.0589
Wet, relative (%) [c]	5 [b]	0.048	0.008	6	0.046	0.007	6	0.046	0.005	6	0.049	0.005	6	0.306	0.106
Blotted, absolute (g)	6	0.0248	0.0032	6	0.0224	0.0070	6	0.0242	0.0028	6	0.0241	0.0042	6	0.1261 * (↑408)	0.0102
Blotted, relative (%) [c]	6	0.043	0.007	6	0.038	0.011	6	0.042	0.006	6	0.043	0.010	6	0.223	0.031

a Data were obtained from Table 4 on page 32 of the study report. Percent difference from controls (calculated by reviewer) is presented in parentheses.
b One vehicle control wet uterine weight (0.3981 g) was excluded from analysis.
c Relative wet and blotted uterine weights were calculated by the reviewer from the individual data (Appendix Table 2, pages 37-38).
BW Body weight
N No. of animals in the group
SD Standard Deviation
* Significantly different from vehicle control at $p < 0.05$.

2. **Microscopic Examination (Optional):** Microscopic examinations were not conducted.

3. **Cholinesterase Activity:** The blood and brain samples were not analyzed for ChE activity.

III. DISCUSSION AND CONCLUSIONS

A. **INVESTIGATOR'S CONCLUSIONS:** There was no animal mortality or treatment-related clinical signs observed in this study. There were no treatment-related changes in body weight in any chlorpyrifos-dosed animals and no significant differences in body weight gains at chlorpyrifos doses less than or equal to 1.5 mg/kg bw/day. At 4 mg/kg bw/day, body weight gain was significantly lower than the vehicle control group during TD 1- 3. Body weight gains for the 4-day study period at 4 mg/kg bw/day were decreased by 22.6% compared to the vehicle control group. There were no treatment-related effects on uterine weights in any chlorpyrifos-treated group compared to weights of the vehicle group with the terminal body weight as a covariate. The positive control group had the expected uterine weight increases without any significant change in body weight or body weight gain. No animal in this study had precocious vaginal opening. Uterine weights of the vehicle-treated animals met the performance criteria outlined in the applicable test guidelines, indicating acceptable assay sensitivity.

   Overall, under the conditions of this study, there was no indication of estrogenicity from chlorpyrifos at doses ≤ 4 mg/kg bw/day, the highest dose level tested in female immature rats.

B. **AGENCY COMMENTS:** All animals survived until scheduled termination. No clinical signs of toxicity were observed in animals for any chlorpyrifos treated groups, and no precocious vaginal opening was observed in the treated females. Body weights and overall body weight gains (Days 1-4) in the chlorpyrifos treated groups were comparable to the controls throughout the study. Uterine weights in the chlorpyrifos treated groups were comparable to the controls. Absolute wet and blotted uterus weights for the EE group were increased ($p<0.05$) by 528% and 408%, respectively, as expected. No statistically significant changes were seen in uterine weight in this study. Chlorpyrifos was negative in the uterotrophic assay.

C. **STUDY DEFICIENCIES:** None