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

OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION

MEMORANDUM

Date: 6/29/2015

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

PC Code: 417300
Decision No.: 460845, 464734
Petition No.: NA
Risk Assessment Type: NA
TXR No.: 0053233
MRID No.: See Table

DP Barcode: D398693, 401747
Registration No.: NA
Regulatory Action: NA
Case No.: NA
CAS No.: 1071-83-6
40 CFR: NA

Ver.Apr. 2010

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

THROUGH: Jess Rowland
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TO: Jolene Trujillo
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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 glyphosate: Test Order # CON-417300-23.

II. RESPONSE

Attached are the EDSP Tier 1 assay DERs for glyphosate.

III. MRID Table

Chemical: Glyphosate		**PC Code: 417300**
Guideline	**Assay**	**MRID**
890.1100	Amphibian Metamorphosis Assay (Frog)	48671309
890.1150	Androgen Receptor Binding (Rat Prostate)	48671301
890.1200	Aromatase Assay (Human Recombinant)	48671303
890.1250	Estrogen Receptor Binding	48671305
890.1300	Estrogen Receptor Transcriptional Activation (Human Cell Line HeLa-9903)	48671307
890.1350	Fish Short-Term Reproduction	48671311
890.1400	Hershberger (Rat)	48617001
890.1450	Female Pubertal (Rat)	48671315
890.1500	Male Pubertal (Rat)	48671313
890.1550	Steroidogenesis (Human Cell Line – H295R)	48617005
890.1600	Uterotrophic (Rat)	48617003

Data Requirement:	EPA DP Barcode	401746
	OECD Data Point	231
	EPA MRID	48671309
	EPA Guideline	890.1100
		Amphibian Metamorphosis Assay (Frog)

Test Material:	Glyphosate		**Purity (%):** 85.14%
Common Name	Glyphosate		
Chemical Name	IUPAC	N-Phosphonomethylglycine	
	CAS Name	Not reported	
	CAS No.	1071-83-6	
	Synonyms	MON77973	
	EPA PC Code	417300	

Primary Reviewer: John Marton **Date:** 12/13/12

Staff Scientist, Cambridge Environmental, Inc.

Secondary Reviewer(s): Teri S. Myers **Date:** 02/25/13

Program Manager, CDM Smith

Additional and Final Reviewer(s): Amy Blankinship **Date:**

EPA/OPP/ERB6

AMY BLANKINSHIP Digitally signed by AMY BLANKINSHIP DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=AMY BLANKINSHIP, dnQualifier=0000040917 Date: 2015.06.16 08:25:58 -04'00'

Additional Reviewer: Justin Housenger **Date:**

EPA/OPP/EFED/ERB5

Digitally signed by JUSTIN HOUSENGER DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=JUSTIN HOUSENGER, dnQualifier=0000044455 Date: 2015.06.16 08:30:23 -04'00'

Date Evaluation Completed: 6/5/15

CITATION: Schneider, S.Z., T.Z. Kendall, and H.O. Krueger. 2012. Glyphosate: Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances. Unpublished study performed by Wildlife International, Ltd., Easton, Maryland 21601. Laboratory report number 707A-103. Study sponsored by Joint Glyphosate Task Force c/o Data Group Management, Inc., Raleigh, North Carolina 27615. Study completed April 11, 2012.

Note: 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 glyphosate (85.14% purity) on amphibian metamorphosis of African clawed frog (*Xenopus laevis*) was conducted under flow-through conditions. Amphibian larvae at Nieuwkoop-Faber (NF) stage 51 (80 per control and treatment group) were exposed to nominal concentrations of 0 (negative control), 0.16, 0.80, 4.0, 20, and 100 mg a.i./L. Mean-measured concentrations were <0.100 (<LOQ; control), 0.13, 0.79, 4.3, 20, and 90 mg a.i./L. The test system was maintained at 21.4 to 22.3°C and a pH of 7.0 to 8.3.

The survival of tadpoles exposed to glyphosate was not significantly affected ($p>0.05$) as it was 100% in the control group and 99% in each of the treatment concentrations. Tail curvature was the only observed clinical sign (*i.e.*, behavioral and other sublethal effects) at test termination and occurred in 46 to 57 (out of 80 exposed) tadpoles in the control and treatment groups (64, 63, 65, 53, 53, and 78% of tadpoles in the negative control, 0.13, 0.79, 4.3, 20, and 90 mg a.i./L treatment groups, respectively), and did not appear to be treatment-related.

Glyphosate caused no significant acceleration or delay of median NF developmental stage throughout the test. Further, no asynchronous development was observed. No tadpoles in the control and treatment groups developed beyond NF stage 57. Glyphosate exposure did not cause significant effects on Day 7 or 21 normalized (for snout-vent length) hind-limb length (HLL) at any concentration tested except a significant decrease ($p<0.05$) at the mid (4.3 mg a.i/L) concentration (↓15.6%) at Day 7. This effect was not observed in any treatment group at Day 21. Snout-vent length (SVL) was not significantly affected at any treatment concentration at Day 7 but was significantly increased ($p<0.05$) in the 4.3, 20, and 90 mg a.i/L treatment concentrations at Day 21 (↑5.2, 2.5, and 6.7%, respectively) compared to the control. Additionally there was a significant increase in Day 21 body weight at 90 mg a.i./L (↑17%).

There were no treatment-related effects on thyroid gland histopathology at any treatment level, with comparable incidence and severity of thyroid gland atrophy and hypertrophy, and follicular cell hypertrophy and hyperplasia in the control and treatment concentrations. While there appeared to be an increased incidence of mild thyroid gland hypertrophy at the highest treatment concentration, the same incidence was observed at the lowest

treatment concentration and the effect was not concentration responsive. Similar findings were observed for follicular cell height increase: an apparent increase in mild severity at the top concentration with a similar incidence at the lowest treatment concentration and no concentration-responsive pattern. Finally, the pathologist report indicated that there were no treatment-related changes in the thyroid glands of tadpoles exposed to glyphosate when compared to those in the negative control.

All performance criteria were met in this study, except for the test solution coefficient of variance (CV) for the 0.13 and 90 mg a.i./L treatment groups, in which the CVs were 41 and 31%, respectively, both greater than the recommended maximum of 20%. However, this deviation did not impact the interpretation of the results.

The assay **satisfies** the EDSP 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 Glyphosate.

Treatment (mg a.i./L) [mean-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
0.13	No	No	No	No	No	No	No
0.79	No	No	No	No	No	No	No
4.3	No	No	Yes	No	No	No	No
20	No	No	No	No	No	No	No
90	No	No	No	No	No	No	No

[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 the U.S. Environmental Protection Agency Series 850- Endocrine Disruptor Screening Program Test Guidelines, OCSPP 890.1100: *Amphibian Metamorphosis (Frog)*; and the OECD Guidelines for Testing of Chemicals, Guideline 231: *Amphibian Metamorphosis Assay*. The following deviations were noted:

1. Using the provided raw data, the reviewer calculated coefficients of variation (CV) of 41% at the low concentration and 31% at the high concentration. The three other treatment levels had CVs of 4.4 to 6.8%.
2. The storage conditions of the test material were not specified.
3. The feeding rate was half of the recommended rate (15 mg/animal/day on Days 0 to 4; 40 mg/animal/day on Days 15 to 21).
4. The flow-rate of the diluter system (69.4 mL/min) exceeded the recommended flow rate (25 mL/min), but provided the appropriate volume turnover (complete volume replacement approximately every 2.4 hours).

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 Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Parts 160 and 792); and OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17), with the following exceptions: periodic analyses of water for potential contaminants were not performed according to Good Laboratory Practice Standards, but were performed using a certified laboratory and standard US EPA analytical methods; and, preliminary analyses of water iodide concentrations were not performed according to Good Laboratory Practice Standards.

A. Test Material MON 77973 (Glyphosate)

Description: 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. : GLP-1103-21149-T (Lot No.)

Purity: 85.14%

Impurities: None reported

Stability of Compound: The mean-measured concentrations yielded recoveries of 78 to 109% of nominal, with coefficients of variation ranging from 4.4 to 41%, with greater than 20% CV in the lowest and highest treatment group. A diluter malfunction was reported in the lowest treatment group on Day 14 in which the measured concentrations were <LOQ. Analytical samples taken on Day 16 showed that test solutions had returned to about 65% of nominal. Additionally, on Day 21 in the highest treatment group (100 mg a.i./L nominal), the measured concentrations were about 50% of nominal for which analysis of backup samples confirmed. The reason for this decline in test concentration at Day 21 was not reported.

Storage Conditions of Test Chemicals: Not specified

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 specified		

[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	Original culture was obtained from Xenopus I (Dexter, Michigan).	*EPA recommends that larvae used in the assay be derived from in-house adults.*
Were parental acclimation conditions same as definitive test?	Yes		
Acclimation period for parental frogs (if applicable):	One week		
Details on parental feeding:	Frogs were fed an adult *Xenopus* diet supplied by Zeigler Brothers, Inc. of Gardners, Pennsylvania at a rate sufficient to maintain the health of the culture.		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Details on parental health:	Culture frogs showed no sign of disease or stress prior to the test.		

Table 3: Larval Selection and Care.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Best single spawn?	Yes	Best single spawn had a viability of 98.5%.	*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):	At least 3		
Number of eggs sampled per spawn:	~250	Eggs were collected and evaluated the day after injecting three adult pairs with hGC to induce spawning.	
NF stage at test initiation	51		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Age at test initiation:	16 days post-fertilization (dpf)		*EPA recommends that the definitive study be initiated with larvae at Nieuwkoop – Faber (NF) developmental stage 51 (≤17 days post-fertilization).*
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:	Three adult male and female frogs were injected with human chorionic gonadotropin (hGC) to induce spawning and each pair was placed in a separate spawning tank overnight.	Eggs were collected the following day and the spawn with the highest viability was used to select tadpoles for use in the definitive test.	

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Loading rate (rearing density):	2 larvae/L	20 larvae per test vessel (10 L)	*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.*
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):	Not reported		
Frequency of feeding:	3 times/day		*EPA recommends that feeding occur at least twice per day.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Details on feeding regime:	The feeding rate was half of the recommended rate (15 mg/animal/day on Days 0 to 4; 40 mg/animal/day on Days 15 to 21).		*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 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.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
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):	~69.4 mL/min	Calculated based on volume of test vessel (10 L) and number of volume additions per day (10). This flow rate provided a complete volume replacement approximately every 2.4 hours.	*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):	Fluid metering pumps were used to deliver volumes of a single test substance stock solution to mixing chambers indiscriminately assigned to each treatment. Stock solutions were diluted with well water in the mixing chambers, and the flow of solution to the test vessels was controlled using rotameters.	Pumps and rotameters were calibrated prior to the test and verified or recalibrated, if necessary, approximately weekly during the test. Delivery of test solutions to the test chambers was initiated five days prior to the introduction of the test organisms in order to achieve equilibrium of the tests substance in the test system. The general operation of the exposure system was checked visually at least two times per day during the test and at least once on the last day of the test.	*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%.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
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.*
Aeration?	No		*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.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Source of dilution water:	Water used for culturing and testing was freshwater obtained from a well ~40 meters deep located on the WLI site. Water was passed through a sand filter to remove particles >25 µm, pumped into a storage tank, and aerated with spray nozzles. Prior to use, water was filtered to 0.45 µm to remove fine particles.	Well water is characterized as moderately-hard water.	*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.*
Was dilution water analyzed for pesticides, heavy metals, and other contaminants?	Yes. See Reviewer's Comments.		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
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).*
Test vessel type/materials:	Glass		*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:	12 L		
Fill volume:	10 L		
Additional details on exposure system:	NA		

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

Parameter	Minimum	Maximum	Mean	Measurement Interval	Guideline Recommendations
Hardness (mg/L as $CaCO_3$)	140	144	142.5	Weekly in control and highest treatment group, alternating among replicates.	*EPA recommends hardness 40 to 48 mg/L as $CaCO_3$.*
pH	7.0	8.3	8.0	Weekly in each test vessel.	*EPA recommends pH 7.5 ± 1, inter-replicate and inter-treatment differentials should not exceed 0.5.*
Dissolved oxygen (mg/L)	7.6	8.7	8.2	Every 2 to 3 days in each test vessel.	*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	22.3	21.9	Weekly in each test vessel. Also measured continuously in one negative control replicate.	*EPA recommends temperature 22±1°C; inter-replicate and inter-treatment differentials should not exceed 0.5°C.*
Iodide	6 µg/L (March 2011, non-GLP)				*EPA recommends aquatic iodide range 0.5 – 10 µg/L (supplemental iodide should not exceed 2 µg/L).*

Parameter	Minimum	Maximum	Mean	Measurement Interval	Guideline Recommendations
Ammonia	Not Reported				*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 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.*
Fluoride	--	--	0.85 mg/L	Periodic screening	
Perchlorate	Not Reported				
Chlorate	Not Reported				
Alkalinity (mg/L as $CaCO_3$)	146	178	161	Weekly in control and highest treatment group, alternating among replicates.	
Conductance (µS/cm)	312	350	330		

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 concentration in the range-finder?	100 mg/L and reported solubility of 10.1 g/L		*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.*
Species:	*Xenopus laevis*		
Life stage:	NF 51		
Test duration:	14 days		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Additional details:	The study was conducted for 14 days under flow-through conditions at nominal concentrations of 0 (negative control), 6.25, 12.5, 25, 50, and 100 mg a.i./L. No signs of toxicity were observed and one incidental mortality occurred at the 50 mg a.i./L level.	Analytical recoveries ranged from 102 to 105% of nominal.	

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.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Method for selecting the highest test concentration in the definitive test:	Range-finder		*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):	NA		
Separation of test concentrations:	5		*EPA recommends that the maximum concentration separation be 0.1 and the minimum be 0.33.*
Number of test concentrations:	5		*EPA recommends a minimum of 3 concentrations and a control, plus solvent control if appropriate.*
Are nominal concentrations adjusted for purity?	Yes	Adjusted based on the acid form	

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Indicate the type of values presented for measured concentrations:	Mean-measured		
Limit of quantification (LOQ):	0.100 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:	Samples were collected for analysis on Days 0. 7, 14, and 21. Additional samples were collected from the lowest level on Day 16, and on Day 1 in the middle (4.0 mg a.i./L) level		*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.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Number of replicates in solvent control (if applicable):	NA		*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		
Was a solvent used?	No		
Solvent type (if applicable):	NA		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
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:	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:	0.911-1.387 Klux		*EPA recommends light intensity 0.6 – 2 Klux (at water's surface).*
Additional details:		Test solutions were clear and colorless with no precipitate observed	

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

Treatment ID	Nominal Concentration (mg a.i./L)	Measured Concentration (mg a.i./L)	Mean CV (%)	Details or Remarks	Guideline Recommendations
Control (dilution water only)	0.00	<0.100	NA		*EPA and OECD recommend that test item concentrations be maintained at a coefficient of variation (CV) ≤20%.*
Treatment 1	0.16	0.13	41		
Treatment 2	0.80	0.79	5.2		
Treatment 3	4.0	4.3	4.4	The Day 0 samples were not included in the mean measured concentrations as the results were due to sampling/analysis error	
Treatment 4	20	20	6.8		
Treatment 5	100	90	31		

Abbreviations: CV Coefficient of variation.

E. Observations

Biological Endpoints: Day 7- NF stage, wet weight, snout-vent length (SVL), hind limb length (HLL), normalized HLL
Day 21- NF stage, wet weight, SVL, HLL, normalized HLL, thyroid pathology
Daily- mortality, clinical observations

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

Mean percent survival on Day 7 was 100% in the negative control and mean-measured 0.13, 0.79, and 4.3 mg a.i./L treatment groups, and 98.8% in the mean-measured 20 and 90 mg a.i./L treatment groups (Table 9). By test termination, survival averaged 98.8% in the negative control, 100, 100, 100, 97.5, and 98.8% in the mean-measured 0.13, 0.79, 4.3, 20, and 90 mg a.i./L treatment groups, respectively.

Table 9: Larval Mortality in *Xenopus laevis*.

Treatment (mg a.i./L) [mean-measured]	Larval Mortality					
	Day 7[1]			Day 21		
	n	Mortality #	Mortality %	n	Mortality #	Mortality %
Negative Control	80	0	0	60	1	1.2
0.13	80	0	0	60	0	0
0.79	80	0	0	60	0	0
4.3	80	0	0	60	0	0
20	80	1[2]	1.2	59	2[4]	2.5
90	80	1[3]	1.2	59	1	1.2

[1] Sample size and cumulative mortality values at Day 7 prior to interim sacrifice.

[2] One mortality in the Replicate C on Day 4; One tadpole inadvertently killed during siphoning of test chamber in Replicate A on Day 2 and not included in overall mortality on Day 7 or 21

[3] One mortality in Replicate A on Day 7

[4] One mortality in Replicate D on Day 21.

Median developmental stage on Day 7 was 53 in all treatment groups (Table 10). On Day 21, median developmental stage was 57 in the control and all treatment groups. No asynchronous development was reported.

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

Treatment (mg a.i./L) [mean-measured]	Developmental Stage					
	Day 7			Day 21		
	n	Median Stage	# Asynchronous	n	Median Stage	# Asynchronous
Negative Control	4	53	0	4	57	0
0.13	4	53	0	4	57	0
0.79	4	53	0	4	57	0
4.3	4	53	0	4	57	0
20	4	53	0	4	57	0
90	4	53	0	4	57	0

Day 7 normalized HLL averaged 0.13 mm in the negative control and 0.13, 0.13, 0.11, 0.13, and 0.13 mm in the mean-measured 0.13, 0.79, 4.3, 20, and 90 mg a.i./L treatment groups, respectively (Table 11). On Day 21, normalized HLL averaged 0.33 mm in the negative control and 0.36, 0.33, 0.34, 0.34, and 0.33 mm in the mean-measured 0.13, 0.79, 4.3, 20, and 90 mg a.i./L treatment groups, respectively.

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

Treatment (mg a.i./L) [mean-measured]	Hind Limb Length (HLL)							
	Day 7				Day 21			
	N	Mean (mm)	±SD	HLL: SVL[1]	n	Mean (mm)	±SD	HLL: SVL[1]
Negative Control	4	2.08	0.10	0.13	4	7.65	0.68	0.33
0.13	4	2.10	0.08	0.13	4	8.48	0.22	0.36
0.79	4	2.15	0.17	0.13	4	7.78	0.43	0.33
4.3	4	1.75	0.21	0.11	4	8.20	0.50	0.34
20	4	2.08	0.10	0.13	4	8.00	0.69	0.34
90	4	2.10	0.14	0.13	4	8.25	0.79	0.33

Abbreviations: SD Standard deviation.

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

Mean SVL on Day 7 ranged from 15.8 mm in the negative control to 16.1 mm in all but the lowest treatment group (Table 12). By Day 21, SVL averaged 23.2 mm in the negative control and 23.6, 23.5, 24.4, 23.8, and 24.8 mm in the mean-measured 0.13, 0.79, 4.3, 20, and 90 mg a.i./L treatment groups, respectively.

Table 12: Larval Growth in *Xenopus laevis*.

Treatment (mg a.i./L) [mean-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	4	15.8	0.98	4	23.2	0.43	4	0.267	0.040	4	0.864	0.038
0.13	4	15.9	0.38	4	23.6	0.66	4	0.273	0.021	4	0.925	0.100
0.79	4	16.1	0.75	4	23.5	0.83	4	0.288	0.031	4	0.907	0.078
4.3	4	16.1	0.80	4	24.4	0.15	4	0.290	0.042	4	0.973	0.037
20	4	16.1	0.34	4	23.8	0.45	4	0.282	0.0	4	0.920	0056
90	4	16.1	0.99	4	24.8	0.38	4	0.300	0.048	4	1.01	0.060

Abbreviations: SD Standard deviation.

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

Tables 13-15 show the histopathological diagnoses reported resulting from the exposure to glyphosate. Table 15 shows additional diagnoses that were examined in the study report, that as reported, do not match up with the standard histopathological diagnoses in Tables 13 and 14. Cells that are left blank in a given column denote that this diagnosis was not examined by the study.

According to the study author, there were no apparent treatment-related trends in thyroid histopathology. Observations and severity of thyroid atrophy and hypertrophy, and follicular cell hypertrophy and hyperplasia were comparable between the control and treatment groups (Table 13). The animals were stage matched. While there appears to be an increased incidence of mild thyroid gland hypertrophy in the highest treatment concentration, the same incidence was observed at the lowest treatment concentration and the effect was not concentration responsive. Similar findings were observed in for follicular cell height increase an apparent increase in mild severity at the top

concentration but again, this incidence was similar to the lowest treatment concentration and no concentration-responsive pattern was seen. Finally, the pathologist report indicated that there were no treatment related changes in the thyroid glands of tadpoles exposed to glyphosate when compared to organisms in the negative control.

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

Treatment (mg a.i./L) [mean-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	0	20	17	20	19				
	1	20	3	20	1				
	2	20	0	20	0				
	3	20	0	20	0				
0.13	0	20	14	20	17				
	1	20	4	20	3				
	2	20	2	20	0				
	3	20	0	20	0				
0.79	0	20	17	20	17				
	1	20	1	20	2				
	2	20	2	20	1				
	3	20	0	20	0				
4.3	0	20	18	20	18				
	1	20	1	20	2				
	2	20	1	20	0				
	3	20	0	20	0				

Treatment (mg a.i./L) [mean-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
20	0	20	18	20	19				
	1	20	0	20	1				
	2	20	2	20	0				
	3	20	0	20	0				
90	0	20	14	20	18				
	1	20	6	20	2				
	2	20	0	20	0				
	3	20	0	20	0				

[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) [mean-measured]	Additional Qualitative Observations[1]										
	Severity	Follicular Lumen Area (Increase)		Follicular Lumen Area (Decrease)		Follicular Cell Height (Increase)[2]		Follicular Cell Height (Decrease)[3]		Follicular Cell Shape	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative Control	0					20	17	20	17		
	1					20	1	20	2		
	2					20	2	20	1		
	3					20	0	20	0		
0.13	0					20	14	20	16		
	1					20	4	20	2		
	2					20	1	20	2		
	3					20	1	20	0		
0.79	0					20	13	20	17		
	1					20	3	20	3		
	2					20	3	20	0		
	3					20	1	20	0		

Treatment (mg a.i./L) [mean-measured]	Additional Qualitative Observations[1]									
	Severity	Follicular Lumen Area (Increase)		Follicular Lumen Area (Decrease)		Follicular Cell Height (Increase)[2]		Follicular Cell Height (Decrease)[3]		Follicular Cell Shape	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
4.3	0					20	16	20	17		
	1					20	2	20	3		
	2					20	2	20	0		
	3					20	0	20	0		
20	0					20	18	20	15		
	1					20	1	20	4		
	2					20	1	20	1		
	3					20	0	20	0		

Treatment (mg a.i./L) [mean-measured]	Additional Qualitative Observations[1]									
	Severity	Follicular Lumen Area (Increase)		Follicular Lumen Area (Decrease)		Follicular Cell Height (Increase)[2]		Follicular Cell Height (Decrease)[3]		Follicular Cell Shape	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
90	0					20	14	20	17		
	1					20	2	20	3		
	2					20	4	20	0		
	3					20	0	20	0		

[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.

[2] Denoted in study report as “Follicle Size Increase”

[3] Denoted in study report as “Follicle Size Decrease”

Table 15: Additional Thyroid Gland Histopathology Observations in *Xenopus laevis*.

Treatment (mg a.i./L) [mean-measured]	Additional Qualitative Observations[1]								
	Severity	Follicle Asymmetry Increase		Follicle Asymmetry Decrease		Gland Asymmetry Increase		Follicular Cell Hyperplasia	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative Control	0	20	17	20	20	20	17		
	1	20	0	20	0	20	3		
	2	20	2	20	0	20	0		
	3	20	1	20	0	20	0		
0.13	0	20	19	20	19	20	19		
	1	20	1	20	1	20	1		
	2	20	0	20	0	20	0		
	3	20	0	20	0	20	0		
0.79	0	20	20	20	20	20	18		
	1	20	0	20	0	20	2		
	2	20	0	20	0	20	0		
	3	20	0	20	0	20	0		
4.3	0	20	20	20	18	20	17		
	1	20	0	20	2	20	3		
	2	20	0	20	0	20	0		
	3	20	0	20	0	20	0		
20	0	20	20	20	19	20	18		
	1	20	0	20	1	20	2		
	2	20	0	20	0	20	0		
	3	20	0	20	0	20	0		

Treatment (mg a.i./L) [mean-measured]	Additional Qualitative Observations[1]								
	Severity	Follicle Asymmetry Increase		Follicle Asymmetry Decrease		Gland Asymmetry Increase		Follicular Cell Hyperplasia	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
90	0	20	19	20	19	20	18		
	1	20	1	20	1	20	2		
	2	20	0	20	0	20	0		
	3	20	0	20	0	20	0		

[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.

Control and treatment tadpoles generally appeared normal and healthy throughout the test. Beginning on Day 2 and continuing until test termination, tail curvature was observed in control and treatment tadpoles. By test termination, tail curvature was observed in 64, 63, 65, 53, 53, and 78% of tadpoles in the negative control, 0.13, 0.79, 4.3, 20, and 90 mg a.i./L treatment groups, respectively (Table 16). Percentages were based on initial number of tadpoles used to initiate the test.

Table 16: Clinical Signs in *Xenopus laevis*.

Treatment (mg a.i./L) [mean-measured]	Clinical Signs[1]		
	Type	n[2]	Incidence
Negative Control	Tail curvature	59	54
0.13	Tail curvature	60	53
0.79	Tail curvature	60	52
4.3	Tail curvature	60	50
20	Tail curvature	57	46
90	Tail curvature	59	57

[1] Note that asynchronous development (unable to stage) is reported previously in Table 10.

[2] Number of surviving tadpoles at test termination

B. **Study Author's Analysis and Conclusions**

Analyses were performed on survival, developmental stage, body weight, SVL, normalized HLL, and incidence and severity of thyroid abnormalities. Unless otherwise noted, the unit of statistical analysis was the replicate test chamber. If necessary, endpoints were analyzed using two complementary statistical approaches. For growth parameters, endpoints were first evaluated for monotonicity. Since responses for these endpoints appeared to be monotonic, a step-down Jonckheere-Terpstra trend test was used to evaluate trends in the ranks of replicate means to determine possible concentration responsive trends among the treatment groups. Body weight and SVL data also were analyzed by performing pair-wise comparisons using Dunnett's multiple comparison test to further evaluate those treatment groups that statistically differed from the control group. Data for endpoints analyzed by Dunnett's test were evaluated for normality using Shapiro-Wilk's test and for homogeneity of variance using Levene's test (α = 0.01).

Survival, developmental stage, and histopathology severity scores were not amenable to the statistical methods used for analysis of other endpoints. In particular, the most suitable unit of statistical analysis for these endpoints was the individual animal. Therefore, survival was analyzed using Fisher's Exact test, and histopathology severity scores of individuals were analyzed using step-down Jonckheere-Terpstra trend tests only.

Statistical tests used to evaluate treatment effects were performed at confidence level of α = 0.05 with SAS software. In regards to the tail curvature, the study authors attributed that was likely related to feeding rate, not exposure to the test material.

Throughout the test, there were no significant effects on developmental stage or normalized HLL. Further, there were no apparent effects on thyroid histopathology. There were significant increases in Day 21 wet weight at the 90 mg a.i./L treatment group and in Day 21 SVL at the 4.3 and 90 mg a.i./L treatment groups. However, since there were no effects on developmental endpoints, the study

authors concluded that the test material was "likely thyroid inactive" to *Xenopus laevis* tadpoles under conditions used in the current study. Glyphosate did not appear to impact the normal function of the HPT axis.

C. Reviewer's Analysis and Conclusions

Statistical Methods: The reviewer visually analyzed mortality data because survival averaged ≥97.5% in all treatment groups. Replicate median values for developmental stage on Days 7 and 21 did not exhibit an increasing or decreasing monotonic trend, therefore, the Mann-Whitney U-test was used to assess differences in developmental stage. Similar to developmental stage, Day 7 SVL and normalized HLL were not monotonic and did not meet the assumptions of ANOVA and were therefore analyzed using the Mann-Whitney test. Day 7 wet weight and HLL were not monotonic but were normally distributed and had equal variance; therefore, these endpoints were analyzed using Dunnett's multiple comparison test. On Day 21, wet weight, HLL, and normalized HLL did not exhibit a monotonic trend but met the assumptions of normality and homogeneity of variance and were therefore analyzed using Dunnett's multiple comparison test. Day 21 SVL exhibited a monotonic trend and was analyzed using the Jonckheere-Terpstra test. No asynchronous development was observed. Unless otherwise indicated, effects were considered statistically significant at $p < 0.05$.

Late-stage tadpoles (NF stage ≥ 61) were excluded from analysis of growth parameters. Only two late-stage tadpoles were found in the study, both in the mean-measured 90 mg a.i./L treatment group.

Conclusions:

On Day 7, HLL was significantly reduced (16% reduction; Dunnett's, $p = 0.017$) at the 4.3 mg a.i./L treatment level. No other growth parameters were affected on Day 7. By Day 21, wet weight was significantly greater in the 90 mg a.i./L treatment group (17% promotion; Dunnett's, $p = 0.023$) and SVL was significantly greater in the three highest treatment groups relative to the

negative control (2-7% increase; Jonckheere-Terpstra, $p \leq 0.031$). No effects were observed for developmental endpoints.

While there appears to be an increased incidence of mild thyroid gland hypertrophy in the highest treatment concentration, the same incidence was observed at the lowest treatment concentration and the effect was not concentration responsive. Similar findings were observed in for follicular cell height increase an apparent increase in mild severity at the top concentration but again, this incidence was similar to the lowest treatment concentration and no concentration-responsive pattern was seen. Finally, the pathologist report indicated that there were no treatment related changes in the thyroid glands of tadpoles exposed to glyphosate when compared to organisms in the negative control.

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

Treatment (mg a.i./L) [mean-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
	Median	*p*	Median	*p*	% Diff.	*p*	% Diff.	*p*	% Diff.	*p*	% Diff.	*p*	Treatment-Related Effects? (Yes/No)
Negative Control	53	--	57	--	--	--	--	--	--	--	--	--	No
0.13	53	>0.99	57	>0.99	1.92	0.48	9.23	0.18	--	--	--	--	No
0.79	53	0.48	57	>0.99	0.00	>0.99	1.54	0.99	--	--	--	--	No
4.3	53	>0.99	57	>0.99	**-15.6**	**0.028**	3.08	0.93	--	--	--	--	No
20	53	>0.99	57	>0.99	0.00	>0.99	3.08	0.93	--	--	--	--	No
90	53	0.48	57	>0.99	0.00	>0.99	2.31	0.98	--	--	--	--	No
Statistical Test	Mann-Whitney		Mann-Whitney		Mann-Whitney		Dunnett's		--		--		--

Abbreviations: [Diff.] Difference.

[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 Glyphosate.

Treatment (mg a.i./L) [mean-measured]	Snout-Vent Length				Body Weight			
	Day 7		Day 21		Day 7		Day 21	
	% Diff.	*p*	% Diff.	*p*	% Diff.	*p*	% Diff.	*p*
Negative Control	--	--	--	--	--	--	--	--
0.13	0.47	0.49	1.51	0.19	2.25	0.99	7.12	0.55
0.79	1.90	0.49	1.08	0.23	7.87	0.87	5.04	0.81
4.3	1.58	0.68	**5.17**	**0.006**	8.62	0.82	12.7	0.11
20	1.74	0.35	**2.48**	**0.031**	5.81	0.96	6.51	0.63
90	1.74	0.41	**6.67**	**<0.001**	12.46	0.55	**16.8**	**0.023**
Statistical Test	Mann-Whitney		Jonckheere		Dunnett's		Dunnett's	

Abbreviations: Diff. Difference.

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

Although there were deviations from the guideline, as noted in the Materials and Methods section of the DER, the study met all validity and performance criteria, except for the test solution CVs in the lowest and highest treatment groups, which were 41 and 31%, respectively.

F. Reviewer's Comments

The reviewer's results agreed with those of the study authors.

There was a diluter malfunction in the low concentration on Day 14 and the measured concentrations were below the LOQ (0.100 mg a.i./L). Therefore, a value of half of the LOQ was used when calculating the mean-measured concentrations. Samples were collected and analyzed on Day 16

following repair of the diluter. Additionally, the Day 0 samples analyzed for the 4.3 mg a.i./L treatment group were <LOQ, and were believed to be due to sampling/analysis error. Additional samples collected on Day 1 were at nominal. The Day 0 samples were not included in the mean measured concentration calculation.

The results from the periodic screening analysis of the dilution water indicated the presence of the following analytes: calcium (34.9 mg/L), chloride (4.5 mg/L), fluoride (0.85 mg/L), magnesium (13.2 mg/L), potassium (7.00 mg/L), sodium (19.0 mg/L), and sulfate (5.7 mg/L).

The in-life portion of the definitive toxicity test was conducted from October 24 to November 14, 2011.

Comparison of EDSP and EFED Statistical Approaches

Both statistical approaches employed the same tests for each endpoint and yielded identical results.

III. REFERENCES

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APPENDIX I. OUTPUT OF REVIEWER'S STATISTICAL VERIFICATION:

test for amphib metamorph screen study - Glyphosate
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 Test Stat	Shapiro-Wilks P-value	Levenes Test Stat	Levenes P-value	Conclusion
0.925	0.075	0.989	0.452	USE PARAMETRIC TESTS

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***
BASIC SUMMARY STATISTICS

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval	
Ctrl	4	0.27	0.04	0.02	14.98	0.20,	0.33
Dose1	4	0.27	0.02	0.01	7.82	0.24,	0.31
Dose2	4	0.29	0.03	0.02	10.52	0.24,	0.34
Dose3	4	0.29	0.04	0.02	14.28	0.22,	0.36
Dose4	4	0.28	0.02	0.01	7.95	0.25,	0.32
Dose5	4	0.30	0.05	0.02	15.89	0.22,	0.38

Level	Median	Min	Max	%of Control(means)	%Reduction(means)
Ctrl	0.25	0.23	0.33	.	.
Dose1	0.28	0.24	0.29	102.25	-2.25
Dose2	0.28	0.26	0.33	107.87	-7.87
Dose3	0.29	0.25	0.34	108.62	-8.62
Dose4	0.28	0.26	0.32	105.81	-5.81
Dose5	0.28	0.27	0.37	112.46	-12.46

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***
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
5	18	0.47	0.795

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     0.27    .         0.28      .        .       .       .       .
.
 Dose1    0.27   0.999      0.28     0.822     .       .       .       .
.
 Dose2    0.29   0.865      0.28     0.852    0.990    .       .       .
.
 Dose3    0.29   0.821      0.28     0.867    0.982   1.000    .       .
.
 Dose4    0.28   0.955      0.28     0.876    0.999   1.000   1.000    .
.
 Dose5    0.30   0.552      0.28     0.882    0.878   0.996   0.998   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
           5                 2.68       0.749

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       0.25                    .                         .
  Dose1      0.28                  0.676                     0.719
  Dose2      0.28                  0.233                     0.907
  Dose3      0.29                  0.491                     0.905
  Dose4      0.28                  0.341                     0.841
  Dose5      0.28                  0.233                     0.887

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

*************************************************************************
***
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
           5           18               0.47        0.795

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	-0.27	.	-0.27	.	.	.	.	.	.
Dose1	-0.27	0.999	-0.27	0.480	.	.	.	.	.
Dose2	-0.29	0.865	-0.29	0.279	0.990	.	.	.	.
Dose3	-0.29	0.821	-0.29	0.289	0.982	1.000	.	.	.
Dose4	-0.28	0.955	-0.29	0.295	0.999	1.000	1.000	.	.
Dose5	-0.30	0.552	-0.30	0.134	0.878	0.996	0.998	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
5	2.68	0.749

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	-0.25	.	.
Dose1	-0.28	0.676	0.281
Dose2	-0.28	0.233	0.093
Dose3	-0.29	0.491	0.095
Dose4	-0.28	0.341	0.159
Dose5	-0.28	0.233	0.113

INCREASING TREND TEST SUMMARY LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
Williams
Jonckheere

test for amphib metamorph screen study - Glyphosate
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
0.665	<.001	3.600	0.020	USE NON-PARAMETRIC TESTS

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***
BASIC SUMMARY STATISTICS

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval
Ctrl	4	53.25	0.50	0.25	0.94	52.45, 54.05
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	53.25	0.50	0.25	0.94	52.45, 54.05
Dose5	4	53.00	0.00	0.00	0.00	. , .

Level	Median	Min	Max	%of Control(means)	%Reduction(means)
Ctrl	53.00	53.00	54.00	.	.
Dose1	53.00	53.00	54.00	100.00	0.00
Dose2	53.00	53.00	53.00	99.53	0.47
Dose3	53.00	53.00	54.00	100.00	0.00
Dose4	53.00	53.00	54.00	100.00	0.00
Dose5	53.00	53.00	53.00	99.53	0.47

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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
5	18	0.40	0.842

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	53.25	.	53.25	.	.	.	.	.	.
Dose1	53.25	1.000	53.25	0.583	.	.	.	.	.

```
 Dose2   53.00   0.853     53.17     0.489    0.950    .       .       .
.
 Dose3   53.25   1.000     53.17     0.506    1.000   0.950    .       .
.
 Dose4   53.25   1.000     53.17     0.517    1.000   0.950   1.000    .
.
 Dose5   53.00   0.853     53.00     0.273    0.950   1.000   0.950   0.950
.

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***
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           5                2.30       0.806

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                 1.000                     0.500
  Dose2     53.00                 0.478                     0.182
  Dose3     53.00                 1.000                     0.391
  Dose4     53.00                 1.000                     0.500
  Dose5     53.00                 0.478                     0.265

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

************************************************************************
***
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
           5           18               0.40        0.842

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   -53.25    .       -53.17      .        .       .       .       .
.
 Dose1  -53.25   1.000    -53.17     0.700     .       .       .       .
.
 Dose2  -53.00   0.853    -53.17     0.735    0.950    .       .       .
.
 Dose3  -53.25   1.000    -53.17     0.753    1.000   0.950    .       .
.
 Dose4  -53.25   1.000    -53.17     0.765    1.000   0.950   1.000    .
.
 Dose5  -53.00   0.853    -53.17     0.773    0.950   1.000   0.950   0.950
.

*************************************************************************
***
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           5                 2.30       0.806

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     -53.00                    .                        .
  Dose1    -53.00                  1.000                    0.500
  Dose2    -53.00                  0.478                    0.818
  Dose3    -53.00                  1.000                    0.609
  Dose4    -53.00                  1.000                    0.500
  Dose5    -53.00                  0.478                    0.735

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams
   Jonckheere

test for amphib metamorph screen study - Glyphosate
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  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
```

```
      0.857         0.003          1.251      0.327    USE NON-PARAMETRIC
TESTS

************************************************************************
***
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    15.83     1.00       0.50        6.35          14.23,   17.42
  Dose1  4    15.90     0.37       0.18        2.30          15.32,   16.48
  Dose2  4    16.13     0.75       0.37        4.62          14.94,   17.31
  Dose3  4    16.08     0.79       0.40        4.93          14.81,   17.34
  Dose4  4    16.10     0.37       0.19        2.32          15.50,   16.70
  Dose5  4    16.10     1.00       0.50        6.23          14.50,   17.70

 Level       Median     Min       Max    %of Control(means)
%Reduction(means)
  Ctrl        15.45    15.10      17.30         .                  .
  Dose1       15.90    15.50      16.30      100.47             -0.47
  Dose2       15.90    15.50      17.20      101.90             -1.90
  Dose3       15.90    15.40      17.10      101.58             -1.58
  Dose4       16.05    15.70      16.60      101.74             -1.74
  Dose5       15.65    15.50      17.60      101.74             -1.74

************************************************************************
***
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
           5           18               0.11        0.989

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    15.83    .        16.02      .        .       .       .       .
.
 Dose1   15.90   1.000     16.02     0.728     .       .       .       .
.
 Dose2   16.13   0.971     16.02     0.762    0.998    .       .       .
.
 Dose3   16.08   0.986     16.02     0.780    0.999   1.000    .       .
.
 Dose4   16.10   0.979     16.02     0.791    0.999   1.000   1.000    .
.
```

```
 Dose5   16.10   0.979     16.02     0.799    0.999   1.000   1.000   1.000
.

************************************************************************
***
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           5                 2.10       0.835

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      15.45                    .                         .
  Dose1     15.90                  0.494                     0.807
  Dose2     15.90                  0.494                     0.830
  Dose3     15.90                  0.678                     0.744
  Dose4     16.05                  0.346                     0.849
  Dose5     15.65                  0.413                     0.769

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

************************************************************************
***
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
           5           18               0.11        0.989

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   -15.83    .       -15.83      .        .       .       .       .
.
 Dose1  -15.90   1.000    -15.90     0.524     .       .       .       .
.
 Dose2  -16.13   0.971    -16.10     0.392    0.998    .       .       .
.
```

```
 Dose3  -16.08   0.986    -16.10     0.406    0.999   1.000    .       .
.
 Dose4  -16.10   0.979    -16.10     0.415    0.999   1.000   1.000    .
.
 Dose5  -16.10   0.979    -16.10     0.421    0.999   1.000   1.000   1.000
.

*************************************************************************
***
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           5                 2.10       0.835

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     -15.45                    .                         .
  Dose1    -15.90                  0.494                     0.193
  Dose2    -15.90                  0.494                     0.170
  Dose3    -15.90                  0.678                     0.256
  Dose4    -16.05                  0.346                     0.151
  Dose5    -15.65                  0.413                     0.231

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams
   Jonckheere

test for amphib metamorph screen study - Glyphosate
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
      0.970         0.676          1.575      0.217    USE PARAMETRIC TESTS

*************************************************************************
***
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
```

```
  Ctrl   4     2.08     0.10       0.05        4.61           1.92,    2.23
  Dose1  4     2.10     0.08       0.04        3.89           1.97,    2.23
  Dose2  4     2.15     0.17       0.09        8.06           1.87,    2.43
  Dose3  4     1.75     0.21       0.10       11.90           1.42,    2.08
  Dose4  4     2.08     0.10       0.05        4.61           1.92,    2.23
  Dose5  4     2.10     0.14       0.07        6.73           1.87,    2.33

 Level       Median     Min       Max    %of Control(means)
%Reduction(means)
  Ctrl         2.05     2.00       2.20         .                .
  Dose1        2.10     2.00       2.20       101.20           -1.20
  Dose2        2.15     2.00       2.30       103.61           -3.61
  Dose3        1.75     1.50       2.00        84.34           15.66
  Dose4        2.05     2.00       2.20       100.00            0.00
  Dose5        2.05     2.00       2.30       101.20           -1.20

*************************************************************************
***
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
           5           18               4.29        0.010

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     2.08    .         2.11      .        .       .       .       .
.
 Dose1    2.10   0.999      2.11     0.717     .       .       .       .
.
 Dose2    2.15   0.907      2.11     0.752    0.995    .       .       .
.
 Dose3    1.75   0.017      1.98     0.216    0.025   0.009    .       .
.
 Dose4    2.08   1.000      1.98     0.220    1.000   0.972   0.041    .
.
 Dose5    2.10   0.999      1.98     0.224    1.000   0.995   0.025   1.000
.

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***
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
```

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     Degrees of Freedom   TestStat    P-value
           5                 8.70       0.122

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       2.05                    .                          .
  Dose1      2.10                0.769                      0.677
  Dose2      2.15                0.656                      0.756
  Dose3      1.75                0.096                      0.050
  Dose4      2.05                1.000                      0.175
  Dose5      2.05                1.000                      0.346

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

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***
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
           5           18               4.29        0.010

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    -2.08    .        -2.02      .        .       .       .       .
.
 Dose1   -2.10   0.999     -2.02     0.795     .       .       .       .
.
 Dose2   -2.15   0.907     -2.02     0.826    0.995    .       .       .
.
 Dose3   -1.75   0.017     -2.02     0.843    0.025   0.009    .       .
.
 Dose4   -2.08   1.000     -2.08     0.648    1.000   0.972   0.041    .
.
 Dose5   -2.10   0.999     -2.10     0.541    1.000   0.995   0.025   1.000
.
```

```
*************************************************************************
***
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           5                 8.70       0.122

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      -2.05                   .                          .
  Dose1     -2.10                0.769                      0.323
  Dose2     -2.15                0.656                      0.244
  Dose3     -1.75                0.096                      0.950
  Dose4     -2.05                1.000                      0.825
  Dose5     -2.05                1.000                      0.654

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams
   Jonckheere

test for amphib metamorph screen study - Glyphosate
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  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.818         <.001          2.508      0.068    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     0.01        0.00        3.77          0.12,    0.14
  Dose2  4     0.13     0.01        0.00        6.28          0.12,    0.14
  Dose3  4     0.11     0.01        0.01       13.32          0.09,    0.13
  Dose4  4     0.13     0.01        0.00        6.28          0.12,    0.14
  Dose5  4     0.13     0.00        0.00        0.00            .  ,     .
```

Level	Median	Min	Max	%of Control(means)	%Reduction(means)
Ctrl	0.13	0.13	0.13	.	.
Dose1	0.13	0.13	0.14	101.92	-1.92
Dose2	0.13	0.12	0.14	100.00	0.00
Dose3	0.12	0.09	0.12	84.42	15.58
Dose4	0.13	0.12	0.14	100.00	0.00
Dose5	0.13	0.13	0.13	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
5	18	4.70	0.006

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	.	0.13	.	.	.	.	.	.
Dose1	0.13	0.988	0.13	0.675	.	.	.	.	.
Dose2	0.13	1.000	0.13	0.618	0.997	.	.	.	.
Dose3	0.11	0.008	0.12	0.158	0.008	0.020	.	.	.
Dose4	0.13	1.000	0.12	0.161	0.997	1.000	0.020	.	.
Dose5	0.13	1.000	0.12	0.163	0.997	1.000	0.020	1.000	.

****
***
NON-PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Kruskal-Wallis test - equality among treatment groups

Degrees of Freedom	TestStat	P-value
5	12.00	0.035

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                 0.478                     0.841
  Dose2      0.13                 1.000                     0.539
  Dose3      0.12                 0.053                     0.010
  Dose4      0.13                 1.000                     0.106
  Dose5      0.13                 1.000                     0.260

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

*****************************************************************************
***
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
           5           18               4.70        0.006

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   0.988     -0.13     0.858     .       .       .       .
.
 Dose2   -0.13   1.000     -0.13     0.884    0.997    .       .       .
.
 Dose3   -0.11   0.008     -0.13     0.898    0.008   0.020    .       .
.
 Dose4   -0.13   1.000     -0.13     0.648    0.997   1.000   0.020    .
.
 Dose5   -0.13   1.000     -0.13     0.656    0.997   1.000   0.020   1.000
.

*****************************************************************************
***
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           5                12.00       0.035
```

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	0.478	0.159
Dose2	-0.13	1.000	0.461
Dose3	-0.12	0.053	0.990
Dose4	-0.13	1.000	0.894
Dose5	-0.13	1.000	0.740

INCREASING TREND TEST SUMMARY LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
Williams
Jonckheere

test for amphib metamorph screen study - Glyphosate
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 Test Stat	Shapiro-Wilks P-value	Levenes Test Stat	Levenes P-value	Conclusion
0.463	<.001	9.000	<.001	USE NON-PARAMETRIC TESTS

****
***
BASIC SUMMARY STATISTICS

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval
Ctrl	4	57.00	0.00	0.00	0.00	. , .
Dose1	4	57.00	0.00	0.00	0.00	. , .
Dose2	4	57.00	0.00	0.00	0.00	. , .
Dose3	4	57.00	0.00	0.00	0.00	. , .
Dose4	4	56.75	0.50	0.25	0.88	55.95, 57.55
Dose5	4	57.00	0.00	0.00	0.00	. , .

Level	Median	Min	Max	%of Control(means)	%Reduction(means)
Ctrl	57.00	57.00	57.00	.	.
Dose1	57.00	57.00	57.00	100.00	0.00
Dose2	57.00	57.00	57.00	100.00	0.00
Dose3	57.00	57.00	57.00	100.00	0.00

```
  Dose4       57.00    56.00      57.00       99.56                0.44
  Dose5       57.00    57.00      57.00      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
           5           18               1.00        0.446

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    57.00    .        57.00      .        .       .       .       .
.
 Dose1   57.00   1.000     57.00     0.583     .       .       .       .
.
 Dose2   57.00   1.000     57.00     0.618    1.000    .       .       .
.
 Dose3   57.00   1.000     57.00     0.636    1.000   1.000    .       .
.
 Dose4   56.75   0.320     56.88     0.269    0.530   0.530   0.530    .
.
 Dose5   57.00   1.000     56.88     0.273    1.000   1.000   1.000   0.530
.

************************************************************************
***
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           5                5.00       0.416

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                      .
  Dose2     57.00                 1.000                      .
  Dose3     57.00                 1.000                      .
  Dose4     57.00                 0.478                    0.079
  Dose5     57.00                 1.000                    0.190
```

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

*****************************************************************************
***
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
           5           18               1.00        0.446

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   -57.00    .       -56.95      .        .       .       .       .
.
 Dose1  -57.00   1.000    -56.95     0.721     .       .       .       .
.
 Dose2  -57.00   1.000    -56.95     0.756    1.000    .       .       .
.
 Dose3  -57.00   1.000    -56.95     0.774    1.000   1.000    .       .
.
 Dose4  -56.75   0.320    -56.95     0.785    0.530   0.530   0.530    .
.
 Dose5  -57.00   1.000    -57.00     0.656    1.000   1.000   1.000   0.530
.

*****************************************************************************
***
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           5                5.00       0.416

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     -57.00                    .                        .
  Dose1    -57.00                  1.000                      .
```

```
  Dose2    -57.00                    1.000                     .
  Dose3    -57.00                    1.000                     .
  Dose4    -57.00                    0.478                   0.921
  Dose5    -57.00                    1.000                   0.810

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams
   Jonckheere

test for amphib metamorph screen study - Glyphosate
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
      0.977         0.844          0.485      0.783    USE PARAMETRIC TESTS

*****************************************************************************
***
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     0.86     0.04        0.02        4.37           0.80,    0.92
  Dose1  4     0.93     0.10        0.05       10.77           0.77,    1.08
  Dose2  4     0.91     0.08        0.04        8.56           0.78,    1.03
  Dose3  4     0.97     0.04        0.02        3.82           0.91,    1.03
  Dose4  4     0.92     0.06        0.03        6.09           0.83,    1.01
  Dose5  4     1.01     0.06        0.03        5.91           0.91,    1.10

 Level       Median     Min       Max    %of Control(means)
%Reduction(means)
  Ctrl         0.86     0.83       0.90         .                  .
  Dose1        0.92     0.81       1.05      107.12              -7.12
  Dose2        0.88     0.85       1.02      105.04              -5.04
  Dose3        0.96     0.94       1.03      112.71             -12.71
  Dose4        0.95     0.84       0.95      106.51              -6.51
  Dose5        1.03     0.92       1.05      116.79             -16.79

*****************************************************************************
***
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
```

```
          5           18              2.46        0.072

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     0.86    .         0.93      .        .       .       .       .
.
 Dose1    0.93   0.551      0.93     0.964     .       .       .       .
.
 Dose2    0.91   0.809      0.93     0.974    0.999    .       .       .
.
 Dose3    0.97   0.105      0.93     0.979    0.895   0.705    .       .
.
 Dose4    0.92   0.628      0.93     0.982    1.000   1.000   0.849    .
.
 Dose5    1.01   0.023      0.93     0.984    0.483   0.284   0.970   0.419
.

*******************************************************************************
***
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           5                10.28       0.068

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       0.86                    .                         .
  Dose1      0.92                  0.346                     0.876
  Dose2      0.88                  0.346                     0.810
  Dose3      0.96                  0.066                     0.984
  Dose4      0.95                  0.154                     0.964
  Dose5      1.03                  0.067                     0.996

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

*******************************************************************************
***
```

```
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
           5           18               2.46        0.072

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    -0.86    .        -0.86      .        .       .       .       .
.
 Dose1   -0.93   0.551     -0.92     0.160     .       .       .       .
.
 Dose2   -0.91   0.809     -0.92     0.171    0.999    .       .       .
.
 Dose3   -0.97   0.105     -0.95     0.056    0.895   0.705    .       .
.
 Dose4   -0.92   0.628     -0.95     0.057    1.000   1.000   0.849    .
.
 Dose5   -1.01   0.023     -1.01     0.003    0.483   0.284   0.970   0.419
.

*****************************************************************************
***
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           5                10.28       0.068

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      -0.86                    .                        .
  Dose1     -0.92                  0.346                    0.124
  Dose2     -0.88                  0.346                    0.190
  Dose3     -0.96                  0.066                    0.016
  Dose4     -0.95                  0.154                    0.036
  Dose5     -1.03                  0.067                    0.004

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams
```

```
   Jonckheere

test for amphib metamorph screen study - Glyphosate
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
      0.965         0.541          3.882      0.015    USE NON-PARAMETRIC
TESTS

*************************************************************************
***
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    23.23     0.43       0.21        1.84          22.55,   23.90
  Dose1  4    23.58     0.69       0.34        2.93          22.48,   24.67
  Dose2  4    23.48     0.85       0.43        3.64          22.12,   24.83
  Dose3  4    24.43     0.17       0.09        0.70          24.15,   24.70
  Dose4  4    23.80     0.47       0.23        1.97          23.05,   24.55
  Dose5  4    24.78     0.36       0.18        1.45          24.20,   25.35

 Level       Median     Min       Max    %of Control(means)
%Reduction(means)
  Ctrl        23.25    22.70      23.70         .                  .
  Dose1       23.65    22.70      24.30      101.51             -1.51
  Dose2       23.40    22.70      24.40      101.08             -1.08
  Dose3       24.45    24.20      24.60      105.17             -5.17
  Dose4       24.00    23.10      24.10      102.48             -2.48
  Dose5       24.85    24.30      25.10      106.67             -6.67

*************************************************************************
***
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
           5           18               4.87        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    23.23    .        23.88      .        .       .       .       .
.
 Dose1   23.58   0.827     23.88     0.977     .       .       .       .
.
 Dose2   23.48   0.946     23.88     0.984    1.000    .       .       .
.
 Dose3   24.43   0.024     23.88     0.988    0.278   0.183    .       .
.
 Dose4   23.80   0.448     23.88     0.989    0.991   0.954   0.591    .
.
 Dose5   24.78   0.003     23.88     0.990    0.055   0.033   0.938   0.164
.

*****************************************************************************
***
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           5                14.86       0.011

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      23.25                    .                         .
  Dose1     23.65                  0.489                     0.810
  Dose2     23.40                  0.780                     0.770
  Dose3     24.45                  0.067                     0.994
  Dose4     24.00                  0.187                     0.969
  Dose5     24.85                  0.067                     0.999

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

*****************************************************************************
***
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
           5           18               4.87        0.005

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	-23.23	.	-23.23	.	.	.	.	.	.
Dose1	-23.58	0.827	-23.53	0.266	.	.	.	.	.
Dose2	-23.48	0.946	-23.53	0.284	1.000	.	.	.	.
Dose3	-24.43	0.024	-24.11	0.020	0.278	0.183	.	.	.
Dose4	-23.80	0.448	-24.11	0.020	0.991	0.954	0.591	.	.
Dose5	-24.78	0.003	-24.78	<.001	0.055	0.033	0.938	0.164	.

****
***
NON-PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Kruskal-Wallis test - equality among treatment groups

Degrees of Freedom	TestStat	P-value
5	14.86	0.011

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	-23.25	.	.
Dose1	-23.65	0.489	0.190
Dose2	-23.40	0.780	0.230
Dose3	-24.45	0.067	0.006
Dose4	-24.00	0.187	0.031
Dose5	-24.85	0.067	<.001

INCREASING TREND TEST SUMMARY LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL
Williams
Jonckheere

test for amphib metamorph screen study - Glyphosate
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
      0.921         0.062          0.622      0.685    USE PARAMETRIC TESTS

*************************************************************************
***
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     7.65     0.68        0.34        8.90           6.57,    8.73
  Dose1  4     8.48     0.22        0.11        2.62           8.12,    8.83
  Dose2  4     7.78     0.43        0.21        5.49           7.10,    8.45
  Dose3  4     8.20     0.50        0.25        6.14           7.40,    9.00
  Dose4  4     8.00     0.69        0.34        8.60           6.91,    9.09
  Dose5  4     8.25     0.79        0.39        9.52           7.00,    9.50

 Level       Median     Min       Max    %of Control(means)
%Reduction(means)
  Ctrl         7.80     6.70       8.30         .                .
  Dose1        8.50     8.20       8.70       110.78           -10.78
  Dose2        7.75     7.30       8.30       101.63            -1.63
  Dose3        8.30     7.50       8.70       107.19            -7.19
  Dose4        8.25     7.00       8.50       104.58            -4.58
  Dose5        8.35     7.20       9.10       107.84            -7.84

*************************************************************************
***
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
           5            18               1.13        0.379

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     7.65    .         8.06      .        .       .       .       .
.
 Dose1    8.48   0.207      8.06     0.901     .       .       .       .
.
```

```
 Dose2    7.78   0.998      8.06     0.920    0.550    .       .       .
.
 Dose3    8.20   0.552      8.06     0.931    0.983   0.901    .       .
.
 Dose4    8.00   0.862      8.06     0.937    0.853   0.993   0.996    .
.
 Dose5    8.25   0.474      8.06     0.941    0.993   0.853   1.000   0.989
.

*************************************************************************
***
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           5                6.59       0.253

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       7.80                   .                         .
  Dose1      8.50                 0.103                     0.978
  Dose2      7.75                 1.000                     0.500
  Dose3      8.30                 0.335                     0.698
  Dose4      8.25                 0.346                     0.680
  Dose5      8.35                 0.283                     0.792

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

*************************************************************************
***
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
           5           18               1.13        0.379

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    -7.65    .        -7.65      .        .       .       .       .
.
 Dose1   -8.48   0.207     -8.11     0.165     .       .       .       .
.
 Dose2   -7.78   0.998     -8.11     0.176    0.550    .       .       .
.
 Dose3   -8.20   0.552     -8.11     0.182    0.983   0.901    .       .
.
 Dose4   -8.00   0.862     -8.11     0.186    0.853   0.993   0.996    .
.
 Dose5   -8.25   0.474     -8.25     0.108    0.993   0.853   1.000   0.989
.

*************************************************************************
***
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           5                 6.59       0.253

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      -7.80                    .                        .
  Dose1     -8.50                  0.103                    0.022
  Dose2     -7.75                  1.000                    0.500
  Dose3     -8.30                  0.335                    0.302
  Dose4     -8.25                  0.346                    0.320
  Dose5     -8.35                  0.283                    0.208

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams
   Jonckheere

test for amphib metamorph screen study - Glyphosate
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
```

```
      0.924         0.072          0.429      0.822    USE PARAMETRIC TESTS

************************************************************************
***
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     0.33     0.03        0.01        7.74           0.28,    0.37
  Dose1  4     0.36     0.01        0.01        3.64           0.33,    0.38
  Dose2  4     0.33     0.01        0.01        4.29           0.31,    0.35
  Dose3  4     0.34     0.02        0.01        5.17           0.31,    0.36
  Dose4  4     0.34     0.02        0.01        7.11           0.30,    0.37
  Dose5  4     0.33     0.03        0.01        7.52           0.29,    0.37

 Level       Median     Min       Max    %of Control(means)
%Reduction(means)
  Ctrl         0.33     0.29       0.35         .                 .
  Dose1        0.36     0.34       0.37       109.23            -9.23
  Dose2        0.33     0.32       0.35       101.54            -1.54
  Dose3        0.34     0.31       0.35       103.08            -3.08
  Dose4        0.35     0.30       0.35       103.08            -3.08
  Dose5        0.34     0.30       0.36       102.31            -2.31

************************************************************************
***
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
           5           18               1.02        0.434

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     0.33    .         0.34      .        .       .       .       .
.
 Dose1    0.36   0.180      0.34     0.908     .       .       .       .
.
 Dose2    0.33   0.996      0.33     0.830    0.528    .       .       .
.
 Dose3    0.34   0.931      0.33     0.846    0.733   0.999    .       .
.
 Dose4    0.34   0.931      0.33     0.856    0.733   0.999   1.000    .
.
```

```
 Dose5    0.33   0.978      0.33     0.847    0.632   1.000   1.000   1.000
.

************************************************************************
***
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           5                5.83       0.323

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       0.33                    .                         .
  Dose1      0.36                  0.123                     0.971
  Dose2      0.33                  1.000                     0.470
  Dose3      0.34                  0.575                     0.425
  Dose4      0.35                  0.481                     0.486
  Dose5      0.34                  0.671                     0.419

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

************************************************************************
***
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
           5           18               1.02        0.434

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    -0.33    .        -0.33      .        .       .       .       .
.
 Dose1   -0.36   0.180     -0.34     0.237     .       .       .       .
.
 Dose2   -0.33   0.996     -0.34     0.254    0.528    .       .       .
.
```

```
 Dose3   -0.34   0.931     -0.34     0.263    0.733   0.999    .       .
.
 Dose4   -0.34   0.931     -0.34     0.268    0.733   0.999   1.000    .
.
 Dose5   -0.33   0.978     -0.34     0.272    0.632   1.000   1.000   1.000
.

*************************************************************************
***
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           5                5.83       0.323

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      -0.33                    .                         .
  Dose1     -0.36                  0.123                     0.029
  Dose2     -0.33                  1.000                     0.530
  Dose3     -0.34                  0.575                     0.575
  Dose4     -0.35                  0.481                     0.514
  Dose5     -0.34                  0.671                     0.581

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams
   Jonckheere
```

DATA EVALUATION RECORD

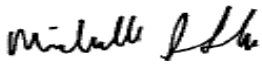
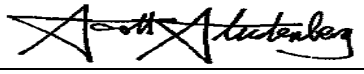
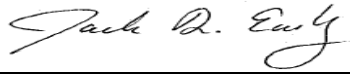
GLYPHOSATE

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

EPA Contract No. EP10H001452
Task Assignment No. 2-74-2012 (MRID 48671301)

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:	
Michelle Sharpe-Kass, M.S.	Date:	6/13/2012
Secondary Reviewer:	Signature:	
Scott D. Studenberg, Ph.D., D.A.B.T.	Date:	6/30/2012
Program Manager:	Signature:	
Jack D. Early, M.S.	Date:	7/16/2012
Quality Assurance:	Signature:	
Jack D. Early, M.S.	Date:	7/03/2012

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: Anwar Y. Dunbar, Ph.D. **Signature:** Anw Y. Dunb
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 05-27-15
Secondary Reviewer: Gregory Akerman, Ph.D. **Signature:**
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 6/15/15

DATA EVALUATION RECORD

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

PC CODE: 417300 **DP BARCODE:** D401747

TXR#: 0053233 **CAS No.:** 1071-83-6

TEST MATERIAL (PURITY): Glyphosate (95.93% glyphosate acid, 85.14% calculated glyphosate content)

SYNONYMS: Roundup, N-(phosphonomethyl)glycine

CITATION: Willoughby, J.A. (2012) Glyphosate: Androgen Receptor Binding (Rat Prostate Cytosol) Screening Assay. CeeTox, Inc., Kalamazoo, MI. Laboratory Study No.: 6500V-100334ARB, March 8, 2012. MRID 48671301. Unpublished

SPONSOR: Joint Glyphosate Task Force, LLC, 8325 Old Deer Trail, Raleigh, NC

TEST ORDER #: CON-417300-23

EXECUTIVE SUMMARY: In an androgen receptor (AR) binding assay (MRID 48617301), ventral prostate cytosol from Sprague Dawley rats was used as the source of AR to conduct saturation and competitive binding experiments. The saturation binding experiment was conducted to demonstrate that the AR in the rat prostate cytosol was present in reasonable numbers and was functioning with appropriate affinity for the radio-labeled reference androgen (R1881). The competitive binding assay measured the binding of a single concentration of [^{3}H]-R1881 (1 nM) in the presence of increasing concentrations (logarithmic increase from 10^{-10} to 10^{-3} M) of glyphosate (95.93% glyphosate acid, 85.14% calculated glyphosate content, Batch # GLP-1103-21149-T). Low-salt TEGD buffer was used as the vehicle for glyphosate. A total of 3 runs were performed, and each run included dexamethasone as a weak positive control, and R1881 as the ligand reference standard.

Saturation binding data were not originally provided in the study report; however, summarized saturation binding data (MRID 48843501) from the performing laboratory were submitted following a request by the Agency. The dissociation constant (K_d) for [^{3}H]-R1881 was 0.613±0.041 nM and the estimated B_{max} was 0.817±0.049 fmol/100 µg protein for the single batch of prostate cytosol that was prepared. The mean and individual K_d values were below the range reported in the EPA validation program (0.685 to 1.57 nM). Confidence in these numbers is high according to the goodness of fit (R^2 = 0.957-0.984) and the small variation among runs.

In the competitive binding experiment, the estimated mean log IC_{50}s for R1881 and the weak positive control (dexamethasone) were −9.0 and −4.6 M, respectively, and the mean relative binding affinity (RBA) for the weak positive control, dexamethasone, was 0.004%. Confidence in the numbers for the reference standards is high as the values as variation between runs was small. All performance criteria were met.

At glyphosate concentrations of 10^{-10} to 10^{-3} M, specific binding of [^{3}H]-R1881 was 92.4-101.3% with the exception of one concentration (10^{-9} M) in Run 1, which had an average binding of 66.5%. Review of the data indicated that this value was a result of a single replicate with a specific binding of 7.5%. Excluding this value yielded a mean specific binding of 96.0%, which concurs with the other runs. As the specific [^{3}H]-R1881 binding was >75% at all concentrations of glyphosate in all runs, an IC_{50} and RBA could not be calculated for glyphosate.

Based on the results from the three runs, glyphosate is classified as a Non-Binder 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 Data Confidentiality, GLP Compliance, and Quality Assurance statements were provided.

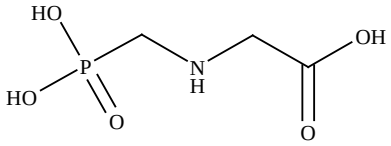
I. MATERIALS AND METHODS

A. MATERIALS

1. Test Facility: CeeTox, Inc.

Location:	4717 Campus Drive, Kalamazoo, MI 49008
Study Director:	Willoughby, J.A.
Other Personnel:	Rutherford, K. (Director of Laboratory Operations); Blakeman, D. (Senior Scientist); Haines, C. (Scientist); McColley, S. (Scientist); B. Meyer, Scientist; Toole, C. (Director of Project Management)
Study Period:	September 22, 2001 to March 8, 2012

2. Test substance: Glyphosate

Description:	White wetcake (white crystalline solid)
Source:	Monsanto, Co, St. Louis, MO
Batch #:	GLP-1103-21149-T (expiry: March 9, 2012)
Purity:	95.93% glyphosate acid, 85.14% calculated glyphosate acid
Solubility:	Not Reported
Volatility:	Not reported
Stability:	One year at room temperature
Storage conditions:	35 to 100°F (Room temperature)
CAS #:	1071-83-6
Molecular weight:	169.01 g/mol
Structure:	

3. Non-labeled ligand (strong positive control): R1881

Supplier:	Sigma-Aldrich, St Louis, MO
Catalog #:	R0908
Batch #:	060M4638
Purity:	98%
CAS #:	965-93-5

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

Supplier:	Perkin-Elmer, Boston MA
Catalog and Batch #:	NET590, Lot # 653698
Date of production:	February 24, 2011
Date of use:	October 25, 27 and 31, 2010,
Radiochemical purity:	>97%
Specific activity:	85.1 Ci/mmol
Concentration of stock:	10 n M

5. Weak positive control: Dexamethasone

Supplier:	Sigma-Aldrich, St Louis, MO
Catalog #	D1756
Batch #:	1419230
Purity:	98.9%
CAS # :	50-02-2

6. Solvent/vehicle control: Low salt TEGD buffer

Justification for choice of solvent:	Glyphosate is soluble (12 g/L) in aqueous solutions, but is not soluble in DMSO
Final Concentration:	Not Applicable

B. METHODS

1. **Preparation of Rat Ventral Prostate Cytosol:** Male Sprague Dawley rats (number not reported) were castrated at 90 days of age and euthanized on the following day. Intact prostates were supplied by Charles River Laboratory, and were transported overnight on dry ice. The ventral prostates were weighed, and placed in ice-cold TEDG (Tris, EDTA, DTT, glycerol) buffer + PMSF (phenylmethylsulfonyl fluoride), homogenized, and centrifuged for 30 min at 30,000 × *g* at 4ºC. Supernatant was pooled, discarding the resulting pellets. Protein concentration of the cytosol was determined to be 8.8 mg/mL using a commercially available protein kit compatible with DTT in the TEDG buffer (e.g., BioRad Protein Assay Kit, Richmond, CA). Cytosol was divided into aliquots for storage at −80º C until use.

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

TABLE 1. Summary of Conditions for Saturation Binding Experiment

Source of receptor		Rat ventral prostate cytosol
Concentration of radioligand (as serial dilutions)		0.25 to 10 nM
Concentration of non-labeled ligand (100X [radioligand])		2 to 1000 nM
Optimization of receptor concentration		Sufficient to bind 25 to 35% of radioligand at 0.25 nM
Temperature		4º C
Incubation time		16 to 20 hours
Composition of assay buffer (TEDG)	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10%
	Phenylmethylsulfonyl fluoride	1 mM
	DTT	1 mM

a Data were obtained from page 2 of the study report (MRID 48843501).

On the day of the assay, the specific activity of the stock solution [^{3}H]-R1881 (originally 85.1 Ci/mmol as manufactured on February 24, 2011) was adjusted for decay over time (adjusted specific activities were not reported), and serial dilutions in low-salt TEDG + PMSF buffer were prepared to achieve the final concentrations in cytosol of 0.25, 0.50, 0.70, 1.0, 1.5, 2.5, 5.0, and 10 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]-

[1] Willoughby, J.A. (2012) Saturation binding information for cytosol isolated 24-Sept-2011. CeeTox, Inc.

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 1.86 mg protein/assay tube, which resulted in the binding of approximately 25-35% of the total radioactivity added. 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 saturation binding experiment consisted of three non-current runs (conducted on September 24, 25, and 26, 2011, respectively). 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
1.0	1.0	100	10	30
1.5	1.5	150	10	45
2.5	2.5	250	100	7.5
5.0	5.0	500	100	15
10	10	1000	100	30

a Data were obtained from page 3 of the study report (MRID 48843501).
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, 21, or 30 µL of 100 nM [^{3}H]-R1881 without cytosol or other components to determine the total counts added.

3. <u>Competitive Binding Experiment</u>: 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		~6.8 mg/mL
Concentration of test substance (as serial dilutions)		10^{-10} to 10^{-3} M
Incubation Temperature		4 ± 2 ºC
Incubation time		16-20 hours
Composition of assay buffer	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10 %
	Protease inhibitor	0.5% v/v
	DTT	1 mM
	Sodium Molybdate	1 mM

a Data were obtained from page 17 of the study report.

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 (adjusted specific activity of 82.0 Ci/mmol for the first run, 82.0 Ci/mmol for the second run and 81.9 mMol for the third run) to the AR in the presence of increasing concentrations of a test substance.

Low salt TEGD buffer was used as a vehicle, and no precipitation was observed by visual inspection at up to 10^{-3} M of glyphosate. Based on data form the competitive binding experiment, the reviewers calculated that the amount of cytosolic protein used in the assay contained enough receptor to bind approximately 9-11% of the [^{3}H]-R1881 at 1 nM.

Dilutions of glyphosate, reference standard (R1881), weak positive control (dexamethasone), and solvent control (TEGD buffer) were prepared to achieve the concentrations shown in Table 4. Each assay consisted of three independent runs on three different days. Each run included triplicate sets of the blank and 1μM R1881 (non-specific binding, NSB) at the beginning and end of each run, along with triplicate tubes of the reference standard, the weak positive control, and the test chemical at each concentration, resulting in a total of 84 samples per run.

TABLE 4. Competitor Final Molar (M) Concentrations in Competitive Binding Assay [a b]				
Solvent Control	**Reference standard**	**Weak positive control**	**Test Chemical**	None
TEGD buffer	R1881	Dexamethasone	Glyphosate	(hot mix)
Tubes 7-12	Tubes 13-33 [c]	Tubes 37-60	Tubes 61-84	Tubes 1-6
	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^{-10}	1×10^{-10}	

a Data were obtained from pages 35-37 of the study report.

b The [^{3}H]-R1881, NSB, and solvent controls were run in 6 replicates and each concentration of each chemical was run in triplicate for a total of 84 tubes per run. Tubes 1-84 contained 50 µL of triamcinolone acetonide and 30 µL [^{3}H]-R1881. Samples were dried, and 300 µL of prostate cytosol were added. Tubes 7-84 also contained 10 µL of the solvent control, reference standard (non-radiolabeled R-1881), weak positive control, or test substance, with the exception of Tubes 13-18 that contained 30 µL of non-radiolabeled R1881 (used to evaluate non-specific binding). Tubes 1-6 contained only 30 µL of [^{3}H]-R1881.

c Tubes 13-18 were used to evaluate non-specific binding by adding 100X of cold (non-radiolabeled) R1881.

Sample tubes were stored 16-20 hours at 4°C to allow the reaction to reach equilibrium, and bound R1881 was separated from free R1881 by washing with HAP buffer and extraction with ethanol, followed by scintillation counting of bound [^{3}H]-R1881.

4. <u>Data Analysis</u>: For the saturation binding experiment, total binding and non-specific binding data were modeled via non-linear regression by using Graph Pad Prism v. 5 (GraphPad Software, Inc., La Jolla, CA)], incorporating automatic outlier elimination according to the method of Motulsky and Brown (2006)[2] implemented by using the ROUT procedure in Prism v. 5 with a Q value of 1.0. Receptor binding data plots were corrected for ligand depletion with the method of Swillens (1995)[3]. For the competitive binding assay, similar methods of nonlinear regression were used to fit a curve (for R1881, the positive control, and the test substance) to the Hill equation formula which incorporated log IC_{50} as a parameter to be estimated. For parameters reported from the saturation binding experiment (K_d and B_{max}) and 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 using Microsoft Excel 2007 (v. 12.0.6557.5000; Microsoft Corporation, Redmond, WA), and mean and standard error were calculated for the composite three runs with Microsoft Excel 2010.

5. <u>Definitions</u>

a. Classification of test material

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

2 Motulsky, H.J. and Brown, R.E. (2006) Detecting outliers when fitting data with nonlinear regression-□ a new method based on robust nonlinear regression and the false discovery rate. BMC Bioinformatics, Vol 7, pp 123-142.
3 Swillens, S. (1995) Interpretation of binding curves obtained with high receptor concentrations: practical aid for computer analysis. *Molec. Pharmacol.* 47(6):1197-1203.

Binder: The average curve for the test chemical across runs crosses 50% of radioligand bound, and the Hill Slope is between −0.6 to −1.4.

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 Hill slope for 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 ÷ IC_{50} of test substance) × 100
Log RBA: Log_{10} (IC_{50} of R1881 ÷ IC_{50} of test substance)

II. RESULTS

A. <u>SATURATION BINDING EXPERIMENT</u>: Saturation binding experiment parameters are presented in Table 6. The dissociation constant (K_d) for [^{3}H]-R1881 was 0.613±0.041 nM and the estimated B_{max} was 0.817±0.049 fmol/100 µg protein for the single batch of prostate cytosol that was prepared. The mean and individual K_d values were below the range reported in the EPA validation program (0.685 to 1.57 nM). Confidence in these numbers is high according to the goodness of fit (R^2 = 0.957-0.984) and the small variation among runs.

TABLE 6. Saturation Binding Experiment of [^{3}H]-R1881 with Androgen Receptor from Rat Prostate Cytosol[a]

Parameter	Run 1	Run 2	Run 3	Mean ± SEM[b]
R^2 (unweighted)	0.984	0.977	0.957	0.957-0.984
B_{max} (nM)	0.011	0.010	0.011	0.011±0.001
B_{max} (fmol/100 µg protein)	0.809	0.773	0.870	0.817±0.049
K_d (nM)	0.565	0.638	0.635	0.613±0.041

a Data were obtained from page 4 of the study report (MRID 48843501).
b The range of R^2 is reported and the mean ± SEM is reported for the other parameters.
R^2 Goodness of fit for curve calculated for specific binding

Figure 1 illustrates the non-specific, specific, and total binding curves for [^{3}H]-R1881 to the androgen receptor for the three independent runs. The specific binding reached a plateau in each run, and non-specific binding was less than 20% of total binding. Figure 2 contains the Scatchard plots that illustrate the binding of [^{3}H]-R1881 to the androgen receptor. The

model fits to the data resulted in linear plots.

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

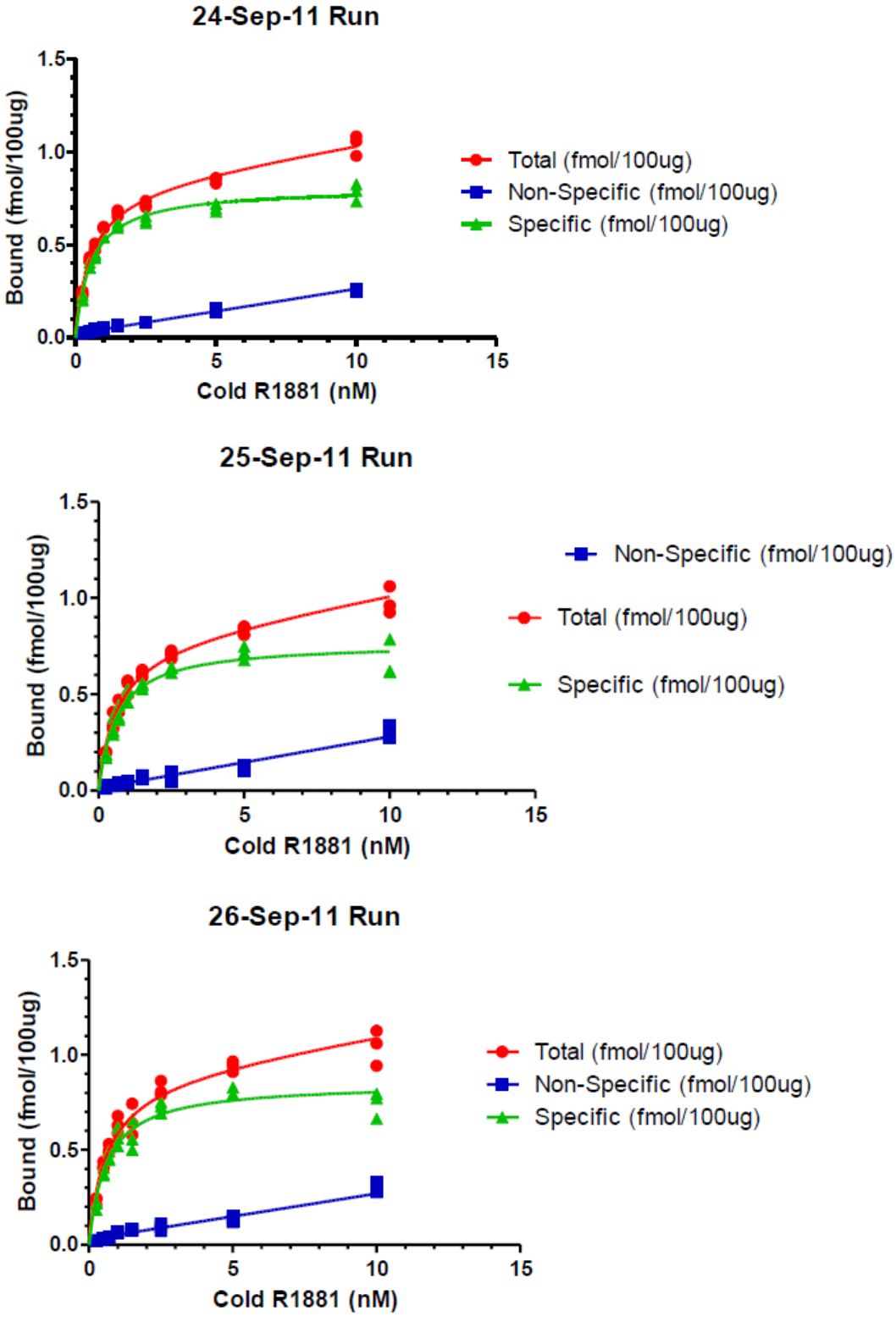
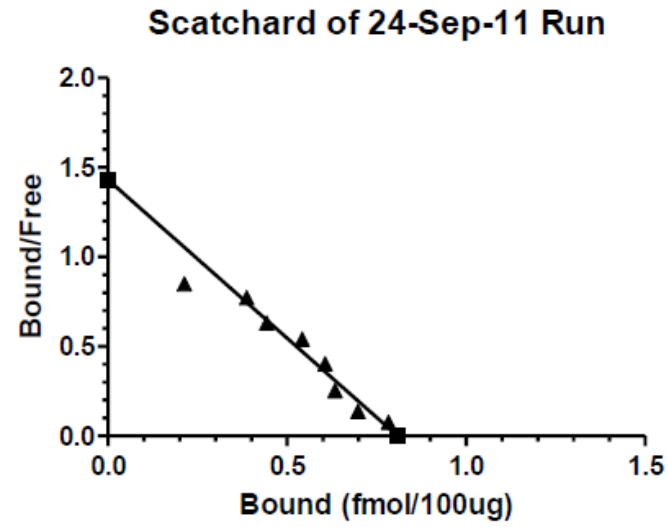
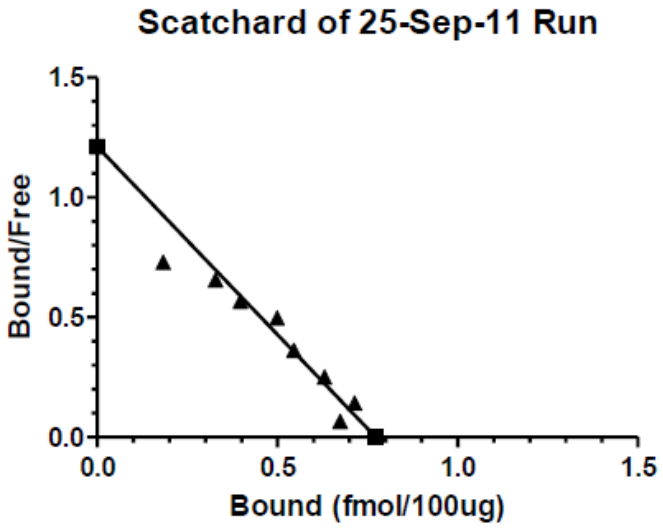
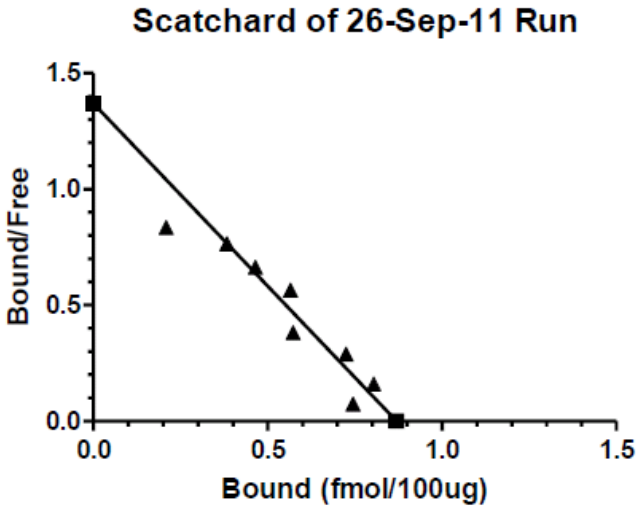


FIGURE 2. Scatchard Plots of the Binding of [^{3}H]-R1881 to the Androgen Receptor.







B. COMPETITIVE BINDING EXPERIMENT: The results from the three competitive binding experiments are summarized in Table 6 and shown graphically in Figures 3-5. No precipitation was observed at glyphosate concentrations up to 10^{-3} M.

In Run 1, specific binding of [^{3}H]-R1881 was 95.5-101.3% at glyphosate concentrations of 10^{-10} to 10^{-3} M, with the exception of 10^{-9}M, which had an average binding of 66.5%. The raw data revealed that this value was a result of a single replicate with a specific binding of 7.5%. Exclusion of this value resulted in a mean specific binding of 96.0%. In this run glyphosate is classified as a "non-binder." In Run 2, specific binding of [^{3}H]-R1881 was 93.8-99.9% at glyphosate concentrations of 10^{-10} to 10^{-3} M, classifying glyphosate as a "non-binder" for binding. In Run 3, specific binding of [^{3}H]-R1881 was 92.4-99.0% at glyphosate concentrations of 10^{-10} to 10^{-3} M, classifying glyphosate as a "non-binder". An IC_{50} and RBA could not be calculated for glyphosate.

The estimated mean log IC_{50}s for R1881 and the weak positive control (dexamethasone) were −9.0 and −4.6 M, respectively, and the mean RBA for dexamethasone was 0.004%. Confidence in the numbers for the reference standards is high as the values were similar between runs.

TABLE 6. Competitive Binding Assay of Glyphosate with AR from Rat Prostate Cytosol

Parameter		Run 1 [b]	Run 2 [b]	Run 3 [b]	Mean ± SE[c]
R^2 (unweighted)	R1881	NR	NR	NR	NA
	Positive control	NR	NR	NR	NA
	Test substance	NR	NR	NR	NA
Log IC_{50} (M)	[a] R1881	−9.0	−9.1	−9.0	−9.03 ± 0.06
	Positive control	−4.6	−4.6	−4.6	−4.6 ± 0.0
	Glyphosate	NA	NA	NA	NA
IC_{50} (M) [c]	R1881	1.0×10^{-9}	7.94×10^{-10}	1.0×10^{-9}	9.31×10^{-10} (± 1.19)
	Dexamethasone	2.51×10^{-5}	2.51×10^{-5}	2.51×10^{-5}	2.51×10^{-5} (± 0.00)
	Glyphosate	NA	NA	NA	NA
Log RBA[c]	Dexamethasone	−4.4	−4.5	−4.4	−4.43 (± 0.06)
	Glyphosate	NA	NA	NA	NA
RBA (%) [c]	Dexamethasone	0.00398	0.00316	0.00398	0.00371 ± 0.00047
	Glyphosate	NA	NA	NA	NA

a Data were obtained from pages 25, 27 and 29 of the study report.
b The mean and standard deviation are reported for the concurrent replicates within each run.
c Calculated by reviewers; for means expressed in scientific notation, the SE values in parentheses are presented in the same order of magnitude as the mean value.
SE Standard Error
NA Not applicable
NR not reported.
R^2 Goodness of fit (R^2 is more appropriately expressed as a range, as opposed to a mean).
RBA (%) relative binding affinity

FIGURE 3. Percentage [^{3}H]-R1881 Bound to the Androgen Receptor in the Presence of R1881, Dexamethasone, or Glyphosate in Assay 1.

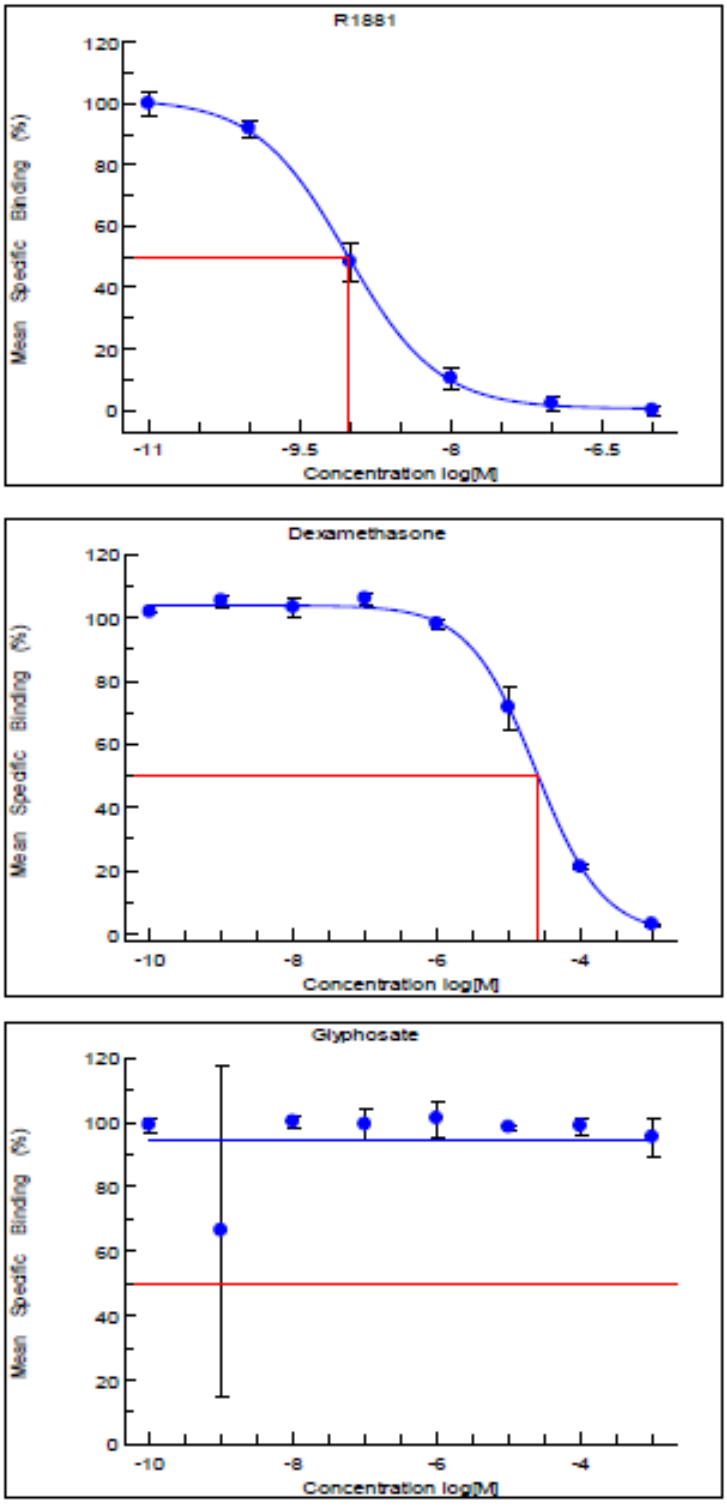


FIGURE 4. Percentage [^{3}H]-R1881 Bound to the Androgen Receptor in the Presence of R1881, Dexamethasone, or Glyphosate in Assay 2.

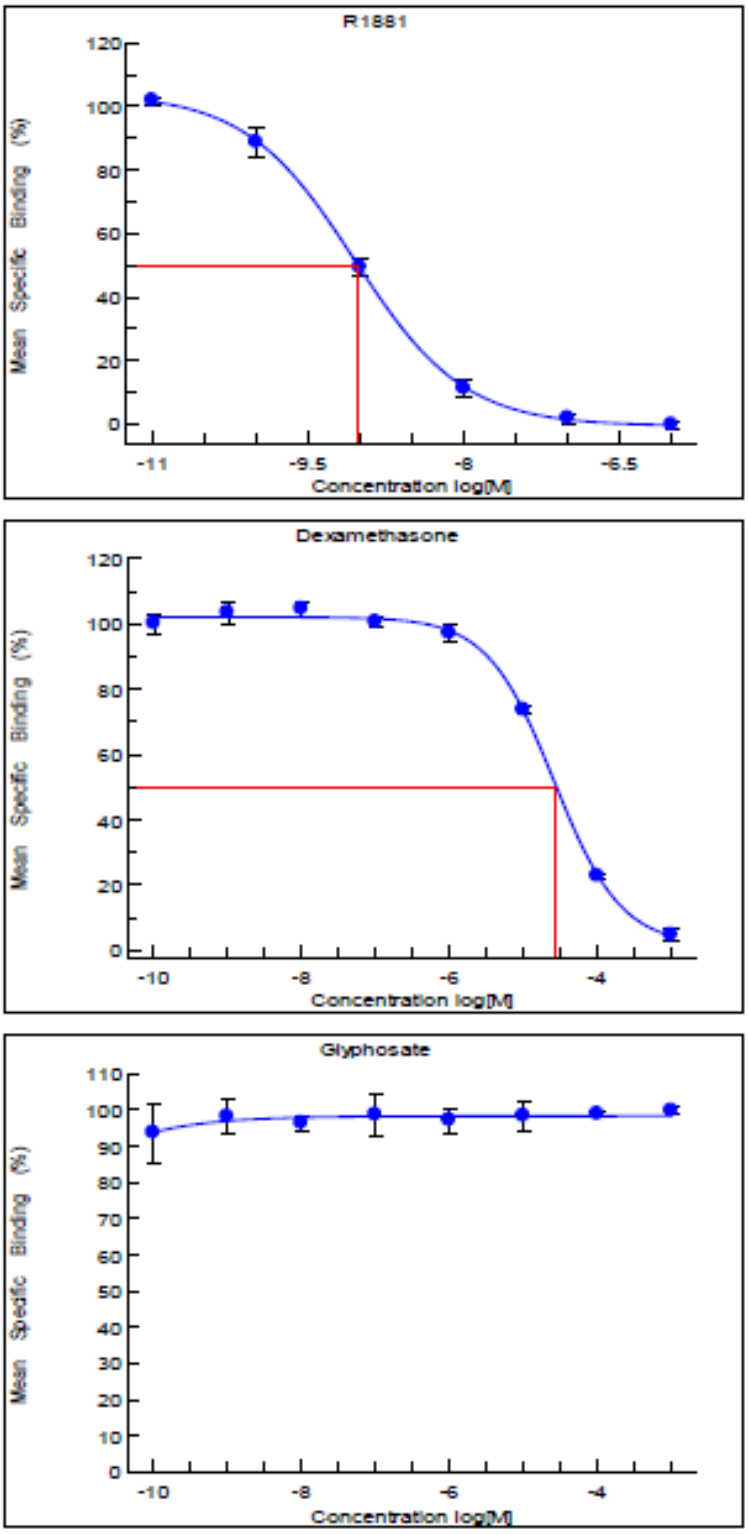
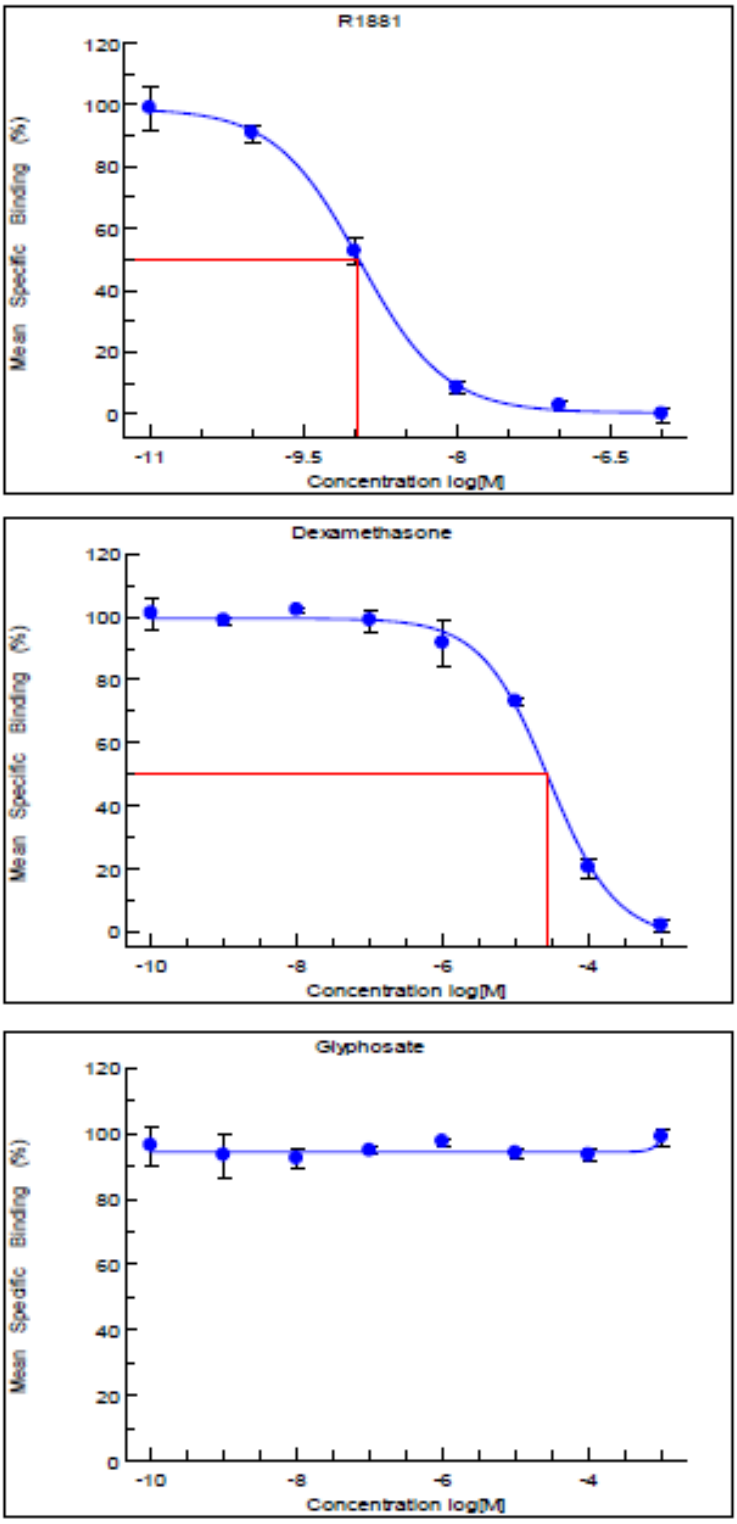


FIGURE 5. Percentage [^{3}H]-R1881 Bound to the Androgen Receptor in the Presence of R1881, Dexamethasone, or Glyphosate in Assay 3.



C. <u>PERFORMANCE CRITERIA</u>: To ensure that the competitive binding assay was functioning properly, each run was evaluated using the 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%	9.7 to 10.9%	X	
Test chemical Top (% binding)	80 to 115	93.5 to 99.2[c]	X	
R1881 fitted curve parameters				
Top (% binding)	82 to 114	99 to 104	X	
Bottom (% binding)	−2.0 to 2.0	0	X	
Hill Slope	−1.2 to −0.8	−0.8 to −1.0	X	
Weak positive control (dexamethasone) fitted curve parameters				
Top (% binding)	87 to 106	100 to 104	X	
Bottom (% binding)	−12 to 12	−2 to 2	X	
Hill Slope	−1.4 to −0.6	−0.9 to −1.0	X	
Saturation Binding Experiment K_d (nM)	0.685 to 1.57	0.565 to 0.638		X
Non-specific binding (%)[d]	≤10.0	0.35 to 0.47	X	

a Data were obtained from pages 25, 27 and 29 of the study report.
b These values represent ranges from the validation study.
c 10^{-10} to 10^{-9} M; excludes the 10^{-9} M value in Assay Run 1 that was 66.5% due to one replicate with a value of 7.5%
d Calculated by the reviewer from data on pages 35-37 of the study report.

The performance criteria were generally met. 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 −0.8 to −1.0. Examination of the data for the two reference standards across the runs indicated consistency of the Hill slope, and top and bottom plateaus, and the placement along the X-axis was consistent.

III. DISCUSSION AND CONCLUSIONS

A. <u>INVESTIGATOR'S CONCLUSIONS</u>: Glyphosate was classified as a "non-binder" in all three independent runs and thus has a final classification of "non-binder."

B. <u>AGENCY COMMENTS</u>: In the competitive binding experiment, no precipitation of glyphosate was observed at any tested concentration; therefore, the suitable top concentration of glyphosate was 10^{-3} M.

In the saturation binding experiments, the mean dissociation constant (K_d) for [^{3}H]-R1881 was 0.613 nM and the mean estimated B_{max} was 0.817 fmol/100 µg protein for the single batch of prostate cytosol that was prepared. The mean and individual K_d values were below the range reported in the EPA validation program (0.685 to 1.57 nM). Confidence in these numbers is high according to the goodness of fit (R^2 = 0.957-0.984) and the small variation among runs.

In the competitive binding experiments, the estimated mean log IC_{50}s for R1881 and the weak positive control (dexamethasone) were −9.0 and −4.6 M, respectively, and the mean

RBA for the dexamethasone was 0.004%. Confidence in the numbers for the reference standards is high as the values were similar between runs. All performance criteria were met.

Specific binding of [^{3}H]-R1881 was 95.5-101.3% at glyphosate concentrations of 10^{-10} to 10^{-3} M in Run 1, with the exception of the 10^{-9} M concentration, which had an average binding of 66.5%. Examination of the raw data indicated that this value was a result of a single replicate with a specific binding of 7.5%. Exclusion of this value results in a mean specific binding of 96.0%. In this run glyphosate is classified as a non-binder. In Runs 2 and 3, specific binding of [^{3}H]-R1881 was ≥92%% at glyphosate concentrations of 10^{-10} to 10^{-3} M, yielding classifications of non-binder for glyphosate.

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

- Curves were not provided showing the average binding of each test substance across all three runs.
- R^2 values for the competitive binding assay were not reported.

DATA EVALUATION RECORD

GLYPHOSATE
Study Type: OCSPP 890.1200, Aromatase Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-74-2012 (MRID 48671303)

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

Prepared by
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Primary Reviewer Joan Harlin, M.S.	Signature: Date:	Joan Harlin 7/5/2012
Secondary Reviewer Scott D. Studenberg, Ph.D., D.A.B.T.	Signature: Date:	Scott D. Studenberg 7/11/2012
Program Manager: Jack D. Early, M.S.	Signature: Date:	Jack D. Early 7/13/2012
Quality Assurance: Jack D. Early, M.S.	Signature: Date:	Jack D. Early 7/13/2012

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: Anwar Y. Dunbar, Ph.D. **Signature:** Anw Y. Dunbar
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 05-27-15
Secondary Reviewer: Greg Akerman, Ph.D. **Signature:**
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 6/15/15

DATA EVALUATION RECORD

STUDY TYPE: Aromatase (Human Recombinant); OCSPP 890.1200

PC CODE: 417300 **DP BARCODE:** D401747

TXR#: 0053233 **CAS No.:** 1071-83-6

TEST MATERIAL (PURITY): Glyphosate (95.93% glyphosate acid (85.14% calculated glyphosate content in sample)

SYNONYMS: *N*-(phosphonomethyl)glycine

CITATION: Wilga, P.C. (2012). Glyphosate: Human Recombinant Aromatase Assay. CeeTox, Inc., Kalamazoo, MI. Laboratory Study No.: 6500V-100334AROM, March 9, 2012. MRID 48671303. Unpublished.

SPONSOR: Joint Glyphosate Task Force LLC, 8325 Old Deer Trail, Raleigh, NC

TEST ORDER #: CON-417300-23

EXECUTIVE SUMMARY: In an *in vitro* aromatase (CYP 19) assay (MRID 48671303), glyphosate (95.93% glyphosate acid; 85.14% calculated glyphosate content in sample; Lot # GLP-1103-21149-T) was incubated with human recombinant aromatase and tritiated androstenedione ([1β-^{3}H(N)]-androst-4-ene-3,17-dione; [^{3}H]ASDN) at log concentrations of 10^{-10} to 10^{-3} M for 15 minutes to assess the potential of glyphosate to inhibit aromatase activity. The solvent vehicle was 0.1 M phosphate buffer for glyphosate, ethanol for ASDN, and dimethyl sulfoxide (DMSO) for 4-OH ASDN, with a final assay volume of ≤1% DMSO.

Aromatase activity was determined by measuring the amount of tritiated water produced at the end of a 15-minute incubation for each concentration of chemical. Tritiated water was quantified using liquid scintillation counting (LSC). Four independent runs were conducted; however, the first run was not used because of incorrect standard preparation. The remaining three runs were conducted and each run included a full activity control, a background activity control, a positive control series (10^{-10} to 10^{-5} M) with a known inhibitor (4-hydroxyandrostenedione; 4-OH ASDN), and the test chemical series (10^{-10} to 10^{-3} M) with three repetitions per concentration.

Aromatase activity in the full activity controls was 0.676 ± 0.072 nmol·mg-protein^{-1}·min^{-1}. The response of each full activity control within a run was between 90 to 110% of the average full activity. Activity in the background controls ranged 0.23 to 0.38% and averaged 0.30% of the

full activity control. The response of the full activity controls and background controls was acceptable for each run.

For the positive control substance (4-OH ASDN), aromatase activity results were within the recommended ranges for the performance criteria. The estimated log IC_{50} for 4-OH ASDN averaged −7.29 M and the Hill slope was −0.96.

For glyphosate, aromatase activity averaged 0.673 ± 0.066 nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration of 10^{-10} M and 0.741 ± 0.100 nmol·mg-protein^{-1}·min^{-1} at the highest tested concentration of 10^{-3} M. The average aromatase activity was ≥99.67% of the control at all tested glyphosate concentrations for all runs.

Based on the data from the average response curve, glyphosate 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).

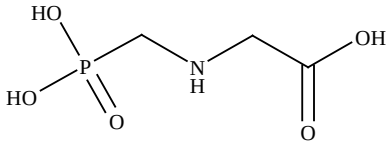
COMPLIANCE: Signed and dated Data Confidentiality, GLP Compliance, and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Substance: Glyphosate

Description:	White crystalline solid
Source:	Monsanto Company, St. Louis, MO
Lot # (expiration date):	GLP-1103-21149-T (March 9, 2012)
Purity:	95.93% glyphosate acid (85.14% calculated glyphosate content in sample)
Volatility:	Not reported
Storage conditions:	Room temperature (e.g. ambient)
Stability:	Not reported
Solvent:	0.1 M sodium phosphate buffer
Solubility (in test solvent):	10^{-3} M
Highest Concentration Tested:	10^{-3} M
Stock Solution Preparation:	Serial dilution
Molecular weight:	169.1 g/mol
CAS #:	1071-83-6
Structure:	



2. Non-Labeled Substrate: Androstenedione (ASDN)

CAS # :	63-05-8
Source:	Steraloids, Inc., Newport, RI (Catalog # A6030-100)
Batch # (expiration date):	L1712 (April 2016)
Purity:	99.8%

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

Source:	Perkin Elmer, Boston, MA (Catalog #NET-926)
Batch # (expiration date):	619344 (January 10, 2012)
Radiochemical Purity (Supplier):	>97%
Specific activity:	26.3 Ci/mmol
Radiochemical Purity (In-lab determination):	Not determined

4. Positive Control: 4-hydroxyandrostenedione (4-OH ASDN)

CAS #	566-48-3
Source:	Sigma-Aldrich, St. Louis, MO (Catalog # F2552)
Batch # (expiration date):	081K2133 (March 2015)
Purity:	99.6%

5. Solvent (Vehicle Control): Dimethyl sulfoxide (DMSO) for 4-OH ASDN; Ethanol for ASDN and [^{3}H]ASDN; 0.1 M Sodium phosphate buffer for glyphosate

Sources:	Not reported
Batch #s (expiration date):	Not reported
Justification for choice of solvents	DMSO and ethanol are listed as vehicles acceptable for use in OCSPP 890.1200. Justification was provided for the use of DMSO as a solvent for 4-OH ASDN and for the use of 0.1 M sodium phosphate buffer as a solvent for glyphosate.
Concentration (% of total volume in assays)	≤1% DMSO; concentration of ethanol was not reported

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

Source:	BD Gentest™, Woburn, MA (Catalog # 456260)
Lot # (expiration date):	19701 (July 2014)
Protein concentration:	3.7 mg/mL
Cytochrome C reductase activity:	540 nmol /mg protein/min
Aromatase activity:	5.7 pmol/pmol P450/min

B. METHODS

1. Assay Components and Preparations: A mixture of non-labeled and radiolabeled [^{3}H]ASDN was prepared such that the final concentration of ASDN in the assay was approximately 0.1 µM, and the amount of tritium added to each incubation tube was 0.1 µCi.

Glyphosate was formulated in the assay buffer (0.1 M sodium phosphate buffer, pH 7.4) based on its high water solubility, and relatively low organic solubility. The positive control, 4-OH ASDN, was formulated in DMSO such that the volume of DMSO used per assay was no more than 1% v/v of the total assay volume to minimize the potential for the solvent to inhibit the enzyme. DMSO was selected because it is listed as one of the solvents of choice detailed in the EPA guideline; it not as volatile as ethanol and so evaporation was less of a concern in the assay, and is more accurate to pipette because of its density and viscosity. ASDN and [^{3}H]ASDN were formulated in ethanol and the assay buffer; no maximum assay concentration for ethanol was reported.

A stock solution of the positive control substance, 4-OH ASDN, was formulated in DMSO. Fresh dilutions of the stock solution were prepared in the same solvent as the stock solution on the day of use. Dilutions were prepared such that the target concentrations of the positive control substance (0.1-10,000 nM; 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 Gentest™, and stored at −80 ± 10ºC (storage interval not reported). Microsomes were thawed and portioned into individual vials based on the protein concentration of the batch (0.008 mg/mL microsomal protein per tube) and returned to the freezer for storage (storage interval not reported) to minimize freeze-thaw cycles to no more than one. The final concentration was approximately 0.004 mg/mL of microsomal protein/assay tube.

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

TABLE 1. Assay Components and Conditions[a]	
Assay Factor	**Values**
0.1M sodium phosphate buffer (pH 7.4)	
Microsomal Protein	0.004 mg/mL[b]
NADPH	0.3 mM
[^{3}H]ASDN	100 nM
Propylene Glycol	5%
Temperature	37 ± 2°C
Incubation Time	15 min

a Data were obtained from p. 18 of the study report.
b The concentration of microsomal protein was optimized for microsomes that produce approximately 540 pmol product/(min x mg protein) and 5.7 pmol product/pmol P450/min.

2. **Suitability Assessments:** The protein concentration was determined from the package information provided by the vendor; protein concentration was not verified on each day the aromatase assay was run.

Aromatase activity in each lot of human recombinant microsomes was determined to demonstrate the presence of sufficient activity for analysis of glyphosate. The aromatase activity was determined to be 0.584-0.771 nmol·mg-protein^{-1}·min^{-1}, which was greater than the minimum recommended aromatase activity of 0.1 nmol·mg-protein^{-1}·min^{-1}.

3. **Aromatase Assay:** Each assay run contained 4 tubes for the full enzyme activity and background activity controls, respectively, and a full concentration curve in duplicate for the positive control, and in triplicate for the test substance were established.

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:** Proficiency testing of the CYP19 aromatase assay was conducted in three independent runs on April 8, 16, and 20, 2010.by the test facility. The raw data from these three runs included evaluation of the positive control, 4-OH ASDN and the four recommended proficiency chemicals (econazole, fenarimol, nitrofen, and atrazine).

Positive Control

(1) **Initial Demonstration of Laboratory Proficiency:** Data from an initial demonstration of laboratory proficiency were not reported. The positive control data from the three acceptable assay runs generally met 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 generally <15%.
- Performance criteria (Table 2) were met, 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): The demonstration of proficiency of a new technician was not indicated. The positive control data for slope, top and bottom percent from the three acceptable assay runs met the criteria as listed in section (i) of OCSPP 890.1200.

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	−1.00	−0.92
Top (%)	90	110	98.36	100.62
Bottom (%)	−5	+6	−0.06	0.76
Log IC_{50} (M)	−7.3	−7.0	−7.30	−7.28

a Data were obtained from pages 19 and 30 of the study report.

b. Proficiency Chemicals: Although the finalized data were not presented (including top and bottom of the curve, Hill slope, and log IC_{50}), the raw proficiency data that were provided (DEST.48671304) appear to support the expected designations of inhibitor or non-inhibitor for each of the proficiency chemicals, as well as the positive control.

TABLE 3. Proficiency Chemicals[a]

Compound	CAS#	Class	Concentrations
Econazole	24169-02-6	Inhibitor	10^{-3} to 10^{-10}
Fenarimol	60168-88-9	Inhibitor	10^{-3} to 10^{-10}
Nitrofen	1836-75-5	Inhibitor	10^{-3} to 10^{-10}
Atrazine	1912-24-9	Non-inhibitor	10^{-3} to 10^{-10}

a Raw data were included in Excel file 890.1200 Aromatase DEST.48671304

5. Determination of Aromatase Activity with Test Chemical(s): The response of aromatase activity to the presence of eight concentrations of glyphosate per run, in triplicate, was tested during three independent runs (Table 4). Solubility was assessed (presence of cloudiness or a precipitate). If insolubility was observed at the highest test concentration for the first run, then the highest test concentration would be adjusted for the second and third runs at the highest test concentration that appeared soluble using log or half-log concentrations. The lowest concentration tested was 10^{-10} M. The full enzymatic activity was obtained at the two lowest concentrations of the test chemical to define the top of the concentration-response curve.

TABLE 4. Test Chemical Study Design for each Test Run[a]			
Sample Type	**Repetitions (Tubes)**	**Description**	**Reference or Chemical (M)**
Full Activity Control	4	All test components[b] 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}
Glyphosate Conc 1[c]	3	All test components plus Glyphosate	1×10^{-3}
Glyphosate Conc 2[c]	3	All test components plus Glyphosate	1×10^{-4}
Glyphosate Conc 3[c]	3	All test components plus Glyphosate	1×10^{-5}
Glyphosate Conc 4[c]	3	All test components plus Glyphosate	1×10^{-6}
Glyphosate Conc 5[c]	3	All test components plus Glyphosate	1×10^{-7}
Glyphosate Conc 6[c]	3	All test components plus Glyphosate	1×10^{-8}
Glyphosate Conc 7[c]	3	All test components plus Glyphosate	1×10^{-9}
Glyphosate Conc 8[c]	3	All test components plus Glyphosate	1×10^{-10}

a Data were obtained from page 20 of the study report.
b The complete assay contained buffer, propylene glycol, microsomal protein, [^{3}H]ASDN, and NADPH.
c Test chemical.

C. DATA ANALYSIS

1. Raw Data: Raw data were converted to aromatase activity (nmol·mg−protein^{-1}·min^{-1}) and percent control 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: For data generated at CeeTox, basic statistical analysis was performed on the data, which included means of replicates, standard deviation of the mean, standard error of the mean, and coefficient of variation.

The response curve was fitted by weighted nonlinear regression analysis using a 4-parameter regression model (XLfit; IDBS; Version 5.2.0.0, Fit Model 208). For each run,

the individual percent of control values were plotted versus logarithm of the test chemical concentration. The fitted concentration response curve was superimposed on the plot, with individual plots prepared for each run. The average percent of control values versus logarithm of test chemical concentration for the individual runs for each test chemical (with different symbols for each run) were included on the same graph with their respective fitted response curves. In addition, the average percent of control values for each run versus the logarithm of test chemical concentration were plotted on a separate graph along with the average concentration response curve across runs were superimposed on the same plot.

3. **Interpretation of Results:** Interpretation of the assay results was based on the average of three runs, using the categories presented in Table 6.

TABLE 6. Interpretation of Results[a]

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 Data obtained from Table 9, p. 23 of the study report.

b 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.

c 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

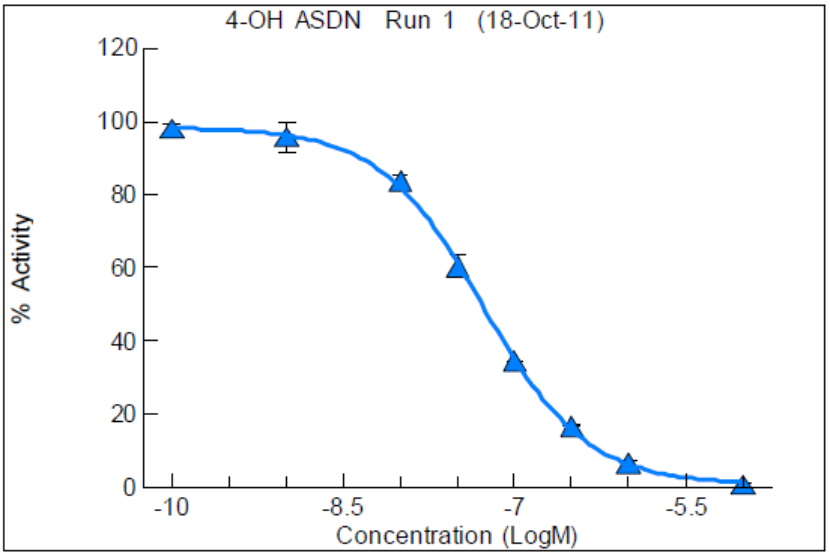
A. CONTROL ACTIVITY: Aromatase activity in the full activity controls ranged from 0.584-0.771 nmol·mg-protein^{-1}·min^{-1} for the 3 test runs, with a mean and standard deviation of 0.676 ± 0.072 nmol·mg-protein^{-1}·min^{-1}. Activity in the background controls ranged 0.23 to 0.38% and averaged 0.30% of the full control activity. The response of the full activity controls and background controls were acceptable for each run.

B. POSITIVE CONTROL: For the positive control substance (4-OH ASDN), aromatase activity averaged 0.668 ± 0.069 nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration 10^{-10} M and 0.005 ± 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 runs is presented in Table 7, along with the overall standard deviation, SEM, and %CV. An example of the inhibition response curve for the positive control from one run is shown in Figure 1.

TABLE 7. Effect of Glyphosate on Aromatase Activity (as percent of control) from Independent Runs[a]						
Chemical	**Concen. (Log M)**	**# Runs**	**Overall Mean[b]**	**Overall SD[b]**	**Overall SEM[b]**	**Overall % CV[b]**
4-OH ASDN (positive control)	−5	3	0.67	0.04	0.02	5.4
	−6	3	5.95	0.45	0.26	7.5
	−6.5	3	15.73	1.07	0.62	6.8
	−7	3	34.02	0.48	0.27	1.4
	−7.5	3	61.47	1.17	0.68	1.9
	−8	3	82.56	1.01	0.58	1.2
	−9	3	98.14	2.63	1.52	2.7
	−10	3	98.77	1.54	0.89	1.6
Glyphosate	−3	3	109.30	5.53	3.19	5.1
	−4	3	109.73	7.16	4.14	6.5
	−5	3	106.72	1.67	0.96	1.6
	−6	3	102.75	2.45	1.41	2.4
	−7	3	104.48	1.05	0.61	1.0
	−8	3	100.67	1.50	0.87	1.5
	−9	3	102.22	0.59	0.34	0.6
	−10	3	99.67	1.86	1.07	1.9

a Data were obtained from Appendix 1, pp. 42-55 of the study report
b Calculated by the reviewers from data presented in this table.
SD Standard Deviation
SEM Standard error of the mean
CV Coefficient of Variance

FIGURE 1. Inhibition Response Curve for 4-OH ASDN From Run 1.



C. <u>TEST SUBSTANCE</u>: For glyphosate, aromatase activity averaged 0.673 ± 0.066 nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration, 10^{-10} M and 0.741 ± 0.100 nmol·mg-protein^{-1}·min^{-1} at the highest tested concentration, 10^{-3} M. The mean aromatase activity of glyphosate (expressed as % full control activity) for each concentration tested across all 3 runs is presented in Table 7 (presented above), along with the overall standard deviation, SEM, and % CV. Inhibition response curves for glyphosate 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 Glyphosate From Each Test Run.

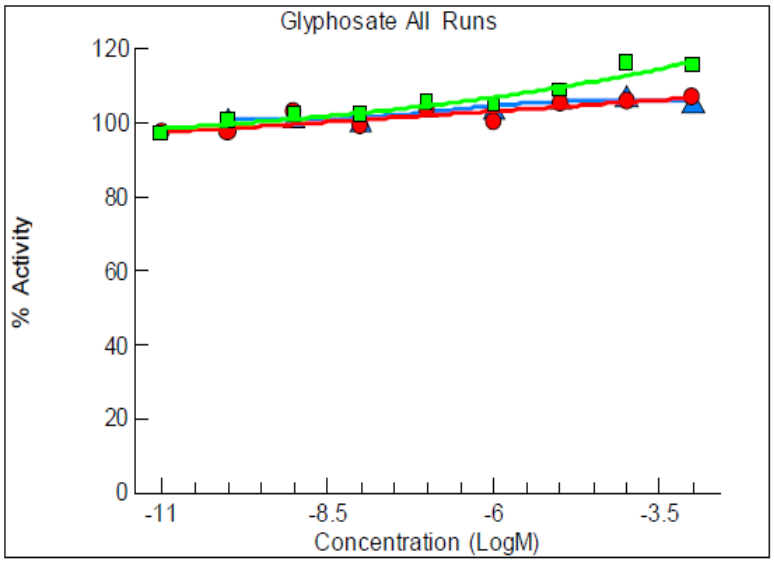
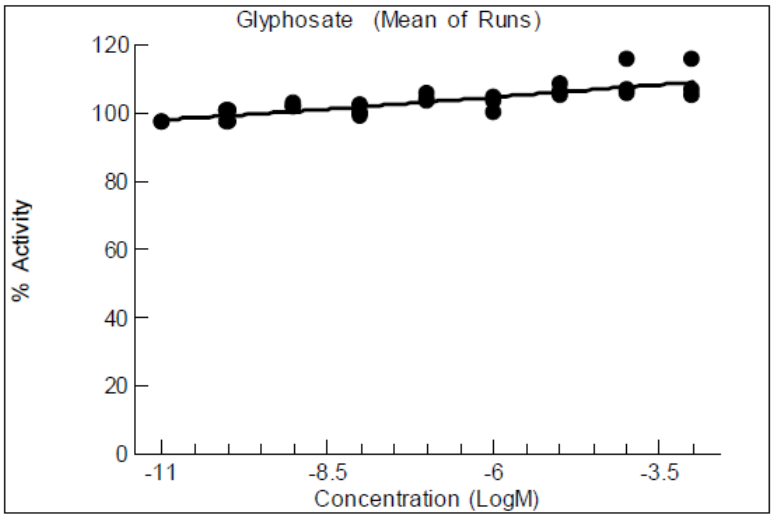


FIGURE 3. Mean Inhibition Response Curves for Glyphosate.



The effect of glyphosate on inhibition of aromatase activity is presented in Table 8. Log IC_{50} and Hill slope estimates were not determined for glyphosate because it never achieved >25% inhibition and could not be fitted by the nonlinear regression model. For 4-OH ASDN, the estimated log IC_{50} averaged −7.29 M and the Hill slope was −0.96 (Table 8). Confidence in these numbers is high due to the relatively small variation.

TABLE 8. Effect of Glyphosate on Aromatase Activity (as Percent of Control) From Independent Runs[a]						
Chemical	**Run 1**	**Run 2**	**Run 3**	**Mean[b]**	**SEM[b]**	**%CV[b]**
Log IC_{50} (M)						
Glyphosate	NA	NA	NA	NA	NA	NA
4-OH ASDN	−7.28	−7.30	−7.29	−7.29	0.01	0.14
Hill slope						
Glyphosate	NA	NA	NA	NA	NA	NA
4-OH ASDN	−0.96	−0.92	−1.00	−0.96	0.04	4.17

a Data were obtained from Table 13, page 30 of the study report
b Calculated by the reviewers from data presented in this table.
SD Standard Deviation
CV Coefficient of Variance
NA Not applicable

Based on the data from the average response curve and the criteria listed above in Table 8, the results support the conclusion that glyphosate is a non-inhibitor in the aromatase assay.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATORS CONCLUSIONS: Glyphosate at the highest soluble concentration of 10−3 M did not inhibit aromatase activity, and had a mean relative activity of 109% (n=3 runs) of vehicle control activity. Therefore, glyphosate was classified as a non-inhibitor of aromatase, as defined by EDSP guideline OCSPP 890.1200.

B. AGENCY COMMENTS: Results of proficiency testing for the aromatase assay were provided as raw data. Although the final calculation of parameters (including top and bottom of the curve, Hill slope, and log IC_{50}) were not provided, the raw proficiency data that were provided appear to support the expected designations of inhibitor or non-inhibitor for each of the proficiency chemicals, as well as the positive control.

Aromatase activity in the full activity controls was 0.676 ± 0.072 nmol·mg-protein^{-1}·min^{-1},and activity in the background controls ranged 0.23 to 0.38% and averaged 0.30% of the full control activity. The response of the full activity controls and background controls were acceptable for each run.

For the positive control substance (4-OH ASDN), aromatase 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. The estimated log IC_{50} for 4-OH ASDN averaged −7.29 M and the Hill slope was −0.96.

For glyphosate, average aromatase activity was ≥99.67% at the lowest and highest tested concentrations tested, 10^{-10} and 10^{-3} M, in each run. Since the lowest portion of the response curve across runs was greater than 75% activity at all concentrations, glyphosate 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:

- The stability of glyphosate was not reported.

- For 4-OH ASDN, the CVs were >15% in separate instances for Runs 1 and 2 (15.9% for 10^{-6} M and 25.5% for 10^{-5} M).

DATA EVALUATION RECORD

GLYPHOSATE

Study Type: OCSPP 890.1250, Estrogen Receptor Binding Assay

EPA Contract No. EP10H001452
Task Assignment No. 3-06-2012

(Revisions to MRID 48671305 to include Saturation binding data;
Main study was originally reviewed under TA 2-74-2012)

Prepared for
Health Effects Division
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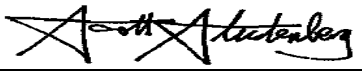
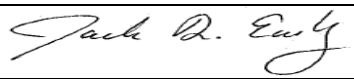
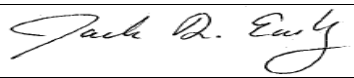
Durham, NC 27713

Primary Reviewer:	Signature:	
Michelle Sharpe-Kass, M.S.	Date:	6/15/2012
Secondary Reviewer:	Signature:	
Scott D. Studenberg, Ph.D., D.A.B.T.	Date:	7/20/2012
Program Manager:	Signature:	
Jack D. Early, M.S.	Date:	7/30/2012
Quality Assurance:	Signature:	
Jack D. Early, M.S.	Date:	7/30/2012

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: Anwar Y. Dunbar, Ph.D. **Signature:** Anw Y. Dunbar
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 05-27-15
Secondary Reviewer: Gregory Akerman, Ph.D. **Signature:**
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 6/15/15

DATA EVALUATION RECORD

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

PC CODE: 417300 **DP BARCODE:** D401747

TXR#: 0053233 **CAS No.:** 1071-83-6

TEST MATERIAL (PURITY): Glyphosate (95.93% glyphosate acid, 85.14% calculated glyphosate content)

SYNONYMS: Roundup, N-(phosphonomethyl)glycine

CITATION: Willoughby, J.A. (2012). Glyphosate: Estrogen Receptor Binding (Rat Uterine Cytosol). CeeTox, Inc., Kalamazoo, MI. Laboratory Study No.: 6500V-100364ERB, March 8, 2012. MRID 48671305. Unpublished.

Willoughby, J.A. (2012). Supplemental Information - Laboratory Proficiency Data for ERTA assays and Saturation Binding Data for AR and ER Binding Assays for Assorted Chemicals. CeeTox, Inc., Kalamazoo, MI. July, 2011. MRID 48843501. Unpublished.

SPONSOR: Joint Glyphosate Task Force, LLC, 8325 Old Deer Trail, Raleigh, NC

TEST ORDER #: CON-417300-23

EXECUTIVE SUMMARY: In an estrogen receptor (ER) binding assay (MRID 48671305) for glyphosate (95.93% glyphosate acid, 85.14% calculated glyphosate content, Batch # GLP-1103-21149-T), uterine cytosol from Sprague Dawley rats was used as the source of ER to conduct saturation and competitive binding experiments. A saturation binding experiment was conducted to demonstrate that the ER in the rat uterine cytosol was present in reasonable numbers and was functioning with appropriate affinity for the radio-labeled reference estrogen prior to conducting ER competitive binding experiments. The competitive binding experiment measured the binding of a single concentration of [^{3}H]-17β-estradiol (1 nM) in the presence of increasing concentrations (10^{-10} to 10^{-3} M) of glyphosate. TEGD buffer was used as the solvent vehicle for glyphosate. A total of 3 runs were performed, and each run included 19-norethindrone as a weak positive control, octyltriethoxysilane as a negative control, and 17-β-estradiol as the natural ligand reference material.

Saturation binding data were not originally provided in the study report; however, summarized saturation binding data (MRID 48843501) from the performing laboratory were submitted

following a request by the Agency. The protein concentrations used in the saturation binding runs varied between each run, and were approximately 3- to 6-fold greater than recommended (160 to 320 µg versus 50±10 µg). The K_d for [^{3}H]-17β-estradiol was 0.331 ± 0.061 nM and the estimated Bmax was 74.55 ± 3.03 fmol/100 µg protein for the prepared rat uterine cytosol. The K_d for each run was within the expected Guideline range of 0.03 to 1.5 nM.

In the competitive binding experiment, the estimated mean log IC_{50}s for 17β-estradiol and 19-norethindrone were −9.0 and −5.5 M, respectively. The mean relative binding affinity (RBA) was 0.032% for 19-norethindrone, compared to the natural ligand.

Glyphosate was tested over a concentration range (10^{-10} to 10^{-3} M) that fully defined the top of the curve. The percent binding at the top plateau (101.2-116.9%) was within 25 percentage points of the lowest concentration of the estradiol standard (98.6-101.8%). Across all runs, the lowest average percent radiolabeled estradiol binding in the presence of glyphosate was >81% (*i.e.* showed less than 25% displacement) at concentrations up to 10^{-3} M indicating that gylphosate was not competing with the natural ligand for binding to the ER.

Based on the results from the three runs, glyphosate 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 Data Confidentiality, GLP Compliance, and Quality Assurance statements were provided.

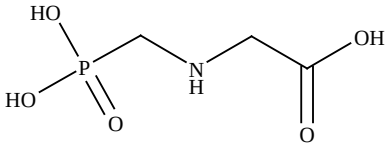
I. MATERIALS AND METHODS

A. MATERIALS

1. Test Facility: CeeTox, Inc.

Location:	4717 Campus Drive, Kalamazoo, MI 49008
Study Director:	Willoughby, J.A.
Other Personnel:	Rutherford, K. (Director of Laboratory Operations); Blakeman, D. (Senior Scientist); Haines, C. (Scientist); McColley, S. (Scientist); Toole, B. Meyer, Scientist; C. (Director of Project Management)
Study Period:	September 28, 2001 to March 8, 2012

2. Test substance: Glyphosate

Description:	White wetcake (white crystalline solid)
Source:	Monsanto, Co, St. Louis, MO
Batch #:	GLP-1103-21149-T (expiry: March 9, 2012)
Purity:	95.93% glyphosate acid, 85.14% calculated glyphosate acid
Solubility:	Not reported
Volatility:	Not reported
Stability:	One year at room temperature
Storage conditions:	35 to 100°F (Room temperature)
CAS #:	1071-83-6
Molecular weight:	169.01 g/mol
Structure:	

3. Non-labeled ligand (strong positive control): 17β-estradiol

Supplier:	Sigma-Aldrich, St Louis, MO
Catalog #:	E8875
Batch #:	110M0138V
Purity:	100 %
CAS #:	50-28-2

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

Supplier:	Perkin-Elmer, Boston, MA
Catalog #:	NET517
Batch#:	650702
Radiochemical purity:	97%
Specific activity:	130.2 Ci/mmol
Concentration of stock:	50 nM

5. Weak positive control: 19-norethindrone

Supplier:	Sigma-Aldrich, St Louis, MO
Catalog #:	N4128
Batch #:	030M1359V
Purity:	99%
CAS #:	68-22-4

6. Negative control: Octyltriethoxysilane

Supplier:	Sigma-Aldrich, St Louis, MO
Catalog #:	440213
Batch #:	24996KK
Purity:	99.34
CAS #:	2943-75-1

7. Solvent/vehicle control: TEGD+PMSF

Justification for choice of solvent: Glyphosate is soluble (12 g/L) in aqueous solutions, but is not soluble in DMSO

B. METHODS

1. Preparation of Rat Uterine Cytosol (RUC): Frozen Sprague Dawley rat uteri were purchased from Harlan Laboratories (Batch #: 210007463). Female Sprague Dawley rats were ovariectomized 7 days prior to being euthanized. Animals were 12-13 weeks old at the time of euthanasia. The uteri were weighed, placed in ice-cold TEDG buffer (Tris, EDTA, DTT, glycerol) + PMSF and used immediately. Uteri were homogenized in buffer and then centrifuged for 10 min at 2500 × *g* at 4º C. The resulting 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 1.10 mg/mL using a protein kit compatible with DTT in the TEDG buffer (BioRad Protein Assay Kit). Cytosol was divided into portions for immediate use or storage at −80º C.

2. Saturation (Radioligand) Binding Experiment: A saturation binding experiment measuring total and non-specific binding of [^{3}H]-17β-estradiol was performed to demonstrate that the ER was present in reasonable concentrations and had the appropriate affinity for the native ligand (MRID 48843501). The conditions for the saturation binding experiments are summarized 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 to 3 nM
Concentration of non-labeled ligand (100X [radioligand])		3 to 300 nM
Concentration of receptor		Sufficient to bind approximately 25 to 35% of radioligand at 0.03 nM
Temperature		4ºC
Incubation time		16 to 20 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 obtained from page 1 of the study report (MRID 48843501).

On the day of the assay, the specific activity of the stock solution [^{3}H]-17β-estradiol (originally 130.2 Ci/mmol as manufactured on May 6, 2011) was adjusted for decay over time (adjusted specific activities were not reported), and 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.

For each batch of cytosol, the optimal protein concentration was determined by testing serial amounts of protein per tube, using 0.03 nM radiolabeled estradiol, until a concentration was reached that bound approximately 25 to 35% of the total radioactivity added. The final protein concentrations were 320 µg, 192 µg and 160 µg per assay tube for the first, second and third saturation binding experiments, respectively (*Note: typically 50 ± 10 µg protein per tube)*. Each assay consisted of three non-concurrent runs (conducted on August 5, 6, and 7, 2011, respectively). Each run included three replicates of each test substance 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	[^{3}H]-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 concentration)
500 µL	500 µL	50 µL	Total volume in each assay tube

a Data were obtained from page 2 of the study report (MRID 48843501).
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.

Tubes were incubated with gentle vortexing for 17.5-19 hours at approximately 4°C. To separate bound from free estradiol, hydroxyapatite (HAP) slurry was added to each tube and vortexed (3 times with 5-min intervals). Subsequently, the contents of each tube were washed three times as follows: 2-mL portions of TEDG + PMSF buffer were added, vortexed, centrifuged for 10 min at 1000 x *g*, and the supernatant decanted and discarded. After the final centrifugation, ethanol (1.5 mL) was added to the HAP pellet remaining in each tube to extract the [^{3}H]-17β-estradiol, followed by vortexing, and centrifugation for 10 min at 1000 x *g*. Aliquots (1 mL) of supernatant were radioassayed by scintillation counting. The temperature was maintained at approximately 4ºC throughout the assay prior to extraction with ethanol.

3. <u>Competitive Binding Experiment</u>: A summary of the experimental 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 Uterine Cytosol
Concentration of radioligand		1 nM
Concentration of receptor		~0.35 mg/mL
Concentration of test substance (as serial dilutions)		10^{-10} to 10^{-3} mM
Temperature		4±2 °C
Incubation time		16-20 hours
Composition of assay buffer	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10% (v/v)
	Phenylmethylsulfonyl fluoride	0.5% (v/v)
	DTT	1 mM

a Data were obtained from page 17 of the study report.

The limit of glyphosate solubility in TEGD buffer was determined by visual observation. On the day of the assay, the specific activity of the stock solution [^{3}H]-17β-estradiol was adjusted for decay over time (126.6 Ci/mmol for the first run; 126.5 Ci/mmol for the second run; and 126.1 Ci/mmol for the third run), and diluted in TEDG buffer + PMSF to the final appropriate concentrations. Serial dilutions of glyphosate, the weak positive control (19-norethindrone), the negative control (octyltriethoxysilane), and the 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 at each concentration, six replicates measuring total activity (50 µL master mix) plus three measuring total binding (solvent control) resulting in a total of 306 samples (102 samples/assay run).

TABLE 4. Molar (M) concentrations in Competitive Binding Assay Run [a b]			
Glyphosate	Positive control	Negative control	Reference Chemical
	19-Norethindrone	Octyltriethoxysilane	Non-labeled 17β-estradiol
Tubes 79-102 [c]	Tubes 31-54 [c]	Tubes 55-78 [c]	Tubes 1-9 and 10-30 [c]
10^{-10}	$10^{-8.5}$	10^{-10}	Solvent control/master mix [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, 37-45 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 Tubes 1-6 contained the master mix ([^{3}H]-17β-estradiol); Tubes 7-9 contained the solvent (TEGD).
d Solvent is TEGD+PMSF buffer for glyphosate, DMSO for the reference chemicals

Tubes were incubated with gentle vortexing for 16-20 hours at 4±2 °C. To separate bound from free estradiol, HAP slurry was added to each tube and the tubes were vortexed (3 times with 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×*g*, and the supernatant decanted and discarded. Ethanol was added to the HAP pellet remaining in each tube to extract the [^{3}H]-17β-estradiol, allowed to sit at room temperature for 15-20 min with vortexing, and centrifugation for 10 min at 1000×*g*. Aliquots of the supernatants were radioassayed by scintillation counting. The temperature was maintained at 4±2°C throughout the assay prior to extraction with ethanol.

C. DATA ANALYSIS: For the saturation binding experiment, total binding and non-specific binding data were modeled via non-linear regression by using Graph Pad Prism v. 5 (GraphPad Software, Inc., La Jolla, CA)], incorporating automatic outlier elimination according to the method of Motulsky and Brown (2006)[1] implemented by using the ROUT procedure in Prism v. 5 with a Q value of 1.0. Receptor binding data plots were corrected for ligand depletion with the method of Swillens (1995)[2]. For the competitive binding assay, similar methods of nonlinear regression were used to fit a curve (for 17β-estradiol, the positive control, and the test substance) to the Hill equation formula which incorporated log IC_{50} as a parameter to be estimated. For parameters reported from the saturation binding experiment (K_d and B_{max}) and 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 using Microsoft Excel 2007 (v. 12.0.6557.5000; Microsoft Corporation, Redmond, WA), and mean and standard error were calculated for the composite three runs with Microsoft Excel 2010.

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

1 Motulsky, H.J. and Brown, R.E. (2006) Detecting outliers when fitting data with nonlinear regression: a new method based on robust nonlinear regression and the false discovery rate. BMC Bioinformatics, Vol 7, pp 123-142.
2 Swillens, S. (1995) Interpretation of binding curves obtained with high receptor concentrations: practical aid for computer analysis. *Molec. Pharmacol.* 47(6):1197-1203.

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. <u>Descriptors for receptor binding</u>:

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 (M) 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
Log RBA: Log_{10} **(**IC_{50} of 17β-estradiol ÷ IC_{50} of test substance)

II. RESULTS

A. <u>SATURATION BINDING EXPERIMENT</u>: Figure 1 illustrates the non-specific, specific, and total binding curves for [^{3}H]-17β-estradiol to the estrogen receptor for the three independent runs. The specific binding reached a plateau in each run, and non-specific binding was less than 20% of total binding. Figure 2 contains the Scatchard plots that illustrate the binding of [^{3}H]-17β-estradiol to the estrogen receptor.

Saturation binding experiment parameters are presented in Table 5. The K_d for [^{3}H]-17β-estradiol was 0.331 nM (± 0.061), and the estimated B_{max} was 74.55 fmol/100 µg protein (± 3.03) for the prepared rat uterine cytosol. The K_d for each run was within the expected range of 0.03 to 1.5 nM. Although the Scatchard plots fit straight lines to the data, the concavity observed in the data sets may indicate issues with ligand depletion. Confidence in these numbers is high due to the goodness of fit and the small variation among runs.

TABLE 5. Saturation Binding Experiment of [^{3}H]-17β-estradiol with Estrogen Receptor from Rat Uterine Cytosol[a]				
Parameter	Run 1	Run 2	Run 3	Mean ± SE[b]
R^2 (unweighted)	0.976	0.982	0.980	0.976-0.982
B_{max} (nM)	0.148	0.102	0.080	0.110±0.035
B_{max} (fmol/100 µg protein)	69.25	79.76	74.65	74.55±3.03
K_d (nM)	0.453	0.286	0.255	0.331±0.061

a Data were obtained from page 3 of the study report (MRID 48843501).

b The range of R^2 is reported and the mean ± SEM is reported for the other parameters.

R^2 Goodness of fit for curve calculated for specific binding

FIGURE 1. Binding of [^{3}H]-17β-estradiol to the Estrogen Receptor during the Saturation Binding Experiment.

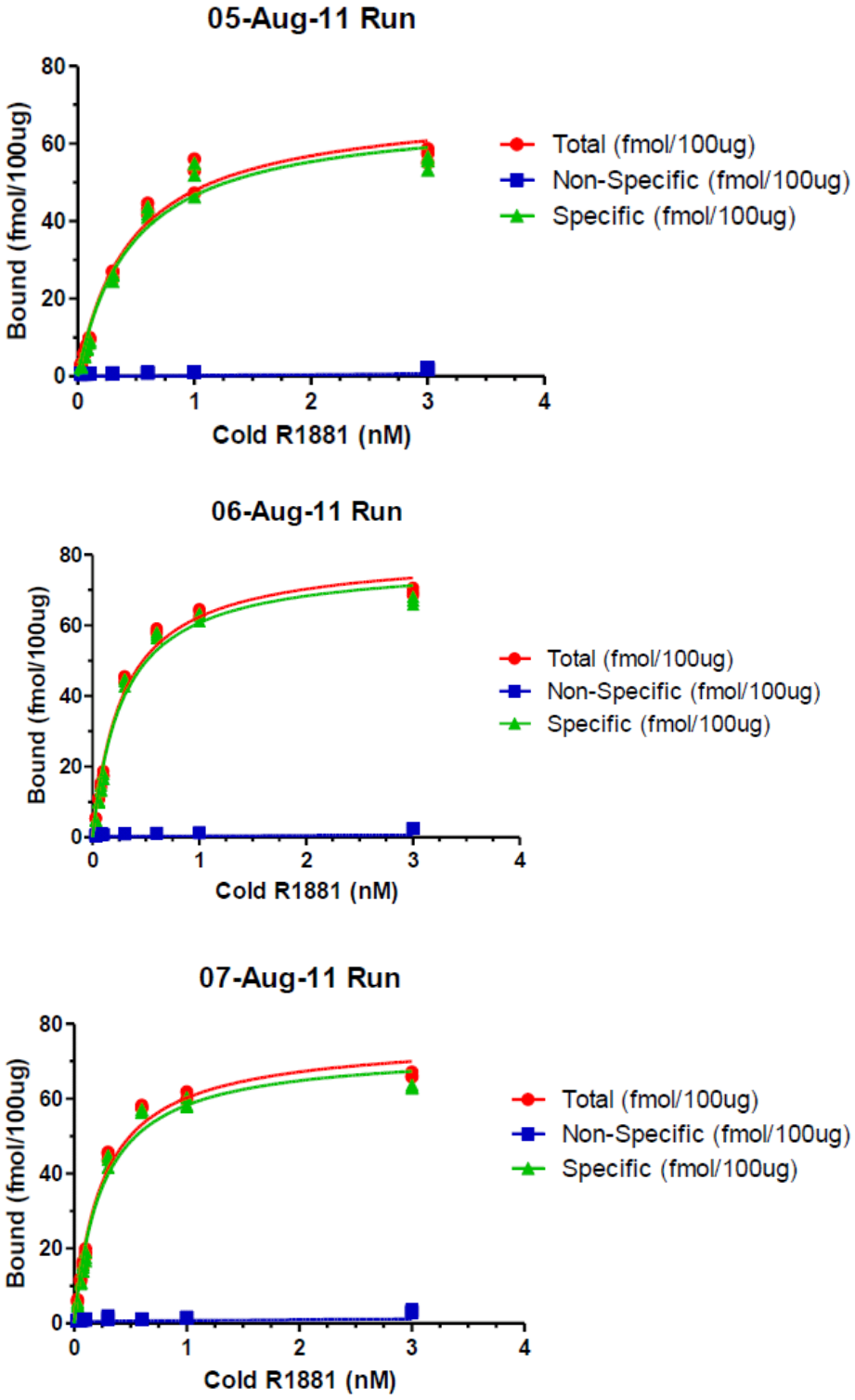
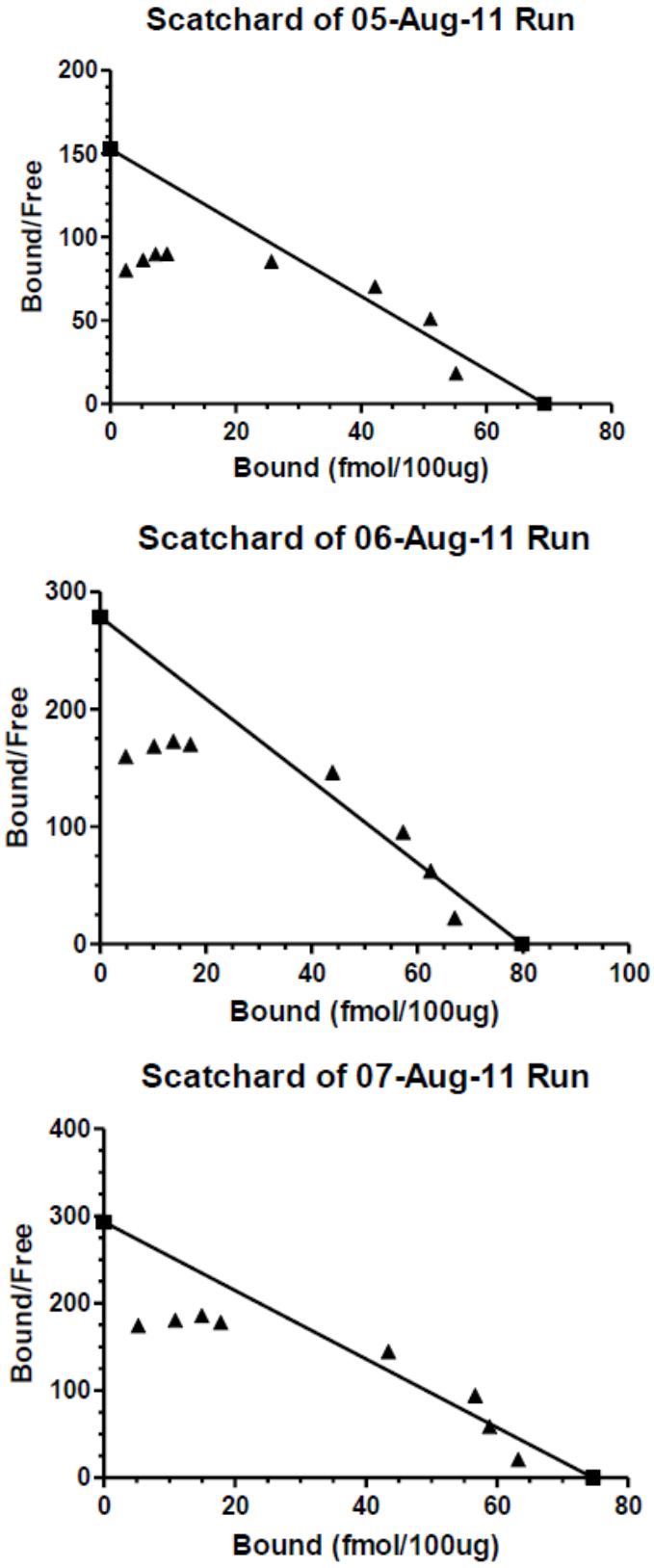


FIGURE 2. Scatchard Plots of the Binding of [^{3}H]-17β-estradiol to the Estrogen Receptor.



B. <u>COMPETITIVE BINDING EXPERIMENT</u>: The results from the three competitive binding experiments are summarized in Table 6 and presented graphically in Figures 3-5. The highest suitable concentration for analysis was 10^{-3} M in all runs. The mean specific binding in the presence of glyphosate was ≥82% at concentrations ≤10^{-3} M in all three runs. The estimated mean log IC_{50} and RBA were not calculated for glyphosate as the percent binding displacement did not reach 50% for any run.

The estimated mean log IC_{50} for 17β-estradiol and 19-norethindrone was −9.0 and −5.5 M, respectively. The mean RBA was 0.032% for 19-norethindrone, compared to the natural ligand. Confidence in these numbers is high due to the small variation. As the lowest average percent binding in the presence of glyphosate was >75% at concentrations up to 10^{-3} M in all three runs, glyphosate is classified as not interactive (0) in this assay (Table 7).

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

Parameter		Run 1 [b]	Run 2 [b]	Run 3 [b]	Mean ± SE [c]
R^2 (unweighted)	17β-estradiol	NR	NR	NR	NA
	Positive control	NR	NR	NR	NA
	Test substance	NR	NR	NR	NA
Log IC_{50} (M)	17β-estradiol	−9.1	−9.0	−8.9	−9.0 ± 0.1
	Positive control	−5.5	−5.5	−5.5	−5.5 ± 0.0
	Test substance	NA	NA	NA	NA
IC_{50} (M)	17β-estradiol	7.94×10^{-10}	1.00×10^{-9}	1.26×10^{-9}	1.02×10^{-9} (±0.23)
	Positive control	3.16×10^{-6}	3.16×10^{-6}	3.16×10^{-6}	3.16×10^{-6} (±0.0)
	Test substance	NA	NA	NA	NA
Log RBA	Positive control	−3.6	−3.5	−3.4	−3.5 ± 0.1
	Test substance	NA	NA	NA	NA
RBA (%)	Positive control	0.025	0.032	0.040	0.032 ± 0.007
	Test substance	NA	NA	NA	NA

a Data were obtained from text on page 23 of the study report.
b The mean and standard deviation are reported for the concurrent replicates within each run.
c The range is reported for r^2, and the mean ± SEM is reported for the remaining parameters.
R^2 Goodness of fit
RBA (%) Relative binding affinity
NA Not applicable.

TABLE 7 Binding Classification of Glyphosate 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 23 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 [^{3}H]-E2 Bound to the Estrogen Receptor in the Presence of Glyphosate, Unlabeled E2, 19-Norethindrone or Octyltriethoxysilane in Assay Run 1.

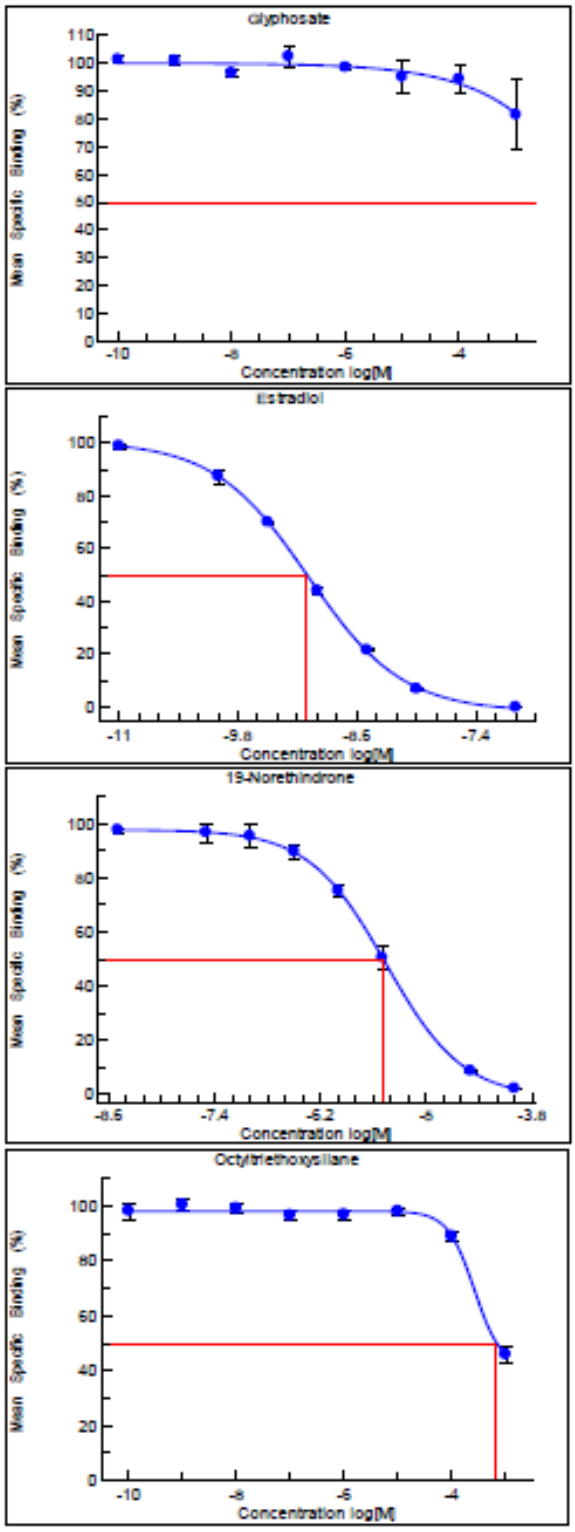


FIGURE 4. Percentage [^{3}H]-E2 Bound to the Estrogen Receptor in the Presence of Glyphosate, Unlabeled E2, 19-Norethindrone or Octyltriethoxysilane in Assay Run 2.

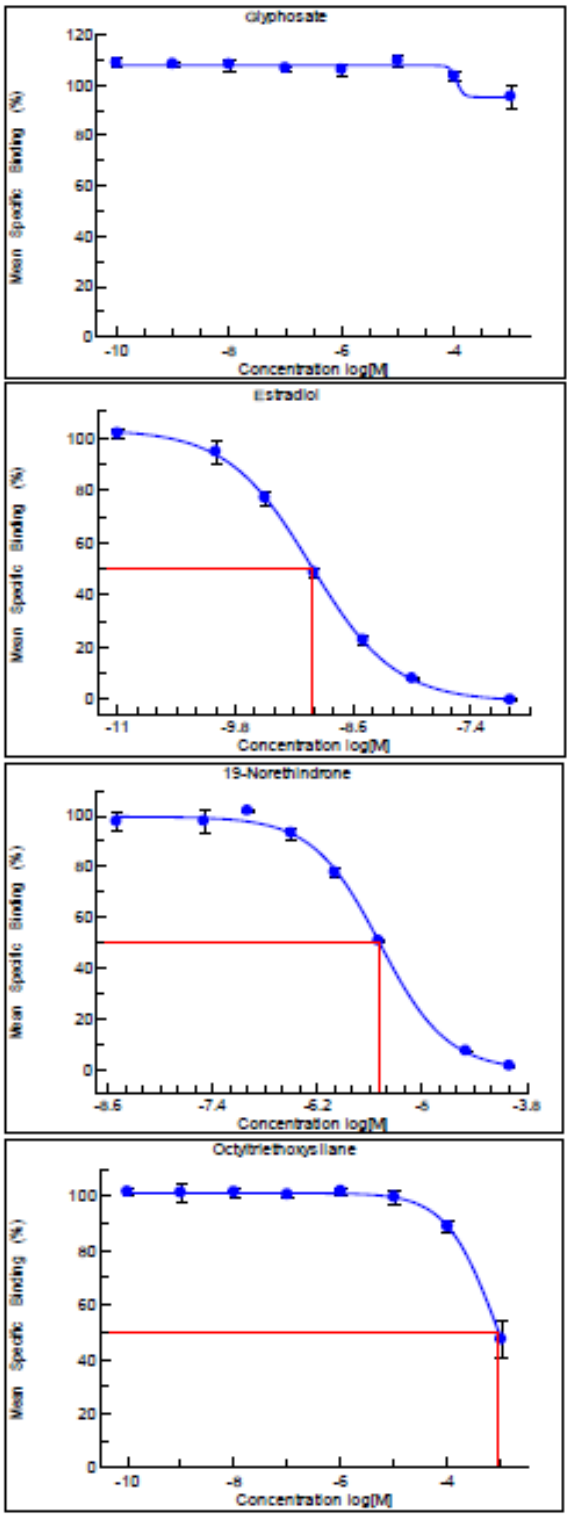
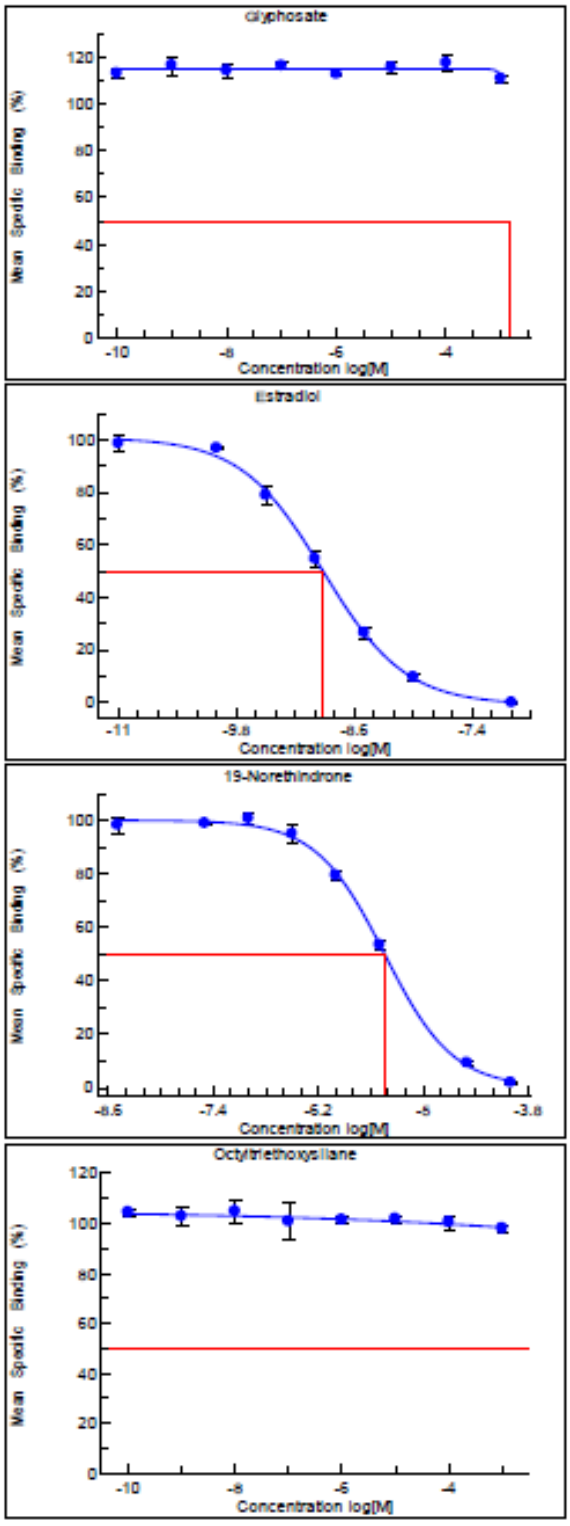


FIGURE 5. Percentage [^{3}H]-E2 Bound to the Estrogen Receptor in the Presence of Glyphosate, Unlabeled E2, 19-Norethindrone or Octyltriethoxysilane in Assay Run 3.



C. PERFORMANCE CRITERIA: To ensure that the competitive binding assay functioned properly, each run was evaluated using the criteria shown in Table 8.

TABLE 8. Criterion [a]	Tolerance Limit(s)	Value	Yes	No
17β-estradiol fitted curve parameters				
Log_e residual SD	≤2.35	0.16 to 0.96	X	
Top (% binding)	94 to 111	101 to 103	X	
Bottom (% binding)	−4 to 1	−1 to 0	X	
Hill Slope ($log_{10}(M)^{-1}$)	−1.1 to −0.7	−1 to −0.9	X	
Weak Positive control (19-norethindrone) fitted curve parameters[b]				
Log_e residual SD	NA	−0.83 to 1.01	NA	
Top (% binding)	NA	98 to 100	NA	
Bottom (% binding)	NA	−1 to 0	NA	
Hill Slope ($log_{10}(M)^{-1}$)	NA	−1.1 to −1	NA	
Solvent concentration				
DMSO[c]	≤10%	4%	X	
Negative control (octyltriethoxysilane) does not displace more than 25% of [^{3}H]-17β-estradiol from the ER on average across all concentrations	≤25%	≤54%[d]		X

a Data were obtained from pages 27, 29 and 31 of the study report.
b The EPA Guideline does not define a set of tolerance limits for 19-norethindrone. Acceptance criteria were only defined for norethynodrel, which cannot be obtained commercially. The values reported were considered acceptable as they show 19-norethindrone to be an acceptable weak positive control.
c DMSO was only used for the reference chemicals.
d For Run 1 and Run 2, octyltriethoxysilane displaced 54% and 52.6% of [^{3}H]-17β-estradiol at 10^{-3} M. Run 3 was within the acceptable parameters.
NA Not applicable

The curve for the reference materials showed that increasing concentrations of unlabeled 17β-estradiol and 19-norethindrone displaced [^{3}H]-17β-estradiol in a manner consistent with one-site binding, as indicated by Hill slopes of −1.1 to −0.9 in the three runs.

Glyphosate was tested over a concentration range that fully defined the top of the curve (10^{-10} to 10^{-9} M). The percent binding at this top plateau (101.2-116.9%) was within 25 percentage points of the lowest concentration of the estradiol standard (98.6-101.8%).

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATOR'S CONCLUSIONS: Glyphosate was classified as "non-interacting" in all three independent runs and thus has a final classification of "non-interacting" for the estrogen receptor.

B. AGENCY COMMENTS: The protein concentrations used in the saturation binding runs varied between each run, and were approximately 3- to 6-fold greater than recommended. The K_d for [^{3}H]-17β-estradiol was 0.331 nM and the estimated B_{max} was 74.55 fmol/100 µg protein for the prepared rat uterine cytosol. The K_d for each run was within the expected Guideline range of 0.03 to 1.5 nM. Although the Scatchard plots fit straight lines to the data, the concavity observed in all of the data sets may indicate issues with ligand depletion.

In the competitive binding experiment, the estimated mean log IC_{50} for 17β-estradiol and 19-norethindrone was −9.0 and −5.5 M, respectively. The mean RBA was 0.032% for 19-norethindrone, compared to the natural ligand.

Glyphosate was tested over a concentration range that fully defined the top of the curve (10^{-10} to 10^{-9} M). The percent binding at this top plateau (101.2-116.9%) was within 25 percentage points of the lowest concentration of the estradiol standard (98.6-101.8%). Glyphosate did not displace more than 25% of the radiolabeled estradiol from the ER at any concentration in the three assay runs. Glyphosate is classified as not interactive 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:

- The protein concentrations used in the saturation binding runs varied between each run, and were approximately 3- to 6-fold greater than recommended.
- Curves were not provided showing the average binding of each test substance across all

DATA EVALUATION RECORD

GLYPHOSATE

Study Type: OCSPP 890.1300, Estrogen Receptor Transcriptional Activation

EPA Contract No. EP10H001452
Task Assignment No. 3-06-2012

(Revisions to MRID 48617307to include laboratory proficiency data;
Main study was originally reviewed under TA 2-74-2012)

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

Prepared by
CSS-Dynamac Corporation
1910 Sedwick Road,
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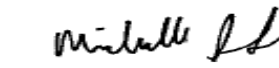
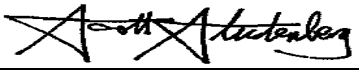
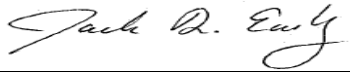
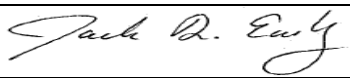
Durham, NC 27713

Primary Reviewer: Michelle Sharpe-Kass, M.S.	Signature: Date:	 7/11/2012
Secondary Reviewer: Scott D. Studenberg, Ph.D., D.A.B.T.	Signature: Date:	 7/13/2012
Program Manager: Jack D. Early, M.S.	Signature: Date:	 7/23/2012
Quality Assurance: Jack D. Early, M.S.	Signature: Date:	 7/23/2012

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 Ackerman, PhD **Signature:**
Health Effects Division **Date:** 6/29/15
Secondary Reviewer: Minerva Mercado PhD, DABT **Signature:**
Health Effects Division **Date:** 6/29/15

Template version 08/2011

DATA EVALUATION RECORD

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

PC CODE: 417300 **DP BARCODE:** D401747

TXR#: Not Provided **CAS No.:** 1071-83-6

TEST MATERIAL (PURITY): Glyphosate (95.93% glyphosate acid, 85.14% calculated glyphosate content)

SYNONYMS: Roundup, N-(phosphonomethyl)glycine

CITATION: Willoughby, J.A. (2012) Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-9903)) Screening Assay with Glyphosate. CeeTox, Kalamazoo, MI. Laboratory Report No.: 6500V-100334ERTA, March 8, 2012. MRID 48671307. Unpublished.

Willoughby, J.A. (2012). Supplemental Information - Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-9903)). Proficiency Data Report. CeeTox, Inc., Kalamazoo, MI. May 15, 2012. MRID 48843501. Unpublished.

SPONSOR: Joint Glyphosate Task Force, LLC, 8325 Old Deer Trail, Raleigh, NC

TEST ORDER #: CON-417300-23

EXECUTIVE SUMMARY: In an estrogen receptor transcriptional activation assay (MRID 48617307), hERα-HeLa-9903 cells cultured *in vitro* were exposed to glyphosate (85.14% a.i. Batch # GLP-1103-21149-T) at logarithmically increasing concentrations from 10^{-10} to 10^{-3} M in cell culture media for 24 hours in three independent runs. The experiments were performed using 96-well plates and each glyphosate concentration was tested in 6 wells/plate in each run. The solvent vehicle was culture media for glyphosate and DMSO (0.1%) for the reference chemicals. Cells were exposed to the test agent for 24±2 hr to induce reporter (luciferase) gene products. Luciferase expression in response to activation of the estrogen receptor was measured using a proprietary luciferase assay.

Glyphosate was tested up to the limit dose, with no precipitation or cytotoxicity observed at any tested concentration. At concentrations up to 10^{-3} M, the relative transcriptional activation of glyphosate was ≤2.4%.

In the main assays, the responsiveness of the cells to the very weak positive control 17α-methyltestosterone was lower than the expected values, indicating a decreased sensitivity of the assay to very weak agonists. Although the conditions of this assay were not optimal to detect very weak activity, glyphosate responses were similar to those of the negative control corticosterone and not comparable to the responses of 17α-methyltestosterone, which was able to reach a maximum of 40.8-42.6% PC. Glyphosate was only able to reach a maximum of 0.8-2.4% PC when tested up to the highest concentration possible based on cytotoxicity. Because the $RPC_{Max} < PC_{10}$ in both assay runs, glyphosate was considered negative 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 Data Confidentiality, GLP Compliance, and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

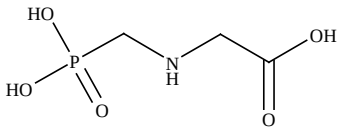
A. MATERIALS

1. Test Facility: CeeTox, Inc.

Location: Kalamazoo, MI
Study Director: J.A. Willoughby
Other Personnel: K. Rutherford (Director Laboratory Operations); D. Blakeman (Senior Scientist); C. Haines (Scientist); S. McColley (Scientist); B. Meyer (Scientist); B. Wallace (Lead Cell Culture Scientist); and C. Toole (Director of Project Management)
Study Period: July 21, 2011 to March 08, 2012

2. Test Substance: Glyphosate

Description: White crystalline solid (white wetcake)
Source (Cat #): Monsanto, Co, St. Louis, MO
Batch # (Exp Date): GLP-1103-21149-T (expiry: March 9, 2012)
Purity: 95.93% glyphosate acid, 85.14% calculated glyphosate acid
Solubility: Not reported
Volatility: Not reported
Stability: NA
Storage conditions: 35 to 100°F (Room temperature)
CAS #: 1071-83-6
Structure:



3. Reference substances

17β-estradiol (strong estrogen; positive control)

Supplier: Sigma-Aldrich, St. Louis MO.
Catalog and Batch #: E8875, 110M0138V
Purity: 100%
CAS # : 50-28-2

17α-estradiol (weak estrogen)

Supplier: Sigma-Aldrich, St. Louis MO.
Catalog and Batch #: E8750, 041M4065V
Purity: 99.72%
CAS # : 57-91-0

Corticosterone (negative compound)

Supplier: Sigma-Aldrich, St. Louis MO.
Catalog and Batch #: 27840, BCBC6322V
Purity: 99.2%
CAS # : 50-22-6

17α-methyltestosterone (very weak agonist)

Supplier: Sigma-Aldrich, St. Louis MO.
Catalog and Batch #: M7252, 060M1543
Purity: 99%
CAS # : 58-18-4

4. Vehicle(s)

Solvent: DMSO (reference chemicals) Sigma-Aldrich Cat # D2650, Lot # RNBB7886 (purity 99.8%, exp. March, 2013; Run 1); Lot # RNBB8623 (purity 100%, exp. May, 2013; Run 2)
Solvent control: DMSO - 0.1% (final concentration)

B. METHODS

1. Cell Culture: Stably-transfected hERα-HeLa-9903 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank and were verified to be free of mycoplasma infection by a DNA fluorochrome assay (Oct. 5, 2010). Cells were maintained in Eagles Minimum Essential Medium without phenol red, supplemented with 60 mg/L kanamycin and 10% dextran-coated charcoal-treated fetal bovine serum (source not reported), in an incubator under 5% CO_2 at 37 ºC. The cells used in this study were passage 26 (range-finder, 9/20/2011), passage 27 (first run, 9/22/2011), and a new vial of cells was brought up from cryopreservation and used in the second run at passage 16 (10/20/2011). Upon reaching 75-90% confluence, cells were subcultured into test plates.

2. Transcriptional Activation Assays: For each test, cells were plated in a 96-well plate (estrogenic activity of plastic not reported) at a density of approximately 1×10^4 cells/100 µL medium/well and allowed to attach for 3 hours. The growth media was replaced with media containing serial log dilutions of glyphosate in cell culture media or of the reference chemicals in DMSO (0.1% final concentration). Cells were incubated for 24±2 hours at 37 ± 1°C. Cytotoxicity was determined by two-read propidium iodide uptake. Transcriptional activation of the estrogen receptor was determined as described in CeeTox Standard Operating Protocol (SOP) 2041. A list of reagents was provided, but the assay reagent was classified as proprietary information, and the SOP was not provided.

a. Preliminary Test: A preliminary test evaluating glyphosate concentrations ranging from $10^{-6.5}$ 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: Responsiveness of the test system was tested on March 5, April 12 and April 28, 2011(MRID 48843501), using cells at passage 15, 25 and 28, respectively. Based on passage numbers and assay dates it is unlikely that cells used for proficiency testing are from the same frozen stock as cells used in main assays. Cells were tested using the following proficiency chemicals, each chemical tested in duplicate on separate days:

Compound	CAS No.	Concentration Range (M)	Expected Response [a]	Notes
Diethylstilbestrol (DES)	56-53-1	10^{-14} to 10^{-8}	Positive	---
17α-Ethynyl estradiol (EE)	57-63-6	10^{-14} to 10^{-8}	Positive	---
Hexestrol	84-16-2	10^{-13} to 10^{-7}	Positive	---
Genistein	446-72-0	10^{-12} to 10^{-5}	Positive	Cytotoxic at 0.01[b], 0.1, and 1 mM
Estrone	53-16-7	10^{-12} to 10^{-6}	Positive	---
Butyl paraben	94-26-8	10^{-11} to 10^{-4}	Positive	Cytotoxic at 0.1[b] and 1 mM
1,3,5-Tris(4-hydroxyphenyl)benzene [c]	15797-52-1	10^{-12} to 10^{-5}	Positive	Cytotoxic at 100µM. PC_{Max} approx. 50% of PC. Binds to hERα and has ER antagonistic activity
Dibutyl phthalate (DBP)	84-74-2	10^{-11} to 10^{-4}	Negative [d]	Cytotoxic at 1 mM
Atrazine	1912-24-9	10^{-11} to 10^{-4}	Negative	Cytotoxic at 1 mM [b]
Corticosterone	50-22-6	10^{-10} to 10^{-4}	Negative	If not cytotoxic at 1 mM, then that should be the highest tested concentration

a Positive = RPC_{Max} ≥10% of the response of the positive control in at least 2 of 2 (or 2 of 3) runs
Negative = RPC_{Max} fails to achieve at least 10% of the response of the positive control in 2 of 2 (or 2 of 3) runs

b Cytotoxicity is expected to be close to 80% at this concentration.

c Compound selected to challenge solubility and cytotoxicity.
d DBP is negative for ERα mediated transcriptional activation, but may not be negative for non-ERβ mediated transcriptional activation. A positive result would indicate that the system is detecting activity other than that due to pure ERα, and is therefore unacceptable.

c. Reference Chemicals: To ensure the stability of the response from the cell line, eight 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^{-15} to 10^{-8}	Strong estrogen
17α-estradiol	57-91-0	10^{-13} to 10^{-6}	Weak estrogen
Corticosterone	50-22-6	10^{-11} to 10^{-4}	Negative compound
17α-methyltestosterone	58-18-4	10^{-12} to 10^{-5}	Very weak agonist

3. Data analysis: To obtain the relative transcriptional activity (RTA) compared 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 the RTA as a percentage of the PC. The test material was defined as negative for inducing estrogen receptor transcriptional activation if the $RPC_{Max} < PC_{10}$ in at least 2 of 2 runs. Log EC_{50} and Hill slope values are calculated only if a positive response is 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: In order to identify a suitable top concentration for use in the transcriptional activation assays, a preliminary cytotoxicity and precipitation assay was conducted (Table 1). No precipitation or cytotoxicity was observed at any tested concentration. Based on these results, logarithmically increasing concentrations from 10^{-10} to 10^{-3} M were selected for the assay.

TABLE 1. Preliminary Test for Solubility, Cytotoxicity, and Concentration-Selection for Glyphosate[a]		
Concentration (M)	**% Viability [b]**	**Comments**
10^{-3}	90	Only concentration assessed for precipitation; negative
$10^{-3.5}$	98	
10^{-4}	95	
$10^{-4.5}$	99	
10^{-5}	97	
$10^{-5.5}$	99	
10^{-6}	103	
$10^{-6.5}$	99	

a Data were obtained from page 21 of the study report.
b If viability is <80%, the concentration is considered cytotoxic.

B. POSITIVE AND NEGATIVE REFERENCE CHEMICALS

1. **Proficiency Chemicals:** The laboratory proficiency assays using the required reference compounds were not included in the original study report, but were provided to the Agency at a later date (MRID 48843501). In addition, the proficiency testing was conducted with cells that were not of the same frozen stock as the cells used in the main assay. The responsiveness of cells to the required proficiency chemicals was performed in duplicate on different days for each chemical. The reported responses are summarized in Table 2a. In the proficiency tests, the reference chemicals 17β-estradiol, 17α-estradiol and 17α-methyltestosterone were tested concurrently with each run of the assay (Table 2b). In the first run, the responsiveness of 17β-estradiol indicated decreased sensitivity to strong agonists, and the response to 17α-methyltestosterone showed an increased responsiveness to very weak agonists; despite the minor deviations this run is considered acceptable. Run 2 was inadequate as the PC_{50} could not be calculated for 17α-methyltestosterone indicating a decreased sensitivity to very weak agonists. Run 3 was acceptable as 17β-estradiol and 17α-methyltestosterone performed within the expected range, but the Hill Slope for 17α-estradiol was higher than expected. The PC-induced fold induction for the three reference chemicals was within the Guideline-recommended historical range of 4- to 30-fold in Runs 1 and 3, but fold induction was 75.1- to 84.7-fold in Run 2 with no explanation given for this 3- to 4-fold increase. Although reportedly performed, the results of the cytotoxicity assay were not provided for review. Raw data pertaining to the RTA of each chemical were not reported, but the scales of the graphs provided indicate genistein and butyl paraben had maximum RTAs well above 400%.

TABLE 2a. Proficiency Chemicals				
Compound	**Expected Response**	**Lab Response**		
		Run 1	**Run 2**	**Run 3**
Diethylstilbestrol	Positive	Positive	Positive	NA
17α-Ethynyl estradiol	Positive	Positive	Positive	NA
Hexestrol	Positive	Positive	Positive	NA
Genistein	Positive	Positive	Positive	NA
Estrone	Positive	Positive	Positive	NA
Butyl paraben	Positive	Positive	Positive	NA
1, 3, 5-Tris(4-hydroxyphenyl)benzene	Positive	NA	Positive	Positive
Dibutyl phthalate	Negative	Negative	Negative	NA
Atrazine	Negative	Negative	NA	Negative
Corticosterone	Negative	Negative	NA	Negative

NA = not applicable. The chemical was not tested at this time.

Table 2b. Performance Criteria for Reference Chemicals in the Proficiency test						
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	−9.6	−11.3	−10.6		Run 1
Log PC_{10}	<−11	−11.5	−12.5	−12.1	X	
Log EC_{50}	−11.3 to −10.1	−9.0	−11.3	−10.6		Run 1
Hill Slope	0.7 to 1.5	1.2	0.9	0.8		
Test range (M)	10^{-14} to 10^{-8}	10^{-14} to 10^{-8}	10^{-14} to 10^{-8}	10^{-14} to 10^{-8}	X	
17α-estradiol						
Log PC_{50}	−9.6 to −8.1	−8.3	−9.4	−8.7	X	
Log PC_{10}	−10.7 to −9.3	−9.3	−10.5	−9.9	X	
Log EC_{50}	−9.6 to −8.4	−8.2	−9.3	−8.9		Run 1
Hill Slope	0.9 to 2.0	0.9	0.9	2.9		Run 3
Test range (M)	10^{-12} to 10^{-6}	10^{-12} to 10^{-6}	10^{-12} to 10^{-6}	10^{-12} to 10^{-6}	X	
17α-methyltestosterone						
Log PC_{50}	−6.0 to −5.1	−6.2	NC	−5.2		Run 1, 2
Log PC_{10}	−8.0 to −6.2	−8.1	−6.3	−7.7		Run 1
Test range (M)	10^{-11} to 10^{-5}	10^{-11} to 10^{-5}	10^{-11} to 10^{-5}	10^{-11} to 10^{-5}	X	

Data were obtained from : Willoughby, J.A. (2012) Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-9903)): Proficiency Data Report. CeeTox, Inc.

2. **Reference Chemicals:** Values derived from the concentration response curve (*e.g.*, Log PC_{50}, Log PC_{10}, Log EC_{50}, Hill slope) for the four reference chemicals that were run concurrently with the test chemical are included in Table 3.

All performance criteria were met for 17β-estradiol, and the only deficiency for 17α-estradiol was a Hill Slope of 2.3 in the third run, which is +0.3 outside the validated range. The cells' responses to 17α-methyltestosterone indicate decrease sensitivity to detect a very weak agonist. This could result in false negative responses from test substances which are weak agonists.

TABLE 3. Performance Criteria for Reference Chemicals[a]					
Reference Chemical Parameter	Acceptable Range	Values		Acceptable	
		Run 1	Run 3	Yes	No
17β-estradiol					
Log PC_{50}	−11.4 to −10.1	−10.4	−10.4	X	
Log PC_{10}	<−11	−11.4	−11.4	X	
Log EC_{50}	−11.3 to −10.1	−10.4	−10.4	X	
Hill Slope	0.7 to 1.5	1.2	1.3	X	
Test range	10^{-14} to 10^{-8} M	10^{-15} to 10^{-8} M	10^{-15} to 10^{-8} M	X	
17α-estradiol					
Log PC_{50}	−9.6 to −8.1	−8.6	−8.6	X	
Log PC_{10}	−10.7 to −9.3	−9.6	−9.6	X	
Log EC_{50}	−9.6 to −8.4	−8.7	−8.8	X	
Hill Slope	0.9 to 2.0	1.6	2.3		Run 2
Test range	10^{-12} to 10^{-6} M	10^{-13} to 10^{-6} M	10^{-13} to 10^{-6} M	X	
Corticosterone					
Test range	10^{-10} to 10^{-4} M	10^{-11} to 10^{-4} M	10^{-11} to 10^{-4} M	X	
17α-methyltestosterone					
Log PC_{50}	−6.0 to −5.1	NA[b]	NA[b]		X
Log PC_{10}	−8.0 to −6.2	−5.9	−5.9		X
Test range	10^{-11} to 10^{-5} M	10^{-12} to 10^{-5} M	10^{-12} to 10^{-5} M	X	

a Data were obtained from page 24 of the study report.
b Not calculable as the responsiveness of the cells was ≤43%

C. DEFINITIVE ASSAY

1. Vehicle and Positive Controls: Data for the vehicle and positive controls are included in Table 4. The overall mean TA value for the vehicle control was 19913 for the first run and 10463 for the third run, and the overall mean TA value for the positive control was 532425 for the first run and 110517 for the third run. The induction for the positive control ranged from 27- to 106-fold. The mean normalized value for the positive control was 512513 for the first run and 1100055 for the third. The PC_{50} (50% of the maximum response) for E2 in this assay is 256257 for the first run and 550027 for the third and the PC_{10} (10% of the maximum response) is 51251 for the first run and 110005 for the third.

TABLE 4. Transcriptional Activation (TA) Response of Vehicle and Positive Control [a]							
Sample	Vehicle Control		Positive Control [b]			Normalized Positive Control [b]	
Runs	Mean	SD	Mean	SD	Fold Induction [c]	Mean	SD
1	19913	4069	532425	130022	26.7	512513	130022
3	10463	2413	1110517	194915	106.1	1100054	194915

a Claculated by reviewer from data were obtained from pages 33 and 34 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 two reported 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} was 2.4% for the first run and 0.8% for the third run, and the associated PC_{Max} was 10^{-5} and 10^{-6} M, respectively. Because the RPC_{Max}<PC_{10} in

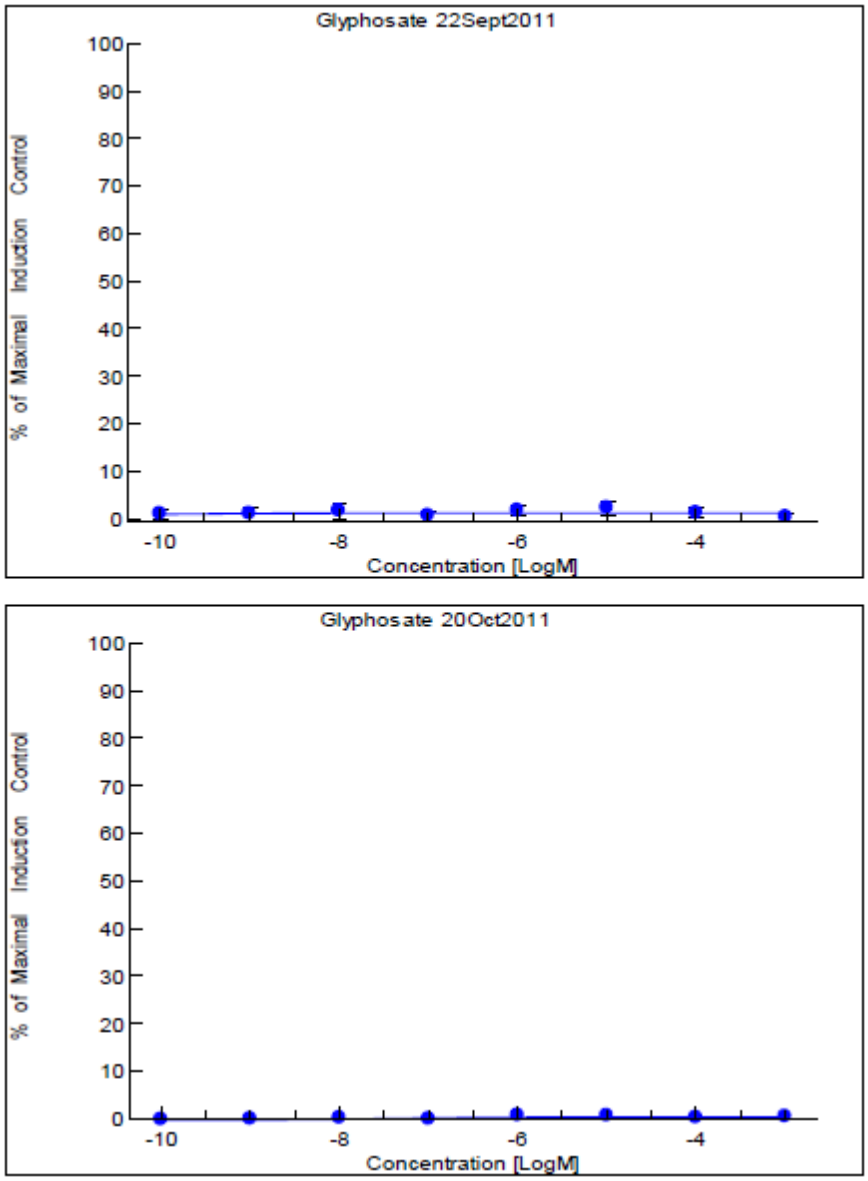
both reported runs, glyphosate was considered negative for estrogen receptor transcriptional activation in this test system.

TABLE 5. Relative Transcriptional Activation (RTA) of Glyphosate[a]

Parameter	RTA (mean ± SD); % of Positive Control (PC)			
	Run 1		Run 2	
Conc. (M)	Mean	SD	Mean	SD
10^{-3}	0.5	0.8	0.5	0.3
10^{-4}	1.3	1.1	0.3	0.2
10^{-5}	2.4	1.4	0.7	0.3
10^{-6}	1.8	1.1	0.8	0.3
10^{-7}	0.8	1.0	0.0	0.3
10^{-8}	1.8	1.5	0.3	0.3
10^{-9}	1.2	1.6	0.0	0.3
10^{-10}	1.1	1.0	-0.1	0.3
Log EC_{50}	NA		NA	
Hill Slope	NA		NA	
RPC_{Max}	2.4%		0.8%	
PC_{Max}	10^{-5}		10^{-6}	
PC_{50}	NA		NA	
PC_{10}	NA		NA	

a Data were obtained from page 22 and 23 of the study report.
NA = Not Applicable

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



3. **Performance Criteria:** For the proficiency chemicals, while the log PC_{50}, log PC_{10}, log EC_{50}, and Hill slope values for the concurrent reference chemicals fell within or near the acceptable ranges (Table 3), the full response of the cells to the reference chemicals was not satisfactory, as detailed above in Section B.2. For the concurrent reference chemicals, while most performance criteria were met for 17β-estradiol, and 17α-estradiol the cells' responses to 17α-methyltestosterone indicate decrease sensitivity to detect a very weak agonist. For 17α-methyltestosterone, the mean RPC_{Max} was 40.8% for the first run and 42.6% for the second run.

III. DISCUSSION AND CONCLUSIONS

A. <u>INVESTIGATORS' CONCLUSIONS</u>: Three independent runs were conducted, however, the second run was considered invalid because the reference substance data, in particular the 17α-estradiol Hill Slope, did not meet the acceptance criteria. These data were not included in the study report. Cytotoxicity and precipitation were not observed at any of the tested concentrations of glyphosate. In the two reported assays, the RPC_{Max} was 2.4% and 0.8% in the first and third run, respectively. Based on these results it can be concluded glyphosate is not an agonist of hERα over the concentration range tested.

B. <u>AGENCY COMMENTS</u>: Glyphosate was tested up to the limit dose, with no precipitation or cytotoxicity observed at any tested concentration. At concentrations up to 10^{-3} M the RTA of glyphosate was ≤2.4%, indicating that it is negative for estrogenic activity under the conditions of this assay

In the main assays, the responsiveness of the cells to the very weak positive control 17α-methyltestosterone was lower than the expected values, indicating a decreased sensitivity of the assay to very weak agonists. Although the conditions of this assay were not optimal to detect very weak activity, glyphosate responses were similar to those of the negative control corticosterone and not comparable to the responses of 17α-methyltestosterone, which was able to reach a maximum of 40.8-42.6% PC. Glyphosate was only able to reach a maximum of 0.8-2.4% PC when tested up to the highest concentration possible based on cytotoxicity. Because the $RPC_{Max} < PC_{10}$ in both assay runs, glyphosate was considered negative for estrogen receptor transcriptional activation in this test system.

C. <u>STUDY DEFICIENCIES</u>: The following deficiencies were noted:

- The cells responded inadequately (<50% RTA) to the very weak estrogen, 17α-methyltestosterone.
- The source of the fetal bovine serum was not provided.

Data Evaluation Record on the Fish Short-Term Reproduction Assay with Glyphosate

EPA MRID Number 48671311

Data Requirement:	EPA DP Barcode	401746
	OECD Data Point	
	EPA MRID	48671311
	EPA Guideline	890.1350
		Fish Short-Term Reproduction Assay

Test Material:	Glyphosate		**Purity (%):** 85.14%
Common Name	Glyphosate		
Chemical Name	IUPAC	N-phosphonomethylglycine	
	CAS Name	Not Reported	
	CAS No.	1071-83-6	
	Synonyms	CP 067573	
	EPA PC Code	417300	

Primary Reviewer: John Marton, Ph.D. **Date:** 2/13/13

Environmental Scientist, CDM Smith

Secondary Reviewer(s): Teri S. Myers, Ph.D. **Date:** 2/25/13

Environmental Scientist, CDM Smith

Additional Reviewer: Amy Blankinship **Date:** AMY BLANKINSHIP Digitally signed by AMY BLANKINSHIP DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=AMY BLANKINSHIP, dnQualifier=0000040917 Date: 2015.06.15 17:35:52 -04'00'

OPP/EFED/ERB6

Additional Reviewer: Robin Sternberg **Date:** Digitally signed by ROBIN STERNBERG DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=ROBIN STERNBERG, dnQualifier=0000039126 Date: 2015.06.08 15:38:21 -04'00'

OPP/EFED/ERB1

Date Evaluation Completed: 6/8/15

CITATION: Schneider, S.Z., K.H. Martin, T.Z. Kendall, and H.O. Krueger. 2012. Glyphosate: Fish Short-Term Reproduction Assay with the Fathead Minnow (*Pimephales promelas*). Unpublished study performed by Wildlife International, Ltd., Easton, MD 21601. Laboratory report number 707A-102A. Study sponsored by Joint Glyphosate Task Force c/o Data Group Management, Inc., Raleigh, NC 27615. Study completed April 11, 2012.

Note: 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 glyphosate with Fathead minnow (*Pimephales promelas*) was conducted under flow-through conditions. Adult fish (2 males and 4 females in each group, 4 groups/treatment, 5.5 months of age) were exposed to glyphosate (85.14% purity) at nominal concentrations of 0 (negative control), 0.048, 0.24, 1.2, 6.0, and 30 mg a.i./L. Mean-measured concentrations were <0.03 (<LOQ; negative control), 0.046, 0.23, 1.2, 6.2, and 33 mg a.i./L. The high test concentration was based on 1/3 of a 96-hr LC_{50} value. The test system was maintained at 24.3 to 29.1°C and a pH of 8.0 to 8.3.

Survival was 100% in the negative control, 0.046, 0.23, 6.2 and 33 mg a.i./L treatment groups and 95.8% in the 1.2 mg a.i./L treatment group. Glyphosate did not result in any significant increases or decreases in weight or length for either sex at any treatment level. There were no observed effects on secondary sex characteristics or clinical signs (*i.e.*, behavioral and other sublethal effects) in males or females in any treatment group.

Spawning and mean fecundity in the negative control were at least every 4 days in each replicate and 23.5 eggs/female/day/replicate (range: 23.2-23.9 eggs/female/day), respectively; fertilization success in the negative control was 97.3%. Fecundity and fertilization success were not significantly different from the negative control for any treatment group.

Plasma vitellogenin (VTG) was significantly decreased 55% ($p<0.05$) in female fish at the mid high-treatment level (6.2 mg a.i./L) compared to the negative control; male VTG was unaffected by treatment. There were no effects in gonado-somatic index (GSI) or nuptial tubercle scores for male or females (none noted in females) in any treatment group relative to the negative control. Although there were gonadal histopathology observations reported for both males and females (*e.g.*, minimal to mild granulomatus inflammation, oocyte atresia, increased mature oocytes), there were no treatment-related patterns in gonadal histopathology for males or females.

All performance criteria were met for this study, except for a slight deviation in temperature. Temperature exceeded the recommended range (25±1°C), for less than 24 hours on Day 7 when the maximum temperature reached 29.1°C (range of 28.6-29.1°C); deviation occurred in three replicates each in the 1.2 mg a.i./L and 6.2 mg a.i./L groups). All fish were reported as normal throughout the test, and there was no mortality during this temperature deviation. This deviation did not impact the ability to interpret the results. It is also noted that there were 3 males and 3 females (as oppose to recommended 2 males and 4 females) in replicate D of the 33 mg a.i./L treatment group due to misidentification during allocation at pre-exposure. Inclusion or exclusion of data from this fish did not affect interpretation of the results in the study.

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

Results Synopsis:

Test organism age at test initiation: 5.5 months
Mean body weight at test initiation: males: 1.6 g; females: 0.9 g (N = 10 at test initiation from a sub-sample of the batch used for the test)
Mean length at test initiation: Not measured
Test type: flow-through

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

Treatment (mg a.i./L) [mean-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
0.046	No	No	No	No	No	No	No	No	No	No	NA	NA	NA	NA
0.23	No	No	No	No	No	No	No	No	No	No	NA	NA	NA	NA
1.2	No	No	No	No	No	No	No	No	No	No	NA	NA	NA	NA
6.2	No	No	No	No	No	No	No	No	No	Yes	NA	NA	NA	NA
33	No	No	No	No	No	No	No	No	No	No	NA	NA	NA	NA

Abbreviations: 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.

I. MATERIALS AND METHODS

Guideline Followed: This study was conducted following guidelines outlined in the U.S. EPA Series 890- Endocrine Disruptor Screening Program Test Guidelines, OCSPP Number 890.1350 *Fish Short-Term Reproduction Assay*; and the OECD Guidelines for Testing of Chemicals, Guideline 229: *Fish Short-Term Reproduction Assay*. The following deviations were noted:

1. Fecundity during the pre-exposure period ranged from 12.1 to 28.3 eggs/female/reproductive day. Guidance recommends fecundity be at least 15 eggs/female/reproductive day. Also, while the breeding groups were ranked by fecundity from highest to lowest and grouped/blocked into groups of 6 starting with the six top performers, the allocation of the breeding groups to the treatment groups did not appear to be random but rather followed the same pattern for each block. However, fecundity rates between replicates during the exposure period were consistent with no observed decrease or increase between treatments.
2. The reviewer-calculated flow rate (41.7 mL/min) was slightly less than recommended (45 mL/min). The study report noted that test solutions were pumped from the mixing chambers into the test chambers at a target rate of 44 mL/min.
3. Temperature exceeded the recommended range 25±1°C, for less than 24 hours on Day 7 when the maximum temperature reached 29.1°C (deviation occurred in replicates B, C, and D in 1.2 mg a.i./L and replicates A, B, and C in 6.2 mg a.i./L where temperatures ranged from 28.6 to 29.1°C). All fish were reported as normal throughout the test and there was no mortality during this temperature deviation.
4. Unionized ammonia and residual chlorine concentrations were not reported.
5. The reported light intensity (450-1976 lux) ranged outside of the recommended minimum (540 lux) and maximum (1080 lux).
6. The ratio of males to females in the highest treatment group, replicate D was 3 males, 3 females, as oppose to 2 males, 4 females due to a male fish mistaken as a female during the

pre-exposure allocation procedure. Fecundity rates for this replicate were adjusted and this mis-sexed fish did not alter the interpretation of the results in this treatment.

These deviations do not impact the acceptability of the study.

Compliance: Signed and dated No Data Confidentiality, GLP, and Quality Assurance statements were provided. This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Parts 160 ad 792); and OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17), with the following exception: periodic analyses of water for potential contaminants were not performed according to Good Laboratory Practice Standards, but were performed using a certified laboratory and standard US EPA analytical methods.

A. Test Material Glyphosate acid

Description: 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. : GLP-1103-21149-T (Lot #)
Purity: 85.14%
Impurities: None reported
Stability of Compound: Stable. Mean-measured concentrations yielded recoveries of 96-110% of nominal.
Storage Conditions of Test Chemicals: Stored under ambient conditions

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	Fish were originally obtained from Osage Catfisheries, Inc., Osage Beach Missouri	*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:	Culture fish were maintained for approximately 2 months prior to the pre-exposure period (which lasted for 19 days).		*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:	During the 7 days prior to pre-exposure, the fish showed no signs of disease or stress and mortality was <5%. The fish did not receive treatment for disease during the acclimation period, the two-week pre-exposure period, or the exposure period.		*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:	Commercial flake food (Sera Vipan) supplemented with brine shrimp nauplii (*Artemia* sp)		*EPA recommends that fish be fed frozen brine shrimp twice per day to promote active reproduction and maintain body condition.*
Source of food:	Flake food was supplied by Sera, North America and brine shrimp were supplied by Brine Shrimp Direct, Ogden, Utah.		
Frequency of feeding:	2 times/day		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Details on feeding:	Fish were not fed for at least 12 hours prior to test termination to allow for clearance of the digestive tracts before terminal weight measurements were made. Feeding rates in each replicate were adjusted approximately weekly, if necessary, to account for mortality. Uneaten food and fecal material were removed from the test chambers daily by siphoning.		

Table 3: Fish Selection and Pre-Exposure Performance.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Age at test initiation:	5.5 months		*EPA recommends reproductively mature (sexually dimorphic) fish, 4.5 - 6 months old.*
Mean weight of males at test initiation (if determined):	1.6 g	Based on sub-sample (N=10) of experimental population	*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):	Not reported	The range of individual weights of male and female fish at the start of the pre-exposure period was within ±20% of the mean weight of each sex.	
Mean weight of females at test initiation (if determined):	0.9 g	Based on sub-sample (N=10) of experimental population	
Range of individual weights (females) at test initiation (if determined):	Not reported	The range of individual weights of male and female fish at the start of the pre-exposure period was within ±20% of the mean weight of each sex.	
Mean length of males at test initiation (if determined):	Not measured		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Mean length of females at test initiation (if determined):	Not measured		
Duration of pre-exposure phase:	19 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:	40	Nearly twice the number of breeding groups was maintained in the pre-exposure period as in the exposure period so that breeding groups with a proven history of spawning could be selected for inclusion in the exposure phase of the test.	*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		
Number of females per tank:	4		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Pre-exposure fecundity:	≥12.1 eggs/female/ reproductive day/ replicate	Of the 40 tanks established during the pre-exposure period, the top 24 tanks (based on fecundity) were selected for use in the definitive test. The top 24 tanks ranged from 12.1 to 28.3 eggs/female/reproductive day.	*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		*EPA recommends that spawning occur at least twice in the 7 days prior to the definitive test.*
Details on pre-exposure:	Pre-exposure was conducted under conditions comparable to those used in the definitive test.	A replicate aquarium was considered suitable for use in the test if spawning in that aquarium occurred at least twice during the 7-day period immediate preceding test initiation. Any pre-exposure aquaria with mortalities, incorrect sex ratio, or poor reproductive performance were excluded from use in the test.	

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 diluter		*Intermittent flow proportional diluters or continuous flow serial diluters are recommended.[2]*

[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
Flow-through rate:	41.7 mL/min	Calculated based on 6 volume additions per day and a test solution volume of 10 L. The test solutions were pumped from the mixing chambers into the test chambers at a target rate of 44 mL/min.	*Recommended flow-through rate is 45 mL/min (2.7 L/hr), or at least 6 total volume exchanges per day.*
Details on toxicant mixing for flow-through systems:	Fluid metering pumps were used to deliver volumes of a single test substance stock solution to mixing chambers indiscriminately assigned to each treatment where it was mixed with well water. Flow was controlled using rotameters.	Pumps and rotameters were calibrated prior to the test and verified or recalibrated, if necessary, approximately weekly during the test. The proportion of the test solution that was pumped into each replicate test chamber was checked prior to the test and approximately weekly during the test to ensure that flow rates varied by no more than ±10% of the mean flow rate for the replicates.	*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 ≤4.9 mg/L (≤ 60% saturation).*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Source of dilution water:	Water was obtained from a well ~40 m deep on-site. Water was passed through a sand filter, pumped into a storage tank, and aerated. Prior to use, water was filtered to 0.45 µm and passed through a UV sterilizer.		*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.*
Was dilution water analyzed for pesticides, heavy metals, and other contaminants?	Yes. See Reviewer's Comments.		
Test vessel type/materials:	Glass		*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:	12 L		*EPA recommends the use of 18 L test chambers (e.g., 40 x 20 x 20 cm).*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Fill volume:	10 L	The depth of the test water in a representative chamber was approximately 13 cm.	*EPA recommends 10 L solution per tank.*
Spawning substrate material:	Tile consisting of an inverted semi-circular section of PVC pipe.	Each aquarium contained three spawning tiles.	*EPA recommends that each tank contain three semi-circular spawning substrates, e.g., aged PVC pipe, 10 - 20 cm in length, split lengthwise.*
Spawning substrate size:	~10 cm		
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)	24.3	29.1[1]	25.4	Weekly (in each test chamber) Continuously (one test chamber during the pre-exposure period and in one negative control replicate during the exposure period)	*EPA recommends temperature 25±1°C; inter-replicate and inter-treatment differentials should not exceed 1°C.*
pH	8.0	8.3	8.1	Weekly (in each test chamber)	*EPA recommends pH 6.5 to 9.0.*
Dissolved oxygen (mg/L)	6.0	7.9	7.2	Weekly (in each test chamber)	*EPA recommends dissolved oxygen (DO) ≥4.9 mg/L (>60% air saturation)*

Parameter	Minimum	Maximum	Mean	Measurement Interval	Guideline Recommendations
Total alkalinity (mg/L as $CaCO_3$)	166	180	173.5	Weekly (one replicate test chamber of the negative control and the highest concentration treatment group, with measurements typically alternating between replicates at each measurement interval)	*EPA recommends total alkalinity >20 mg/L as $CaCO_3$.*
Hardness (mg/L as $CaCO_3$)	140	148	144.5		
Total organic carbon (mg/L)	--	--	<1	Once in the 4-week period prior to test	*EPA recommends that total organic carbon in dilution water be ≤2 mg/L.*
Unionized ammonia (µg/L)	Not reported				*EPA recommends that unionized ammonia in the dilution water be ≤1 µg/L.*
Residual chlorine (µg/L)	Not reported				*EPA recommends that residual chlorine in dilution water be <10 µg/L.*

Parameter	Minimum	Maximum	Mean	Measurement Interval	Guideline Recommendations
Specific conductance	281	387	341.5	Weekly (one replicate test chamber of the negative control and the highest concentration treatment group, with measurements typically alternating between replicates at each measurement interval)	*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.*

[1] On Day 7, temperature measurements were out of range in replicates B, C and D of the 1.2 mg a.i./L treatment group and in replicates A, B and C of the 6.2 mg a.i./L treatment group. Temperature measurements in the test chambers ranged from 28.6 to 29.1°C. It was determined that the temperature probe wiring connecting the heating plates to the temperature control unit had become loose. After the probe connection was corrected, temperature in these tanks returned to target temperature and ranged from 24.4 to 24.7°C. This temperature variation was of short duration (<24 hours), and did not have any adverse impact on the study.

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?	1/3 of the reported LC_{50} from a previous acute toxicity study.	In a 96-hour acute toxicity test, a nominal glyphosate concentration of 87 mg a.i./L resulted in 100% mortality while a nominal concentration of 81 mg a.i./L resulted in no mortality.	*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*		
Life stage:	Not specified		*EPA recommends that range-finding tests be performed with fish of similar age and size to those that would be utilized in the test.*
Test duration:	14 days	Reference acute toxicity study was conducted for 96 hours.	*EPA recommends a 96-hour exposure.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Additional details:	The range-finding study was conducted with nominal concentrations of 1.9, 3.8, 7.5, 15, and 30 mg a.i./L. One incidental mortality occurred at 15 mg a.i./L with no other mortalities occurring in any control or treatment group. No signs of toxicity were observed in any control or treatment group throughout the duration of the range-finder.	Test solutions in the mixing and test chambers appeared clear and colorless throughout the rangefinder. Analytical recoveries ranged from 92 to 128% of nominal on Day 14.	*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.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Method for selecting the highest test concentration in the definitive test:	Range-finder		*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):	EG & G Bionomics. 1975. Chronic toxicity of glyphosate to the fathead minnow (*Pimephales promelas Rafinesque*). Monsanto unpublished study BN-75-129. MRID 108171.		
Separation of test concentrations:	0.2		*EPA suggests that a concentration separation of between 0.33 (or three-fold) and 0.1 (or ten-fold) is scientifically acceptable[1].*
Number of test concentrations:	5		*EPA recommends a minimum of 3 concentrations and a control, plus solvent control if appropriate.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Are nominal concentrations adjusted for purity?	Yes		
Indicate the type of values presented for measured concentrations:	Mean-measured		
Limit of quantification (LOQ):	0.0300 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:	Samples were collected for analysis on Days 0, 7, 14, and 21.		*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?	Unsure	Blocks of breeding groups were set up based on pre-exposure fecundity rates with one replicate for each treatment including controls included in each block. However, it does not appear that within the blocks, that the treatment replicates were randomized as the same pattern was used for all blocks.	*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	In the 33 mg a.i./L replicate D, there were 3 males and 3 males due to a mis-sexed fish at pre-exposure allocation.	*EPA recommends 2 males per replicate.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Number of female fish per replicate at test initiation:	4		*EPA recommends 4 females per replicate.*
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		

[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
Photoperiod:	16 hrs light : 8 hrs dark	A 30-minute transition period of low light intensity was provided between light and dark phases to avoid sudden changes in lighting.	*EPA recommends photoperiod 16:8 (light:dark).*
Light intensity at water's surface:	450-1976 lux	Fluorescent light bulbs that emit wavelengths similar to natural sunlight	*EPA recommends light intensity 540 – 1080 lux (at water's surface).*
Additional details:	NA	The stock solution was clear and colorless in appearance with no visible precipitates. Test solutions in the mixing chambers and test chambers were reported as clear and colorless with no evidence of precipitation.	

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

Treatment ID	Nominal Concentration (mg a.i./L)	Measured Concentration (mg a.i./L)	Mean CV (%)	Details or Remarks	Guideline Recommendations
Negative Control	0.00	<LOQ	NA	NA	*EPA recommends that test item concentrations be maintained at a coefficient of variation (CV) ≤20%.*
Treatment 1	0.048	0.046	9.0	± 0.004	
Treatment 2	0.24	0.23	9.9	± 0.022	
Treatment 3	1.2	1.2	2.3	± 0.029	
Treatment 4	6.0	6.2	2.4	± 0.148	
Treatment 5	30	33	7.8	NA	

Abbreviations: ^{CV} Coefficient of variation.

LOQ=0.0300 mg a.i./L

E. Observations

Biological Endpoints: Survival, fecundity, fertility success, and clinical signs were observed daily. On Day 21 total length, wet weight, secondary sex characteristics, plasma concentrations of vitellogenin (VTG), gonadal sex, GSI, and tubercle scores were evaluation

Were raw (individual) data provided? Yes

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

By test termination, a single incidental male mortality was observed in the mean-measured 1.2 mg a.i./L treatment group (Table 9). Male survival was 100% in the remaining treatment groups, and no female mortalities were observed. Overall survival was 100% in the negative control, 0.046, 0.23, 6.2 and 33 mg a.i./L treatment groups and was 95.8% in the 1.2 mg a.i./L treatment group.

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

Treatment (mg a.i./L) [mean-measured]	Males			Females		
	n	# Surviving	% Survival	n	# Surviving	% Survival
Negative Control (<LOQ)	8	8	100	16	16	100
0.046	8	8	100	16	16	100
0.23	8	8	100	16	16	100
1.2	8	7	87.5	16	16	100
6.2	8	8	100	16	16	100
33	9[1]	9	100	15[1]	15	100

[1] In replicate D of this treatment, there were 3 males and 3 females instead of the recommended 2 and 4 due to a mis-sexing error.

LOQ=0.0300 mg a.i./L

After 21 days of exposure, male wet weight ranged from 2.05 g in the mean-measured 33 mg a.i./L treatment group to 2.28 g in the mean-measured 0.23 mg a.i./L treatment group (Table 10). Total length ranged from 52 mm in the highest concentration to 55 mm in the negative control and mean-measured 0.23 mg a.i./L treatment group. Mean female wet weight ranged from 1.04 to 1.14 g in the control and treatment groups at test termination, and mean total length ranged from 45 to 46 mm (Table 10).

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

Treatment (mg a.i./L) [mean-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	2.20	0.462	4	1.14	0.047	4	55	2.8	4	46	0.7
0.046	4	2.17	0.343	4	1.10	0.053	4	53	1.8	4	46	0.9
0.23	4	2.28	0.397	4	1.04	0.070	4	55	3.5	4	45	1.1
1.2	4	2.20	0.186	4	1.12	0.053	4	54	1.6	4	46	0.2
6.2	4	2.14	0.273	4	1.07	0.108	4	53	2.3	4	45	1.3
33	4	2.04[1]	0.208	4	1.13	0.091	4	52[1]	1.0	4	46	0.8

Abbreviations: SD Standard deviation.

[1] In replicate D of this treatment, there were 3 males instead of the recommended 2 due to a mis-sexing error. If this fish is removed from analysis of body weight and length, the treatment means for length are the same as when retained and are similar for body weight (2.05 when retained and 2.04 when removed). The values in this table reflect data excluding the mis-sexed male.

LOQ=0.0300 mg a.i./L

Mean fecundity ranged from 22.6 to 29.3 eggs/reproductive female/day in the mean-measured 0.23 and 0.046 mg a.i./L treatment groups, respectively (Table 11). Mean fertilization success ranged from 96.0% in the mean-measured 1.2 mg a.i./L treatment group to 98.4% in the mean-measured 0.23 mg a.i./L treatment group.

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

Treatment (mg a.i./L) [mean-measured]	Fecundity[1]		Fertilization Success (%)[2]	
	Mean	± SD	Mean	± SD
Negative Control (<LOQ)	23.5	0.33	97.3	0.36
0.046	29.3	5.3	97.6	1.0
0.23	22.6	5.4	98.4	1.4
1.2	24.9	0.89	96.0	2.7
6.2	28.1	6.4	98.1	1.1
33	23.6	2.2	96.7	2.0

[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.

LOQ=0.0300 mg a.i./L

The reviewer-calculated treatment medians based on replicate medians (Table 12). Male median treatment tubercle scores ranged from 15 to 19. No nuptial tubercles were observed for females.

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

Treatment (mg a.i./L) [mean-measured]	Males		Females	
	n	Median Tubercle Score	n	Median Tubercle Score
Negative Control (<LOQ)	4	17	4	0
0.046	4	19	4	0
0.23	4	16	4	0
1.2	4	19	4	0
6.2	4	17	4	0
33	4	15[1]	4	0

[1] In replicate D of this treatment there were 3 males instead of the recommended 2 due to a mis-sexing error. If this fish is removed from analysis of tubercle score, the treatment medians are the same as when retained.

LOQ=0.0300 mg a.i./L

Mean GSI ranged from 1.11 to 1.52% in males and from 13.1 to 15.8% in females (Table 13).

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

Treatment (mg a.i./L) [mean-measured]	Males			Females		
	n	Mean GSI[1] (%)	±SD	n	Mean GSI[1] (%)	±SD
Negative Control (<LOQ)	4	1.47	0.198	4	14.7	3.27
0.046	4	1.11	0.212	4	14.4	2.05
0.23	4	1.42	0.371	4	13.1	1.67
1.2	4	1.32	0.081	4	14.0	2.58
6.2	4	1.36	0.335	4	15.5	2.05
33	4	1.52[2]	0.330	4	15.8	3.14

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

[2] In replicate D of this treatment there were 3 males instead of the recommended 2 due to a mis-sexing error. If this fish is removed from analysis of GSI, the treatment means are very similar as when retained (1.51 when retained vs. 1.52 when removed). The values in this table reflect data excluding the mis-sexed male.

LOQ=0.0300 mg a.i./L

Median gonadal stages were 2.0 in males from 2 to 3 in females (Table 14). There were no apparent treatment-related trends in gonadal staging.

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

Treatment (mg a.i./L) [mean-measured]	Males		Females	
	n	Median Stage[1]	n	Median Stage[2]
Negative Control (<LOQ)	8	2	16	3
0.048	8	2	16	3
0.24	8	2	16	3
1.2	7	2	15	2
6.2	8	2	16	3
33	9	3[3]	15	3

[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.

[3] In replicate D of this treatment there were 3 males instead of the recommended 2 due to a mis-sexing error. If this fish is removed from median treatment analysis, the treatment median are very similar as when retained (2.5 (rounded to 3) when all three analyzed vs. 2.25 (rounded to 2) when mis-sexed removed). As the evaluation of this endpoint relies heavily on the pathologists report, this table reflects all three fish.

LOQ=0.0300 mg a.i./L

The gonads from a total of 48 males and 94 females were studied. Because the mis-sexed male in replicate D of the mean-measured 33 mg a.i./L treatment group was not explicitly identified, it is included in the tables (*i.e.*, n = 9 males for the 33 mg a.i./L treatment group). Testes and ovaries from the five treatment groups showed no changes in gonadal staging or increased abnormalities when compared with the negative control (Tables 15-18). Minimal and mild granulomatous inflammation was found in male gonads from the mean-measured 0.046 mg a.i./L treatment group, though these observations were not considered to be treatment-related.

Mild increased oocyte atresia in females was observed in the negative control, low, and mid-concentration treatments, and a single incident of moderate increased oocyte atresia was noted in the high-concentration treatment group (Table 17). Moderate to marked increases in mature oocytes were observed in two, five, and one females in the negative control and mean-measured 1.2 and 33 mg a.i./L treatment groups, respectively. Mild granulomatous inflammation was noted in a single female in the negative control and mean-measured 6.2 mg a.i./L treatment group (Table 18). No other female gonadal histopathological observations were made.

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

Treatment (mg a.i./L) [mean-measured]	Diagnostic Observations[1]								
	Severity	Increased Proportion of Spermatogonia		Presence of Testis-Ova		Increased Testicular Degeneration		Interstitial Cell Hypertrophy/ Hyperplasia	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative Control (<LOQ)	0	8	8	8	8	8	8	8	8
	1	8	0	8	0	8	0	8	0
	2	8	0	8	0	8	0	8	0
	3	8	0	8	0	8	0	8	0
	4	8	0	8	0	8	0	8	0
0.046	0	8	8	8	8	8	8	8	8
	1	8	0	8	0	8	0	8	0
	2	8	0	8	0	8	0	8	0
	3	8	0	8	0	8	0	8	0
	4	8	0	8	0	8	0	8	0
0.23	0	8	8	8	8	8	8	8	8
	1	8	0	8	0	8	0	8	0
	2	8	0	8	0	8	0	8	0
	3	8	0	8	0	8	0	8	0
	4	8	0	8	0	8	0	8	0

Treatment (mg a.i./L) [mean-measured]	Diagnostic Observations[1]								
	Severity	Increased Proportion of Spermatogonia		Presence of Testis-Ova		Increased Testicular Degeneration		Interstitial Cell Hypertrophy/ Hyperplasia	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
1.2	0	7	7	7	7	7	7	7	7
	1	7	0	7	0	7	0	7	0
	2	7	0	7	0	7	0	7	0
	3	7	0	7	0	7	0	7	0
	4	7	0	7	0	7	0	7	0
6.2	0	8	8	8	8	8	8	8	8
	1	8	0	8	0	8	0	8	0
	2	8	0	8	0	8	0	8	0
	3	8	0	8	0	8	0	8	0
	4	8	0	8	0	8	0	8	0
33	0	8	8	8	8	8	8	8	8
	1	8	0	8	0	8	0	8	0
	2	8	0	8	0	8	0	8	0
	3	8	0	8	0	8	0	8	0
	4	8	0	8	0	8	0	8	0

[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.

LOQ=0.0300 mg a.i./L

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

Treatment (mg a.i./L) [mean-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	8	8	8	8	8	8	8	8	8	8
	1	8	0	8	0	8	0	8	0	8	0
	2	8	0	8	0	8	0	8	0	8	0
	3	8	0	8	0	8	0	8	0	8	0
	4	8	0	8	0	8	0	8	0	8	0
0.046	0	8	8	8	8	8	8	8	8	8	4
	1	8	0	8	0	8	0	8	0	8	1
	2	8	0	8	0	8	0	8	0	8	3
	3	8	0	8	0	8	0	8	0	8	0
	4	8	0	8	0	8	0	8	0	8	0

Treatment (mg a.i./L) [mean-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
0.23	0	8	8	8	8	8	8	8	8	8	8
	1	8	0	8	0	8	0	8	0	8	0
	2	8	0	8	0	8	0	8	0	8	0
	3	8	0	8	0	8	0	8	0	8	0
	4	8	0	8	0	8	0	8	0	8	0
1.2	0	7	7	7	7	7	7	7	7	7	7
	1	7	0	7	0	7	0	7	0	7	0
	2	7	0	7	0	7	0	7	0	7	0
	3	7	0	7	0	7	0	7	0	7	0
	4	7	0	7	0	7	0	7	0	7	0
6.2	0	8	8	8	8	8	8	8	8	8	8
	1	8	0	8	0	8	0	8	0	8	0
	2	8	0	8	0	8	0	8	0	8	0
	3	8	0	8	0	8	0	8	0	8	0
	4	8	0	8	0	8	0	8	0	8	0

Treatment (mg a.i./L) [mean-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
33	0	8	8	8	8	8	8	8	8	9	9
	1	8	0	8	0	8	0	8	0	9	0
	2	8	0	8	0	8	0	8	0	9	0
	3	8	0	8	0	8	0	8	0	9	0
	4	8	0	8	0	8	0	8	0	9	0

[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.

LOQ=0.0300 mg a.i./L

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

Treatment (mg a.i./L) [mean-measured]	Diagnostic Observations[1]						
	Severity	Increased Oocyte Atresia		Perifollicular Cell Hyperplasia/ Hypertrophy		Decreased Yolk Formation	
		n	Incidence	n	Incidence	n	Incidence
Negative Control (<LOQ)	0	16	15	16	16	16	16
	1	16	0	16	0	16	0
	2	16	1	16	0	16	0
	3	16	0	16	0	16	0
	4	16	0	16	0	16	0
0.046	0	16	15	16	16	16	16
	1	16	0	16	0	16	0
	2	16	1	16	0	16	0
	3	16	0	16	0	16	0
	4	16	0	16	0	16	0
0.23	0	16	16	16	16	16	16
	1	16	0	16	0	16	0
	2	16	0	16	0	16	0
	3	16	0	16	0	16	0
	4	16	0	16	0	16	0
1.2	0	15	13	15	15	15	15
	1	15	0	15	0	15	0
	2	15	2	15	0	15	0
	3	15	0	15	0	15	0
	4	15	0	15	0	15	0

Treatment (mg a.i./L) [mean-measured]	Diagnostic Observations[1]						
	Severity	Increased Oocyte Atresia		Perifollicular Cell Hyperplasia/ Hypertrophy		Decreased Yolk Formation	
		n	Incidence	n	Incidence	n	Incidence
6.2	0	16	16	16	16	16	16
	1	16	0	16	0	16	0
	2	16	0	16	0	16	0
	3	16	0	16	0	16	0
	4	16	0	16	0	16	0
33	0	15	14	15	15	15	15
	1	15	0	15	0	15	0
	2	15	0	15	0	15	0
	3	15	1	15	0	15	0
	4	15	0	15	0	15	0

[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.

LOQ=0.0300 mg a.i./L

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

Treatment (mg a.i./L) [mean-measured]	Additional Diagnostic Observations[1]									
	Severity	Interstitial Fibrosis		Egg Debris in Oviduct		Granulomatous Inflammation		Decreased Post-Ovulatory Follicles		Increased Mature Oocytes	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative Control (<LOQ)	0	16	16	16	16	16	15	16	16	16	14
	1	16	0	16	0	16	0	16	0	16	0
	2	16	0	16	0	16	1	16	0	16	0
	3	16	0	16	0	16	0	16	0	16	0
	4	16	0	16	0	16	0	16	0	16	2
0.046	0	16	16	16	16	16	16	16	16	16	16
	1	16	0	16	0	16	0	16	0	16	0
	2	16	0	16	0	16	0	16	0	16	0
	3	16	0	16	0	16	0	16	0	16	0
	4	16	0	16	0	16	0	16	0	16	0

Treatment (mg a.i./L) [mean-measured]	Additional Diagnostic Observations[1]									
	Severity	Interstitial Fibrosis		Egg Debris in Oviduct		Granulomatous Inflammation		Decreased Post-Ovulatory Follicles		Increased Mature Oocytes	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
0.23	0	16	16	16	16	16	16	16	16	16	16
	1	16	0	16	0	16	0	16	0	16	0
	2	16	0	16	0	16	0	16	0	16	0
	3	16	0	16	0	16	0	16	0	16	0
	4	16	0	16	0	16	0	16	0	16	0
1.2	0	15	15	15	15	15	15	15	15	15	10
	1	15	0	15	0	15	0	15	0	15	0
	2	15	0	15	0	15	0	15	0	15	0
	3	15	0	15	0	15	0	15	0	15	1
	4	15	0	15	0	15	0	15	0	15	4
6.2	0	16	16	16	16	16	15	16	16	16	16
	1	16	0	16	0	16	0	16	0	16	0
	2	16	0	16	0	16	1	16	0	16	0
	3	16	0	16	0	16	0	16	0	16	0
	4	16	0	16	0	16	0	16	0	16	0

Treatment (mg a.i./L) [mean-measured]	Additional Diagnostic Observations[1]											
	Severity	Interstitial Fibrosis		Egg Debris in Oviduct		Granulomatous Inflammation		Decreased Post-Ovulatory Follicles		Increased Mature Oocytes	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
33	0	15	15	15	15	15	15	15	15	15	14
	1	15	0	15	0	15	0	15	0	15	0
	2	15	0	15	0	15	0	15	0	15	0
	3	15	0	15	0	15	0	15	0	15	0
	4	15	0	15	0	15	0	15	0	15	1

[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.

LOQ=0.0300 mg a.i./L

Male plasma vitellogenin (VTG) concentrations ranged from 299 ng/mL in the mean-measured 33 mg a.i./L treatment group to 1340 ng/mL in the mean-measured 0.23 mg a.i./L treatment group (Table 19). Female VTG concentrations ranged from 1442000 ng/mL in the mean-measured 6.2 mg a.i./L treatment group to 3191000 ng/mL in the negative control.

Table 19: Plasma Vitellogenin in Fathead Minnow (*Pimephales promelas*).

Treatment (mg a.i./L) [mean-measured]	Plasma Vitellogenin (VTG)					
	Males			Females		
	n	Mean (ng/mL plasma)	±SD	n	Mean (ng/mL plasma)	±SD
Negative Control (<LOQ)	4	1010	1140	4	3191000	1170000
0.046	4	774	310	4	2124000	807000
0.23	4	1340	2070	4	2226000	624000
1.2	4	752	1240	4	2195000	403000
6.2	4	385	367	4	1442000	550000
33	4	299[1]	231	4	2142000	356000

Abbreviations: SD Standard deviation.

[1] In replicate D of this treatment there were 3 males instead of the recommended 2 due to a mis-sexing error. If this fish is removed from analysis of VTG, the treatment means are very similar as when retained (327 when retained vs. 299 when mis-sexed removed). The values in this table reflect data excluding the mis-sexed male.

LOQ=0.0300 mg a.i./L

No plasma sex steroids were measured (Table 20).

Table 20: Plasma Sex Steroids in Fathead Minnow (*Pimephales promelas*). Not Measured.

Treatment (mg a.i./L) [mean-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	ND	ND	4	ND	ND	4	ND	ND	4	ND	ND
0.046	4	ND	ND	4	ND	ND	4	ND	ND	4	ND	ND
0.23	4	ND	ND	4	ND	ND	4	ND	ND	4	ND	ND
1.2	4	ND	ND	4	ND	ND	4	ND	ND	4	ND	ND
6.2	4	ND	ND	4	ND	ND	4	ND	ND	4	ND	ND
33	4	ND	ND	4	ND	ND	4	ND	ND	4	ND	ND

Abbreviations: ND Not determined. SD Standard deviation.

LOQ=0.0300 mg a.i./L

No secondary sex characteristics or clinical signs were observed in any males or females in the control or treatment groups (Table 21). All fish reported as normal during exposure.

Table 21: Secondary Sex Characteristics and Clinical Signs in Fathead Minnow (*Pimephales promelas*).

Treatment (mg a.i./L) [mean-measured]	Secondary Sex Characteristics and Clinical Signs					
	Males			Females		
	Type	n	Incidence	Type	n	Incidence
Negative Control (<LOQ)	None	8	--	None	16	--
0.046	None	8	--	None	16	--
0.23	None	8	--	None	16	--
1.2	None	7	--	None	16	--
6.2	None	8	--	None	16	--
33	None	9	--	None	15	--

LOQ=0.0300 mg a.i./L

B. Study Author's Analysis and Conclusions

Analyses were performed to evaluate differences between treatment and control groups for each of the following endpoints: survival, wet weight, total length, fecundity, fertility, GSI, VTG concentration, tubercle score, gonad developmental stage, and incidence and severity of gonad abnormalities.

Measurements of VTG are inherently variable, and boxplots of log transformed VTG values were used to identify potential outliers (Tukey's method) that might need special handling in the analyses. No outliers were excluded from analyses in this study.

Unless otherwise noted, replicate test chambers were used as the unit of statistical analysis. Males and females were analyzed separately for each endpoint when appropriate. Endpoints were first evaluated for monotonicity. Since the responses for all endpoints except male tubercle scores appeared to be monotonic, a step-down Jonckheere-Terpstra trend test was used to evaluate possible trends in the ranks of replicate means to determine concentration responsive trends among the treatment groups. Dunnett's test was used to evaluate male tubercle scores.

Survival and histopathology severity scores and stages were not amenable to the statistical methods used for analysis of other endpoints. In particular, the most suitable unit of statistical analysis for these endpoints was the individual animal. Therefore, survival was analyzed using Fisher's Exact test, and histopathology severity scores and stages of individuals were analyzed using step-down Jonckheere-Terpstra trend tests.

Statistical tests used to evaluate treatment effects were performed at confidence level of α=0.05 with SAS software.

There were no apparent effects on survival, growth, reproduction, secondary sex characteristics, GSI, VTG, or gonad histopathology in male or female fish exposed to glyphosate for 21 days up to a concentration of 33 mg a.i./L. Based on these endpoints, glyphosate does not appear to impact the

function of the hypothalamus-pituitary-gonadal (HPG) endocrine axis in *Pimephales promelas* under conditions and concentrations employed in the current test.

C. Reviewer's Analysis and Conclusions

Statistical Methods: A single male mortality occurred and was not considered to be treatment-related. Therefore, survival was not statistically analyzed. No endpoint exhibited a monotonic trend, and all endpoints were tested for homogeneity of variance and normality using Bartlett's Test (α=0.01) and the Shaprio-Wilks Test (α=0.01), respectively. Female median nuptial tubercle score, male median tubercle score, and male mean VTG concentration failed both tests and were analyzed using the Mann-Whitney U Two-Sample test. Fecundity was normally distributed but had unequal variance and was analyzed using the Dunnett T3 Multiple Comparison Test. All remaining endpoints were normally distributed with equal variance and were analyzed using the Dunnett Multiple Comparison Test.

Unless otherwise indicated, effects were considered statistically significant at p<0.05. Analyses were completed using CETIS.

Conclusions:

Female VTG concentrations were significantly reduced 55% at the 6.2 mg a.i./L treatment concentration. No other significant effects were observed in any treatment group relative to the negative control.

Table 22: Reproductive and HPG Endpoints[1,2] for Male Fathead Minnow (*Pimephales promelas*) in the FSTRA with Glyphosate.

Treatment (mg a.i./L) [mean-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)	17	NA	NA	NA	No	NA	NA	NA	NA	NA	NA
0.046	19	0.17	-24.8	0.26	No	-23.5	1.0	NA	NA	NA	NA
0.23	16	1.0	-3.4	1.0	No	32.0	0.89	NA	NA	NA	NA
1.2	19	0.09	-9.86	0.91	No	-25.7	1.0	NA	NA	NA	NA
6.2	17	1.0	-7.82	0.96	No	-61.9	0.69	NA	NA	NA	NA
33	15	0.86	2.89	1.0	No	-70.4	0.69	NA	NA	NA	NA
Statistical Test	Mann-Whitney		Dunnett		NA	Mann-Whitney		NA		NA	

Abbreviations: Diff. Difference. E2 17β-estradiol. GSI Gonado-Somatic Index. Histo. Histopathology. NA Not applicable. T Testosterone. VTG Vitellogenin.

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 $p<0.05$.

LOQ=0.0300 mg a.i./L

Table 23: Reproductive and HPG Endpoints[1,2] for Female Fathead Minnow (*Pimephales promelas*) in the FSTRA with Glyphosate.

Treatment (mg a.i./L) [mean-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)	NA	NA	NA	NA	0	NA	NA	NA	No	NA	NA	NA	NA	NA	NA
0.046	24.8	0.34	0.33	1.0	0	1.0	-2.22	1.0	No	-33.5	0.16	NA	NA	NA	NA
0.23	-3.41	1.0	1.18	0.77	0	1.0	-10.8	0.84	No	-30.3	0.23	NA	NA	NA	NA
1.2	6.08	0.14	-1.36	0.67	0	1.0	-4.85	0.99	No	-31.3	0.21	NA	NA	NA	NA
6.2	19.6	0.62	0.87	0.91	0	1.0	5.38	0.99	No	-54.8	0.01	NA	NA	NA	NA
33	-0.53	1.0	-0.64	0.97	0	1.0	7.40	0.96	No	-32.9	0.17	NA	NA	NA	NA
Statistical Test	Dunnett T3		Dunnett		Mann-Whitney		Dunnett		NA	Dunnett		NA		NA	

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

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 $p<0.05$.

LOQ=0.0300 mg a.i./L

Table 24: Growth Endpoints[1,2] in the Fish Short-Term Reproduction Assay (FSTRA) with Glyphosate.

Treatment (mg a.i./L) [mean-measured]	Body Weight				Length			
	Males		Females		Males		Females	
	% Diff.	*p*	% Diff.	*p*	% Diff.	*p*	% Diff.	*p*
Negative Control (<LOQ)	--	--	--	--	--	--	--	--
0.046	-1.48	1.0	-3.08	0.94	-2.74	0.83	-0.41	1.0
0.23	3.41	1.0	-8.57	0.26	0.23	1.0	-2.44	0.30
1.2	-0.23	1.0	-1.32	1.0	-2.28	0.90	-1.31	0.80
6.2	-2.61	1.0	-5.93	0.58	-2.51	0.87	-1.62	0.65
33	-7.26	0.93	0.66	1.0	-5.25	0.31	-0.54	0.99
Statistical Test	Dunnett		Dunnett		Dunnett		Dunnett	

Abbreviations: Diff. Difference.

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 $p<0.05$.

LOQ=0.0300 mg a.i./L

E. Study Deficiencies

There was one deviation from the performance criteria: Temperature exceeded the recommended range 25±1°C, for less than 24 hours on Day 7 when the maximum temperature reached 29.1°C (deviation occurred in replicates B, C, and D in 1.2 mg a.i./L and replicates A, B, and C in 6.2 mg a.i./L where temperatures ranged from 28.6 to 29.1°C). All fish were reported as normal throughout the test, and there was no mortality during this temperature deviation. This deviation did not have an impact on the interpretation of the results of this study.

F. Reviewer's Comments

The reviewer's results were slightly more conservative than those of the study authors in that the review detected a significant reduction in female VTG concentration at the mean-measured 6.2 mg a.i./L treatment level whereas the study authors found no differences. Therefore, the reviewer's results are reported in the Executive Summary of this DER.

Results from the periodic screening analysis of the dilution water indicated the presence of the following metals: calcium (34.9 mg/L), chloride (4.5 mg/L), fluoride (0.85 mg/L) magnesium (13.2 mg/L), potassium (7.00 mg/L), sodium (19.0 mg/L), and sulfate (5.7 mg/L). The TOC concentration during the 4-week period prior to the test was <1 mg/L, and no pesticides or organics were detected.

It is also noted that there were 3 males and 3 females (as oppose to recommended 2 males, 4 females) in replicate D of the 33 mg a.i./L treatment group due to misidentification during allocation at pre-exposure. Inclusion or exclusion of gender-specific data from this fish did not affect interpretation of the results in the study.

The in-life portion of the definitive toxicity was conducted from December 21, 2011 to January 11, 2012.

III. REFERENCES

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The SAS System for Windows. 1999-2001. Version 8.2. SAS Institute, Inc., Cary, North Carolina.

OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Wildlife International**

Analysis ID:	16-0647-3496	**Endpoint:**	Fecundity	**CETIS Version:**	CETISv1.8.7
Analyzed:	22 Feb-13 10:37	**Analysis:**	Parametric-Multiple Comparison	**Official Results:**	Yes
Batch ID:	09-5292-6152	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	21 Dec-11	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	Well Water
Ending Date:	11 Jan-12	**Species:**	Pimephales promelas	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Osage Catfisheries, Osage Beach, MI	**Age:**	6 mo

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	23.5%	33	>33	NA	

Dunnett T3 Multiple Comparison Test

Control	vs	C-mg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.046	2.18	5	13.4	3	0.3425	CDF	Non-Significant Effect
		0.23	0.298	5	13.4	3	0.9985	CDF	Non-Significant Effect
		1.2	3.02	4.3	2.03	4	0.1429	CDF	Non-Significant Effect
		6.2	1.45	5	15.9	3	0.6192	CDF	Non-Significant Effect
		33	0.111	4.87	5.5	3	1.0000	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	147.8537	29.57075	5	1.71	0.1827	Non-Significant Effect
Error	310.7525	17.26403	18			
Total	458.6063		23			

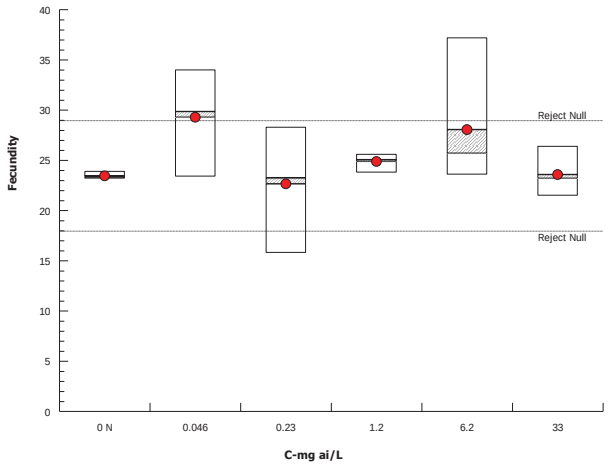
Distributional Tests

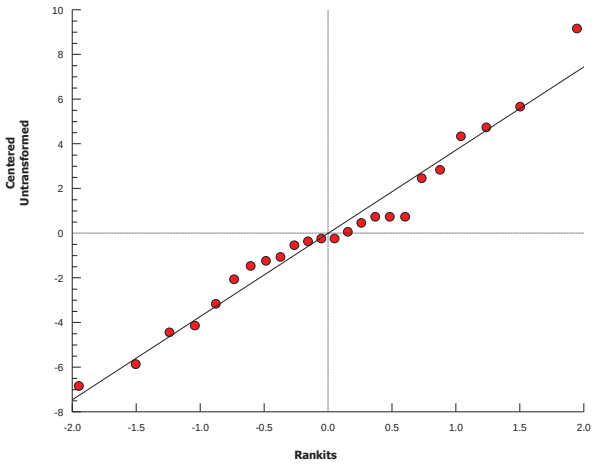
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	20	15.1	0.0013	Unequal Variances
Distribution	Shapiro-Wilk W Normality	0.971	0.884	0.6823	Normal Distribution

Fecundity Summary

C-mg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	23.5	22.9	24	23.4	23.2	23.9	0.166	1.41%	0.0%
0.046		4	29.3	20.8	37.8	29.8	23.4	34	2.67	18.2%	-24.8%
0.23		4	22.6	14.1	31.2	23.3	15.8	28.3	2.68	23.7%	3.41%
1.2		4	24.9	23.5	26.3	25	23.8	25.6	0.442	3.56%	-6.08%
6.2		4	28.1	17.9	38.2	25.7	23.6	37.2	3.18	22.6%	-19.6%
33		4	23.6	20	27.1	23.2	21.5	26.4	1.12	9.48%	-0.53%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Wildlife International**

Analysis ID:	17-4558-5072	**Endpoint:**	FemaleBodyWt	**CETIS Version:**	CETISv1.8.7
Analyzed:	22 Feb-13 10:36	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	09-5292-6152	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	21 Dec-11	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	Well Water
Ending Date:	11 Jan-12	**Species:**	Pimephales promelas	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Osage Catfisheries, Osage Beach, MI	**Age:**	6 mo

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	12.7%	33	>33	NA	

Dunnett Multiple Comparison Test

Control	vs	C-mg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.046	0.671	2.76	0.144	6	0.9391	CDF	Non-Significant Effect
		0.23	1.87	2.76	0.144	6	0.2573	CDF	Non-Significant Effect
		1.2	0.288	2.76	0.144	6	0.9984	CDF	Non-Significant Effect
		6.2	1.29	2.76	0.144	6	0.5782	CDF	Non-Significant Effect
		33	0.144	2.76	0.144	6	0.9999	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.02927083	0.005854166	5	1.08	0.4057	Non-Significant Effect
Error	0.097825	0.005434722	18			
Total	0.1270958		23			

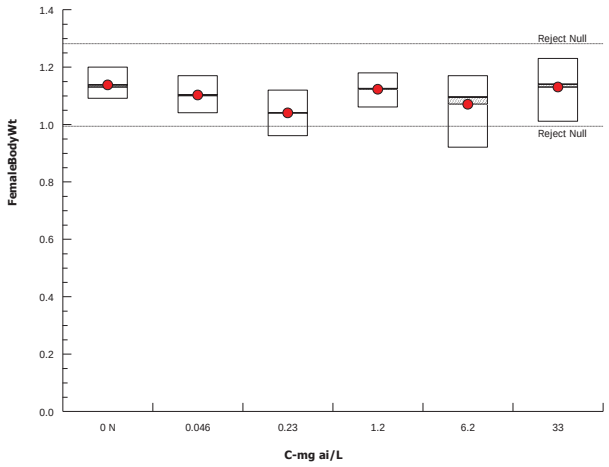
Distributional Tests

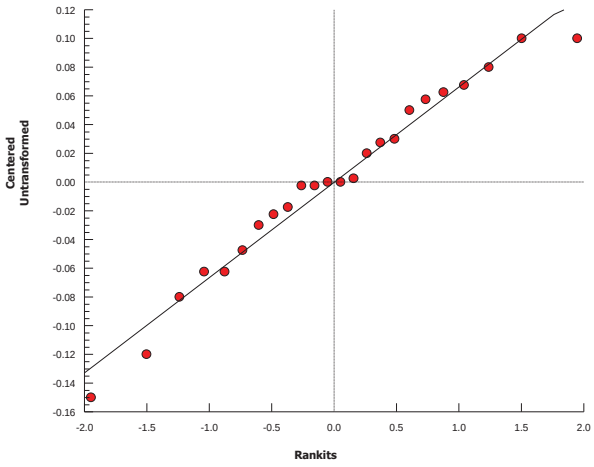
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	3.08	15.1	0.6879	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.969	0.884	0.6380	Normal Distribution

FemaleBodyWt Summary

C-mg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	1.14	1.06	1.21	1.13	1.09	1.2	0.0232	4.08%	0.0%
0.046		4	1.1	1.02	1.19	1.1	1.04	1.17	0.0266	4.82%	3.08%
0.23		4	1.04	0.929	1.15	1.04	0.96	1.12	0.0349	6.71%	8.57%
1.2		4	1.12	1.04	1.21	1.13	1.06	1.18	0.0266	4.74%	1.32%
6.2		4	1.07	0.898	1.24	1.1	0.92	1.17	0.054	10.1%	5.93%
33		4	1.13	0.985	1.27	1.14	1.01	1.23	0.0455	8.05%	0.66%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Wildlife International**

Analysis ID:	06-5595-3988	**Endpoint:**	FemaleGSI	**CETIS Version:**	CETISv1.8.7
Analyzed:	22 Feb-13 10:36	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	09-5292-6152	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	21 Dec-11	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	Well Water
Ending Date:	11 Jan-12	**Species:**	Pimephales promelas	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Osage Catfisheries, Osage Beach, MI	**Age:**	6 mo

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	33.6%	33	>33	NA	

Dunnett Multiple Comparison Test

Control	vs	C-mg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.046	0.183	2.76	4.94	6	0.9998	CDF	Non-Significant Effect
		0.23	0.887	2.76	4.94	6	0.8418	CDF	Non-Significant Effect
		1.2	0.399	2.76	4.94	6	0.9930	CDF	Non-Significant Effect
		6.2	0.443	2.76	4.94	6	0.9889	CDF	Non-Significant Effect
		33	0.609	2.76	4.94	6	0.9582	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	19.44667	3.889335	5	0.607	0.6959	Non-Significant Effect
Error	115.4119	6.411773	18			
Total	134.8586		23			

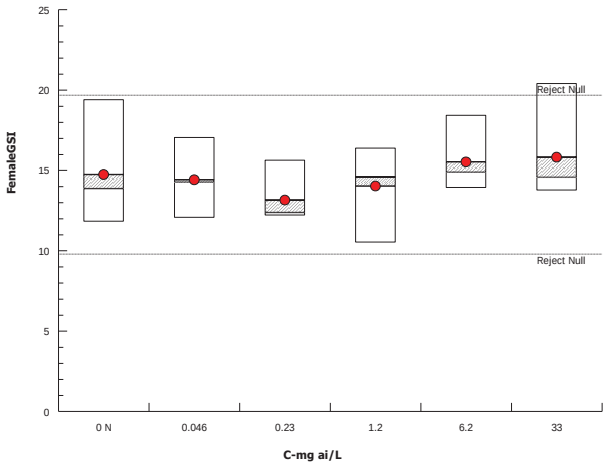
Distributional Tests

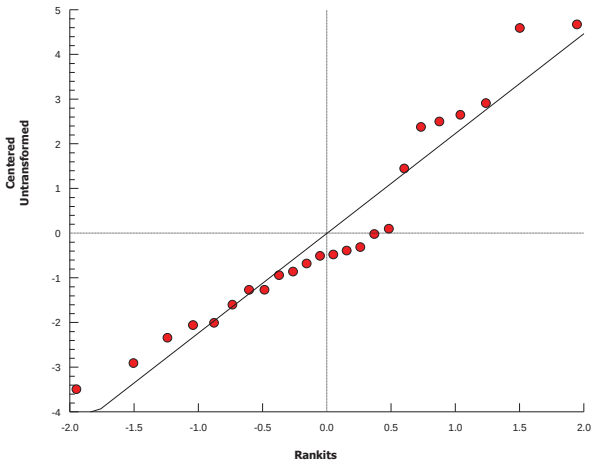
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	1.83	15.1	0.8722	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.928	0.884	0.0876	Normal Distribution

FemaleGSI Summary

C-mg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	14.7	9.53	19.9	13.9	11.8	19.4	1.64	22.2%	0.0%
0.046		4	14.4	11.1	17.7	14.3	12.1	17	1.03	14.3%	2.22%
0.23		4	13.1	10.5	15.8	12.4	12.2	15.6	0.833	12.7%	10.8%
1.2		4	14	9.9	18.1	14.6	10.5	16.4	1.29	18.4%	4.85%
6.2		4	15.5	12.3	18.8	14.9	13.9	18.4	1.03	13.2%	-5.38%
33		4	15.8	10.8	20.8	14.6	13.8	20.4	1.57	19.9%	-7.4%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) **Wildlife International**

Analysis ID:	00-3851-8392	**Endpoint:**	FemaleLength	**CETIS Version:**	CETISv1.8.7
Analyzed:	22 Feb-13 10:35	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	09-5292-6152	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	21 Dec-11	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	Well Water
Ending Date:	11 Jan-12	**Species:**	Pimephales promelas	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Osage Catfisheries, Osage Beach, MI	**Age:**	6 mo

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	3.79%	33	>33	NA	

Dunnett Multiple Comparison Test

Control	vs	C-mg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.046	0.296	2.76	1.75	6	0.9982	CDF	Non-Significant Effect
		0.23	1.78	2.76	1.75	6	0.2987	CDF	Non-Significant Effect
		1.2	0.955	2.76	1.75	6	0.8020	CDF	Non-Significant Effect
		6.2	1.18	2.76	1.75	6	0.6528	CDF	Non-Significant Effect
		33	0.395	2.76	1.75	6	0.9933	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	3.492687	0.6985374	5	0.871	0.5198	Non-Significant Effect
Error	14.43917	0.8021764	18			
Total	17.93186		23			

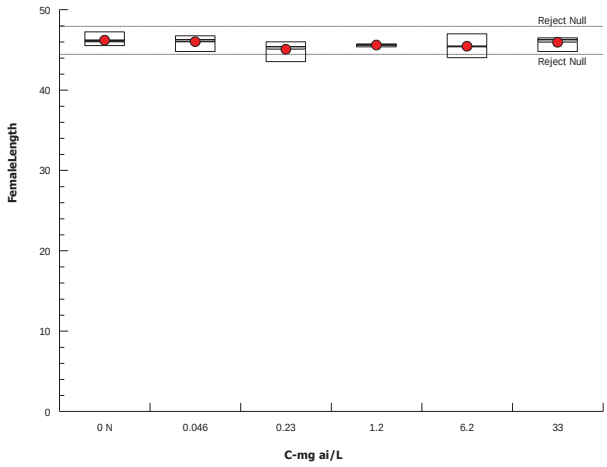
Distributional Tests

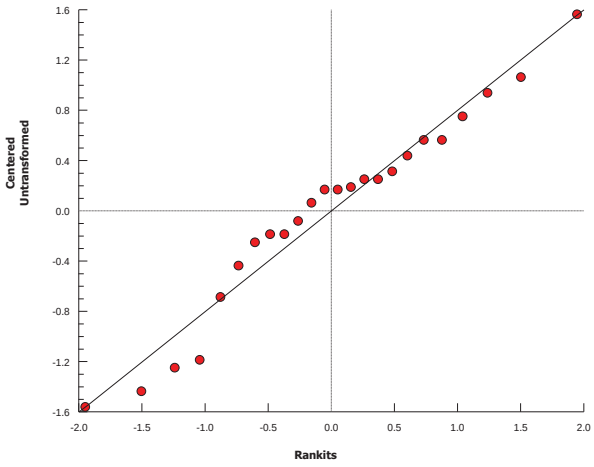
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	6.53	15.1	0.2577	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.959	0.884	0.4282	Normal Distribution

FemaleLength Summary

C-mg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	46.2	45	47.4	46	45.5	47.3	0.373	1.62%	0.0%
0.046		4	46	44.6	47.4	46.3	44.8	46.8	0.433	1.88%	0.41%
0.23		4	45.1	43.3	46.8	45.4	43.5	46	0.544	2.41%	2.44%
1.2		4	45.6	45.3	45.9	45.6	45.3	45.8	0.103	0.45%	1.31%
6.2		4	45.4	43.4	47.4	45.4	44	47	0.632	2.78%	1.62%
33		4	45.9	44.6	47.3	46.3	44.8	46.5	0.413	1.8%	0.54%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) **Wildlife International**

Analysis ID:	18-9864-2048	**Endpoint:**	FemaleMedianTubercleScore	**CETIS Version:**	CETISv1.8.7
Analyzed:	22 Feb-13 10:35	**Analysis:**	Nonparametric-Two Sample	**Official Results:**	Yes
Batch ID:	09-5292-6152	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	21 Dec-11	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	Well Water
Ending Date:	11 Jan-12	**Species:**	Pimephales promelas	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Osage Catfisheries, Osage Beach, MI	**Age:**	6 mo

Data Transform	Zeta	Alt Hyp	Trials	Seed	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	33	>33	NA	

Mann-Whitney U Two-Sample Test

Control	vs	C-mg ai/L	Test Stat	Critical	Ties	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.046	8	NA	1	6	1.0000	Exact	Non-Significant Effect
		0.23	8	NA	1	6	1.0000	Exact	Non-Significant Effect
		1.2	8	NA	1	6	1.0000	Exact	Non-Significant Effect
		6.2	8	NA	1	6	1.0000	Exact	Non-Significant Effect
		33	8	NA	1	6	1.0000	Exact	Non-Significant Effect

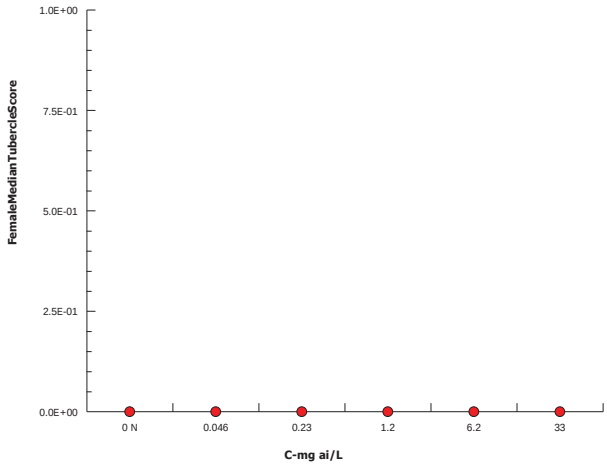
ANOVA Table

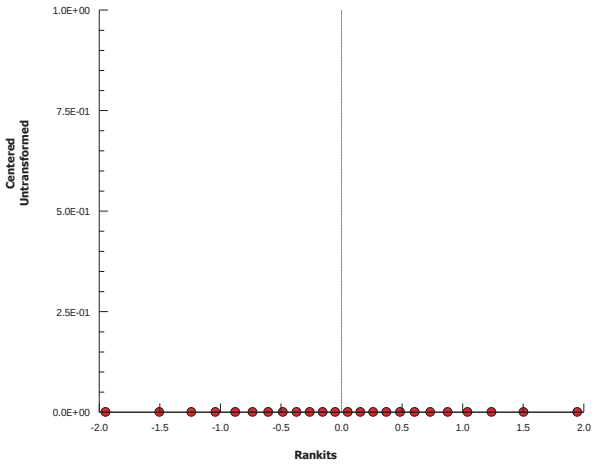
Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0	0	5	65500	<0.0001	Significant Effect
Error	0	0	18			
Total	0		23			

FemaleMedianTubercleScore Summary

C-mg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	0	0	0	0	0	0	0		
0.046		4	0	0	0	0	0	0	0		
0.23		4	0	0	0	0	0	0	0		
1.2		4	0	0	0	0	0	0	0		
6.2		4	0	0	0	0	0	0	0		
33		4	0	0	0	0	0	0	0		

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) **Wildlife International**

Analysis ID:	20-1443-6213	**Endpoint:**	FemaleVTG	**CETIS Version:**	CETISv1.8.7
Analyzed:	22 Feb-13 10:34	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	09-5292-6152	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	21 Dec-11	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	Well Water
Ending Date:	11 Jan-12	**Species:**	Pimephales promelas	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Osage Catfisheries, Osage Beach, MI	**Age:**	6 mo

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	43.3%	1.2	6.2	2.728	

Dunnett Multiple Comparison Test

Control	vs	C-mg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.046	2.14	2.76	1E+06	6	0.1638	CDF	Non-Significant Effect
		0.23	1.93	2.76	1E+06	6	0.2329	CDF	Non-Significant Effect
		1.2	2	2.76	1E+06	6	0.2092	CDF	Non-Significant Effect
		6.2*	3.5	2.76	1E+06	6	0.0108	CDF	Significant Effect
		33	2.1	2.76	1E+06	6	0.1745	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	6.26239E+12	1.252478E+12	5	2.5	0.0690	Non-Significant Effect
Error	9.012601E+12	5.007001E+11	18			
Total	1.527499E+13		23			

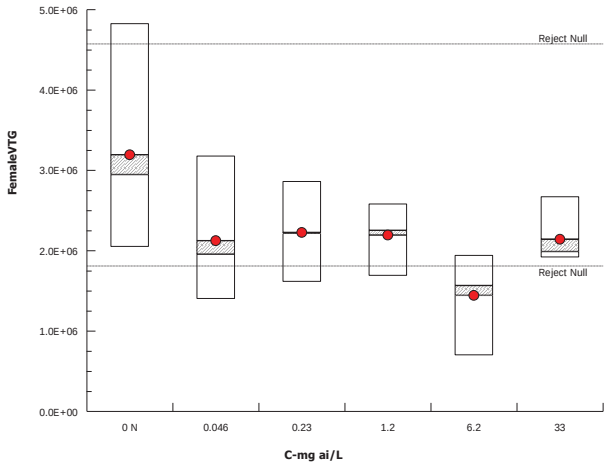
Distributional Tests

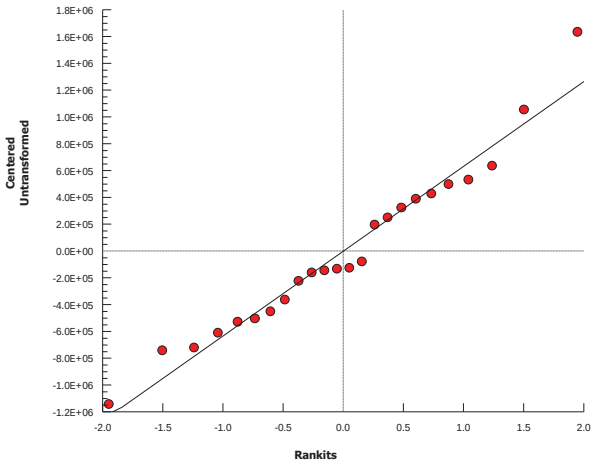
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	5.29	15.1	0.3816	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.967	0.884	0.6036	Normal Distribution

FemaleVTG Summary

C-mg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	3.19E+6	1.33E+6	5.06E+6	2950000	2.05E+6	4.83E+6	5.86E+5	36.7%	0.0%
0.046		4	2.12E+6	8.40E+5	3.41E+6	1960000	1.40E+6	3.18E+6	4.03E+5	38.0%	33.5%
0.23		4	2.23E+6	1.24E+6	3.22E+6	2210000	1.62E+6	2.86E+6	3.11E+5	28.0%	30.3%
1.2		4	2.19E+6	1.55E+6	2.83E+6	2250000	1.69E+6	2.58E+6	2.01E+5	18.3%	31.3%
6.2		4	1.44E+6	5.67E+5	2.32E+6	1570000	7.01E+5	1.94E+6	2.75E+5	38.1%	54.8%
33		4	2.14E+6	1.58E+6	2.71E+6	1990000	1.92E+6	2.67E+6	1.78E+5	16.6%	32.9%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Wildlife International**

Analysis ID:	04-9288-1468	**Endpoint:**	Fertility	**CETIS Version:**	CETISv1.8.7
Analyzed:	22 Feb-13 10:34	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	09-5292-6152	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	21 Dec-11	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	Well Water
Ending Date:	11 Jan-12	**Species:**	Pimephales promelas	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Osage Catfisheries, Osage Beach, MI	**Age:**	6 mo

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	3.24%	33	>33	NA	

Dunnett Multiple Comparison Test

Control	vs	C-mg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.046	0.285	2.76	3.15	6	0.9985	CDF	Non-Significant Effect
		0.23	1.01	2.76	3.15	6	0.7699	CDF	Non-Significant Effect
		1.2	1.16	2.76	3.15	6	0.6690	CDF	Non-Significant Effect
		6.2	0.744	2.76	3.15	6	0.9114	CDF	Non-Significant Effect
		33	0.547	2.76	3.15	6	0.9727	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	17.09373	3.418745	5	1.31	0.3034	Non-Significant Effect
Error	46.9425	2.607917	18			
Total	64.03622		23			

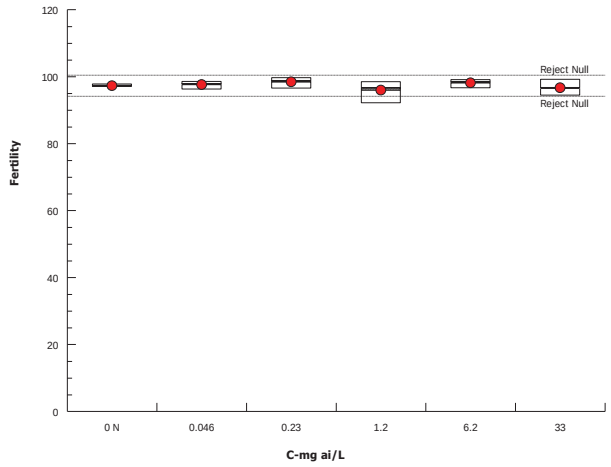
Distributional Tests

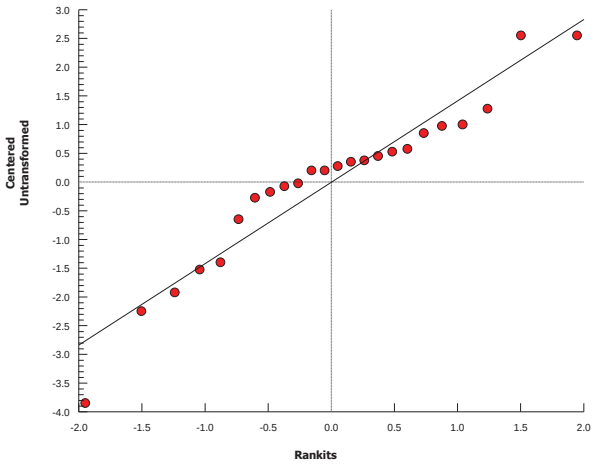
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	9.52	15.1	0.0899	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.934	0.884	0.1212	Normal Distribution

Fertility Summary

C-mg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	97.3	96.7	97.8	97.1	97	97.8	0.18	0.37%	0.0%
0.046		4	97.6	96	99.2	97.8	96.2	98.6	0.503	1.03%	-0.33%
0.23		4	98.4	96.3	101	98.8	96.5	99.7	0.68	1.38%	-1.18%
1.2		4	96	91.6	100	96.6	92.1	98.5	1.36	2.84%	1.36%
6.2		4	98.1	96.4	99.9	98.4	96.6	99.1	0.548	1.12%	-0.87%
33		4	96.6	93.5	99.8	96.5	94.4	99.2	1	2.08%	0.64%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) **Wildlife International**

Analysis ID:	06-1661-0832	**Endpoint:**	MaleBodyWt	**CETIS Version:**	CETISv1.8.7
Analyzed:	22 Feb-13 10:34	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	09-5292-6152	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	21 Dec-11	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	Well Water
Ending Date:	11 Jan-12	**Species:**	Pimephales promelas	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Osage Catfisheries, Osage Beach, MI	**Age:**	6 mo

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	29.0%	33	>33	NA	

Dunnett Multiple Comparison Test

Control	vs	C-mg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.046	0.141	2.76	0.638	6	1.0000	CDF	Non-Significant Effect
		0.23	0.324	2.76	0.638	6	0.9973	CDF	Non-Significant Effect
		1.2	0.0216	2.76	0.638	6	1.0000	CDF	Non-Significant Effect
		6.2	0.249	2.76	0.638	6	0.9992	CDF	Non-Significant Effect
		33	0.671	2.76	0.638	6	0.9394	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.1157334	0.02314668	5	0.217	0.9508	Non-Significant Effect
Error	1.9232	0.1068444	18			
Total	2.038934		23			

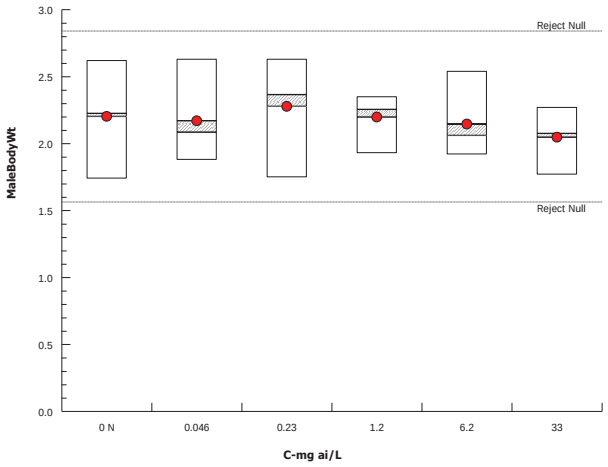
Distributional Tests

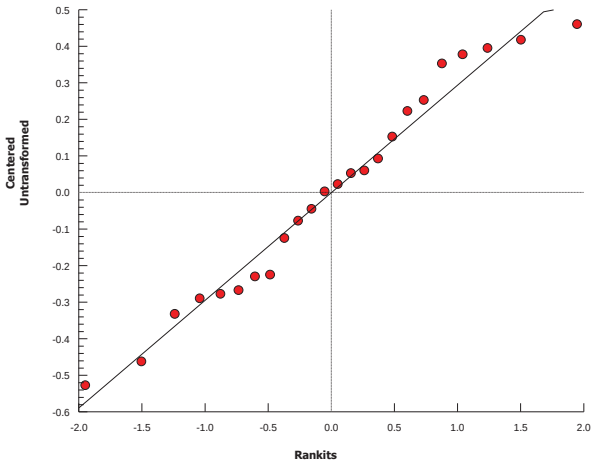
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	3.23	15.1	0.6644	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.961	0.884	0.4643	Normal Distribution

MaleBodyWt Summary

C-mg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	2.2	1.47	2.94	2.22	1.74	2.62	0.231	21.0%	0.0%
0.046		4	2.17	1.62	2.72	2.09	1.88	2.63	0.171	15.8%	1.48%
0.23		4	2.28	1.65	2.91	2.37	1.75	2.63	0.198	17.4%	-3.41%
1.2		4	2.2	1.9	2.49	2.26	1.93	2.35	0.093	8.47%	0.23%
6.2		4	2.14	1.71	2.58	2.06	1.92	2.54	0.137	12.7%	2.61%
33		4	2.05	1.72	2.38	2.07	1.77	2.27	0.104	10.1%	7.04%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Wildlife International**

Analysis ID:	13-6344-6785	**Endpoint:**	MaleGSI	**CETIS Version:**	CETISv1.8.7
Analyzed:	22 Feb-13 10:33	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	09-5292-6152	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	21 Dec-11	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	Well Water
Ending Date:	11 Jan-12	**Species:**	Pimephales promelas	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Osage Catfisheries, Osage Beach, MI	**Age:**	6 mo

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	36.6%	33	>33	NA	

Dunnett Multiple Comparison Test

Control	vs	C-mg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.046	1.87	2.76	0.538	6	0.2557	CDF	Non-Significant Effect
		0.23	0.257	2.76	0.538	6	0.9991	CDF	Non-Significant Effect
		1.2	0.745	2.76	0.538	6	0.9114	CDF	Non-Significant Effect
		6.2	0.591	2.76	0.538	6	0.9629	CDF	Non-Significant Effect
		33	0.218	2.76	0.538	6	0.9996	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.4204208	0.08408416	5	1.11	0.3904	Non-Significant Effect
Error	1.365375	0.07585417	18			
Total	1.785796		23			

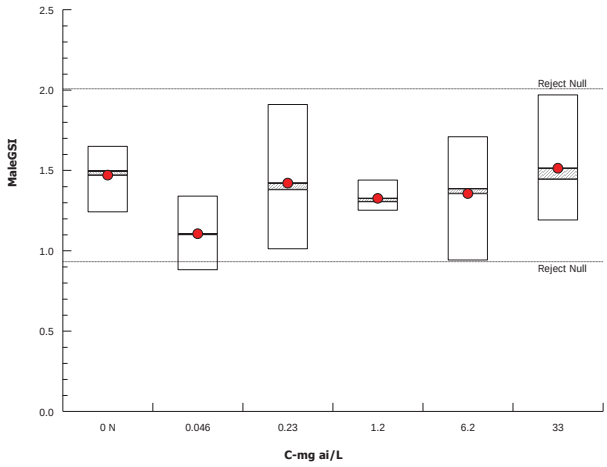
Distributional Tests

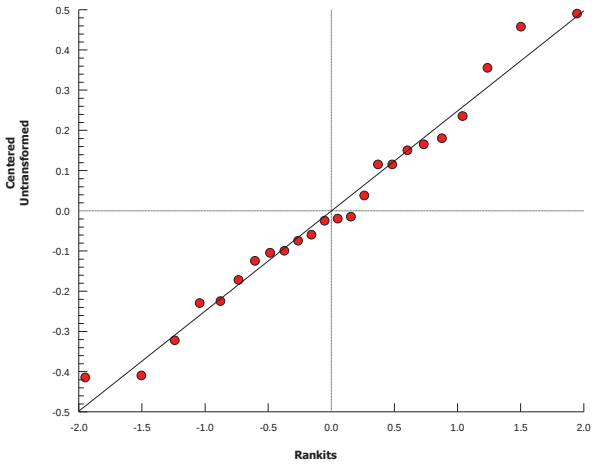
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	5.92	15.1	0.3139	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.974	0.884	0.7686	Normal Distribution

MaleGSI Summary

C-mg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	1.47	1.15	1.79	1.5	1.24	1.65	0.0991	13.5%	0.0%
0.046		4	1.11	0.768	1.44	1.1	0.88	1.34	0.106	19.2%	24.8%
0.23		4	1.42	0.83	2.01	1.38	1.01	1.91	0.185	26.1%	3.4%
1.2		4	1.32	1.2	1.45	1.3	1.25	1.44	0.0405	6.12%	9.86%
6.2		4	1.36	0.822	1.89	1.38	0.94	1.71	0.167	24.7%	7.82%
33		4	1.51	0.973	2.05	1.44	1.19	1.97	0.169	22.4%	-2.89%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) **Wildlife International**

Analysis ID:	04-9763-6661	**Endpoint:**	MaleLength	**CETIS Version:**	CETISv1.8.7
Analyzed:	22 Feb-13 10:32	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	09-5292-6152	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	21 Dec-11	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	Well Water
Ending Date:	11 Jan-12	**Species:**	Pimephales promelas	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Osage Catfisheries, Osage Beach, MI	**Age:**	6 mo

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	8.26%	33	>33	NA	

Dunnett Multiple Comparison Test

Control	vs	C-mg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.046	0.916	2.76	4.52	6	0.8252	CDF	Non-Significant Effect
		0.23	0.0763	2.76	4.52	6	1.0000	CDF	Non-Significant Effect
		1.2	0.763	2.76	4.52	6	0.9033	CDF	Non-Significant Effect
		6.2	0.84	2.76	4.52	6	0.8669	CDF	Non-Significant Effect
		33	1.76	2.76	4.52	6	0.3086	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	24.42708	4.885417	5	0.911	0.4960	Non-Significant Effect
Error	96.5625	5.364583	18			
Total	120.9896		23			

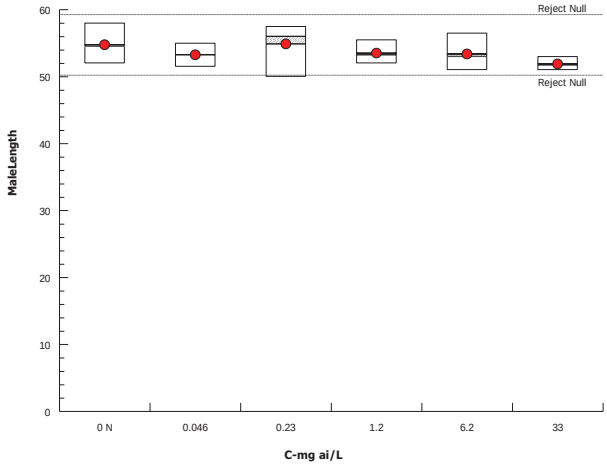
Distributional Tests

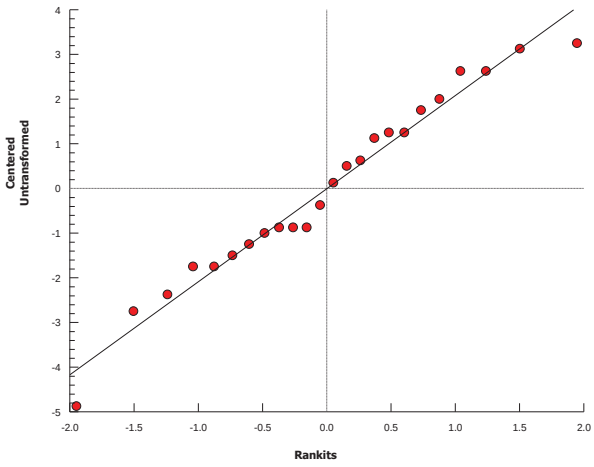
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	4.6	15.1	0.4661	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.969	0.884	0.6512	Normal Distribution

MaleLength Summary

C-mg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	54.8	50.4	59.1	54.5	52	58	1.38	5.03%	0.0%
0.046		4	53.3	50.5	56	53.3	51.5	55	0.878	3.3%	2.74%
0.23		4	54.9	49.2	60.5	56	50	57.5	1.77	6.46%	-0.23%
1.2		4	53.5	51	56	53.3	52	55.5	0.791	2.96%	2.28%
6.2		4	53.4	49.7	57.1	53	51	56.5	1.16	4.35%	2.51%
33		4	51.9	50.2	53.5	51.8	51	53	0.515	1.99%	5.25%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Wildlife International**

Analysis ID:	01-6732-3416	**Endpoint:**	MaleMedianTubercleScore	**CETIS Version:**	CETISv1.8.7
Analyzed:	22 Feb-13 10:32	**Analysis:**	Nonparametric-Two Sample	**Official Results:**	Yes
Batch ID:	09-5292-6152	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	21 Dec-11	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	Well Water
Ending Date:	11 Jan-12	**Species:**	Pimephales promelas	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Osage Catfisheries, Osage Beach, MI	**Age:**	6 mo

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	64.7%	33	>33	NA	

Mann-Whitney U Two-Sample Test

Control	vs	C-mg ai/L	Test Stat	Critical	Ties	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.046	13	NA	0	6	0.1714	Exact	Non-Significant Effect
		0.23	8	NA	0	6	1.0000	Exact	Non-Significant Effect
		1.2	15	NA	1	6	0.0857	Exact	Non-Significant Effect
		6.2	8	NA	0	6	1.0000	Exact	Non-Significant Effect
		33	9	NA	0	6	0.8571	Exact	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	108.125	21.625	5	0.577	0.7173	Non-Significant Effect
Error	675	37.5	18			
Total	783.125		23			

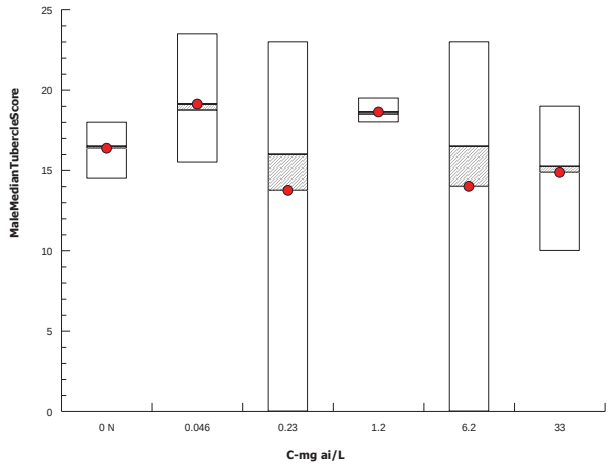
Distributional Tests

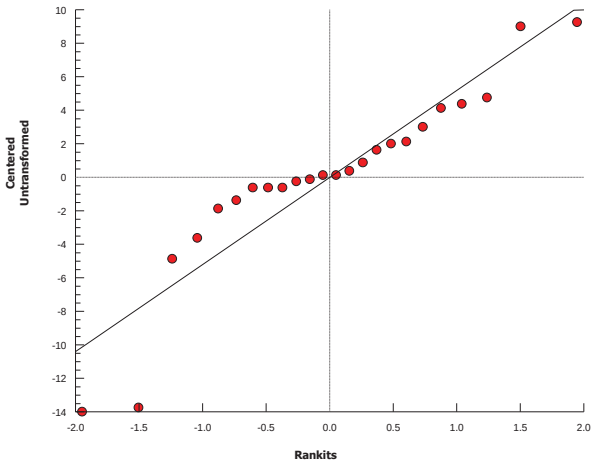
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	19.4	15.1	0.0017	Unequal Variances
Distribution	Shapiro-Wilk W Normality	0.874	0.884	0.0064	Non-normal Distribution

MaleMedianTubercleScore Summary

C-mg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	16.4	14.1	18.7	16.5	14.5	18	0.718	8.77%	0.0%
0.046		4	19.1	13.9	24.4	18.8	15.5	23.5	1.65	17.3%	-16.8%
0.23		4	13.8	-2.09	29.6	16	0	23	4.98	72.4%	16.0%
1.2		4	18.6	17.4	19.8	18.5	18	19.5	0.375	4.03%	-13.7%
6.2		4	14	-1.64	29.6	16.5	0	23	4.92	70.2%	14.5%
33		4	14.9	8.56	21.2	15.3	10	19	1.98	26.7%	9.16%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) **Wildlife International**

Analysis ID:	03-8602-7723	**Endpoint:**	MaleVTG	**CETIS Version:**	CETISv1.8.7
Analyzed:	22 Feb-13 10:31	**Analysis:**	Nonparametric-Two Sample	**Official Results:**	Yes
Batch ID:	09-5292-6152	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	21 Dec-11	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	Well Water
Ending Date:	11 Jan-12	**Species:**	Pimephales promelas	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Osage Catfisheries, Osage Beach, MI	**Age:**	6 mo

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	190.0%	33	>33	NA	

Mann-Whitney U Two-Sample Test

Control	vs	C-mg ai/L	Test Stat	Critical	Ties	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.046	8	NA	0	6	1.0000	Exact	Non-Significant Effect
		0.23	9	NA	0	6	0.8857	Exact	Non-Significant Effect
		1.2	8	NA	0	6	1.0000	Exact	Non-Significant Effect
		6.2	10	NA	0	6	0.6857	Exact	Non-Significant Effect
		33	9	NA	0	6	0.8857	Exact	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	2891146	578229.2	5	0.469	0.7942	Non-Significant Effect
Error	22184360	1232464	18			
Total	25075500		23			

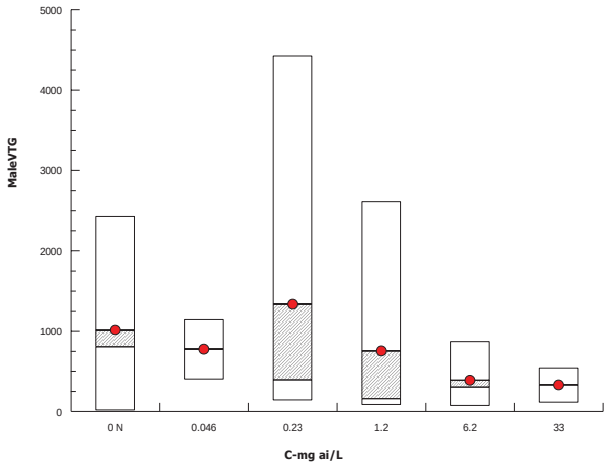
Distributional Tests

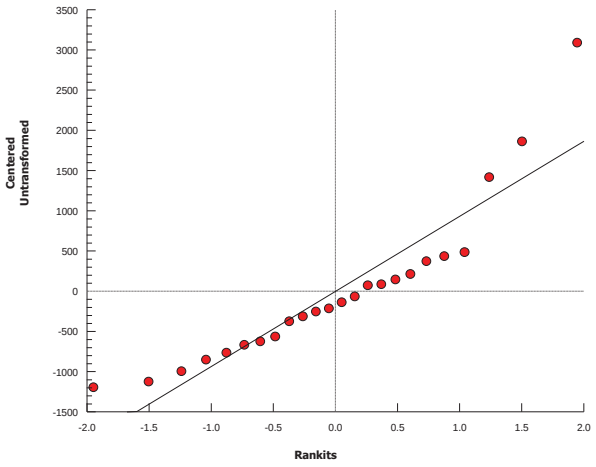
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	17.5	15.1	0.0037	Unequal Variances
Distribution	Shapiro-Wilk W Normality	0.859	0.884	0.0033	Non-normal Distribution

MaleVTG Summary

C-mg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	1010	-806	2830	803	15	2430	571	113.0%	0.0%
0.046		4	774	280	1270	777	398	1150	155	40.1%	23.5%
0.23		4	1340	-1950	4630	390	139	4420	1030	155.0%	-32.0%
1.2		4	752	-1220	2730	156	83	2610	620	165.0%	25.7%
6.2		4	385	-199	970	301	71	869	184	95.3%	61.9%
33		4	327	-6.7	661	329	111	539	105	64.1%	67.7%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) **Wildlife International**

Analysis ID:	13-5082-8160	**Endpoint:**	MaleVTG	**CETIS Version:**	CETISv1.8.7
Analyzed:	22 Feb-13 10:38	**Analysis:**	Nonparametric-Control vs Ord. Treatments	**Official Results:**	Yes
Batch ID:	09-5292-6152	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	21 Dec-11	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	Well Water
Ending Date:	11 Jan-12	**Species:**	Pimephales promelas	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Osage Catfisheries, Osage Beach, MI	**Age:**	6 mo

Data Transform	Zeta	Alt Hyp	Trials	Seed	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	33	>33	NA	

Jonckheere-Terpstra Step-Down Test

Control	vs	C-mg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.046	8	NA		-2	1.0000	Exact	Non-Significant Effect
		0.23	26	NA		-2	0.8311	Exact	Non-Significant Effect
		1.2	58	NA		-2	0.3819	Exact	Non-Significant Effect
		6.2	99	NA		-2	0.2226	Exact	Non-Significant Effect
		33	146	NA		-2	0.2010	Exact	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	2891146	578229.2	5	0.469	0.7942	Non-Significant Effect
Error	22184360	1232464	18			
Total	25075500		23			

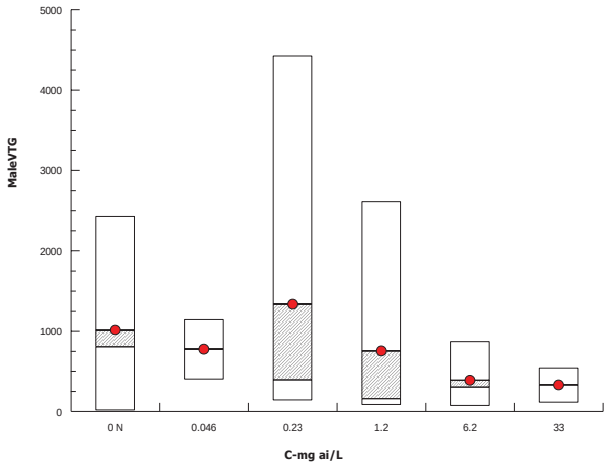
Distributional Tests

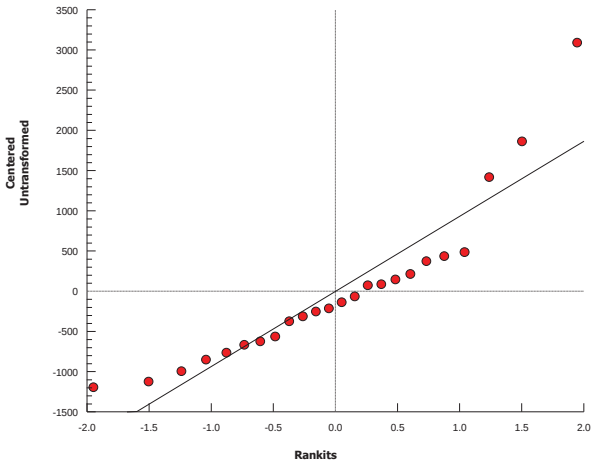
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	17.5	15.1	0.0037	Unequal Variances
Distribution	Shapiro-Wilk W Normality	0.859	0.884	0.0033	Non-normal Distribution

MaleVTG Summary

C-mg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	1010	-806	2830	803	15	2430	571	113.0%	0.0%
0.046		4	774	280	1270	777	398	1150	155	40.1%	23.5%
0.23		4	1340	-1950	4630	390	139	4420	1030	155.0%	-32.0%
1.2		4	752	-1220	2730	156	83	2610	620	165.0%	25.7%
6.2		4	385	-199	970	301	71	869	184	95.3%	61.9%
33		4	327	-6.7	661	329	111	539	105	64.1%	67.7%

Graphics





DATA EVALUATION RECORD

GLYPHOSATE

Study Type: OCSPP 890.1400, *In vivo* Hershberger Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-34-2012 (MRID 48617001)

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 Luck, M.S.	Date:	04/10/2012
Secondary Reviewer:	Signature:	
David A. McEwen, B.S.	Date:	04/16/2012
Program Manager:	Signature:	
Jack D. Early, M.S.	Date:	4/18/2012
Quality Assurance:	Signature:	
Jack D. Early, M.S.	Date:	4/18/2012

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: Anwar Y. Dunbar, Ph.D. **Signature:**
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 05-27-15
Secondary Reviewer: Greg Akerman, Ph.D. **Signature:**
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 6/15/15

DATA EVALUATION RECORD

STUDY TYPE: *In Vivo* Hershberger Assay (Rat); OCSPP 890.1400; OECD 441

PC CODE: 417300 **DP BARCODE:** D398693

TXR#: 0053233 **CAS#:** 1071-83-6

TEST MATERIAL (PURITY): Glyphosate (85.1% a.i.)

SYNONYMS: N-(phosphonomethyl)glycine

CITATION: Stump, D. G. (2012). A Hershberger Assay of Glyphosate Administered Orally in Peripubertal Orchidoepididymectomized Rats. WIL Research Laboratories, LLC, Ashland, OH. Laboratory Report No.: WIL-843003, January 6, 2012. MRID 48617001. Unpublished.

SPONSOR: Joint Glyphosate Task Force, LLC, 8325 Old Deer Trail, Raleigh, NC 27615

TEST ORDER #: CON-417300-23

EXECUTIVE SUMMARY: In a Hershberger Assay (MRID 48617001) screening for androgenic activity, glyphosate (85.1% a.i., Batch/lot# GLP-1103-21149-T) in 0.5% methylcellulose (w/v) was administered daily via oral gavage (5 mL/kg) to groups of six 54- or 55-day old, castrated male Sprague Dawley rats at dose levels of 0 (vehicle), 100, 300, or 1,000 (limit dose) mg/kg/day. The androgenic positive control group consisted of 6 castrated rats exposed to 0.2 mg/kg/day of testosterone propionate (TP) by subcutaneous (s.c.) injection.

To screen for potential anti-androgenic activity, glyphosate in 0.5% methylcellulose (w/v) was administered daily via oral gavage to groups of six 54- or 55-day old, castrated male Sprague Dawley rats at dose levels of 0 (vehicle), 100, 300, or 1,000 mg/kg/day in conjunction with a daily dose of reference androgen TP at 0.2 mg/kg/day by s.c. injection. The anti-androgenic positive control group consisted of 6 castrated rats exposed to 0.2 mg/kg/day TP by s.c. injection and 3 mg/kg/day flutamide (FT) via oral gavage. TP alone was used as the anti-androgenic negative control.

For both components of the assay, body weights were determined daily. The animals were dosed for 10 consecutive days and terminated approximately 24 hours after the final dose. At necropsy, the five androgen-dependent tissues were collected and weighed.

All animals survived until scheduled termination. No animals exhibited any dose-related clinical signs of toxicology and there were no treatment-related gross pathological findings.

In the androgen agonist assay, there were no treatment-related effects on body weights, overall body weight gains, or the weights of accessory sex organs for any glyphosate dose group. Animals in the positive TP control group had increased ($p<0.01$) accessory sex organ weights as follows: 437% in seminal vesicles; 728% in ventral prostate; 200% in levator ani-bulbocavernosus (LABC); 361% in Cowper's gland; and 45% in glans penis. The performance criteria indicated that this assay was performing as expected.

In the anti-androgen assay, there were no treatment-related effects on body weights, overall body weight gains, or the weights of accessory sex organs for any glyphosate dose group. Animals dosed with TP + FT (positive control) had decreased ($p<0.01$) accessory sex organ weights as follows: 76% in seminal vesicles; 80% in ventral prostate; 63% in LABC; 70% in Cowper's gland; and 29% in glans penis. The performance criteria indicated that this assay was performing as expected.

Statistically significant changes were not seen in two or more of the five androgen sensitive tissue weights. Glyphosate 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, Data Confidentiality and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

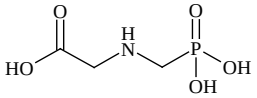
A. MATERIALS

1. Test Facility: WIL Research Laboratories, LLC

Location: Ashland, OH
Study Director: D. G. Stump
Other Personnel: E. S. Bodle (Assistant Director, Analytical Chemistry), S. A. Keets (Senior Operations Manager, Vivarium), C. A. Kopp (Manager, Gross Pathology and Developmental Toxicology Laboratory), G. M. Maginnis (Clinical Veterinarian), T. M. Rafeld (Group Manager, Formulations Laboratory), C. S. Wally (Group Supervisor, Sample Processing Laboratory), R. A. Wally (Operations Manager, Reporting & Technical Support Services), M. E. Haubenstricker (Participating Scientist, Analyses of Dosing Formulations), L. Freshwater (Contributing Scientist, Statistical Analysis)
Study Period: June 14, 2011 - January 6, 2012

2. Test Substance: Glyphosate

Description: White powder
Source: Monsanto (St. Louis, MO)
Lot/Batch #: GLP-1103-21149-T (expiration date 3/9/2012)
Purity: 85.14% (95.93% dried)
Stability: Stable in vehicle for up to 15 days at room temperature
CAS #: 1071-83-6
Structure:



3. Reference Androgen: Testosterone propionate (TP)

Supplier: AK Scientific, Inc. (Mountain View, CA)
Lot/Batch #: 70321J (expiration date 8/9/2012)
Purity: 98.3%
CAS #: 57-85-2

4. Reference Anti-androgen: Flutamide (FT)

Supplier: Spectrum Chemical Manufacturing Corporation (New Brunswick, NJ)
Lot/Batch #: 2AC0144 (expiration date 3/1/2013)
Purity: 100%
CAS #: 13311-84-7

5. Solvent/Vehicle Control (test substance): Methylcellulose

Supplier: Sigma Chemical (St. Louis, MO)
Lot #: 060M0123V (expiration date 5/1/2013)
Rationale (if other than water): Test substance not soluble in water at the concentrations used in the study
Final concentration: 0.5% (w/v)

Solvent/Vehicle Control (TP and FT): Ethanol/Corn oil

Supplier: Spectrum Chemical Manufacturing Corporation (New Brunswick, NJ)
Lot #: Ethanol: ZT0426 (expiration date 8/2/2013)
Corn oil: ZT1301 (expiration date 7/1/2012)
Rationale (if other than water): Not applicable
Final concentration: TP and FT were dissolved in minimal amounts of 95% ethanol and then diluted with corn oil (ratio of ethanol to corn oil not reported)

6. Test Animals:

Species:	Rat (castrated males only)	
Strain:	Sprague Dawley [Crl:CD(SD)]	
Age/weight at dose initiation:	Post-natal day (PND) 54-55 (approximate)/ 211.3-279.2 g	
Source:	Charles River Laboratories (Portage, MI)	
Housing:	Rats were individually housed in stainless steel wire-mesh cages suspended above cage board.	
Diet:	Certified Rodent LabDiet® 5002, PMI International, *ad libitum* Phytoestrogen content was not reported	
Water:	Reverse-osmosis purified drinking water, *ad libitum*	
Environmental conditions:	**Temperature:**	21.3-21.6 ºC (mean daily temperature)
	Humidity:	51.8-55.2% (mean daily humidity)
	Air changes:	10/hr
	Photoperiod:	12 hrs light/12 hrs dark
Acclimation period:	Rats were castrated at the supplier and received at the facility approximately one week post-castration; rats were then allowed a 6-day acclimation period at the facility	

B. STUDY DESIGN

1. In life dates: Start: June 27, 2011 End: July 7, 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. To screen for the potential anti-androgenic activity, the test substance was also administered daily via oral gavage to castrated male rats in conjunction with a daily dose of TP (0.2 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.

3. Study Schedule: Male rats were castrated on PND 42 (approximate age) according to standard procedures and allowed approximately 13 days for recovery and regression of accessory sex organ weights prior to initiation of dosing. The dose administration period was from PND 54 or 55 through PND 63 or 64 (approximate age). Rats were euthanized 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. Statistical analysis indicated that there were no significant differences in group means at study initiation. Furthermore, 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	6
Low (Glyphosate)	100	6
Mid (Glyphosate)	300	6
High (Glyphosate)	1000	6
Testosterone propionate (TP), positive control[b]	0.2	6
Anti-Androgen Assay		
Low (Glyphosate + TP)	100 + 0.2	6
Mid (Glyphosate + TP)	300 + 0.2	6
High (Glyphosate + TP)	1000 + 0.2	6
Flutamide + TP, positive control	3 + 0.2	6

a Data were obtained from page 26 of the study report. Glyphosate concentrations are expressed as free base equivalents.

b This dose group served as the positive control for the androgen agonist assay and the negative control for the anti-androgen assay.

5. **Dose Selection Rationale:** The dose levels used in this study were chosen based on the results of a dose range-finding study.[1] In the study, the test substance was administered by oral gavage to four groups of five adult male rats (strain not identified) at 0, 200, 500, and 1,000 mg/kg/day once daily for 10 days. All males survived to the scheduled necropsy. Mean body weight gain was decreased at 500 and 1,000 mg/kg/day, resulting in mean body weights that were 9% and 5% lower, respectively, than the control group on Day 10. Mean liver, adrenal gland, and kidney weights in all test substance-treated groups were comparable to the control group. Therefore, the high-dose level of 1,000 mg/kg/day (limit dose) was selected for the current study.

6. **(a) Dose Preparation:** Dose formulations were prepared twice during the study as single formulations for each dose level, by mixing appropriate amounts of test substance with 0.5% methylcellulose. Formulations of TP and FT were prepared once by dissolving the material in a small amount of 95% ethanol and diluting to volume with corn oil. Analyses to demonstrate homogeneity, stability, and resuspension homogeneity were conducted previously for dose formulations at 1 and 200 mg/mL following up to 15 days of room temperature storage.[2] During the study, samples of each test substance dosing formulation (middle stratum of each) prepared during the in-life phase were analyzed for achieved concentration.

(**b) Dose Analysis:**

Results

Homogeneity: Not provided

1 Stump, D.G. A Dose Range-Finding Oral (Gavage) Toxicity Study of Glyphosate in Young Adult Rats for the Endocrine Disruption Screening Program (Study No. WIL-843001). WIL Research Laboratories, LLC, Ashland, OH, 2011.

2 Haubenstricker, M.E. Analytical Validation and Stability Study of Glyphosate in Aqueous Formulations (Study No. WIL-843004). WIL Research Laboratories, LLC, Ashland, OH, 2011.

Stability: It was stated that glyphosate in 95% methylcellulose at 1 and 200 mg/mL was stable at room temperature for 15 days.

Concentration (percent of nominal): 104-114%

The analytical data indicated that the variation between nominal and actual dosage to the animals was acceptable. The study referenced above should be submitted for verification of the homogeneity and stability findings.

7. **Dosage administration:** Test formulations were administered to the animals daily via oral gavage (5 mL/kg) for 10 days. TP was given via s.c. injection at 0.5 mL/kg, and FT was administered via oral gavage at 5 mL/kg. Dose volumes were adjusted daily based on the concurrent body weight measurement.

8. **Statistics:** Statistical analyses were conducted for organ weights, daily body weights, and body weight gains. Each endpoint was tested for homogeneity of variance using Levene's test. If that test was significant at p=0.01, then a log transformation was applied and Levene's test conducted on the transformed data. If that test was still significant, then the square root transformation was applied to the raw data (except cumulative body weight gain) and Levene's test conducted again. If the test was still significant, then a nonparametric test, as described below, was used to analyze the data. One-sided tests were conducted for in-life data and two-sided tests were conducted for organ weight data.

   If variances were homogeneous, the one- or two-sided t-test (for comparing positive control data to data for negative control) or an ANOVA (for comparing dose groups to the negative control) was performed on data; the ANOVA test was followed by a one- or two-sided Dunnett's test. If the transformations were unsuccessful in making the variances homogeneous, the nonparametric one- or two-sided Wilcoxon rank sum test was used to compare data for the positive control to the negative control. For comparison of dose groups to the negative control, if the transformations were unsuccessful in making the variances homogeneous, the nonparametric Kruskal-Wallis test was used, followed by a one- or two-sided Dunn's test. Significance was denoted at $p<0.05$. Statistical analyses were performed using SAS software (version 9.2 or higher). The statistical analyses were considered adequate.

C. METHODS

1. **Clinical Examinations:** Cage-side checks for mortality and moribundity were conducted twice daily. Individual clinical observations (hand-held physical examinations) were recorded daily through termination. Each rat was also observed for signs of toxicity approximately 4 hours following dosing.

2. **Body Weight:** Animals were weighed at randomization, daily throughout the dosing period, and on the day of termination. Mean body weight changes were calculated for each corresponding interval and also for the overall dosing period (Days 1-11).

3. **Food Consumption (Optional):** Food consumption was not measured.

4. **Serum Hormone Measurements (Optional):** At study termination, each animal was anesthetized with isoflurane, and blood was collected from the vena cava for potential future serum hormone analyses; however, serum hormone analyses were not conducted for this study.

5. **Dissection and Measurement of Tissue and Organ Weights:** On PND 64 or 65 (approximately 24 hours after the final administration of the test substance), all animals were anesthetized with isoflurane, exsanguinated, and examined. The five mandatory androgen-dependent organs (ventral prostate, seminal vesicles with coagulating glands, LABC, Cowper's gland, and glans penis) were excised and weighed fresh (unfixed) according to the standard operating procedures detailed in the U.S. EPA Guideline (OCSPP 890.1400). The accessory sex organs were placed in 10% neutral buffered formalin.

II. RESULTS

A. OBSERVATIONS

1. **Mortality:** All animals survived until scheduled termination.

2. **Clinical signs of toxicity:** No remarkable clinical signs of toxicity were observed in animals for any dose groups. Any clinical findings noted in the dose groups occurred infrequently, in a manner that was not dose related, and/or at similar frequency as the vehicle control group.

B. BODY WEIGHT AND WEIGHT GAIN: Selected body weight and body weight gain data for the androgen agonist assay are presented in Table 2. Body weights in the treatment groups and positive control group were comparable to controls throughout the duration of the study.

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			Positive Control Vehicle + TP (0.2)			Glyphosate (100)			Glyphosate (300)			Glyphosate (1000)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
1	6	243.3	14.7	6	237.7	18.4	6	240.9	15.7	6	240.9	13.5	6	240.9	13.7
4	6	261.7	14.3	6	257.0	21.4	6	258.5	17.3	6	259.5	17.2	6	258.2	13.2
7	6	281.2	14.0	6	278.2	23.6	6	276.7	17.6	6	279.3	20.2	6	271.7	23.5
11	6	309.1	13.2	6	311.1	31.8	6	306.6	17.5	6	308.8	23.2	6	300.5	20.9
Body Weight Gain (Days 1-11)	6	65.8	6.9	6	73.4	15.2	6	65.7	5.9	6	67.9	13.9	6	59.6	13.2

a Data were obtained from Table 4 on page 93 of the study report.
N Number of animals in the group
SD Standard Deviation

Selected body weight and body weight gain data for the anti-androgen agonist assay are presented in Table 3. Body weights in the treatment groups and positive control group were comparable to controls 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 (0.2)			Positive Control TP + FT (0.2 + 3)			Glyphosate + TP (100 + 0.2)			Glyphosate + TP (300 + 0.2)			Glyphosate + TP (1000 + 0.2)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
1	6	237.7	18.4	6	240.6	17.8	6	238.3	16.1	6	239.6	16.5	6	242.1	20.4
4	6	257.0	21.4	6	262.0	21.1	6	257.6	16.8	6	264.9	17.3	6	265.2	22.7
7	6	278.2	23.6	6	281.8	26.7	6	281.6	17.8	6	286.5	17.7	6	286.3	24.1
11	6	311.1	31.8	6	310.6	28.5	6	317.2	16.7	6	322.4	19.5	6	320.1	26.5
Body Weight Gain (Days 1-11)	6	73.4	15.2	6	70.0	12.2	6	78.9	6.4	6	82.8	6.2	6	78.0	10.6

a Data were obtained from Table 5 on page 94 of the study report.
N Number of animals in the group
SD Standard Deviation

C. FOOD CONSUMPTION (Optional): Food consumption was not measured.

D. SERUM HORMONE CONCENTRATIONS (Optional): Serum hormone concentrations were not measured.

E. ORGAN WEIGHTS: Accessory sex organ weights for the androgen agonist assay are presented in Table 4. There were no treatment related effects in accessory sex organs for any glyphosate dose group. Animals in the TP group had increased ($p<0.01$) accessory sex organ weights as follows: 437% in seminal vesicles; 728% in ventral prostate; 200% in LABC; 361% in Cowper's gland; and 45% in glans penis.

Percent CVs for the vehicle control and the glyphosate treatment groups were compared to the performance criteria in the Guidelines. All %CV values were less than the maximum permissible values.

TABLE 4. Accessory Sex Organ Weights (mg) from Androgen Agonist Assay in Sprague Dawley Rats[a]																				
Organ	**Dose (mg/kg/day)**																			
	Vehicle Control				**Positive Control Vehicle + TP (0.2)**				**Glyphosate (100)**				**Glyphosate (300)**				**Glyphosate (1000)**			
	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Seminal vesicles	6	86.4	12.1	14.0	6	463.6** (↑437)	35.9	7.8	6	89.3	14.0	15.7	6	93.1	14.7	15.8	6	84.4	19.3	22.9
Ventral prostate	6	16.2	3.7	23.0	6	134.2** (↑728)	22.2	16.5	6	21.5	9.4	43.7	6	15.1	5.5	36.5	6	17.2	4.3	24.8
LABC	6	157.3	22.0	14.0	6	471.6** (↑200)	63.1	13.4	6	160.0	30.5	19.0	6	151.8	14.2	9.4	6	164.3	19.3	11.8
Cowper's glands	6	6.2	1.3	21.5	6	28.6** (↑361)	6.4	22.2	6	6.6	2.0	30.4	6	7.0	2.0	28.8	6	6.4	1.5	23.0
Glans penis	6	105.0	15.9	15.2	6	152.1** (↑45)	20.9	13.7	6	102.0	14.4	14.1	6	103.6	20.1	19.4	6	100.8	16.4	16.2

a Data were obtained from Tables 6 and S18 on pages 85, 86, and 95 of the study report. Percent differences from controls are included in parentheses.
N Number of animals in the group
SD Standard Deviation
CV Coefficient of Variation
** Significantly different from controls at $p<0.01$

Accessory sex organ and liver weights for the anti-androgen assay are presented in Table 5. There were no treatment related effects in accessory sex organs for any glyphosate dose group. Animals dosed with TP + FT (positive control) had decreased ($p<0.01$) accessory sex organ weights as follows: 76% in seminal vesicles; 80% in ventral prostate; 63% in LABC; 70% in Cowper's gland; and 29% in glans penis. Liver weights in the positive control group were comparable to the vehicle control.

Percent CVs for the vehicle control and the glyphosate treatment groups were compared to the performance criteria in the Guidelines. All %CV values were less than the maximum permissible values.

TABLE 5. Accessory Sex Organ Weights (mg) from Anti-Androgen Agonist Assay in Sprague Dawley Rats[a]																				
Organ	**Dose (mg/kg/day)**																			
	Vehicle Control + TP (0.2)				**Positive Control TP + FT (0.2 + 3)**				**Glyphosate + TP (100 + 0.2)**				**Glyphosate + TP (300 + 0.2)**				**Glyphosate + TP (1000 + 0.2)**			
	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Seminal vesicles	6	463.6	35.9	7.8	6	111.9** (↓76)	17.5	15.6	6	411.0	71.9	17.5	6	417.5	99.5	23.8	6	391.6	75.3	19.2
Ventral prostate	6	134.2	22.2	16.5	6	27.0** (↓80)	8.2	30.3	6	112.2	18.5	16.5	6	110.6	26.5	24.0	6	125.3	25.3	20.2
LABC	6	471.6	63.1	13.4	6	173.9** (↓63)	31.3	18.0	6	405.4	49.0	12.1	6	461.0	92.1	20.0	6	471.8	83.1	17.6
Cowper's glands	6	28.6	6.4	22.2	6	8.7** (↓70)	1.9	22.1	6	31.1	3.1	10.0	6	28.5	9.2	32.4	6	29.9	5.0	16.6
Glans penis	6	152.1	20.9	13.7	6	108.3** (↓29)	5.9	5.4	6	150.6	17.9	11.9	6	148.8	23.7	15.9	6	168.2	15.4	9.2

a Data were obtained from Tables 7 and S19 on pages 87, 88, and 95 of the study report. Percent differences from controls are included in parentheses.
N Number of animals in the group
SD Standard Deviation
CV Coefficient of Variation
** Significantly different from controls at $p<0.01$

III. DISCUSSION AND CONCLUSIONS

A. <u>INVESTIGATOR'S CONCLUSIONS</u>: Based on the results of this study, no androgenic or anti-androgenic effects of glyphosate were noted on the androgen-sensitive organs at any dosage level up to 1,000 mg/kg/day (the limit dose and highest level tested) when administered orally by gavage to male Crl:CD(SD) rats. The anti-androgenic positive control substance, flutamide, elicited the expected responses (lower weights of androgen-dependent organs). The androgenic positive control substance, testosterone propionate, elicited the expected responses (higher weights of androgen-dependent organs) as defined in the test guidance for this study design.

B. <u>AGENCY COMMENTS</u>: All animals survived until scheduled termination. No animals exhibited any dose-related clinical signs of toxicology.

In the androgen agonist assay, there were no treatment-related effects on body weights, overall body weight gains, or the weights of accessory sex organs for any dose group. Animals in the positive control group had the expected increases accessory sex organ weights. The performance criteria indicated that the assay was performing as expected.

There were no treatment-related effects on body weights, overall body weight gains, or the weights of accessory sex organs for any dose group in the anti-androgen assay. Animals dosed with TP + FT (positive control) had the expected decreases in accessory sex organ weights. The performance criteria indicated that the assay was performing as expected.

Statistically significant changes were not seen in two or more of the five androgen sensitive tissue weights. Glyphosate was negative for androgenicity and anti-androgenicity in the Hershberger assay.

C. STUDY DEFICIENCIES: None

DATA EVALUATION RECORD

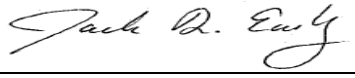
GLYPHOSATE

Study Type: OCSPP 890.1450, Female Pubertal Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-74-2012 (MRID 48671315)

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, B.S.	Date:	6/20/2012
Secondary Reviewer:	Signature:	
Scott D. Studenberg, Ph.D., D.A.B.T.	Date:	75/2012
Program Manager:	Signature:	
Jack D. Early, M.S.	Date:	7/13/2012
Quality Assurance:	Signature:	
Steven Brecher, Ph.D., D.A.B.T.	Date:	7/13/2012

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: Anwar Y. Dunbar, Ph.D. **Signature:** Anw Y. Dunbar
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 05-27-15
Secondary Reviewer: Elizabeth Mendez, Ph.D. **Signature:**
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 6/9/15

DATA EVALUATION RECORD

STUDY TYPE: Female Pubertal Assay; OCSPP 890.1450; OECD None.

PC CODE: 417300 **DP BARCODE:** D401747

TXR #: 0053233 **CAS No:** 1071-83-6

TEST MATERIAL (PURITY): Glyphosate technical (95.93% a.i.)

SYNONYMS: *N*-(phosphonomethyl)glycine

CITATION: Stump, D.G. (2012) A Pubertal Development and Thyroid Function Assay of Glyphosate Administered Orally in Intact Juvenile/Peripubertal Female Rats. WIL Research Laboratories, LLC, Ashland, OH, Laboratory Project ID: WIL-843007, April 10, 2012. MRID 48671315. Unpublished.

SPONSOR: Joint Glyphosate Task Force, LLC. c/o Data Group Management, 8325 Old Deer Trail, Raleigh, NC

TEST ORDER #: CON-417300-23

EXECUTIVE SUMMARY: In a Female Pubertal Assay (MRID 48671315), 15 Crl:CD(SD) Sprague-Dawley rats/dose group were treated daily via oral gavage with glyphosate technical (95.93% a.i., Lot #: GLP-1103-21149-T) in 0.5% methylcellulose at doses of 0, 100, 300 or 1000 mg/kg/day (limit dose) from post-natal day (PND) 22 to 42. Animals were examined for vaginal opening (VO) daily beginning on PND 22, and age and weight at day of attainment were recorded. Following sacrifice on PND 42, blood was collected for clinical chemistry analyses, including total thyroxine (T_4) and thyroid stimulating hormone (TSH) levels, which were analyzed using an electrochemiluminescent immunoassay (T_4) and a magnetic [^{125}I]rTSH gamma counter immunoassay (TSH). Liver, adrenal glands, thyroid, pituitary, and urogenital organ weights were recorded, and microscopic examinations were performed on the thyroid, kidneys, ovary and uterus.

One animal in the control group was sacrificed *in extremis* on PND 27 due to impairment of the right forelimb (due to possible mechanical injury). All other animals survived until scheduled sacrifice. Treatment-related clinical signs were limited to rales in 4/15 and 13/15 females in the 300 and 1000 mg/kg/day groups, respectively, at approximately 4 hours post-dosing. This finding did not persist to the daily examinations. No other treatment-related clinical signs were noted during the 4-hour post-dosing or daily examinations at any dose level. There were no treatment-related differences in age of attainment of VO, body weight at VO, final body weights, or body weight gains in the treated groups relative to controls. One female each in the control

and 300 mg/kg/day groups failed to attain VO. There were no statistically significant differences in mean age at first vaginal estrus, mean cycle length, or percent cycling. The cycle status at necropsy was similar among all groups. Serum T_4 and TSH were not affected by treatment, and no adverse treatment-related effects on any clinical chemistry parameter were observed at any dose. There were no treatment-related microscopic findings in the thyroid, ovaries, uterus, or kidneys at any dose.

The dose levels tested were adequate since the high dose was the Limit Dose (1000 mg/kg/day).

The assay **satisfies** the EDSP Tier 1 Test Order requirements for a Female Pubertal Assay (OCSPP 890.1450).

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

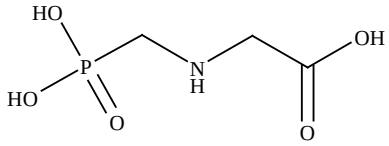
I. MATERIALS AND METHODS

A. MATERIALS

1. Test Facility: WIL Research Laboratories, LLC

Location:	Ashland, OH
Study Director:	D.G. Stump
Other Personnel:	C.A. Picut, Pathologist
	M.E. Haubenstricker, Analytical Chemist
	L. Freshwater, Statistics
Study Period:	August 23, 2011 – April 10, 2012

2. Test Substance: Glyphosate

Description:	White powder
Source:	Monsanto (St. Louis, MO)
Lot/Batch #:	GLP-1103-21149-T (exp. March 9, 2012)
Purity:	95.93% (dried)
Stability:	Stable in 0.5% methylcellulose for up to 15 days at room temperature
CAS #:	1071-83-6
Structure:	

3. Vehicle: 0.5% Methylcellulose in deionized water

4. Test Animals:

Species:	Rat	
Strain:	Crl:CD(SD) Sprague-Dawley	
Age/Weight at Study Initiation:	PND 22/41.4 – 51.3 g females only	
Source:	Charles River Laboratories (Portage, MI)	
Housing:	2-3/cage in plastic cages with heat treated laboratory-grade pine shavings for bedding.	
Diet:	Harlan Laboratories 2016CM Teklad Global 16% protein rodent diet, ad libitum Phytoestrogen content average 11 ppm total isoflavones (genistein+daidzein+glycitein)	
Water:	Municipal water, reverse-osmosis filtered (on site), *ad libitum*	
Environmental Conditions:	**Temperature:**	21.4-22.1 °C
	Humidity:	41.2-58.9%
	Air changes:	≥10/hr
	Photoperiod:	14 hrs light/ 10 hrs dark

B. STUDY DESIGN

1. In-Life Dates: Start: September 28, 2011 End: October 19, 2011

2. Mating: Time-mated pregnant dams were received from the supplier on gestation day (GD) 7. The day evidence of mating was confirmed was designated GD 0. To reduce variability, litters were culled to 5 pups/sex (when possible) on PND 4. The male offspring were used for a concurrently submitted male pubertal assay (MRID 48671313).

3. Animal Assignment: Following weaning on PND 21, animals were randomly assigned (stratified by weight in a block design) to the test groups noted in Table 1. Littermates were not assigned to the same treatment group.

TABLE 1. Study Design [a]		
Test group	**Dose (mg/kg/day)**	**# of Females**
Control	0	15
Low	100	15
Mid	300	15
High	1000	15

a Data were obtained from page 26 of the study report.

4. **Dose Selection Rationale:** The dose levels for the current study were selected based on the results of a 7-day oral gavage range-finding study (WIL Study No. WIL-843006, data not provided) in juvenile female rats. The animals (5/dose) were administered glyphosate daily via oral gavage from PND 22-28 at dose levels of 200, 500, and 1000 mg/kg/day. No treatment-related effects were observed in clinical observations, body weights, or body weight gains at any dose level. Slightly lower food consumption was noted at 1000 mg/kg/day. Based on these results, dose levels of 100, 300, and 1000 mg/kg/day (limit dose) were selected for this female pubertal assay. Although the data for the dose range-finding study (WIL Study No. WIL-843006) were not provided, the animals were dosed up to the limit dose of 1000 mg/kg/day and it was concluded that the animals were dosed at a sufficiently high level.

5. **Dose Preparation and Analysis:** Dose formulations were prepared approximately weekly by mixing appropriate amounts of test substance with 0.5% methylcellulose in deionized water, and then were divided into aliquots for daily dispensation. It was stated that analyses to demonstrate homogeneity and stability of the test substance in formulations for up to 15 days of room temperature storage at concentrations of 1 and 200 mg/mL (which bracketed those used in the current study) were conducted in a previous study (Haubenstricker, 2011, WIL-843004); however, no data were provided. Concentration of the test material as administered to the study animals was assessed from the middle stratum of each dosing formulation prepared during the in-life phase of the study. Additionally, the reviewers derived the homogeneity (% RSD) of the samples collected for concentration analysis.

   Results of Dose Analysis

   Homogeneity (% RSD): 0.12-2.7%

   Stability (% of initial): Data not provided

   Concentration (% of nominal): 98.9-112%

   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:** All doses were administered once daily by oral gavage, from PND 22 through PND 42, in a volume of 5 mL/kg of body weight. Dosing was performed between 0700 and 0900 hours daily.

7. **Statistics:** Statistics were conducted at WIL Research and at BioSTAT Consultants, Inc. At WIL, cycling status (cycling vs. non-cycling) and percent of animals cycling were assessed using Chi-square analysis. Estrous cycle length and day of first estrus were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's test when intergroup differences were indicated by ANOVA. Dichotomous pathology data were analyzed with pairwise Fisher's exact test. Tests were conducted at the $p \leq 0.05$ significance level.

   At BioSTAT, the data that were statistically analyzed included organ weights (liver, kidneys, pituitary and adrenals), organ weight to final body weight ratios, daily body weights, weight gains, serum chemistries, hormones and vaginal opening data. Each endpoint was tested for homogeneity of variance using Levene's test. If this was positive ($p \leq 0.01$) then log or square root transformations were applied to the data; if these were positive then a non-parametric test was used. Organ weights, age at vaginal opening and weight at vaginal opening data, when parametric, were analyzed using analysis of covariance (ANCOVA) followed by Dunnett's test using PND 21 body weights as the covariate. Other parameters were analyzed with ANOVA followed by Dunnett's test. Linear trend tests were used in conjunction with both ANOVA and ANCOVA. For non-parametric data the Kruskal-Wallis test was used followed by Dunn's test. Pathology data presented as a graded response were analyzed with a pairwise Mann-Whitney U test.

C. METHODS

1. **Mortality and Clinical Examinations:** All animals were examined twice daily for mortality and moribundity. Clinical examinations were conducted daily (prior to dosing) through the day of euthanasia. Additionally, animals were examined approximately 4 hours post-dosing for clinical signs.

2. **Body Weight:** Animals were weighed on the day of randomization, daily prior to dosing and the day of euthanasia. Mean body weight changes were calculated for each corresponding interval and also for the overall treatment period (PND 22-42).

3. **Vaginal Opening:** Beginning on PND 22, all animals were examined daily for onset of vaginal opening. The appearance of a small "pin hole", a vaginal thread, and complete vaginal opening were recorded for all days they were observed. Age and weight on the day of completion of vaginal opening were recorded.

4. **Estrous Cyclicity:** Beginning on the day of vaginal opening, up to and including the day of necropsy, daily vaginal smears were obtained and evaluated to determine the stage of estrus for each female. Cycle length was determined for a complete estrous cycle by counting the number of days from one diestrus to the next diestrus or from one metestrus to the next metestrus. The overall pattern for each female was characterized as regular, irregular, non-cycling or insufficient data. The definitions for these terms are as follows:

   - Regular cycling (RC): the animal has at least 6 days of data collected and displays 1 complete cycle with no cycles greater than 5 days of duration, no cycles with 3 or more days of proestrus (P) and/or estrus (E), and no cycles less than 4 days in duration.

- Irregular cycling (IC): 1) the animal does not display 1 complete cycle but has at least 1 E and/or P and a partial cycle greater than 5 days, 2) the animal has at least 1 E and either at least 1 cycle (complete or partial) greater than 5 days in duration or at least 1 cycle with 3 or more consecutive days of P and/or E, 3) the animal has at least 1 cycle less than 4 days in duration, or 4) the animal has 1 irregular cycle and 1 regular cycle.
- Non-cycling (NC): no E or P present on any days of estrous cycle determination and at least 5 days of data collected.
- Insufficient data (ID): 1) the animal does not display at least 1 complete cycle but has at least 1 E and/or P and a partial cycle of 5 days or fewer, 2) no E is present on any days of estrous cycle determination and 4 or fewer days of data collected, or 3) at least 1 E or P present and only 1-4 days of data collected.

Percent cycling and percent regularly cycling were calculated as follows:

Percent cycling: [(RC + IC + ID) / (RC + IC + ID + NC)] X 100

Percent cycling regularly: [RC / (RC + IC)] X 100

5. **Sacrifice and Pathology:** On the day before euthanasia, rats were moved to a holding room separate from where the necropsies were to be performed. Dosing was performed in the holding room on the day of termination and the animals were moved to the necropsy room one at a time for terminal procedures. Approximately 2 hours following dosing, animals were sacrificed by decapitation without anesthesia and all sacrifices were completed by 1300 hours to minimize hormonal variability due to normal diurnal fluctuation. Immediately following decapitation, trunk blood samples were taken for T_4, TSH and serum chemistry analyses. The sample for TSH analysis was stored at approximately −20°C and the sample for T_4 was analyzed fresh.

a. **Hormone Analysis:** Total T_4 was analyzed using an electrochemiluminescent assay on the Cobas e411 (Roche Diagnostics, Indianapolis, IN) and TSH levels were analyzed using an [^{125}I] rTSH kit (Izotop, Institute of Isotopes Ltd., Budapest, Hungary). For both assays, multiple quality control samples were run prior to analysis.

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	Globulins
X	Sodium	X	Glucose
	ENZYMES	X	Total bilirubin
X	Alkaline phosphatase (ALP)	X	Total protein
	Cholinesterase (ChE)	X	Triglycerides
	Creatine phosphokinase		Serum protein electrophoresis
	Lactic acid dehydrogenase (LDH)	X	Albumin globulin ratio (A/G Ratio, by calculation)
X	Alanine aminotransferase (ALT/also SGPT)	X	Bile acids
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. **<u>Organ Weights and Histopathology</u>:** The following CHECKED (X) tissues were collected and weighed. The (XX) organs, in addition, were subjected to histological examination.

	UROGENITAL		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 except the thyroid/trachea were weighed prior to fixation. Paired organs (kidneys, adrenals, and ovaries) were weighed together. The uterus was separated from the vagina and weighed. The uterus was weighed "wet" and then again following removal of the fluid in the lumen (blotted weight).

The ovaries (left or right not specified), uterus and kidneys were fixed in 10% buffered formalin, rinsed and stored in 70% ethanol prior to embedding. The thyroid was fixed in 10% buffered formalin for at least 24 hrs. 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 were noted. At least two sections of the thyroid were examined. Five random sections of an ovary were evaluated for follicular development and any abnormalities/lesions (such as atrophy). Uterine histology documented cases of uterine hyper- or hypotrophy as characterized by changes in uterine horn diameter and myometrial, stromal, or endometrial gland development. The stage of the estrous cycle of the female at the time of necropsy was evaluated.

II. RESULTS

A. **Mortality:** One animal in the control group (Animal No. 24840-10) was sacrificed *in extremis* on PND 27 due to impairment of the right forelimb (due to possible mechanical injury). All other rats survived until scheduled sacrifice.

B. **Clinical Signs of Toxicity:** Treatment-related clinical findings were limited to rales in 4/15 and 13/15 females in the 300 and 1000 mg/kg/day groups, respectively, approximately 4 hours post-dosing. This finding did not persist to the daily examinations. No other treatment-related clinical signs were noted during the 4-hour post-dosing or daily examinations at any dose level. Additional clinical signs noted during the study, including red material around the nose, occurred infrequently, at similar frequencies in the controls, and/or in a manner that was not dose-dependent.

C. **General Growth and Vaginal Opening:** Body weights, body weight gains, age at attainment of vaginal opening and weight at day of attainment are presented in Table 2. There were no treatment-related differences in age of attainment, body weight at vaginal opening, final body weights, or body weight gains in the treated groups relative to controls. One female each in the control and 300 mg/kg/day groups failed to attain vaginal opening.

The mean age at vaginal opening in the control group (36.4 days) exceeded the performance criteria maximum (35.62 days); however, the CV value (6.52%) was within the acceptable range (0-6.52%).

TABLE 2. General Growth and Vaginal Opening (VO)[a]																
Parameter Evaluated		**Vehicle Control**				**Glyphosate 100 mg/kg/day**				**Glyphosate 300 mg/kg/day**				**Glyphosate 1000 mg/kg/day**			
		N	**Mean**	**SD**	**CV (%)**	**N**	**Mean**	**SD**	**CV (%)**	**N**	**Mean**	**SD**	**CV (%)**	**N**	**Mean**	**SD**	**CV (%)**
Initial Body Weight (PND 22; g)	U	15	45.9	1.8	3.9	15	45.3	2.2	4.8	15	45.3	2.1	4.7	15	45.3	2.5	5.4
Weight at VO (g)	U	14	119.6	12.6	10.6	15	119.2	16.3	13.6	15	125.1	15.6	12.4	15	117.4	16.8	14.3
	A	14	119.2	12.6	10.6	15	119.4	16.3	13.6	15	124.8	15.6	12.4	15	118.0	16.8	14.3
Final Body Weight (PND 42; g)	U	14	147.3	11.7	7.9	15	147.0	10.3	7.0	15	152.1	6.4	4.2	15	141.3	10.6	7.5
Final Body Weight (% of control)	U	NA					-0.20				3.26				-4.07		
Body Weight Gain (g)	U	14	101.5	10.6	10.4	15	101.7	9.0	8.9	15	106.8	5.9	5.5	15	96.0	9.7	10.1
Age at VO (PND)	U	14	36.4	2.4	6.5	15	36.1	2.5	7.0	15	36.8	2.5	6.8	15	36.3	2.6	7.2
	A	14	36.5	2.4	6.5	15	36.1	2.5	7.0	15	36.8	2.5	6.8	15	36.3	2.6	7.2
Proportion unopened (#/N)		1/15				0/15				1/15				0/15			

a Data were obtained from page 56 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
NA Not applicable

D. <u>Organ Weights</u>: Organ weights at necropsy are presented in Table 3. No treatment-related effects were observed in the unadjusted, adjusted, or relative organ weights at any dose.

The control group means and CVs for all organ weights were within the acceptable ranges of the performance criteria.

TABLE 3. Organ Weights at Necropsy[a]																	
Organ		Vehicle Control				Glyphosate 100 mg/kg/day				Glyphosate 300 mg/kg/day				Glyphosate 1000 mg/kg/day			
		N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Liver (g)	U	14	6.72	0.85	12.66	15	6.75	0.65	9.61	15	7.03	0.47	6.68	15	6.47	0.52	8.11
	A	14	6.70	0.85	12.66	15	6.76	0.65	9.61	15	7.01	0.47	6.68	15	6.50	0.52	8.11
	R	14	4.55	0.32	7.07	15	4.58	0.22	4.78	15	4.62	0.20	4.43	15	4.58	0.18	4.02
Kidneys (g)	U	14	1.17	0.10	8.88	15	1.16	0.12	10.3	15	1.17	0.10	8.53	15	1.14	0.10	8.96
	A	14	1.16	0.10	8.88	15	1.16	0.12	10.3	15	1.17	0.10	8.53	15	1.15	0.10	8.96
	R	14	0.79	0.05	5.85	15	0.79	0.05	6.25	15	0.77	0.05	6.34	15	0.81	0.06	7.01
Pituitary (mg)	U	14	8.3	0.92	11.10	15	9.2	1.68	18.4	15	8.8	0.98	11.1	15	8.6	1.29	15.1
	A	14	8.2	0.92	11.10	15	9.2	1.68	18.4	15	8.8	0.98	11.1	15	8.7	1.29	15.1
	R	14	5.6	0.59	10.47	15	6.2	0.98	15.8	15	5.8	0.69	11.9	15	6.1	0.90	14.8
Adrenals (mg)	U	14	33.3	5.66	17.03	15	33.1	5.19	15.7	15	33.2	3.84	11.6	15	31.8	5.63	17.7
	A	14	33.2	5.66	17.03	15	33.2	5.19	15.7	15	33.1	3.84	11.6	15	31.9	5.63	17.7
	R	14	22.5	3.26	14.47	15	22.5	3.61	16.0	15	21.8	2.44	11.2	15	22.5	3.72	16.5
Ovaries (mg)	U	14	68.3	9.32	13.65	15	67.9	11.01	16.2	15	67.8	9.31	13.7	15	69.1	10.8	15.6
	A	14	67.9	9.32	13.65	15	68.0	11.01	16.2	15	67.7	9.31	13.7	15	69.6	10.8	15.6
Uterus, wet (mg)	U	14	263.6	91.13	34.57	15	316.9	130.03	41.0	15	342.6	171.3	50.0	15	238.9	116.8	48.9
	A	14	262.5	91.13	34.57	15	315.6	130.03	41.0	15	343.7	171.3	50.0	15	238.6	116.8	48.9
Uterus, blotted (mg)	U	14	235.7	68.25	28.95	15	256.8	56.78	22.1	15	260.2	72.29	27.8	15	205.9	73.1	35.5
	A	14	235.2	68.25	28.95	15	256.4	56.78	22.1	15	260.5	72.29	27.8	15	206.0	73.1	35.5
Thyroid, fixed (mg)	U	14	8.97	2.63	29.31	15	8.72	1.91	21.9	15	9.33	1.24	13.3	15	8.63	1.89	21.8
	A	14	8.88	2.63	29.31	15	8.76	1.91	21.9	15	9.27	1.24	13.3	15	8.76	1.89	21.8

a Data were obtained from page 57 of the study report.
U Unadjusted for body weight on PND 22
A Adjusted for 100 g 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. <u>Estrous Cyclicity</u>: Estrous cycle data are provided in Table 4. There were no statistically significant differences in mean age at first vaginal estrus, mean cycle length, or percent cycling. The cycle status at necropsy was similar among groups. The percent of females regularly cycling was lower ($p<0.05$) at 300 and 1000 mg/kg/day (40 and 60%, respectively) compared to the controls (75%). However, a large number of females (9-10 per group) in these dose groups had insufficient estrous cycle data, which resulted in only 5 females/dose available for evaluation of estrous cyclicity. Therefore this finding was considered to be unrelated to treatment.

TABLE 4. Estrous Cyclicity[a]									
Treatment Groups	N	Mean Age at First Vaginal Estrus (PND)	Mean Cycle Length (days)	Cycling (%)	Regularly Cycling (%)	Cycle Status at Necropsy (# Females)			
						Diestrus	Proestrus	Estrus	Not Cycling
Vehicle	13	36.5	5.0	100	75	9	1	3	0
Glyphosate 100 mg/kg/day	15	37.5	4.8	100	80	8	1	6	0
Glyphosate 300 mg/kg/day	14	37.8	5.0	100	40*	7	2	5	0
Glyphosate 1000 mg/kg/day	15	38.3	5.0	100	60*	11	0	4	0

a Data were obtained from pages 57 and 58 of the study report.
N Number of animals
* Significantly different from controls at $p<0.05$.

F. <u>Clinical Chemistry and Hormone Levels</u>: Mean clinical chemistry and hormone levels are presented in Table 5. In addition, the study report provided historical control ranges on pages 353-355 for the clinical chemistry parameters, but not hormone measures. There were no treatment-related effects on serum T_4 or TSH levels at any dose.

No adverse treatment-related effects on any clinical chemistry parameter were observed at any dose. The statistically significant differences from controls noted in AST, ALP, potassium, chloride, and phosphorous levels were considered unrelated to treatment and/or not adverse because they were minor, within the historical control range, the change in direction was one not normally associated with a toxic effect, unrelated to dose, and/or there were no corroborative histopathological findings in the associated organs.

It is noted that 11/20 of the control clinical chemistry means are outside the historical control range for the performing laboratory.

TABLE 5. Hormone Levels and Clinical Chemistry[a]																
Parameter	Vehicle Control				Glyphosate 100 mg/kg/day				Glyphosate 300 mg/kg/day				Glyphosate 1000 mg/kg/day			
	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Thyroid Hormones																
Total T_4 (µg/dL)	14	4.12	0.54	13.1	15	4.38	0.47	10.6	15	4.64	0.68	14.7	15	3.76	0.66	17.7
TSH (ng/mL)	14	3.74	1.92	51.2	14	3.86	1.63	42.1	15	3.85	2.17	56.5	14	2.69	1.27	47.1
Clinical Chemistry																
A/G Ratio	14	3.08	0.49	15.9	15	3.17	0.36	11.2	15	2.92	0.34	11.6	15	2.88	0.30	10.6
Albumin (g/dL)	14	4.2	0.28	6.81	15	4.2	0.16	3.82	15	4.0	0.27	6.66	15	4.2	0.26	6.21
ALP (U/L)	14	252	30.8	12.2	15	260	51.1	19.7	15	283	38.1	13.5	15	303* (↑20)	50.0	16.5
ALT (U/L)	14	126	31.4	24.9	15	102	17.2	16.9	15	113	11.6	10.3	15	126	42.8	34.1
AST (U/L)	14	506	164.6	32.5	15	366* (↓28)	89.7	24.5	15	374* (↓26)	39.7	10.6	15	383* (↓24)	109.6	28.6
Bile Acids (µmol/L)	14	39.4	30.21	76.7	15	26.0	11.85	45.6	15	41.2	28.59	69.3	15	38.4	24.23	63.1
Calcium (mg/dL)	14	11.2	0.31	2.81	15	11.3	0.26	2.28	15	11.3	0.39	3.41	15	11.2	0.57	5.06
Chloride (mEq/L)	14	100	1.6	1.6	15	101	1.0	1.0	15	100	1.7	1.7	15	103* (↑3)	2.3	2.2
Cholesterol (mg/dL)	14	87	13.1	15.1	15	90	14.1	15.7	15	90	15.0	16.7	15	92	11.2	12.2
Creatinine (mg/dL)	14	0.1	0.05	35.95	15	0.2	0.05	31.7	15	0.1	0.05	35.2	15	0.2	0.05	26.4
GGT (U/L)	14	0.3	0.26	98.01	15	0.1	0.00	0.00	15	0.1	0.00	0.00	15	0.1	0.00	0.00
Globulin (g/dL)	14	1.4	0.19	13.64	15	1.3	0.15	11.2	15	1.4	0.15	11.0	15	1.5	0.15	9.96
Glucose (mg/dL)	14	147	9.6	6.5	15	151	8.0	5.3	15	148	7.8	5.3	15	146	10.7	7.3
Phosphorous (mg/dL)	14	11.7	1.00	8.56	15	10.8* (↓8)	0.59	5.44	15	11.4	0.84	7.35	15	11.8	0.58	4.97
Potassium (mEq/L)	14	8.08	0.98	12.09	15	7.00* (↓13)	0.49	7.0	15	7.11* (↓12)	0.56	7.85	15	7.55	0.64	8.46
Sodium (mEq/L)	14	137	1.6	1.2	15	139	1.5	1.1	15	139	1.8	1.3	15	138	3.2	2.3
Total Bilirubin (mg/dL)	14	0.06	0.02	37.18	15	0.06	0.013	23.7	15	0.06	0.02	28.7	15	0.06	0.02	35.2
Total Protein (g/dL)	14	5.6	0.35	6.23	15	5.6	0.22	4.02	15	5.4	0.32	5.85	15	5.6	0.32	5.60
Triglycerides (mg/dL)	14	70	16.1	23.0	15	63	23.1	36.8	15	62	16.0	25.6	15	58	16.7	29.0
Urea Nitrogen (mg/dL)	14	14.8	3.00	20.25	15	15.1	2.32	15.3	15	14.7	1.92	13.0	15	14.4	2.10	14.6

a Data were obtained from pages 59 and 60 of the study report. Percent differences from controls, calculated by the reviewers, are included in parentheses.
N Number of animals examined
SD Standard Deviation
CV Coefficient of Variation
* Significantly different from controls at $p<0.05$.

G. <u>Histopathology</u>: The incidences of histopathological findings of the thyroid gland are presented in Table 6. There were no treatment-related microscopic findings in the thyroid, ovaries, uterus, or kidneys at any dose.

TABLE 6. 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	14	1	5	14
	2	0	14	2	9	14
	3	1	14	3	0	14
	4	10	14	4	0	14
	5	3	14	5	0	14
Glyphosate 100 mg/kg/day	1	0	15	1	7	15
	2	0	15	2	8	15
	3	1	15	3	0	15
	4	8	15	4	0	15
	5	6	15	5	0	15
Glyphosate 300 mg/kg/day	1	0	15	1	6	15
	2	0	15	2	9	15
	3	2	15	3	0	15
	4	8	15	4	0	15
	5	5	15	5	0	15
Glyphosate 1000 mg/kg/day	1	0	15	1	7	15
	2	0	15	2	8	15
	3	2	15	3	0	15
	4	9	15	4	0	15
	5	4	15	5	0	15

a Data were obtained from pages 113 of the study report.
b Thyroid histopathology is graded on a 1 – 5 scale: Follicular cell height,1 = lowest, 5 = highest; and Colloid area, 1 = most colloid, 5 = least colloid.
O Number Observed
E Number Examined

The incidence of histopathological findings of the ovaries, uterus and kidneys are presented in Table 7. The ovaries appeared normal histopathologically. There were no microscopic findings in the uterus related to treatment with the test material. Single occurrences of findings in the kidneys (cyst, mild pelvis dilatation, and mild chronic inflammation) were considered to be incidental.

TABLE 7. Incidence of Histopathological Lesions of the Ovaries, Uterus, and Kidney [a]								
Findings	Dose Level (mg/kg/day)							
	Vehicle Control		Glyphosate 100 mg/kg/day		Glyphosate 300 mg/kg/day		Glyphosate 1000 mg/kg/day	
	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined
Ovaries								
Unremarkable	14	14	15	15	15	15	15	15
Uterus								
Unremarkable	14	14	15	15	15	15	15	15
Kidney								
Unremarkable	13	14	15	15	13	15	12	15
Cyst	1	14	0	15	1	15	1	15
Dilatation, pelvis mild	0	14	0	15	1	15	1	15
Inflammation, chronic, mild	0	14	0	15	0	15	1	15

a Data were obtained from pages 103-106 of the study report.

III. DISCUSSION AND CONCLUSIONS

A. <u>INVESTIGATORS' CONCLUSIONS</u>: The Investigators concluded that there was no evidence of any direct test substance-related estrogenic or anti-estrogenic effects, nor was there any evidence of direct test substance-related effects on pubertal development or thyroid function in the juvenile/peripubertal female rat following oral administration of glyphosate at dose levels up to 1000 mg/kg/day (limit dose).

B. <u>AGENCY COMMENTS</u>: One animal in the control group was sacrificed *in extremis* on PND 27 due to impairment of the right forelimb; all other animals survived until scheduled sacrifice. Treatment-related clinical signs were limited to rales in 4/15 and 13/15 females in the 300 and 1000 mg/kg/day groups, respectively, approximately 4 hours post-dosing, but this finding did not persist to the daily examinations. There were no treatment-related differences in any general growth or vaginal opening parameters relative to controls. One female each in the control and 300 mg/kg/day groups failed to attain vaginal opening. There were no statistically significant differences in any estrous cyclicity parameters, and the cycle status at necropsy was similar among all groups. Serum T_4 and TSH were not affected by treatment, and no adverse treatment-related effects on any clinical chemistry parameter were observed at any dose. There were no treatment-related microscopic findings in the thyroid, ovaries, uterus, or kidneys at any dose.

C. <u>STUDY DEFICIENCIES</u>: The following minor deficiency was noted that is not considered to have had an adverse impact on the results, interpretation or conclusions of this study:

- The mean age at vaginal opening in the control group (36.4 days) exceeded the recommended performance criteria maximum (35.62 days).

DATA EVALUATION RECORD

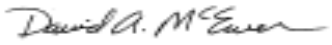
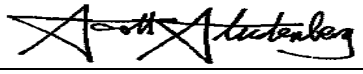
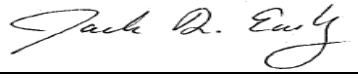
GLYPHOSATE

Study Type: OCSPP 890.1500, Male Pubertal Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-74-2012 (MRID 48671313)

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: David A. McEwen, B.S.	Signature: Date:	 7/03/2012
Secondary Reviewer: Scott D. Studenberg, Ph.D., D.A.B.T.	Signature: Date:	 7/08/2012
Program Manager: Jack D. Early, M.S.	Signature: Date:	 7/13/2012
Quality Assurance: Steven Brecher, Ph.D., D.A.B.T.	Signature: Date:	 7/13/2012

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: Anwar Y. Dunbar, Ph.D. **Signature:** Anwar Y. Dunbar
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 05-27-15
Secondary Reviewer: John Liccione, Ph.D. **Signature:**
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 6/18/15

DATA EVALUATION RECORD

STUDY TYPE: Male Pubertal Assay; OCSPP 890.1500

PC CODE: 417300 **DP BARCODE:** D401747

TXR #: 0053233 **CAS No:** 1071-83-6

TEST MATERIAL (PURITY): Glyphosate technical (95.93% a.i.)

SYNONYMS: *N*-(phosphonomethyl)glycine

CITATION: Stump, D.G. (2012) A Pubertal Development and Thyroid Function Assay of Glyphosate Administered Orally in Intact Juvenile/Peripubertal Male Rats. WIL Research Laboratories, LLC, Ashland, OH. Laboratory Project ID: WIL-843005, April 10, 2012. MRID 48671313. Unpublished.

SPONSOR: Joint Glyphosate Task Force, LLC. c/o Data Group Management, 8325 Old Deer Trail, Raleigh, NC

TEST ORDER #: CON-417300-23

EXECUTIVE SUMMARY: In a Male Pubertal Assay (MRID 48671313), 15 Crl:CD(SD) Sprague-Dawley rats/dose group were treated daily via oral gavage (5 mL/kg) with glyphosate technical (95.93% a.i., Lot #: GLP-1103-21149-T) in 0.5% methylcellulose at doses of 0, 100, 300 or 1000 mg/kg/day (limit dose) from post-natal day (PND) 23 to 53. Animals were examined for preputial separation (PPS) daily beginning on PND 30, and age and weight at day of attainment were recorded. Following sacrifice on PND 53, blood was taken for total thyroxine (T_4), testosterone, thyroid stimulating hormone (TSH), and clinical chemistry analysis. The hormones were analyzed by radioimmunoassay (RIA) or chemiluminescence. Weights were recorded for the liver, adrenal glands, thyroid, pituitary, and urogenital organs, and microscopic examinations were performed on the thyroid, kidneys, right testis and epididymides.

One male in the 1000 mg/kg/day group was found dead prior to dosing on PND 24; no significant clinical or macroscopic findings were observed in this animal. All other rats survived until scheduled sacrifice. Treatment-related clinical findings were limited to rales in 9/15 and 14/15 males in the 300 and 1000 mg/kg/day groups, respectively, approximately 4 hours post-dosing. This finding persisted in the daily examinations in 7/15 males at 1000 mg/kg/day throughout the study.

Treatment-related decreases in overall (PND 23-53) body weight gains were observed at 300 mg/kg/day (↓8%, not significant; NS) and 1000 mg/kg/day (↓12%, $p<0.01$). On PND 53,

final body weights in the 300 and 1000 mg/kg/day groups were decreased ($p<0.05$) by 7-10%. A treatment-related delay in the mean age at attainment of complete PPS was noted at 1000 mg/kg/day (48.0 days) compared to controls (45.9 days). However, it was determined that the delay in attainment of complete PPS at this dose was a result of the treatment-related decrease in body weight, rather than a direct anti-androgenic effect.

No compound-related effects on organ weights were observed at any dose. No treatment-related effects on T_4, TSH, or testosterone levels were observed at any dose. T_4 and TSH levels were lower than the control group in all treated groups and testosterone was lower at 300 and 1000 mg/kg/day. However, these changes were not statistically significant and were not associated with any histopathological findings. At 1000 mg/kg/day, the increases ($p<0.01$) in ALT (also at 300 mg/kg/day), sodium, albumin, ALP, AST, chloride, phosphorous, and total protein, and the decrease in urea nitrogen were considered to be related to treatment.

At 1000 mg/kg/day, there was a slight increase in the number of animals with colloid area Grade 4 (5 treated vs. 1 control) and Grade 5 (1 treated vs. 0 controls). There were no treatment-related effects on follicular cell height at any dose compared to controls. There were no treatment-related findings in the testes, epididymides or kidneys.

The highest dose tested showed evidence of overt toxicity based on the decreases in terminal body weight, clinical signs, and mortality at 1000 mg/kg/day; the dose concentrations used in the study are considered adequate.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for a Male Pubertal Assay (OCSPP 890.1500).

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

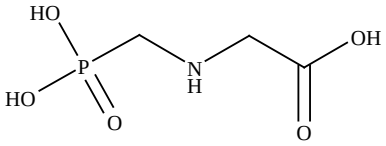
I. MATERIALS AND METHODS

A. MATERIALS

1. Test Facility: WIL Research Laboratories, LLC

Location:	Ashland, OH
Study Director:	D.G. Stump
Other Personnel:	C.A. Picut, Pathologist
	M.E. Haubenstriker, Analytical Chemist
	L. Freshwater, Statistics
Study Period:	August 23, 2011 – April 10, 2012

2. Test Substance: Glyphosate

Description:	White powder
Source:	Monsanto (St. Louis, MO)
Lot/Batch #:	GLP-1103-21149-T (exp. March 9, 2012)
Purity:	95.93% (dried)
Stability:	Stable in 0.5% methylcellulose for up to 15 days at room temperature
CAS #:	1071-83-6
Structure:	

3. Vehicle: 0.5% Methylcellulose in deionized water

4. Test Animals

Species:	Rat	
Strain:	Crl:CD(SD) Sprague-Dawley	
Age/Weight at Study Initiation:	PND 23 /44.3 – 56.6 g males only	
Source:	Charles River Laboratories (Portage, MI)	
Housing:	2-3/cage in plastic cages with heat treated laboratory-grade pine shavings for bedding	
Diet:	Harlan Laboratories 2016CM Teklad Global 16% protein rodent diet, ad libitum Phytoestrogen content average 11 ppm total isoflavones (genistein+daidzein+glycitein)	
Water:	Municipal water, reverse-osmosis filtered (on site), *ad libitum*	
Environmental Conditions:	**Temperature:**	21.2-22.2 °C
	Humidity:	40.1-58.9%
	Air changes:	≥10/hr
	Photoperiod:	14 hrs light/ 10 hrs dark

B. STUDY DESIGN

1. In-Life Dates: Start: September 29, 2011 End: October 30, 2011

2. Mating: Time-mated pregnant dams were received from the supplier on gestation day (GD) 7. The day evidence of mating was confirmed as designated GD 0. To reduce variability, litters were culled to 5 pups/sex (when possible) on PND 4. The female offspring were used for a concurrently submitted female pubertal assay (MRID 48671315).

3. Animal Assignment: Following weaning on PND 21, animals were randomly assigned (stratified by weight in a block design) to the test groups noted in Table 1. Littermates were not assigned to the same treatment group.

TABLE 1. Study Design [a]		
Test group	**Dose (mg/kg/day)**	**# of Males**
Control	0	15
Low	100	15
Mid	300	15
High	1000	15

a Data were obtained from page 26 of the study report.

4. **Dose Selection Rationale:** The dose levels for the current study were selected based on the results of a 7-day oral gavage range-finding study (WIL Study No. WIL-843006, data not provided) in juvenile male rats. The animals (5/dose) were administered glyphosate daily via oral gavage from PND 23-29 at dose levels of 200, 500, and 1000 mg/kg/day. At 1000 mg/kg/day, one male was found dead. No treatment-related effects were observed in clinical observations, body weights, or body weight gains at any dose level. Slightly lower food consumption was noted at 1000 mg/kg/day. Based on these results, dose levels of 100, 300, and 1000 mg/kg/day (limit dose) were selected for this male pubertal assay.

5. **Dose Preparation and Analysis:** Dose formulations were prepared approximately weekly by mixing appropriate amounts of test substance with 0.5% methylcellulose in deionized water. It was stated that analyses to demonstrate homogeneity and stability of the test substance in formulations for up to 15 days of room temperature storage at concentrations of 1 and 200 mg/mL (which bracketed those used in the current study) were conducted in a previous study (Haubenstricker, 2011, WIL-843004); however, no data were provided. Concentration of the test material as administered to the study animals was assessed from the middle stratum of each dosing formulation prepared during the in-life phase of the study. Additionally, the reviewers derived the homogeneity (% RSD) of the samples collected for concentration analysis.

   Results of Dose Analysis

   Homogeneity (% RSD): 0.04-1.9%

   Stability (% of initial): Data not provided

   Concentration (% of nominal): 97.5-114%

   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:** All doses were administered once daily by gavage from PND 23 through PND 53, in a volume of 5mL/kg of body weight. Dosing was performed between 0700 and 0900 hours daily.

7. **Statistics:** Statistics were conducted by BioSTAT Consultants, Inc. using SAS® software. Data statistically analyzed included the organ weights, organ weight to body weight ratio (liver, kidneys, pituitary, and adrenal glands), daily body weights, cumulative body weight gain, serum chemistries, hormones, and weight and age at PPS. When an animal did not attain PPS, PND 54 (last Study Day + 1) and terminal body weight were used as the age and body weight at PPS, respectively. Gamma glutamyltransferase values under range were assigned a value of 0.1 (half the LLQ) for statistical analysis and reporting.

   Endpoints were tested for homogeneity of variance using Levene's test. If that test was significant (p=0.01), then a log or square root transformation was applied to the raw data; if the test was still significant then a nonparametric test was used to analyze the data. If the variances were homogeneous then an analysis of variance (ANOVA) was conducted. The statistical model contained a factor for treatment group and a blocking factor based on the time of study start. A two-sided Dunnett's test was conducted, regardless of the outcome of the ANOVA, looking for significant differences in the test article groups when compared with the vehicle control. If the variances were not homogeneous then the nonparametric Kruskal-Wallis test was used, ignoring the blocking factor, followed by Dunn's test, to compare each of the test article groups with the vehicle control. Since these were pre-planned pairwise comparisons, Dunn's test was conducted regardless of the outcome of the Kruskal-Wallis test. The tests were two-sided (p=0.05), looking for significant differences from the vehicle control. In addition, organ weights, age and weight at PPS were subject to the following analyses if they met the homogeneity of variance criteria: 1) Analysis of covariance (ANCOVA) with Dunnett's test, using body weight at PND 21 as the covariate; 2) linear trend test using the ANOVA model; and 3) linear trend test using the ANCOVA model.

   Histopathology findings presented as a graded response were analyzed with pairwise Mann-Whitney U tests. The tests were one-sided at p=0.05 for increased severity. The tests were two-sided for graded responses presented on a Grade 1-5 scale at p=0.05 for increased or decreased severity.

C. METHODS

1. **Mortality and Clinical Examinations:** All animals were examined twice daily for mortality and moribundity. Clinical examinations were conducted daily (prior to dosing) through the day of euthanasia. Additionally, animals were examined approximately 4 hours post-dosing for clinical signs.

2. **Body Weight:** Animals were weighed on the day of randomization, daily prior to dosing and the day of euthanasia. Mean body weight changes were calculated for each corresponding interval and for the overall treatment period (PND 23-53).

3. **Preputial Separation (PPS):** Beginning on PND 30, all animals were examined daily for onset of PPS. The appearance of a partial or a persistent thread of tissue between the glans and prepuce was recorded. Age and weight on the day of completion of PPS were recorded.

4. **Sacrifice and Pathology:** On the day before euthanasia, rats were moved to a holding room separate from where the necropsies were to be performed. Dosing was performed in the holding room on the day of termination and the rats were moved to the necropsy room one at a time for terminal procedures. Approximately 2 hours following dosing, animals were sacrificed by decapitation without anesthesia and all sacrifices were completed by 1300 hours to minimize hormonal variability due to normal diurnal fluctuation. Immediately following decapitation, trunk blood samples were taken for T_4, TSH, testosterone, and serum chemistry analyses. The hormone samples were analyzed fresh or stored frozen ($\leq -20°C$) for subsequent analyses.

a. **Hormone Analysis:** Total T_4 and testosterone were analyzed using an electrochemiluminescent assay on the Cobas e411 (Roche Diagnostics, Indianapolis, IN) and TSH levels were analyzed using an [^{125}I] rTSH kit (Izotop, Institute of Isotopes Ltd., Budapest, Hungary). For both assays, multiple quality control samples were run prior to analysis.

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	Globulins (by calculation)
X	Sodium	X	Glucose
	ENZYMES	X	Total bilirubin
X	Alkaline phosphatase (ALK)	X	Total protein
	Cholinesterase (ChE)	X	Triglycerides
	Creatine phosphokinase		Serum protein electrophoresis
	Lactic acid dehydrogenase (LDH)	X	A/G ratio (by calculation)
X	Alanine aminotransferase (ALT/also SGPT)	X	Bile acids
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. **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*+
XX	Epididymides (left and right separately)*+	X	Liver*
X	Seminal vesicle plus coagulating glands (with fluid)*	X	Adrenals (paired)*
X	Ventral prostate*	X	Pituitary*
X	Dorsolateral prostate*		
X	Levator ani-bulbocavernosus (LABC) muscle complex*		
XX	Kidneys (paired)*+		

* Weights required based on guideline 890.1500
\+ Histopathological examination required based on guideline 890.1500

The right testis, right epididymis and kidneys were weighed prior to fixation. Following weighing, the testis and epididymis were fixed in Bouin's solution (duration of fixation not specified). The thyroid (with parathyroid) was collected with the trachea and fixed in 10% buffered formalin for at least 24 hrs. Following fixation, the thyroid was dissected free of the trachea and weighed. All collected tissues were routinely processed into paraffin blocks, sectioned, stained with hematoxylin and eosin (additionally, PAS was used for the testes and epididymides), 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. Two sections from the thyroid were examined in order to obtain representative samples of the thyroid tissue.

II. RESULTS

A. Mortality: One male in the 1000 mg/kg/day group (Animal No. 24843-05) was found dead prior to dosing on PND 24. This death following a single dose was considered to be related to treatment because no deaths were observed in the control group, lower body weight gain was observed in this group following the first day of treatment, and mortality was observed in the 7-day range-finding study at 1000 mg/kg/day. All other animals survived until scheduled sacrifice.

B. Clinical Signs of Toxicity: Treatment-related clinical findings were limited to rales in 9/15 and 14/15 males in the 300 and 1000 mg/kg/day groups, respectively, approximately 4 hours post-dosing (Table 2). This finding persisted to the daily examinations in 7/15 males at 1000 mg/kg/day. No other treatment-related clinical signs were noted during the 4-hour post-dosing or daily examinations at any dose level. Additional clinical signs noted during the study, including impaired use of forelimb, red material around the nose, yellow material around the urogenital area, and marked struggling during the dosing procedure, occurred infrequently, at similar frequencies in the controls, and/or in a manner that was not dose-dependent.

TABLE 2. Incidence of Clinical Observations [a]								
Observation	Dose Level (mg/kg/day)							
	Vehicle Control		Glyphosate 100 mg/kg/day		Glyphosate 300 mg/kg/day		Glyphosate 1000 mg/kg/day	
	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined	# Observed	# Examined
Daily Observations								
No clinical observations	464[b]/15	15	463[b]/15	15	455[b]/15	15	415[b]/15	15
Found dead	0	15	0	15	0	15	1	15
Rales	0	15	0	15	1	15	20[b]/7	15
4-Hours Post-dosing								
No clinical observations	448[b]/15	15	445[b]/15	15	426[b]/15	15	343[b]/15	15
Rales	0	15	0	15	13[b]/9	15	75[b]/14	15

a Data were extracted from pages 64 & 65 of the study report
b Total number of observations.

C. <u>General Growth and Preputial Separation</u>: Body weights, body weight gains, age at attainment of PPS and weight at day of PPS are presented in Table 3. Treatment-related decreases in overall (PND 23-53) body weight gains were observed at 300 (↓8%, not significant) and 1000 mg/kg/day (↓12%, $p<0.01$). On PND 53, final body weights in the 300 and 1000 mg/kg/day groups were decreased ($p<0.05$) by 7-10%. The control group body weight at PND 21 (44.7 g) was slightly below the performance criteria acceptable value of 45.472 g; however, the % CV value was within the acceptable range.

A treatment-related delay in the mean age at attainment of complete PPS was noted at 1000 mg/kg/day (48.0 days) compared to controls (45.9 days). It was reported that Ashby (2000)[1] demonstrated that body weight differences of approximately 12% could delay balano-preputial separation by 1-2 days. The delay observed at 1000 mg/kg/day occurred at a dose that produced a 10% lower final body weight. Additionally, mean body weight at attainment of PPS was similar to controls in all treated groups. Furthermore, it was stated that if the test material was producing an anti-androgenic response, the testosterone level in the 1000 mg/kg/day group would have been expected to be higher than the control group. However, the testosterone level at 1000 mg/kg/day (1.57 ng/mL) was lower than the control group (2.86 ng/mL). Based on these findings, it was determined that the delay in attainment of complete PPS at 1000 mg/kg/day was a result of the treatment-related decrease in body weight, rather than a direct anti-androgenic effect.

The CV value for the body weight at attainment of PPS (9.77%) in the control group was above the acceptable performance criteria range (7.57%). However, the mean weight at attainment of PPS and the mean and CV values for age of attainment of PPS were within the acceptable ranges.

[1] Ashby, J and Lefevre, P.A. The pubertal male rat assay as an alternative to the Hershberger castrated male rat assay for the detection of anti-androgens, estrogens and metabolic modulators. J Appl Toxicol. **2000**, 20(1), 35-47.

TABLE 3. General Growth and Preputial Separation (PPS)[a]																
Parameter Evaluated		Vehicle Control				Glyphosate 100 mg/kg/day				Glyphosate 300 mg/kg/day				Glyphosate 1000 mg/kg/day			
		N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Initial Body Weight (PND 23; g)	U	15	51.7	2.48	4.79	15	50.8	3.38	6.66	15	50.3	3.42	6.79	15	50.8	2.91	5.73
Weight at PPS (g)	U	15	216.8	21.2	9.77	15	227.1	22.6	9.96	15	213.1	18.4	8.64	14	214.6	26.2	12.2
	A	15	216.5	21.2	9.77	15	226.9	22.6	9.96	15	213.1	18.4	8.64	14	214.9	26.2	12.2
Final Body Weight (PND 53; g)	U	15	273.1	14.0	5.14	15	276.3	25.4	9.18	15	255.0* (↓7)	22.0	8.64	14	244.8** (↓10)	23.2	9.47
Final Body Weight (% of control)	U	NA					1				-7				-10		
Body Weight Gain (g)	U	15	221.4	13.9	6.30	15	225.6	24.4	10.8	15	204.7 (↓8)	19.8	9.69	14	194.0** (↓12)	21.6	11.1
Age at PPS (PND)	U	15	45.9	2.17	4.72	15	46.9	2.42	5.16	15	47.4	3.07	6.47	14	48.0	1.66	3.47
	A	15	45.9	2.17	4.72	15	46.8	2.42	5.16	15	47.5	3.07	6.47	14	47.9	1.66	3.47
Proportion unseparated (#/N)		0/15				0/15				1/15				0/14			

a Data were obtained from page 59 of the study report. Percent difference from control (calculated by reviewers) is included in parentheses.
U Unadjusted for body weight on PND 23
A Adjusted for body weight on PND 23
N Number of animals examined
SD Standard Deviation
CV Coefficient of Variation
NA Not applicable
* Significantly different from controls at $p<0.05$.
** Significantly different from controls at $p<0.01$.

D. <u>Organ Weights</u>: Organ weights at necropsy are presented in Table 4. No compound-related effects on organ weights were observed at any dose. At 1000 mg/kg/day, the decreases ($p<0.05$) noted in absolute liver (↓15%), pituitary gland (↓16%), LABC (↓16%), ventral prostate (↓23%), and seminal vesicle with fluid (↓19%) and without fluid (↓16%) weights were considered to be secondary to decreased body weights, because there were no histologic findings in any tissues. Likewise, the decreased ($p<0.05$) absolute liver weight (↓10%) noted at 300 mg/kg/day was considered to be secondary to decreased body weights. The decrease in dorsolateral prostate weight (↓14%, $p<0.05$) noted at 300 mg/kg/day was unrelated to dose.

The mean control thyroid (13.73 mg) and kidney weights (1.93 g) were below the performance criteria minimum acceptable values (14 mg and 2.242 g, respectively), but were within the laboratory's historical control ranges for rats of this age, and the CV values were within the acceptable range.

TABLE 4. Organ Weights at Necropsy[a]

Organ		Vehicle Control				Glyphosate 100 mg/kg/day				Glyphosate 300 mg/kg/day				Glyphosate 1000 mg/kg/day			
		N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Liver (g)	U	15	12.69	1.06	8.38	15	13.09	1.56	11.94	15	11.45* (↓10)	1.51	13.20	14	10.77* (↓15)	1.51	13.99
	A	15	12.67	1.06	8.38	15	13.12	1.56	11.94	15	11.42* (↓10)	1.51	13.20	14	10.82* (↓15)	1.51	13.99
	R	15	4.64	0.26	5.53	15	4.73	0.287	6.060	15	4.47	0.27	6.091	14	4.39	0.31	7.03
Kidneys (g)	U	15	1.93	0.15	7.57	15	1.99	0.259	13.02	15	1.83	0.22	12.17	14	1.83	0.23	12.76
	A	15	1.92	0.15	7.57	15	1.99	0.259	13.02	15	1.83	0.22	12.17	14	1.84	0.23	12.76
	R	15	0.71	0.04	6.08	15	0.72	0.043	6.064	15	0.72	0.05	6.949	14	0.74	0.04	5.24
Pituitary (mg)	U	15	10.9	1.14	10.4	15	10.5	1.50	14.27	15	10.0	1.36	13.62	14	9.2* (↓16)	1.71	18.59
	A	15	10.9	1.14	10.4	15	10.5	1.50	14.27	15	9.9	1.36	13.62	14	9.2* (↓16)	1.71	18.59
	R	15	4.0	0.41	10.3	15	3.8	0.48	12.50	15	3.9	0.46	11.88	14	3.8	0.53	13.98
Adrenals (mg)	U	15	45.6	7.28	16.0	15	46.8	6.76	14.45	15	41.7	6.39	15.35	14	41.4	5.02	12.12
	A	15	45.4	7.28	16.0	15	46.9	6.76	14.45	15	41.5	6.39	15.35	14	41.7	5.02	12.12
	R	15	16.7	2.44	14.7	15	16.9	1.86	10.95	15	16.3	1.78	10.93	14	16.9	1.68	9.91
Seminal vesicle + CG, with fluid (mg)	U	15	630.0	106	16.8	15	619.8	140.9	22.73	15	545.5	136.8	25.08	14	513.2* (↓19)	88.2	17.19
	A	15	627.1	106	16.8	15	621.2	140.9	22.73	15	543.3	136.8	25.08	14	518.6* (↓17)	88.2	17.19
Seminal vesicle + CG, without fluid (mg)	U	15	387.5	44.7	11.6	15	387.5	67.84	17.51	15	344.9	59.80	17.34	14	323.8* (↓16)	52.4	16.19
	A	15	385.9	44.7	11.6	15	388.0	67.84	17.51	15	343.9	59.80	17.34	14	326.5* (↓15)	52.4	16.19
Ventral prostate (mg)	U	15	257.5	37.8	14.7	15	264.0	61.11	23.14	15	236.3	44.33	18.76	14	199.2* (↓23)	46.0	23.11
	A	15	256.5	37.8	14.7	15	264.8	61.11	23.14	15	235.4	44.33	18.76	14	201.2* (↓22)	46.0	23.11
Dorsolateral prostate (mg)	U	15	122.8	17.6	14.3	15	115.3	21.53	18.68	15	105.3* (↓14)	15.41	14.64	14	112.2	18.9	16.81
	A	15	122.4	17.6	14.3	15	115.6	21.53	18.68	15	105.0* (↓14)	15.41	14.64	14	113.0	18.9	16.81
LABC (mg)	U	15	539.6	61.5	11.4	15	527.8	107.7	20.40	15	491.4	97.15	19.77	14	453.9* (↓16)	67.0	14.77
	A	15	537.9	61.5	11.4	15	528.9	107.7	20.40	15	490.0	97.15	19.77	14	457.2* (↓15)	67.0	14.77
Epididymis, left (mg)	U	15	201.9	19.0	9.4	15	199.4	18.61	9.33	15	187.5	29.15	15.54	14	182.0	23.1	12.67
	A	15	201.2	19.0	9.4	15	199.3	18.61	9.33	15	187.2	29.15	15.54	14	183.0	23.1	12.67
Epididymis, right (mg)	U	15	201.1	21.5	10.7	15	201.0	15.32	7.62	15	193.4	30.89	15.97	14	182.0	17.2	9.45
	A	15	200.2	21.5	10.7	15	201.2	15.32	7.62	15	192.9	30.89	15.97	14	183.4	17.2	9.45
Testis, left (mg)	U	15	1366	61.3	4.49	15	1407	86.78	6.17	15	1352	93.40	6.91	14	1300	189	14.51
	A	15	1364	61.3	4.49	15	1408	86.78	6.17	15	1351	93.40	6.91	14	1305	189	14.51
Testis, right (mg)	U	15	1393	72.9	5.23	15	1420	87.27	6.15	15	1349	136.7	10.13	14	1337	167	12.48
	A	15	1389	72.9	5.23	15	1420	87.27	6.15	15	1347	136.7	10.13	14	1341	167	12.48
Thyroid, fixed (mg)	U	15	13.73	2.76	20.1	15	13.91	2.098	15.08	15	13.32	2.27	17.04	14	12.63	2.81	22.24
	A	15	13.71	2.76	20.1	15	13.91	2.098	15.08	15	13.31	2.27	17.04	14	12.66	2.81	22.24

a Data were obtained from page 60 of the study report. Percent differences from controls (calculated by reviewers) are included in parentheses.
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)
N Number of animals examined
SD Standard Deviation
CV Coefficient of Variation
* Significantly different from controls at $p<0.05$.

E. <u>Clinical Chemistry and Hormone Levels</u>: Mean hormone and clinical chemistry levels are presented in Table 5. No treatment-related effects on T_4, TSH, or testosterone levels were observed at any dose. T_4 and TSH levels were lower than the control group in the 100, 300, and 1000 mg/kg/day groups and testosterone was lower at 300 and 1000 mg/kg/day. However, these changes were not statistically significant and were not associated with any histopathological findings.

The CV value for mean TSH level in the control group (75.059%) exceeded the maximum value in the performance criteria (58.29%); however, the mean TSH value was within the acceptable range of the performance criteria.

Treatment-related differences ($p<0.01$) from controls in the following clinical chemistry parameters were noted at 1000 mg/kg/day: (i) ALT (↑51%); (ii) sodium (↑2%); (iii) albumin (↑5%); (iv) ALP (↑22%); (v) AST (↑35%); (vi) chloride (↑4%); (vii) phosphorus (↑7%); (viii) total protein (↑5%); and (ix) urea nitrogen (↓17%). Additionally, increased ALT (↑22%, $p<0.05$) was noted at 300 mg/kg/day. However, as these differences were minor and/or in a direction not usually associated with a toxicological effect (urea nitrogen), none of the findings at 300 or 1000 mg/kg/day were considered adverse. No other statistically significant clinical chemistry findings were noted.

TABLE 5. Hormone Levels and Clinical Chemistry[a]																
Parameter	Vehicle Control				Glyphosate 100 mg/kg/day				Glyphosate 300 mg/kg/day				Glyphosate 1000 mg/kg/day			
	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Thyroid Hormones																
Total T_4 (µg/dL)	15	6.22	1.08	17.3	15	6.02	0.43	7.06	15	5.92	0.87	14.8	14	5.68	0.74	13.0
TSH (ng/mL)	15	8.30	6.23	75.1	15	6.77	3.73	55.0	15	6.91	2.82	40.8	14	5.37	3.40	63.2
Testosterone (ng/mL)	15	2.86	1.57	54.9	15	3.97	3.23	81.2	15	2.31	1.54	66.4	14	1.57	0.80	50.7
Clinical Chemistry																
A/G Ratio	15	2.28	0.20	8.86	15	2.27	0.20	8.59	15	2.17	0.26	12.1	14	2.38	0.25	10.5
Albumin (g/dL)	15	4.1	0.12	3.05	15	4.1	0.14	3.27	15	4.1	0.18	4.35	14	4.3** (↑5)	0.22	5.16
ALP (U/L)	15	277	36.8	13.3	15	276	34.5	12.5	15	287	59.6	20.8	14	339** (↑22)	63.6	18.7
ALT (U/L)	15	59	7.7	13.0	15	66	8.7	13.2	15	72* (↑22)	13.4	18.4	14	89** (↑51)	23.1	26.0
AST (U/L)	15	111	17.1	15.4	15	129	21.7	16.8	15	131	28.6	21.9	14	150* (↑35)	59.7	39.7
Bile Acids (µmol/L)	15	22.7	13.9	61.2	15	16.9	12.1	71.3	15	28.1	25.7	91.4	14	20.7	8.32	40.3
Calcium (mg/dL)	15	12.3	0.37	2.98	15	12.5	0.32	2.55	15	12.4	0.19	1.52	14	12.4	0.45	3.66
Chloride (mEq/L)	15	100	1.5	1.5	15	100	1.2	1.2	15	101	1.1	1.1	14	104** (↑4)	2.4	2.3
Cholesterol (mg/dL)	15	91	12.7	14.0	15	88	15.0	17.1	15	83	13.2	15.9	14	85	11.4	13.4
Creatinine (mg/dL)	15	0.2	0.05	33.7	15	0.1	0.05	35.2	15	0.1	0.05	35.2	14	0.1	0.05	36.6
GGT (U/L)	15	0.2	0.14	88.4	15	0.1	0.03	24.2	15	0.2	0.29	153	14	0.1	0.00	0.00
Globulin (g/dL)	15	1.8	0.15	8.36	15	1.9	0.15	8.12	15	1.9	0.21	11.1	14	1.8	0.15	8.39
Glucose (mg/dL)	15	152	6.0	4.0	15	154	9.7	6.3	15	150	9.7	6.5	14	156	11.5	7.4
Phosphorous (mg/dL)	15	10.1	0.61	6.03	15	10.3	0.61	5.90	15	10.4	0.70	6.77	14	10.8** (↑7)	0.50	4.64
Potassium (mEq/L)	15	6.39	0.39	6.03	15	6.37	0.27	4.25	15	6.40	0.28	4.40	14	6.43	0.384	5.98
Sodium (mEq/L)	15	142	1.3	0.9	15	143	1.7	1.2	15	144	1.3	0.9	14	145** (↑2)	2.1	1.5
Total Bilirubin (mg/dL)	15	0.05	0.01	29.9	15	0.05	0.02	41.8	15	0.05	0.02	40.3	14	0.06	0.021	35.2
Total Protein (g/dL)	15	5.9	0.21	3.57	15	6.0	0.21	3.47	15	6.0	0.21	3.49	14	6.2** (↑5)	0.26	4.15
Triglycerides (mg/dL)	15	124	33.1	26.6	15	158	48.8	30.8	15	124	37.4	30.2	14	122	51.4	42.1
Urea Nitrogen (mg/dL)	15	15.4	1.57	10.2	15	14.7	1.98	13.5	15	14.0	2.27	16.2	14	12.8** (↓17)	2.36	18.4

a Data were obtained from pages 61 and 62 of the study report. Percent differences from controls (calculated by reviewers) are included in parentheses.
N Number of animals examined
SD Standard Deviation
CV Coefficient of Variation
* Significantly different from controls at $p<0.05$.
** Significantly different from controls at $p<0.01$.

F. <u>Macro- and Histopathology</u>: At the scheduled euthanasia, no treatment-related macroscopic findings were noted. The incidences of histopathological findings of the thyroid gland are presented below in Table 6. At 1000 mg/kg/day, there was a slight increase in the number of animals with colloid area Grade 4 (5 treated vs. 1 control) and

Grade 5 (1 treated vs. 0 controls). There were no treatment-related effects on follicular cell height at any dose compared to controls. There were no treatment-related findings in the testes, epididymides or kidneys.

TABLE 6. 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	15	1	0	15
	2	6	15	2	13	15
	3	8	15	3	2	15
	4	1	15	4	0	15
	5	0	15	5	0	15
Glyphosate 100 mg/kg/day	1	0	15	1	0	15
	2	3	15	2	13	15
	3	10	15	3	2	15
	4	2	15	4	0	15
	5	0	15	5	0	15
Glyphosate 300 mg/kg/day	1	0	15	1	0	15
	2	3	15	2	13	15
	3	10	15	3	2	15
	4	2	15	4	0	15
	5	0	15	5	0	15
Glyphosate 1000 mg/kg/day	1	0	14	1	1	14
	2	3	14	2	13	14
	3	5	14	3	0	14
	4	5	14	4	0	14
	5	1	14	5	0	14

a Data were obtained from page 116 of the study report.
b Thyroid histopathology is graded on a 1 – 5 scale: Follicular cell height,1 = lowest, 5 = highest; and Colloid area, 1 = most colloid, 5 = least colloid.
O Number Observed
E Number Examined

III. DISCUSSION AND CONCLUSIONS

A. <u>INVESTIGATOR'S CONCLUSIONS</u>: The investigators concluded that there was no evidence of any direct test substance-related androgenic or anti-androgenic effects, nor was there any evidence of direct test substance-related effects on pubertal development or thyroid function in the juvenile/peripubertal male rat at up to 1000 mg/kg/day (limit dose).

B. **AGENCY COMMENTS:** One male in the 1000 mg/kg/day group was found dead prior to dosing on PND 24; no significant clinical or macroscopic findings were observed in this animal. All other rats survived until scheduled sacrifice. Treatment-related clinical findings were limited to rales in 9/15 and 14/15 males in the 300 and 1000 mg/kg/day groups, respectively, approximately 4 hours post-dosing. This finding persisted in the daily examinations in 7/15 males at 1000 mg/kg/day through PND 52.

Treatment-related decreases in overall (PND 23-53) body weight gains were observed at 300 mg/kg/day (↓8%, not significant) and 1000 mg/kg/day (↓12%, $p<0.01$). On PND 53, final body weights in the 300 and 1000 mg/kg/day groups were decreased ($p<0.05$) by 7-10%. A treatment-related delay in the mean age at attainment of complete PPS was noted at 1000 mg/kg/day (48.0 days) compared to controls (45.9 days). It was reported that body weight differences of approximately 12% could delay balanopreputial separation by 1-2 days. The delay observed occurred at a dose that produced a 10% lower final body weight. Additionally, mean body weight at attainment of PPS was similar to controls in all treated groups. Furthermore, it was stated that if the test material was producing an anti-androgenic response, the testosterone level in the 1000 mg/kg/day group would have been expected to be higher than the control group. However, the testosterone level at 1000 mg/kg/day (1.57 ng/mL) was lower than the control group (2.86 ng/mL). Based on these findings, it was determined that the delay in attainment of complete PPS at 1000 mg/kg/day was a result of the treatment-related decrease in body weight, rather than a direct anti-androgenic effect.

No compound-related effects on organ weights were observed at any dose. Because there were no histologic findings in any tissues examined, the decreases noted in various absolute organ weights at 300 and 1000 mg/kg/day were considered to be secondary to decreased body weights.

No treatment-related effects on T_4, TSH, or testosterone levels were observed at any dose. T_4 and TSH levels were lower than the control group in the 100, 300, and 1000 mg/kg/day groups and testosterone was lower at 300 and 1000 mg/kg/day. However, these changes were not statistically significant and were not associated with any histopathological findings. Treatment-related differences ($p<0.01$) from controls in the following clinical chemistry parameters were noted at 1000 mg/kg/day: (i) ALT (↑51%); (ii) sodium (↑2%); (iii) albumin (↑5%); (iv) ALP (↑22%); (v) AST (↑35%); (vi) chloride (↑4%); (vii) phosphorus (↑7%); (viii) total protein (↑5%); and (ix) urea nitrogen (↓17%). Additionally, increased ALT (↑22%, $p<0.05$) was noted at 300 mg/kg/day.

At 1000 mg/kg/day, there was a slight increase in the number of animals with colloid area Grade 4 (5 treated vs. 1 control) and Grade 5 (1 treated vs. 0 controls). There were no treatment-related effects on follicular cell height at any dose compared to controls. There were no treatment-related findings in the testes, epididymides or kidneys.

The highest dose tested (1000 mg/kg/day) showed evidence of overt toxicity based on the decreases in terminal body weight, clinical signs and mortality.

C. <u>STUDY DEFICIENCIES</u>: 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 control group body weight at PND 21 (44.7 g) was slightly below the performance criteria recommended value of 45.472 g; however, the % CV value was within the acceptable range.
- The CV value for the body weight at attainment of PPS (9.77%) in the control group was above the recommended performance criteria range (7.57%). However, the mean weight at attainment of PPS and the mean and CV values for age of attainment of PPS were within the acceptable ranges.
- The mean control thyroid (13.73 mg) and kidney weights (1.93 g) were below the recommended performance criteria minimum acceptable values (14 mg and 2.242 g, respectively) but were within the laboratory's historical control ranges for rats of this age, and the CV values were within the acceptable range.

DATA EVALUATION RECORD

GLYPHOSATE

Study Type: OCSPP 890.1550, Steroidogenesis Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-57-2012 (MRID 48617005)

Prepared for
Health Effects Division
Office of Pesticide Programs
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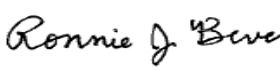
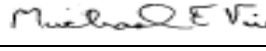
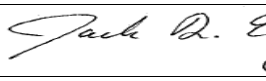
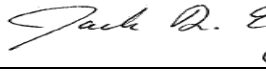
Primary Reviewer: Ronnie J. Bever Jr., Ph.D., D.A.B.T.	Signature: Date:	 06/12/2012
Secondary Reviewer Michael E. Viana, Ph.D., D.A.B.T.	Signature: Date:	 06/19/2012
Program Manager: Jack D. Early, M.S.	Signature: Date:	 06/26/2012
Quality Assurance: Jack D. Early, M.S.	Signature: Date:	 06/26/2012

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: Anwar Y. Dunbar, Ph.D. **Signature:**
Risk Assessment Branch 1 Health Effects Division (7509P) **Date:** 05-27-15
Secondary Reviewer: John Liccione, Ph.D. **Signature:**
Risk Assessment Branch 1 Health Effects Division (7509P) **Date:** 6/8/15

DATA EVALUATION RECORD

STUDY TYPE: Steroidogenesis Assay (H295R Cells); OCSPP 890.1550

PC CODE: 417300 **DP BARCODE:** D398693

TXR#: 0053233 **CAS No.:** 1071-83-6

TEST MATERIAL (PURITY): Glyphosate (not reported)

SYNONYMS: *N*-(phosphonomethyl)glycine

CITATION: Hecker, M., Hollert H., Cooper, R., *et al.* (2011) The OECD validation program of the H295R steroidogenesis assay: phase 3. Final inter-laboratory validation study. MRID 48617005. Published: Environ. Sci. Pollut. Res. (2011) 18:503-515.

SPONSOR: Not applicable

TEST ORDER #: CON-417300-23

EXECUTIVE SUMMARY: The purpose of this study was to validate the use of a standardized steroidogenesis assay as detailed in *OECD Guideline for the Testing of Chemicals: Draft Proposal for a New Guideline 4XX – The H295R Steroidogenesis Assay* (available on-line at http://www.oecd.org/dataoecd/56/11/44285292.pdf). In this validation study, 28 chemicals were selected as a screen for potential effects of endocrine-disrupting chemicals on the production of testosterone (T) and 17β-estradiol (E2). These chemicals were selected based on their known or suspected endocrine activity, or lack thereof, and included inhibitors and inducers of different potencies as well as positive and negative controls. These chemicals were selected and approved by the OECD Validation and Management Group for Non-Animal Testing (VMG NA). Glyphosate was one of the chemicals evaluated. A total of seven laboratories from the USA, Denmark, Germany, Japan, Hong Kong, and Canada, each with different levels of experience in conducting the H295R steroidogenesis assay, were invited to participate in this validation study. Inclusion of laboratories with different levels of proficiency in conducting the assay was essential to evaluate the completeness of the test protocols and their transferability. Each laboratory was assigned a random code number (1–7) as part of the study. However, part way through the study, two of the seven laboratories decided to cease their participation in the validation studies. Thus, with the exception of the QC exposure data, only the data for the remaining five laboratories that completed the validation studies are presented (Labs 1, 2, 3, 4, and 6). One laboratory evaluated all 28 chemicals, and one other laboratory (#4) also evaluated glyphosate. The laboratories were not identified.

In this steroidogenesis assay (MRID 48617005), H295R cells cultured *in vitro* in 24-well plates were incubated with glyphosate (purity and lot # not provided) at seven concentrations between 0.0001 and 100 μM (specific concentrations not reported) for 48 hours in triplicate for three independent experiments. The test chemical's vehicle was not identified. The presence or absence of precipitation and/or cytotoxicity was not reported. 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 concentrations; positive controls included the known inhibitor (prochloraz) and inducer (forskolin) of estradiol and testosterone production. T and E2 levels were measured using radioimmunoassays or ELISA; responses of the QC plates measured by these assays were confirmed by LC-MS (at Lab 1).

The report stated that with a few exceptions, all of the laboratories met the key quality performance parameters for conducting the H295R assay protocol. The report stated that two laboratories demonstrated that glyphosate exposure does not affect testosterone or estradiol levels in this assay; however, data were not presented.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for a Steroidogenesis assay (OCSPP 890.1550).

COMPLIANCE: Signed and dated GLP Compliance and Quality Assurance statements were not provided in this published article.

I. MATERIALS AND METHODS

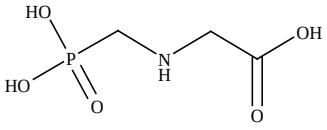
The following information was obtained from the cited article (MRID 48617005). Additionally, the cited article stated that a standardized H295R steroidogenesis assay protocol was developed and presented as a proposed draft guideline (http://www.oecd.org/dataoecd/56/11/44285292.pdf). The reviewers assume that this protocol was followed without deviation, as the point of this study was the validation of this protocol. These assumptions apply not only to the methodology, but the recommended criteria and required protocol compliance of the results as well. Consequently, some of the materials and methods from this report come from the OECD protocol referenced by the hyperlink.

A. MATERIALS: A total of 28 chemicals were selected in this study to validate the H295R steroidogenesis assay as a screen for potential effects of endocrine-disrupting chemicals on the production of T and E2. These chemicals were selected based on their known or suspected endocrine activity, or lack thereof, and included inhibitors and inducers of different potencies as well as positive and negative controls. Where possible, the test set of chemicals was harmonized with those used in other steroidogenesis assays currently under development or in validation [e.g., the Registration, Evaluation, Authorization and Restriction of Chemical substances (REACH) program]. These chemicals were selected and approved by the OECD VMG NA. Glyphosate was one these chemicals.

1. Test Facility: Not reported

Location:	USA, Denmark, Germany, Japan, Hong Kong, and Canada (laboratories from seven countries were initially involved, but two decided to cease participation; the seven laboratories were not identified)
Study Director:	Not reported
Other Personnel:	Not reported
Study Period:	Not reported

2. Test Substance: Glyphosate

Description (molecular weight):	White crystalline powder (169.07)
Batch # (expiration date):	Not reported
Purity:	Not reported
Solubility:	Not reported; water soluble (1.01 g/100 mL at 20ºC)
Vapor pressure:	Not reported; $<1 \times 10^{-5}$ Pa at 25ºC
Stability:	Not reported
Storage conditions:	Not reported
CAS #:	1071-83-6
Structure:	

3. Positive Control: Forskolin

Description (molecular weight):	White powder (410.50)
Source:	Not reported
Lot # (expiration date):	Not reported
Purity:	Not reported
Solubility (in solvent):	Soluble at tested concentrations in DMSO
Storage conditions:	Room temperature
CAS #:	66575-29-9

4. Negative Control: Prochloraz

Description (molecular weight):	White powder (376.67)
Source:	Not reported
Lot # (expiration date):	Not reported
Purity:	Not reported
Solubility (in solvent):	Soluble at tested concentrations in DMSO
Storage conditions:	Room temperature
CAS #:	67747-09-5

5. Solvent/Vehicle Control: Not reported (DMSO listed in the proposed draft guideline)

6. Stock Medium: Dulbecco's modified Eagle's medium/nutrient mixture F12 Ham with 15 mM HEPES, sodium bicarbonate, ITS+Premix, and 2.5% Nu-Serum (assumed by reviewer)

Source:	Not reported
Lot # (expiration date):	Not reported

7. Test Cells: H295R human adrenocortical carcinoma cells (ATCC CLR-2128) were cultured for a minimum of four to five passages to ensure sufficient basal E2 production (cell age was not to exceed ten passages). Cells were incubated in the stock medium at 5% CO_2 and 37°C for approximately 24 hours prior to exposure.

The following performance criteria were met (indicated by an "x"):

x	Cell passage identifier. Cell Passage #: Not reported
x	Cells frozen down at passage 5
x	Frozen cells cultured for 4 additional passages
x	Total number of passages does not exceed 10

B. METHODS: A total of seven laboratories from the USA, Denmark, Germany, Japan, Hong Kong, and Canada, each with different levels of experience in conducting the H295R steroidogenesis assay, were invited to participate in this validation study. Inclusion of laboratories with different levels of proficiency in conducting the assay was essential to evaluate the completeness of the test protocols and their transferability. Each laboratory was assigned a random code number (1–7) as part of the study. However, part way through the study, two of the seven laboratories decided to cease their participation in the validation studies. Thus, with the exception of the QC exposure data, only the data for the remaining five laboratories that completed the validation studies are presented (Labs 1, 2, 3, 4, and 6). One laboratory evaluated all 28 chemicals, and one other laboratory (#4) also evaluated glyphosate. The laboratories were not identified.

1. Pre-Test Information: The report stated that laboratories were required to demonstrate competence in performing all of the procedures that are part of the H295R steroidogenesis assay prior to testing chemicals. The QC that was part of the actual conduct of the assay to allow for the evaluation of the assay performance during each experiment also served as a benchmark for determining laboratory competence prior to the initiation of chemical testing. Prior to initiation of the actual exposure experiments, each chemical was tested for potential interference with the hormone detection system used. This was of particular relevance for antibody-based assays such as enzyme-linked immunoassays (ELISAs) and radio immunoassays (RIAs), because it has been previously shown that some chemicals can interfere with these tests. The following performance criteria were to be met, and the report

stated that all laboratories met the key quality performance parameters with few exceptions (details not reported):

System	Parameter	Comparison to/between	T	E2
Hormone detection system	Sensitivity	Detectable fold decrease relative to SC	≥2-fold	≥2-fold
	Precision	CV among replicate measures (absolute concentrations) of the same well for SCs	≤25%	≤25%
Cell assay	Basal hormone production in SCs	Fold greater than LOQ of hormone detection system	≥5-fold	≥2.5-fold
	Precision (SCs)	CV among absolute concentrations of replicate wells	≤30%	≤30%
	Sensitivity (induction @ 10 μM forskolin)	Fold greater than SC	≥2-fold	≥7.5-fold
	Sensitivity (inhibition @ 3 μM prochloraz)	Fold less than SC	≥0.5-fold	≥0.5-fold

Induction and inhibition refer to the relative change in hormone production after exposure to 10 μM forskolin or 3 μM prochloraz, respectively, in the QC plates

CV Coefficient of variation (%), *LOQ* limit of quantification, *SC* solvent control

Resulting data from the pre-test assays were not presented to allow for independent verification.

a. **Hormone Assay Interference Test:** No data from the hormone assay interference test were provided.

b. **Hormone Extraction:** No data from pre-test hormone extraction were provided.

c. **Laboratory Proficiency Test:** No laboratory proficiency test data were provided.

2. **Test Solutions:** Details on the preparation of the glyphosate test solution (including the solvent used) were not provided. The presence or absence of precipitation was not reported.

3. **Cell Plating and Preincubation:** Cells were maintained in the Stock Medium described above. H295R cells were grown for five passages, frozen in liquid nitrogen, then thawed and cultured for at least four or five additional passages prior to use in the assay. The cells were plated into wells of a 24-well cell culture plate at a density of approximately 200,000 to 300,000 cells/mL. The cells were then placed into a 5% CO_2 incubator at 37°C for approximately 24 hours prior to chemical exposure. Prior to dosing, the cells were checked microscopically for attachment and proper morphology. Each experiment was repeated three times with exception of Labs 1 and 3, where one and two replicate experiments were conducted per chemical, respectively.

4. **Exposure:** Cells were exposed for 48 h to seven concentrations between 0.0001 and 100 μM of the test chemical in triplicate. Although these concentrations were not presented in the study report, the concentrations are typically as shown in Table 1.

TABLE 1. Dosing Schematic for the Exposure of H295R Cells to Glyphosate (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 Not included in the study report.

A concurrent QC plate was included with each of the 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 as the test plate 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	Blank	Blank	Blank + MeOH[b]	Blank + MeOH[b]	Blank + MeOH[b]
B	DMSO	DMSO	DMSO	DMSO + MeOH[b]	DMSO + MeOH[b]	DMSO + MeOH[b]
C	Forskolin 1 µM	Forskolin 1 µM	Forskolin 1 µM	Prochloraz 0.1 µM	Prochloraz 0.1 µM	Prochloraz 0.1 µM
D	Forskolin 10 µM	Forskolin 10 µM	Forskolin 10 µM	Prochloraz 1 µM	Prochloraz 1 µM	Prochloraz 1 µM

a Data were not included in the study report, but were reported in the OECD protocol on page 11.

b MeOH = methanol was added to these wells for 30 minutes at room temperature following medium removal (end of exposure).

Following dosing, the plates were incubated for 48 hours under the conditions previously described. After the 48 hour exposure, each well was examined under the microscope for cell condition (attachment, morphology, degree of confluence) and signs of cytotoxicity. The media was collected from all wells in two equal portions and stored at −80°C until analyzed.

5. **Cell Viability/Cytotoxicity Assay:** After media removal, cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosman, 1983) or the Live/Dead® variability assay (Invitrogen, Carlsbad, CA). All concentrations, where cell viability was less than or equal to 80%, were excluded from the data analysis.

6. **Hormone Measurement System:** At the end of the exposure period, the medium was removed from each well, and hormones were generally extracted using ethyl ether; however, the medium was used without extraction in the RIA assay. The other laboratories used the ELISA detection system, with one laboratory confirming the QC plate hormone results using LC-MS analysis. The ELISA and RIA detection systems used commercially available hormone detection kits. The lower limit of quantification (LLQ) was ≤100 pg/mL for testosterone and ≤10 pg/mL for estradiol.

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 ≤30% of nominal concentration)

x	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
x	Test compound tested for interference with measurement system

C. DATA ANALYSIS: All data were expressed as mean ± standard error of the mean (SEM). To examine the relative changes in hormone production, results were normalized to the mean solvent control (SC) value for each assay (i.e., each 24-well plate of cells used to test a given chemical), and results were expressed as percent change relative to the SC. Prior to conducting statistical analyses, the assumptions of data normality and variance of homogeneity were evaluated. Normality was evaluated using standard probability plots or the Shapiro–Wilk's test. If the data were normally distributed or approximated a normal distribution, differences between chemical treatments and SCs were analyzed using one-way analysis of variance (ANOVA) followed by a two-sided Dunnett's test. If data were not normally distributed, the Kruskal–Wallis test followed by the Mann–Whitney U test were used. Data analysis was conducted using pooled replicate experiments. All statistical analyses were conducted using SYSTAT 11 (SYSTAT Software, Point Richmond, CA). Differences were considered significant at $p<0.05$.

II. RESULTS

A. TEST COMPOUND: Data were not presented. Two labs demonstrated that the testosterone and estradiol levels in glyphosate-treated cells were similar to SC. These results were in stated to be in agreement with *in vivo* (fish) data[1]. No additional information was provided.

B. CYTOTOXICITY: The presence or absence of cytotoxicity in the glyphosate-treated cells was not reported.

C. QC PLATE: The report stated that with a few exceptions, all of the laboratories met the key quality performance parameters for conducting the H295R assay protocol. It is not clear if this statement was referring to the concurrent QC plates, the Pre-Test, or both. However, the results provided were shown in Figure 1 of the study report on page 507 (copied below).

[1] Soso, AB, Barcellos, LJG, Ranzani-Paiva, MJ, *et al.* (2007) Chronic exposure to sub-lethal concentration of a glyphosate-based herbicide alters hormone profiles and affects reproduction of female Jundi'a (*Rhamdia quelen*). Environ. Toxicol. Pharm. **23**:308–313.

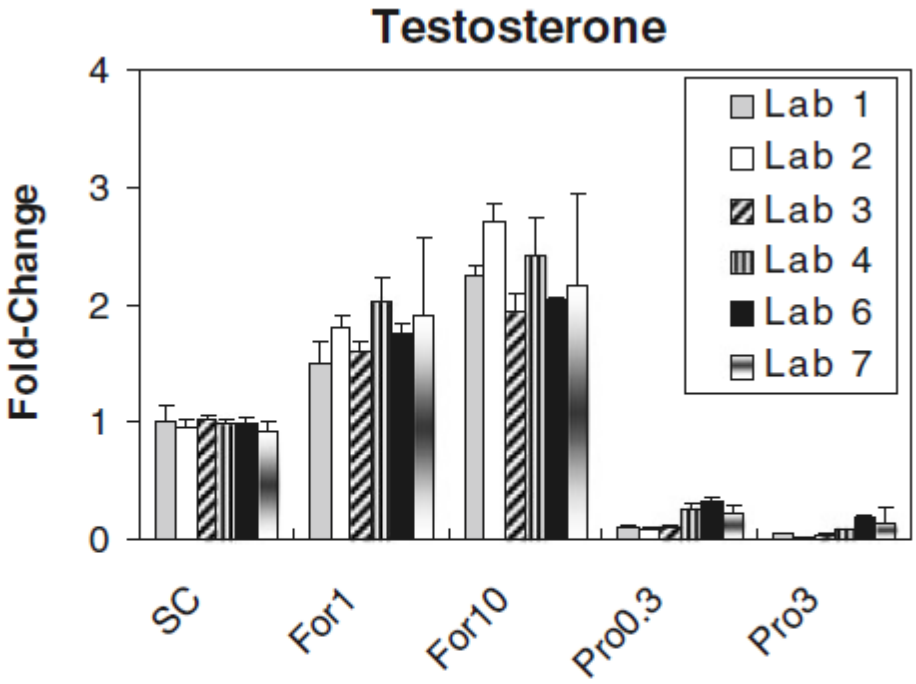
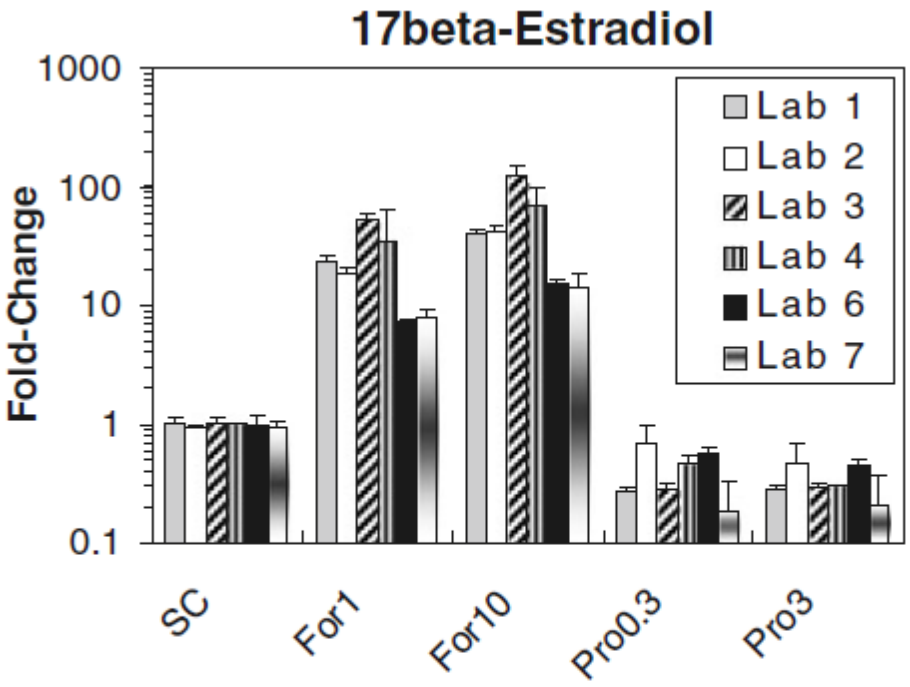


Fig. 1 Comparison of changes in the concentrations of testosterone (T) and estradiol (E2) relative to the solvent controls (SC=1) in the QC plates among laboratories (Lab). For1=1 μM Forskolin; For10=10 μM Forskolin; Pro0.3=0.3 μM Prochloraz; Pro3=3 μM



Prochloraz. Error bars = 1 × standard deviation. *Bars* represent means of four independent experiments. (Lab 5: only T data from two experiments.)

III. DISCUSSION AND CONCLUSIONS

A. **<u>INVESTIGATOR'S CONCLUSIONS</u>:** Glyphosate exposure did not affect testosterone or estradiol levels in this assay.

B. **<u>AGENCY COMMENTS</u>:** Glyphosate was evaluated by two laboratories as part of the OECD validation program of the H295R steroidogenesis assay. Both laboratories reported that glyphosate exposure does not affect testosterone or estradiol levels in this assay.

C. **<u>STUDY DEFICIENCIES</u>:** In lieu of detailed reporting of the methodology, many assumptions were made regarding the conduct of the assay according to the OECD guideline.

DATA EVALUATION RECORD

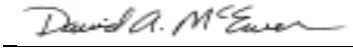
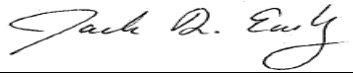
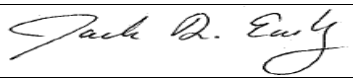
GLYPHOSATE

Study Type: OCSPP 890.1600, *In vivo* Uterotrophic Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-34-2012 (MRID 48617003)

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 Luck, M.S.	Date:	4/11/2012
Secondary Reviewer:	Signature:	
David A. McEwen, B.S.	Date:	4/13/2012
Program Manager:	Signature:	
Jack D. Early, M.S.	Date:	4/18/2012
Quality Assurance:	Signature:	
Jack D. Early, M.S.	Date:	4/18/2012

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: Anwar Y. Dunbar, Ph.D. **Signature:** Anw Y. Dah
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 05-27-15
Secondary Reviewer: Jess Rowland **Signature:** Jess Rowland
Health Effects Division (7509P) **Date:** 5/22/15

Template version 09/2011

DATA EVALUATION RECORD

STUDY TYPE: Uterotrophic Assay (Rat); OCSPP 890.1600; OECD 440

PC CODE: 417300 **DP BARCODE:** D398693

TXR#: 0053233 **CAS#:** 1071-83-6

TEST MATERIAL (PURITY): Glyphosate (85.1% a.i.)

SYNONYMS: N-(phosphonomethyl) glycine

CITATION: Stump, D. G. (2012). A Uterotrophic Assay of Glyphosate Administered Orally in Ovariectomized Rats. WIL Research Laboratories, LLC, Ashland, OH. Laboratory Report No.: WIL-843002, January 6, 2012. MRID 48617003. Unpublished.

SPONSOR: Joint Glyphosate Task Force, LLC, 8325 Old Deer Trail, Raleigh, NC 27615

TEST ORDER #: CON-417300-23

EXECUTIVE SUMMARY: In a Uterotrophic Assay (MRID 48617003) conducted to screen for potential estrogenic activity, glyphosate (85.1% a.i., Batch/lot# GLP-1103-21149-T) in 0.5% methylcellulose (w/v) was administered daily via oral gavage to groups of six ovariectomized female Sprague Dawley rats at dose levels of 0 (vehicle), 100, 300, or 1,000 (limit dose) mg/kg/day on post-natal days (PND) 66/67 to 68/69. The positive control group was treated with a daily dose of 17α-ethynyl estradiol (EE) at 3 μg/kg/day by oral gavage. Body weights were determined daily. All animals were terminated and necropsied on PND 69/70 approximately 24 hours after the final dose administration to determine wet and blotted uterine weights.

All animals survived until scheduled termination and no treatment-related clinical findings were observed in glyphosate dosed animals. Body weights, body weight gains, and uterine weights in the glyphosate groups were comparable to the vehicle control.

In the positive control (EE) group, mean body weights decreased on Days 3 and 4 (not significant, NS), leading to an overall body weight loss during the study of 5.6 g ($p<0.01$) compared to a gain of 11.3 g in the controls. Absolute wet and blotted uterus weights for the positive control (EE) group were increased ($p<0.01$) by 758% and 256%, respectively, as expected.

No statistically significant changes were seen in uterine weight in this assay. Glyphosate is 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 GLP Compliance, Data Confidentiality and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

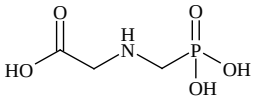
A. MATERIALS

1. Test Facility: WIL Research Laboratories, LLC

Location: Ashland, OH
Study Director: D. G. Stump
Other Personnel: E. S. Bodle (Assistant Director, Analytical Chemistry), S. A. Keets (Senior Operations Manager, Vivarium), C. A. Kopp (Manager, Gross Pathology and Developmental Toxicology Laboratory), T. M. Rafeld (Group Manager, Formulations Laboratory), C. S. Wally (Group Supervisor, Sample Processing Laboratory), R. A. Wally (Operations Manager, Reporting & Technical Support Services), M. E. Haubenstricker (Participating Scientist, Analyses of Dosing Formulations), L. Freshwater (Contributing Scientist, Statistical Analysis)
Study Period: June 14, 2011 - January 6, 2012

2. Test Substance: Glyphosate

Description: White powder
Source: Monsanto (St. Louis, MO)
Lot/Batch #: GLP-1103-21149-T (expiration date 3/9/2012)
Purity: 85.14% (95.93% dried)
Stability: Stable in vehicle for up to 15 days at room temperature
CAS #: 1071-83-6
Structure:



3. Reference Estrogen: 17α-ethynyl estradiol (EE)

Supplier: Sigma Aldrich (St. Louis, MO)
Lot/Batch #: 028K1411 (expiration date 8/4/2011)
Purity: 99.0%
CAS #: 57-63-6

4. Solvent/Vehicle Control (test substance): Methylcellulose

Supplier: Sigma Chemical (St. Louis, MO)
Lot/Batch #: 060M0123V (expiration date 5/1/2013)
Rationale (if other than water): Test substance not soluble in water at the concentrations used in the study
Final concentration: 0.5% (w/v)

Solvent/Vehicle Control (EE): Ethanol/Corn oil

Supplier: Spectrum Chemical Manufacturing Corporation (New Brunswick, NJ)
Lot #: Ethanol: ZT0426 (expiration date 8/2/2013)
Corn oil: 2AD0465 (expiration date 2/1/2013)
Rationale (if other than water): Not applicable
Final concentration: EE was dissolved in minimal amounts of 95% ethanol and then diluted with corn oil (ratio of ethanol to corn oil was not reported)

5. Test Animals:

Species:	Rat (ovariectomized females only)	
Strain:	Sprague Dawley [Crl:CD(SD)]	
Age/weight at dose initiation:	PND 66-67/ 245.6 – 301.2 g	
Source:	Charles River Laboratories (Portage, MI)	
Housing:	Rats were individually housed in stainless steel wire-mesh cages suspended above cage board.	
Diet:	2016C*M* Teklad Global 16% Protein Rodent Diet, Harlan Laboratories, *ad libitum* Genistein equivalent content = 29.0 ppm total isoflavones (genistein + daidzein + glycitein)	
Water:	Reverse-osmosis purified drinking water, *ad libitum*	
Environmental conditions:	**Temperature:**	21.3-21.6 ºC (mean daily temperature)
	Humidity:	51.8-55.2% (mean daily humidity)
	Air changes:	10/hr
	Photoperiod:	12 hrs light/12 hrs dark
Acclimation period:	11 days	

B. STUDY DESIGN

1. In-Life Dates: Start: July 2, 2011 End: July 5, 2011

2. Study Design: Sexually mature ovariectomized female rats were received from Charles River Laboratories; rats were ovariectomized at PND 49 by the supplier. Animals were received approximately one week following ovariectomy (PND 55-56) and acclimated for 11 days prior to initiation of dosing. Vaginal smears were taken daily for five days prior to assignment of animals to study, to verify that females were in persistent diestrus. The dose administration period was from PND 66-67 through 68-69. Rats were euthanized approximately 24 hours later on PND 69-70 and necropsied for uterine weight measurements

3. Animal Assignment: Animals were randomly assigned, stratified by body weight, to the test groups noted in Table 1. Statistical analysis indicated that there were no significant differences between group mean weights at study initiation. Furthermore, 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
Estrogen Agonist Assay		
Vehicle Control	0	6
Low Glyphosate	100	6
Mid Glyphosate	300	6
High Glyphosate	1000	6
17α-ethynyl estradiol (EE), Reference Estrogen	0.003	6

a Data were obtained from page 25 of the study report. Glyphosate concentrations are expressed as free base equivalents.

4. **Dose Selection Rationale:** The dose levels used in this study were chosen based on the results of a dose range-finding study.[1] In the study, the test substance was administered by oral gavage to four groups of female rats [Crl:CD(SD)] at 0, 200, 500, and 1,000 mg/kg/day once daily for 3 consecutive days; the 0, 200, and 500 mg/kg/day dosing groups consisted of 5 rats each and the 1,000 mg/kg/day group consisted of 8 rats. All females survived to the scheduled necropsy. Mean body weights, body weight gains, and food consumption in all treatment groups were similar to the control group. Therefore, the high-dose level of 1,000 mg/kg/day (limit dose) was selected for the current study.

5. **Dose Preparation and Analysis:** Dose formulations were prepared once as single formulations for each dosage level by mixing appropriate amounts of test substance with 0.5% methylcellulose. A stock solution of EE was prepared once by dissolving the material in a small amount of 95% ethanol and diluting to volume with corn oil; dosing formulations were prepared daily by diluting the stock solution. Analyses to demonstrate homogeneity, stability, and resuspension homogeneity were conducted previously for dose formulations at 1 and 200 mg/mL following up to 15 days of room temperature storage.[2] During the study, samples of each test substance dosing formulation (middle stratum of each) prepared during the in-life phase were analyzed for achieved concentration.

Results of Dose Analysis

Homogeneity: Not provided

Stability: It was stated that glyphosate in 95% methylcellulose at 1 and 200 mg/mL was stable at room temperature for 15 days.

Concentration (% of nominal): 104-105%

The analytical data indicated that the variation between nominal and actual dosage to the animals was acceptable. The study referenced above should be submitted for verification of the homogeneity and stability findings.

6. **Dosage Administration:** Animals were administered the test formulations and/or EE or vehicle daily via oral gavage for three consecutive days in a dose volume of 5 mL/kg body weight. Dose volumes were adjusted daily based on the concurrent body weight measurement.

7. **Statistics:** Statistical analyses were conducted for organ weights, daily body weights, and body weight gains. Each endpoint was tested for homogeneity of variance using Levene's test. If that test was significant at p=0.01, then a log transformation was applied and

1 Stump, D.G. A Dose Range-Finding Oral (Gavage) Toxicity Study of Glyphosate in Young Adult Rats for the Endocrine Disruption Screening Program (Study No. WIL-843001). WIL Research Laboratories, LLC, Ashland, OH, 2011.

2 Haubenstricker, M.E. Analytical Validation and Stability Study of Glyphosate in Aqueous Formulations (Study No. WIL-843004). WIL Research Laboratories, LLC, Ashland, OH, 2011.

Levene's test conducted on the transformed data. If that test was still significant, then the square root transformation was applied to the raw data and Levene's test conducted again. If the test was still significant, then a nonparametric test, as described below, was used to analyze the data. One-sided tests were conducted for uterine weights and two-sided tests were conducted for body weights and body weight gains.

For uterine weights, if variances were homogeneous, the analysis of covariance (ANCOVA), using the body weight at termination as the covariate was performed; the two groups were compared using a one-sided Dunnett's test. For body weight and body weight gain data, if variances were homogeneous, an ANOVA was performed on data; the ANOVA test was followed by a two-sided Dunnett's test. If the transformations were unsuccessful in making the variances homogeneous, the nonparametric one- or two-sided Wilcoxon rank sum test was used to compare data for the positive control to the negative control. For comparison of dose groups to the negative control, if the transformations were unsuccessful in making the variances homogeneous, the nonparametric Kruskal-Wallis test was used, followed by a one- or two-sided Dunn's test. Significance was denoted at $p<0.05$. Statistical analyses were performed using SAS software (version 9.2 or higher). The statistical analyses were considered adequate.

C. METHODS

1. **Clinical Examinations:** Cage-side checks for mortality and moribundity were conducted twice daily. Individual clinical observations (hand-held physical examinations) were recorded daily through termination. Each rat was also observed for signs of toxicity approximately 4 hours following dosing.

2. **Body Weight:** Animals were weighed at randomization, daily throughout the dosing period, and at termination. Mean body weight changes were calculated for each corresponding interval and also for the overall treatment period (Days 0-3).

3. **Food Consumption (Optional):** Food consumption was not measured.

4. **Necropsy and Measurement of Uterine Weight:** On PND 69-70 (approximately 24 hours after final administration of the test substance), all surviving animals were euthanized by carbon dioxide inhalation 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. The uterus and vagina were preserved in 10% neutral buffered formalin for possible future histopathologic examination.

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 test-substance related clinical signs of toxicity were observed in animals for any dose groups. Findings noted in the treated groups were limited to observation of red material around the nose in one rat in the 300 mg/kg/day dose group on one study day.

B. BODY WEIGHT AND WEIGHT GAIN: Body weight and body weight gain data are presented in Table 2. Body weights in the treatment groups were comparable to controls throughout the duration of the study. In the positive control (EE) group, mean body weights decreased (NS) on Days 3 and 4, leading to an overall body weight loss during the study of −5.6 g ($p<0.01$) compared to a gain of 11.3 g in the controls.

TABLE 2. Selected Group Body Weights and Cumulative Body Weight Gains (g) In the Estrogen Agonist Assay [a]

Study Day #	Dose (mg/kg/day)														
	Vehicle Control			Glyphosate (100)			Glyphosate (300)			Glyphosate (1000)			Reference Estrogen EE (0.003)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
1	6	277.0	19.2	6	277.6	14.6	6	275.2	15.6	6	278.3	15.5	6	278.7	18.8
2	6	279.7	19.5	6	282.4	15.8	6	279.2	16.9	6	282.8	16.6	6	280.2	21.1
3	6	286.4	21.1	6	288.2	16.3	6	284.8	15.5	6	287.5	16.5	6	278.2	19.4
4	6	288.3	21.0	6	292.9	16.0	6	284.3	19.4	6	291.6	17.5	6	273.2	20.9
Body Weight Gain (1 - 3)	6	11.3	3.6	6	15.3	3.0	6	9.1	5.1	6	13.3	6.0	6	-5.6**	3.4

a Data were obtained from Tables S6-S9 on pages 49-54 of the study report.
N Number of animals in the group
SD Standard Deviation
** Significantly different from controls at $p<0.01$

C. FOOD CONSUMPTION (Optional): Food consumption was not measured.

D. PATHOLOGY

1. Uterine Weights: Uterine weight data are presented in Table 3. Uterine weights in the glyphosate treatment groups were comparable to the vehicle controls. Absolute wet and blotted uterus weights for the positive control (EE) group were increased ($p<0.01$) by 758% and 256%, respectively. The increased uterine weights were within the expected range.

No macroscopic findings in the uterus were observed in the glyphosate treatment groups or the positive control group.

TABLE 3. Uterine Weights from Estrogen Agonist Assay in Sprague Dawley Rats[a]

Parameter	Dose (mg/kg/day)														
	Vehicle Control			Glyphosate (100)			Glyphosate (300)			Glyphosate (1000)			Reference Estrogen EE (0.003)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
Terminal BW	6	289	21	6	293	16	6	284	19	6	292	18	6	273	21
Wet, absolute (mg)	6	111.0	10.8	6	110.7	12.5	6	118.3	16.5	6	113.6	9.7	6	953.1** (↑758)	90.4
Wet, relative (%)	6	0.038	0.0025	6	0.038	0.0056	6	0.042	0.0061	6	0.039	0.0044	6	0.352	0.055
Blotted, absolute (mg)	6	98.2	11.7	6	98.7	10.6	6	103.0	11.6	6	102.4	8.9	6	349.3** (↑256)	31.1
Blotted, relative (%)	6	0.034	0.0024	6	0.034	0.0048	6	0.0.36	0.0040	6	0.035	0.0038	6	0.129	0.017

a Data were obtained from Tables S11-S14 on pages 56-59 of the study report. Percent differences from controls, calculated by the reviewers, are included in parentheses.
BW Body weight
N Number of animals in the group
SD Standard Deviation
** Significantly different from controls at $p<0.01$

2. Microscopic Examination (Optional): Microscopic examinations were not conducted.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATOR'S CONCLUSIONS: Based on the lack of effects on mean uterine weights (wet and blotted), glyphosate did not demonstrate or mimic biological activities consistent with agonism of natural estrogens when administered orally to ovariectomized female rats at dosage levels of 100, 300, and 1,000 mg/kg/day. The positive control substance (17α-ethynylestradiol) elicited the expected increases in wet and blotted uterine weights (8.6- and 3.6-fold, respectively).

B. AGENCY COMMENTS: All animals survived until scheduled termination and no treatment-related clinical findings were observed in glyphosate dosed animals. Body weights, body weight gains, and uterine weights in the glyphosate dosing groups were comparable to the vehicle controls.

In the positive control (EE) group, mean body weights decreased (NS) on Days 3 and 4, leading to an overall body weight loss during the study of 5.6 g ($p<0.01$) compared to a gain of 11.3 g in the controls. Absolute wet and blotted uterus weights for the positive control (EE) group were increased ($p<0.01$) by 758% and 256%, respectively, as expected. No statistically significant changes were seen in uterine weight in this assay. Glyphosate is negative in the uterotrophic assay.

C. STUDY DEFICIENCIES: None