STUDY REPORT
Original: Draft

STUDY TITLE

ACUTE TOXICITY STUDY OF SOLBERE ON EARTHWORM - *EISENIA FETIDA*

STUDY NUMBER: BIO-ETX 139

Study Completion Date: DD.MM.YY

SPONSOR

CO2 SOLVED LLC,
30301 RIVERVIEW Dr. JUNCTION CITY,
OR 97448, USA

TEST FACILITY

BIONEEDS INDIA PRIVATE LIMITED
DEVARAHOSAHALLY
SOMPURA HOBLI, NELAMANGALA TALUK
BANGALORE RURAL DISTRICT, PIN - 562 111
KARNATAKA, INDIA
E-mail: bioneeds@bioneeds.in
Website: www.bioneeds.in
Tel No.: +91 816 - 2214400
Fax: +91 816 – 2214444

CONFIDENTIAL

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QUALITY ASSURANCE STATEMENT

The Study No.: BIO-ETX 139 entitled "Acute Toxicity Study of Solbere on Earthworm - *Eisenia fetida*" has been inspected as per OECD Principles of Good Laboratory Practice [C (97)186/Final].

The dates of inspections and dates of reporting to the Study Director and the Test Facility Management have been listed below:

Inspection Dates	**Inspection Phases**	**Reporting Dates**	
		Study Director	**Test Facility Management**
Initiation Phase			
	Study plan verification		
In-life Phase			
	Test item formulation preparation and exposure - Limit test		
Reporting Phase			
	Draft report inspection		
	Final report inspection		

Inspections were performed according to the Standard Operating Procedures of the test facility's Quality Assurance Unit. The study report was inspected against the approved study plan and pertinent raw data and accurately reflects the raw data.

....

(Signature)
Mr. PRAVEEN B.
Quality Assurance Unit

(Date)

STATEMENT OF GLP COMPLIANCE

The Study No.: BIO-ETX 139 entitled "Acute Toxicity Study of Solbere on Earthworm - *Eisenia fetida*" was performed in compliance with the OECD Principles of Good Laboratory Practice [C (97)186/Final].

DECLARATION

I hereby declare that the work was performed under my supervision and in accordance with the described procedures. It is assured that the reported results faithfully represent the raw data obtained during the experimental work. No circumstances have been left unreported which may have affected the quality or integrity of the data or which might have a potential bearing on the validity and reproducibility of this study.

I accept overall responsibility for the technical conduct of the study as well as the interpretation, analysis, documentation and reporting of the results.

....

(Signature) **(Date)**

Dr. T. S. SADANANDA

Study Director

STATEMENT OF CONFIDENTIALITY

This Report Contains **CONFIDENTIAL** and **PROPRIETARY** Information of **CO2 SOLVED LLC,** and will not be disclosed to anyone without the expressed or written approval of sponsor, except to the employees of the test facility wherever necessary and to persons authorized by law or judicial judgment.

....

(Signature) **(Date)**
Dr. T. S. SADANANDA
Study Director

....

(Signature) **(Date)**
Dr. K. R. RAGHUNATHA REDDY
Deputy Test Facility Management

ABBREVIATIONS OF COMMONLY USED UNITS AND SYMBOLS

AET	:	Acute Earthworm Toxicity
cm	:	Centimeter
Conc.	:	Concentration
h/hr	:	Hour
kg	:	Kilogram
LC_{50}	:	Median Lethal Concentration
LOEC	:	Lowest Observed Effect Concentration
mg	:	Milligram
mg/kg	:	Milligram per Kilogram
min	:	Minute
NA / -	:	Not Applicable
No.	:	Number
NOEC	:	No Observed Effect Concentration
OECD	:	Organization for Economic Co-operation and Development
R	:	Replicate
SD	:	Standard Deviation
%	:	Percentage
°C	:	Degree Celsius
Min	:	Minimum
Max	:	Maximum

1. STUDY DETAILS

1.1	**Study Title**	:	Acute Toxicity Study of Solbere on Earthworm - *Eisenia fetida.*
1.2	**Study Number**	:	BIO-ETX 139
1.3	**Study Code**	:	AET
1.4	**Sponsor Details**		
	Sponsor	:	CO2 Solved LLC, 30301 Riverview Dr. Junction city, OR 97448, USA
	Sponsor's Representative and Monitoring Scientist	:	George Baker CO2 Solved LLC, 30301 Riverview Dr. Junction city, OR 97448, USA
1.5	**Test Facility**	:	Bioneeds India Private Limited Devarahosahally, Sompura Hobli, Nelamangala Taluk, Bangalore Rural District, PIN - 562 111 Karnataka, India
1.6	**Study Responsibilities**		
	Study Director	:	Dr. T. S. Sadananda., M.Sc., Ph. D. Bioneeds India Private Limited, Devarahosahally, Sompura Hobli, Nelamangala Taluk, Bangalore Rural District, PIN - 562 111 Karnataka, India E-mail: bioneeds@bioneeds.in
	Study Co-ordinator	:	Mr. Suresh C. S., M.Sc.
	Study Personnel	:	Mr. Lavakumar C., M.Sc. Mr. H. Mahantesh., M.Sc. Mr. Thiayagaraj M., M.Sc.
1.7	**Study Schedule**		
	Study Initiation Date	:	26 April 2019
	Experiment Start Date	:	28 April 2019
	Acclimatization Date	:	28 April 2019 to 29 April 2019
	Treatment Date (DRF)	:	29 April 2019
	Experiment End Date (DRF)	:	13 May 2019
	Main Study Acclimatization Date	:	15 May 2019 to 16 May 2019
	Treatment Date	:	16 May 2019
	Experiment End Date	:	30 May 2019

Experimental Completion Date	:	30 May 2019
Draft Report Submission Date	:	31 May 2019
Study Completion Date	:	DD.MM.YYY

2. SUMMARY

The test item, Solbere obtained from CO2 Solved LLC, was evaluated for acute toxicity on earthworms as per the OECD Guidelines for Testing of Chemicals (Section 2), Effects on Biotic Systems, Guideline No. 207, "Earthworm, Acute Toxicity Tests" adopted on 04 April 1984.

Adult earthworms with well-developed clitellum were selected for toxicity testing.

Dose Range finding study

During the dose range finding study, no clinical signs of toxicity or mortality were observed at the tested concentrations 0.01, 0.1, 1.0, 10.0 and 100.0. Hence limit test was conducted as main study at the test concentration of 1000.0 mg of Solbere/kg dry weight of artificial soil along with the solvent control group.

Limit Test

During the limit test, no clinical signs of toxicity were observed in the negative and solvent control group and no clinical signs of toxicity were observed at the tested concentration of 1000.0 mg of Solbere/kg dry artificial soil during the 14 days of exposure period.

During the 14 days of exposure period, no mortalities were observed in controls and at the tested concentration of 1000.0 mg of Solbere/kg dry artificial soil during the 14 days of exposure period.

Conclusion

Based on earthworm body weights on Day 14, the No Observed Effect Concentration (NOEC) for Solbere is 1000 mg/kg dry artificial soil and Lowest Observed Effect Concentration (LOEC) for Solbere is > 1000 mg/kg dry artificial soil, respectively.

The acute median lethal concentration (LC_{50}) of Solbere after a 14 day exposure period is > 1000 mg/kg dry artificial soil.

3. STUDY COMPLIANCE

3.1 GLP Compliance

The study was performed:

a. In compliance with the OECD Principles of Good Laboratory Practice [C (97)186/Final].

b. In accordance with the Standard Operating Procedures at Bioneeds India Private Limited and as per the mutually agreed study plan with the sponsor.

3.2 Regulatory Guideline

The study was performed in accordance with the OECD Guidelines for Testing of Chemicals (Section 2), Effects on Biotic Systems, Guideline No. 207, "Earthworm, Acute Toxicity Tests", adopted by the Council on 4 April 1984.

4. SAFETY PRECAUTIONS

Gloves, head cap, face mask and goggles were used in addition to protective body garments and shoes to ensure adequate personnel health and safety and to avoid inhalation and skin contact with the test item.

5. OBJECTIVE

The objective of this study is to determine the Lowest Observed Effect Concentration (LOEC), the No Observed Effect Concentration (NOEC) and the median lethal concentration (LC_{50}) of Solbere to Earthworm *Eisenia fetida* in the artificial soil medium.

6. VALIDITY CRITERIA OF THE TEST

Mortality in the control (negative) group was 0.0% at the termination of the test. Hence the test is considered valid.

7. MATERIALS AND METHODS

7.1 Test Item Information

The test item information provided by sponsor as per Test Item Data Sheet and Certificate of Analysis is presented below:

Name of Test Item	:	Solbere
Chemical Name (IUPAC)	:	Calcium Carbonate
CAS No.	:	471-34-1
Physical appearance (with color)	:	White Liquid
Batch No.	:	NA
Lot No.	:	19029
Purity (Declared by sponsor and / or as per Certificate of Analysis)	:	58%
Batch produced by (Name and address)	:	CO2 Solved LLC, 30301 Riverview Dr. Junction city, OR 97448, USA

Date of Manufacture	:	1/29/19
Date of Expiry	:	1/29/21
Storage Conditions	:	Ambient (21 to 29°C)
Test Item Code by Test Facility	:	D807-001

The responsibility for the correct identity and stability of the test item rests with the sponsor. The Certificate of Analysis of Solbere is attached as Annexure 1.

7.2 Test System

Test Species : Earthworm (*Eisenia fetida*)

Justification for Selection of Earthworm Species : *Eisenia fetida* was selected as the test system, because it is one of the non-target beneficial invertebrates inhabiting the soil. They are readily available and can be easily cultured in controlled laboratory conditions. It is a suitable model for acute toxicity assessment of soil macro organisms and is recommended by the OECD Guideline No. 207 and other regulatory authorities.

Source of Supply : In house maintained earthworms were originally obtained from Mount Carmel College (Autonomous), Centre for Scientific Research and Advanced Learning, No. 58, Palace Road, Bangalore - 560 052, Karnataka, India.

Age and Body Weight at Treatment : Adult earthworms about two months old with clitellum with individual body weights ranged from 301.6 to 398.6 mg and 301.9 to 362.2 mg during dose range finding study and limit test, respectively.

Groups, Replicates and Number of Earthworms per Replicate : During the dose range finding study, seven groups (1 solvent control group and 6 treatment groups) with 2 replicates each were used.

During limit test thee groups [1 negative control, 1 solvent control and 1 treatment group)] consisting of four replicates in each group were used.

Ten worms per replicate were used during the dose range finding study and limit test.

7.3 Husbandry

a. Environmental Condition : The duration of the exposure was 14 days and pH of the artificial soil was 6.03 and 6.06 at the start of the dose range finding and limit test, respectively. The temperature ranged from 19.9 to 20.3°C and continuous lighting with an illumination ranged between 629 to 683 Lux during dose range finding study.

Temperature of 19.9 to 20.8°C and continuous lighting with an illumination ranged between 615 to 659 Lux was

maintained during limit test respectively.

b. Test Vessel : All glass test containers (spoutless beakers) of approximately 3 L capacity covered with perforated plastic film were used in the experiment. The test vessels were cleaned thoroughly and properly labeled for identification prior to experiment. Each glass beaker contained 750 g wet weight of artificial soil as the test medium for ten earthworms. Each test vessel was labeled with the minimum details of the study: study no., group no. and replicate no., concentration, exposure start and end dates.

7.4 Artificial Soil Preparation

A mixture of sphagnum peat (10%), kaolin clay (20%) and industrial sand (70%) constitutes the artificial soil. Prior to test item addition, the background moisture content and pH of the dry artificial soil was measured on day 0. Moisture corrected weight of artificial soil was used during test medium preparation. pH was adjusted to 6.03 and 6.06 by addition of calcium carbonate during dose range finding study and limit test respectively.

7.5 Grouping

On the day of treatment, the earthworms conditioned for 24 hours prior in artificial soil was separated, washed with distilled water and dried with blotting paper. Matured earthworms with a body weight between 301.6 to 398.6 mg and 301.9 to 362.2 mg during dose range finding study and limit test was considered for testing. Individual worms were weighed and impartially grouped into groups of 10.

7.6 Test Concentrations and Justification for Selection

During dose range finding study as per guideline, six concentrations were selected with the concentrations of 0.01, 0.1, 1.0, 10.0, 100.0 and 1000.0 mg/kg dry weight of artificial soil along with a control group.

In dose range finding study, no mortalities was observed in control and at the tested concentrations of 0.01, 0.1, 1.0, 10.0, 100.0 and 1000.0 mg/kg dry weight of artificial soil. Hence, the limit test was conducted as main study at the concentration of 1000.0 mg/kg dry weight of artificial soil.

7.7 Acclimatization

Adult earthworms with well-developed clitellum were acclimatized in dry artificial soil for a period of 24 hours prior to exposure during the dose range finding study and limit test.

7.8 Duration of Test

Duration of the test was 14 days (assessment of mortality and clinical signs of toxicity on the day 7 and 14).

7.9 Selection of Vehicle and Justification for Selection

The test item was miscible in distilled water containing acetone (0.1 mL/L) as

evidenced by an in-house solubility/suspensibility test. Hence, distilled water containing acetone has been selected as vehicle for test item dose formulation preparation.

7.10 Test Medium Preparation

Prior to the treatment, the required quantity of test item was taken in a beaker and required volume of acetone (0.1 mL/L) and a small quantity of distilled water was added and then mixed using a glass rod until complete suspension of the test item. After complete suspension, the mixture was transferred to a measuring cylinder and the beaker was rinsed with distilled water and transferred again to measuring cylinder and the rinsing procedure was repeated to ensure complete transfer of contents. The volume was brought up to the required volume using distilled water. The formulated test item sample was introduced into the dry artificial soil.

Dose range finding study

The artificial soil was prepared prior to exposure. The moisture content was found to be 27.8%.

Pre weighed test item of 3.0 mg was dissolved in 2.997 mL of distilled water + 0.0003 mL of acetone to obtain the concentration of 1 mg/mL, from that 15 µL and 150 µL was taken and added to 25 mL of distilled water and added to 1.5 kg of soil separately to obtain test concentration of 0.01 and 0.1 mg/kg dry artificial soil.

Pre-weighed test item of 1.5, 15.0, 150.0 and 1500.0 mg was dissolved in 25 mL distilled water and thoroughly mixed with 1.5 kg of dry artificial soil (separately taken for each concentration) to obtain the test concentrations of 1.0, 10.0, 100.0 and 1000.0 mg/kg dry artificial soil.

Limit Test

The artificial soil was prepared prior to exposure. Pre-weighed test item (3000.4 mg) was dissolved in 49.995 mL of distilled water + 0.0005 mL of acetone and thoroughly mixed with 3000 g of dry artificial soil (separately taken for each concentration) to obtain test concentration of 1000.0 mg test item/kg dry artificial soil.

The dry soil constituents was blended in the correct proportions followed by addition of distilled water to achieve an overall moisture content of 28.5% of the dry weight of soil. The artificial soil medium for each test group at one batch was blended thoroughly using double cone blender for about 10 minutes. The complete mixture was moistened but not so wet that water appeared when the artificial soil was compressed. From these prepared soil samples, about 750 g dry weight of artificial soil was used for each replicate as the test medium (4 replicates per test concentration).

Refer Appendix 3, 4, 5 and 6.

7.11 Selection of Route of Administration and Justification for Selection

Test item formulation prepared in distilled water was mixed thoroughly with required quantity of artificial soil using laboratory mixer (a double cone blender) for 10 minutes as per the recommendations of OECD Test Guideline No. 207, Earthworm Acute Toxicity Test.

7.12 Exposure of Earthworms to Test Item

Each group of 10 earthworms were released into the respective treatment group replicates (glass beakers filled with 750 g dry weight of artificial soil containing test item with defined moisture level) and covered with perforated transparent polythene film during dose range finding and limit test respectively.

7.13 Dose Range Finding Study

The dose range finding study (DRF) with 6 concentrations of 0.01, 0.1, 1.0, 10.0, 100.0 and 1000.0 mg of Solbere /kg dry weight of artificial soil was tested along with a control to determine the range of toxicity.

7.14 Limit Test

During dose range finding study no mortalities were observed in the tested concentrations of 0.01, 0.1, 1.0, 10.0, 100.0 and 1000.0 mg of Solbere/kg dry weight of artificial soil, hence limit test was conducted as the main study at the concentration of 1000.0 mg of Solbere/kg dry weight of artificial soil along with a control group.

7.15 Positive Control

For the evaluation of the quality of the Earthworm and the experimental conditions, 2-Chloroacetamide is tested twice a year to demonstrate satisfactory test conditions. The detail of the last study is attached as Annexure 2.

8. OBSERVATIONS

8.1 Clinical Signs of Toxicity and Mortality

Clinical signs of toxicity and mortality were assessed on days 7 and 14. Test medium was emptied onto a plate, worms were sorted from the medium and their reaction to a mechanical stimulus at the front end was recorded to assess behavioral or pathological symptoms. After the Day 7 assessment worms and medium were placed back in the test container. Earthworms were disposed of along with the treated soil after the completion of the experiment.

8.2 Body Weight

Body weights were recorded for individual earthworms on days 0 and 14 (survivors on day 14). Before recording the body weight, earthworms were washed with distilled water and kept on blotting paper to remove the soil particles and excess distilled water.

8.3 Moisture Content

Three randomly selected, unused artificial soil samples, intended for exposure was taken from any of the three groups for determination of moisture content on Days 0 and 14 (after earthworm observation).

9. STATISTICAL ANALYSIS OF RESULTS

Statistical analysis of mortality for the test item was not required because no mortalities were observed during the 14 days exposure as it was a limit test.

10. STUDY REPORT PREPARATION AND RESULTS

The computer printout of the data (in the form of appendix) was verified with the original raw data. Individual animal data were summarized and presented as tables

along with results of statistical analyses. All findings were presented in the study report as per the standard reporting procedure.

11. AMENDMENTS AND DEVIATIONS

No Amendment was raised and no deviations occurred during the conduct of the study.

12. STUDY REPORT DISTRIBUTION

Original: 1/2 - Sponsor

Original: 2/2 - Archives, Bioneeds India Private Limited

13. ARCHIVING

All materials and data generated from the experiment will be stored at archives of the test facility. The study plan, raw data, study report will be maintained in the archives of Bioneeds India Private Limited for 9 years from the date of completion of the study. The soft copies of the study plan, study report and data compilation will be copied to compact disc and will be archived for a period of 9 years from the date of completion of the study. At the end of archiving period, the Sponsor's instructions will be sought to either extend the archiving period or to return the archived material to the Sponsor or for the material to be disposed off.

14. REFERENCES

- The OECD Guidelines for Testing of Chemicals (Section 2), Effects on Biotic Systems, Guideline No. 207, "Earthworm, Acute Toxicity Tests", adopted by the Council on 4 April 1984.
- Finney DJ, 1971. Probit Analysis, 3rd ed., Cambridge, The University press, pp. 333.
- The Analysis of Agricultural Materials, 1981. Ministry of Agriculture, Fisheries and Food, Reference Book 427, HMSO, London.

15. RESULTS AND DISCUSSION

15.1 Clinical Signs of Toxicity and Mortality

Dose Range Finding Study

During the dose range finding study, no clinical signs of toxicity and no mortalities were observed in the control group and at the tested concentrations of 0.01, 0.1, 1.0, 10.0, 100.0 and 1000.0 mg of Solbere / kg dry weight of artificial soil during the 14 days of exposure.

Refer Table 1.

Limit Test

During the limit test, no clinical signs of toxicity and no mortalities were observed in control and at the tested concentration of 1000.0 mg of Solbere / kg dry weight of artificial soil during the 14 days of exposure period.

Refer Table 2.

15.2 Body Weight

Body weights were recorded for individual earthworms on days 0 and 14.

Refer Tables 3, 4, 5 and 6, Appendix 7, 8, 9 and 10.

15.3 Moisture Content

The average moisture content of three randomly selected artificial soil samples intended for exposure on days 0 and 14 (after the observation) was found to be 27.8 and 26.4% during dose range finding study and 28.5 and 25.0% during limit test.

Refer Appendix 5 and 6.

16. CONCLUSION

Based on earthworm body weights on Day 14, the No Observed Effect Concentration (NOEC) for Solbere is 1000 mg/kg dry artificial soil and Lowest Observed Effect Concentration (LOEC) for Solbere is >1000 mg/kg dry artificial soil, respectively.

The acute median lethal concentration (LC_{50}) of Solbere after a 14 day exposure period is > 1000 mg/kg dry artificial soil.

17. TABLES

TABLE 1. OBSERVATION OF EARTHWORMS ON DAYS 7 AND 14 DURING DOSE RANGE FINDING STUDY

Group	Conc. (mg/kg dry artificial soil)	R	Signs of Toxicity and Mortality Observed on				Mortality on Day 14	
			Day 7		Day 14		CM	%M
			Toxic Signs	DW	Toxic Signs	DW		
G1	0.0 (SC)	R1	N(10)	0	N(10)	0	0	0.0
		R2	N(10)	0	N(10)	0	0	
G2	0.01	R1	N(10)	0	N(10)	0	0	0.0
		R2	N(10)	0	N(10)	0	0	
G3	0.1	R1	N(10)	0	N(10)	0	0	0.0
		R2	N(10)	0	N(10)	0	0	
G4	1.0	R1	N(10)	0	N(10)	0	0	0.0
		R2	N(10)	0	N(10)	0	0	
G5	10.0	R1	N(10)	0	N(10)	0	0	0.0
		R2	N(10)	0	N(10)	0	0	
G6	100.0	R1	N(10)	0	N(10)	0	0	0.0
		R2	N(10)	0	N(10)	0	0	
G7	1000.0	R1	N(10)	0	N(10)	0	0	0.0
		R2	N(10)	0	N(10)	0	0	

N: Normal (without any signs of toxicity); SC: Solvent control; R: Replicates; DW: Dead worms; CM: Cumulative mortality; % M: Percent mortality; Concentration is expressed as mg test item/kg dry artificial soil; Alphabet outside the parentheses represent the clinical symptom code and values inside the parentheses represent the number of earthworms exhibiting that particular symptom.

TABLE 2. OBSERVATION OF EARTHWORMS ON DAYS 7 AND 14 DURING LIMIT TEST

Group	Conc. (mg/kg dry artificial soil)	R	Signs of Toxicity and Mortality Observed on				Mortality on Day 14	
			Day 7		Day 14		CM	%M
			Toxic Signs	DW	Toxic Signs	DW		
G1	0.0 (N.C)	R1	N(10)	0	N(10)	0	0	0.0
		R2	N(10)	0	N(10)	0	0	
		R3	N(10)	0	N(10)	0	0	
		R4	N(10)	0	N(10)	0	0	
G2	0.0 (S.C)	R1	N(10)	0	N(10)	0	0	0.0
		R2	N(10)	0	N(10)	0	0	
		R3	N(10)	0	N(10)	0	0	
		R4	N(10)	0	N(10)	0	0	
G3	1000.0	R1	N(10)	0	N(10)	0	0	0.0
		R2	N(10)	0	N(10)	0	0	
		R3	N(10)	0	N(10)	0	0	
		R4	N(10)	0	N(10)	0	0	

N: Normal (without any signs of toxicity); SC: Solvent control; NC: Negative control; R: Replicates; DW: Dead worms, CM: Cumulative mortality; % M: Percent mortality; Concentration is expressed as mg test item/kg dry artificial soil; Alphabet outside the parentheses represent the clinical symptom code and values inside the parentheses represent the number of earthworms exhibiting that particular symptom.

TABLE 3. MEAN BODY WEIGHTS OF EARTHWORMS ON DAY 0 DURING DOSE RANGE FINDING STUDY

Refer Appendix 7

Group	Conc. (mg/kg dry artificial soil)	Mean Body Weight (mg) on Day 0	±SD
G1	**0.0 (SC)**	351.0	25.2
G2	**0.01**	352.7	28.5
G3	**0.1**	354.4	31.8
G4	**1.0**	360.5	29.7
G5	**10.0**	358.2	25.6
G6	**100.0**	344.8	30.3
G7	**1000.0**	357.2	29.1

±SD: Standard deviation.

TABLE 4. MEAN BODY WEIGHTS OF EARTHWORMS ON DAY 14 DURING DOSE RANGE FINDING STUDY

Refer Appendix 8

Group	Conc. (mg/kg dry artificial soil)	Mean Body Weight (mg) on Day 14	±SD
G1	**0.0 (SC)**	330.9	11.0
G2	**0.01**	335.3	9.3
G3	**0.1**	336.9	10.8
G4	**1.0**	341.7	9.3
G5	**10.0**	339.5	9.7
G6	**100.0**	332.5	7.8
G7	**1000.0**	328.5	12.5

±SD: Standard deviation; SC: Solvent control.

TABLE 5. MEAN BODY WEIGHTS OF EARTHWORMS ON DAY 0 DURING LIMIT TEST

Refer Appendix 9

Group	Conc. (mg/kg dry artificial soil)	Mean Body Weight (mg) on Day 0	±SD
G1	**0.0 (NC)**	361.5	26.6
G2	**0.0 (SC)**	371.1	26.4
G3	**1000.0**	368.5	20.4

±SD: Standard deviation; NC: Negative Control; SC: Solvent control.

TABLE 6. MEAN BODY WEIGHTS OF EARTHWORMS ON DAY 14 DURING LIMIT TEST

Refer Appendix 10

Group	Conc. (mg/kg dry artificial soil)	Mean Body Weight (mg) on Day 14	±SD
G1	**0.0 (NC)**	337.2	13.2
G2	**0.0 (SC)**	327.8	13.0
G2	**1000.0**	336.2	14.6

±SD: Standard deviation, NC: Negative Control, SC: Solvent Control.

TABLE 7. LIGHT INTENSITY AND TEMPERATURE DURING DOSE RANGE FINDING STUDY

Day	Temperature (°C)	Light intensity (Lux)			
		A	B	C	D
0	20.3	**662**	**653**	**683**	**629**
1	20.1				
2	20.2				
3	20.0				
4	20.3				
5	20.1				
6	20.2				
7	19.9				
8	20.1				
9	20.3				
10	19.9				
11	20.2				
12	20.1				
13	19.9				
14	20.2	**652**	**630**	**650**	**654**
Mean	**20.1**	**651.6**			
± SD	**0.14**	**17.2**			
Min	**19.9**	**629**			
Max	**20.3**	**683**			

Note: A, B, C and D represents light intensity at different spatial points; ±SD: Standard deviation; Min: Minimum; Max: Maximum.

TABLE 8. LIGHT INTENSITY AND TEMPERATURE DURING LIMIT TEST

Day	Temperature (°C)	Light intensity (Lux)			
		A	B	C	D
0	19.9	643	658	646	637
1	20.1				
2	20.3				
3	19.9				
4	20.2				
5	20.1				
6	20.4				
7	20.2				
8	20.5				
9	20.7				
10	20.4				
11	20.3				
12	20.6				
13	20.5				
14	20.8	659	644	615	642
Mean	**20.3**	**643.0**			
± SD	**0.27**	**13.7**			
Min	**19.9**	**615**			
Max	**20.8**	**659**			

Note: A, B, C and D represents light intensity at different spatial points; ±SD: Standard deviation; Min: Minimum; Max: Maximum.

18. APPENDICES

APPENDIX 1. ARTIFICIAL SOIL PREPARATION AND pH DURING DOSE RANGE FINDING STUDY

Artificial Soil Components	Usage (g)	pH
Industrial sand (70%)	7700	6.03
Kaolin clay of 30% kaolinite content (20%)	2200	
Sphagnum peat (10%)	1100	
Calcium carbonate	1.0430	
Total	11001.043	

Note: Values in parentheses are the percentage of artificial soil components used in the preparation of artificial soil. pH of the artificial soil was determined by thoroughly mixing air dried artificial soil in 1 Molar solution of analytical grade potassium chloride (1:5 ratio). After 2 hour of settling period the pH of the supernatant was measured.

APPENDIX 2. ARTIFICIAL SOIL PREPARATION AND pH DURING LIMIT TEST

Artificial Soil Components	Usage (g)	pH
Industrial sand (70%)	7000	6.06
Kaolin clay of 30% kaolinite content (20%)	2000	
Sphagnum peat (10%)	1000	
Calcium carbonate	1.0239	
Total	10001.239	

Values in parentheses are the percentage of artificial soil components used in the preparation of artificial soil. pH of the artificial soil was determined by thoroughly mixing air dried artificial soil in 1 Molar solution of analytical grade potassium chloride (1:5 ratio). After 2 hours of settling period the pH of the supernatant was measured.

APPENDIX 3. TEST MEDIUM PREPARATION DURING DOSE RANGE FINDING STUDY

Group	Concentration (mg/kg dry artificial soil)	*Dry weight of artificial soil used per group (g)	Amount of test item used per group (mg)	Amount of distilled water # (mL)
G1 (SC)	**0.0 (Control)**	1500	0.0	25
****G2**	**0.01**	1500	-	25
****G3**	**0.1**	1500	-	25
G4	**1.0**	1500	1.5	25
G5	**10.0**	1500	15.0	25
G6	**100.0**	1500	150.0	25
G7	**1000.0**	1500	1500.0	25

*: Moisture corrected weight of dry artificial soil. #: Distilled water was used only for test item formulation; SC: solvent control (24.9975mL of distilled water and 0.0025 mL of acetone was mixed and total volume was made up to 25 mL); **: For G2 & G3: 3.0 mg of test item was weighed and dissolved in 2.9975 mL of distilled water + 0.0025 mL of acetone and volume was made up to 3.0 mL to obtain test concentration of 1 mg/mL, from this stock 15 µL and 150 µL was taken and added to 1.5 kg artificial soil to obtain test concentration of 0.01 and 0.1 mg/kg dry artificial soil.

APPENDIX 4. TEST MEDIUM PREPARATION DURING LIMIT TEST

Group	Concentration (mg/kg dry artificial soil)	*Dry weight of artificial soil used per group (kg)	Amount of test item used per group (mg)	Amount of distilled water # (mL)
G1	**0.0 (NC)**	3000	0.0	50
G2	**0.0 (SC)**	3000	0.0	50
G3	**1000**	3000	3000.4	50

*: Moisture corrected weight of dry artificial soil. SC: Solvent Control (49.99 5 +0.005 mL of acetone was mixed and volume was made up to 50 mL; #: Distilled water was used only for test item formulation.

APPENDIX 5. MOISTURE OF ARTIFICIAL SOIL DURING DOSE RANGE FINDING STUDY

Particulars	Moisture on Day 0			Moisture on Day 14		
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
Mass of empty container (W1)	130.8	132.2	129.8	128.9	127.3	126.6
Mass of container + wet soil (W2)	230.8	232.2	229.8	228.9	227.3	226.6
Mass of container + dry soil (W3)	208.9	210.8	207.8	207.3	206.2	206.7
Mass of dry soil (W3-W1)	78.1	78.6	78.0	78.4	78.9	80.1
% Moisture*	**28.0**	**27.2**	**28.2**	**27.6**	**26.7**	**24.8**
Mean*		**27.8**			**26.4**	

Note: Mass is expressed in grams (g); Asterisks (*) indicate the values were rounded off
Method Adopted: Oven drying at the temperature of 105 ± 5°C for 6 hours.

Calculation of Moisture Content in Test Medium:

The percent of moisture content (water) was calculated as follows:

$$\%W = \frac{W2-W3}{W3-W1} \times 100$$

Where,

%W : Percent of moisture content.

W1 : Mass (g) of container.

W2 : Mass (g) of container and wet soil.

W3 : Mass (g) of container and dry soil.

APPENDIX 6. MOISTURE OF ARTIFICIAL SOIL DURING LIMIT TEST

Particulars	Moisture on Day 0			Moisture on Day 14		
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
Mass of empty container (W1)	140.9	141.8	140.5	141.9	143.9	142.7
Mass of container + wet soil (W2)	239.1	242.9	242.1	244.2	241.9	243.3
Mass of container + dry soil (W3)	217.2	220.5	219.7	223.9	222.3	223.1
Mass of dry soil (W3-W1)	76.3	78.7	79.2	82	78.4	80.4
% Moisture*	**28.7**	**28.5**	**28.3**	**24.8**	**25.0**	**25.1**
Mean*		**28.5**			**25.0**	

Note: Mass is expressed in grams (g); Asterisks (*) indicate the values were rounded off
Method Adopted: Oven drying at the temperature of 105 ± 5 °C for 6 hours.

Calculation of Moisture Content in Test Medium:

The percent of moisture content (water) was calculated as follows:

$$\%W = \frac{W2-W3}{W3-W1} \times 100$$

Where,

%W : Percent of moisture content.

W1 : Mass (g) of container.

W2 : Mass (g) of container and wet soil.

W3 : Mass (g) of container and dry soil.

APPENDIX 7. INDIVIDUAL BODY WEIGHT OF EARTHWORMS ON DAY 0 DURING DOSE RANGE FINDING STUDY

Group	Conc. (mg/kg dry artificial soil)	R	1	2	3	4	5	6	7	8	9	10	Mean	±SD
G1	0.0 (SC)	R1	385.5	327.4	335.6	372.9	376.2	360.7	321.6	361.2	316.5	380.9	**353.9**	**26.2**
		R2	321.2	360.6	376.9	380.5	362.2	352.9	337.2	310.2	362.3	317.2	**348.1**	**25.1**
G2	0.01	R1	360.1	354.9	376.2	392.5	376.2	310.9	380.6	317.2	327.6	362.9	**355.9**	**28.2**
		R2	312.2	320.9	370.3	362.5	330.2	392.7	370.9	323.2	386.1	326.5	**349.6**	**29.9**
G3	0.1	R1	360.2	320.9	386.6	322.5	382.6	392.9	327.5	386.9	372.1	396.5	**364.9**	**30.3**
		R2	301.6	353.9	346.2	310.1	320.6	339.8	321.9	366.2	386.9	392.2	**343.9**	**31.2**
G4	1.0	R1	308.7	390.6	380.2	362.6	310.3	390.1	380.6	362.9	320.5	380.9	**358.7**	**33.0**
		R2	371.6	330.2	380.9	337.6	352.2	380.5	310.6	379.6	392.7	386.2	**362.2**	**27.8**
G5	10.0	R1	321.9	380.8	326.2	353.1	346.9	332.5	330.3	370.2	376.9	380.2	**351.9**	**23.6**
		R2	359.8	346.2	361.5	396.8	322.9	356.1	392.9	329.2	380.5	398.6	**364.5**	**27.2**
G6	100.0	R1	380.1	310.3	360.9	310.6	316.3	326.2	380.5	392.3	316.9	312.3	**340.6**	**33.7**
		R2	323.4	380.9	326.7	392.3	311.2	362.5	317.9	354.2	361.7	359.3	**349.0**	**27.7**
G7	1000.0	R1	383.0	350.2	360.9	322.5	362.3	380.6	383.9	310.3	380.5	376.3	**361.1**	**26.2**
		R2	373.2	336.2	310.8	390.6	310.9	385.4	393.2	353.9	318.2	360.4	**353.3**	**32.6**
		Min							**301.6**					
		Max							**398.6**					

R: Replicates; ±SD: Standard Deviation; SC: Solvent control; Weight of earthworm in milligrams (mg); Numerical values 1 to 10 are for the immediate reference for assessing the number of earthworms; Min: Minimum; Max: Maximum.

APPENDIX 8. INDIVIDUAL BODY WEIGHT OF EARTHWORMS ON DAY 14 DURING DOSE RANGE FINDING STUDY

Group	Conc. (mg/kg dry artificial soil)	R	1	2	3	4	5	6	7	8	9	10	Mean	±SD
G1	0.0 (SC)	R1	318.9	326.2	340.1	312.4	320.2	325.3	335.2	331.3	320.1	333.6	326.3	8.6
		R2	320.1	330.3	324.2	346.2	324.6	341.2	353.2	346.2	327.3	341.9	335.5	11.5
G2	0.01	R1	333.8	340.3	332.4	329.2	326.4	331.3	318.9	340.2	332.4	344.3	332.9	7.4
		R2	330.2	332.7	328.9	340.1	325.1	330.4	342.3	348.9	360.1	338.9	337.8	10.7
G3	0.1	R1	350.2	340.3	332.4	338.9	329.4	330.4	343.2	362.2	338.1	329.3	339.4	10.5
		R2	325.4	318.6	326.8	340.2	339.1	324.2	353.2	342.2	329.5	345.3	334.5	11.1
G4	1.0	R1	348.2	339.2	352.3	349.6	339.2	358.2	327.9	344.2	350.3	340.2	344.9	8.6
		R2	344.3	342.3	341.2	336.2	326.4	334.2	352.3	346.6	339.1	321.5	338.4	9.3
G5	10.0	R1	336.9	360.2	340.2	338.8	342.3	336.1	343.5	340.2	350.3	340.2	342.9	7.3
		R2	331.8	344.2	329.4	344.3	353.2	341.5	324.5	346.2	321.5	324.9	336.2	11.0
G6	100.0	R1	321.8	338.2	342.6	344.2	339.2	328.2	342.3	339.2	327.2	330.2	335.3	7.8
		R2	340.3	337.2	329.3	334.2	325.3	324.2	318.5	334.2	331.2	321.8	329.6	7.1
G7	1000.0	R1	338.5	352.3	340.3	344.3	326.3	337.2	343.2	318.9	321.2	320.9	334.3	11.6
		R2	316.2	321.3	324.2	321.2	332.4	329.9	340.1	327.2	312.4	301.9	322.7	10.8

R: Replicates; ±SD: Standard Deviation; SC: Solvent control; Weight of earthworm in milligrams (mg); Numerical values 1 to 10 are for the immediate reference for assessing the number of earthworms.

APPENDIX 9. INDIVIDUAL BODY WEIGHT OF EARTHWORMS ON DAY 0 DURING LIMIT TEST

Group	Conc. (mg/kg dry artificial soil)	R	1	2	3	4	5	6	7	8	9	10	Mean	±SD
G1	0.0 (NC)	R1	324.3	391.3	357.3	327.3	368.5	361.5	334.5	327.4	343.3	385.4	**352.1**	**24.5**
		R2	338.2	373.9	377.3	316.5	351.1	392.2	365.2	370.5	324.8	360.6	**357.0**	**24.2**
		R3	394.0	391.8	395.9	391.3	326.6	380.1	349.4	379.5	374.4	353.0	**373.6**	**23.2**
		R4	333.8	347.5	396.4	311.6	385.5	391.3	399.5	325.3	357.4	384.2	**363.3**	**32.3**
G2	0.0 (SC)	R1	348.8	346.3	398.3	339.8	396.7	382.3	350.1	388.3	380.2	370.9	**370.2**	**22.2**
		R2	423.2	370.5	344.2	323.5	450.2	370.6	329.8	333.6	350.7	377.2	**367.4**	**41.3**
		R3	358.6	372.3	391.6	380.2	370.0	393.7	380.2	393.6	363.1	399.1	**380.2**	**14.0**
		R4	329.7	390.6	339.8	395.1	358.6	377.1	350.3	380.7	362.6	383.2	**366.8**	**22.1**
G3	1000.0	R1	374.5	399.2	348.1	375.2	380.9	364.1	395.1	405.9	398.1	366.6	**380.8**	**18.5**
		R2	382.2	341.4	368.5	338.8	352.6	354.2	325.2	347.3	341.8	387.0	**353.9**	**19.7**
		R3	348.3	353.2	379.8	360.4	395.5	368.5	398.8	368.8	371.5	355.5	**370.0**	**17.1**
		R4	368.5	340.9	382.4	328.5	384.9	369.8	383.6	373.7	382.5	377.9	**369.3**	**19.3**
		Min						**301.9**						
		Max						**362.2**						

R: Replicates; ±SD: Standard Deviation; Weight of earthworm in milligrams (mg); Numerical values 1 to 10 are for the immediate reference for assessing the number of earthworms. NC: Negative Control; SC: Solvent Control.

APPENDIX 10. INDIVIDUAL BODY WEIGHT OF EARTHWORMS ON DAY 14 DURING LIMIT TEST

Group	Conc. (mg/kg dry artificial soil)	R	1	2	3	4	5	6	7	8	9	10	Mean	±SD
G1	0.0 (NC)	R1	309.2	366.5	324.5	311.5	342.5	330.2	315.2	307.5	312.2	345.2	**330.4**	**11**
		R2	312.5	333.8	325.5	301.5	322.5	328.5	314.5	325.7	304.2	342.5	**339.4**	**13**
		R3	341.2	336.5	354.8	372.4	311.5	347.8	319.5	336.5	374.4	322.5	**337.8**	**16**
		R4	315.4	324.1	366.5	298.9	354.2	345.2	366.5	301.4	344.5	365.4	**341.1**	**12**
G2	0.0 (SC)	R1	324.5	325.1	365.4	321.5	325.4	352.9	315.5	358.2	340.5	349.7	**332.8**	**11**
		R2	400.1	347.8	314.2	303.5	409.8	321.5	308.5	311.2	324.5	341.2	**317.8**	**6**
		R3	322.5	362.5	371.2	354.2	358.2	366.2	345.2	382.5	345.2	362.1	**324.5**	**10**
		R4	311.5	360.2	318.5	366.2	341.2	314.2	321.5	344.6	358.7	366.5	**336.1**	**8**
G3	1000.0	R1	345.7	365.2	321.5	324.5	358.2	355.2	378.7	397.2	347.5	322.4	**351.6**	**25**
		R2	341.7	315.2	324.5	308.8	325.4	322.7	301.4	315.8	322.4	318.2	**319.6**	**11**
		R3	322.5	336.5	345.2	330.5	328.5	341.5	364.5	358.2	335.2	329.2	**339.2**	**13**
		R4	344.2	325.1	366.2	319.5	354.2	340.2	347.3	305.6	326.5	314.5	**334.3**	**19**

R: Replicates; ±SD: Standard Deviation; Weight of earthworm in milligrams (mg); Numerical values 1 to 10 are for the immediate reference for assessing the number of earthworms. NC: Negative Control; SC: Solvent Control.

19. ANNEXURES

ANNEXURE 1. CERTIFICATE OF ANALYSIS OF SOLBERE

Camena Lab
1025 NW 11th Street
Corvallis, OR 97330
Phone: 541-990-9910
E-mail: casoro3@hotmail.com

Certificate of Analysis

SOLBERE
Lot 19029
Manufacture 01-29-19
Assayed 02-25-19
Calcium assay: Method ASTM C25.27147 section 33

Test	Result
Appearance	Aqueous slurry
Color	White
Solubility in water	0.015 g/L @ 25° C
Specific Gravity	1.58-1.63 g/mL
pH 5% in water	9.0-10.0
Calcium	21.8-22.8 % by weight
Flash Point	Non-flammable
Freezing Point	1° C

Tested by Carole Jubert: Carole Jubert

Date: 2-28-19

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ANNEXURE 2. SUMMARY OF TOXIC STANDARD

The toxic standard 2-Chloroacetamide, was tested in order to validate the test system. Test concentration of 10.0, 13.0, 16.9, 22.0 and 28.6 mg/kg dry artificial soil was selected for exposure.

No clinical signs were observed at the tested concentration of 10.0 mg/kg dry artificial soil during the 14 days of exposure. Clinical signs of lethargy was observed on day 7 and day 14 and clinical signs of stunted growth and withered was observed on day 14 at the tested concentration of 13.0 mg/kg dry artificial soil during the 14 days of exposure.

Clinical sign of lethargy, stunted growth and withered was observed on day 7 and on day 14 at the tested concentration of 16.9 mg/kg dry artificial soil during the 14 days of exposure. Clinical signs of lethargy, stunted growth and withered were observed on day 7 and clinical signs of withered and stunted growth on day 14 at the tested concentration of 22.0 mg/kg dry artificial soil during the 14 days of exposure.

Clinical signs of lethargy and stunted growth were observed on day 7 at the tested concentration of 28.6 mg/kg dry artificial soil during the 14 days of exposure.

Mortality of 0.0, 30.0, 62.5, 87.5 and 100% were observed in the tested concentration of 10.0, 13.0, 16.9, 22.0 and 28.6 mg/kg dry artificial soil during the 14 days of exposure period.

The acute median lethal concentration (LC_{50}) of 2-Chloroacetamide was calculated by using the Probit analysis (Finney, 1971). The LC_{50} is 16.32 mg/kg dry artificial soil, with the 95% confidence limits of 15.72 to 16.94 mg/kg dry artificial soil.

This 14 days LC_{50} of 2-Chloroacetamide lie in the validity criteria (2-Chloroacetamide LC_{50} sholuld be below 80 mg/kg dry artificial soil) towards test system response and test procedure. Hence this test with reference standard establishes the acceptability of test system response and test procedure followed.

ANNEXURE 3. GLP CERTIFCATE

सत्यमेव जयते

GOVERNMENT OF INDIA

Department of Science and Technology

National Good Laboratory Practice (GLP) Compliance Monitoring Authority (NGCMA)

Certificate of GLP Compliance

Based on the Inspection and the subsequent follow-up actions

Bioneeds India Private Limited

Devarahosahally, Sompura Hobli, Nelamangala Taluk
Bangalore Rural District - 562111 (Karnataka)

is certified capable of conducting the below-mentioned tests/studies in compliance with Organization for Economic Co-operation & Development (OECD) Principles of GLP:

- **Physical-chemical Testing including Five Batch Analysis**
- **Toxicity Studies**
- **Mutagenicity Studies**
- **Environmental Toxicity Studies on Aquatic and Terrestrial Organisms**
- **Residue Studies**
- **Analytical and Clinical Chemistry Testing**
- **Others**

The specific areas of expertise, types of chemicals and test systems are listed in annexure overleaf.

Validity: September 23, 2017 – September 22, 2020

This certificate is subject to the condition that the test facility complies with the NGCMA's Document No. GLP-101 "Terms & Conditions of NGCMA for obtaining and maintaining GLP certification by a test facility" and OECD Principles of GLP.

Certificate No.: GLP/C-109/2017
Issue Date : 20-10-2017

(Dr. Neeraj Sharma)
Head, NGCMA

National GLP Compliance Monitoring Authority (NGCMA)

Annexure to Certificate of GLP Compliance No. GLP/C-109/2017

Areas of Expertise:

Physical-chemical Testing including Five Batch Analysis

Toxicity Studies

- Acute Toxicity
- Sub-acute Toxicity
- Chronic Toxicity
- Reproductive and Developmental Toxicity
- Inhalation Toxicity
- Local Lymph Node Assay
- Neurotoxicity

Mutagenicity Studies

- Bacterial Reverse Mutation Test
- Chromosome Aberration Test (*In-vivo/ In-vitro*)
- Micronucleus Test (*In-vivo/ In-vitro*)
- *In-vitro* Mammalian Cell Gene Mutation Test Using HPRT and XPRT Genes

Environmental Toxicity Studies on Aquatic and Terrestrial Organisms

- Acute Immobilization Test in Daphnia
- Acute Fish Toxicity Test
- Avian Acute Oral and Dietary Toxicity Test
- Acute Toxicity Study in Earthworms
- Acute Oral and Contact Toxicity Test in Honeybee
- Fish-embryo Toxicity Test
- Earthworm and Daphnia Reproduction Toxicity Test
- Acute Silkworm Toxicity Test
- Acute Trichogramma Toxicity Test

Residue Studies

Analytical and Clinical Chemistry Testing

Others

- Bioanalytical Studies
- Toxicokinetics Studies
- Biocompatibility Studies
- *In-vitro* 3T3 NRU Photo Toxicity Test
- Mouse Lymphoma Assay
- *In-vitro* Skin Absorption Study
- Bovine Corneal Opacity and Permeability Test
- *In-vitro* Skin Irritation: Reconstructed Human Epidermis Test
- *In-vitro* Ocular Irritation Test
- In Chemico Skin Sensitization: Direct Peptide Reactivity Assay
- Cytotoxicity Assay

Types of Chemicals:

Industrial Chemicals, Pesticides, Pharmaceuticals, Veterinary Drugs, Cosmetics, Food Additives, Feed Additives and Medical Devices.

Test Systems:

Rat (Wistar and Sprague dawley) and Mice (Swiss albino, BALB/c, CBA/J and C57/BL6), Rabbit (New Zealand White), Guinea Pig (Dunkin hartley), Alga, Daphnia Magna, Fish, Honeybee, Earthworm, Japanese quail, Chicken, Pigeon, Silkworm, Trichogramma, Tester Strains (*Salmonella typhimurium and E. coli*), Cell Lines (L929, AA8, CHO-K1, L5178Y, A-549, SK-MEL-28, BALB/3T3, $HEPG_2/C_2A$, MDA-MB-231, MCF-7, BT-549 and V79-4), EpiDerm™ (EPI-200-SIT), EpiOcular™ (OCL-200-EIT), Human Cadaver Skin, Human Lymphocytes, Plasma and Tissues.

(Dr. Neeraj Sharma)
Head, NGCMA