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FINAL REPORT

ACUTE TOXICITY OF STABILROAD IN EARTHWORM (Eisenia fetida)

TEST ITEM: STABILROAD

STUDY NO.: CSIR-IITR/GLP/052

STUDY COMPLETED ON
NOVEMBER 02, 2018

SPONSOR

VISHWA SAMUDRA ENGINEERING PVT.LTD
AVANI ECOPROJECTS PVT.LTD, PLOT NO: 46
AMAR COOPERATIVE SOCIETY, JUBILEE HILLS
HYDERABAD-500033

TEST FACILITY

VISHAKTATA PARIKSHAN: GLP ANUROOP SUVIDHA CSIR-LNDIAN INSTITUTE OF TOXICOLOGY RESEARCH GHERU CAMPUS, SAROJINI NAGAR INDUSTRIAL AREA KANPUR ROAD, LUCKNOW-226008
UTTAR PRADESH
INDIA

सीएसआईआर-भारतीय विषविज्ञान अनुसंधान संस्थान
CSIR-INDIAN INSTITUTE OF TOXICOLOGY RESEARCH
विषविज्ञान भवन, 31, महात्मा गाँधी मार्ग, लखनऊ -226001, उ.प्र., भारत
VISHVIGYAN BHAWAN, 31, MAHATMA GANDHI MARG, , LUCKNOW-226001, U.P. INDIA

STUDY REPORT

STUDY TITLE

ACUTE TOXICITY OF STABILROAD IN EARTHWORM (*Eisenia fetida*)

TEST ITEM: STABILROAD

STUDY NO.: CSIR-IITR/GLP/052

STUDY COMPLETED ON:
02 November 2018

SPONSOR

VISHWA SAMUDRA ENGINEERING PVT.LTD
AVANI ECOPROJECTS PVT.LTD, PLOT NO: 46
AMAR COOPERATIVE SOCIETY, JUBILEE HILLS
HYDERABAD-500033

TEST FACILITY

VISHAKTATA PARIKSHAN: GLP ANUROOP SUVIDHA
CSIR-INDIAN INSTITUTE OF TOXICOLOGY RESEARCH
GHERU CAMPUS, SAROJINI NAGAR INDUSTRIAL AREA
KANPUR ROAD, LUCKNOW-226008
INDIA

TABLE OF CONTENTS

STATEMENT OF CONFIDENTIALITY

The report contains confidential and proprietary information belonging to Vishwa Samudra Engineering Pvt.Ltd., Avani Ecoprojects Pvt. Ltd, Plot No: 46, Amar Cooperative Society, Jubilee Hills, Hyderabad-500033.The contents of this report will not be disclosed to anyone without an expressed or a written approval of competent authority of Vishwa Samudra Engineering Pvt.Ltd., Hyderabad.

STATEMENT OF GLP COMPLIANCE

The study was performed in compliance with the OECD Principles of Good Laboratory Practice for the testing of chemicals as specified by International [C (97) 186/Final] Legislation. This study was conducted in accordance with the Standard Operating Procedures of Vishaktata Parikshan: GLP Anuroop Suvidha, CSIR-Indian Institute of Toxicology Research and the mutually agreed study plan which was signed by the Study Director on August 20, 2018 for which email approval was received from the sponsor on August 19, 2018.

DECLARATION

The Study Director hereby declares that the work was performed under his 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.

Study Director
Date: 02/11/2018

Test Facility Management
Date: 02/11/2018

Deputy Test Facility Management
Vishaktata Parikshan : GLP Anuroop Suvidha
Toxicity Testing:GLP Compliant Facility CSIR-IITR Lucknow, India

QUALITY ASSURANCE STATEMENT

Study No.: CSIR-IITR/GLP/052, "Acute Toxicity of Stabilroad in Earthworm (*Eisenia fetida)*" has been inspected in accordance with the OECD Principles of Good Laboratory Practice for the testing of chemicals as specified by International [C (97) 186/Final] Legislation.

This study was inspected and findings reported to the Management and Study Director on the dates shown below:

INSPECTION DATE	PHASE	REPORTING DATE
	Initiation Phase	
16/08/2018	Study Plan review	16/08/2018
	In-Life Phase	
11/09/2018	Test Item dispensing in artificial soil and earthworms exposure, moisture content determination and record of earthworms body weight, light intensity and temperature.	11/09/2018
13/09/2018	Record of temperature during the study	13/09/2018
18/09/2018	Day '7' Observation - Mortality and toxicity signs and symptoms in control and treatment groups. Record of temperature and light intensity.	18/09/2018
25/09/2018	Day '14' Observation - Mortality and toxicity signs and symptoms, body weight of earthworms and moisture content determination of artificial soil.	25/09/2018
	Reporting Phase	
12/10/2018	Draft Report Review	15/10/2018
02/11/2018	Final Report Review	02/11/2018

Inspections were performed according to the Standard Operating Procedures of the Test Facility's Quality Assurance Unit. The report was inspected as per the approved study plan and pertinent raw data is accurately reflected in the report.

Date: 2nd Nov 2018

(_____)
Quality Assurance Unit,
Vishaktata Parikshan: GLP Anuroop Suvidha,
CSIR-Indian Institute of Toxicology Research
Gheru Campus, Sarojini Nagar Industrial Area
Kanpur Road, Lucknow-226008, India

LIST OF COMMONLY USED ABBREVIATIONS AND SYMBOLS

CSIR	Council of Scientific and Industrial Research
GLP	Good Laboratory Practices
IITR	Indian Institute of Toxicology Research
LC_{50}	Median Lethal Concentration
NAD	No Abnormalities Detected
OECD	Organization for Economic Cooperation and Development
TIIS	Test Item Information Sheet
°C	Degree Celsius
g	Gram
mg	Milligram
ml	Millilitre
kg	Kilogram
SD	Standard Deviation

1. STUDY DETAILS

Study Title	:	Acute Toxicity of Stabilroad in Earthworm (*Eisenia fetida*)
Test Item	:	Stabilroad
Study Number	:	CSIR-IITR/GLP/052
Sponsor	:	Vishwa Samudra Engineering Pvt.Ltd Avani Ecoprojects Pvt.Ltd, Plot No: 46 Amar Cooperative Society, Jubilee Hills Hyderabad-500033
Sponsor's Representative	:	Mr Srinivas Vallabhaneni Vishwa Samudra Engineering Pvt.Ltd Avani Ecoprojects Pvt.Ltd, Plot No: 46 Amar Cooperative Society, Jubilee Hills Hyderabad-500033 E-Mail: srinivas.email@gmail.com
Test Facility	:	Vishaktata Parikshan: GLP Anuroop Suvidha, CSIR-Indian Institute of Toxicology Research, Gheru Campus Sarojini Nagar Industrial Area Kanpur Road, Lucknow-226008, India
Study Schedule **Range finding Study** Experiment Start Date Acclimatization Dosing Day 7 Observation Day 14 Observation Experiment Completion Date **Main Study** Experiment Start Date Acclimatization Dosing Day 7 Observation Day 14 Observation Experiment Completion Date	 : : : : : : : : : : : :	 23/08/2018 23/08/2018 24/08/2018 31/08/2018 07/09/2018 07/09/2018 10/09/2018 10/09/2018 11/09/2018 18/09/2018 25/09/2018 25/09/2018

2. STUDY PERSONNEL

The following personnel participated in the conduct of the study.

Name	Function
Dr Anbumani Sadasivam	Study Director
Ms Monika Seth	Study Personnel
Ms Alina Zehra	Study Personnel

3. SUMMARY

Acute toxicity study in Earthworm (*Eisenia fetida*) was performed as per the OECD Guideline for the testing of Chemicals, Number 207 with the test item stabilroad. The earthworms were acclimatized one day prior to the exposure in the artificial soil. All earthworms were normal during the acclimatization period. The test item suspended with distilled water.

Range finding experiment was conducted with test concentrations of 0.1, 1.0, 10, 100 and 1000 mg/kg (dry weight of artificial soil) along with a concurrent control group. Two replicates were maintained for treatment and control group with 10 earthworms per replicate. The test item was suspended in distilled water. After the exposure on day 0, earthworms were observed for mortality and toxicity signs on days 7 and 14. No mortality was observed in control and treatment groups throughout the study duration. All exposed earthworms appeared normal in control and treatment groups throughout the study duration.

Based on the range finding experiment results, limit test for stabilroad was conducted with a test concentration of 1000 mg/kg (dry weight of artificial soil) along with a concurrent control group. Four replicates were maintained for treatment and control group with 10 earthworms per replicate. The test item was suspended in distilled water. After the exposure on day 0, earthworms were observed for mortality and toxicity signs on days 7 and 14. No mortality was observed in control and treatment groups throughout the study duration. All exposed earthworms appeared normal in control and treatment groups throughout the study duration.

CONCLUSION

Based on the test results, the LC_{50} of stabilroad in earthworms (*Eisenia fetida*) was found to be greater than 1000 mg/kg, dry weight of artificial soil.

4. OBJECTIVE

The purpose of this study was to assess the acute toxicity of test item stabilroad in the earthworm by artificial soil test method (*Eisenia fetida)* for a period of 14 days.

5. MATERIALS AND METHODS

5.1 Materials

5.1.1 Test Item Information

(As furnished by the Sponsor)

Test Item	:	Stabilroad
Common Name	:	Stabilroad
Chemical Name	:	Not Applicable
Accession No	:	Not Applicable
Batch / Lot Details	:	Batch 1
Product Number	:	Not Applicable
Date of Manufacture	:	October 2017
Date of Expiry / Retest	:	October 2019
CAS Number	:	Not Applicable
Physical Appearance	:	Powder
Recommended storage	:	Dry; should be air tight away to moisture from air always
Purity	:	Not Known
Solubility	:	Not Known
Intended Usage	:	For road construction as stabilizer along with cement
Manufacturer	:	B&K Industries UG & Co. KG Siedlerstraße 1A 85774 Unterföhring Germany

5.1.2 Identity of the Test Item

The physico-chemical properties of the test Item have been provided by the sponsor as mentioned in the test item information sheet. Responsibility for the correct identity and purity rests with the sponsor. The authenticity of the test Item was not conducted at the test facility.

5.1.3 Test System & Test Conditions

Species	:	*Eisenia fetida*
Age	:	Adult earthworms with an individual weight of 300 to 600 mg
Source	:	Earthworm Culture, Ecotoxicology Laboratory, CSIR-Indian Institute of Toxicology Research, Gheru Campus, Sarojini Nagar Industrial Area, Lucknow.
Justification for the selection of species	:	Recommended by the regulatory guideline (OECD 207) for terrestrial toxicity assessment
Test Room Details	:	Cooling incubator (CSIR-IITR/EQP/ECO/BOD/002), Ecotoxicology Laboratory, Room No:47
Test Method	:	Artificial Soil Test
Test Vessel	:	1 litre crystallizing glass dishes covered with perforated plastic film.
No. of Replicates	:	Range Finding Experiment: 2 replicates Limit Test: 4 replicates
Temperature (°C)	:	19.8 - 20.2
Light (Lux)	:	587- 693

Test Medium	:	Artificial Soil – 750g/replicate (10% Peat, 20% Kaolin clay and 70% Industrial sand)
Test Medium pH on Day zero	:	5.74
Acclimatization	:	24 hour prior to test, earthworms were conditioned in the artificial soil
Test Duration	:	14 days.

5.2 Methods

5.2.1 Artificial Soil Preparation

10% sphagnum peat, 20% kaolin clay and 70% industrial sand were used for artificial soil preparation. The dry constituents were blended in correct proportions and hand mixed thoroughly. 200 ml of distilled water was added to 750 g dry weight of artificial soil and the medium was thoroughly mixed. The complete mixture was moistened but not wet as assessed by no appearance of water when artificial soil was compressed.

5.2.2 Moisture Content

At the beginning and end of the test, the moisture content of the test medium was assessed. Approximately, 10 g of soil sample was dried in a previously weighed clean oven dried glass Petri plate in a thermostatically controlled oven with a temperature range of 105 ± 2°C for approximately 3 hours. The beakers were placed in a dessicator and the moisture content of the artificial soil was calculated as percentage of the dry soil weight using the formula:

Moisture content (MC %) = (W2-W3) / (W3-W1) x 100
Where,
W1 = Weight of the glass Petri plate (g)
W2 = Weight of the moist soil + glass Petri plate (g)
W3 = Weight of the dried soil + glass Petri plate (g)

5.2.3 Acclimatization of Earthworms

Sufficient numbers of healthy earthworms were collected from the in-house laboratory culture breeding box, acclimatized for one day in artificial soil for range finding and limit test.

5.2.4 Weight of Earthworms

Prior to the acclimatization, approximate weight of earthworms were checked. 10 earthworms were approximately weighed between 300 and 600 mg on the day of acclimatization. Body weight of all earthworms (control and treatments) was recorded on day 0 and the remaining live worms on day 14 of exposure.

5.2.5 Vehicle

Distilled water was selected as vehicle for test item exposure in artificial soil.

5.2.6 Dose Formulation

For range finding experiment the concentrations selected were 0.1, 1.0, 10, 100 & 1000 mg/kg dry weight of artificial soil. For limit test, 1000 mg/kg dry weight of artificial soil was selected. A total of 770g of artificial soil was used. 200mL of distilled water was added to 750 g of artificial soil (dry weight) and then thoroughly mixed to make it moist. The artificial soil was transferred to the respective labeled dishes. Remaining 20 g was used for pH and moisture content determinations. The procedure was conducted in two replicates for range finding study and four replicates for limit test.

5.2.7 Treatment

For each test, 750 g of the test medium was added into the test vessel. The earthworms acclimatized for 24 hours in artificial soil were washed with distilled water before use, weighed and placed on the test medium surface. One litre crystallizing glass dishes were used as test vessels covered with perforated plastic film to prevent the test medium from drying and kept under the test conditions for 14 days in a cooling incubator.

5.2.8 Range Finding Experiment

Initially, a range finding experiment was conducted by exposing earthworms to concentrations of 0.1, 1.0, 10, 100 and 1000 mg/kg (dry weight of artificial soil) of the test tem with concurrent control group. Two replicates were maintained for treatment and control groups with 10 earthworms per replicate.

5.2.9 Limit Test

Based on the results of range finding experiment, limit test was conducted by exposing earthworms to 1000 mg/kg dry weight of artificial soil, test concentration with concurrent control group. Four

replicates were maintained for treatment and control groups with 10 earthworms per replicate.

5.2.10 Mortality Assessment

The test duration was 14 days and the mortality was assessed on day 7 and day 14 by emptying the test medium into a tray, sorting worms from the medium and testing their reaction for behavior using a mechanical stimulus at the front end. After the day 7 assessment, live worms and the same medium were replaced in the corresponding test vessel(s) and the experiment was continued for another 7 days. After the day 14 assessment, all the exposed earthworms were collected and disposed off through a third party contract.

6. OBSERVATIONS

On the day of acclimatization and day zero, pH of artificial soil was checked for all the test groups during range finding experiment and limit test.

Light intensity and temperature was recorded on the day of acclimatization and once daily during the 14 day exposure period of the range finding experiment and limit test.

Moisture content of artificial soil was checked on days 0 and 14 during range finding experiment and limit test.

Earthworms were assessed for mortality, behavior and toxicity symptoms on days 7 and 14 during the range finding and limit test.

Body weight of earthworms was recorded on day 0 and live worms on day 14 (Table 17 & 22).

7. RESULTS

7.1 Moisture Content

In range finding experiment, moisture content of artificial soil on day zero was recorded to be 40.26% in control group and 42.25%, 40.28%, 39.73% in 0.1, 10 & 1000 mg/kg dry weight of artificial soil, treatment groups (Table 18).

On day 14, moisture content of artificial soil was recorded to be 37.50% in control and 41.09%, 43.52%, 46.37 in 0.1, 10 & 1000 mg/kg dry weight of artificial soil, treatment groups (Table 18).

In limit test, moisture content of artificial soil on day zero was recorded to be 36.78% in control group and 40.00% in 1000 mg/kg dry weight of artificial soil, treatment group (Table 23).

On day 14, moisture content of artificial soil was recorded to be 35.29% in control and 41.09% in 1000 mg/kg (dry weight of artificial soil) treatment group (Table 23).

7.2 pH of Artificial Soil

pH of artificial soil was found to be 5.72 and 5.52 on the day of acclimatization during range finding and limit test (Table 14 & 19).

During range finding experiment, pH of the artificial soil was recorded as 5.76, 5.78, 5.79, 6.01, 6.21 and 6.12 for control, 0.1, 1.0, 10, 100 and 1000 mg/kg dry weight of artificial soil, test concentrations (Table 14).

During the limit test, pH of the artificial soil was recorded as 5.74 and 5.99 for control and 1000 mg/kg dry weight of artificial soil, test concentration (Table 19).

7.3 Mortality and Toxicity Signs

During range finding study, no mortality was observed in control and in worms exposed to 0.1, 1.0, 10, 100 & 1000 mg/kg dry weight of artificial soil, test concentrations on day 7 and day 14 (Table 15).

No abnormal behavior or toxicity signs were observed in control and worms exposed to 0.1, 1.0, 10, 100 & 1000 mg/kg dry weight of artificial soil, test concentrations on day 7 and day 14 (Table 16).

In limit test, no mortality was observed in control and in worms exposed to 1000 mg/kg dry weight of artificial soil, test concentration on day 7 and day 14 (Table 20).

No abnormal behavior or toxicity signs were observed in control and worms exposed to 1000 mg/kg dry weight of artificial soil, test concentration on day 7 and day 14 (Table 21).

8. VALIDITY CRITERIA

A validity criterion was met, as there was no mortality found in the control group.

9. STATISTICAL ANALYSIS

No statistical analysis was carried out as the study was concluded with limit test.

10. DATA COMPILATION

Data are summarized in tabular form for the following:
The number of earthworms used, average body weight of live worms (day 0 and 14), mortality and toxicity signs on days 7 and 14, pH of artificial soil on the day of acclimatization and day 0; moisture content data on days 0 and 14.

11. CONCLUSION

Based on the test results, the LC_{50} of Stabilroad in earthworms (*Eisenia fetida*) was found to be greater than 1000 mg/kg dry weight of artificial soil.

12. ARCHIVING

The following has been archived at the test facility for 9 years after completion of the study: all raw data, study plan, draft and final reports. A representative sample of test Item has been sent from the Test Item Control Office to the Archives in the test facility. The sample shall be stored for a period of 9 years from the date of this final report. Before discarding of any archived study materials, the Sponsor will be contacted for the disposal.

13. REPORT DISTRIBUTION

The study report will be distributed as follows:

Test Facility	:	One signed final report in original (Copy No. 1/2) and an electronic copy in the PDF format.
Sponsor	:	One signed final report in original (Copy No. 2/2) and an electronic copy in the PDF format.

14. pH DATA : RANGE FINDING EXPERIMENT

Details	pH Value
Acclimatization	5.72
At the start of the test (Control)	5.76
0.1 mg/kg dry weight of artificial soil	5.78
1.0 mg/kg dry weight of artificial soil	5.79
10 mg/kg dry weight of artificial soil	6.01
100 mg/kg dry weight of artificial soil	6.21
1000 mg/kg dry weight of artificial soil	6.12

15. MORTALITY DATA: RANGE FINDING EXPERIMENT

Test Item Concentration (mg/kg dry weight of artificial soil)	No. of Earthworms/ Replicate	Day 7		Average (%)	Day 14		Average (%)
		Replicates			Replicates		
		1	2		1	2	
Control	10	0	0	0	0	0	0
0.1	10	0	0	0	0	0	0
1.0	10	0	0	0	0	0	0
10	10	0	0	0	0	0	0
100	10	0	0	0	0	0	0
1000	10	0	0	0	0	0	0

16. TOXICITY SIGNS : RANGE FINDING EXPERIMENT

Test Item Concentration (mg/kg dry weight of artificial soil)	No. of Earthworms/ Replicate	Symptom Code	Day 7 Replicates		Symptom Code	Day 14 Replicates	
			1	2		1	2
Control	10	NAD	10	10	NAD	10	10
0.1	10	NAD	10	10	NAD	10	10
1.0	10	NAD	10	10	NAD	10	10
10	10	NAD	10	10	NAD	10	10
100	10	NAD	10	10	NAD	10	10
1000	10	NAD	10	10	NAD	10	10

Note: Symptom Code: NAD – No abnormalities detected

17. BODY WEIGHT OF EARTHWORMS (mg): RANGE FINDING EXPERIMENT

Concentration: Control		Day: 0
S. No.	Replicate	
	1	2
1	452	387
2	577	469
3	577	530
4	404	390
5	514	432
6	565	575
7	496	583
8	589	421
9	432	451
10	547	539
Mean	**515.30**	**477.70**
SD	**66.86**	**73.83**

17a. BODY WEIGHT OF EARTHWORMS (mg): RANGE FINDING EXPERIMENT

Concentration: 0.1 mg/kg dry weight of artificial soil		Day: 0
S. No.	Replicate	
	1	2
1	575	428
2	469	544
3	521	515
4	368	532
5	566	574
6	530	531
7	387	447
8	366	490
9	360	475
10	436	446
Mean	**457.80**	**498.20**
SD	**85.84**	**48.56**

Concentration: 1.0 mg/kg dry weight of artificial soil		Day: 0
S. No.	Replicate	
	1	2
1	348	409
2	515	468
3	580	407
4	482	436
5	448	488
6	391	455
7	542	542
8	450	508
9	545	446
10	386	399
Mean	**468.70**	**455.80**
SD	**77.55**	**46.70**

17b. BODY WEIGHT OF EARTHWORMS (mg): RANGE FINDING EXPERIMENT

Concentration: 10 mg/kg dry weight of artificial soil		Day: 0
S. No.	Replicate	
	1	2
1	372	457
2	486	522
3	368	444
4	429	366
5	337	592
6	442	375
7	363	405
8	345	449
9	523	484
10	448	577
Mean	**411.30**	**467.10**
SD	**63.53**	**77.82**

Concentration: 100 mg/kg dry weight of artificial soil		Day: 0
S. No.	Replicate	
	1	2
1	594	450
2	586	471
3	473	340
4	467	439
5	495	414
6	498	477
7	469	518
8	388	318
9	425	488
10	470	513
Mean	**486.50**	**442.80**
SD	**63.50**	**68.01**

17c. BODY WEIGHT OF EARTHWORMS (mg): RANGE FINDING EXPERIMENT

Concentration: 1000 mg/kg dry weight of artificial soil		Day: 0
S. No.	Replicate	
	1	2
1	415	506
2	518	584
3	423	426
4	484	387
5	586	583
6	485	559
7	470	512
8	467	560
9	527	432
10	436	465
Mean	**481.10**	**501.40**
SD	**52.28**	**70.93**

17d. BODY WEIGHT OF EARTHWORMS (mg): RANGE FINDING EXPERIMENT

Concentration: Control		Day: 14
S. No.	Replicate	
	1	2
1	396	464
2	393	432
3	511	428
4	469	489
5	455	482
6	383	513
7	366	534
8	463	400
9	475	362
10	397	352
Mean	**430.80**	**445.60**
SD	**49.14**	**61.67**

Concentration: 0.1 mg/kg dry weight of artificial soil		Day: 14
S. No.	Replicate	
	1	2
1	411	461
2	426	340
3	443	405
4	446	453
5	472	347
6	360	566
7	469	482
8	448	321
9	388	302
10	393	355
Mean	**425.60**	**403.20**
SD	**36.89**	**84.99**

17e. BODY WEIGHT OF EARTHWORMS (mg): RANGE FINDING EXPERIMENT

Concentration: 1.0 mg/kg dry weight of artificial soil		Day: 14
S. No.	Replicate	
	1	2
1	391	430
2	498	338
3	342	505
4	427	392
5	369	423
6	450	393
7	390	423
8	469	378
9	386	441
10	417	472
Mean	**413.90**	**419.50**
SD	**47.90**	**47.71**

Concentration: 10 mg/kg dry weight of artificial soil		Day: 14
S. No.	Replicate	
	1	2
1	377	427
2	320	570
3	328	417
4	415	304
5	472	324
6	486	392
7	358	318
8	318	483
9	328	510
10	297	569
Mean	**369.90**	**431.40**
SD	**66.78**	**99.66**

17f. BODY WEIGHT OF EARTHWORMS (mg): RANGE FINDING EXPERIMENT

Concentration: 100 mg/kg dry weight of artificial soil		Day: 14
S. No.	Replicate	
	1	2
1	484	434
2	439	269
3	336	425
4	447	467
5	374	328
6	531	398
7	436	429
8	465	387
9	327	465
10	361	479
Mean	**420.00**	**408.10**
SD	**67.57**	**66.20**

Concentration: 1000 mg/kg dry weight of artificial soil		Day: 14
S. No.	Replicate	
	1	2
1	492	489
2	474	501
3	452	360
4	518	466
5	411	330
6	388	405
7	418	412
8	468	399
9	356	425
10	465	439
Mean	**444.20**	**422.60**
SD	**49.90**	**53.94**

18. MOISTURE CONTENT DATA: RANGE FINDING EXPERIMENT

Concentration (mg/kg dry weight of artificial soil)	Moisture Content (%)	
	Day 0 (Start of the experiment)	Day 14 (End of the experiment)
Control	40.26	37.50
0.1	42.25	41.09
10	40.28	43.52
1000	39.73	46.37

19. pH DATA : LIMIT TEST

Details	pH Value
Acclimatization	5.52
At the start of the test (Control)	5.74
1000 mg/kg dry weight of artificial soil	5.99

20. MOISTURE CONTENT DATA : LIMIT TEST

Concentration (mg/kg dry weight of artificial soil)	Moisture Content (%)	
	Day 0 (Start of the experiment)	Day 14 (End of the experiment)
Control	36.78	35.29
1000	40.00	41.09

21. MORTALITY DATA : LIMIT TEST

Test Item Concentration (mg/kg dry weight of artificial soil)	No. of Earthworms/ Replicate	Day: 7					Day: 14				
		Replicates				Average (%)	Replicates				Average (%)
		1	2	3	4		1	2	3	4	
Control	10	0	0	0	0	0	0	0	0	0	0
1000	10	0	0	0	0	0	0	0	0	0	0

22. TOXICITY SIGNS (LIMIT TEST)

Test Item Concentration (mg/kg dry weight of artificial soil)	No. of Earthworms/ Replicate	Symptom Code	Day 7 Replicates		Symptom Code	Day 14 Replicates	
			1	2		1	2
Control	10	NAD	10	10	NAD	10	10
1000	10	NAD	10	10	NAD	10	10

Note: Symptom Code: NAD – No abnormalities detected

23a. BODY WEIGHT OF EARTHWORMS (mg): LIMIT TEST

Concentration: Control				Day: 0
S. No.	Replicate			
	1	2	3	4
1	467	503	596	428
2	464	562	455	368
3	466	384	353	419
4	411	460	328	506
5	560	543	596	592
6	553	420	324	454
7	498	482	464	596
8	574	428	388	544
9	378	434	355	385
10	414	475	333	350
Mean	**478.50**	**469.10**	**419.20**	**464.20**
S.D.	**67.50**	**55.88**	**105.46**	**90.58**

Concentration: 1000 mg/kg dry weight of artificial soil				Day: 0
S. No.	Replicate			
	1	2	3	4
1	346	471	350	468
2	531	353	344	531
3	352	440	414	561
4	467	584	446	564
5	561	518	433	562
6	590	562	546	577
7	414	341	428	495
8	450	556	509	358
9	451	370	581	506
10	522	418	559	404
Mean	**468.40**	**461.30**	**461.00**	**502.60**
S.D.	**83.19**	**90.88**	**84.23**	**73.75**

ANNEXURE – I

STUDY PLAN

Vishaktata Parikshan: GLP Anuroop Suvidha
Toxicity Testing: GLP Test Facility, CSIR-IITR, India

STUDY PLAN

STUDY No. : CSIR-IITR/GLP/052

ACUTE TOXICITY OF STABILROAD IN EARTHWORM (*EISENIA FETIDA*)

TEST ITEM: STABILROAD

SPONSOR

VISHWA SAMUDRA ENGINEERING PVT.LTD
AVANI ECOPROJECTS PVT.LTD, PLOT NO: 46
AMAR COOPERATIVE SOCIETY, JUBILEE HILLS
HYDERABAD-500033

TEST FACILITY

VISHAKTATA PARIKSHAN: GLP ANUROOP SUVIDHA
CSIR-INDIAN INSTITUTE OF TOXICOLOGY RESEARCH
GHERU CAMPUS, SAROJINI NAGAR INDUSTRIAL AREA
KANPUR ROAD, LUCKNOW-226008
INDIA

Vishaktata Parikshan: GLP Anuroop Suvidha
Toxicity Testing: GLP Test Facility, CSIR-IITR, India

TABLE OF CONTENTS

Vishaktata Parikshan: GLP Anuroop Suvidha
Toxicity Testing: GLP Test Facility, CSIR-IITR, India

Vishaktata Parikshan: GLP Anuroop Suvidha
Toxicity Testing: GLP Test Facility, CSIR-IITR, India

1. STUDY DETAILS

Study Title	:	Acute Toxicity of Stabilroad in Earthworm (*Eisenia fetida*)
Test Item	:	Stabilroad
Study Number	:	CSIR-IITR/GLP/052
Sponsor	:	Vishwa Samudra Engineering Pvt.Ltd Avani Ecoprojects Pvt.Ltd, Plot No: 46 Amar Cooperative Society, Jubilee Hills Hyderabad-500033
Sponsor's Representative	:	Mr Srinivas Vallabhaneni Vishwa Samudra Engineering Pvt.Ltd Avani Ecoprojects Pvt.Ltd, Plot No: 46 Amar Cooperative Society, Jubilee Hills Hyderabad-500033 E-Mail: remella@yahoo.com
Test Facility	:	Vishaktata Parikshan: GLP Anuroop Suvidha CSIR-Indian Institute Of Toxicology Research Gheru Campus, Sarojini Nagar Industrial Area Kanpur Road, Lucknow-226008, India
Study Director	:	Dr. Anbumani Sadasivam Ecotoxicology Vishaktata Parikshan: GLP Anuroop Suvidha, CSIR-Indian Institute Of Toxicology Research, Gheru Campus, Sarojini Nagar Industrial Area, Kanpur Road, Lucknow-226008, India Contact Details: +91-522-2476051, +91-8005494565 E Mail: anbumani@iitr.res.in
Study Personnel	:	Ms. Monika Seth Ms. Alina Zehra

Vishaktata Parikshan: GLP Anuroop Suvidha
Toxicity Testing: GLP Test Facility, CSIR-IITR, India

Study Schedule (Tentative)		
Range finding Study		
Experiment Start Date	:	23/08/2018
Acclimatisation	:	23/08/2018
Dosing	:	24/08/2018
Day 7 Observation	:	31/08/2018
Day 14 Observation	:	07/09/2018
Experimental Completion Date	:	07/09/2018
Main Study		
Experiment Start Date	:	10/09/2018
Acclimatisation	:	10/09/2018
Dosing	:	11/09/2018
Day 7 Observation	:	18/09/2018
Day 14 Observation	:	25/09/2018
Experimental Completion Date	:	25/09/2018
Draft Report (proposed)	:	At least 15 days from the date of completion of Main study experiment.

2. **QUALITY ASSURANCE**
The Quality Assurance Unit of the Test Facility will inspect the study, the raw data, the draft and final reports. Findings of all inspections will be reported to the Management and to the Study Director. The details of phase inspected, Inspection dates and reporting dates will be entered as QA-statement in the study report.

The Quality Assurance Unit has reviewed the study plan and will receive a copy thereof.

3. **STUDY COMPLIANCE**
The study will be performed in accordance with the following:

- OECD Principles of Good Laboratory Practice for the testing of chemicals as specified by International [C (97) 186/Final] Legislation
- OECD Test Guideline No. 207 – Earthworm Acute Toxicity Test.

Vishaktata Parikshan: GLP Anuroop Suvidha
Toxicity Testing: GLP Test Facility, CSIR-IITR, India

- The mutually agreed study plan and the Standard Operating Procedures of the test facility (CSIR-IITR/ECO/002, Revision 2).

4. AMENDMENT PROCEDURES

This study plan may be amended or subjected to alterations. In each case, any amendment to the approved study plan and the reasons for such amendments will be documented and realized only after written / telephonic / e mail consent from the study Sponsor and review by the Quality Assurance Unit and Test Facility Management. If immediate action is necessary, verbal agreement from the Sponsor will be confirmed as soon as possible by study plan amendment. Minor changes (unplanned) of the study plan which do not influence the procedures or the outcome of the study may be subject to the discretion of the Study Director, but will be mentioned in the report as deviations.

5. SAFETY PRECAUTIONS

Gloves, face mask and goggles (if required) will be used in addition to protective body garments and shoes to ensure adequate personal health and safety. In case of eye contact, the eye will be washed thoroughly with water and medical treatment will be sought. In case of skin contact, it will be washed with soap and water with subsequent medical aid.

6. OBJECTIVE

The purpose of the study is to assess the acute toxicity of test item stabilroad by artificial soil test method to the earthworm, *Eisenia fetida* with an observation period of 14 days.

7. MATERIALS AND METHODS

7.1 Materials

7.1.1 Test Item Information

(As furnished by the Sponsor)

Test Item	:	Stabilroad
Common Name	:	Stabilroad
Formula	:	Not Applicable
Chemical Name	:	Not Applicable

Vishaktata Parikshan: GLP Anuroop Suvidha
Toxicity Testing: GLP Test Facility, CSIR-IITR, India

Batch / Lot Details	:	Batch 1
Product Number	:	Not Applicable
Date of Manufacture	:	October 2017
Date of Expiry / Retest	:	October 2019
CAS Number	:	Not Applicable
Physical Appearance	:	Powder
Recommended storage	:	Dry; should be air tight away from moisture
Purity	:	Not Known
Solubility	:	Not Known
Intended Usage	:	For road construction as stabilizer along with cement
Manufacturer	:	B&K Industries UG & Co. KG Siedlerstraße 1A 85774 Unterföhring Germany

7.1.2 Identity of the Test Item

The physico-chemical properties of the Test Item have been provided by the sponsor. The responsibility for the correct identity and purity rests with the Sponsor. The authenticity of the test item will not be conducted at the test facility.

Vishaktata Parikshan: GLP Anuroop Suvidha
Toxicity Testing: GLP Test Facility, CSIR-IITR, India

7.1.3 Test System & Test Conditions

Species	:	*Eisenia fetida*
Age	:	Adult earthworms with an individual weight of 300 to 600 mg.
Justification for the selection of species	:	Recommended by the regulatory guideline (OECD) for the toxicity assessment.
Source	:	Earthworm culture, Ecotoxicology Lab, Gheru Campus, CSIR-Indian Institute of Toxicology Research (IITR).
Test Room Details	:	Room No: 47, Cooling Incubator (18-22 °C), Ecotoxicology Laboratory.
Test Method	:	Artificial Soil Test
Test Vessel	:	1litre crystallizing glass dishes covered with a perforated plastic film.
Number of Replicates	:	Range Finding Study: 2 replicates for each test concentration and control. Main Study: 4 replicates for each test concentration and control.
Light	:	Continuous light (400-800 Lux)
Test Medium	:	Artificial soil - 750g/replicate (10% Peat, 20% Kaolin clay, 70% Industrial sand)
Test Medium pH Range	:	5.50 - 6.50 (Measured at the start of the test)
Test Duration	:	14 days for Range Finding and 14 days for Main Study

Vishaktata Parikshan: GLP Anuroop Suvidha
Toxicity Testing: GLP Test Facility, CSIR-IITR, India

7.2 Methods

7.2.1. Artificial Soil Preparation

Artificial soil is a mixture of 10 percent sphagnum peat, 20 percent kaolin clay and 70% industrial sand. The dry constituents will be blended in the correct proportions and hand mixed thoroughly and stored for use. pH of the medium will be adjusted to 6.0 ± 0.5 (if needed) by addition of calcium carbonate. The dry mixture will be moistened before use but not so wet that water appears when the artificial soil is compressed. After the preparation of artificial soil, the moisture content will be determined.

7.2.2. Moisture Content

Moisture content will be determined at the beginning and end of the test. Determined quantity of distilled water will be added to give an overall moisture content of about 35 percent of the dry weight.

A clean oven dried glass petriplate will be taken and its initial weight will be taken. To this, approximately 10 g of soil will be added and dried in a thermostatically controlled oven with a temperature range of 105 ± 2°C for approximately 3 hours. The beaker will be placed in a desiccator and the moisture content of the soil is calculated as a percentage of the dry soil weight.

Moisture content (%) = (W2-W3) / (W3-W1) x 100
Where,
W1 = Weight of the glass beaker (g)
W2 = Weight of the moist soil + glass beaker (g)
W3 = Weight of the dried soil + glass beaker (g)

7.2.3 Acclimatization of Earthworm

Before start of the experiment, sufficient number of earthworms will be collected from the breeding box, washed with distilled water and acclimatized for minimum of one day in artificial soil. Bodyweight of minimum 10 earthworms will be recorded on the day of acclimation.

7.2.4 Earthworm Weighing

Prior to the release of earthworms on the test medium surface, body weight of earthworms will be checked and recorded in the raw data. Earthworm weighing between 300 and 600 mg will be

Vishaktata Parikshan: GLP Anuroop Suvidha
Toxicity Testing: GLP Test Facility, CSIR-IITR, India

released into the test vessel containing test medium with appropriate test concentrations.

7.2.5 Vehicle

Since the test item solubility is not known by the sponsor, solubility test conducted prior to the study shows that it is insoluble in water and other solvents (acetone, DMSO). Hence the test item will be directly mixed with the artificial soil before earthworms exposure.

7.2.6 Test Item Preparation

Required quantity of test item will be weighed and directly mixed with the artificial soil for uniform distribution. Homogeneity analysis will be not performed since the test item is insoluble.

7.2.7 Range Finding and Main Study

A preliminary range finding experiment will be performed by exposing earthworms to a range of 0.1, 1.0, 10.0, 100.0 and 1000.0 mg/kg (dry weight of artificial soil) test concentrations. Based on the results of the range finding experiment, main experiment will be conducted at minimum five test concentrations to determine the LC_{50} of stabilroad. The details of test concentrations selected for the main study will be given in the study report.

7.2.8 Treatment

During range finding study, two replicates will be maintained for each test concentration including control and each replicate will contain 10 earthworms. For each replicate, 750 g of the artificial soil will be placed in the respective test vessel. Ten earthworms in the body weight ranging between 300 and 600 mg will be placed on the test medium surface. The test containers will be covered with perforated plastic film to prevent the test medium from drying and will be kept under the test conditions for 14 days to indentify the test concentration range that results in 0% and 100% mortality.

During main study, four replicates will be maintained for each test concentration including control and each replicate will contain 10 earthworms. For each replicate, 750 g of the artificial soil will be placed in the respective test vessel. Ten earthworms in the body weight ranging between 300 and 600 mg will be placed on the test medium surface. The test containers will be covered with perforated

Vishaktata Parikshan: GLP Anuroop Suvidha
Toxicity Testing: GLP Test Facility, CSIR-IITR, India

plastic film to prevent the test medium from drying and will be kept under the test conditions for 14 days for LC_{50} determination.

7.2.9 **Limit Test**

If no mortality is observed during the range finding study at all the exposed test concentrations, a limit test will be performed to demonstrate that the LC_{50} will be greater than 1000 mg/kg (dry weight of the artificial soil) of the test item.

During limit test, four replicates will be maintained at 1000 mg/kg (dry weight of artificial soil) along with the control and each replicate will contain 10 earthworms. For each replicate, 750 g of the artificial soil will be placed in the respective test vessel. Ten earthworms in the body weight ranging between 300 and 600 mg will be placed on the test medium surface. The test containers will be covered with perforated plastic film to prevent the test medium from drying and will be kept under the test conditions for 14 days.

8. **OBSERVATIONS**

pH of the blended artificial soil will be recorded on the day of acclimation and day "0" of treatment and control groups.

Moisture content of artificial soil will be checked on day "0" and day "14" in control and treatment groups (low, medium and high test concentrations) during range finding study whereas in main study, moisture content will be determined at all test concentrations including the control group on day "0" and day "14".

Light intensity will be recorded everyday (day "0" to day "14") during the range finding and main study.

Mortality and toxicity signs will be recorded on day 7 and 14 after treatment during the range finding and main study. The mortality will be assessed by emptying the test medium onto a tray, sorting worms from the medium and testing their reaction to a mechanical stimulus at the front end. After the 7th day assessment, worms and test medium will be replaced in the corresponding test vessel and incubate for additional 7 days. Any behavioral or toxicity symptoms, if observed will be recorded in the raw data. Body weight of live earthworms will be recorded on day "0" and day "14" during the range finding and main study.

Vishaktata Parikshan: GLP Anuroop Suvidha
Toxicity Testing: GLP Test Facility, CSIR-IITR, India

9. **VALIDITY CRITERIA**

The mortality in controls should not exceed 10 percent at the end of the test.

10. **STATISTICAL ANALYSIS**

The mortality/concentration data will be used to calculate the median lethal concentration (LC_{50}) and its confidence limits. Finney's Probit Analysis will be applied to calculate the LC_{50} with 95% confidence limits and graph showing concentration/effect curve will also be plotted. Statistical analysis will be not performed if the study is conducted as a limit test.

11. **DATA COMPILATION**

Data will be summarized in a tabular form, the number of earthworms' used, average live weight and number of live worms per treatment at start and on completion of the experiment. Moisture content data for day 0 and day 14 and mortality data for day 0 and day 14 will also be tabulated.

12. **DATA AND FINAL REPORT**

The data will be summarized in tabular form, showing for each test group the number of earthworms at the start and end of the test, death of individual earthworm at different concentration levels and description of toxic effects. The final report will be prepared in compliance to the principles of GLP and normally include, but not limited to the following:

- A descriptive title.
- The name and address of the Sponsor and the test facility along with the details of study schedule.
- The names of all personnel involved in the study.
- A compliance statement signed by the Study Director that all applicable GLP regulations were followed in the conduct of the study.
- Quality Assurance (QA) statement; that states that the report accurately reflects the raw data obtained during the

Vishaktata Parikshan: GLP Anuroop Suvidha
Toxicity Testing: GLP Test Facility, CSIR-IITR, India

performance of the study and including the dates of QA activities and the dates reported to study director and management.

- The Test Item and its code, composition and other appropriate characteristics and vehicle with identification by name.
- Complete description of the test system including species, source, number, test conditions, photoperiod, and acclimation.
- Statistical analysis of the results (if applicable).
- Method of preparation of stock and test solutions.
- Graph of the concentration mortality curve at the end of the test (if applicable).
- LC_{50} values, with 95% confidence limits at each of the recommended observation times (day 7 and 14) (if applicable).
- Highest concentration causing no mortality, lowest concentration causing 100% mortality (if applicable).
- Moisture content of artificial soil at the start and end of the test, pH at start of the test.
- A description of the results; discussion and conclusion.
- A description of all study plan deviations, if any.
- A description of all circumstances that may have affected the quality or integrity of the study.
- The storage locations of all raw data, specimens, reports, Test Item reference sample and the archiving period.

13. ARCHIVING

The following will be archived at the test facility for at least 9 years (3 cycles of GLP) after completion of the study: study plan, all raw data, draft and final reports, a representative sample of Test Item

Vishaktata Parikshan: GLP Anuroop Suvidha
Toxicity Testing: GLP Test Facility, CSIR-IITR, India

(approximately one gram), etc. Before discarding of any archived study materials, the Sponsor will be contacted for the disposal.

14. STUDY PLAN DISTRIBUTION

The final study plan (original copies) will be distributed as follows:

Test Facility : One signed study plan in original (Copy No. 1/2)
Sponsor : One signed study plan in original (Copy No. 2/2)
Document Control: One controlled copy
Quality Assurance Unit: One controlled copy
Study Personnel: One controlled copy

15. REPORT DISTRIBUTION

The study report will be distributed as follows:

Test Facility : One signed final report in original (Copy No. 1/2) and an electronic copy in the PDF format.
Sponsor : One signed final report in original (Copy No. 2/2) and an electronic copy in the PDF format.

Vishaktata Parikshan: GLP Anuroop Suvidha
Toxicity Testing: GLP Test Facility, CSIR-IITR, India

16. AGREEMENT

This study plan for Study No.: CSIR-IITR/GLP/052, "Acute Toxicity Study of Stabilroad in Earthworm (*Eisenia fetida*)" has been mutually agreed:

for **TEST FACILITY**	for **STUDY SPONSOR**
1. _____	1. _____
STUDY DIRECTOR	Mr Srinivas Vallabhaneni Viswa Samudra Engineering Pvt. Ltd., Hyderabad - 5000033
Date: 20/08/2018	Date:

2. _____

QUALITY ASSURANCE UNIT

Date: 20/08/2018

3. _____ 20/08/2018

TEST FACILITY MANAGEMENT

Date:

ANNEXURE – II

Test Item Information Sheet

TEST ITEM INFORMATION SHEET

Test Item Name	:	STABILROAD
Common Name (If Applicable)	:	STABILROAD
Chemical Name (If Applicable)	:	NOT APPLICABLE
Accession No. (If Applicable)	:	NOT APPLICABLE
Test Item Manufactured By (Name and Address)	:	B&K Industries UG & Co. KG Siedlerstraße 1a 85774 Unterföhring Germany
Test Item Supplied By (Name and Address)	:	Vishwa Samudra Engineering Pvt Ltd – Avani Ecoprojects Pvt Ltd, Plot No 46, Amar Cooperative Society, Jubilee Hills, Hyderabad 500033
Batch / Lot Details	:	BATCH 1
Date of Manufacture	:	OCTOBER 2017
Date of Expiry / Retest	:	OCTOBER 2019
Physical Appearance	:	Powder
Purity (as per Analysis Certificate)	:	NOT KNOWN
Physico Chemical Properties including Solubility	:	NOT KNOWN
Storage Conditions Recommended	:	Dry Should be air tight away to moisture from air always
Quantity of Test Item Submitted	:	500 GMS
Analysis Certificate Submitted with Test Item	:	NO
Material Safety Data Sheet Submitted with Test Item	:	Non Hazardous
Intended usage	:	For Road Construction as Stabilizer along with Cement
Name and Address of Sponsor / Client	:	Vishwa Samudra Engineering Pvt Ltd – Avani Ecoprojects Pvt Ltd, Plot No 46, Amar Cooperative Society, Jubilee Hills, Hyderabad 500033
Name and Signature with Date	:	R KRISHNA MADHAV 26/07/2018

AVANI ECOPROJECTS PVT. LTD.

Avani EcoProjects Private Limited
Plot No. 46, Amar Co-Operative Society, Jubilee Hills, Hyderabad-500 033.
Phone: 040-64642919

ANNEXURE – III

GLP Certificate

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

Toxicity Testing: GLP Test Facility
CSIR-Indian Institute of Toxicology Research
Gheru Campus, Sarojini Nagar Industrial Area
Kanpur Road, Lucknow-226008 (Uttar Pradesh)

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

- **Toxicity Studies**
- **Mutagenicity Studies**
- **Environmental toxicity studies on aquatic & terrestrial organisms**
- **Analytical and Clinical Chemistry Testing**

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

Validity: June 5, 2017 – June 4, 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-102/2017
Issue Date : 01-08-2017

(Dr. Neeraj Sharma)
Head, NGCMA