STUDY REPORT

STUDY TITLE

ACUTE IMMOBILIZATION TEST OF STABILROAD IN *DAPHNIA MAGNA*

TEST ITEM: STABILROAD

STUDY NO.: CSIR-IITR/GLP/050

STUDY COMPLETED ON:
01 October 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

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STATEMENT OF CONFIDENTIALITY

This report contains confidential and proprietary information belonging to Vishwa Samudra Engineering Pvt. Ltd, Plot No: 46, Amar Cooperative Society, Jubilee Hills, Hyderabad-500033, India. 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, India.

STATEMENT OF GLP COMPLIANCE

This 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 27, 2018 for which email approval was received from the sponsor on the same day.

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: 01/10/2018

01/10/2018
Test Facility Management
Date:

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/050, "Acute Immobilization Test of Stabilroad in *Daphnia magna*" 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	
23/08/2018	Study Plan review	23/08/2018
	In-Life Phase	
11/09/2018	Acclimatization of Daphnia brooders, measurement of physico-chemical parameters	11/09/2018
12/09/2018	Exposure of daphnia neonates to test item and zero hour observation – Immobility and toxicity signs and symptoms in control and treatment groups.	12/09/2018
14/09/2018	48 hour observation – immobility, toxicity signs and symptoms in control and treatment groups; record of physico-chemical parameters.	14/09/2018
	Reporting Phase	
19/09/2018	Draft Report Review	20/09/2018
01/10/2018	Final Report Review	01/10/2018

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: 01/10/2018

(AHKhan)

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
CoA	Certificate of Analysis
GLP	Good Laboratory Practices
IITR	Indian Institute of Toxicology Research
OECD	Organization for Economic Cooperation and Development
TIIS	Test Item Information Sheet
ºC	Degree Celsius
h	Hour
l	Litre
mg	Milligram
ml	Millilitre

1. STUDY DETAILS

Study Title	:	Acute Immobilization Test of Stabilroad in *Daphnia magna*
Test Item	:	Stabilroad
Study Number	:	CSIR-IITR/GLP/050
Sponsor	:	Vishwa Samudra Engineering Pvt.Ltd Avani Ecoprojects Pvt.Ltd, Plot No: 46 Amar Cooperative Society, Jubilee Hills Hyderabad-500033, India
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, India 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 24 Hour Observation 48 Hour Observation Experiment Completion Date **Limit Test** Experiment Start Date Acclimatization Dosing 24 Hour Observation 48 Hour Observation Experiment Completion Date	 : : : : : : : : : :	 03/09/2018 03/09/2018 05/09/2018 06/09/2018 07/09/2018 07/09/2018 10/09/2018 10/09/2018 12/09/2018 13/09/2018 14/09/2018 14/09/2018

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 immobilization toxicity study in *Daphnia magna* was conducted in accordance with OECD guideline 202 for the test item Stabilroad. The daphnids with brood chambers were acclimatized 48 h prior to the exposure in M4 medium. Less than 24 h old second instar neonate daphnids were collected from the acclimatized gravid females and exposed to test item. After the exposure on day zero, daphnids were observed for immobilization at 24 and 48 h and the respective data were recorded.

Range finding experiment was conducted with test concentrations of 1.0, 10, 25, 50 and 100 mg/l along with a concurrent control group. Two replicates were maintained for treatment and control groups with 5 daphnids per replicate. The test item was formulated in M4 medium. After the exposure on day zero, daphnids were observed for immobility and toxicity signs at 24 and 48 h. All exposed daphnids appeared normal in control and treatment groups throughout the study duration as no immobility was observed.

Based on the range finding experiment results, a limit test was conducted with a test concentration of 100 mg/l along with a concurrent control group. Four replicates were maintained for treatment and control groups with 5 daphnids per replicate. The test item was formulated in M4 medium. After the exposure on day zero, daphnids were observed for immobility and toxicity signs at 24 and 48 h. All exposed daphnids appeared normal in control and treatment groups throughout the study duration as no immobility was observed.

CONCLUSION

Based on the test results, the EC_{50} of Stabilroad for *Daphnia magna* observed for a period of 48 h was found to be greater than 100 mg/l.

4. OBJECTIVE

The purpose of this study was to assess the acute toxicity of Stabilroad to young daphnids aged less than 24 h.

The sensitivity and reliability of the experimental technique was assessed with potassium dichromate (reference substance) as recommended by the OECD 202 guideline. The result from the most recent test run is included in this report.

5. MATERIALS AND METHODS

5.1 Materials

5.1.1 Test Item Information

(As furnished by the Sponsor)

Test Item	:	Stabilroad
Common Name	:	Stabilroad
Formula	:	Not Applicable
Chemical Name	:	Not Applicable
CAS Number	:	Not Applicable
Batch / Lot Details	:	Batch 1
Product Number	:	Not Applicable
Date of Manufacture	:	October 2017
Date of Expiry / Retest	:	October 2019
Physical Appearance	:	Powder
Recommended storage	:	Dry; should be air tight away from moisture
Purity	:	Not Known

Solubility	:	Dispersed in aqueous solution
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. The 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	:	*Daphnia magna*
Age	:	Young daphnids aged less than 24 hours
Source	:	*Daphnia magna* Culture, Ecotoxicology Laboratory, CSIR-Indian Institute of Toxicology Research, Gheru Campus Lucknow.
Justification for the selection of species	:	Recommended by the regulatory guideline (OECD 202) for aquatic toxicity assessment
Test Room Details	:	Cooling incubator (CSIR-IITR/EQP/ECO/BOD/004), Ecotoxicology Laboratory, Room No:47
Test Method	:	Static
Test Vessel	:	100 ml glass beakers
Volume of Test Solution	:	50 ml

No. of Replicates	:	Range Finding Experiment: 2 replicates Limit Test: 4 replicates
Number of Daphnids / replicate	:	5 daphnids / replicate
Light	:	16 hour light and 8 hour darkness
Culture Medium	:	M4 Medium (Stock minerals)
Temperature (˚C)	:	Control: 20.4 – 20.8 °C Treatment: 20.1 – 21.1 °C
pH	:	Control: 7.81 – 7.94 Treatment: 7.60 – 8.08
Dissolved Oxygen (DO)	:	Control: 6.42 – 5.72 mg/l Treatment: 5.90 – 6.24 mg/l
Hardness (M4 Medium)	:	158 mg/l
Acclimatization	:	48 h prior to test item exposure
Test Duration	:	48 h

5.2 Methods

5.2.1 Acclimatization of Daphnids

Sufficient number of adult daphnids with full brood chambers were collected from the in-house laboratory culture and acclimatized for 48 hour in M4 medium. The daphnids were fed with algal (*Pseudokirchneriella subcapitata*) suspension. Daphnids aged not more than 24 hours (second instar) were separated on the day of exposure and used for the study.

5.2.2 Vehicle

Since the compound is inert and neither soluble in water nor in solvents, the required quantity of the test item in M4 media is stirred using magnetic stirrer for 48 h prior to the exposure. The solution appeared to

be cloudy with small suspended particles. From this solution, required volume is dispensed in to the treatment vessels before exposure of daphnids.

5.2.3 Dose Formulation

For range finding experiment the concentrations selected were 1.0, 10.0, 25.0, 50.0 and 100.0 mg/l. A stock concentration of 1.0 mg/ml was prepared by dissolving 50 mg of the test item in 50 ml of distilled water. From this, serial dilutions were made to obtain concentrations of 1.0, 10.0, 25.0, 50.0 and 100.0 mg/l. From the above prepared concentrations, 50 ml of the test solution was transferred into each replicate beaker.

For limit test, 100 mg/l test concentration was selected by dissolving 30 mg of the test item in 300 ml of M4 medium. From the above prepared concentration, 50 ml of the test solution was transferred into each replicate beaker.

5.2.4 Treatment

Test vessels were filled with 50 ml of the test solution at different concentrations. Daphnids were then released into the respective test vessels. During limit test, 20 daphnids were divided into four groups of 5 daphnids each, one group in each test replicate. In range finding experiment, 10 daphnids were divided into two groups of 5 daphnids each, one group in each test replicate. Control group daphnids were exposed to 50 ml of M4 medium without the test item.

5.2.5 Physico-chemical Parameters

The test medium was analyzed for pH, temperature and dissolved oxygen at the beginning and end of the study in all the test groups, during range finding **(Table 1)** and limit test **(Table 3)**. Hardness was analyzed once at the start of the experiment in control group.

6. OBSERVATIONS

Each test vessel was checked for immobilised daphnids and any abnormal behaviour at 24 and 48 hours after test item exposure. Daphnids that were not able to swim within 15 seconds after gentle agitation of the test vessel were considered to be immobilised (even if they can still move their antennae).

7. RESULTS

7.1 Immobilization

In range finding experiment, no immobilization was observed in control and daphnids exposed to 1.0, 10.0, 25.0, 50.0 and 100.0 mg/l of test item throughout the study duration. Thus, the percent immobilization at the end of 48 hour was recorded to be 0% in control and above mentioned treatment groups **(Table 2)**.

Similarly, in limit test, the percent immobilization at the end of 48 hour was recorded to be 0% in control and 100 mg/l Stabilroad treatment groups **(Table 4)**.

8. VALIDITY CRITERIA

The study is considered to be valid since the following criterion was met:

1. In control, no immobilization was observed throughout the experiment period.
2. The dissolved oxygen concentration at the end of the test was ≥ 3 mg/l in control and test replicate vessels.

9. STATISTICAL ANALYSIS

No statistical analysis was carried out as only the limit test study was performed.

10. DATA COMPILATION

Data are summarized in tabular form for each treatment and control groups. The number of daphnids used and immobilization at each observation are reported.

11. CONCLUSION

Based on the test results, the EC_{50} of the test item Stabilroad in *Daphnia magna* was found to be greater than 100 mg/l.

12. ARCHIVING

The following has been archived at the test facility for 9 years after completion of the study: study plan, all raw data, 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. Sponsor's approval would be sought before discarding of any archived study materials.

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.

TABLE 1: PHYSICO-CHEMICAL PARAMETERS – RANGE FINDING EXPERIMENT

Concentration	Control		1.0 mg/l		10.0 mg/l	
	0 h	48 h	0 h	48 h	0 h	48 h
pH	7.77	7.39	7.75	7.31	7.64	7.30
Temperature (ºC)	20.2	20.0	20.0	20.2	19.8	20.3
Dissolved Oxygen (mg/l)	6.50	6.30	6.37	6.14	6.37	6.21
Hardness* (mg/l)	156	-	-	-	-	-
Concentration	**25.0 mg/l**		**50.0 mg/l**		**100.0 mg/l**	
pH	7.56	7.30	7.62	7.26	7.50	7.33
Temperature (ºC)	20.0	19.9	20.4	20.5	20.5	20.5
Dissolved Oxygen (mg/l)	6.50	6.21	6.58	6.27	5.95	6.08
Hardness* (mg/l)	-	-	-	-	-	-

Note: * - Analysed at the start of the experiment; h - hour

TABLE 2: IMMOBILIZATION DATA – RANGE FINDING EXPERIMENT

Test Item Concentration (mg/l)	No. of Daphnids/ Replicate	24 h		Average (%)	48 h		Average (%)
		Replicates			Replicates		
		1	2		1	2	
Control	5	0	0	0	0	0	0
1.0	5	0	0	0	0	0	0
10.0	5	0	0	0	0	0	0
25.0	5	0	0	0	0	0	0
50.00	5	0	0	0	0	0	0
100.00	5	0	0	0	0	0	0

Note: h - hour

TABLE 3: PHYSICO-CHEMICAL PARAMETERS – LIMIT TEST

Concentration	Control		100.00 mg/l	
	0 h	48 h	0 h	48 h
pH	7.82	7.92	7.71	8.07
Temperature (ºC)	20.7	20.8	20.9	20.6
Dissolved Oxygen (mg/l)	6.50	5.78	5.92	6.21
Hardness* (mg/l)	160	-	-	-

Note: * - Analysed at the start of the experiment; h - hour

TABLE 4: IMMOBILIZATION DATA – LIMIT TEST

Test Item Concentration (mg/l)	No. of Daphnids / Replicate	24 h					48 h				
		Replicates				Average (%)	Replicates				Average (%)
		1	2	3	4		1	2	3	4	
Control	5	0	0	0	0	0	0	0	0	0	0
100.00	5	0	0	0	0	0	0	0	0	0	0

Note: h - hour

14. RESULTS OF REFERENCE STUDY

Study No: CSIR-IITR/GLP/045

Validity Period: June 2018 – December 2018

Summary

Acute immobilization test in *Daphnia magna* was performed as per the OECD Guideline for the testing of Chemicals, Number 202 with potassium dichromate, as reference item. Healthy brood adult daphnids were acclimatized 48 h prior to the exposure in M4 media. All daphnids were normal during the acclimatization period and daphnids less than 24 h old (second instar neonates) were separated from the adult daphnids for reference item exposure. The reference item was formulated with distilled water.

Less than 24 hour old daphnids were exposed to 0.50, 0.85, 1.45, 2.46, 4.18 mg/l concentrations of reference item. A stock concentration of 1 mg/ml was prepared by dissolving 20 mg of potassium dichromate in 20 ml of deionised water. From this serial dilution were made to obtain concentrations of 0.50, 0.85, 1.45, 2.46, 4.18 mg/l. From this, 50 ml of the test solution was transferred into four replicate beakers for each concentration containing 5 daphnids in each beaker.

No immobilization was observed in control and 0.50 mg/l potassium dichromate treatment group of Daphnia throughout the study period. At the end of 24 hours, 4/20, 6/20, 14/20 and 20/20 daphnids were immobilized at 0.85, 1.45, 2.46 and 4.18 mg/l concentrations. At the end of 48 hours, 2/20, 3/20 and 18/20 daphnids were immobilized at 0.85, 1.45 and 2.46 mg/l concentrations. The cumulative percent immobilization at the end of 48 hours in the control and 0.5 mg/l group was 0%. The cumulative percent immobilization at the end of 48 hours in 0.85, 1.45, 2.46 and 4.18 mg/l potassium dichromate treatment groups were 30, 45, 90 and 100% respectively. Results of potassium dichromate concentration analysis are mentioned in section 19.

CONCLUSION

EC_{50} of reference item, potassium dichromate in *Daphnia magna* observed for a period of 48 hours was calculated as 1.33 mg/l with upper confidence limits 1.59 mg/l and lower confidence limits of 1.11 mg/l and regression equation Y = 9.7924ln(x)+6.9839.

15. POTASSIUM DICHROMATE CONCENTRATION ANALYSIS: REFERENCE STUDY

Department : Formulation Analysis		Page 7 Of 7
TITLE: Formulation Analysis using Liquid Chromatograph		
SOP No.: CSIR-IITR/ANL/006	Revision No.: 02	
Effective Date: 13/02/2017	Revision Date: 12/02/2020	

Annexure I

Result Format

Dated: 02/07/2018

1. Test item code: CSIR-IITR/GLP/ 045 (I,II,III, IV, V)
2. Details provided by Sponsorer: No
3. Sample receiving date: 04/06/2018
4. No of test item received: 05 nos
5. Solubility of test item: Water
6. Result:

S No.	Test item code	Instrument used	Calculated concentration of Potassium Dichromate	Compound identified	Remarks if any
1	CSIR-IITR/GLP/ 045-I (0.5ppm)	AAS, Analytik Jena ZEEnet 700 To estimate the Chromium concentration	**0.40ppm**	NA	Concentration of Potassium Dichromate were calculated by measuring Chromium
2	CSIR-IITR/GLP/ 045-II (0.85ppm)		**0.76 ppm**	NA	
3	CSIR-IITR/GLP/ 045-III (1.45ppm)		**1.30 ppm**	NA	
4	CSIR-IITR/GLP/ 045-IV (2.46ppm)		**2.45 ppm**	NA	
5	CSIR-IITR/GLP/ 045-V (4.18ppm)		**4.49 ppm**	NA	

Anuroop 5/7/2018

Vishaktata Parikshan: GLP Anuroop Suvidha, CSIR-Indian Institute Of Toxicology Research, Gheru Campus, Sarojini Nagar Industrial Area, Kanpur Road, Lucknow-226008, India: Phone: 91-522-2611547, 2628228, Mobile: 91-9453309900, Fax: 91-522-2611547, E-mail: tfm.glp@iitr.res.in

ANNEXURE – I

STUDY PLAN

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

STUDY PLAN

STUDY No. : CSIR-IITR/GLP/050

ACUTE IMMOBILIZATION TEST OF STABILROAD IN *DAPHNIA MAGNA*

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

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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 Immobilization Test of Stabilroad In *Daphnia magna*
Test Item	:	Stabilroad
Study Number	:	CSIR-IITR/GLP/050
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 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. Alina Zehra Ms. Monika Seth

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

Study Schedule (Tentative)		
Range Finding Study	:	03/09/2018
Acclimatization	:	05/09/2018
Dosing	:	06/09/2018
24 Hour Observation	:	07/09/2018
48 Hour Observation	:	07/09/2018
Experimental Completion Date		
Main Study		
Acclimatization	:	10/09/2018
Dosing	:	12/09/2018
24 Hour Observation	:	13/09/2018
48 Hour Observation	:	14/09/2018
Experimental Completion Date	:	14/09/2018
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. 202 – "*Daphnia* sp. Acute Immobilization Test", adopted on 13th April 2004.
- The mutually agreed study plan and the Standard Operating Procedures of the test facility (CSIR-IITR/ECO/018).

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

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 BXGE when exposed to young daphnids aged less than '24' hours at the start of the test, followed by an observation period of '48' hours under static condition and immobilization is recorded at 24 and 48 hours.

The sensitivity and reliability of the experimental technique employed will assessed with potassium dichromate as positive control which is recommended by the OECD guideline 202. The result from the most recent test run will be included in the study report.

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

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
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

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

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.

7.1.3 Test System & Test Conditions

Species	:	*Daphnia magna*
Age	:	Less than 24 hour old neonates.
Justification for the selection of species	:	Recommended by the regulatory guideline (OECD) for the toxicity assessment.
Source	:	Daphnia 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	:	Static
Test Vessel	:	100 ml glass beakers.
Test Volume	:	50 ml
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	:	16 hours light and 8 hours darkness.
Feeding	:	During the test, daphnids will not

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

		be fed.
Test Medium	:	M4 Media
Hardness of Medium	:	140-250 mg/l
Test Medium pH Range	:	6 to 9 (should not vary by more than 1.5 units)
Dissolved Oxygen		≥3 mg/L
Test Duration	:	48 hours

7.2 Methods

7.2.1 Homogeneity Analysis

Homogeneity and dose formulation analysis will not be conducted at the test facility, since standard for this test item is not available with the sponsor.

7.2.2 Acclimatization

Sufficient numbers of healthy daphnia brooders will be collected minimum two days prior to the study from the culture vessels and kept in a clean beaker with sufficient feed. The daphnids (neonates) released on the next day will be collected and separated into another vessel. The second batch of the neonates (<24 h old) will be used for the experiment and the information will be recorded on raw data sheet. Dissolved oxygen, pH, total hardness and temperature of the test medium will be recorded on day of acclimatization. Remaining parent daphnids will be shifted to the stock culture vessel.

7.2.3 Vehicle

M4 medium prepared in distilled water will be used to prepare the stock solution for the study and the same will be documented in the raw data and report.

7.2.4 Test Item Preparation

The test item is neither soluble in distilled water nor in solvents (acetone and DMSO). Hence, the stock solution will be prepared two days prior to the exposure by stirring them continuously. The

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aqueous portion is then collected once the particles settle down and will be used for daphnia exposure. The details will be recorded in the raw data and report.

7.2.5 Test and Control Groups

For range finding study, two replicates will be maintained for each treatment and control having five daphnids for each replicate.
For main study, four replicates will be maintained for each treatment and control having five daphnids for each replicate.

7.2.6 Range Finding Study

A preliminary range finding study will be conducted with a series of widely spaced concentrations like 1.0, 10.0, 25, 50 and 100.0 mg/l. A stock solution prepared by using M4 medium will then be added to each test group to produce requisite test group concentrations.
For each replicate, 50 ml of the test solution will be placed in the respective test vessel of 100 ml capacity. 05 daphnids will be placed in each test vessel using a wide mouth pipette carefully. The vessels will then be kept under the test conditions for 48 hours.

7.2.7 Limit Test

If the highest concentration of the test item (i.e. 100 mg/l) shows '0' percent immobilization at the end of 48 hour exposure period in a range finding study, then the study is restricted to a limit test.
Four replicates will be maintained for highest concentration and control having five daphnids for each replicate.

7.2.8 Main Study

Based on the results of the range finding study, the main study will be conducted with minimum five test concentrations with a separation factor preferably not exceeding 2.2. The doses selected for main study will be provided in study report and recorded in the raw data sheet.

During the main study, four replicates will be maintained for each test concentration and control groups where each replicate will contain 05 daphnids. For each replicate, 50 ml of test solution will be placed in the respective test vessel. 05 daphnids will be placed in each test vessel using a wide mouth pipette carefully. The vessels will then be kept under the test conditions for 48 hours.

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8. OBSERVATIONS

During the range finding and main study, dissolved oxygen, pH and temperature of the test medium will be recorded on day of acclimatization, "0" hour and "48" hours of the treatment and control groups.

Daphnids that are not able to swim for a few seconds, after gentle agitation of the vessel will be considered to be immobilized (even if they can still move their antennae).

In addition to immobility, any abnormal behavior or appearance will be recorded at 24 and 48 hours. Hardness will be checked in control group on day 0.

9. VALIDITY CRITERIA

Daphnids in control should not show more than 10 percent immobilization.

The dissolved oxygen concentration at the end of the test should be ≥ 3 mg/L in control and test vessels.

10. STATISTICAL ANALYSIS

The mortality/concentration data will be used to calculate the Median Effective Concentration (EC_{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. No statistical analysis will be performed for the limit test and range finding experiment.

11. DATA COMPILATION

Data will be summarized in a tabular form, the number of daphnids used, and number of dead and live daphnids per treatment at start and during the course of the experiment (24 h and 48 h). Dissolved oxygen, pH and temperature at the start and end of the experiment 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 daphnids at the start and end of the test, death of individual daphnids 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:

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- 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 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.
- EC_{50} values, with 95% confidence limits at each of the recommended observation times (24 hour and 48 hour), if possible.
- 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.

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- 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 (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.

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16. AGREEMENT

This study plan for Study No.: CSIR-IITR/GLP/050, "Acute Immobilization Test of Stabilroad in *Daphnia magna*" has been mutually agreed:

for **TEST FACILITY**

1. _____

STUDY DIRECTOR

Date: 27/08/2018

for **STUDY SPONSOR**

1. _____

Mr Srinivas Vallabhaneni
Viswa Samudra Engineering Pvt. Ltd.
Hyderabad - 5000033

Date:

2. _____

QUALITY ASSURANCE UNIT

Date: 27/08/2018

3. _____ 27/08/2018

TEST FACILITY MANAGEMENT

Date:

ANNEXURE – II

TEST ITEM INFORMATION SHEET

TEST ITEM INFORMATION SHEET

AVANI
BUILDING GREEN ROADS

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

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

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