

**STUDY REPORT**  
**Original: Draft**

**STUDY TITLE**

**ACUTE TOXICITY STUDY OF SOLBERE ON FISH, *CYPRINUS CARPIO***

**STUDY No.: BIO-ETX 138**

**Study Completion Date: DD.MM.YY**

**SPONSOR**  
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30301 RIVERVIEW Dr. JUNCTION CITY,  
OR 97448, USA

**TEST FACILITY**

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**CONFIDENTIAL**

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## QUALITY ASSURANCE STATEMENT

The Study No.: BIO-ETX 138, entitled “Acute Toxicity Study of Solbere on fish, *Cyprinus carpio*” 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
<b>Initiation Phase</b>			
12 March 2019	Study plan verification	12 March 2019	12 March 2019
16 May 2019	Study plan amendment no. 1 verification	16 May 2019	16 May 2019
<b>In-life Phase</b>			
23 May 2019	Test item formulation preparation, exposure and dose concentration analysis - limit test	23 May 2019	23 May 2019
<b>Reporting Phase</b>			
30 May 2019	Draft report inspection	30 May 2019	30 May 2019
	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 138, "Acute Toxicity Study of Solbere on fish, *Cyprinus carpio*" 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)

**Dr. T. S. SADANANDA**  
Study Director

.....  
(Date)

**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 test facility wherever necessary and to persons authorized by law or judicial judgment.

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

.....  
**(Date)**

.....  
**(Signature)**  
**Dr. S. N. VINAYA BABU**  
**Test Facility Management**

.....  
**(Date)**

## ABBREVIATIONS OF COMMONLY USED UNITS AND SYMBOLS

cm	: Centimeter
DRF	: Dose Range Finding
g	: Gram
GLP	: Good Laboratory Practice
h/hr	: Hour
L	: Liter
LC <sub>50</sub>	: Lethal Concentration 50%
mg	: Milligram
mg/L	: Milligram per Liter
min	: Minute
Min	: Minimum
Max	: Maximum
No.	: Number
OECD	: Organization for Economic Co-operation and Development
-	: Not Applicable
%	: Percentage
°C	: Degree Celsius



## 1. STUDY DETAILS

- 1.1 Study Title** : Acute Toxicity Study of Solbere on fish, *Cyprinus carpio*.
- 1.2 Study Number** : BIO-ETX 138
- 1.3 Study Code** : AFT
- 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** : Bionees 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.  
Bionees India Private Limited,  
Devarahosahally,  
Sompura Hobli, Nelamangala Taluk,  
Bangalore Rural District, PIN - 562 111  
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E.mail: bionees@bionees.in
- Study Co-ordinator : Mr. Lavakumar C., M.Sc.
- Study Personnel : Mr. Abilash T. S., B.Sc.  
Mr. H. Mahanthesh, M.Sc.  
Mr. Thiyagaraj M., M.Sc.
- 1.7 Study Schedule**
- Study Initiation Date : 02 May 2019
- Experimental Starting Date : 02 May 2019
- Acclimatization Start Date (DRF) : 02 May 2019 to 08 May 2019
- Treatment Date (DRF) : 09 May 2019
- Observation End Date (DRF) : 13 May 2019
- Observation End Date (DRF) : 13 May 2019

Observation End Date (DRF) : 13 May 2019  
Acclimatization Start Date (MS) : 02 May 2019 to 22 May 2019  
Treatment Date (MS) : 23 May 2019  
Observation End Date (MS) : 27 May 2019  
Experimental Completion date : 27 May 2019  
Draft Report Submission date : 30 May 2019  
Study Completion Date : DD.MM.YY

## 2. SUMMARY

The test item Solbere, obtained from CO2 Solved LLC., was tested for acute toxicity on fish as per the OECD Guidelines for Testing of Chemicals (Section 2), Effects on Biotic Systems, Guideline No. 203, "Fish Acute Toxicity Test" adopted on 17 July 1992.

The freshwater fish *Cyprinus carpio* was exposed over 96 hours to Solbere to determine the 96 hours median lethal concentration (LC<sub>50</sub>).

### Range Finding Study

Range finding study was conducted with 5 concentrations of 1.0, 10.0, 50.0, 75.0 and 100.0 mg/L of Solbere along with control group.

No clinical sign of toxicity or mortality were observed in control group and at the tested concentrations of 1.0, 10.0, 50.0, 75.0 and 100.0 mg/L during the 96 hour exposure period.

### Limit Test

Based on the results of dose range finding study, limit test was conducted as main study at the concentration of 100.0 mg/L of Solbere along with the control groups.

During limit test, no clinical signs of toxicity or mortality were observed in the control groups and at the tested concentration of 100.0 mg/L during the 96 hour of exposure period

### Analytical Measurements

During limit test, test media sample of Solbere was analyzed for test concentrations by HPLC method. The results are found to be within the acceptable range of  $\pm 20\%$  to the nominal concentration.

### Conclusion

The 96 hours No Observed Effect Concentration (NOEC) is 100 mg/L, Lowest Observed Effect Concentration (LOEC) and acute median lethal concentration (LC<sub>50</sub>) value of Solbere is >100 mg/L.

### **3. STUDY COMPLIANCE**

#### **3.1 GLP Compliance**

The study was performed:

- a. In compliance with the OECD Principles of Good Laboratory Practices [C (97)186/Final].
- b. In accordance with the Standard Operating Procedures at Bioneds 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. 203, "Fish Acute Toxicity Test" adopted on 17 July 1992.

### **4. SAFETY PRECAUTIONS**

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

### **5. OBJECTIVE**

The acute toxicity study of Solbere on fish (*Cyprinus carpio*) was performed to determine the No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) along with 96 hour LC<sub>50</sub> value.

### **6. MATERIALS AND METHODS**

#### **6.1 Test Item Information**

The test item information 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
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 provided by sponsor is attached as Annexure 1.

## 6.2 Selection of Vehicle and Justification for Selection

Based on the dissolution test, test item Solbere was miscible in the Reverse Osmosis (RO) purified water (Tap water) containing acetone (100 µL/L). Hence, reverse osmosis water containing acetone was used as vehicle.

## 6.3 Selection of Route of Exposure and Justification for Selection

Test item was introduced homogeneously into the test medium as per recommendations of OECD Test Guideline No. 203 for testing of chemicals by aqueous exposure “Acute toxicity-Fish”.

## 6.4 Test System

- Fish Species** : *Cyprinus carpio*
- Justification for Selection of Species** : *Cyprinus carpio* (common carp) was readily available species and has been historically shown to be a suitable model for acute toxicity studies. Moreover, it is recommended by OECD Test Guideline No. 203, and by other regulatory test guidelines.
- Source of Supply** : In-house maintained *Cyprinus carpio* (common carp) procured from Fisheries Research and Information Center (Inland), Hebbal, Bangalore, Karnataka, India.
- Total Length of Fish** : The length was measured a day prior to the exposure. The length of fish used during range finding study and limit test ranged from 3.0 to 3.7 cm.  
Refer Appendix 1 & 4.
- No. of Groups and No. of Fish per Group** : Six groups [1 solvent control and 5 treatment groups] consisting of 7 fish in each group were used during the range finding study.  
Three groups [1 negative control, 1 solvent control and 1 treatment group] consisting of 7 fish in each group were used during the limit test.

## 6.5 Acclimatization

One hundred healthy fish with an additional ten fish were collected for acclimatization. Prior to test initiation healthy fish were acclimatized for 7 days during range finding study and 21 days during limit test. No mortality was observed during acclimatization period.

Fish were fed ad libitum daily with commercial feed pellets (Kijaro Grow). Feeding was stopped approximately 24 hour prior to commencement of the exposure to test item.

Water temperature during acclimatization was between 22.1 to 23.4°C. Hardness of the water was measured once during the period of acclimatization (at start) was 222 mg CaCO<sub>3</sub>/L. Prior to acclimatization the glass aquaria was labeled for identification

(study no., study code, no. of fish acclimatized, acclimatization start and acclimatization end date).

#### **6.6 Diluent Water**

Reverse osmosis purified water (Tap water) with the pH 6.78 to 7.38, total hardness 216 to 226 mg CaCO<sub>3</sub>/L with dissolved oxygen content of >76.1% was used for holding the fish and as diluent medium during acclimatization and exposure periods.

#### **6.7 Test Vessels**

Thoroughly cleaned rectangular glass aquaria having the water holding capacity of 50 liters was used as test vessels. The test vessels were labeled for identification (study no., test item code, study code, group no. and concentration, exposure start and exposure end) prior to experiment initiation.

#### **6.8 Feeding**

Fish was fed *ad libitum* daily with commercial feed pellets (Kijaro Grow). Feeding was stopped approximately 24 hours prior to commencement of the exposure to test item.

#### **6.9 Test Condition**

Duration of the experiment was 96 hours. Temperature was maintained between 22.1 to 23.9°C during range finding and limit test respectively. A photoperiod of 12 hour light and 12 hour darkness was maintained.

#### **6.10 Grouping**

A day prior to exposure, the fish were randomly and impartially selected for all the groups (grouping) and weighed in groups to determine the biomass (loading rate) during the test.

A maximum loading of fish was 0.61 and 0.62 g wet weight of fish/L during range finding study and limit test respectively.

#### **6.11 Test Medium Preparation**

Based on the dissolution trial Solbere was miscible in Reverse Osmosis (RO) purified water (Tap water) containing acetone (100 µL/L).

The test item stock solution was prepared by weighing required quantity of test item in a beaker, to this required volume of acetone was added and small volume of tap water (reverse osmosis water) was added and stirred well, after complete miscibility, test volume was transferred to measuring cylinder, beaker was rinsed with tap water (reverse osmosis water) and transferred again to measuring cylinder, the rinsing process was repeated until the complete transfer of test contents. Finally the volume made up to required volume using tap water (reverse osmosis water). A homogeneous distribution of the test item within the stock solution was achieved by means of stirring on magnetic stirrer shortly before releasing to the test vessels. The stock solution prepared was released to test vessel containing known volume of tap water (reverse osmosis water) and stirred well using glass rod to obtain desired concentration.

The preparation of test media on day 0 during range finding study and limit test are presented as Appendix 2 and Appendix 5 respectively.

## **6.12 Active Ingredient Content Analysis**

- Static test: Concentrations were determined at the beginning and at the end of the test period.

During limit test, samples from all the test concentrations were collected and analyzed for test concentration on day 0 (0 hour, Fresh) and stability on day 4 (96 hours, aged). 15 mL of test samples were collected in duplicates from the central point of the tank. The validated analytical method for determination of active content analysis of Solbere was used. (Bioneds Study Number: BIO-ANM 1367).

## **6.13 Dose Range Finding Study**

A dose range finding (DRF) study with 5 doses of 1.0, 10.0, 50.0, 75.0 and 100.0 mg/L of Solbere was used along with solvent control to find out the range of the lethal dose. During range finding study, test was conducted under semi static renewal conditions and test media was renewed once in 24 hours.

## **6.14 Limit Test**

During dose range finding study no clinical signs of toxicity or mortality were observed, hence limit test was conducted as main study at the test concentration of 100 mg/L.

## **6.15 Euthanasia and Disposal**

Unused fishes, kept for acclimatization in excess for replacement and all the treated fish at termination of the study were euthanized with an over dose of MS-222 (Tricaine methane sulfonate) and Sodium bicarbonate. All euthanized fishes were collected at the end of the treatment and sent for disposal.

# **7. OBSERVATIONS**

## **7.1 Clinical Signs of Toxicity and Mortality**

Fish was observed for signs of toxicity (behavioral and morphological responses) and mortality at 3, 6, 24, 48, 72 and 96 hours of exposure.

## **7.2 Environmental Parameters**

### **Dose Range Finding Study**

Total hardness of the diluent water prior to its use for exposure was analyzed. Temperature, pH and dissolved oxygen were recorded at beginning and at 24 hour intervals in fresh and spent solutions until completion of the test during dose range finding study.

### **Limit Test**

Total hardness of the diluent water prior to its use for exposure was analyzed. Temperature, pH and dissolved oxygen were recorded at beginning and at 24 hours intervals in spent solutions until completion of the test.

# **8. STUDY REPORT PREPARATION AND RESULTS**

Data is summarized in tabular form showing clinical signs, cumulative percent mortalities, physico-chemical parameters and analytical measurement. Individual values were presented as appendices.

**9. STATISTICAL ANALYSIS OF RESULTS**

The acute median lethal concentration ( $LC_{50}$ ) of Solbere was not evaluated as it was a limit test.



## 10. RESULTS AND DISCUSSION

### 10.1 Signs of Toxicity and Mortality

#### Range Finding Study

No clinical sign of toxicity were observed in control group and at the tested concentrations of 1.0, 10.0, 50.0, 75.0 and 100.0 mg/L during the 96 hour exposure period.

Refer Tables 1 & 2

#### Limit Test

Based on the results of dose range finding study, limit test was conducted as main study at the concentration of 100.0 mg/L of Solbere along with negative and solvent control groups.

No clinical signs of toxicity or mortality were observed in the negative and solvent control groups and at the tested concentration of 100.0 mg/L during the 96 hour of exposure period.

Refer Tables 3 and 4

### 10.2 Environmental Parameters

The environmental parameters during range finding and limit test are summarized as below:

Test	Environmental Parameters			
	Temp. (°C)	Dissolved oxygen (%)	pH	Hardness (mg/L CaCO <sub>3</sub> )
Range Finding Study	22.3	76.1	6.78	216
	to	to	to	to
	23.9	89.9	7.29	226
Limit Test	22.1	80.4	6.81	220*
	to	to	to	
	22.9	85.6	7.02	

\*Limit test performed under static condition hence hardness was measured once at the starting of the study

Refer Appendix 3 and 6

### 10.3 Active Ingredient Content Analysis

During limit test media samples of Solbere was analyzed for test concentrations by HPLC method. The results are found to be within the acceptable range of  $\pm 20\%$  to the nominal concentration.

Refer Table 5 and Appendix 7

## 11. STATISTICAL ANALYSIS OF RESULTS

As it was a limit test statistical analysis was not performed.

## **12. CONCLUSION**

The 96 hours No Observed Effect Concentration (NOEC) is 100 mg/L, Lowest Observed Effect Concentration (LOEC) and acute median lethal concentration (LC<sub>50</sub>) value of Solbere is >100.0 mg/L.

## **13. VALIDITY CRITERIA OF THE TEST**

The acute fish toxicity test fulfills the validity criteria of the test as given below:

- Mortality in the control (negative and solvent vehicle control) group was 0% at the termination of the test during limit test (validity criterion: should not exceed 10%).
- Dissolved oxygen concentration in the test media was above 80.4% of the air saturation value during limit test (validity criterion: should be at least 60%).
- The results are found to be within the acceptable range of  $\pm 20\%$  to the nominal concentration for Solbere.

## **14. AMENDMENTS AND DEVIATIONS**

One study plan amendment was raised during the conduct of the study to include the details of active ingredient content analysis and details of environmental parameters analysis. No deviation occurred during the conduct of the study.

## **15. STUDY REPORT DISTRIBUTION**

Original: 1/2 - Sponsor

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

## **16. 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 Bionees 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 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.

## **17. REFERENCES**

- Finney DJ, 1971. Probit Analysis, 3<sup>rd</sup> Edition, Cambridge, University Press. pp. 333.
- OECD Guidelines for Testing of Chemicals (Section 2), Effects on Biotic Systems, Guideline No. 203, "Fish Acute Toxicity Test" adopted on 17 July 1992.
- L. W. Huson, A rapid approximate method of estimating the median of a dose-tolerance distribution, tropical pest management, volume 29, issue 2, 1983.
- Bionees study number: BIO-ANM 1367 entitled: Validation of analytical method to determine the content of Solbere in reverse osmosis water by HPLC Method.

## 18. TABLES

**TABLE 1. SUMMARY OF CLINICAL SIGNS DURING RANGE FINDING STUDY**

Group	Test Conc. (mg/L)	No. of Fish/ Group at Start	Clinical Signs of Toxicity at					
			3 h (±20 Mins)	6 h (±20 Mins)	24 h (±20 Mins)	48 h (±20 Mins)	72 h (±20 Mins)	96 h (±20 Mins)
G1 (SC)	0.0	7	1(7)	1(7)	1(7)	1(7)	1(7)	1(7)
G2	1.00	7	1(7)	1(7)	1(7)	1(7)	1(7)	1(7)
G3	10.0	7	1(7)	1(7)	1(7)	1(7)	1(7)	1(7)
G4	50.0	7	1(7)	1(7)	1(7)	1(7)	1(7)	1(7)
G5	75.0	7	1(7)	1(7)	1(7)	1(7)	1(7)	1(7)
G6	100.0	7	1(7)	1(7)	1(7)	1(7)	1(7)	1(7)

SC: Solvent control; 1: Normal; h: hour; min: Minutes; Values outside and inside the parentheses represent the clinical symptom and the number of fish that exhibit a particular symptom respectively.

**TABLE 2. SUMMARY OF MORTALITY AND 96 HOUR % MORTALITY  
DURING RANGE FINDING STUDY**

Group	Test Conc. (mg/L)	No. of Fish/ Group at Start	Mortality (No.) Observed at						96 hours Percent Mortality
			3 h (±20 Mins)	6 h (±20 Mins)	24 h (±20 Mins)	48 h (±20 Mins)	72 h (±20 Mins)	96 h (±20 Mins)	
<b>G1 (SC)</b>	<b>0.0</b>	<b>7</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
<b>G2</b>	<b>1.0</b>	<b>7</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
<b>G3</b>	<b>10.0</b>	<b>7</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
<b>G4</b>	<b>50.0</b>	<b>7</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
<b>G5</b>	<b>75.0</b>	<b>7</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
<b>G6</b>	<b>100.0</b>	<b>7</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>

SC: Solvent control; h: hour; mins: Minutes

**TABLE 3. SUMMARY OF CLINICAL SIGNS DURING LIMIT TEST**

Group	Test Conc. (mg/L)	No. of Fish/Group at Start	Clinical Signs of Toxicity at					
			3 h (±20 Mins)	6 h (±20 Mins)	24 h (±20 Mins)	48 h (±20 Mins)	72 h (±20 Mins)	96 h (±20 Mins)
<b>G1 (NC)</b>	<b>0.0</b>	7	1(7)	1(7)	1(7)	1(7)	1(7)	1(7)
<b>G2 (SC)</b>	<b>0.0</b>	7	1(7)	1(7)	1(7)	1(7)	1(7)	1(7)
<b>G3</b>	<b>100.0</b>	7	1(7)	1(7)	1(7)	1(7)	1(7)	1(7)

NC: Negative control; SC: Solvent control; 1: Normal; h: hour; mins: Minutes.

**TABLE 4. SUMMARY OF MORTALITY AND 96 HOUR % MORTALITY DURING LIMIT TEST**

Group	Test Conc. (mg/L)	No. of Fish/Group at Start	Mortality (No.) Observed at						96 hours Percent Mortality
			3 h (±20 Mins)	6 h (±20 Mins)	24 h (±20 Mins)	48 h (±20 Mins)	72 h (±20 Mins)	96 h (±20 Mins)	
<b>G1 (NC)</b>	<b>0.0</b>	<b>7</b>	0	0	0	0	0	0	0
<b>G2 (SC)</b>	<b>0.0</b>	<b>7</b>	0	0	0	0	0	0	0
<b>G3</b>	<b>100.0</b>	<b>7</b>	0	0	0	0	0	0	0

NC: Negative control; SC: Solvent control; h: hour; mins: Minutes

**TABLE 5. SUMMARY OF TEST CONCENTRATION ANALYSIS DURING LIMIT TEST**

<b>Group</b>	<b>Nominal Conc. (mg/L)</b>	<b>Conc. Obtained for Solbere (mg/L)</b>	<b>Recovery (%)</b>
<b>Day 0 (0 hour - Fresh)</b>			
<b>G1 (NC)</b>	<b>0.0</b>	-	-
<b>G2 (SC)</b>	<b>0.0</b>	-	-
<b>G3</b>	<b>100.0</b>	99.60	99.60
<b>Day 4 (96 hours - Spent)</b>			
<b>G1 (NC)</b>	<b>0.0</b>	-	-
<b>G2 (SC)</b>	<b>0.0</b>	-	-
<b>G3</b>	<b>100.0</b>	97.88	97.88

NC: Negative control; SC: Solvent control; Conc.: Concentration; -: Not applicable.



## **19. APPENDICES**

**APPENDIX 1. INDIVIDUAL FISH LENGTH DURING RANGE FINDING STUDY**

Group	Test Conc. (mg/L)	Fish Length (cm)						Mean	±SD	Cummulative Weight (g)	Loading rate (g)
<b>G1 (SC)</b>	<b>0.0</b>	3.4	3.1	3.5	3.6	3.2	3.1	3.4	0.2	14.4	
<b>G2</b>	<b>1.0</b>	3.3	3.3	3.4	3.2	3.0	3.4	3.3	0.1	15.1	
<b>G3</b>	<b>10.0</b>	3.6	3.3	3.6	3.4	3.2	3.5	3.4	0.1	14.6	
<b>G4</b>	<b>50.0</b>	3.1	3.2	3.4	3.1	3.0	3.2	3.6	0.2	14.5	<b>0.61</b>
<b>G5</b>	<b>75.0</b>	3.0	3.3	3.0	3.3	3.0	3.2	3.1	0.1	15.2	
<b>G6</b>	<b>100.0</b>	3.3	3.2	3.3	3.6	3.2	3.1	3.5	0.2	15.3	
<b>Min</b>					<b>3.0</b>						
<b>Max</b>					<b>3.6</b>						

SC: Solvent control; ±SD: Standard deviation; g: Gram.; min: Minimum; max: Maximum

**APPENDIX 2. TEST MEDIUM PREPARATION DURING RANGE FINDING STUDY**

Day - 0

Test Item Quantity (mg)	For Stock Solution				Test Media Preparation			
	Vol. of Acetone (mL)	Vol. of Dilution Medium (mL)	Volume made up to	Final Conc. (mg/mL)	Vol. of Stock Solution used (mL)	Final Volume of Test Media (L)	Group	Final Test Conc. (mg/L)
7000.1	0.070	699.93	700	10	0.0	25	G1 (SC)	0.0
-	-	-	-	-	2.5	25	G2	1.0
-	-	-	-	-	25.0	25	G3	10.0
-	-	-	-	-	125.0	25	G4	50.0
-	-	-	-	-	187.5	25	G5	75.0
-	-	-	-	-	250.0	25	G6	100.0

NC: Negative control; SC: Solvent control (2.5 mL of Acetone + 24997.5 mL Reverse osmosis water).

**APPENDIX 3. PHYSICO-CHEMICAL PARAMETERS OF TEST MEDIA DURING RANGE FINDING STUDY**

**Dissolved Oxygen (%)**

Group	Test Conc. (mg/L)	0 to 24 h		24 to 48 h		48 to 72 h		72 to 96 h	
		Fresh 0 h	Spent 24 h	Fresh 24 h	Spent 48 h	Fresh 48 h	Spent 72 h	Fresh 72 h	Spent 96 h
<b>G1 (SC)</b>	<b>0.0</b>	81.2	79.6	81.5	78.4	83.8	82.2	83.3	80.4
<b>G2</b>	<b>1.00</b>	79.5	78.2	80.6	79.6	84.9	81.6	84.4	82.3
<b>G3</b>	<b>10.0</b>	79.5	77.3	81.8	80.1	83.6	81.0	82.6	81.1
<b>G4</b>	<b>50.0</b>	80.1	78.2	83.4	81.3	84.5	83.2	81.8	80.1
<b>G5</b>	<b>75.0</b>	78.5	76.1	80.5	79.6	85.2	83.3	82.9	79.6
<b>G6</b>	<b>100.0</b>	81.4	78.5	79.9	78.3	89.9	85.6	80.4	78.4
<b>Min</b>						<b>76.1</b>			
<b>Max</b>						<b>89.9</b>			

SC: Solvent control; h: hour; Minimum; Max: Maximum.

**Temperature (°C)**

Group	Test Conc. (mg/L)	0 to 24 h		24 to 48 h		48 to 72 h		72 to 96 h	
		Fresh 0 h	Spent 24 h	Fresh 24 h	Spent 48 h	Fresh 48 h	Spent 72 h	Fresh 72 h	Spent 96 h
<b>G1 (SC)</b>	<b>0.0</b>	23.6	23.4	23.0	22.6	23.1	22.9	23.2	22.9
<b>G2</b>	<b>1.00</b>	23.5	23.2	23.5	22.4	22.9	22.3	23.0	22.6
<b>G3</b>	<b>10.0</b>	23.7	23.6	23.6	23.1	22.6	22.4	23.1	22.4
<b>G4</b>	<b>50.0</b>	23.9	23.5	23.2	22.4	22.9	22.6	22.9	22.3
<b>G5</b>	<b>75.0</b>	23.5	23.4	23.7	22.3	22.3	22.4	22.8	22.4
<b>G6</b>	<b>100.0</b>	23.6	23.3	23.2	22.6	22.4	22.3	22.7	22.3
<b>Min</b>						<b>22.3</b>			
<b>Max</b>						<b>23.9</b>			

SC: Solvent control; h: hour; min: Minimum; Max: Maximum.

**APPENDIX 3 (Contd...). PHYSICO-CHEMICAL PARAMETERS OF TEST MEDIA  
DURING RANGE FINDING STUDY**

**pH**

Group	Test Conc. (mg/L)	0 to 24 h		24 to 48 h		48 to 72 h		72 to 96 h	
		Fresh 0 h	Spent 24 h	Fresh 24 h	Spent 48 h	Fresh 48 h	Spent 72 h	Fresh 72 h	Spent 96 h
<b>G1 (SC)</b>	<b>0.0</b>	6.90	6.82	6.99	6.89	6.94	6.82	6.99	6.84
<b>G2</b>	<b>1.00</b>	6.84	6.80	6.94	6.90	6.96	6.88	6.94	6.91
<b>G3</b>	<b>10.0</b>	6.95	6.81	6.93	6.91	6.92	6.80	6.99	6.92
<b>G4</b>	<b>50.0</b>	6.97	6.86	6.81	6.80	6.85	6.81	6.86	6.85
<b>G5</b>	<b>75.0</b>	7.01	6.93	6.92	6.83	6.82	6.79	6.85	6.83
<b>G6</b>	<b>100.0</b>	7.29	6.91	6.99	6.82	6.83	6.78	6.92	6.82
<b>Min</b>		<b>6.78</b>							
<b>Max</b>		<b>7.29</b>							

SC: Solvent control; h: hour; min: Minimum; Max: Maximum.

**Total Hardness (as mg CaCO<sub>3</sub>/L)**

Day	0	1	2	3
Total Hardness	216	226	220	224

**APPENDIX 4. INDIVIDUAL FISH LENGTH DURING LIMIT TEST**

Group	Test Conc. (mg/L)	Fish Length (cm)						Mean	±SD	Cummulative Weight (g)	Loading Rate (g/L)	
		3.3	3.5	3.2	3.4	3.1	3.2					3.5
<b>G1</b> (NC)	<b>0.0</b>	3.3	3.5	3.2	3.4	3.1	3.2	3.5	3.3	0.2	15.2	
<b>G2</b> (SC)	<b>0.0</b>	3.4	3.6	3.4	3.3	3.5	3.4	3.1	3.4	0.2	15.6	
<b>G3</b>	<b>100.0</b>	3.2	3.5	3.7	3.6	3.2	3.0	3.2	3.4	0.3	15.4	<b>0.62</b>
	<b>Min</b>						<b>3.0</b>					
	<b>Max</b>						<b>3.7</b>					

NC: Negative control; SC: Solvent control; ±SD: Standard deviation; g: Gram; min: Minimum; Max: Maximum.

**APPENDIX 5. TEST MEDIUM PREPARATION DURING LIMIT TEST**

For Stock Solution				Test Media Preparation				
Test Item Quantity (mg)	Vol. of Acetone (mL)	Vol. of Dilution Medium (mL)	Volume made up to	Final Conc. (mg/mL)	Vol. of Stock Solution used (mL)	Final Volume of Test Media (L)	Group	Final Test Conc. (mg/L)
7000.2	0.070	699.93	700	10	-	25	G1 (NC)	0.0
-	-	-	-	-	-	25	G2 (SC)	0.0
-	-	-	-	-	250.0	25	G3	100.0

NC: Negative control; SC: Solvent control (2.5 mL of Acetone + 24997.5 mL Reverse osmosis water).

**APPENDIX 6. PHYSICO-CHEMICAL PARAMETERS OF TEST MEDIA  
DURING LIMIT TEST**

**Dissolved Oxygen (%)**

Group	Test Conc. (mg/L)	0 to 24 h		48 h	72 h	96 h
		Fresh 0 h	Spent 24 h	Spent 48 h	Spent 72 h	Spent 96 h
G1 (NC)	0.0	85.6	84.2	82.6	81.8	80.8
G2 (SC)	0.0	84.9	83.1	82.9	81.6	80.4
G3	100.0	83.6	83.2	83.0	82.4	81.1
<b>Min</b>				<b>80.4</b>		
<b>Max</b>				<b>85.6</b>		

h: Hour

**Temperature (°C)**

Group	Test Conc. (mg/L)	0 to 24 h		48 h	72 h	96 h
		Fresh 0 h	Spent 24 h	Spent 48 h	Spent 72 h	Spent 96 h
G1 (NC)	0.0	22.4	22.5	22.8	22.5	22.4
G2 (SC)	0.0	22.6	22.1	22.6	22.9	22.7
G3	100.0	22.1	22.5	22.4	22.6	22.6
<b>Min</b>				<b>22.1</b>		
<b>Max</b>				<b>22.9</b>		

h: Hour

**pH**

Group	Test Conc. (mg/L)	0 to 24 h		48 h	72 h	96 h
		Fresh 0 h	Spent 24 h	Spent 48 h	Spent 72 h	Spent 96 h
G1 (NC)	0.0	7.02	7.00	7.01	6.99	6.95
G2 (SC)	0.0	6.93	6.91	6.89	6.85	6.84
G3	100.0	6.92	6.89	6.84	6.81	6.82
<b>Min</b>				<b>6.81</b>		
<b>Max</b>				<b>7.02</b>		

h: Hour; min: Minimum; Max: Maximum.



**APPENDIX 6 (Contd...). PHYSICO-CHEMICAL PARAMETERS OF TEST MEDIA  
DURING LIMIT TEST**

**Total Hardness (as mg CaCO<sub>3</sub>/L)**

<b>Day</b>	<b>0</b>	<b>1</b>	<b>2</b>	<b>3</b>
Total Hardness	220	-	-	-

-: Not applicable; Limit test was performed under static condition; hence only once hardness has been measured.

## APPENDIX 7. TEST CONCENTRATION ANALYSIS REPORT

**(will be included during finalization)**

## **20. 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*

## ANNEXURE 2. CONTAMINANT ANALYSIS REPORT OF FISH FEED



INSTITUTE FOR ANALYSIS OF DAIRY, FOOD, CULTURES & PHARMA  
 #8, Siddhi Vinayaka Complex, Nagarabhatta 2nd Stage, 2nd Block  
 Near BDA Complex, 90 Feet Ring Road, Bangalore-560072  
 Ph: +91-80-2318 6906 to 10, Cell: +91 9845900842/8152881444  
 Mail: iadfac@gmail.com/ qnaidfac@gmail.com/bdiadfac@gmail.com

### CERTIFICATE OF ANALYSIS

BOOKING NO: 6818  
 CERTIFICATE NO.: 5824/2019 - 2020

<b>NAME OF MANUFACTURER/PARTY:</b>		<b>BIONEEDS INDIA PRIVATE LIMITED</b> Devarahosahally - 562111, Somapura Hobli, NH-4, Nalamangala Taluk, Bangalore Rural BANGALOPRE - 562111 KARNATAKA	
<b>DETAILS OF RAW MATERIAL/FINAL PRODUCTS</b>			
1. Nature of sample	Fish Feed	9. Your Ref. No.	NM
2. Condition of sample	Good	10. Customer order No.	NM
3. Sample package	Zip lock cover	11. sample receipt date	11/03/2019
4. Brand name	Kjuro grow feed	12. start of analysis date	16/03/2019
5. Grade/Variety/Type/Class/Size	500 g	13. completion date	18/03/2019
6. B.No./DoM	FP102/2019, mfg dt: 19/02/2019 Exp dt: 18/02/2020	14. Deviation if any	NE
7. Sampled by	By Customer	15. Attachments	NM
8. Date of sampling	14/03/2019	16. Sampling protocol	N.A

SR	TEST NAME	UNIT	RESULTS	SPECIFICATIONS	METHOD OF TEST
1	Total plate count/g	g	260 cfu	-	APHA 4 <sup>th</sup> Edition
2	Salmonella/25g	g	Not Detected	-	APHA 4 <sup>th</sup> Edition
3	E.coli/25g	g	Absent	-	IS 5487 (I) : 2005
4	Pseudomonas/g	g	< 10 cfu	-	IS 14843 : 2000
5	Yeast & Mould Count/g	g	40 cfu	-	IS 3403 : 2005
6	Arsenic (mg/kg)	mg/kg	Not Detected	-	IADFAC/INC-158
7	Lead (mg/kg)	mg/kg	Not Detected	-	IADFAC/INC-158
8	Cadmium (mg/kg)	mg/kg	Not Detected	-	IADFAC/INC-158
9	Mercury (mg/kg)	mg/kg	Not Detected	-	IADFAC/INC-158
10	Hexachlorocyclohexane & Isomers (mg/kg)	mcg/kg	Not Detected	-	PAM Vol-4 Edition
11	Lindane (mg/kg)	mcg/kg	Not Detected	-	
12	Heptachlor (mg/kg)	mcg/kg	Not Detected	-	
13	Epoxide (mg/kg)	mcg/kg	Not Detected	-	
14	Chlordane (mg/kg)	mcg/kg	Not Detected	-	
15	Aldrin (mg/kg)	mcg/kg	Not Detected	-	
16	Dieldrin (mg/kg)	mcg/kg	Not Detected	-	
17	Endrin (mg/kg)	mcg/kg	Not Detected	-	
18	DDE (mg/kg)	mcg/kg	Not Detected	-	
19	DDT (mg/kg)	mcg/kg	Not Detected	-	
20	DDT (mg/kg)	mcg/kg	Not Detected	-	
21	Endosulfan & Sulphate (mg/kg)	mcg/kg	Not Detected	-	
22	Fenitrothion Malathion (mg/kg)	mcg/kg	Not Detected	-	
23	PCB (mg/kg)	mcg/kg	Not Detected	-	

Remarks	 AUTHORIZED SIGNATORY
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**Note :**

1. The results listed, refer only to the samples analysed & applicable parameters, Endorsement products is neither inferred nor implied.
2. Total liability of our institute is limited to the invoiced amount.
3. The report cannot be reproduced, completely or in part, in any form (including print) without an explicit written permission from IADFAC Lab. P. Ltd
4. Sample drawn and submitted by the party for Analysis unless otherwise stated.
5. Analysed sample destroyed after one month.

*Accepted and released on 22/03/19*

## ANNEXURE 2 (Contd...), CONTAMINANT ANALYSIS REPORT OF FISH FEED



INSTITUTE FOR ANALYSIS OF DAIRY, FOOD, CULTURES & PHARMA  
 #8, Siddhi Vinayaka Complex, Nagarabhavi 2nd Stage, 2nd Block  
 Near BDA Complex, 80feet Ring Road, Bangalore-560072  
 Ph.:+91-80-2318 6906 to 10, Cell: +91 9845900842/8152881444  
 Mail: iadfac@gmail.com/ qmaidfac@gmail.com/bdiadfac@gmail.com

### CERTIFICATE OF ANALYSIS

BOOKING NO: 2356  
 CERTIFICATE NO. : 1958/2016 - 2017

<b>NAME OF MANUFACTURER/PARTY:</b>		<b>BIONEEDS INDIA PRIVATE LIMITED</b> Devirahosahally - 562111, Sampura Hobli, NH-4, Nelamangata Taluk, Bangalore Rural BANGALOPRE - 562111 KARNATAKA	
<b>DETAILS OF RAW MATERIAL/FINAL PRODUCTS</b>			
1. Nature of sample	Fish Feed	9. Your Ref. No.	NM
2. Condition of sample	Good	10. Customer code No.	Nil
3. Sample package	Zip lock cover	11. sample receipt date	11/03/2019
4. Brand name	Kjaro grow feed	12. start of analysis date	15/03/2019
5. Grade/Variety/Type/Class/Size	500 g	13. completion date	19/03/2019
6. B.No./DoM	FF/02/2019 .mg dt: 19/02/2019 Exp dt: 18/02/2020	14. Deviation if any	Nil
7. Sampled by	By Customer	15. Attachments	Nil
8. Date of sampling	14/03/2019	16. Sampling protocol	N.A

SR	TEST NAME	UNIT	RESULTS	SPECIFICATIONS	METHOD OF TEST
24	Ochratoxina	mcg/kg	Not Detected	-	PAM Ver-I Edition
25	Pirimiphos	mcg/kg	Not Detected	-	
26	Chloropyrifos	mcg/kg	Not Detected	-	
27	Aflatoxins B1	mg/kg	Not Detected	-	IADFAC/III-C-188
28	Aflatoxins B2	mg/kg	Not Detected	-	IADFAC/III-C-188
29	Aflatoxins G1	mg/kg	Not Detected	-	IADFAC/III-C-188
30	Aflatoxins G2	mg/kg	Not Detected	-	IADFAC/III-C-188
31	Ochratoxins	mg/kg	Not detected	-	IADFAC-III-C-110
32	Moisture (%)	%	7.00	-	IS 7874 (P-1) 1975
33	Crude protein (%)	%	16.2	-	IS 7874 (P-1) 1975
34	Crude fat (%)	%	1.92	-	IS 7874 (P-1) 1975
35	Calcium (%)	%	0.357	-	IS 7874 (P-8) 1975
36	Phosphorous (%)	%	0.47	-	IS 7874 (P-8) 1975
37	Total ash (%)	%	4.1	-	IS 7874 (P-1) 1975

Remarks

**AUTHORISED SIGNATORY**

**Note :**

1. The results listed, refer only to the samples analysed & applicable parameters, Endorsement products is neither inferred nor implied.
2. Total liability of our institute is limited to the invoiced amount.
3. The report cannot be reproduced, completely or in part, in any form media (including print) without an explicit written permission from IADFAC Lab. P. Ltd.
4. Sample drawn and submitted by the party for Analysis unless otherwise stated.
5. Analysed sample destroyed after one month.

Accepted and Released on 22/03/19  
 14/03/2019

### ANNEXURE 3. 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

**Bioneds 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

## ANNEXURE 3 (Contd...), GLP CERTIFICATE

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
<ul style="list-style-type: none"><li>o Acute Toxicity</li><li>o Sub-acute Toxicity</li><li>o Chronic Toxicity</li><li>o Reproductive and Developmental Toxicity</li><li>o Inhalation Toxicity</li><li>o Local Lymph Node Assay</li><li>o Neurotoxicity</li></ul>
Mutagenicity Studies
<ul style="list-style-type: none"><li>o Bacterial Reverse Mutation Test</li><li>o Chromosome Aberration Test (<i>In-vivo/ In-vitro</i>)</li><li>o Micronucleus Test (<i>In-vivo/ In-vitro</i>)</li><li>o <i>In-vitro</i> Mammalian Cell Gene Mutation Test Using HPRT and XPRT Genes</li></ul>
Environmental Toxicity Studies on Aquatic and Terrestrial Organisms
<ul style="list-style-type: none"><li>o Acute Immobilization Test in Daphnia</li><li>o Acute Fish Toxicity Test</li><li>o Avian Acute Oral and Dietary Toxicity Test</li><li>o Acute Toxicity Study in Earthworms</li><li>o Acute Oral and Contact Toxicity Test in Honeybee</li><li>o Fish-embryo Toxicity Test</li><li>o Earthworm and Daphnia Reproduction Toxicity Test</li><li>o Acute Silkworm Toxicity Test</li><li>o Acute Trichogramma Toxicity Test</li></ul>
Residue Studies
Analytical and Clinical Chemistry Testing
Others
<ul style="list-style-type: none"><li>o Bioanalytical Studies</li><li>o Toxicokinetics Studies</li><li>o Biocompatibility Studies</li><li>o <i>In-vitro</i> 3T3 NRU Photo Toxicity Test</li><li>o Mouse Lymphoma Assay</li><li>o <i>In-vitro</i> Skin Absorption Study</li><li>o Bovine Corneal Opacity and Permeability Test</li><li>o <i>In-vitro</i> Skin Irritation: Reconstructed Human Epidermis Test</li><li>o <i>In-vitro</i> Ocular Irritation Test</li><li>o In Chemico Skin Sensitization: Direct Peptide Reactivity Assay</li><li>o Cytotoxicity Assay</li></ul>

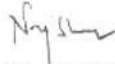
### Types of Chemicals:

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, A48, CHO-K1, L5178Y, A-549, SK-MEL-28, BALB/3T3, HEPG/C, A, 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