A Comparative Study on the Methods of Acute Toxicity, Median Lethal Concentration and Safe Concentration of Terramycin 200 to an Indian Snake-Headed Murrel, *Channa punctatus* (Bloch)

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Abstract

The acute toxicity, median lethal concentration and safe concentration of Terramycin 200 in an Indian snake-headed Murrel, *Channa punctatus* (Bloch) of body weight 40-50g were evaluated applying various methods. Lorke and Enegide*et al.*,estimated the 96hr-LC_{50} value to be 2711.09 and 2800.0 mg/L. The Up-and-Down method yielded a 96hr-LC_{50} range of 2216.67 to 2683.33mg/L as a rough estimate. Furthermore, the Behren-Karber, Miller and Tainter and Finney methods had 96hr-LC_{50} values of 2450.0, 2390.0, and 2511.88mg/L, respectively. Finally, the Reed-Muench method was used to calculate the 96hr-LC_{50} dose as well as the median ideal dose of 3129.68mg/L. The cumulative median lethal average of 96hr-LC_{50} dose in *Channa punctatus* was 2450.0+2390.0+2511.88+3129.68 = 2620.39 mg/L of Terramycin 200. Among the various methods used, the Chinedu method was found to be the most accurate. There was a significant relationship between the dose of Terramycin 200 and fish mortality. Terramycin 200 accumulates in aquatic bodies, disrupting the physiological activities of this fish, according to the findings. As a result, caution should be exercised when using high concentrations of Terramycin to treat *Channa punctatus*.

It can be deduced that this fish is more resistant to Terramycin 200 toxicity than other fishes. The calculated safety level value indicated that determining an acceptable dose of Terramycin 200 for this fish is feasible. Terramycin 200 may even be classified as a slightly/moderately cytotoxic substance to *Channa punctatus* based on its toxicity range.

Keywords: Ecotoxicity, Median Lethal Concentration, Terramycin 200, *Channa punctatus.*

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Introduction

To meet the world's demand for fish, intensive culture has been introduced. In this context, many antibiotics are used. Antibiotics appear to be necessary for preventing or treating fish diseases, as well as promoting fish growth and health (Romero *et al.*, 2012). Most fish farmers use Terramycin, Chloromycetin, and Erythromycin. Terramycin is used in pisciculture to treat bacterial infections in fish as well as for other purposes (Kreutzmann, 1977; Barros-Becker *et al.,* 2012). However, serious concerns have been raised about the use of antibiotics due to their negative effects on aquatic ecosystems. Fish, on the other hand, are subjected to stressful conditions during fish farm management and stress is a reaction to any situation that alters their normal metabolism, resulting in changes in the physiological state and biochemical alterations.

Acute toxicity is defined as an adverse effect that occurs within 24 hours of a single or multiple toxicant administrations (Saganuwan, 2016). The median lethal concentration (LC_{50}) has been used as an important parameter to measure acute toxicity and as an initial screening procedure to determine the toxicity of a substance. It appears that using software to improve traditional methods is a current requirement (Ramakrishnan, 2016; Erhirhie*et al.,* 2018)

Channa punctatus (Bloch), a freshwater Indian Snake-headed Murrel, is one of the most important fish species. In many Indian university laboratories, it is used as a model for dealing with the alimentary canal of a teleost and a test animal (Mishra and Behera, 2019). The effect of Terramycin 200, on the other hand, has not been studied in Indian air-breathing fishes (Pandit and Kumari 2020; Choudhary, 2022).

As a result, the current study sought to ascertain the median lethal dose of Terramycin 200 on *Channa punctatus* applying various methods. The study will help determine the toxicity level of Terramycin 200 for the increased yield of this fish. The research will also be useful in determining differences in fish health caused by Terramycin 200 toxicity, the suitability of environmental conditions for fish, the relative sensitivity of fish to Terramycin 200, and the physicochemical conditions of water bodies.

However, there is no evidence that Terramycin 200 affects Indian air-breathing fishes (Choudhary, 2022). As a result, *Channa punctatus* (Bloch), a common Indian air-breathing fish, was chosen to study its acute toxicity and safe level of Terramyicn 200.

Materials and Methods

The investigation was carried out in a controlled laboratory setting by the ethics of the Department and VKS University, Arrah, using a static renewable method.

During March-April 2023, live healthy specimens of *Channa punctatus* (Bloch) (BW: 40.0-50.0g) were purchased from Arrah (Bhojpur), Bihar's local market. Before being transferred to large aquaria, the fish were disinfected with dilute $KMnO_4$. The fish were fed goat liver and fish food purchased from the local market. Following APHA (2022), they were acclimatised for a fortnight at VKS University Departmental Laboratory in Arrah.

In this experiment, Terramycin 200 for fish (Syndel, USA) was used. It is a bacterial disease treatment premix for aquaculture. The dose schedule chosen was based on preliminary research with a variety of doses as well as a previous report of Chloramphenicol in fish (Kasagala and Pathiratne, 2008). Twenty specimens were fed Terramycin 200 (0, 400, 1000, 2000, 4000, 8000, and 16000 mg/L) and another set of controls at random. Lockwood (1976) defined death as total immobility with no abdominal flexion upon forced extensions.

The following methods were used to calculate the LC_{50} dose, from estimation to confirmation:

1. Median lethal dose method (Lorke, 1983; Enegide *et al.*, 2013)
2. Up-and-Down (Staircase) method (Dixon and Mood, 1948; Bruce, 1985).
3. Behren-Karber method (Behren, 1929; Karber, 1931)
4. Regression Analysis method
5. Finney (1971) probit analysis method
6. Reed-Muench (1938) method and
7. Chinedu *et al.,* (2013) method

Terramycin 200's toxicity was classified using a standard table (Loomis, 1996). Various methods were used to calculate the safe levels of Terramycin 200 (CCREM, 1991; CWQC, 1972; IJC, 1977; Hart and Weston, 1948; NAS/NAE, 1972 and 1973; Sprague, 1971).
For statistical analysis, Graph Pad Prism 5 software was used. The data was input into a Microsoft Excel spreadsheet. All entries were double-checked for potential typing errors. The observations were expressed as a mean for each group. The information was then used in group analysis and linear regression.

Results and Discussion

Physico-chemical characteristics of water: The physicochemical characteristics of test water having a temperature of 24.0±2.0^{0}C, pH: 7.32±0.18, dissolved oxygen: 6.4±0.8 mg/L, total alkalinity: 65.0±6.45 mg/L, hardness: 150.6±5.2 mg/L and chloride: 16.7±0.2 mg/L was recorded during the experimental period. The observed parameters are within the range of favourable growth performances (Boyd, 1981). Physiological parameters such as antibiotic concentration and formulation, as well as exposure, have a significant impact on such studies (Gupta *et al.*,1981).

Ethological observations: Different doses of Terramycin 200 resulted in uncoordinated behaviour in the fish. In response to sudden changes in the surrounding environment, fish were alert, stopped swimming, and remained static in position during preliminary exposure. After a while, they tried to avoid the toxic water by swimming and jumping quickly. Because of the stretching of their body muscles, their fins became tough and stretched. They secreted massive amounts of mucus from their entire body constantly and a thick layer of mucus was soon discovered deposited inside the buccal cavity and gills. The pigmentation of the body increased. Finally, the fish exhibited vertical posture for a few minutes, with its mouth close to the surface of the water attempting to gulp air and its tail pointing downward. They soon settled at the bottom of the tank, and after a while, their bellies grew upward and the fish died, while the opercula remained wide open, exposing the gills.
Stress-induced behavioural changes are also recognised as the most sensitive indicator of potential toxic effects. The numerous behavioural changes observed in *Channa punctatus*, such as restlessness, abnormal swimming behaviour, vigorous jerks of the body, loss of balance, myotonia and anorexia, are similar to previous observations in fish exposed to various toxicants (Hussein *et al.*, 1996; Nwani*et al.*, 2013).

96hr-LC50 dose determination: LC_{50} values recorded in this study were attributed to the size of fish with potentially immune systems for biotransformation of Terramycin 200 from the body.

(1) Lorke (1983) and Enegide *et al.*, (2013) method of median lethal dose: The procedure is divided into two stages.

(A) Three groups of three fishes were formed from the nine fishes. Each group of fish received a different dose of Terramycin 200 (700, 1400, and 2100 mg/L). The fish are kept under observation for 96 hours to see how they behave and if they will die.

(B) Three fish are used in this stage, which is divided into three groups of one fish each. The fish are given higher doses of Terramycin 200 (3500, 7000, and 14000 mg/kg) and then observed for 96 hours for behaviour and mortality.

Therefore, 96hr-LC50 = $\sqrt{C_0\ x\ C_{100}}$ = $\sqrt{2100\ x\ 3500}$ = 2711.09mg/LTerramycin 200 (Table 1). Where, C_0 = the maximum concentration with no mortality of fish and C_{100} = the minimum concentration with total mortality of fish.

Furthermore, Enegide *et al.*, (2013) median lethal dose method was used to calculate 96hr-LC_{50} dose = $\frac{M_0 + M_1}{2}$ = $\frac{2100+3500}{2}$ = 2800.0mg/L (Table 1). Where, M_0 = maximum concentration with no mortality of fish and M_1 = minimum concentration with total mortality of fish.

(2) Dixon-Mood (1948) Up-and-Down (Staircase) Method: The Terramycin

200 is dosed sequentially to single fish in this method. Following the administration of the first dose, the next dose is determined by the outcome of the subsequent dose. If the fish survives the next dose, the dose is adjusted upward; if mortality is recorded at the next dose, the dose is adjusted downward. A constant factor is used to adjust the dose either upward or downward. Testing is stopped when the upper limit (2000-5000 mg/L) is reached without mortality or when the LD_{50} is determined from the test. It gives an approximation of the MLC_{50} dose.

If a fish survives, a constant factor is added to the dose for the next fish and vice versa. It estimates the median lethal dose (MLC_{50}) in 6 fish over 96 hours using arithmetic, geometric, and harmonic means. Using this method, MLC50 was calculated to be 2450.0mg/L Terramycin 200 (Table 2). When no mortality is observed, even at concentrations of 2000-5000 mg/L, the method is abandoned (Bruce, 1985; OECD, 2013).

(3) Behren (1929)-Karber (1931) method: It is a non-parametric method requiring at least partial mortality, but the data does not fit the probit model. This method used equal spacing between dose intervals and an equal number of fish at each dose level for observations ranging from 0% to 100%. Many fish were sacrificed because the calculated dose did not kill a single fish. For the first four hours, 24 hours, and daily for 14 days, the fish were doused with the test substance and monitored for signs of toxicity. Using this method, the 96hr-LC_{50} dose of Terramycin 200 was calculated to be 2450.0 mg/L (Table 3).

(4) Regression analysis method:It is used when there is no partial mortality. The Terramycin 200 doses were converted into a mortality rate. This method resulted in the death of many fish. Terramycin 200 24, 48, 72, and 96hr-LC_{50} doses calculated using this method were 4944.62, 3357.00, 2797.50, and 2390.00 mg/L, respectively (Table 4). Terramycin 200 dosage was gradually reduced during treatment.

(5) Finney's (1971) probit analysis method: After calculating percent mortality, it is a parametric maximum likelihood method that calculates net/corrected percent mortality from 10% to 100%. Then, based on the straight line obtained in the graph, values of empirical probit ranging from 3.72 to 8.72 (Table-5) were noted from the Fischer and Yates table. Using empirical probit values ranging from 4.40 to 6.80, the expected/provisional probit was calculated. The working probit (from 3.81 to 7.45) and weighing coefficient (from 0.248 to 0.547) values were calculated to determine the mean and deviation of the Terramycin 200 doses and mortality. Finally, Terramycin 200's median lethal concentration was calculated to be 96hr-LC_{50} = Antilog 3.40 = 2511.88 mg/L. Because the calculated value of χ^2 is less than the tabulated value, the data is homogeneous, indicating that the 'eye fit' line is in good agreement.

The 96hr-LC50 calculated using the Behren (1929)-Karber (1931) method, the regression analysis method, and the Finney probit analysis method are nearly identical. These are the most common methods for determining 96hr-LC_{50} used by workers. The Behren-Karber method, on the other hand, can be used to calculate the 24, 48, 72, and 96hr-LC50 doses from the same set of experiments

(6) Reed-Muench's (1938) method: If the log of the dose is not spaced symmetrically around log LC_{50}, they cause a bias in LC_{50} estimation. This study requires experimentation with 10 fish per dose for a better correlation. Furthermore, the smallest test dose must kill one fish, and only four test doses should be used. Finally, the method was tweaked by calculating the percentage of test animals that lived and died (Saganuwan, 2011). The 95% confidence level, however, cannot be calculated using this method.

According to Reed-Muench's (1938) method, the median lethal dose of Terramycin 200 is 3129.68 mg/L. It demonstrated the arithmetic mean precision, validity and dependability as a rough estimate of LD_{50}. The geometric mean is used in toxicological studies for exponential data. In situations where an arithmetic mean would be incorrect, such as rates and ratios, the harmonic mean is used (Dawson and Trapp, 2004; Saganuwan, 2015). As a result, for a rough estimate of LD_{50} dose, arithmetic or harmonic mean can be used instead of geometric mean.

Table 1 Lorke's (1983) method for LC_{50} dose (mg/L) of Terramycin 200 in *Channa punctatus*

S. No.	Dose of Terramycin 200	Log dose of Terramycin 200	96hr % mortality (n= 3 in each group)	S. No.	Dose of Terramycin 200	Log dose of Terramycin 200	96hr % mortality (n = 1 in each group)
1.	700	2.845	0	1.	14000	4.146	1
2.	1400	3.146	0	2.	7000	3.845	1
3.	2100	3.322	0	3.	3500	1.875	1

Lorke's (1983) method: 96hr-LC_{50} = = $\sqrt{C_0 \ x \ C_{100}}$ = $\sqrt{2100 \ x \ 3500}$ = 2711.09 mg/L-Terramycin 200.

Enegide et al, (2013) method: 96hr-LC_{50} = $\frac{M_0 + M_1}{2}$ = $\frac{2100+3500}{2}$ = 2800 mg/L-Terramycin 200.

Table 2 Up-and-Down method (Dixon-Mood, 1948; Bruce, 1985) for determination of LC_{50} dose (mg/L) of Terramycin 200 in *Channa punctatus*

Number	Dose (mg/L)	Survival/Mortality	Number	Dose (mg/L)	Survival/Mortality
1st	1400	Survival	1st	4200	Mortality
2nd	2100	,,.	2nd	3500	,,
3rd	2800	Mortality	3rd	2800	..
4th	2100	Survival	4th	2100	Survival
5th	2800	Mortality	5th	1400	,,
6th	2100	,,	6th	2100	,,
Arithmetic Mean	2216.67	LC_{50}	Arithmetic Mean	2683.33	LC_{50}
Geometric Mean	2160.31	LC_{50}	Geometric Mean	2516.75	LC_{50}
Harmonic Mean	2100.00	LC_{50}	Harmonic Mean	2355.14	LC_{50}

Mean LC_{50} = $\frac{2216.67+2683.33}{2}$ = 2450 mg/L-Terramycin 200.

Table 3 Behren-Karber method for 96hr-LC_{50} determination of Terramycin 200 in *Channa punctatus*

Group	Dose g/L)of erramy-cin 200	Difference between two consecutive dose (A)	No. of fish exposed	Mortality				Overall mortality at 96hr	Mean mortality between two consecutive dose (B)	A x B
				24hr	48hr	72hr	96hr			
1	0	0	20	0	0	0	0	0	0	0
2	1400	700	20	0	0	0	2	2	1	700
3	2100	700	20	0	2	4	7	7	4.5	3150
4	2800	700	20	2	7	10	13	13	10.0	7000
5	3500	700	20	6	13	16	18	18	15.5	10850
6	4200	700	20	12	16	18	20	20	19	13300
			N = 20							35000

96hrLC50 = LC100 -$\frac{\Sigma AB}{N}$

= 4200 – $\frac{35000}{20}$ = 4200 – 1750 = 2450 mg/L

Table 4 Statistical relationship between dose (mg/L) of Terramycin 200 and mortality of *Channa punctatus* (Miller and Tainter)

Sl. No.	Exposure period (hours)	Regression equation y=bx+a (% mortality)	Lethal Concentration (mg/L)		Toxicity Factor	t value (df=4)	F value (u = 1; v = 4)
1	24	y= 0.013x – 14.28 ($R^2 = 0.685$)	LC_{10}	1867.69	1.000	2.952 (p<0.05)	14.12 (p<0.05)
			LC_{50}	4944.62			
			LC_{90}	8021.54			
2	48	y = 0.020x – 17.14 ($R^2 = 0.843$)	LC_{10}	1357.00	1.477	4.636 (p<0.01)	45.00 (p<0.01)
			LC_{50}	3357.00			
			LC_{90}	5357.00			
3	72	y = 0.024x – 17.14 ($R^2 = 0.879$)	LC_{10}	1130.83	1.664	5.385 (p<0.01)	49.59 (p<0.01)
			LC_{50}	2797.50			
			LC_{90}	4464.17			
4	96	y = 0.026x – 12.14 ($R^2 = 0.937$)	LC_{10}	851.54	2.108	7.713 (p<0.01)	158.8 (p<0.01)
			LC_{50}	2390.00			
			LC_{90}	3928.46			

Table 5 Probit analysis for toxicity of Terramycin 200 in *Channa punctatus*

Dose (mg/L) of Terramycin 200	Log dose (mg/L) of Terramycin 200	Number of fish exposed	Mortality of fish	% of mortality of fish	Net/corrected mortality of fish	Empirical probit	Expected/Provisional probit	Working probit	Weighing Coefficient	nw	nwx	nwy	nwx^2	nwy^2	nwxy
	x	n		p			Y	y	w						
0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0
1400	3.146	20	2	10	10	3.72	4.40	3.81	0.246	4.92	15.45	18.75	48.60	71.44	58.97
2100	3.322	20	7	35	35	4.51	5.60	4.82	0.565	10.30	37.54	54.47	124.70	262.53	180.94
2800	3.447	20	13	65	65	5.45	6.20	5.46	0.628	12.56	43.29	68.58	149.23	374.43	236.39
3500	3.544	20	18	90	90	6.28	6.40	6.12	0.544	10.88	38.56	66.59	136.65	407.50	235.98
4200	3.623	20	20	100	100	8.72	6.80	7.45	0.507	10.14	36.74	75.54	133.10	562.79	273.69
										49.80	171.58	283.93	592.28	1678.94	985.97

Following calculations made from the table:
(a) $\bar{x}$ = 3.45, (b) $\bar{y}$ = 5.70, (c) S_{xx}= 1.12, (d) S_{yy}= ., (e) S_{xy}= 0.99, (f) χ^2= 5.14 (tabulated value 16.266 at p=0.001 and df = 4), (g) b = 0.88 and (h) S = 17.27.
96hr-LC_{50} = Antilog 3.4 = 2511.88mg/L (0.8511ppm) of Terramycin 200.
96hr-LC_{95} = Antilog 4.546 = 31622.77mg/L of Terramycin 200.
96hr-LC_{84} = Antilog 3.767 = 5847.90mg/L of Terramycin 200.
96hr-LC_{16} = Antilog 1.897 = 77.80mg/L of Terramycin 200.

95% confidence limit (fudicial limit) = $S^{\frac{2.77}{\sqrt{20}}}$ = 10.02

Table 6 Reed-Muench (1938) method for 96hr-LC_{50} determination of Terramycin 200 in *Channa punctatus*

Sl. No.	Dose (mg/L)	Log dose (mg/L)	Experiment		Specific Cumulative			Rate of mortality	% mortality	% survival
			No of mortality	No. of survival	Mortality	Survival	Total			
1	2100	3.322	2	18	2	40	42	$\frac{2}{42}$	4.76	95.24
2	2800	3.447	7	13	9	22	31	$\frac{9}{31}$	29.03	70.97
3	3500	3.544	13	7	22	9	31	$\frac{22}{31}$	70.97	29.03
4	4200	3.623	18	2	30	2	32	$\frac{30}{32}$	93.75	6.25

Calculation of median lethal concentration (MLC_{50})	Calculation of median survival concentration (MSC_{50})
$\frac{50.0-29.03}{70.97-29.03} = \frac{20.97}{41.94} = 0.50$	$\frac{50.0-29.03}{70.97-29.03} = \frac{20.97}{41.94} = 0.50$
3.544 – 3.447 = 0.097	3.544 – 3.447 = 0.097
0.50 x 0.097 = 0.0485	0.50 x 0.097 = 0.0485
3.447 + 0.0485= 3.4955	3.447 + 0.0485= 3.4955
Antilog of 3.4955 = 3129.68	Antilog of 3.4955 = 3129.68
MLC_{50} = 3129.68 mg/L	**MSC_{50} = 3129.68 mg/L**

Table 7 Recommended doses for the proposed method (Chinedu *et. al.* 2013)

Sagess	Recommended doses (mg/L)			
	Group 1	Group 2	Group 3	Group 4
1.	10	100	300	600
	50	200	400	800
2.	1000	1500	2000	-
3.	3000	4000	5000	-

Table 8 Comparison of different methods

Sl. No.	Parameters	Lorke's method	Up and Down method	Karber's method	Chinedu method
1.	Duration	Less	May take more time	Less	Less
2.	Expenditure	Moderate	Less	High	Less
3.	No. of fishes	Few	Few	Much	Few
4.	Nature of experiment	Simple	Simple	Complicated	Simple
5.	Accuracy of results	Uncertain	Accurate	Uncertain	Accurate

Table 9 Classification of substances/toxicants on the basis of toxicity range

Sl. No.	Toxicity range (mg/L)	Toxicant Classification (Loomis and Hayes, 1996)	Sl No.	Toxicity range (mg/L)	Toxicant Classification EPA,1970, FIFRA, 1947 and Globally Harmonized System of Classification and Labeling of Chemicals (GHS, 1992)
1.	<5	Extremely Toxic	1.	<50	Most Toxic
2.	5-50	Highly Toxic	2.	>50 and <500	Highly Toxic
3.	50-500	Moderately Toxic	3.	<5000	Moderately Toxic
4.	500-5000	Slightly Toxic	4.	>5000	Least Toxic
5.	5000-15000	Practically Non-toxic		-	
6.	>15000	Relatively Harmless		-	

Table 10 Estimation of safe concentrations (mg/L) of Terramycin 200 at 48/96hr exposure of *Channa punctatus*

Sl. No.	Method	Dose of Terramycin 200 (mg/L)	Accumulation Factor	Safe level (mg/L) of Terramycin 200
1.	CCREM (1991)	96hr-LC50= 2390.00	0.05	2390.00x 0.05 = 119.5
2.	CWQC (1972)	48hr-LC50= 3357.00	0.01	3357.00x 0.01 = 335.7
3.	Hart et al, (1948)	96hr-LC50= 2390.00	$0.03 \text{ x}\left(\frac{24\text{hr}-\text{LC50}}{48\text{hr}-\text{LC50}}\right)^2$	$2390.00\text{x } 0.03 \text{ x } \left(\frac{4947.62}{3357.00}\right)^2 = 155.47$
4.	IJJ (1977)	96hr-LC50= 2390.00	0.05	2390.00x 0.05 = 119.5
5.	NAS/NAE (1973)	96hr-LC50= 2390.00	0.00001 to 0.1	2390.00x 0.01 = 0.0239to239.0
6.	Sprague (1971)	96hr-LC50= 2390.00	0.1	2390.00x 0.01 = 239.0

(7) Chinedu *et al.,* (2013) method: This method is divided into three stages. The results of each stage determine the next step. The fish should be observed for 1 hour after administration, followed by 10 minutes every 2 hours for the next 24 hours. Toxic behavioural signs and mortality should be recorded (Table 7).

Stage 1 or Initial Stage: Four fish are required. These fish were divided into four groups, each with one fish. The different fishes were then given different doses of Terramycin 200. No mortality is recorded at this stage, we move on to stage 2.

Stage 2: Three fish are involved in this stage, which is divided into three groups of one fish each. Different Terramycin 200 doses were higher than those used in stage 1. Because there was no death, the testing was moved to stage 3.

Stage 3:Three fish are also required for this stage, which is divided into three groups of one fish each. The different fishes received Terramycin 200 doses ranging from 3000 to 5000 mg/L. At each dose, mortality was recorded. Where no mortality is recorded at this stage, the Terramycin 200 LC_{50} is said to be greater than 5000 mg/kg, indicating a high level of safety.

Confirmatory (or confidence) test

If mortality was recorded at a given dose in any of the stages, a confirmatory test should be performed to ensure that the Terramycin 200 was the cause of the death.

This test simply involves giving two animals the dose of Terramycin 200 that caused death (or the lowest dose that caused death in a situation where more than one death was recorded). Then, for the next 24 hours, observe for 1 hour after administration and 10 minutes every 2 hours. If at least one of the two animals dies, this should serve as confirmation and validation of the test result.

The confirmatory test can also be performed on a substance whose LD_{50} has been clearly stated in the literature.

Calculation

After the test procedure, the formula that should be employed in the calculation of the LD_{50} is shown below:

$LC50 = \frac{[2000+3000]}{2} = 2500$mg/L of Terramycin 200

Where M_0 = Highest dose of Terramycin 200 that gave no mortality,

M_1 = Lowest dose of Terramycin 200 that gave mortality.

Any of the two-dose ranges in stage 1 (which are 10, 100, 300, and 600 or 50, 200, 400, and 800) may be used to test acute toxicity using this method. The doses for stages 2 and 3 are documented in Table 7. To ensure the accuracy of the results, a wide range of doses has been recommended for this method. This method has some advantages over other methods, which are documented in 8. This includes the fact that it is accurate, only involves a few fish, the cost is moderate, and the process is simple and takes less time.

In different fishes, the 96hr-LC_{50} dose of Terramycin 500 ranged from 62.5-100.0mg/l (Brain *et al.,* 2004; Ankley *et al.,* 2007). Because of its >1 quotient, Terramycin 500 causes environmental intoxication risk at both the lowest (50.0 mg/kg) and highest predicted environmental concentration (1750 mg/kg), according to Carraschi *et al.,* (2011). This account suggests that differences in LC_{50} values reflect differences in the sensitivity of different fish species to different types of toxicant exposures. It appears to be influenced by the type and age of fish used in the toxicity tests. But, according to Pandit and Kumari (2020), the average 96hr-LC_{50}Terramycin 500 dose for *Clarias batrachus* is 663.8 mg/L. Therefore, the current study supports the findings of Wheeler (1969) and Pandit and Kumari (2020).

There was a significant relationship between the dose of Terramycin 200 and fish mortality (Table 4). According to these findings, *Channa punctatus* is more resistant to Terramycin 200 toxicity than most other fishes. The findings are consistent with previous research. The LC_{50} dose difference between species is determined by the body weight, age, sex, and feeding conditions of the test fishes, as well as ambient conditions, water temperature, and regional variations. The toxicity of Terramycin 200 is also influenced by its shape, size, surface area, surface charges, and chemical composition.

A comparison of the Lorke, Up and Down, Karber, and Chinedu *et al.*, methods revealed that the Chinedu *et al.*, method is superior in terms of duration, expenditure, number of fish required, and nature of results (Table 8).

The cumulative median lethal average 96hr-LC_{50} dose for Terramycin 200 in *Channa punctatus*was calculated using the Behren (1929)-Karber (1931) method, the Regression analysis method, the Finney (1971) probit analysis method, and the Reed-Muench (1938) method is 2450.0+2390.0+2511.88+3129.68 = 2620.39 mg/L). Terramycin 200 is slightly/moderately toxic to *Channa punctatus*, according to the value (Table 9).

Safe levels, according to reports are included to account for uncertainties in data and evaluation processes. In the absence of acute toxicity data, the safe level is also employed. In *Channa punctatus*, the Terramycin 200 safety level is 239.0 mg/L (Table 10). Furthermore, Terramycin 200 has a safe level of 23.9 mg/L in rats and 2.39 mg/L in humans (https://en.wikipedia.org/wiki/Toxicity).

Conclusion

Determining the LC_{50} dose is an important step in ecotoxicological research. Use the Lorke and Enegide*et al.*, method first, followed by the Up-and-Down method for range finding and a rough estimate of the LC_{50} dose for better results. Following that, the LC_{50} dose should be calculated using the Behren-Karber or Regression Analysis method, as well as the Finney probit method. Finally, using various cross-checks, Chinedu *et al.*,should be used to confirm the LC_{50} dose value. The current study indicates that determining the acceptable concentration of Terramycin 200 in *Channa punctatus* is difficult based on the range of safe levels. However, Terramycin 200 may be classified as slightly/moderately toxic to *Channa punctatus* based on the toxicity range.

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