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Evaluation of ideal median lethal concentration (LC_{50}) of a biofertilizer in an Indian air-breathing fish, *Channa gachua* (Ham.)

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ABSTRACT

This is first report on estimation, determination, confirmation, toxicity status and safe level of a biofertilizer (mustard oil cake) in *Channa gachua* (Ham.). The work was conducted during 2018 by the use of static renewal method. In this work, 24hr-LC_{50} value from Lorke and Enegide *et al.* method was estimated 14142.16 and 15000.0 mg/L respectively. A range of 96hr-LC_{50} value of 4258.3 to 4600.0mg/L was calculated from Up-and-Down method and used as to find and rough estimation of LC_{50} dose. After that Behren-Karber or regression analysis method and Finney probit method used to determine the 96hr-LC_{50} value and the respective value was found 4900.0, 5344.0 and 5012.0mg/L. Finally, Reed-Muench method used to confirm 96hr-LC_{50} dose by various cross checks and median ideal lethal dose was found 4933.0mg/L. Range of tabulated safe level indicates that it is difficult to decide the acceptable concentration of mustard oil cake in *Channa gachua* based on the present study. However, on the basis of standard toxicity range, mustard oil cake may be treated as a substance of slightly toxic or practically non-toxic to *Channa gachua.* The work will help in deciding the optimum does of a biofertilizer as well as its safe level and toxicity status for higher yield of this fish.

Keywords: LC_{50}, Biofertilizer, *Channa gachua*, various methods.

INTRODUCTION

Biofertilizers contain microorganisms that are able to convert macronutrients from unavailable to available form, during biological processes and affect the development of root system and better seed germination (Rajendran and Devaraj 2004). Mustard oil cake is one of the randomly used biofertilizers in pisciculture in India (Nath *et al.* 2018).

Acute toxicity is defined as the adverse effect occurring after administration of a toxicant within 24 hours (Saganuwan, 2016). The assessment of the median lethal concentration (LC_{50}) has been used as a major parameter in measuring acute toxicity and also as an initial procedure to screen toxicity of a toxicant. The term LC_{50} was introduced by Trevan (1927) to estimate the concentration of a substance that produces 50% death in test animals. Acute toxicity study gives information about LC_{50}, therapeutic index, degree of safety of a toxicant and toxicity status of the test substance (Akhila *et al.* 2007; Arwa and Vladimir 2016).

Various methods such as Arithmetical method (Karber 1931; Reed and Muench 1938), Graphical method, Lorke (1983) method and up and down method (OECD 2013) are used in determination of LC_{50}. Improvement of the conventional methods through application of software is the issue of the present day (Ramakrishnan 2016; Gertrude-Emilia 2017; Erhirhie *et al.* 2018). Although, sometimes software used may not provide 95% confidence limit.

Channa gachua (Dwarf Snakehead) is a hardy fish inhabiting in paddy fields and ponds in India and Shahabad region of Bihar. The IUCN status of this fish indicates that its number is decreasing (Chaudhry 2010).

Therefore, the aim of this work is to use certain methods to test acute toxicity of a biofertilizer in *Channa gachua*, which if adopted, should produce more accurate and reproducible results using few fishes. The work will help in deciding the optimum does of a biofertilizer as well as its safe level and toxicity status for higher yield of this fish.

MATERIALS AND METHODS

Fresh and healthy specimens of *Channa gachua* (Hamilton 1822) (55-75g body weight, 13-16cm total length) were obtained from local market, Arrah (Bhojpur), Bihar during 2018. They were acclimatized for a fortnight in Departmental Laboratory of VKS University, Arrah.

Fishes were randomly selected from the stock and divided into two groups. One group was taken to control or 'C' and other as treated or 'T'. Powder of mustard (*Brassica napus*) oil cake was selected as a biofertilizer for the experiment.

The investigation was performed using static renewable method in controlled laboratory conditions following ethics of the Department and University. Temperature (30.0±1.0), pH (8.24±0.4), dissolved oxygen (3.47±0.69mg/L), total alkalinity (325.00± 8.57mg/L) and total hardness (184.66±3.63mg/L) were recorded daily at exposure times of 24, 48, 72 and 96 hours following APHA (2009). Following methods were used to calculate LC_{50} dose from its estimation to confirmation:

(1) Lorke's (1983) and Enegide *et al.* (2013) median lethal dose method

(2) Up-and-Down (Staircase) method (Dixon-Mood 1948; Bruce 1985)

(3) Behren-Karber (1931) method

(4) Regression Analysis method

(5) Finney (1971) probit analysis method and

(6) Reed-Muench (1938) method.

Loomis and Hayes (1996) table was followed to categories toxicity nature of mustard oil cake.

The safe level estimation of the mustard oil cake was calculated following the methods of CCREM (1991), CWQC (1972), IJC (1977), Hart *et al.* (1948), NAS/NAE (1973) and Sprague (1971).

Statistical analysis was done with Graph Pad Prism 5 software.

RESULTS AND DISCUSSION

(1) Lorke's (1983) median lethal dose method: The method is divided into following two phases. The behaviour and the mortality of fishes were observed for 24 hours in each phase.

(A) Three fishes each in three groups were given 100, 1000 and 10000mg/L mustard oil cake.

(B) One fish each in three groups were given 15000, 20000 and 25000mg/L mustard oil cake.

24hr-LC_{50} = $\sqrt{C_0 \text{ x } C_{100}} = \sqrt{10000 \text{ x } 20000}$ = 14142.16mg/L mustard oil cake (Table 1). Where, C_0 = maximum concentration with no mortality and C_{100} = minimum concentration with complete mortality.

Further, Enegide *et al.* (2013) median lethal dose method was used as confirmatory test to determine 24hr-LC_{50} dose = $\frac{M_0 + M_1}{2} = \frac{10000+20000}{2}$ = 15000.0mg/L mustard oil cake (Table 1). Where, M_0 = maximum concentration with no mortality and M_1 = minimum concentration with complete mortality. These values indicate relatively harmless nature of mustard oil cake for *Channa gachua* (Table 7). Although, less number of fishes was sacrificed in this method, but the accuracy and reliability of these methods is not good due to observation for 24hours only.

(2) Up-and-Down (Staircase) Method (Dixon-Mood 1948; Bruce 1985):

It is based on the reduction in the number of fishes. In this method, if fish survives, the dose for the next is increased by a constant factor and vice-versa. It applies arithmetic, geometric and harmonic mean for estimation of median lethal dose (MLC_{50}) using 6 fishes for 24-96 hours. In this method, log of standard deviation was used for its default value progression. The formula $\frac{\bar{x}}{SE}$ = a and $\frac{a}{\log of\ SD}$ was used to calculate confidence interval or upper and lower boundary of standard error of mean. By this method, MLC_{50} was calculated $\frac{4600.0+4258.33}{2}$ = 4429.165mg/L of mustard oil cake (Table 2). The value indicates slightly toxic nature of mustard oil cake for *Channa gachua* (Table 7). The method is terminated when no mortality is observed even at 2000-5000 mg/L (Bruce 1985; OECD 2013). It gives rough estimation of MLC_{50} dose.

(3) Behren-Karber (1931) method:

It is non-parametric method that requires at least one partial mortality but data do not fit in probit model. In this method, equal spacing of interval of log dose and equal number of fishes at each dose level was applied for observation from 0 to 100%. Many fishes were sacrificed because the dose calculated was not killed a single fish. The fishes were dosed with the test substance and observed for the first four hours, 24 hours and daily for 14-days for signs of toxicity. By this method, 96hr-LC_{50} of mustard oil cake was calculated 4900.0 mg/L (Table 3). The value indicates slightly toxic nature of mustard oil cake for *Channa gachua* (Table 7).

(4) Miller and Tainter (1944) graphical/regression analysis method:

It is applied when there is no partial mortality. The dose of mustard oil cake was converted into log dose and percent mortality as probit mortality. In this method, many fishes were sacrificed. By this method, 24, 48, 72 and 96hr-LC_{50} of mustard oil cake was calculated 17222.0, 9478.23, 5803.61 and 4900.0 mg/L respectively (Table 4). These values indicate slightly toxic, practically non-toxic to relatively harmless nature of mustard oil cake for *Channa gachua* (Table 7).

(5) Finney (1971) probit analysis maximum likelihood method:

It is a parametric method in which after calculating percent mortalities, net/corrected percent mortalities from 25% to 100% were calculated. Then, values of empirical probit from 4.33 to 8.22 (Table-5) were noted from Fischer and Yates's table depending upon the straight line obtained in the graph. Values of empirical probit were followed by the calculation of expected/provisional probit from 4.30 to 5.60. The values of working probit (from 4.326 to 6.423) and weighing coefficient (from 0.532 to 0.616) were calculated to determine the values of mean and deviation of dose of mustard oil cake and mortalities.

From the above values, slope was calculated to be 2.907. Finally the median lethal concentration was calculated to be LC_{50} = Antilog 3.700= 5012 mg/L of mustard oil cake (range: 3855 to 6666 mg/L). The value indicates slightly toxic to practically non-toxic nature of mustard oil cake for *Channa gachua* (Table 7).

The value of 96hr-LC_{50} calculated from Behren-Karber (1931) method, Miller and Tainter (1944) method and Finney (1971) probit analysis method is almost similar. These are most common methods used by the worker for the determination of 96hr-LC_{50}. However, out of these three methods, Behren-Karber (1931) method is useful for calculation of 24, 48, 72 and 96hr-LC_{50} dose from the same set of experiment.

(6) Reed-Muench (1938) method:

Reed and Muench lead to a bias in the estimation of the LC_{50} if the log of the doses is not spaced symmetrically about log LC_{50}. For this study, experimentation with 10 fishes per each dose is necessary for better correlation. Also the least test dose must kill one fish and there should be only four test doses. The method was modified by Saganuwan (2011) in calculating survival and mortality of percent of test animals to arrive a conclusion. Although, 95% confidence limit cannot be calculated with this method.

The ideal LC_{50} is calculated from the mean of MLC_{50} and MSC_{50} = $\frac{4446.0+5420.0}{2}$ = 4933.0 mg/L (Table 6).

Table 1. Lorke's (1983) method for LC_{50} dose of mustard oil cake in *Channa gachua*.

S. No.	Concentration of mustard oil cake (mg/L)	Log conc. of mustard oil cake (mg/L)	24hr % mortality (n = 3 in each group)	S. No.	Concentration of mustard oil cake (mg/L)	Log conc. of mustard oil cake (mg/L)	24hr % mortality (n = 1 in each group)
1.	100	1	0	1	25000	3.398	1
2.	1000	2	0	2	20000	3.699	1
3.	10000	3	0	3	15000	3.875	0

Lorke's (1983) method: 24hr-LC_{50} = $\sqrt{C_0 \ x \ C_{100}}$ = $\sqrt{10000 \ x \ 20000}$ = 14142.16mg/L mustard oil cake.

Enegide et al, (2013) method: 24hr-LC_{50} = $\frac{M_0 + M_1}{2}$ = $\frac{10000+20000}{2}$ = 15000mg/L mustard oil cake.

Table 2. Up-and-Down method (Dixon-Mood, 1948; Bruce, 1985) for determination of LC_{50} dose of mustard oil cake in *Channa gachua.*

Number	Dose (mg/L)	Survival/Mortality	Number	Dose (mg/L)	Survival/Mortality
1st	4000	Survival	1st	5000	Mortality
2nd	4500	„.	2nd	4550	„
3rd	5000	Mortality	3rd	4000	..
4th	4550	Survival	4th	3500	Survival
5th	5000	Mortality	5th	4000	„
6th	4550	„	6th	4500	„
Arithmetic Mean	4600.0	LC_{50}	Arithmetic Mean	4258.33	LC_{50}
Standard deviation	372.83	2.57 (Default dose)	Standard deviation	529.54	2.72 (Default dose)
Standard error of Mean	152.175	11.76 (Confidence interval)	Standard error of Mean	216.139	7.24 (Confidence interval)
Geometric Mean	4587.11	LC_{50}	Geometric Mean	4230.55	LC_{50}
Harmonic Mean	4573.93	LC_{50}	Harmonic Mean	4202.51	LC_{50}

Mean LC_{50} = $\frac{4600.0+4258.33}{2}$ = 4429.165mg/L mustard oil cake.

Table 3. Behren-Karber method for 96hr-LC_{50} determination of mustard oil cake in *Channa gachua*.

Group	Dose of mustard oil cake (mg/l)	Difference between two consecutive dose (A)	No. of fish exposed	Mortality				Overall mortality at 96hr	Mean mortality between two consecutive dose (B)	AxB
				24hr	48hr	72hr	96hr			
1	0	0	20	0	0	0	0	0	0	0
2	2000	2000	20	1	3	5	5	5	2.5	5000
3	4000	2000	20	2	6	7	8	8	6.5	13000
4	6000	2000	20	4	7	11	12	12	10.0	20000
5	8000	2000	20	5	9	14	16	16	14.0	28000
6	10000	2000	20	7	12	17	20	20	18.0	36000
								61		102000

$$96hr\text{-}LC_{50} = LC10000 - \frac{\Sigma AB}{N} = 10000 - \frac{102000}{20} = 10000 - 5100 = 4900 \text{mg/L mustard oil cake.}$$

Table 4. Statistical relationship between dose of Mustard Oil Cake (mg/L) and mortality of *Channa gachua*.

Sl. No.	Exposure period (hours)	Regression equation y=bx+a	LC_{50} (mg/L)	Toxicity Factor	t value (df=4)	F value ($u_1=1, v_1=4$)	95% Confidence limit	
							lower	higher
1	24	y=1.104x-0.0554	17222.0	1.000	13.0 ($p<0.001$)	347 ($p<0.001$)	13312.0	21132.0
2	48	y=1.257x-0.0345	9478.23	1.817	12.5 ($p<0.01$)	728.4 ($p<0.01$)	5568.23	13388.23
3	72	y=1.045x-0.0793	5803.61	2.967	14.7 ($p<0.001$)	198.9 ($p<0.001$)	1893.61	9713.61
4	96	y=1.627x-0.2615	5344.00	3.223	12.0 ($p<0.01$)	18.14 ($p<0.01$)	1434.0	9254.0

Table 5. Probit analysis for toxicity of mustard oil cake in *Channa gachua*.

Dose of mustard oil cake (mg/L)	Log dose of mustard oil cake (mg/L)	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
2000	3.301	20	5	25	25		4.33	4.30	4.326	0.532	10.64	35.123	46.029	115.940	198.558	151.941
4000	3.602	20	8	40	40		4.75	4.80	4.747	0.627	12.54	45.169	59.527	162.699	282.576	214.418
6000	3.778	20	12	60	60		5.25	5.30	5.253	0.616	12.32	46.545	64.717	175.847	339.958	244.501
8000	3.903	20	16	80	80		5.84	5.50	5.809	0.581	11.62	45.353	67.501	177.012	392.111	263.455
10000	4.000	20	20	100	100		8.22	5.60	6.423	0.558	11.16	44.640	71.681	178.560	460.405	286.723
	-	-	-	-	-		-	-	-	-	58.28	216.830	309.455	810.058	1673.608	1161.038

LC_{50} = Antilog 3.7000= 5012.0mg/L of Mustard oil cake.

Table 6. Reed-Muench (1938) method for 96hr-LC_{50} determination of Mustard oil cake in *Channa gachua*.

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	2000	3.301	3	7	3	36	39	$\frac{3}{39}$	7.70	92.30
2	4000	3.602	4	6	9	12	21	$\frac{9}{21}$	42.86	57.14
3	6000	3.778	6	4	15	6	21	$\frac{15}{21}$	71.43	28.57
4	8000	3.903	8	2	23	2	25	$\frac{23}{25}$	92.00	8.00

Estimation of median lethal concentration (MLC_{50})	Estimation of median survival concentration (MSC_{50})
$\frac{50.0-42.86}{71.43-42.86} = \frac{7.14}{28.57} = 0.2499$	$\frac{50.0-28.57}{57.14-28.57} = \frac{21.43}{28.57} = 0.7501$
3.778 – 3.602 = 0.176	3.778 – 3.602 = 0.176
0.2499 x 0.176 = 0.398	0.7501 x 0.176 = 0.132
3.602 + 0.398 = 3.6418	3.602 + 0.132 = 3.6418
Antilog of 3.6418 = 4446.0 mg/L	Antilog of 3.734 = 5420.0 mg/L
MLC_{50} = 4446.0 mg/L	MSC_{50} = 5420.0 mg/L

Table 7. Classification of substances on the basis of toxicity range (After Loomis and Hayes, 1996).

Sl. No.	Toxicity range (mg/L)	Toxicant Classification	Sl. No.	Toxicity range (mg/L)	Toxicant Classification
1.	<5	Extremely Toxic	2.	5-50	Highly Toxic
3.	50-500	Moderately Toxic	4.	500-5000	Slightly Toxic
5.	5000-15000	Practically Non-toxic	6.	>15000	Relatively Harmless

Table 8. Safe level estimates of mustard oil cake in *Channa gachua*.

Sl. No.	Method	Dose of mustard oil cake (mg/L)	Factor	Calculation of Safe level (mg/L)
1.	Canadian Council of Resource and Environment Minister (=CCREM) (1991)	96hr-LC50 = 5344.0	0.05	5344.0 x 0.05 = 267.2
2.	CWQC (1972)	48hr-LC50 = 9478.23	0.01	9478.23 x 0.01 = 94.78
3.	Hart et al, (1948)	96hr-LC50 = 5344.0	$0.03 \times \left(\frac{24hr-LC50}{48hr-LC50}\right)^2$	$5344.0 \times 0.03 \times \left(\frac{17222.0}{9478.23}\right)^2 =$ 529.30
4.	IJJ (1977)	96hr-LC50 = 5344.0	0.05	5344.0 x 0.05 = 267.2
5.	NAS/NAE (1973)	96hr-LC50 = 5344.0	0.1 to 0.00001	5344.0 x 0.1 = 534.40
6.	Sprague (1971)	96hr-LC50 = 5344.0	0.1	5344.0 x 0.1 = 534.40

Ideal Median Lethal Dose (IMLD50) was found similar to the Effective Dose 50 (ED50) as calculated by Aguilar *et al.* (2014) using Behren and Karber's formula. Therefore, it may be inferred that, any of the five cross checks may be used to confirm LD_{50} in addition to using the average of MSC_{50} and MLC_{50}.
The average 96hr-LC_{50} dose from Behren-Karber (1931) method, Miller and Tainter (1944) method, Finney (1971) probit analysis method and Reed-Muench (1938) method is 4900.0+4900.0+ 5012.0+ 4933.0 = 4936.25mg/L of mustard oil cake in *Channa gachua*. The value indicates slightly toxic nature of mustard oil cake for *Channa gachua* (Table 7).

The median lethal dose of mustard oil cake between 4446.0 to 5420.0mg/L from Reed-Muench (1938) method is comparable with the 4258.33 to 4600.0mg/L from up-and-down method. It showed precision, validity and reliability of using arithmetic mean as rough estimate of LD_{50}. In toxicological studies, the geometric mean is used for exponential data. The harmonic mean is applied for things like rates and ratios where an arithmetic mean would actually be incorrect (Dawson and Trapp 2004; Saganuwan 2015). Therefore, it may be inferred that arithmetic or harmonic mean can be used for rough estimation of LD_{50} dose in place of geometric mean.

96hr-LC_{50} dose of mustard oil cake varies from 170-200, 610 and 730 mg/L in fry of major carps, *Cyprionus carpio* and *Tilapia mossambica* respectively (www. sodhganga.infibnet.ac.in). Nath *et al.* (2017) reported a sublethal dose of mustard oil cake 420 mg/L in *Channa punctatus*. Therefore, it seems that the present observation resembles earlier findings. The difference in dose of mustard oil cake may be due to differences in the species, ambient temperature, locality of fish collection, month of the year etc.

On the basis of average 96hr-LC_{50} dose of a biofertilizer in *Channa gachua*, mustard oil cake may be treated as a substance of slightly toxic (500-5000mg/L) or practically non-toxic (5000-15000mg/L) to *Channa gachua* (Table 7). EPA (2015) lists aquatic toxicity as practically non-toxic when concentration is greater than 100mg/L. Thus, the toxicity nature of mustard oil cake is also confirmed by EPA (2015).

It is reported that, safe levels are added to account for uncertainties in data and evaluation processes. It is also used in case of lacking of data on acute toxicity. A range of safe level of mustard oil cake was calculated from 94.78 to 534.40 mg/L in *Channa gachua* (Table 8). The range indicates that it is difficult to decide the acceptable concentration of mustard oil cake in *Channa gachua* based on the present study. Morecver, 9.478 to 53.44 mg/L and 0.9478 to 5.344 mg/L of mustard oil cake allow a safe level for rat and man respectively (https://en.wikipedia.org/wiki/Toxicity).

CONCLUSIONS

It may be inferred that for ecotoxicological work, determination of LC_{50} dose is one of the basic step. For better result, one should proceed with the Lorke and Enegide *et al.,* method followed by Up-and-Down method respectively for range finding and rough estimation of LC_{50} dose. After that Behren-Karber or Regression Analysis method and Finney probit method should be used to determine the dose of LC_{50}. Finally, Reed-Muench method should be used to confirm the value of LC_{50} dose by various cross checks. Range of safe level indicates that it is difficult to decide the acceptable concentration of mustard oil cake in *Channa gachua* based on the present study. However, on the basis of toxicity range, mustard oil cake may be treated as a substance of slightly toxic or relatively harmless to *Channa gachua.*

CONFLICT OF INTEREST:
The Authors declare no conflict of interest.

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