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Ecotoxicity of silver nanoparticles in an Indian air-breathing catfish, *Clarias batrachus* (Linnaeus)

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Abstract
This is an initial report on ecotoxicity of silver nanoparticles for *Clarias batrachus* (Linnaeus). The work was conducted by the use of static renewal method during 2018. In this experiment, 24hr-LC_{50} value from Lorke and Enegide *et al*, technique was estimated severally 158.11 and 175.0 mg/L. A range of 96hr-LC_{50} value of 158.33 to 191.67mg/L was calculated from Up-and-Down technique as a rough estimate. Moreover, from Behren-Karber, Miller and Tainter and Finney technique varied 96hr-LC_{50} value was found 177.5, 171.6 and 171.4mg/L. Finally, Reed-Muench technique used to confirm 96hr-LC_{50} dose and a median ideal dose was found 173.25mg/L. The findings suggest that silver nanoparticles are accumulated in aquatic bodies and distressing the physiological activities of *Clarias batrachus*. The findings inferred that this fish incorporates a ton of capability to resist silver nanoparticles toxicity compared to completely different fishes. Vary of tabulated safety level indicated that it's robust to settle on the acceptable dose of silver nanoparticles for this fish. However, on the premise of traditional toxicity varies; silver nanoparticles may even be treated as moderately cytotoxic substance to *Clarias batrachus*.

Keywords: Ecotoxicity, silver nanoparticles, *Clarias batrachus*

Introduction
Since the recent past, scientific studies are focusing on silver nanoparticles toxicity. The toxicity of silver nanoparticles in living organisms range from μgl^{-1} to mgl^{-1} including major carps and other fish [1-4]. Fish exposed to silver nanoparticles led to deformation of the embryo, inflammation, cytotoxicity, dampened mitochondrial activity, oxidative stress mechanism and apoptosis [5].

Acute toxicity may be defined as the adverse effect occurring after the administration of a toxicant within 24 hours [6]. The assessment of the median lethal concentration (LC_{50}) has been used as an important parameter to measure acute toxicity and an initial procedure to screen toxicity of a substance. The study gives information about LC_{50}, therapeutic index, degree of safety level and toxicity status of a substance [7, 8]. Various methods are used in determination of LC_{50}. It seems that improvement of the conventional methods through application of software is the need of the present day [9, 10]. But, sometimes software used may not provide 95% confidence limit. However, some countries including UK have taken steps to ban the LD50. *Clarias batrachus*, a freshwater Indian air breathing catfish is one of the important fish species. It is treated as a typical example to deal with the alimentary canal of a teleost and a test animal in many laboratories of Indian Universities [11]. However, the effect of silver nanoparticles on Indian Air-breathing fishes is lacking.

Therefore, the present work was designed to evaluate the median lethal dose of silver nanoparticles on *Clarias batrachus*. The work will help in deciding the toxicity level of silver nanoparticles for the higher yield of this fish. The study will also be helpful to evaluate variation in health of the fish due to toxicity of silver nanoparticles, suitability of environmental conditions for fish, relative sensitivity of fish to silver nanoparticles and also physico-chemical conditions of water bodies.

Materials and Methods
Live specimens of *Clarias batrachus* (Linnaeus) (46.0-68.0g body weight, 18.0-22.0cm total length and 16.2-19.2cm standard length) were purchased from the local market of Arrah (Bhojpur), Bihar during 2018. They were acclimatized for a fortnight in Departmental

Laboratory of VKS University, Arrah.

The silver nanoparticle (size 60nm, TEM; Product No. 730815) was procured from Sigma Aldrich and stored at 6 ^{0}C temperature in a freeze in the laboratory. The particles of nano silver bear nearly spherical appearance and generally yellowish hues colour. It has electrical, optical and thermal properties. The concentration and density of silver nanoparticles is 0.02 mg/mL in aqueous buffer and 0.9976 g/mL at 25 °C respectively. It contains sodium citrate as stabilizer. Stock suspension of the uncoated powder was made using dechlorinated tap water to obtain various concentrations of the test solution. Respective grams of the metallic oxides were dissolved in 1 L of the dechlorinated tap water to obtain appropriate mixtures to obtain stock solutions. From the stock solution, solutions of desired concentrations were prepared for experimental purposes.

The investigation was performed using a static renewable method in controlled laboratory conditions following the ethics of Department and VKS University, Arrah. Temperature (25.0±1.0), pH (7.84±0.08), dissolved oxygen (4.56 ± 0.64mg/L), total alkalinity (334.00±8.62mg/L) and total hardness (190.66±8.66mg/L) were recorded daily at exposure times of 24, 48, 72 and 96 hours [12]. The following methods were used to calculate LC_{50} dose from its estimation to confirmation:

1. Median lethal dose method [13, 14]
2. Up-and-Down (Staircase) method [15, 16]
3. Behren-Karber method [17, 18]
4. Regression Analysis method
5. Finney probit analysis method [19] and
6. Reed-Muench method [20].

A standard table was followed to categorize the toxicity nature of silver nanoparticles [21]. The safe level estimation of the silver nanoparticles was calculated different methods [22 - 27].

Statistical analysis was done with Graph Pad Prism 5 software. Data were entered in a Microsoft Excel spreadsheet. All the entries were double checked for any possible key board error. The observations were expressed in mean for each group. The data were used for group analysis followed by its linear regression.

Results and Discussion

(1) Lorke's [13] and Enegide *et al*, [14] median lethal dose method: The method is divided into following two phases. The behaviour and the mortality of fish were observed for 24 hours in each phase to estimate the lethal dose.

(A) Three fish taken in three groups were given 1, 10 and 100mg/L silver nanoparticles.

(B) One fish each from three groups were given 250, 500 and 1000mg/L silver nanoparticles.

Therefore, 24hr-LC_{50} = $\sqrt{C_0 \times C_{100}}$ = $\sqrt{100 \times 250}$ = 158.114mg/L silver nanoparticles (Table 3). Where, C_0 = maximum concentration with no mortality of fish and C_{100} = minimum concentration with total mortality of fish.

Further, Enegide *et al*, [14] median lethal dose method was used to determine 24hr-LC_{50} dose = $\frac{M_0 + M_1}{2}$ = $\frac{100 + 250}{2}$ = 175.0mg/L silver nanoparticles (Table 3). Where, M_0 = maximum concentration with no mortality of fish and M_1 = minimum concentration with total mortality of fish. Although, less number of fish was sacrificed in this method, but the accuracy and reliability of these methods is not good due to observation for 24hours only.

(2) Dixon-Mood [15] Up-and-Down (Staircase) Method: It is based on the reduction in the number of fish. In this method, if fish survived, the dose for the next is increased by a constant factor and vice-versa. It applies arithmetic, geometric and harmonic mean for the estimation of the median lethal dose (MLC_{50}) using 6 fish 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 $\frac{158.33 + 191.67}{2}$ = 175.0mg/L of silver nanoparticles (Table 4). The method is terminated when no mortality is observed even at 2000-5000 mg/L [16, 28]. It gives rough estimation of MLC_{50} dose.

(3) Behren-Karber [17, 18] method: It is a non-parametric method that requires at least one partial mortality but data do not fit in the probit model. In this method, equal spacing of the interval of log dose and the equal number of fish at each dose level was applied for observation from 0 to 100%. Many fish were sacrificed because the dose calculated was not killed a single fish. The fish were doused 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} dose of silver nanoparticles was calculated 177.5 mg/L (Table 5).

(4) Regression analysis method: It is applied when there is no partial mortality. The dose of silver nanoparticles was converted into log dose and percent mortality as probit mortality. In this method, many fish were sacrificed. By this method, 24, 48, 72 and 96hr-LC_{50} dose of silver nanoparticles was calculated 346.20, 237.33, 201.28 and 171.16 mg/L respectively (Table 6). A gradual decrease was found in the dose of silver nanoparticles in relation to treatment.

(5) Finney [19] probit analysis method: It is a parametric maximum likelihood method in which after calculating percent mortality, net/corrected percent mortality from 10% to 100% were calculated. Then, values of empirical probit from 3.72 to 8.22 (Table-7) were noted from Fischer and Yates table depending upon the straight line obtained in the graph. Values of empirical probit were applied in the calculation of expected/provisional probit from 3.70 to 6.40. The values of working probit (from 4.90 to 6.20) and weighing coefficient (from 0.302 to 0.627) were calculated to determine the values of mean and deviation of the dose of silver nanoparticles and mortality.

Finally, the median lethal concentration was calculated to be LC_{50} = Antilog 2.234= 171.4 mg/L of silver nanoparticles.

The value of 96hr-LC_{50} calculated from Behren-Karber method, regression analysis method and Finney probit analysis method is almost similar [17 - 19]. These are the most common methods used by the worker for the determination of 96hr-LC_{50}. However, out of these three methods, the Behren-Karber [17, 18] method is useful for the calculation of 24, 48, 72 and 96hr-LC_{50} dose from the same set of experiment.

(6) Reed-Muench [20] method: They lead to a bias in the estimation of the LC_{50} if the log of the dose is not spaced symmetrically about log LC_{50}. For this study, experimentation with 10 fish 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 in calculating survival and mortality of percent of test animals to arrive a conclusion [29]. Although, 95% confidence limit cannot be calculated with this method.

Confirmation: The conformation of LC_{50} was done applying following cross checks:

(a) The ideal LC_{50} is calculated from the mean of MLC_{50} and MSC_{50} = $\frac{170.7 + 175.8}{2}$ = 173.25 mg/L (Table 8). The value indicates moderately toxic nature of silver nanoparticles for *Clarias batrachus* (Table 1).

(b) Sum of doses and sum of mortalities: Its value was $\frac{100+150+200+250}{1+3+6+9}$ = $\frac{700}{19}$ = 36.84. Multiplication of 36.84 x 5 is 184.21. Therefore, LC_{50} = 184.21mg/L.

The median lethal dose of silver nanoparticles between 170.70 to 175.80mg/L from Reed-Muench [20] method is comparable with the 158.33 to 191.67mg/L from up-and-down method. It showed precision, validity and reliability of using arithmetic mean as the 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 [30, 31]. Therefore, it may be inferred that arithmetic or harmonic mean can be used for the rough estimation of LD_{50} dose in place of geometric mean.

Earlier reports inferred that 48hr-LC_{50} dose of silver nanoparticles dispersed from 63-300µg/L in *Perca fluviatilis* [32] to 1000mg/L in *Danio rerio* [33].

The median lethal dose of silver nanoaprticles ranged from 0.202mg/L in *Hypophthalmichthys molitrix*, 0.34mg/L in *Hypophthalmichthys molitrix* and 0.53mg/L in *Carassius auratus*, 100mg/L in *Oreochromis niloticus* and *Labeo rohita*, 164.02mg/L in *Clarias batrchus* and 250mg/L in *Danio rerio* [34 - 39]. These findings suggest that *Clarias batrachus* has capacity to resist silver nanoparticles toxicity more in comparison to most of the other fishes. The observations of are comparable with earlier work [38]. The difference in LC_{50} dose among the same species depends upon the body weight, age, sex and feeding conditions of the test fishes; ambient conditions, water temperature and regional variations [34, 35]. The toxicity level of silver nanoaprticles also depends upon its shape, size, surface area, surface charges and chemical composition [34].

The average 96hr-LC_{50} dose from Behren-Karber [17, 18] method, Regression analysis method, Finney [19] (1971) probit analysis method and Reed-Muench [20] method is 177.50+171.60+171.40+173.25 = 173.44mg/L of silver nanoparticles in *Clarias batrachus*. The value indicates the moderately toxic nature of silver nanoparticles for *Clarias batrachus* (Table 1).

It is reported that, safe levels are added to account for uncertainties in data and evaluation processes. The safe level is also used in case of lack of data on acute toxicity. A range of safety level of silver nanoparticles was calculated from 2.37 to 17.12 mg/L in *Clarias batrachus* (Table 2). The range indicates that it is difficult to decide the acceptable concentration of silver nanoparticles in *Clarias batrachus*. Moreover, 0.237 to 1.712 mg/L and 0.0237 to 0.1712 mg/L of silver nanoparticles allow a safe level for rat and man respectively (https://en.wikipedia.org/wiki/Toxicity).

Table 1: 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 2: Safety level estimates of silver nanoparticles in *Clarias batrachus*

Sl. No.	Method	Dose of mustard oil cake (mg/L)	Factor	Calculation of Safety level (mg/L) of silver nanoparticles
1.	Canadian Council of Resource and Environment Minister (=CCREM) (1991)	96hr-LC50 = 171.16	0.05	171.16 x 0.05 = 8.558
2.	CWQC (1972)	48hr-LC50 = 237.33	0.01	237.33 x 0.01 = 2.37
3.	Hart *et al*, (1948)	96hr-LC50 = 171.16	0.03 x $\left(\frac{24hr-LC50}{48hr-LC50}\right)^2$	171.16 x 0.03 x $\left(\frac{346.20}{237.33}\right)^2$ = 10.93
4.	IJJ (1977)	96hr-LC50 = 171.16	0.05	171.16 x 0.05 = 8.558
5.	NAS/NAE (1973)	96hr-LC50 = 171.16	0.1 to 0.00001	171.16 x 0.05 = 17.12
6.	Sprague (1971)	96hr-LC50 = 171.16	0.1	171.16 x 0.05 = 17.12

Table 3: Lorke's (1983) method for LC_{50} dose of LC_{50} dose of silver nanoparticles in *Clarias batrachus*

S. No.	Dose of silver nanoparticles (mg/L)	Log dose of silver nanoparticles (mg/L)	96hr % mortality (n = 3 in each group)	S. No.	Dose of silver nanoparticles (mg/L)	Log concentration of mustard oil cake (mg/L)	96hr % mortality (n = 1 in each group)
1.	1	0	0	1.	1000	3.000	1
2.	10	1	0	2.	500	2.699	1
3.	100	2	0	3.	250	2.398	1

Lorke's (1983) method: 24hr-LC_{50} = = $\sqrt{C_0 \; x \; C_{100}}$ = $\sqrt{100 \; x \; 250}$ = 158.114mg/L silver nanoparticles.

Enegide *et al*, (2013) method: 24hr-LC_{50} = $\frac{M_0 + M_1}{2}$ = $\frac{100+250}{2}$ = 175.0mg/L silver nanoparticles.

Table 4: Up-and-Down method (Dixon-Mood, 1948; Bruce, 1985) for determination of LC_{50} dose of silver nanoparticles in *Clarias batrachus*

Number	Dose (mg/L)	Survival/Mortality	Number	Dose (mg/L)	Survival/Mortality
1st	100	Survival	1st	300	Mortality
2nd	150	,,.	2nd	250	,,
3rd	200	Mortality	3rd	200	..
4th	150	Survival	4th	150	Survival
5th	200	Mortality	5th	100	,,
6th	150	,,	6th	150	,,
Arithmetic Mean	158.33	LC_{50}	Arithmetic Mean	191.67	LC_{50}
Standard deviation	37.64	1.5756 (Default dose)	Standard deviation	73.60	1.8669 (Default dose)
Standard error of Mean	15.36	6.54 (Confidence interval)	Standard error of Mean	31.04	3.307 (Confidence interval)
Geometric Mean	154.31	LC_{50}	Geometric Mean	179.77	LC_{50}
Harmonic Mean	150.00	LC_{50}	Harmonic Mean	168.22	LC_{50}

Mean LC_{50} = $\frac{158.33+191.67}{2}$ = 175.0mg/L silver nanoparticles.

Table 5: Behren-Karber method for 96hr-LC_{50} determination of silver nanoparticles in *Clarias batrachus*

Group	Dose of silver nanoparticles (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	10	0	0	0	0	0	0	0
2	100	50	10	0	0	0	1	1	0.5	25
3	150	50	10	0	1	2	3	3	2	100
4	200	50	10	1	3	4	6	6	5	250
5	250	50	10	3	6	8	9	9	7.5	375
6	300	50	10	6	8	9	10	10	9.5	475
	1000							61		1225

96hrLC50 = LC100 - $\frac{\Sigma AB}{N}$ = 300 – $\frac{1225}{10}$ = 350 – 122.5 = 177.5 mg/L silver nanoparticles.

Table 6: Regression analysis method for LC_{50} dose of silver nanopartcles (mg/L) of *Clarias batrachus*

Sl. No.	Exposure period (hours)	Regression equation y=bx+a	Lethal Concentration (mg/L)		Toxicity Factor	t value (df=5)	F value (u = 1; v = 4)	95% Confidence limit	
								lower	Higher
1	24	y=0.01857x-1.429	LC_{10}	130.80	1.000	2.945 (p<0.05)	8.734 (p<0.05)	98.54	146.68
			LC_{50}	346.20				307.65	424.00
			LC_{90}	561.90				468.61	749.47
2	48	y=0.02829x-1.714	LC_{10}	95.93	1.458	4.47 (p<0.01)	20.01 (p<0.05)	67.76	113.85
			LC_{50}	237.33				229.49	249.56
			LC_{90}	378.72				345.13	431.56
3	72	y=0.03371x-1.785	LC_{10}	82.62	1.720	5.01 (p<0.01)	25.19 (p<0.01)	54.98	101.06
			LC_{50}	201.28				200.00	203.86
			LC_{90}	319.93				298.93	351.39
4	96	y=0.03714x-1.357	LC_{10}	63.62	2.023	7.13 (p<0.001)	50.64 (p<0.01)	42.17	79.51
			LC_{50}	171.16				167.48	173.93
			LC_{90}	278.86				268.37	292.79

Table 7: Probit analysis for toxicity of silver nanoparticles in *Clarias batrachus*

Dose of silver nano particles (mg/L)	Log dose of silver nano particles (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	10	0	0	0	0	0	0	0	0	0	0	0	0	0
100	2.000	10	1	10	10	3.72	3.70	4.90	0.336	3.36	6.72	16.46	13.44	80.67	32.93
150	2.176	10	3	30	30	4.48	4.80	5.40	0.627	6.27	13.64	33.86	29.69	182.83	73.67
200	2.301	10	6	60	60	5.25	5.60	5.80	0.558	5.58	12.84	32.36	29.54	187.71	74.47
250	2.398	10	9	90	90	6.28	6.20	6.00	0.370	3.70	8.87	22.20	21.28	133.20	53.24
300	2.477	10	10	100	100	8.72	6.40	6.20	0.302	3.02	7.48	18.72	18.53	116.09	46.40
	-	-	-	-	-	-	-	-	-	21.93	49.55	123.60	112.48	700.50	280.71

96hr-LC_{50} = Antilog 2.234= 171.4mg/L of silver nanoparticles.

Table 8: Reed-Muench (1938) method for 96hr-LC_{50} determination of silver nanoparticles in *Clarias batrachus*

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	100	2.000	1	9	3	36	39	$\frac{3}{39}$	7.70	92.30
2	150	2. 176	3	7	6	12	18	$\frac{6}{18}$	33.33	66.67
3	200	2.301	6	4	12	5	17	$\frac{12}{17}$	70.59	29.41
4	250	2.398	9	1	21	1	22	$\frac{21}{22}$	95.45	4.55

Calculation of median lethal concentration (MLC_{50})	Calculation of median survival concentration (MSC_{50})
$\frac{50.0-33.33}{70.59-33.33} \quad \frac{16.67}{37.26} = 0.45$	$\frac{50.0-29.41}{66.67-29.41} \quad \frac{20.59}{37.26} = 0.553$
2.301 – 2.176 = 0.125	2.301 – 2.176 = 0.125
0.45 x 0.125 = 0.05625	0.553 x 0.125 = 0.06912
2.176 + 0.05625 = 2.2323	2.176 + 0.06912 = 2.2451
Antilog of 2.2323 = 170.7 mg/L	Antilog of 2.2451 = 175.8 mg/L
MLC_{50} = 170.7 mg/L	MSC_{50} = 175.8 mg/L

Conclusion

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, the 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 silver nanoparticles in *Clarias batrachus* based on the present study. However, on the basis of toxicity range, silver nanoparticles may be treated as a substance of moderately toxic to *Clarias batrachus.*

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