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DETERMINATION OF MEDIAN TOLERANCE LIMIT (LC_{50}) OF *GAMBUSIA AFFINIS* FOR MERCURIC CHLORIDE AND ITS BEHAVIORAL IMPACTS

Gupta S. S. and Jawale C. S.*
P.G. Department of Zoology, H.P.T. Arts and R.Y.K. Science College, Nasik 422005, Maharashtra, India.
***P.G. Department of Zoology, H.P.T. Arts and R.Y.K. Science College, Nasik 422005, Maharashtra, India.
(E-mail: supriya_hm@yahoo.co.in; csjawale@hotmail.com)

ABSTRACT
In this study 96-h LC_{50} value of mercuric chloride ($HgCl_2$), a metal salt widely used in industry, was determined for the guppy (*Gambusia affinis*). The experiments were planned in four series of a total of 450 guppies employing the static test method of acute toxicity. 10 fish were placed in each replicate of each dose. The experiments were performed as four replicates, and behavioral changes in the *G.affinis* were determined for each mercuric chloride metal salt concentration. The data obtained were statistically evaluated by the use of software based on Finney's Probit Analysis Method and a 96-h LC_{50} value for *Gambusia affinis* was found to be 97.099 µg/L in a static bioassay test system. The water temperature during experiments was kept between 21 and 23^{o}C. The behavioral changes observed in fish were, vertical and downward swimming pattern, capsizing, surfacing phenomenon, dark pigmentation, hemorrhagic regions, increased distance between gill and operculum, mucus secretion, and decrease level of sensitization.
KEY WORDS: Bioassay; Acute toxicity; LC_{50}; Mercuric chloride; *Gambusia affinis*

INTRODUCTION
Toxic chemicals generated through man's industrial, agricultural and domestic activities eventually reach aquatic environment and cause a major threat to the inhabiting organisms, since the aquatic environment is fragile and hence, sensitive to the toxic effects of chemical pollutants (Jaffi, 1991). Some of the industrial effluents release inorganic mercuric compounds like mercuric chloride, which is converted into more toxic organic form viz., methyl mercury, through bacterial action (Wiener and Spry, 1996). These compounds are likely to get transferred to the fish from water and enter the food chain.

Exposure to toxic mercury is growing health hazard throughout the world today and it is considered as one of the most potent neurotoxin known, having a number of adverse health effects in animals and humans. Recent studies show that mercury exposure may occur in the environment, and increasingly in occupational and domestic settings. Children are particularly vulnerable to mercury intoxication, which may lead to impairment of the developing central nervous system, as well as pulmonary and nephritic damage (Counter and Buchanon, 2004). The decision whether a certain xenobiotic is dangerous for the aquatic system and the food cycle, can only be made after the acute toxicity test on mammals, bacteria, fish and biological dissociation tests have been carried out in detail (Ardalı, 1990). The fact that increasing use of contaminating chemicals in many industrialized parts of the world makes the development of ecotoxicity measurement techniques an absolute necessity (Brando et al., 1992). The first step is the acute toxicity test on algae; fish etc. in order to show the potential risks of these chemicals (OECD, 1993). Although the initial aquatic toxicity tests were carried by the use of bacteria, invertebrates like Cladocera, Rotifer and other groups, they can in no way replace the actual test performed on fish. What is important is the toxicity in fish which is the last chain in the food cycle (Castano *et al.*, 1996).

As fishes are continuously exposed to comparatively low concentrations of metal and other chemicals affecting their behavioral responses, and there is a growing interest in the development of behavioral markers to access the sub lethal effects of toxicant. Any change in the behavior and physiology of the fish indicates the deterioration of water quality, since fishes are the biological indicators of water quality. Behavior is considered a promising tool in ecotoxicology and these studies are becoming prominent in toxicity assessments in unicellular organisms, insects, fishes and even rodents. Locomotors behavior is commonly affected by contaminants and the pattern of swimming is highly organized species-specific responses. (Goel and Sharma, 1996). Hence in present study 96-h LC_{50} value of mercuric chloride, a metal salt widely used in industry, was determined for the guppy (*Gambusia affinis*). The experiments were performed to determine behavioral pattern of fish at various dose concentrations of mercuric chloride.

MATERIALS AND METHOD
Adult guppies were obtained from a local fish breeder and brought to the laboratory within 30 minutes in plastic bags with sufficient air. The plastic bags were placed into the aquariums of about 25 liter capacity for 30-35 minutes for acclimatization. Then the bags were cut open and the fish were allowed to swim into the aquarium water. The aquaria

were aerated with a central aeration system and the fish were exposed to 15 days conditioning period at room temperature. The fish were fed with commercial pellet food at least once a day during this period. Experimental fish were not fed 24-h before the start of the tests. Care was taken to keep the mortality rate of fish not more than 5% in the last four days before the experiment was started. Different concentrations of Mercuric Chloride ($HgCl_2$) in dechlorinated tap water were used in the static bioassays. For the acute bioassay tests, 10 fishes were used per concentration. A total of four replicates were carried out for each dose and control group. The containers were not aerated at the dosing time. Mercuric Chloride concentrations administered in 20 L capacity test tanks were 60, 70, 80, 90, 100, 110, 120, 130, 140 and 150 μg/L. There was a simultaneous control group maintained in dechlorinated water without adding the Mercuric Chloride ($HgCl_2$) and keeping all other conditions constant. The mortality rate in the control group did not exceed 10% and 90% of the fish looked healthy throughout the experiment. The experiment was carried out in two series and a total of 450 *Gambusia affinis* were used. This species was selected for static bioassays because it can be easily cultured and raised under laboratory conditions through a complete life cycle, and it is one of the standard test species used for laboratory toxicity studies (TSE, 1998) and (OECD, 1993).

Water quality parameters (temperature, dissolved oxygen (DO), $CaCO_3$ hardness and pH) in the aquaria were periodically determined before the bioassay tests. The water temperature was kept between 21-23^{o}C. The average pH was 7.4 ± 0.3, average hardness was 68mg/L and dissolved oxygen was 7.2 mg/L. The experimental medium was aerated in order to keep the amount of oxygen not less than 4 mg/l.

All experiments were carried out for a period of 96 hours. The number of dead fish were counted every 12 hours and removed from the aquaria as soon as possible. The mortality rate was determined at the end of the 24, 48, 72 and 96 hours. No food was given to the fish during the experiments. The behavioral changes of the healthy fish and the fish exposed to various doses of mercuric chloride ($HgCl_2$) were photographed and evaluated as regard to behavioral anomalies (TSE, 1998).

The experiments were carried out with static acute experimental method. In this method the experimental solution and the samples (i.e. fish) are put in a suitable experimental cell (i.e. aquarium) and kept like that for a certain period. Since the decreased amount of oxygen and increased metabolic waste become a problem in long term experiments, the duration of such experiments are usually kept at 96 hours or less (TSE, 1998). The bioassay system was as described in standardized methods (OECD, 1993) and (APHA, 1997). Data generated during experimentation was subjected to Finney's Probit Analysis LC_{50} Determination Method [lognormal Distribution] (Finney, 1971). The computer analysis was carried on for LC_{50} on software Biostat (2009).

RESULTS

The relation between the mercuric chloride concentration and the mortality rate of *G.affinis* according to Finney's Probit Analysis using Biostat (2009) Computer Program is shown in Table 1. The results obtained from acute static 96-h toxicity experiments of mercuric chloride for *G.affinis* and Probit (Y) and weight (Z) values are listed in table 2. Regression statistics showing different LC values (LC_{10}, LC_{16}, LC_{50}, LC_{84}, LC_{90} and LC_{100}), LC_{50} standard error and intercept are shown in table 2. The mean LC_{50} value of mercuric chloride on *G.affinis* individuals was found to be 97.099μg/L by the use of Biostat (2009) computer program based on Finney's Probit Analysis-least squares [normal Distribution] Method. Fig. 1 shows the plot of Dose percentile and Actual response in percent verses Log dose concentrations of $HgCl_2$.

The change in behavioral patterns:

Behavioral changes are the most sensitive indication of potential toxic effects. Optomotor responses are very useful in evaluating the behavioral changes of fish (Richmonds and Dutta, 1992). In this study when *G. Affinis* exposed to various concentrations of Mercuric chloride at microgram/liter level displayed behavioral disorders. The magnitude and duration of behavioral changes increased with increased concentration. The behavioral changes observed in fish are as follows:

Control group: There were no behavioral changes and deaths observed in the control group throughout the experiment. The theoretical spontaneous response in the control was zero.

10 µg/L- 50 µg/L: Behavior of fish was normal with no mortality till 96 hours.

60 µg/L: Swimming disorders such as vertical and downward swimming pattern was observed. The fishes gathered at the surface in initial 24hours. The swimming speed decreased with time during the experimental span. Dark pigmentation was also observed between 72-96 hours.

70 µg/L: Same observations were seen as in 60 μg/L concentration.

80 µg/L: Vertical and downward swimming pattern was observed. The fishes gathered at the surface in initial 24 hours. The swimming speed decreased with time during the experimental span. Dark pigmentation was also observed between 72-96 hours. Decrease in the level of sensitization (response to stimuli) and settlement of fishes at the bottom was observed, at the end of experiment.

90 µg/L: Same observations were seen as in 80 µg/L concentration.

100 µg/L: Vertical and downward swimming pattern was observed. The fishes gathered at the surface in initial 24 hours. The swimming speed decreased with time during the experimental span. Dark pigmentation was also observed between 48-72 hours. Increased distance between gill and operculum was observed. Decrease in the level of sensitization (response to stimuli) and settlement of fishes at the bottom was observed, at the end of experiment.

110 µg/L: Same observations were seen as in 100 µg/L concentration.

120 µg/L: Similar observations were noted as in concentration 100 µg/L .

130 µg/L: Vertical and downward swimming pattern was observed. The fishes gathered at the surface in initial 24 hours. The swimming speed decreased with time during the experimental span. Dark pigmentation, mucous secretion, hemorrhage region and increased distance between gill and operculum was observed between 48-72 hours. Decrease in the level of sensitization (response to stimuli) and settlement of fishes at the bottom was observed, at the end of experiment.

140 µg/L: Vertical and downward swimming pattern was observed. The fishes gathered at the surface in initial 24 hours. The swimming speed decreased with time during the experimental span. Increased distance between gill and operculum was observed 24 hours. Dark pigmentation, mucous secretion and hemorrhage region was seen between 24-48 hours. The fishes gathered in a corner at bottom after 72 hours. Decrease in the level of sensitization (response to stimuli) and settlement of fishes at the bottom was observed, at the end of experiment. The first fish died within the first 24 hours and the remaining at 96 hours.

150 µg/L: Vertical and downward swimming pattern was observed. The fishes gathered at the surface in initial 24 hours. The swimming speed decreased with time during the experimental span. Increased distance between gill and operculum, mucous secretion and hemorrhage region was observed after 24 hours. Dark pigmentation, decrease in the level of sensitization and gathering of fishes in a corner at bottom was observed after 48 hours. The two fishes died within the first 24 hours and the remaining at 96 hours.

DISCUSSION AND CONCLUSION

The toxicity of particular pollutants depends on many factors such as animal weight, time of exposure, temperature, pH and hardness of water. The evaluation of LC_{50} concentration of pollutant is an important step, before carrying further studies on physiological changes in animals. Finney's Probit analysis gave 96 hours LC_{50} value for *Gambusia Affinis* exposed to different concentrations of Mercuric Chloride as 97.077 µg/L. The toxicity increases as the exposure time as well as concentration of pollutant increases. Control mortality was zero. In the present study the control fish behaved in natural manner i.e., they were active with well-coordinated movements. They were alert to the slightest disturbance, but in the toxic environment the fishes exhibited irregular, erratic movements, with vertical and downward swimming patterns which were also reported by Rabia Sarikaya (2003). Whereas decreased level of sensitization reported, may be the result of inhibition of AchE activity, leading to accumulation of acetylcholine in the cholinergic synapses, leading to hyperstimulation and was also reported by Shinde et al.(2007), koprucu *et al.* (2006), Shambanagouda *et al.,* (2009), Mehmet Yilmaz *et al.,* (2004), Rabia Sarikaya *et al.,* (2003), and Shivkumar and David (2007).

Dark pigmentation observed, was also noticed by Prashanth *et al.,* (2011) and Koprucu *et al.,* (2006). Increased distance between gill operculum and mucus secretion was also seen. Prashanth *et al.* (2011) and Saxena *et al.* (2005) observed the mucus secretion in fishes, which forms a barrier between the body and the toxic media thereby probably reduces contact with the toxicant so as to minimize the irritating effect, or to eliminate it through epidermal mucus. Hemorrhage regions observed during the experiments were also observed by Saxena *et al.,* (2005). Surfacing phenomenon noticed, was also reported by Rabia Sarikaya *et al.* (2003), Mehmet Yilmaz *et al*. (2004) and Prashanth *et al.* (2011). This surfacing phenomenon, i.e, significant preference to upper layer in exposed groups was explained by Katja *et al.* (2005).

Acute and Chronic effects of Mercury have been widely described for different aquatic organisms and exposure routes. This metal is an important constituent in industrial effluents (Schaperclaus, 1991) into fresh water and seas. The result obtained in this study clearly indicates the fact that behavioral changes are the most sensitive indication of potential toxic effects and it is necessary to perform behavioral investigations of all heavy metal poisoning on fish. This study could lead to the enrichment of the behavioral information during the heavy metal poisoning, which in future may come up as an effective technique to know the toxicity of an unknown compound.

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Table 1. Probit analysis of $HgCl_2$ toxicity for the 96 hrs. using Finney method Lognormal distribution.

Probit Analysis - Finney Method [Lognormal Distribution]							
Log10[Dose (Stimulus)]	**Actual Percent (%)**	**Probit Percent(%)**	**Total number of Individuals**	**R**	**E(R)**	**Difference**	**Chi-square**
1.7782	0.1	0.0972	10	1.	0.9725	0.0275	0.0008
1.8451	0.3	0.1984	10	3.	1.984	1.016	0.5202
1.9031	0.3	0.3237	10	3.	3.2367	-0.2367	0.0173
1.9542	0.4	0.4548	10	4.	4.5478	-0.5478	0.066
2.	0.5	0.5769	10	5.	5.7692	-0.7692	0.1026
2.0414	0.7	0.6816	10	7.	6.8164	0.1836	0.0049
2.0792	0.7	0.7662	10	7.	7.6618	-0.6618	0.0572
2.1139	0.8	0.8315	10	8.	8.3148	-0.3148	0.0119
2.1461	0.9	0.8803	10	9.	8.8028	0.1972	0.0044
2.1761	0.975	0.9159	10	9.75	9.1587	0.5913	0.0382

(Alpha value (For confidence interval = 0.05)

Chi-square

Chi-square	0.8235
Degrees Of Freedom	8
p-level	0.9991

Table 2. Probit analysis and regression statistics of $HgCl_2$ toxicity on *Gambusia affinis*

Probit Analysis - Least squares [Normal Distribution]				
Dose (Stimulus) ug/lit	**Actual Percent (%)**	**N**	**Probit (Y)**	**Weight (Z)**
60.	0.1	10.	3.7183	2.6548
70.	0.3	10.	4.476	4.452
80.	0.3	10.	4.476	4.452
90.	0.4	10.	4.7471	4.7471
100.	0.5	10.	5.	5.
110.	0.7	10.	5.524	4.452
120.	0.7	10.	5.524	4.452
130.	0.8	10.	5.8415	3.8171
140.	0.9	10.	6.2817	2.6548
150.	0.975	10.	6.9604	1.

Regression Statistics:

LC50	97.099 ug/lit
LC50 LCL	86.8578 ug/lit
Beta	0.0289
Beta Standard Error	0.0066
LC50 Standard Error	5.1541
LC50 UCL	107.3401
Intercept	2.1916
LC10	52.7834 ug/lit
LC84	131.6738 ug/lit
LC100	148.9612 ug/lit
LC16	62.5241 ug/lit
LC90	141.4146 ug/lit

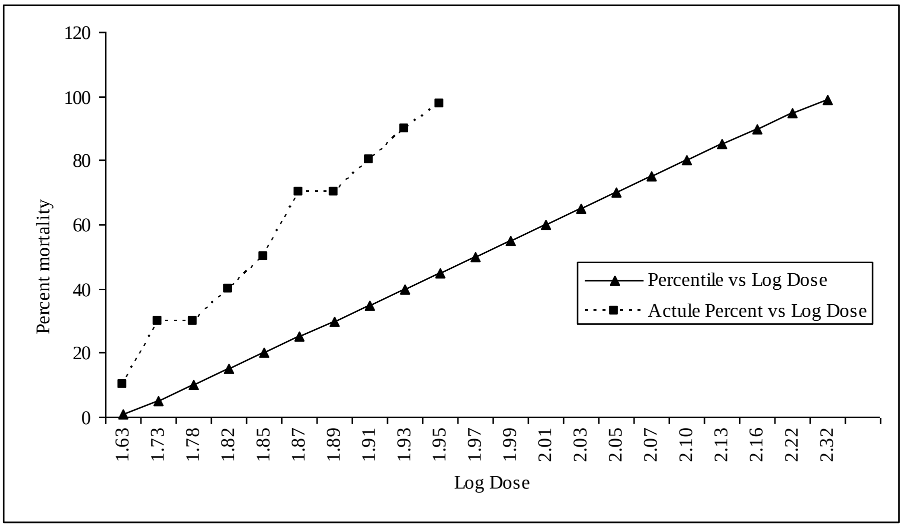

Figure 1. Graph showing Dose percentile and Actual response in percent verses Log dose concentrations of $HgCl_2$ on Gambusia affinis.

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