Acute toxicity and behavioral response of, *Gambusia affinis* (a non-target organism) to Menadione, a Synthetic analogue of a natural insecticide (Plumbagin)

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Abstract:

Menadione, (2 methyl 1,4 naphthoquinone) has a potential to be developed as an effective insecticide from quinone group, which is explored for its effect on non target species. In the light of this, the acute toxicity of Menadione was investigated in the present study for *Gambusia affinis*. Fishes were exposed to different concentrations (0.018 to 0.09 mg/L) of Menadione for 96 hrs. The acute toxicity value was found to be 0.0536 mg/L. Acute toxicity and behavioral patterns were studied in lethal and sub lethal concentrations. *Gambusia* exhibited several distinct behavioral patterns like, erratic swimming pattern, surfacing phenomenon, decreased sensitization, hemorrhage regions, decreased opercular movements, increased distance between gill and operculum, hypo-pigmentation and then hyper pigmentation before death. Fish in sublethal concentrations were found to be stressed but it was not fatal. Hence, it can be concluded that the present compound, Menadione has a potential to be developed as a safer and effective insecticide for non target species and the target species respectively. This acute toxicity value is 50 times higher than which is required for insecticidal property, so Menadione can be considered safe for aquatic non target species.

Key words: *Gambusia affinis*, behavioral patterns, Menadione.

1. Introduction

The increasing human population and the demand for food material have urged the massive usage of pesticides in the crops. The production of pesticides started in India in 1952 with the establishment of a plant for the production of BHC near Calcutta, and in 1999 India became the second largest manufacturer of pesticides in Asia after China and ranked twelfth globally (Mathur, 1999). In India 76% of the pesticide used is insecticide, as against 44% globally (Mathur, 1999). There has been a steady growth in the production of technical grade pesticides in India, from 5,000 metric tons in 1958 to 102,240 metric tons in 1998. In 1996–97 the demand for pesticides in terms of value was estimated to be around Rs. 22 billion (USD 0.5 billion), which is about 2% of the total world market (Aktar, 2009). The pattern of pesticide usage in India is different from that for the world in general. The use of herbicides and fungicides is correspondingly less heavy. The main use of pesticides in India is for cotton crops (45%), followed by paddy and wheat. Every year around 50% cotton crop in India is damaged by pests. So the man has been forced to rely increasingly upon the use of chemicals for the pest control by the demand for more high quality food, fiber and better protection. Use of insecticides has increased tremendously during the last two decades under the pressure of these demands. However, benefits that have occurred to mankind from their use have been attended by some grave problem. One of those problem points towards the non target organisms which are seriously affected in many situations.

Aquatic system serves as main sink for these insecticides. There are mainly two means by which these insecticides reaches to the system first, as agricultural runoffs and second, directly by washing the applicators and other things associated with insecticides. Once the insecticide reaches the aquatic system the whole aquatic system is affected.

Fish are exposed to pesticides in three basic ways first, dermal; second, breathing and third, orally. Poisoning by consuming another animal that has been poisoned by a pesticide is termed "secondary poisoning." For example, fish feeding on dying insects poisoned by insecticides may themselves be killed if the insects they consume contain large quantities of pesticides or their toxic byproducts (Louis, 2009).

So it is the need of hour to design the insecticide keeping in mind both the protection of crops from pest and protection of non target species from insecticide. A fine balance needs to be created between the two, as we cannot progress by leaving one behind. So in the present study we have kept *Gambusia affinis*, a non target organism on test against one of the future potential insecticide: Menadione (2 methyl 1, 4- naphthoquinone).

Fishes are continuously exposed to low concentrations of chemicals which in turn affect their behavioral responses. Hence, there is a growing interest in the development of behavioral markers to assess the sublethal affects of toxicants. Any change in behavior and physiology of fish indicates the deterioration of water quality, since fish are the biological indicators of water quality. Behavior is considered a promising tool in eco toxicology and these studies are becoming prominent in toxicity assessment of unicellular organisms, insects, fish and even rodents. Locomotary behavior is commonly affected by contaminants and the pattern of fish swimming, which is highly organized species specific response (Mehmet et al.2004)

Therefore, the present work has been undertaken to assess the toxicity of a potential insecticide "Menadione" on a non-target species, *Gambusia affinis* by determining the LC_{50} value for 96 hours with special emphasis on behavioral changes on exposure to lethal & sub-lethal concentrations.

2. Materials & methods

This study was carried out in department of Zoology, HPT Arts RYK Science College, Nasik, Maharashtra, India. The fish used for toxicity testing was *Gambusia affinis*. They were provided by a local fish breeder and transported to laboratory in plastic bags filled with water and air. The fishes were acclimatized to laboratory conditions in 50L capacity aquaria. The healthy mature fish of uniform size (wt=0.250mg, length =2.9cm, width =3.1mm) were sorted out from the tank and then transferred to another aquarium where they were acclimatized for 15 days in dechlorinated water. Overcrowding was avoided by keeping fish in two acclimatization tank.

During this period the fishes were fed with commercial pelleted food at least once a day. The water in the aquarium was changed on alternate day to avoid the influence of the excreta and left over food material. The medium was aerated intermittently to avoid the effect of stratification and to maintain of dissolved oxygen not less than 4mg/lit. The temperature for the acclimatization & test was maintained as 27±2 °C. Acclimatized fish were not fed 24 hours before the start of tests. Care was taken in order to keep the mortality rate of fish not more than 5% in the last four days before the experiment was started.

The toxicant used in static bioassays was a potential pesticide under research, Menadione (Company: Laboratory Reagent) initially dissolved in 1ml acetone and final volume was made by dechlorinated tap water. The test organisms were distributed in different concentrations of Menadione. For the tests, 10 fish were used per concentration per replicate. A total of four replicates were carried out for each dose and control group. The aquaria were not aerated during the experiments. Different Menadione concentrations administered in the tanks were 0.018, 0.024, 0.03, 0.036, 0.042, 0.054, 0.06, 0.066, 0.072, 0.078 and 0.09 mg/L.

There was simultaneous control group together with these experimental groups. The control group was without the Menadione, keeping other conditions same. All experiments were carried out for a period of 96 hours. The water of experimental and control groups was replaced daily with their respective concentrations. The number of dead fish were observed every 12 hours and removed immediately from the aquaria. The mortality rate was determined at the end of 24, 48, 72 and 96 hours. The behavioral changes of the healthy fish and fish exposed to various doses of Menadione were observed for behavioral changes and photographed.

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 Probit Analysis LC_{50} Determination Method [lognormal Distribution]. The computer analysis was carried on for LC_{50} on software Biostat (2009).

3. Result

Relation between the Menadione concentration and the mortality rate of *G.affinis* according to Probit Analysis using Biostat (2009) Computer Program is shown in Table No.1. The results obtained from acute static 96-h toxicity experiments of Menadione for *G.affinis* and Probit (Y) and weight (Z) values along with 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 1. The mean LC_{50} value of Menadione on *G. affinis* individuals was found to be 0.0536 mg/L based on least squares [normal Distribution] probit analysis. Figure No. 1 shows the plot of Dose percentile and Actual response in percent.

Probit Analysis - Least squares [Normal Distribution]				
Dose (Stimulus)	Actual Percent (%)	N	Probit (Y)	Weight (Z)
0.018	0.1	10	3.71827124349729	2.65481373049187
0.024	0.2	10	4.15854328264522	3.81708656529043
0.03	0.3	10	4.47599812961732	4.45199625923464
0.036	0.2	10	4.15854328264522	3.81708656529043
0.042	0.4	10	4.74706673217342	4.74706673217342
0.048	0.4	10	4.74706673217342	4.74706673217342
0.054	0.5	10	5	5
0.06	0.6	10	5.25293326782658	4.74706673217342
0.066	0.6	10	5.25293326782658	4.74706673217342
0.072	0.7	10	5.52400187038268	4.45199625923464
0.078	0.8	10	5.84145671735478	3.81708656529043
0.09	0.9	10	6.28172875650271	2.65481373049187

Regression Statistics			
LD_{50}	0.0536522186954734	**LD_{50} Standard Error**	0.00406174313744943
LD_{50} LCL	0.04560956161081	**LD_{50} UCL**	0.0616948757801369
Beta	31.784246444163	**Intercept**	3.29470465870694
Beta Standard Error	7.21954566629126		
LD_{10}	0.0133263057072769	**LD_{16}**	0.0221900916396456
LD_{84}	0.0851143457513013	**LD_{90}**	0.0939781316836699
LD_{100}	0.100845409279215		

Table 1: The relationship between concentration of toxicant and mortality by Least squares [Normal Distribution]-Probit analysis and regression statistics providing estimated LC values.

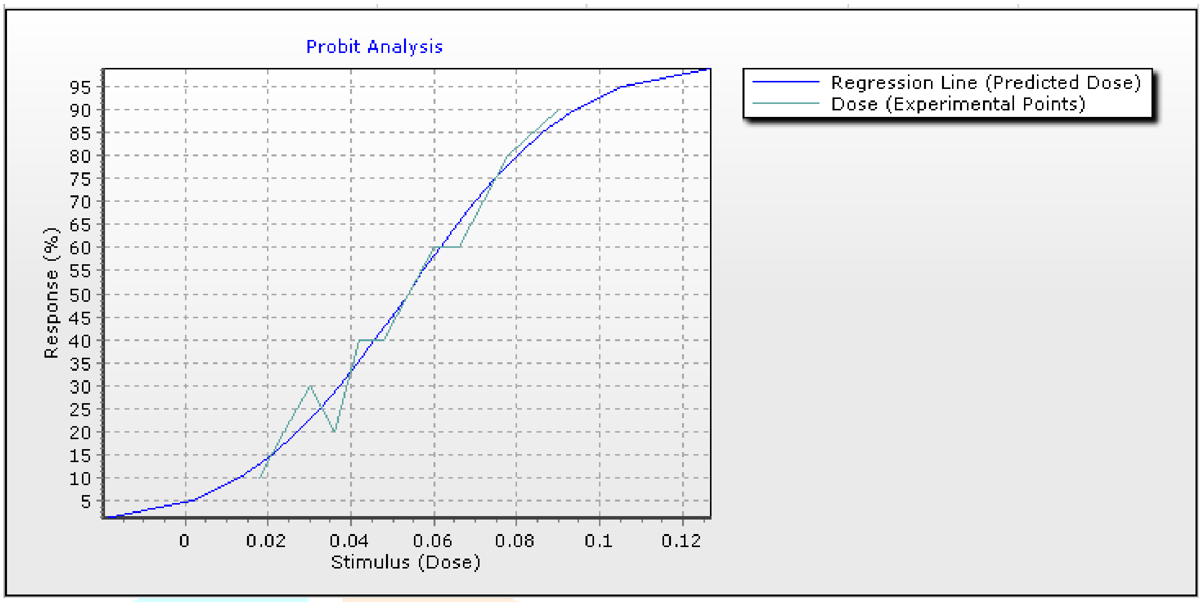

Figure No. 1. The plot of stimulus and percent response showing regression line of predicted doses.

3.1 The changes in behavioral patterns.

Behavioral changes are the most sensitive indication of potential toxic effects. Optomer responses are very useful in evaluation of the behavioral changes of fish (Richmonds and Dutta 1992).

In the present study, *G affinis* exposed to various concentrations of Menadione exhibited behavioral anomalies as, shown in Figure No. 2. The behavioral changes of *Gambusia affinis* exposed to various concentrations of Menadione were as follows.

Control group: There were no behavioral changes and death observed in the control group throughout the experiment (Figure No. 2A).

0.018-0.03 mg/L: Swimming disorders such as verticals and downward swimming pattern was observed. The fish tended to gather at the surface in initial 24 hours. The swimming speed decreased with time during the experimental span.

0.036 mg/L: Vertical and downward swimming pattern was observed. The fish tended to gather at the surface in initial 24 hours. The swimming speed decreased with time during the experimental span. Hypo-pigmentation was observed between 48-72 hours (Figure No. 2B).

0.042mg/L: Vertical and downward swimming pattern was observed. The fish tended to gather at the surface in initial 24 hours with temporarily increased opercular movements. The swimming speed decreased with time during the experimental span. Hypo-pigmentation was observed between 48-72 hours. The fish tended to gather in corner at bottom after 48 hours (Figure No. 2C).

0.048 mg/L: Vertical and downward swimming pattern was observed. The fish tended to gather at the surface in initial 24 hours with temporarily increased opercular movements. The swimming speed decreased with time during the experimental span and latter jerky movements were also seen. Hypo pigmentation was seen and the fish tended to gather in corner at bottom after 48 hours.

0.054mg/L: Vertical and downward swimming pattern was observed. The fish tended to gather at the surface in initial 24 hours with temporarily increased opercular movements. The swimming speed decreased with time during the experimental span. Hypo pigmentation and increased distance between gill and operculum was observed after 24 hours (Figure No. 2D). Decreased level of sensitization and gathering in corner at the bottom was observed after 48 hours.

0.06 mg/L: Vertical and downward swimming pattern was observed. The fish tended to gather at the surface in initial 24 hours. The swimming speed decreased with time during the experimental span. Mucous secretion (Figure No. 2E) and Hypo-pigmentation, was observed after 24 hour. Hemorrhage region above head (Figure No. 2F), increased distance between gills and operculum were observed after 48 hours of exposure. Whereas, decreased sensitization and gathering in corner at the bottom was observed after 72 hours.

0.066 mg/L, 0.072 mg/L, 0.078 mg/L and 0.09 mg/L: Vertical and downward swimming pattern was observed. The fish tended to gather at the surface in initial 24 hours with temporarily increased opercular movements. The swimming speed decreased with time during the experimental span. Mucous secretion, hypo-pigmentation, hemorrhage region and increased distance between gill and operculum were observed after 24 hour. However, decreased sensitization and gathering in corner at the bottom was observed after 48 hours.

Changes seen in fishes along the increasing concentration:

With increasing concentrations of toxicant the fish exhibited varied behavioral and morphological abnormalities, like as the fish were exposed to toxicant they showed vertical and downward swimming pattern. However, within 1-2 hours of exposure they calmed down and start swimming slowly. Surfacing phenomenon was also shown by the fishes occasionally. The swimming speed of the fishes decreased with increase in time of exposure and increase in concentration of toxicant. Distance between gill and operculum increased in all concentrations at and above LC_{50}. The mucous secretion and hemorrhage region over head was seen beyond LC_{50} value, i.e at lethal concentrations. Hypo pigmentation and decreased level of sensitization was seen in sub lethal and lethal concentrations as the time exposure increased. Gathering of fish school at the corner was also observed in sublethal and lethal concentrations with the increase in exposure time. In lethal concentrations before death the fish showed hyper-pigmentation with least of the body and opercular movements, finally they sank down and die.

4. 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. The mean LC_{50} value of Menadione on *G.affinis* individuals was found to be 0.0536 mg/L by the use of Biostat (2009) computer program based on Probit Analysis: least squares [normal Distribution] analysis. The safe concentration for our test compound is considered to be 0.012 mg/L, where zero mortality was seen with no behavioral changes. The toxicity increased as the exposure time and concentration of pollutant increased. Control mortality was zero. In the present study the control fish behaved in a natural manner i.e., they were active with well–coordinated movement. They were alert to the slightest disturbance, but in the toxic environment the fishes exhibited irregular, erratic movement, with jerky swimming movements, vertical and downward swimming pattern, decrease in the level of sensitization is probably due to inhibition of AchE activity, leading to accumulation of acetyl choline in the cholinergic synapsis further leading to hyper stimulation (Rao et al. 2005a). The similar observations were made by Gupta and Jawale (2013), Sindhe et al. (2007), Sarkariya and Yilmaz (2003), Gul et al.(2009), Yilmaz et al. (2004), Kopruco et al. (2006), Shambanagouda et al. (2009) and Shivkumar and David

(2007). They slowly became lethargic, hyper excited, restless and secrete excess mucus all over the bodies. Mucus secretion in fish form a barrier between the body and toxic media thereby probably reduces contact with the toxicant so as to minimize its irritating effect, or to eliminate it through epidermal mucus. Similar behavioral pattern was observed by Rao et al. (2005b), Sindhe et al. (2007), Sarkariya and Yilmaz (2003), Gupta and Jawale (2013), Olaifa et al. (2004), Prashanth et al. (2011) and Saxena et al. (2005)

Opercular movements increased initially in all exposure periods but decreased later, steadily in lethal concentration compared to sub lethal exposure periods. The increased opercular gill movements observed initially may possibly compensate for increased physiological activity under stressful conditions (Shivakumar and David, 2004). Beyond LC_{50} value hemorrhage region was observed (Saxena et al.**,** 2005). Hypo-pigmentation of the fish occurred at higher concentrations beyond LC_{50}. A similar result of hypo pigmentation was observed by Shahi and Singh (2010) in *Channa punctatus* using plant based pesticide. This change in body color was explained by Rao et al. (2005), as impairment of pituitary functions reflected by reduction in number and size of the chromatophores and their pigment content. Distance between gills and operculum was increased at and beyond LC_{50} concentration (Khunyakari et al., 2001). Gathering at the surface was seen on the first day itself in sub-lethal exposure periods and continued the same more intensely, which is in accordance with the observation made by Sarikaya and Yilmaz (2003), Yilmaz (2004), Prashanth et al. (2011). Gathering at the surface may help to avoid contact with the toxic medium. Surfacing phenomenon i.e., significant preference of upper layers in the exposed group might be the result of the need for higher oxygen levels during the exposure period as seen by Katja et al. (2005). Finally fish sank to the bottom with the least opercular movements and died with their mouths open.

The present test compound, Menadione has a potential to replace the expensive, short lived natural insecticide known as Plumbagin. Menadione has LC_{50} of 1.5 µg for *Dysdercus cingulatus*, *a* cotton pest and on the other hand it has the LC_{50} value of 0.053 mg/L for *Gambusia affinis*, a small non target aquatic species. This shows that LC_{50} value for *Gambusia* is too high as compared to the LC_{50} for *Dysdercus cingulatus*. Although some intense behavioral changes were observed at sublethal doses of Menadione but even that concentration is far too higher than the LC_{50} value of 1.5 µg for *Dysdercus cingulatus*. So it can be concluded that the concentration which will be used to combat *Dysdercus* in fields will be very low and should not affect the non target species. We may predict so, as the test compound has less leaching capacity, it is light sensitive, doesn't show bioaccumulation (pubchem, NCBI) and even if it reaches the aquatic system a harmless quantity will reach there. Hence, it can be concluded that the present compound, Menadione has a potential to be developed as a safer and effective insecticide for non target species and the target species respectively.

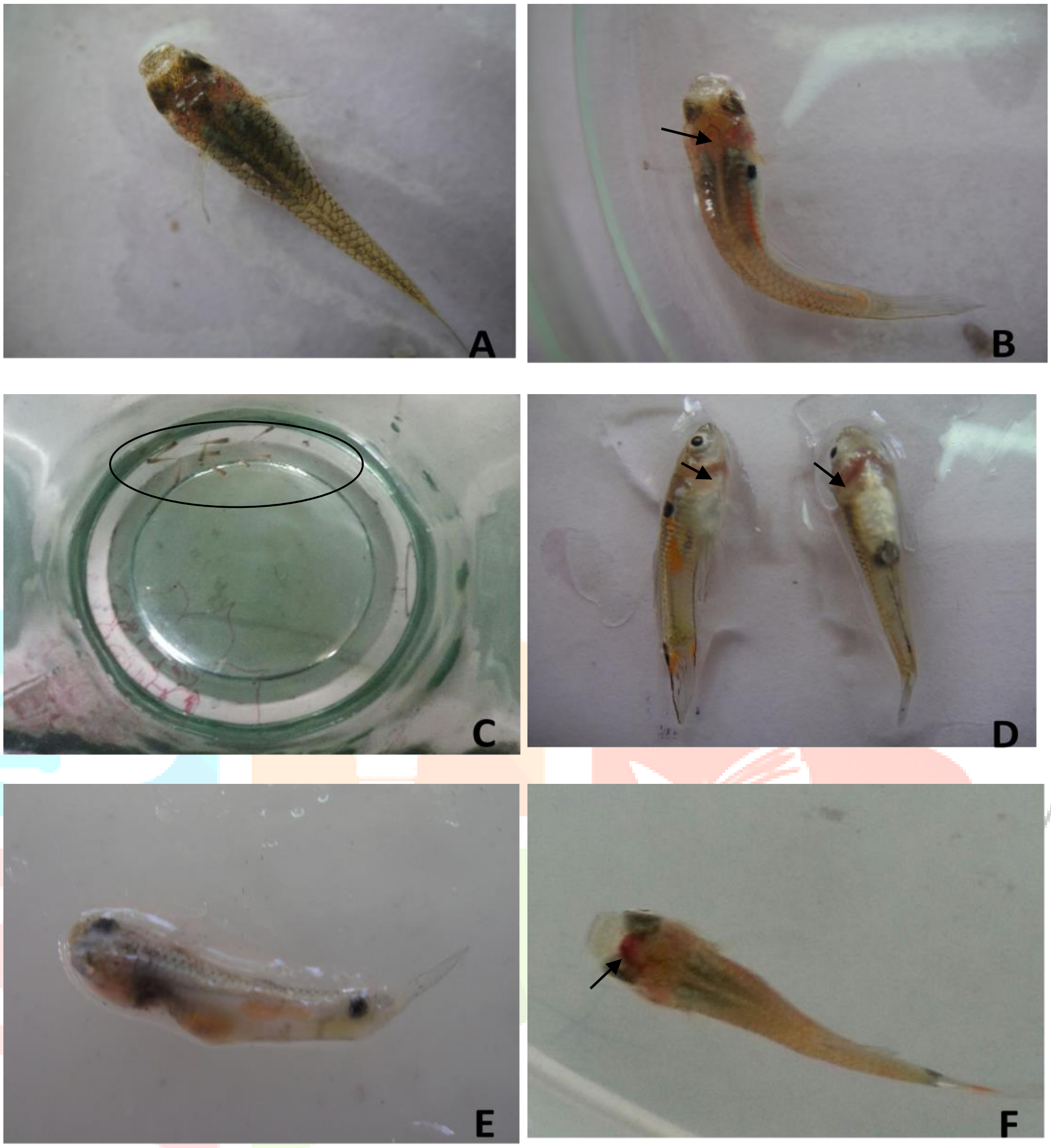

Figure 1: Images of *Gambusia affinis* showing the various physiological effects on exposure to Menadione **A)** Shows the control healthy *Gambusia affinis*. **B)** Hypo-pigmented fish during the experiment. **C)** Gathering shown by fish under the effect of Menadione**. D)** Increased distance between gill and operculum during respiratory stress in fish of experimental tanks. **E)** Thick mucus secretion and degenerated body under the influence of toxicant. **F)** Hemorrhage region over the head after the treatment of *Gambusia affinis* with Menadione.

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