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Identification of a sensitive index during fish bioassay of an azo dye methyl red (untreated and treated)

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Abstract: *Acute and chronic toxicity of methyl red (untreated) was examined on a freshwater fish Poecilia reticulata, using indices viz; mortality, reduction in RBC counts and their morphological abnormality (poikilocytosis and anisocytosis). Similar studies (acute toxicity) were also made in physicochemically and biologically treated methyl red. Data comparison of these four indices revealed poikilocytosis as the most sensitive index, since it measured higher toxicity of methyl red when fish mortality was either minimum at its low concentration (5 ppm) during both acute and chronic toxicity or even nil in the biologically treated 100 ppm methyl red, during acute toxicity. Mortality was next to poikilocytosis though it ranked 1st at higher concentration of methyl red during acute toxicity. The reduction in RBC counts however, was found to be the most sensitive parameter only in case of prolonged exposure (4 weeks) to 5 ppm methyl red. Amongst the four indices used for quantifying toxicity; anisocytosis was found to be the least expressive. Based on these findings we recommend quantification of data on fish mortality and poikilocytosis during acute toxicity whereas reduction in RBC counts and poikilocytosis during chronic exposure to methyl red.*

Key words**:** *Fish bioassay, Methyl red, Mortality, RBC counts, Poikilocytosis, Anisocytosis.*

Introduction

In most of the ecotoxicological studies, fish has been considered as an ideal test organism to examine both acute and chronic toxicity of pollutants. During fish bioassay, a great emphasis has been laid on its mortality. It is likely that the low concentration of pollutant may not result in fish mortality, while pollutant may still be toxic to them. It is important to note here that blood is quite sensitive to pollutants. The erythrocyte membrane has been reported permeable to pollutants (Moss and Hathway, 1964), which not only destroys membrane, but also alters its shape and size by casting adverse effects on structure and function. (Bielinska and Terlecki, 1984; Udden, 2000; Suwalsky *et al.,* 2004). Thus, alongwith mortality, studies on erythrocyte morphology and its count may serve as important indices for assessing and monitoring pollutant toxicity.

Several workers have examined toxicity of dyes and textile wastewaters on fish in relation to their mortality (Rana and Raizada, 1999; Karanjkar *et al.,* 2000), and changes in RBC counts (Goel *et al.,* 1981) and their morphological abnormalities (Murugesan *et al.,* 1989). Sharma *et al.* (2003) reported toxic effects of methyl red on fish mortality and their RBC counts. It is thus evident that toxic effects of dyes/textile wastewaters were not assessed simultaneously on fish mortality, their RBC counts and morphological abnormalities, inspite of the fact that such studies may decipher pollutant toxicity better. With this backdrop, we have examined short (acute toxicity) as well as long-term (chronic toxicity) toxic effects of methyl red (untreated and treated) on mortality and RBC (morphology and count) in a freshwater fish *Poecillia reticulata* Peters, with the objective to identify the most sensitive parameter amongst the four indices chosen for this purpose. The important findings of this study are reported in the present communication.

Materials and Methods

A stock solution of methyl red was prepared by dissolving its weighed amount in 10-15ml of ethanol made up to 1000ppm using tap water. The fish caught from a tank were acclimatized for 15 days in a trough (40 litre) containing good plankton population to serve as food and *Ceratophyllum demersum* (a submerged hydrophyte) to oxygenate water. Thereafter, 70 healthy mature fish of uniform size (Length=2.2 $\pm$ 0.06cm; Width=2.5 $\pm$ 0.014cm) starved for 24hr in de-chlorinated tap water were exposed to six dilutions of methyl red (5, 10, 20, 30, 40 and 50ppm) and tap water (control) for assessing acute toxicity (96hr) as per APHA (1989). Chronic toxicity however, was assessed by exposing the fish to two sublethal concentrations (5 and 10ppm) for 4 weeks in the microcosms, as described by Sharma *et al.,* 2003. Dead fish were counted and removed immediately. LC50 value of methyl red was calculated by probit analysis using Compaq Personal Computer Basic Version 1.13 (Finney, 1971). Autopsy of fish was done after 96hr exposure in case of acute exposure, whereas at weekly intervals during chronic exposure for RBC counts (Dacie and Lewis, 1982) and blood smear preparation (Lee *et al.,* 1993). The RBC measurements were made by an occulometer standardized with a micrometer scale as parallel magnification (10x X 100x). The percentage of morphological abnormal RBCs in methyl red treatments was calculated by observing approximately 200 RBCs in 20 microscopic fields (10x X 100x) using an oil immersion.

Since textile dye wastewaters in the industries are treated by physicochemical and biological methods, therefore reduction in toxicity of treated methyl red (30ppm and 100ppm) was also examined. During physicochemical treatment, about 100mg of bentonite dissolved in 10ml tap water was added to 1litre acidified (by H_2SO_4) 30ppm /100ppm methyl red solution

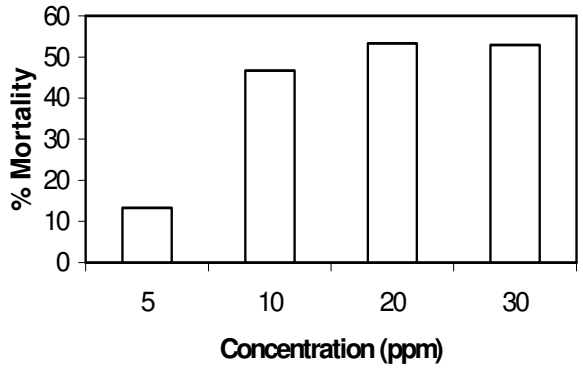

Fig. 1: Percentage mortality of fish at different concentrations of methyl red during acute toxicity.

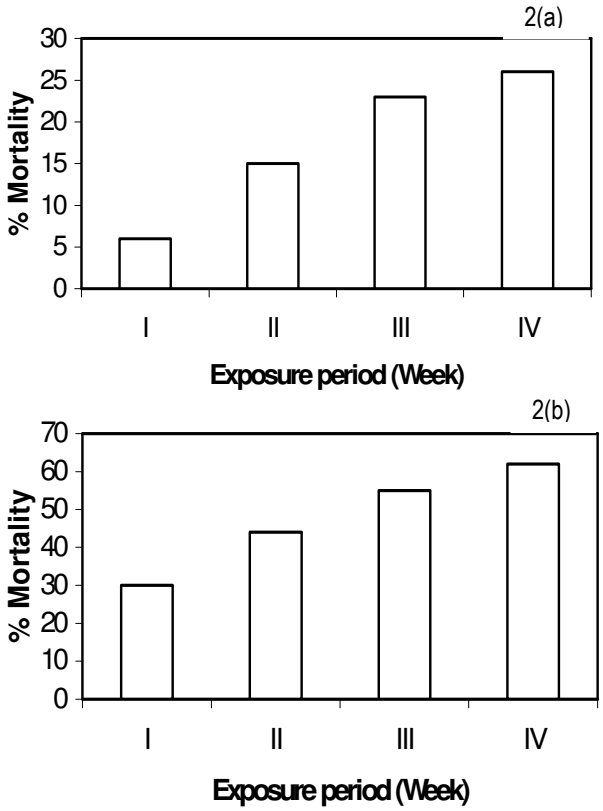

Fig. 2: Percentage mortality of fish during chronic exposure (Fig.2a: 5 ppm; Fig. 2b: 10 ppm) of methyl red.

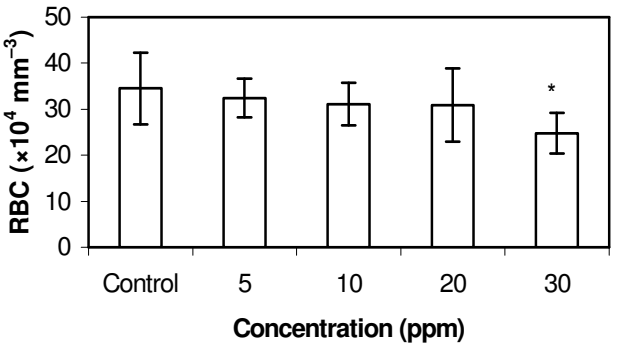

Fig. 3: RBC counts of fish at different concentrations of methyl red during acute toxicity.
*Significant at 5% probability; ± = SD.

(pH 4.0) and stirred for 15 minutes. The colored precipitate formed was allowed to settle for about 12hr. The clear supernatant solution was separated and neutralized (pH 7.0) using NaOH (N/10). The toxicity of treated methyl red was examined as described earlier.

Methyl red (30 and 100ppm) was also treated biologically in two separate studies. In the first study (study 1); both 30ppm and 100ppm methyl red (pH 8.1) supplemented with 5ppm phosphorus (PO_4-P) were taken separately, and initially treated in the gravel bed fixed film bioreactors developed in 15 litre sized plastic bucket. The bioreactor outflows were then polished in the constructed wetlands of *Phragmites* at 3 days-retention period. The microbial communities inoculated, both in bioreactor and *Phragmites* wetland rhizosphere were isolated from the habitats polluted by textile dye wastewaters. In order to compensate evapotranspiration losses both from bioreactor and constructed wetland, tap water was added into the final wetland outflow to raise its volume equal to initial untreated methyl red. Acute toxicity of both 30ppm and 100ppm treated methyl red was estimated as described earlier.

In another set of biological treatment (study-2), acidified 100ppm methyl red (pH ≅ 5.0) supplemented with NO_3-N (50ppm), PO_4-P (5ppm) and K (10ppm) was treated only in the constructed wetland of *Phragmites* at one day retention period and toxicity of treated outflow was estimated as descried earlier. The Student 't' test and correlation (r) calculated using computer program (Systat Version-5).

Table – 1: Fish mortality, % reduction in RBC counts in comparison to control fish and percentage of abnormal RBC in the physicochemically and biologically treated methyl red.

Treatment process	Mortality (%)	% reduction in RBC counts	% abnormal RBC
1. Physicochemical : 30 ppm	23±4	15 ±3	33 ± 6
100 ppm	67 ± 11	27 ±7	51 ± 10
2. Biological: study 1. 30 ppm	38±6	Nil	18 ± 3
100 ppm	100	-	-
study 2. 100 ppm	Nil	6 ±1.7	35 ± 7

Data in parenthesis indicate % reduction in count in comparison to control fish.

Table – 2: Measurement (diameter) of RBCs in the control and methyl red treatments (acute toxicity).

Treatments	Control	Methyl red			
		5ppm	10ppm	20ppm	30ppm
RBC diameter	6.58± 0.41	5.91**± 0.62 (-10)	6.77 ± 0.64 (+ 2.9)	7.53**± 0.57 (+14)	6.81 ± 0.56 (+3.5)

**Significant at 1% probability; data in parenthesis indicate % change in diameter in comparison to control

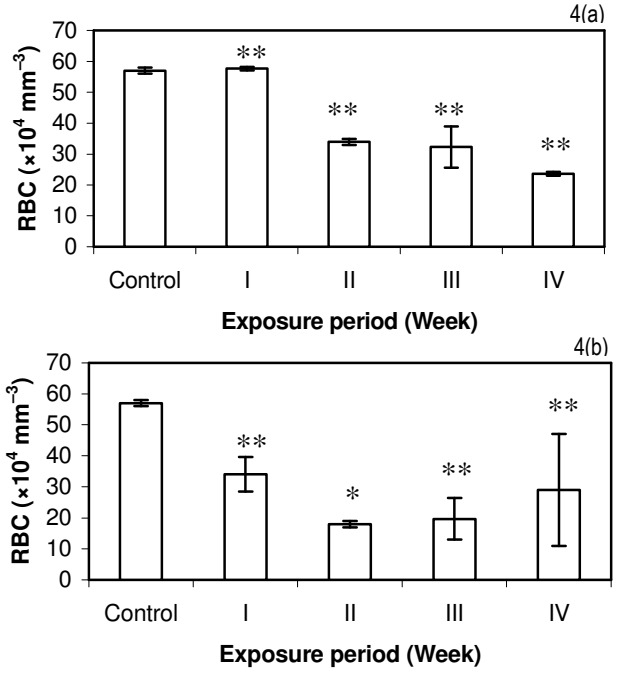

Fig. 4: RBC counts of fish during chronic exposure (Fig.4a: 5 ppm ; Fig. 4b: 10ppm) of methyl red.

*Significant at 5% probability; **Significant at 1% probability; ± = SD

Results and Discussion

Mortality: Fish mortality was dose dependent during acute exposure, while it was found to be dependent both on dose and exposure period during chronic exposure (Fig. 1, 2a, b). LC_{50} value of methyl red was 27.2ppm during acute exposure. The toxicity of methyl red decreased after its treatment, the reduction being higher after biological treatment in comparison to physico-chemical treatment, especially when acidified methyl red (supplemented with nutrients) was treated in the constructed wetland (study 2) (Table 1).

RBC counts: In comparison to control fish, RBC counts decreased in fish exposed to methyl red; being dose dependent during acute toxicity (6-28%) (Fig. 3), whereas it depended both on concentration and exposure period (5ppm: nil - 58%; 10ppm: 40-68%) of methyl red during chronic toxicity (Fig.4a, b). Contrary to acute toxicity; cytotoxicity of methyl red was lesser during chronic exposure, since reduction in RBC count was nil even after one-week exposure of fish at 5ppm concentration of methyl red in the microcosms (Fig. 4a).

In comparison to untreated methyl red (30ppm), cytotoxicity of physicochemically and biologically treated methyl red decreased markedly, especially in the latter (Table 1).

RBC shape (Poikilocytosis): RBCs in control fish were oval whereas in methyl red treatments they were spherical, beaked, triangular, quadrilateral and pentagonal (Fig. 5). They were however, normochromic both in control and methyl red treatments. During acute exposure, the percentage of morphologically abnormal RBCs increased, with an increase in concentration of methyl red (r = + 0.62) (Fig. 6), while both with concentration and exposure period of methyl red (r: 5ppm = + 0.96; 10ppm = + 0.99) during chronic toxicity (Fig. 7a, b). Interestingly, during acute toxicity the percentage of abnormal RBCs at both 5 and 10ppm was significantly higher than those recorded at the same concentrations even after two weeks of exposure in chronic toxicity. Thus, magnitude of methyl red toxicity decreased in microcosm exposed fish, as also noted earlier for RBC count. In comparison to untreated methyl red (30ppm), the percentage of morphologically abnormal RBCs decreased markedly in the treated methyl red of even higher concentration (100ppm), more particularly after biological treatment (Table 1).

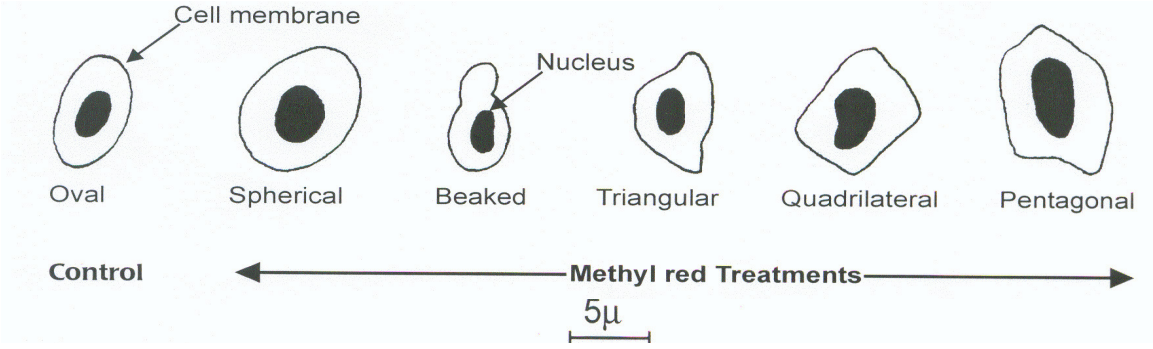

Fig. 5: Camera lucida diagram of morphologically abnormal RBC's in the methyl red exposed fish.

Table – 3: Measurement (diameter) of RBCs in the control and methyl red treatments (chronic toxicity).

Treatments	Control	Exposure period (week)			
		I	II	III	IV
5ppm	7.87 ± 0.41	8.11± 0.74 (+3)	9.26** ± 0.97 (+18)	7.77 ± 1.29 (-1.3)	8.5** ± 0.59 (+8)
10ppm	-do-	7.66 ± 0.4 (-2.7)	8.83** ± 0.95 (+11)	7.9 ± 0.6 (<1)	8.1 ± 1.1 (+2.9)

**Significant at 1% probability; data in parenthesis indicate % change in diameter in comparison to control

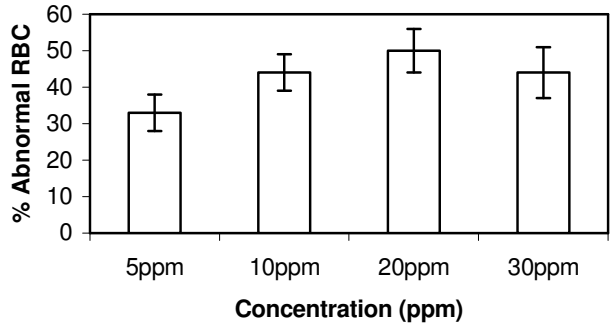

Fig. 6: Percentage of morphologically abnormal RBCs at different concentrations of methyl red during acute toxicity. ± = SD

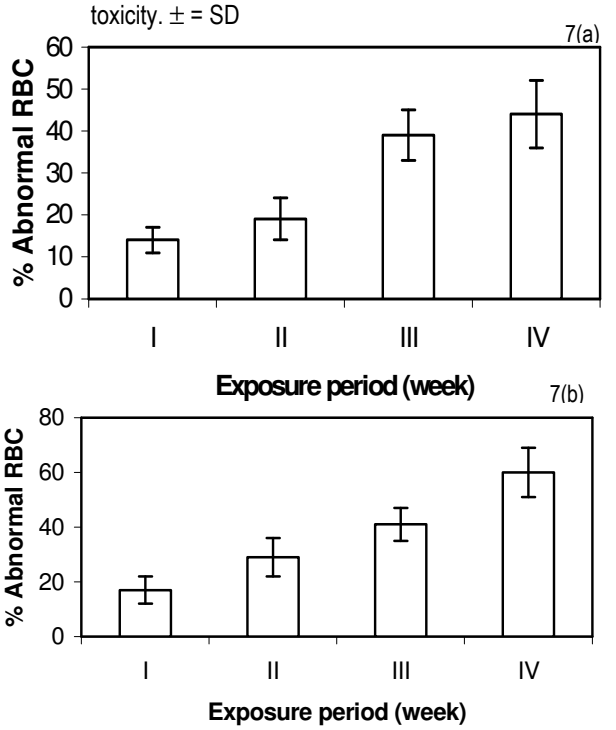

Fig. 7. Percentage of morphologically abnormal RBCs during chronic exposure (Fig.7a: 5ppm; Fig. 7b: 10ppm) of methyl red. ± = SD

RBC measurements (Anisocytosis): During acute exposure, the mean diameter of RBCs decreased (microcytic condition) at lower concentration (5ppm), while an opposite trend (macrocytic condition) was observed at higher concentrations (>5ppm) of methyl red (Table 2). During chronic exposure also, both conditions were observed at 5ppm and 10ppm and maximum change in size was recorded during second week of exposure (Table 3). Similar to acute exposure, anisocytosis was not governed by methyl red concentrations during chronic exposure.

RBC size was similar to control fish, after both physicochemical and biological treatment of 30ppm methyl red, whereas it increased a little (7- 8%) in 100ppm treatments. The present study has thus revealed toxic effects of both untreated and treated methyl red on fish, being higher in the former in comparison to the latter. During both acute and chronic exposure studies, a comparison of mortality, reduction in RBC count, poikilocytosis and anisocytosis at a lower concentration (5ppm) revealed their values in the following order:

Acute: Poikilocytosis (33%) > Mortality (13.3%) > Anisocytosis (10%) > RBC count (6%)

Chronic- I week: Poikilocytosis (14%) > Mortality (6%) > Anisocytosis (3%) > RBC count (Nil)

IV week: RBC count (58%) > Poikilocytosis (44%)> Mortality (26%)> Anisocytosis (8%)

Thus, variations in RBC were found to be the maximum, thereby establishing it to be the most sensitive index for expressing methyl red toxicity at lower concentration.

The order of values for the aforesaid parameters at a higher concentration (30ppm) during acute toxicity was:

Mortality (52.9%) > Poikilocytosis (44%) > RBC count (28%) > Anisocytosis (3.5%)

As evident, mortality took a front seat in expressing methyl red toxicity at its higher concentration. This may possibly be on account of severe toxic effects on nervous system, which were expressed as jumping of fish, and their faster swimming and opercular movements finally terminating in to their death during 24hr of exposure to methyl red.

In physicochemically treated methyl red, the order of parameter sensitivity was;

30ppm: Poikilocytosis (33%) > Mortality (23%) >RBC count (15%) > Anisocytosis (nil)

100ppm: Mortality (67%) > Poikilocytosis (51%) > RBC count (27%) > Anisocytosis (7.6%)

It is likely that reduction of methyl red concentration in 30ppm treatment decreased its knock down effect as noted during acute exposure. As a result, RBC detected methyl red toxicity better, similar to 5ppm treatments of both acute and

chronic studies. In 100ppm treatment, despite of reduction in methyl red concentration its knock down effect continued, similar to 30ppm treatment of acute toxicity, possibly due to higher concentration of toxic pollutant/s in the treated effluent.

The sensitivity of various parameters in biologically treated methyl red was:

30ppm: Mortality (38%) > Poikilocytosis (8%) > RBC count (nil) = Anisocytosis (nil)

100ppm: Poikilocytosis (35%) > Anisocytosis (8%) ≅ RBC count (6%) > Mortality (nil)

Mortality was more effective in detecting toxicity in 30ppm treatment. This may be ascribed to better degradation of methyl red in acidic medium in the wetland rhizosphere, despite of its higher concentration.

From the ongoing trends, it is evident that poikilocytosis is the most sensitive index, since it measures toxicity in a situation, when mortality is either very low as noted at 5ppm during chronic exposure or nil as found in biologically treated methyl red (100ppm) (Fig. 2a; Table1). Mortality is next to poikilocytosis, though it may be an important index at higher concentration of methyl red. The measure of reduction in RBC counts assumes importance only in case of prolonged exposure (4 weeks) to 5ppm methyl red. Although anisocytosis expresses toxic effects of methyl red but its importance as toxicity marker is less pronounced in comparison to others.

Present study infers poikilocytosis to be the best parameter for quantifying acute and chronic toxicity of methyl red. Other important parameters ranking second in order of priority are; mortality (for acute toxicity) and reduction in RBC counts (for chronic toxicity).

Acknowledgments

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